Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids

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PREFACE

This book resulted from presentations at an international conference on bacterial plasmids held January 5-9, 1981 in Santo Domingo, Dominican Republic. This was the first meeting of its kind in the Southern Hemisphere. The meeting place was selected for its relaxed and comfortable climate, conducive to interactions among participants. More importantly the locale facilitated the participation of nearby Latin American clinical and research scientists who deal directly with the health manifestations of pathogenic plasmids. Diseases and socio-economic practices of developing countries exist in the Dominican Republic whose scientific community could directly benefit from having the meeting there.

The book includes the talks as well as extended abstracts of poster presentations from the meeting. This combination, which provides readers with reviews as well as recent findings, captures the full scientific exchange which took place during the 5-day meeting.

As one indication of pathogenicity related to plasmids, the conferees were surveyed for gastro-intestinal problems during and after their stay in the Dominican Republic. The results are summarized at the end of this book.

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Royston C. Clowes
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EVOLUTION AMONG ANTIBIOTIC RESISTANCE PLASMIDS IN THE HOSPITAL ENVIRONMENT

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Plasmid mediated resistance to antibiotics was first discovered about 25 years ago in Japan because of the unexpected appearance of multiple drug resistance during an outbreak of bacillary dysentery (1). Ever since this time the unexpected appearance of a new or unusual drug resistance marker or an unusual pattern of multiple drug resistance has been a clue that plasmids might be involved as carriers of the resistance genes, and in many cases the 'epidemic strain' of the pathogen involved in the outbreak has been found to contain one or more resistance plasmids. Spectacular examples of this are the extensive epidemic of bacillary dysentery due to Shigella dysenteriae Type I in Central America and southern Mexico during 1969-70, investigated by Mata, et al. (2) and the somewhat smaller but still dramatic epidemic of typhoid fever which occurred in and around Mexico City in 1972, investigated by Olarte et al. (3). In both instances the epidemic strain was found to contain a plasmid which conferred resistance to multiple antibiotics. On a smaller scale, many outbreaks of hospital-associated infection have been shown to be due to a particular strain of a gram-negative organism which contains one or more plasmids.

More recently, within the last four or five years, with the simplification of some of the methods of molecular biology which can be used to investigate plasmids as physical entities, it has become possible for individuals whose interests and backgrounds lie primarily in the clinical and epidemiological aspects of antibiotic resistance, and who are not card-carrying molecular biologists, to investigate plasmids more directly, and to adequately compare plasmids isolated in clinical surroundings with one another. During this time there have been several well-documented cases in which outbreaks of infection in hospitals have been shown to be due to two or more different species of gram-negative bacilli harboring a common resistance plasmid. Examples of this are the finding by Elwell et al. (4) at Seattle of a plasmid conferring resistance to tobramycin and other antibiotics in both Klebsiella pneumoniae and Enterobacter cloacae in a burn unit, and the finding by Sadowski et al. (5) at Minneapolis of a plasmid conferring resistance to gentamicin in four different species of gram-negative bacilli. In this outbreak one patient was found to be inhabited by three different species of microorganism harboring this plasmid, strongly suggesting that transmission of the plasmid from one bacterial species to another occurred in vivo. In Boston, O'Brien et al. (6) have found a plasmid conferring resistance to gentamicin and other antibiotics in six different species of gram-negative bacilli at the Peter Bent Brigham Hospital, and again at Seattle Tompkins et al. (7) have also found a common resistance plasmid in six species of gram-negative pathogens at one hospital. In these instances it is appropriate to speak of an 'epidemic plasmid' rather than an 'epidemic strain'.

We have recently had the opportunity to investigate an extensive outbreak of hospital-associated infections due to gentamicin-resistant organisms in which the common element appeared to be a transposable DNA sequence carrying the gentamicin resistance gene, which moved from one plasmid to another, these plasmids in turn spreading among several bacterial species. The molecular biological studies were carried out in my laboratory at the Medical University of South Carolina, primarily by Craig Rubens, as part of a doctoral research project. The epidemiological aspects of the study were performed by Zell McGee and William Schaffner at Vanderbilt University, where this outbreak took place.

Beginning in late 1973, an increase in hospital-associated infections due to gentamicin-resistant organisms was seen at Vanderbilt University Medical Center (Figure 1). At this time most of these infections were due to Serratia marcescens and Pseudomonas aeruginosa. Later, in 1976, an increase in infections due to getamicin-resistant strains of Klebsiella pneumoniae was observed, with a few cases caused by gentamicin-resistant strains of Enterobacter cloacae. No increase in infections due to Escherichia coli was seen. The outbreak eventually involved four hospitals in the Nashville area, interhospital spread apparently taking place on the hands of the medical staff (8). At one hospital an outbreak of infections due to Serratia marcescens was immediately followed by an upsurge of cases due to Klebsiella pneumoniae resistant to the same group of antibiotics (9).

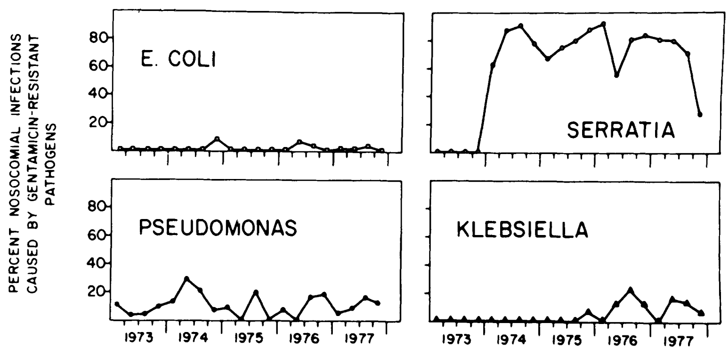

Figure 1. Per cent of nosocomial infections caused by gentamicin-resistant pathogens at Vanderbilt University Medical Center, 1973 - 1977.

From the several hundred strains isolated during this outbreak, a group of 25 strains was selected which represented each of the different species of bacteria involved, different times during the five-year period, and different hospitals and wards where infections with gentamicin-resistant bacteria were occurring (Table 1). These strains were examined for plasmid DNA content, antibiotic resistance markers, and presence of aminoglycoside modifying enzymes. (The enzyme studies were kindly performed by Kenneth Price and Peter Kressel at Bristol Laboratories). Gentamicin-resistant strains of Pseudomonas aeruginosa all contained a single 9.8 Md plasmid, and were also resistant to most other commonly used antibiotics with the exception of amikacin. They produced an aminoglycoside acetyltransferase (either AAC 3-1 or AAC 3-3) and the aminoglycoside phosphotransferase APH 3-1. Gentamicin-sensitive strains of P. aeruginosa lacked this plasmid, were sensitive to gentamicin and tobramycin, and did not contain aminoglycoside-modifying enzymes. Gentamicin-resistant strains of Serratia marcescens isolated during the first three years of the outbreak (Groups I and II) also contained a 9.8 Md plasmid, plus another larger plasmid of either 80 or 100 Md. These strains were also resistant to numerous other antibiotics and elaborated aminoglycoside-modifying enzymes. A single strain of gentamicin-resistant S. marcescens isolated in 1976 (Group III) contained a single 105 Md plasmid, but exhibited resistance to aminoglycosides and produced the AAC 3-3 enzyme. Strains of Klebsiella pneumoniae and Enterobacter cloacae, all isolated late in the outbreak, contained plasmids of either 105 or 110 Md, were resistant to multiple antibiotics including gentamicin and tobramycin, and were found to possess aminoglycoside modifying enzymes. Thus, a preliminary assessment indicated that gentamicin-resistant strains of all species contained either a small 9.8 Md plasmid, or a very large plasmid of 105 Md or larger.

In order to separate the different plasmids from one another, we took advantage of the fact that small plasmids (approximately 10 Md or less) are likely to be nonself-transmissible, but readily transform $CaCl_2$ treated E. coli, whereas larger plasmids (approximately 30 Md or larger) are likely to be self-transmissible but not readily taken up by $CaCl_2$ treated E. coli. Table 2 shows the results of transformation experiments with $CaCl_2$ treated E. coli C600. In every case it was possible to obtain transformants containing the small 9.8 Md plasmid. As seen in this table, this plasmid conferred resistance to β-lactam antibiotics and to the aminoglycosides, along with the ability to produce aminoglycoside-modifying enzymes. Table 3 shows the results of conjugation experiments between gentamicin-resistant strains containing large plasmids and rif^r E. coli SF186. Plasmids of 80 Md and 100 Md size do not confer resistance to aminoglycoside antibiotics nor ability to elaborate

Table 1. Antibiotic Resistance Patterns, Plasmid DNA Content and Aminoglycoside-modifying Enzymes of Representative Gram-negative Bacilli from Nosocomial Infections.

Organism	No. Tested	Antibiotic Resistance Markers[a]											Plasmid DNA (Mass)	Aminoglycoside Modifying Enzymes
		Ap	Cb	Cd	Tc	Sm	Cm	Su	Gm	Tb	Km	Ak		
P. aeruginosa	5	+	+	+	+	+	+	+	+	+	+	-	9.8 Md	AAC 3-1 or AAC 3-3 & APH 3-1
S. marcescens														
Group I	5	+	+	+	+	+	-	-	+	+	+	-	80 Md & 9.8 Md	AAC 3-1 or AAC 3-3 & APH 3-1
Group II	8	+	+	+	+	+	+	+	+	+	+	-	100 Md & 9.8 Md	AAC 3-1, AAC 3-3, AAC 6'-2 & APH 3-1
Group III	1	+	+	+	+	+	+	+	+	+	+	-	105 Md	AAC 3-3
K. pneumoniae	4	+	+	+	+	+	+	+	+	+	+	-	105 Md	AAC 3-1 or AAC 3-3
E. cloacae	2	+	+	+	+	+	+	+	+	+	+	-	110 Md	AAC 3-1 or AAC 3-3 & APH 3-1

a) Ampicillin (Ap), Carbenicillin (Cb), Cephaloridine (Cd), Tetracycline (Tc), Streptomycin (Sm), Chloramphenicol (Cm), Sulfamethoxazole (Su), Gentamicin (Gm), Tobramycin (Tb), Kanamycin (Km), Amikacin (Ak). (+) Denotes presence of the resistance marker.

Table 2. Transformation of *E. coli* C600 by Plasmid DNA from Gentamicin-resistant Epidemic Strains.

Plasmid DNA From	Mass of Plasmid Transformed into Recipient *E. coli*	Antibiotic Resistance Transformed into *E. coli*										Aminoglycoside-Modifying Enzymes Transformed into *E. coli*
		Ap	Cb	Cd	Tc	Sm	Cm	Su	Cm	Tb	Km	
P. aeruginosa	9.8 Md	+	+	+	-	-	-	-	+	I[a]	+	AAC 3-1 & APH 3-1
		+	+	+	-	-	-	-	+	+	+	AAC 3-3 & APH 3-1
S. marcescens												
Group I	9.8 Md	+	+	+	-	-	-	-	+	I	+	AAC 3-1 & APH 3-1
Group II	9.8 Md	+	+	+	-	-	-	-	+	I	+	AAC 3-1 & APH 3-1
	9.8 Md	+	+	+	-	-	-	-	+	+	+	AAC 3-3 or AAC 6'-2 & APH 3-1

a) AAC 3-1 mediates intermediate tobramycin resistance (MIC 4-16 μg/ml) in *E. coli*

Table 3. Conjugation Experiments Between Gentamicin-resistant Epidemic Strains and Recipient rif^r *E. coli* SF186.

Donor	Mass of Plasmid Transferred	Antibiotic Resistance Transferred into SF186										Aminoglycoside-Modifying Enzyme
		Ap	Cb	Cd	Te	Sm	Cm	Su	Gm	Tb	Km	
S. marcescens												
Group I	80 Md	+	+	+	+	+	-	-	-	-	-	None
Group II	100 Md	+	+	+	+	+	+	+	-	-	-	None
Group III	105 Md	+	+	+	+	+	+	+	+	+	+	AAC 3-3
K. pneumoniae	105 Md	+	+	+	+	+	+	+	+	I[a]	-	AAC 3-1
	105 Md	+	+	+	+	+	+	+	+	+	+	AAC 3-3
E. cloacae	110 Md	+	+	+	+	+	+	+	+	I	-	AAC 3-1 & APH 3-1
	110 Md	+	+	+	+	+	+	+	+	+	+	AAC 3-3 & APH 3-1

a) AAC 3-1 mediates intermediate tobramycin resistance (4-16 μg/ml) in *E. coli* aminoglycoside-modifying enzymes, whereas plasmids of 105 Md or 110 Md confer aminoglycoside resistance along with ability to elaborate enzymes which modify these agents.

We next looked for evidence of relatedness among the various plasmids found in these strains. Digests were prepared from 9.8 Md plasmids obtained from five different strains, using the restriction endonuclease Hinc II. These included three strains of S. marcescens and two of P. aeruginosa. Agarose gel electrophoresis revealed that the pattern of fragments produced was the same in all of these plasmids. When large plasmids (80 to 110 Md) were cleaved with Hind III, many common fragments were observed, with the largest number of fragments produced from the 110 Md plasmids. Similar results were obtained with Bam HI. DNA-DNA hybridization studies also revealed a high degree of DNA base sequence homology among these large plasmids (Table 4). When plasmids of the same molecular size were hybridized, essentially complete homology was found.

These findings led us to believe that a transposable DNA sequence containing the genes for aminoglycoside resistance, originally present on the 9.8 Md plasmid, had been translocated to a larger (probably 100 Md) plasmid, resulting in the formation of a composite plasmid containing all the resistance markers observed in the strains isolated from this outbreak. Experiments to test this hypothesis were performed and have been described previously (10). Briefly, a 105 Md plasmid from a strain of S. marcescens was put into E. coli containing the small plasmid pMB8. Hybrid plasmids were found in these cells, which appeared to represent concatameric forms of pMB8 into which was inserted a transposon corresponding to a molecular size of 6.2 Md, containing genes for resistance to aminoglycosides and β-lactam antibiotics. Heteroduplex analysis confirmed this interpretation. The transposition event was shown to be independent of the rec A system. Similar experiments were done with a 105 Md plasmid from a strain of K. pneumoniae, with identical results. Finally, heteroduplex analysis using pBM8::6.2 Md transposon and a 9.8 Md plasmid from S. marcescens revealed that these two plasmids shared a contiguous 6.2 Md region. The findings thus indicate that not only do the 105 Md plasmids contain a transposon carrying the genes for gentamicin resistance, but that this transposable sequence originated on the 9.8 Md plasmids found in strains isolated in the early stages of the outbreak.

It is interesting to view these events from the perspective of a selfish gentamicin resistance gene. As long as this gene was restricted to a small non-conjugative plasmid, its horizon was quite limited. An opportunity to enter a new bacterial species

Table 4. Hybridization between ^{3}H-labeled Plasmid DNA and Unlabeled DNA.

Source of Unlabeled DNA	Molecular Weight of Plasmid (Md)	Relative DNA Sequence Homology				
		^{3}H-SM80	^{3}H-SM100	^{3}H-SM105	^{3}H-KP105	^{3}H-EC110
S. marcescens	80	100	84	77	77	62
S. marcescens	100	100	100	80	82	78
S. marcescens	105	99	77	100	95	88
K. pneumoniae	105	100	92	96	100	91
E. cloacae	110	100	100	100	100	100

would arise only if a large, conjugative plasmid happened to enter the cell in which it was residing. This might result in its mobilization and transfer into a new bacterial host. However, when the DNA sequence of which it is a part underwent transposition to a larger conjugative plasmid, it acquired the ability to spread to additional bacterial species, with opportunity to inhabit a greater variety of patients and a wider diversity of niches within the hospital environment.

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R FACTORS PRESENT IN EPIDEMIC STRAINS OF *SHIGELLA* AND *SALMONELLA* SPECIES FOUND IN MEXICO

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The appearance of *Shigella flexneri* resistant to common antibiotics (tetracycline, chloramphenicol and streptomycin) was detected in Mexico as early as 1955 (1,2). Of particular importance was the clinical failure observed in the treatment with tetracycline of children with acute dysentery caused by strains of *S. flexneri* resistant in vitro to this antibiotic. After the discovery of R plasmids in Japan, we tested our culture collection of *Shigella*, *Salmonella* and *Escherichia coli*, including strains which were isolated between the years 1955 and 1969, and found that a large proportion of the resistances observed in these cultures was transmisible to *E. coli* K-12 (3).

The proportion of the number of multiple resistant strains increased during the following years, particularly in the *Shigella* group (Table 1). However, the process has not been steady showing ups and downs. Shortly after the introduction of new antibiotics such as ampicillin, aminoglycosides and cephalosporins, strains resistant to them also appeared.

SHIGELLA DYSENTERIAE TYPE 1 EPIDEMICS

In spite of the fact that the clinicians became concerned about the negative repercussions observed in the treatment of infections caused by multiple resistant strains of enteropathogenic bacteria, if was not until 1969-1970 when the true epidemic importance of this kind of resistance became evident due to the dysentery outbreak originated by a multiple resistant strain of *S. dysenteriae* type 1 which spread through Central America and Mexico (4,5). The organism showed a uniform and persistent resistance pattern representing a single clone.

Table 1. Shigella Strains Isolated in Mexico City from 1953 to 1976, Resistant to Various Antibiotics [a]

Year of isolation	No. of strains tested	Per cent resistant to 10 mcg/ml or more [b]			
		T	C	S	A
1953	31	0	0	74	
1955	26	19	8	50	
1956	41	34	7	39	
1957	33	39	3	39	
1959	71	38	21	63	
1960	44	43	20	73	0
1961	41	34	27	41	
1962-1964	53	36	32	53	3
1965	57	30	19	61	7
1967	55	35	35	58	
1971	42	48	36	100	14
1975	69	52	16	99	7
1976	25	76	36	96	56

[a] Source: Olarte, J. 1978 Bol.Med. Hosp.Infant. Mex., 35:295-309.
[b] T: tetracycline, C: chloramphenicol, S: streptomycin, A: ampicillin

As seen in Table 2, it was found that the R factors conferring resistance to chloramphenicol, streptomycin, tetracycline, and sulfonamides, present in all samples tested of the epidemic strain of Shiga 1 belonged to the incompatibility group O plasmid (6). This incompatibility group was described by Hedges, Datta et al. (7), and was originally found in two strains of enteropathogenic Escherichia coli, serotypes O86:B7 and O126:B16, isolated in our Hospital from babies with acute diarrhea in 1956; it was of interest since it confers resistance to ampicillin, yet its host strains were isolated before ampicillin was ever used. The corresponding beta-lactamases were studied at the laboratory of Stanley Falkow (8). Group O plasmids have been also found in S. flexneri and are apparently common in Central America and Mexico (6). The Shiga 1 epidemic, which extended from 1968 to 1970 attacking over 100,000 persons, almost entirely subsided during the following years.

In Mexico City on June 1972 (Table 2), an outbreak of dysentery due to S. dysenteriae type 1 took place in a hospital ward lodging children under treatment for tuberculosis. This time the causative organism was resistant to ampicillin, in addition to chloramphenicol, tetracycline, streptomycin, and sulfonamides. The ampicillin resistance could be transferred independently from the other drug resis-

Table 2. R Factors Found in _Shigella dysenteriae_ 1 and _Escherichia coli_

ORGANISM	STRAIN	ORIGIN	RESISTANCE[a]	TRANSFERRED	COMPATIBILITY GROUP
S. DYSENTERIAE 1	EPIDEMIC (SEVERAL HUNDRED ISOLATED)	CENTRAL AMERICA AND MEXICO 1968 - 1970	C S T Su	C S T Su	O
E. COLI 086:B7	SPORADIC CASE (E - 997)	MEXICO CITY, 1956	A S T Su	A S T Su	Iω AND O
E. COLI 0126:B16	SPORADIC CASE (E - 1235)	MEXICO CITY, 1956	A C S T Su	A C S T Su	Iω AND O
S. DYSENTERIAE 1	HOSPITAL OUTBREAK (5 STRAINS)	MEXICO CITY, 1972	A C S T Su	C S T Su[b] A[c]	O
S. DYSENTERIAE 1	COMMUNITY OUT-BREAK (762)	COSTA RICA, 1974	A C S T Su	C S T Su[b] A[c]	
S. DYSENTERIAE 1	COMMUNITY OUT-BREAK (6986)	BANGLA DESH, 1972 - 1973	A C S T Su	A C S T Su A[c]	

a C: CHLORAMPHENICOL, S: STREPTOMYCIN, T: TETRACYCLINE, Su: SULFONAMIDES, A: AMPICILLIN
b 80 - MDAL CONJUGATIVE R - PLASMID
c 5.5 - MDAL NONCONJUGATIVE PLASMID

SOURCE: COMPILED FROM REFS. 6, 9, 10

tances from five recovered strains of _S. dysenteriae_ type 1 to _E. coli_ K-12 (9). It was found that these strains were carrying two different plasmids, the O 80 megadaltons plasmid detected in the former epidemic strain, and a 5.5 megadaltons plasmid which contained the ampicillin transposon (TnA) sequences and was nonconjugative (10). It is interesting that strains of Shiga bacillus with the same resistance pattern coded by identical plasmids were also isolated in Costa Rica, and strains of Shiga with the same ampicillin 5.5 megadaltons plasmid were simultaneously found in Bangla Desh. The ubiquity of this small ampicillin plasmid is noteworthy.

The practical implications of such resistant strains have been previously emphasized (9,10).

TYPHOID FEVER EPIDEMIC

Though strains of _Salmonella typhi_ resistant to chloramphenicol were found in different parts of the world since the early sixties (11), it was until 1972 when a strain resistant to multiple antibiotics, including chloramphenicol, caused a large and rapid spreading epidemic of typhoid fever in Mexico City, Pachuca and other communities of Mexico. Over 10,000 cases were seen (12).

As shown in Table 3, all chloramphenicol resistant strains of _S. typhi_ isolated during the outbreak, which were studied in various laboratories in Mexico (12,13), the United States (14), and England (6,11), were uniformly resistant to chloramphenicol, streptomycin, tetracicline, and sulfonamides, and had the same phage sensitivity pattern, representing a single clone. The resistance was caused by a transmissible plasmid of the incompatibility group H. Considering that the outbreak of typhoid fever in Mexico followed the severe epidemic of bacillary dysentery in Central America, already mentioned, in which the causative organism was resistant to the same four drugs, the question was raised of whether the same plasmid was present in both epidemic pathogens (15). However, the studies accomplished indicate that each organism carried a phylogenetically distinct plasmid, H and O incompatibility group, respectively (6,14).

In the course of the typhoid outbreak seven strains of S. typhi were isolated in different localities, which were resistant to ampicillin in addition to chloramphenicol, streptomycin, tetracycline, and sulfonamides (Table 3). One of these (H-185), was also resistant to kanamycin. All seven strains were infected with the H plasmid conferring resistance to the four drugs to which the epidemic strain was resistant, but in addition were carrying several different R plasmids. In four strains (H-185, La Raza 2, LA, and JM) the ampicillin resistance plasmid was nonconjugative, but was mobilized by the

Table 3.R Factors Found in Salmonella typhi in Mexico - 1972

STRAIN	ORIGIN	RESISTANCE	TRANSFERRED	COMPATIBILITY GROUP	PHAGE TYPE[a]
EPIDEMIC (MORE THAN 10 000 CASES)	MEXICO-CENTRAL STATES AND WEST COST	C S T Su	C S T Su	H	D V S - 2[b]
SPORADIC CASES:					
H - 185	MEXICO CITY	A K C S T Su	K A K[c] C S T Su	I_ω AND O - H	D V S - 10
LA RAZA 1	MEXICO CITY	A C S T Su	A C S (K) C S T Su	A AND C H	D V S - 11
PUEBLA 12	PUEBLA	A C S T Su	A S C S T Su	I_α H	D V S - 2[b]
J R R	ACAPULCO	A C S T Su	A C S T Su	I_δ H	Vi NEGATIVE
LA RAZA 2	MEXICO CITY	A C S T Su	C S T Su A C S T Su[c]	H -	
L A	MEXICO CITY	A C S T Su	C S T Su A C S T Su[c]	H -	D V S - 10
J M	TULANCINGO	A C S T Su	C S T Su A C S T Su[c]	H -	D V S - 2[b]

a ACCORDING TO E. S. ANDERSON - LONDON (PERSONAL COMMUNICATION)
b EPIDEMIC STRAIN (Vi DEGRADED APPROACHING GROUP A - ACCORDING TO CDC)
c THE A RESISTANCE WAS NOT SELF-TRANSMISSIBLE, BUT MOBILIZED BY ACCOMPANYING PLASMID

SOURCE: COMPILED FROM REFS. 6, 12

accompanying transmissible plasmid. The other three strains had conjugative ampicillin resistance plasmids, all different from one another in compatibility (A and C in strain La Raza 1, $I\alpha$ in strain Puebla 12, and $I\delta$ in strain JRR). The kanamycin resistance in strain H-125 was plasmid determined and transmissible, coded by incompatibility group $I\omega$ and O (6).

The epidemic strain of S. typhi was of a Vi degraded phage type. Two ampicillin resistant strains (Puebla 12 and JM) were of the same phage type of the epidemic strain, one (JRR) was Vi negative, another (La Raza 2) was not phage typed. The remaining three ampicillin resistant strains (H-185, La Raza 1, and LA) showed different phage sensitivities. According to E.D. Anderson the epidemic strain and these three strains correspond to new phage types to be described (personal communication).

These findings show that the carrying of the H plasmid of the epidemic strain did not prevent the acquisition of other R factors by the same strain. This was also true in the case of the O plasmid of the epidemic strain of Shiga bacillus. There is the possibility that the acquisition of some R factors could influence in some way the phage sensitivity of S. typhi; however, Alfaro, Martuscelli and Mendoza (16), have obtained some experimental results contrary to this hypothesis.

The typhoid outbreak was apparently a self-limited event. Typhoid fever has been endemic in Mexico City for a long time, but its ocurrence in epidemic form has been rather unusual for the last 30 years. The explosive outbreak began in early 1972, reached a peak by the middle of the year and then declined to almost disappear in 1973. It is not clear how the epidemic started, but it is even more difficult to understand why it did not spread to the whole country and Central and South America, considering the large number of residual carriers that would be expected from such a large number of cases, combined with epidemiological conditions over this large area propicious for its propagation.

As seen in Table 4, the proportion of S. typhi chloramphenicol resistant strains isolated in Mexico City was very high (94.7 %) in 1972, decreased through 1973, and then, with the exception of 1975 (23.5 %), it has declined dramatically. It is not known when the resistant strain first make its appearance in Mexico. We detected it at the time the outbreak was obvious, but the search for resistant strains during the years immediately before the outbreak was neglected, since we become complacent, believing that the susceptibility of the organism to chloramphenicol was very stable.

The decreasing incidence of the resistant strain could be due to either genetic, ecological, or epidemiological causes, or a combination of them. Unfortunately, we can not place too much trust

Table 4. Decreased Incidence of *S. typhi* Resistant to Chloramphenicol, Mexico City - 1972 to 1980

Year		Number of strains	Per cent resistant
1972 [a]		226	94.7
1973	Jan-Jun	604	60.7
	Jul-Dec	179	41.3
1974 [b]		109	3.5
1975 [b]		230	23.5
1976 [b]		142	4.9
1977 [b]		89	6.2
1978 [b]		60	3.6
1979 [b]		95	0.0
1980 [b]		108	3.7

[a] From Ref. 13. [b] Data provided by J. Martuscelli, UNAM, Mexico.

in the epidemiological data collected during the outbreak. The behavior of resistant strains in nature is not well understood.

SALMONELLOSIS OTHER THAN TYPHOID FEVER

Regarding the infections caused by species of *Salmonella* called of animal origin, strains resistant to multiple drugs have been detected in Mexico for many years (3). From time to time we have observed outbreaks of limited importance which mainly affect young children. No doubt that these outbreaks have spread through the community in general; however, they have been only studied in pediatric hospitals. The extension that these organisms have affected different animal species in Mexico is not well known.

As shown in Table 5, a strain of *S. poona* resistant to nine drugs was isolated from 154 children with acute gastroenteritis attending the Hospital Infantil de Mexico, from May to July, 1976. Twenty-three had septicemia, and nine septicemia and meningitis; seven of the latter died (17). From June, 1979 to May, 1980, a strain of *S. newport* resistant to eight drugs was isolated in the same Hospital from 51 children with acute gastroenteritis. Five had septicemia and two septicemia and meningitis. In both outbreaks some of the children were already infected with the salmonella at the time of admission, whereas others acquired the salmonella in the hospital

Table 5. Salmonellosis in Children Caused by Multiple Resistant Strains of S. poona and S. newport, Hospital Infantil de Mexico

ORGANISM	ILLNESS		RESISTANCE
S. POONA (MAY - JULY, 1976) (From Ref.17)	GASTROENTERITIS	122 CASES	AMPICILLIN, CHLORAMPHENICOL, TETRACYCLINES, CEPHALOTHIN, KANAMYCIN, STREPTOMYCIN, GENTAMICIN, CARBENICILLIN, SULFANOMIDES
	GASTROENTERITIS COMPLICATED WITH SEPTICEMIA	23 CASES	
	GASTROENTERITIS COMPLICATED WITH SEPTICEMIA AND MENINGITIS	9 CASES	
S. NEWPORT (JUNE 1959 - MAY 1980)	GASTROENTERITIS	44 CASES	AMPICILLIN, CHLORAMPHENICOL, CEPHALOTHIN, STREPTOMYCIN, KANAMYCIN, GENTAMICIN, SULFONAMIDES
	GASTROENTERITIS COMPLICATED WITH SEPTICEMIA	5 CASES	
	GASTROENTERITIS COMPLICATED WITH SEPTICEMIA AND MENINGITIS	2 CASES	

as a result of cross infection. The resistance present in both strains of Salmonella is transmissible; the R factors involved are under study.

Similar outbreaks of gastroenteritis caused by multiresistant Salmonella serotypes of animal origin have been reported in various regions. Of particular importance have been certain strains of S. wien which have spread through various countries in Southern Europe and North Africa (18), and S. typhimurium in South America (19), the Middle East and Great Britain (18,20).

The possibility that epidemic multiresistant strains of enteropathogenic bacteria possess, in addition to the R factors, an enhanced virulence or a factor which facilitates its transmissibility, has been the subjet of much speculation and remains to be resolved.

The data presented in this review are a good example of what likely could occur in any country that, like Mexico, meets the conditions for the propagation of enteric infections, together with its high incidence and the indiscriminate use of antimicrobial drugs.

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TRIMETHOPRIM-RESISTANT BACTERIA IN HOSPITAL AND IN THE COMMUNITY: SPREAD OF PLASMIDS AND TRANSPOSONS

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INTRODUCTION

Trimethoprim is a very effective synthetic antibacterial drug that was introduced for use in human and veterinary medicine about 10 years ago in Europe (7 years in the US). Until recently it has been used always in conjunction with a sulfonamide. Trimethoprim and sulfonamides act synergistically, at different points upon the folic acid cycle of bacteria and using both drugs together should prevent the emergence of resistant mutants[1]. Trimethoprim-sulfonamide preparations are effective against a wide range of bacteria and have been extensively used in treating urinary, respiratory and, to a lesser extent, gastrointestinal infections in hospitals and in the community.

Combining trimethoprim with sulfonamide could not entirely prevent the emergence of trimethoprim-resistance since resistance to sulfonamides, often plasmid-determined[2] was already common. Using trimethoprim-sulfonamide to treat infection caused by bacteria highly resistant to sulfonamide is equivalent to treating the infection with trimethoprim alone[3]. Laboratory-selected trimethoprim-resistant mutants are frequently thymine-requiring. Some clinical isolates are of this kind while others show quantitative or qualitative alterations in dihydrofolate reductase activity[4].

Although most published reports on trimethoprim resistance refer to members of the Enterobacteriaceae, resistance in *Staphylococcus aureus*[5], *S. albus*[6], *Streptococcus faecalis*[5] and in occasional strains of *Haemophilus influenzae*[7] has been noted.

Plasmids determining trimethoprim resistance were first identified in 1972[8] and plasmid-mediated resistance permits normal bacterial growth at high concentrations of trimethoprim. The plasmids determine production of trimethoprim-insensitive dihydrofolate reductase (DHFR) that the bacterium uses when its native enzyme is inhibited by the drug[9,10]. Two types of plasmid-determined DHFR have been identified[11] and sometimes plasmid DHFR is encoded by transposable DNA sequences[12,13]. We have collected bacteria from various sources and characterised trimethoprim-resistance plasmids and transposons, the aim being to gain understanding of the routes of dissemination of the resistance genes.

MATERIALS AND METHODS

Sources of bacterial strains

Three sets of urinary isolates of *Escherichia coli* were tested: 1) 93 from schoolgirls taking part in a long-term study of bacteriuria in schoolgirls, 1979-80[14]; 2) 187 from specimens sent by general practitioners to the diagnostic laboratory, Department of Bacteriology, Royal Postgraduate Medical School, January-August 1980; and 3) 269 from inpatients in Hammersmith Hospital, London, July and August 1980.

The strains were identified by conventional biochemical methods and tested for sensitivity using a disc diffusion method[15].

Plasmid transfer and characterization

These were as described previously using *E. coli* K12 strain J62-2 as primary recipient and the *recA* strains PB1150 and HH26[16,17].

Tests for transposition of trimethoprim resistance

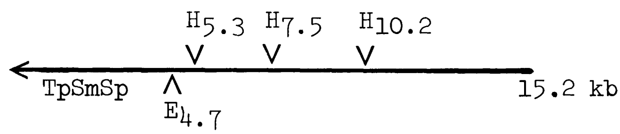
1) *Genetic analysis*. Transposition was indicated if trimethoprim resistance was retained in *E. coli* K12 after a plasmid that had carried it was eliminated during incompatibility tests or in "curing" experiments. If the new locus was in the chromosome, this was indicated by non-transmissibility of the resistance and lack of plasmid bands after gel electrophoresis of lysates. A second transposition event could then be shown by introducing a conjugative plasmid (without trimethoprim resistance) and retesting for transfer of trimethoprim resistance. Transpostion to another plasmid, at the first or second transposition event, was shown by linkage in transfer experiments and by DNA analysis. Serial transfer from plasmid to chromosome, chromosome to plasmid or plasmid to plasmid was looked for in Rec+ or *recA* hosts.

2) *Restriction enzyme analysis*. Having identified a transposon, its mass was determined (from the increase in molecular weight upon

its acquisition by another plasmid) and its susceptibility to cutting by restriction enzymes (from the altered restriction pattern yielded by a plasmid upon its acquisition). Enzymes used were *EcoR1* and *HindIII*, from BRI Inc. When Tn7 is digested with *HindIII*, two characteristic internal fragments (Fig. 1) can be recognised by their migration in gel electrophoresis.

For the purposes of this paper, we took transposition of trimethoprim-streptomycin/spectinomycin resistance, mass of DNA transposed and identification of the characteristic *HindIII* fragments, as evidence for the presence of Tn7 or a closely similar transposon, without testing each example for transpostion in a *recA* host. Methods for DNA extraction, digestion with enzymes and gel electrophoresis were as described by Datta et al[18].

Figure 1 Map of Tn7



Cut sites H = *HindIII* E = *EcoR1*

RESULTS

The incidence of trimethoprim-resistance in enterobacteria

1) In the community. Strains of *E. coli* isolated from urinary tract infections outside hospital are rarely trimethoprim-resistant. Table 1 shows our latest results of tests for sensitivity to ampicillin, sulfonamide and trimethoprim on *E. coli* strains from two groups of non-hospitalized people. Table 2 shows the trend over the last 2 decades for the sensitivity of comparable bacteria to ampicillin and trimethoprim.

Table 1. Resistance to trimethoprim (Tp), sulfonamide (Su), and ampicillin (Ap) in urinary isolates of *E. coli*

	Numbers (%) from		
Total resistant to	Schoolgirls (Cardiff) 1979-80	General practice (Hammersmith) Jan-Aug 1980	Inpatients (Hammersmith) July-Aug 1980
Tp	0	3 (1.6)	27 (10.0)
Su	12 (12.9)	42 (22.5)	81 (30.1)
Ap	16 (17.2)	47 (25.1)	73 (27.1)
Sensitive to Tp Su Ap	75 (80.6)	122 (65.2)	157 (58.4)
Total	93 (100)	187 (100)	269 (100)
Combinations of resistance			
Tp	0	1 (0.5)	2 (0.7)
Su	2 (2.2)	17 (9.1)	31 (11.5)
Ap	6 (6.5)	22 (11.8)	25 (9.3)
SuTp	0	0	6 (2.2)
ApTpSu	0	2 (1.1)	15 (5.6)
ApTp	0	0	5 (1.5)
ApSu	10 (10.8)	23 (12.3)	29 (10.8)

All Tp-resistant strains, but not all Tp-sensitive ones, were tested for sensitivity to a wide range of antibacterial drugs, not shown here.

2) In hospital. Trimethoprim resistance has been increasing in frequency in hospital infections. Table 1 shows our results for *E. coli* from urinary infections. When all enterobacteria from all infected sites are included, the incidence of trimethoprim resistance in the same hospital was 20%.

Table 2. E. coli from urinary-tract infections in the community

Year	Percentage resistant to: ampicillin	trimethoprim
1960	0	0
1970	5	1
1980	25	1

Data, from refs. 19,20 and 21 and this paper, summarize findings in several groups of patients in England and Wales.

Trimethoprim-resistance plasmids

The first trimethoprim resistance plasmids were very uniform, though found in a variety of bacterial species. They were of incompatibility group W (IncW), had molecular masses of about 25 Md and determined resistance to sulfonamides(Su) and trimethoprim (Tp) (example, R388). In a collection of trimethoprim-resistant bacteria made in 1972 these were the only trimethoprim resistance plasmids found. They were found only in bacteria isolated in London hospitals: no trimethoprim resistance plasmids were detected in isolates from other parts of England and Wales[22]. In several later studies, plasmids of many different incompatibility groups carried trimethoprim resistance (Table 3) and the resistance patterns were different from the original TpSu. Bacteria carrying these plasmids were nearly always sulfonamide-resistant but sulfonamide resistance genes were not necessarily determined by the trimethoprim resistance plasmids. Some plasmids, e.g. R751, carried no resistance except to trimethoprim[24], others carried multiresistance e.g. pTH1[18].

Table 3. Trimethoprim resistance plasmids: range of Incompatibility (Inc) Groups

Year	Inc Groups found
1972	W
1980	W B C D I FII N P X

Data in refs. 16, 18, 22 and 23 and unpublished results.

Trimethoprim-resistance plasmids have usually been identified in bacteria from environments where the drug is most used i.e. from hospital patients and farm animals. Of the 27 trimethoprim E. coli strains from hospital infections, listed in Table 1, 24 carried trimethoprim resistance plasmids. Although resistance to trimethoprim is unusual in bacteria causing human infections outside hospital, when it does occur it is often plasmid-determined. From urinary tract infections in the community, we had only 3 trimethoprim-resistant E. coli from a total of 280 (Table 1). In 2 of the 3, the resistance was plasmid-borne.

Trimethoprim-resistance transposons

Two transposons carrying trimethoprim-resistance genes are known, Tn7[12] and Tn402[13].

Tn7 determines a low level of resistance to streptomycin/spectinomycin and a high level of trimethoprim resistance. It is approximately 15 kilobases (kb) and a restriction map of it is shown in Fig. 1. It transposes very readily e.g. if a plasmid carrying Tn7 is transferred to E. coli K12 and then eliminated, a high proportion (between 1% and 50%) of the "cured" clones still carry the transposon, now integrated into the chromosome.

We have identified Tn7 in plasmids and chromosomes of many naturally-occurring bacteria, isolated from man and animals. The first example[12] came from E. coli from a calf that had been fed large doses of a trimethoprim-sulfonamide combination for experimental purposes. Later examples from farm animals were in E. coli[17] and Salmonella[25]. Trimethoprim-sulfonamide combinations have been extensively used in animals in England for both therapy and prophylactic purposes. Smith[26] has shown that an increasing proportion of faecal E. coli from healthy market pigs carry trimethoprim resistance plasmids. We have examined 15 such plasmids (received from H. W. Smith) and have positively identified Tn7 in 6 of them.

In hospital infections, Tn7 has been found in plasmids of different genera[16,18] and also in the chromosomes of infecting bacteria[18]. Of the 27 trimethoprim E. coli strains from hospital infections (Table 1) trimethoprim resistance plasmids were identified in 24 strains and 18 of these carried Tn7-like sequences.

In the two cases of plasmid-determined resistance in community infections, Tn7 was identified in both. There was no epidemiological connexion between the patients and the Tn7-bearing plasmids were different in their resistance patterns and molecular masses.

Tn7 determines DHFR type I of Pattishall et al[11]. Tn402, identified in plasmid R751[13,24], determines DHFR type II[27]. Tn402 transposes at a frequency too low to be detected in our studies, but DHFR II is carried by naturally occurring plasmids of at least four incompatibility groups[27], indirect evidence for the spread of Tn402 in nature.

DISCUSSION

Despite widespread use of trimethoprim-sulfonamide combinations, trimethoprim-resistance is still uncommon (frequency 1%) in strains of E. coli isolated from urinary tract infections in the community outside hospitals. This is our finding in the new isolates described here; it confirms the experience of others[21,28]. In the same strains, the incidence of resistance to sulfonamides and to ampicillin is higher (Table 1). The frequency of acquired resistance to any antibacterial drug evidently depends upon various factors among which are: 1) the ability of the bacteria to become resistant by mutation, 2) access to a pool of resistance genes that may be transferred from one bacterium to another and 3) the degree of selection for resistance in the environment.

In the case of trimethoprim, acquisition of resistance, by mutation can be demonstrated in the laboratory[1] but during short courses of therapy in man, infecting or commensal enterobacteria do not commonly mutate to resistance. Lacey et al[6] studied the effects of 5-day courses of trimethoprim-sulfonamide or of trimethoprim alone upon the bacteria carried by 279 patients and found no trimethoprim-resistant Enterobacteriaceae. Resistant mutants of Streptococcus faecalis, however, readily appear on exposure to trimethoprim[5].

During the use of long-term co-trimoxazole for the control of intractable urinary infections, some strains of E. coli were found that had become trimethoprim-resistant by mutation[29].

Our research is concerned with the second factor determining the incidence of resistance, the availability of a pool of plasmid genes that may be acquired by contact with other, already-resistant bacteria. The low incidence of trimethoprim-resistant E. coli in urinary infections in the community indicates that this pool, in the intestinal bacteria of people outside hospitals, is still small. Had it been greater, resistant Enterobacteriaceae might have been isolated from the patients studied by Lacey et al[6] after short-term courses of therapy. From patients on long-term prophylaxis studied by Pearson et al[29], strains of Enterobacteriaceae carrying trimethoprim resistance plasmids were isolated in a few cases. Here the third factor was operative, there being strong selection for resistance when trimethoprim was taken for months rather than days.

We have found transposons resembling, or identical with, Tn7 in plasmids of many incompatibility groups in bacteria of various genera in a variety of environments in England i.e. in hospital infections, in E. coli from urinary infections in the community, in salmonella from man and animals and in the normal intestinal E. coli of market pigs. Tn7 has been found in the chromosomes of naturally-occurring bacteria from which loci it transposes very readily, in the laboratory, to plasmids that did not previously carry trimethoprim-resistance. Tn402, though it does not transpose so readily in laboratory experiments, is found on a variety of naturally-occurring plasmids. These transposons possess a potential for world-wide dissemination. Such a thing has already happened with Tn1 and related transposons that determine resistance to penicillins, including ampicillin and carbenicillin, mediated by the TEM β-lactamase[30]. Tn1-related genes are carried by plasmids of many types and are now common in bacteria, isolated in all continents, and of many genera including all the Enterobacteriaceae, Pseudomonas aeruginosa, Haemophilus influenzae and Neisseria gonorrhoeae. The spread of Tn1-determined β-lactamase genes is largely responsible for the high incidence of ampicillin resistance in E. coli and other Enterobacteriaceae both in and out of hospitals (Tables 1 and 2). The very successful spread of this DNA element can be related to its facility in transposition and by heavy use of ampicillin in the treatment of many kinds of infections, trivial or severe. Trimethoprim resembles ampicillin in its wide spectrum of activity, low toxicity and convenient oral dosage. Until recently it has been used in combination with a sulfonamide but it is now available for use alone, in which form it is more acceptable to patients, having fewer unpleasant side-effects. Since neither Tn7 nor Tn402 determines sulfonamide resistance their dissemination may be favoured by use of trimethoprim alone.

The frequency of resistance acquired by gene transfer depends upon the extent of the pool of transmissible or transposable resistance genes and the selection of resistant bacteria depends upon use of the drug. With the use of trimethoprim alone, we should look for changes in these variables.

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ECOLOGICAL FACTORS THAT AFFECT THE SURVIVAL, ESTABLISHMENT, GROWTH AND GENETIC RECOMBINATION OF MICROBES IN NATURAL HABITATS

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Despite the remarkable advances in the isolation, analysis, reconstruction,and methods of introducing new genes into organisms, the ultimate fate of natural and manipulated genetic material is dependent on the survival, establishment, and growth of the organismal vectors (usually microbes) that house the genetic material in the natural habitats into which the vectors are introduced. Survival, establishment, and growth are, in turn, dependent on the genetic constitution of the microbes and on the physical (temperature, pressure, electromagnetic radiation, surfaces, spatial relations), chemical (carbonaceous substrates, ironganic nutrients, growth factors, ionic composition, available water, pH, oxidation-reduction potential, gaseous composition, toxicants), and biological (characteristics of and positive and negative interactions between microbes) factors of the various habitats (Fig. 1). Limitations of space preclude a detailed discussion of and an extensive bibliography to these ecological factors and to the genetical aspects of this report. Consequently, reference is made to reviews wherever possible.

The relative influence of these individual ecological factors differs with the recipient habitat and is usually greater on introduced than on indigenous microbes. Furthermore, none of these factors operates individually but in concert with numerous other factors, and although one or a few factors may be dominant in a specific habitat, their influences may have indirect, but cascading, effects on other characteristics. Consequently, an alteration in one environmental factor may result in simultaneous or subsequent changes in other factors, and ultimately, the habitat and the ability of both introduced microbes and of portions

HABITAT \ FACTOR	SUBSTRATES (carbon)	INORGANIC NUTRIENTS	GROWTH FACTORS	IONIC COMPOSITION	WATER	TEMPERATURE	PRESSURE	RADIATION (e-m spectrum)	GASEOUS COMPOSITION	pH	Eh (redox potential)	SURFACES (particulates)	SPATIAL RELATIONS	MAGNETIC FIELDS	TOXICANTS	CHARACTERISTICS OF ORGANISMS	POSITIVE INTERACTIONS	NEGATIVE INTERACTIONS	OTHERS
SOILS	X				X							X							
PLANTS		X						X											
ATMOSPHERE					X			X								X			
FRESH WATERS		X												X					
SALT WATERS							X	X											
ESTUARIES & SEASHORES				X						X									
WASTE WATERS (sewage)											X				X				
RUMEN & CECUM									X										
GASTROINTESTINAL TRACT										X		X							
GENITOURINARY TRACT										X									
RESPIRATORY TRACT																X			
ORAL CAVITY												X							
SKIN				X	X													X	
INSECTS & OTHER INVERTEBRATES									X										
FOODS							X			X									
PETROLEUM																			
MATÉRIELS					X														
INDUSTRIAL FERMENTATIONS									X	X						X			
LABORATORY					X											X			
OTHERS																			

Fig. 1. Physical, chemical, and biological factors that affect the ecology of microorganisms in various habitats. For illustrative purposes, some of the dominant factors in some habitats have been indicated.

of the indigenous microbiota to survive are changed. Inasmuch as the possible permutations of interactions between these environmental factors are essentially unlimited, the relative success of microbes containing new genetic information to survive, establish and grow in these natural habitats cannot be easily predicted.

The heightened activity in recombinant DNA technology increases the probability that genetically engineered microbes will eventually be introduced - either accidentally or deliberately - into natural habitats, such as soils, waters, and sediments, which are the major final repositories for all microbes. Inasmuch as such microbes will contain new DNA sequences - some inadvertently inserted along with desired and, presumably, harmless sequences - there are potential dangers to the health of plants and animals, including humans, and to other aspects in the biosphere, especially if such microbes are able to grow better in the recipient environment than the indigenous microbiota or the experimental parental strains (1-5)and as even minor changes in a single biosynthetic capability can apparently result in significantly increased growth rates (6) and, hence, presumably in greater survival and colonization by introduced microbes. For example, will bacteria with an acquired ability to fix N_2, coupled with existing capabilities for rapid growth, efficient metabolism, and survival value in natural habitats, reduce the N_2 content of the atmosphere, enhance pollution of ground-waters with NO_3^-, and deplete the ozone layer due to formation of NO_x from NO_3^- ? Will organisms engineered to destroy oil-spills remain restricted to these spills or will they spread and eventually also degrade petroleum products in the refinery and the gas station, especially if they also acquired other genes that will enhance their ability to survive in these habitats?

The survival value of manipulated microbes in natural habitats is presumably low and there should, therefore, be little danger of their establishment and proliferation in natural habitats, and some "constructed" host organisms are so auxotrophic and debilitated that they should "self-destruct" outside of enriched laboratory media (7). However, there have been few studies on the survival of such microbes in natural habitats and on the ability of debilitated recipients to acquire the genes from the natural habitat into which they may be deposited that will reduce their degree of auxotrophy and enhance their survival. There have apparently been no studies on the influence of the physicochemical characteristics of the recipient environment on the survival of and the acquisition of genes by these microbes. These characteristics have major roles in determining the survival, establishment, and growth of both indigenous and introduced microorganisms in natural habitats (8).

Most studies on genetic recombination in bacteria have been conducted in vitro, and there are few data showing that gene transfer occurs in situ. A few in vivo studies have been conducted with zxenic animals or with animals in which the normal biota, usually of the intestinal tract, had been greatly reduced or eliminated by antibiotic pretreatment, and these have foçused on conjugation, primarily R-factor transfer, as the mechanism of gene transfer (9-32).

A few studies have investigated the transfer of R-factors in non-animal habitats. Smith (33) showed that 373 strains from 435 strains of Escherichia coli that were isolated from 90 river water samples in Great Britain and were resistant to chloramphenicol could transfer this resistance to F^- strains of E. coli K12, and 179 of these strains were resistant to five or more antibiotics. Furthermore, 208 of these strains could also transfer the resistance to chloramphenicol to Salmonella typhimurium. Antibiotic-resistant bacteria containing conjugative R-factor plasmids have also been isolated in sewage-impacted waters in the United States (e.g., in the Hudson River, the new York Bight (34,35(, and in Chesapeake Bay (36). Many of these strains contained plasmids that conferred resistance not only to antibiotics but also to heavy metals (37) and to other antibacterial agents, such as the algal product, chlorellin (38).

Transfer of R-factor genes by transduction has been demonstrated in Staphylococcus aureus (39-42) and in Pseudomonas aeruginosa (43). Certain soil-borne bacteria (e.g., species of Pseudomonas, Arthrobacter, and Acinetobacter) and some non-soil bacteria (e.g., species of Klebsiella and Serratia) appear to be evolving genetic competence, via plasmid transfer, for the utilization of a spectrum of aromatic hydrocarbons that were assumed to be not only recalcitrant but also toxic to these organisms (44-46). Furthermore, conjugation in vitro in soil-borne bacteria, such as pseudomonads, has been demonstrated (47-49).

Although there is empirical evidence (i.e., increase in nosocomial infections by drug-resistant bacteria) to indicate that the transfer of genes conferring resistance to antibiotics and heavy metals occurs in natural habitats, there is little experimental evidence to verify this, as most of these studies have been restricted to either isolating such resistant bacteria from natural habitats or demonstrating the transfer and expression of such genetic material under controlled laboratory conditions. Essentially no studies have attempted to bridge these experimental extremes, probably because of the lack of both techniques to study genetic recombination in natural habitats and interest on the part of scientists trained in microbial genetics.

Both auxotrophic and prototrophic strains of *E. coli* K12 can survive, multiply, and conjugate in sterile soils (50). The presence of clay minerals, especially montmorillonite, increased the frequency of recombination, probably because clays enhance bacterial growth. This enhancement is due, in great part, to the ability of clays to buffer soils against changes in pH, which, in turn, is a function of the cation exchange capacity of the clays. Many of the mechanisms whereby clay minerals affect the survival, establishment, growth, and metabolic activities of microbes in natural habitats have been defined (8,51). Preliminary studies on conjugation in non-sterile soils have indicated that the frequency of recombination is significantly less than in sterile soils (Krasovsky and Stotzky, unpublished).

The decrease in frequency of recombination in non-sterile soils supports results obtained with the transfer of drug-resistance plasmids in an animal system (21,22). The frequency of transfer of a multiple drug-resistance plasmid from *Salmonella typhosa* to *E. coli* in the bladder of healthy rabbits was as high (and, in some instances, higher) as in *in vitro* systems containing either sterile urine or synthetic mating media. However, in the presence of other bacteria (exogens; i.e., *Proteus mirabilis* and non-conjugative *E. coli*), the frequency of transfer decreased significantly (Fig. 2). This decrease was not the result of a physical (i.e., steric) interference of the exogens in the conjugation process, as polystyrene latex particles of the same size and at the same concentration as the exogens had essentially no effect on the frequency of plasmid transfer, suggesting that the exogens caused a chemical interference with conjugation. Whether such interference was responsible for the lower frequencies of conjugation in non-sterile than in sterile soils is not known, but as a variety of species may be in close proximity in various natural microbial habitats (52-54), such interference could be possible.

The studies of conjugation in sterile soil also indicated that bacteria auxotrophic for different nutrients could co-exist, both in soil and on replica-plated agar media, by cross-feeding (syntrophism), rather than by having undergone genetic recombination (50). This observation emphasizes the need to investigate carefully both claims for apparent genetic recombination in natural habitats and the possibility that auxotrophs can survive in natural habitats, despite their apparent fragility and debilitation, if other microbes in the same habitat serve as commensals to provide the nutrients that the auxotrophs are incapable of synthesizing. Sagik and Sorber (55) indicated that such auxotrophs (e.g., the EK2 host, DP50supF) can survive in a nutrient-rich environment (i.e., a model sewage treatment plant). This survival appeared to be associated with the solid portion of the waste stream, again indicating that particulates and the resultant

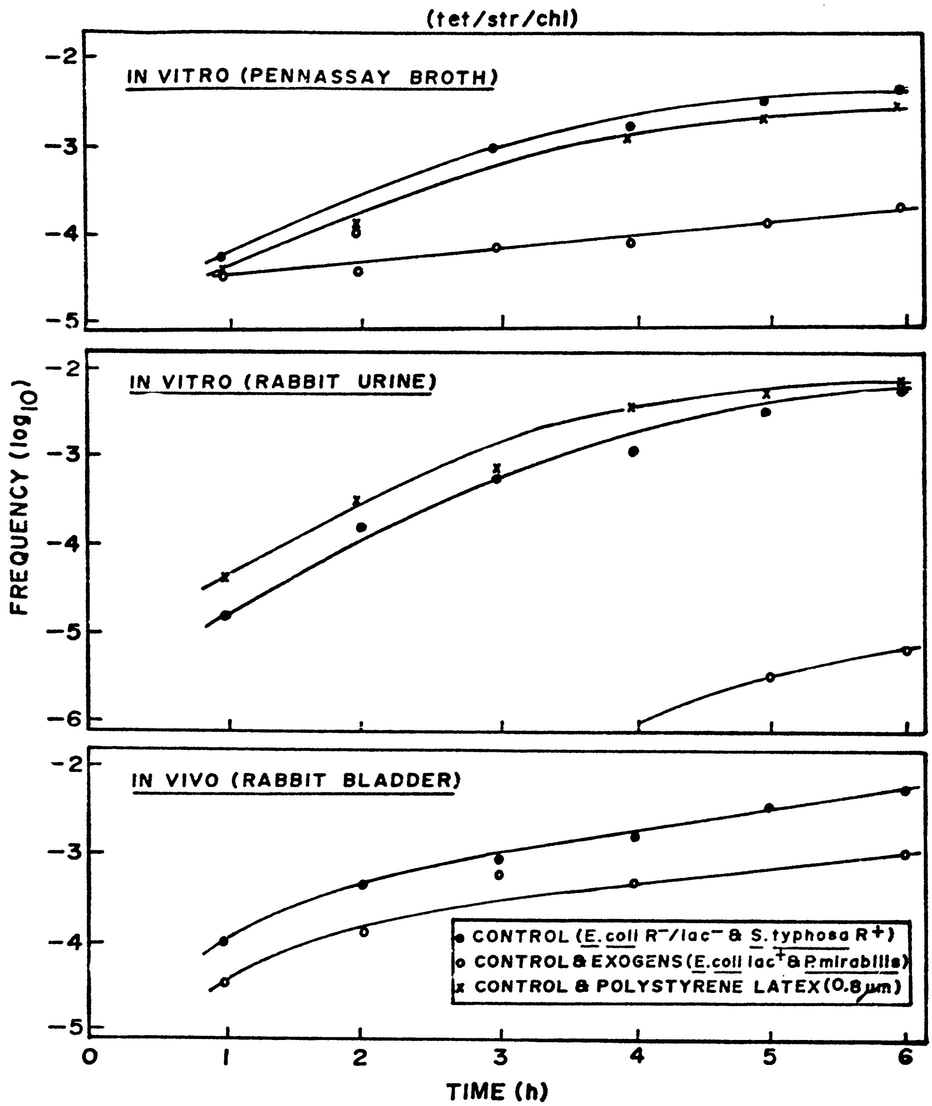

Fig. 2. Frequency of transfer of a plasmid conferring resistance to tetracycline, streptomycin, and chloramphemicol from Escherichia coli to Salmonella typhosa in vivo and in vitro, in the presence and absence of Proteus mirabilis and a non-conjugative E. coli or polystyrene latex particles (ref. 21,22).

increases in surface area enhance the growth and survival of bacteria (8,51). Furthermore, cometabolism or "shared" detoxification" of inhibitors can contribute to the survival of toxin-sensitive microbes in the absence of any genetic recombination (54,56,57,58).

There is little documentation that transformation occurs in natural microbial habitats (4,5,59,60). Although this lack of information is a reflection primarily of the paucity of studies on transformation in situ, it may also reflect an unsubstantiated concept; namely, that "naked" DNA is very susceptible to enzymic degradation in natural habitats. However, Greaves and Wilson (61,62) have indicated that nucleic acids adsorb to clay minerals in soil, especially to montmorillonite, and that this sorption provides protection to the nucleic acids against enzymic degradation. Similarly, viruses, proteins, peptides, and amino acids adsorbed to clays are protected to various degrees against microbial degradation (8,51). Consequently, both naked DNA (involved in transduction) may persist in natural habitats in the absence of an appropriate host.

This apparent protection against degradation of soluble organics and of viruses as a result of adsorption to clay minerals is an important consideration in any potential genetic exchange in habitats containing clays and, probably, other surface-active particulates. It might be expected that transforming DNA and transducing viruses would not long survive in natural habitats in the absence of hosts and be rapidly degraded by the indigenous microbiota, as nucleic acids and viruses should be ideal substrates for non-host microbes (i.e., they contain C, N, and P and, in the case of viruses, also S). However, evidence is accumulating that DNA and viruses persist in natural habitats as a result of being adsorbed to clay minerals, which protects against both physicochemical and biological inactivation. Furthermore, this adsorption does not reduce the catalytic activity of enzymes (in fact, it may increase it (8); or the ability of viruses to infect their hosts (51,63-65). Consequently, if viruses and transforming DNA (no studies have apparently been conducted on the transforming ability of adsorbed DNA) persist in natural habitats, it is possible that their genetic information could eventually be transmitted to any suitable host that may be introduced, inadvertently or deliberately, into these habitats.

The survival and subsequent establishment of microbes that are not inhabitants of a particular habitat have been sporadically studied; e.g., the survival of enteric bacteria (including E. coli, Salmonella sp., Shigella sp) that could be introduced into soils and waters by wastewater or sludge applications (66-68) and of Listeria monocytogenes (69) and Clostridium botulinum (70).

These studies have generally indicated that soil and natural waters are not particularly hospitable habitats for these microbes, although there are reports of some exceptions, especially when these habitats have been carefully examined (71). Although many of the bacterial species currently used in recombinant DNA technology are not normal inhabitants of soil and water and, therefore, do not survive long in these habitats, the spectrum of organisms that are increasingly being used include species that are indigenous to these habitats. No data are apparently available on the ability of introduced microbes to transfer genetic information to indigenous microbes in various natural habitats and vice versa, although this is, obviously, a very important consideration in the survival of genetically manipulated microbes and of their genetic informati-n in such habitats.

When survival, establishment, and subsequent growth of introduced microbes have occurred, some physiochemical factor has usually been implicated. For example, the establishment of Fusarium oxysporum f. cubense, the causal agent of Fusarium wilt of banana, in soils (more than 140) throughout the banana growing areas of the world was correlated with the absence in these soils of a specific clay jineral that had the characteristics of montmorillonite. Similarly, Histoplasma capsulatum, a fungus pathogenic to humans and which has a discrete geographic distribution, was isolated essentially only from soils (131 from 134 soils) that did not contain this clay mineral. Preliminary studies with some other fungal pathogens of humans (e.g., Cryptococcus neoformans, Blastomyces dermatitidis) showed similar patterns, although the geographic distribution of Coccidioides immitis appeared not to be correlated with the clay mineralogy but rather with the salinity of the soils (8). Introduction of these fungi into the habitat was not the limiting factor for their subsequent growth and survival (e.g., healthy banana plantations were routinely irrigated with surplus waters from diseased plantations; birds and bats, the presumed spreading vectors of H. capsulatum, defecate everywhere), but rather, the limiting factor was their establishment as members of the soil microbiota.

Studies on various levels of experimental complexity have shown that clay minerals affect the establishment and growth of fungi in soil primarily by influencing the activities of indigenous bacteria, which, in turn, exert a biological control on the fungi (8). Furthermore, competition between bacteria and fungi in soil and other habitats is mediated by pH, osmotic pressure, nutrient levels, oxygen content, and toxicants, and the types of clay minerals present modulate the effects of these physicochemical factors (8,37,51,72,73).

Consequently, clay minerals, as only one example of an ecological factor, have a major role in the establishment and growth of microbes, in the survival of viruses, in the persistence of readily degradable organics, and in the genetic recombination in bacteria in habitats that contain clays. The pH, which is an important factor in genetic recombination in vitro (74-77), also appears to affect genetic recombination in soil (Fig. 3), and the effect of pH, in turn, is modified by the buffering capacity of different clays (8).

These interactions are indicative of how individual ecological factors can influence other factors, which, in turn, can affect a spectrum of microbial events in natural habitats.

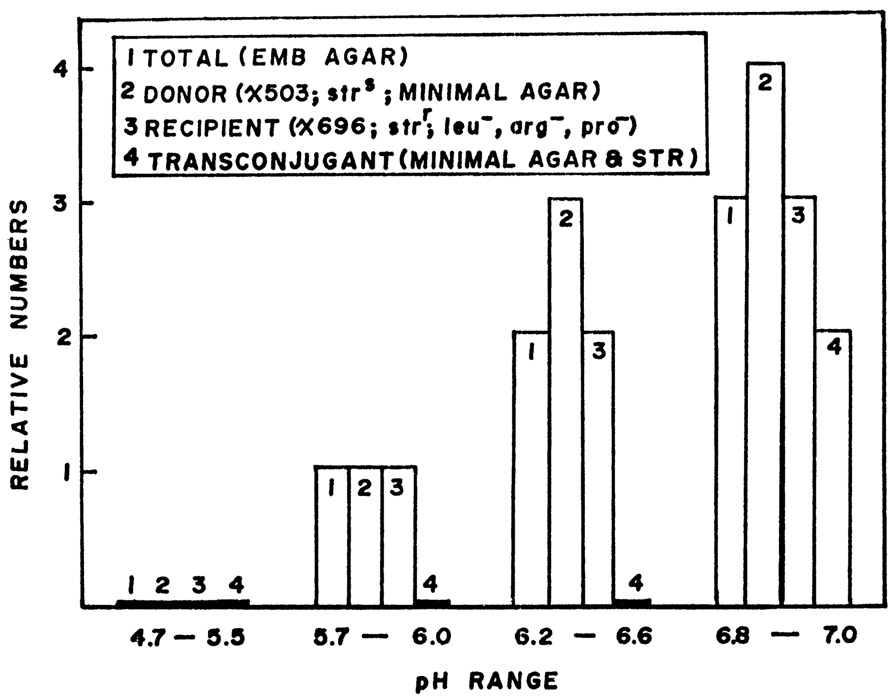

Fig. 3. Effect of pH on survival and growth of auxotrophic and prototrophic strains of Escherichia coli and on their conjugation in soil (Krasovsky and Stotzky, unpublished).

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EPIDEMIOLOGY AND GENETICS OF HEMOLYSIN FORMATION IN ESCHERICHIA COLI

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Hemolysins or cytolysins are extracellular toxic proteins that disrupt the membranes of erythrocytes and other differentiated eucaryotic cells.[1] Most hemolysins seem to have little or no effect on procaryotic cells. The hemolytic phenotype is frequently associated with pathogenic strains of a given bacterial species. There is clear evidence for the involvement in pathogenesis for cytolysins in Gram-positive pathogenic bacteria, such as streptolysins produced by Streptococcus pyogenes, α-, β-, γ- and δ-toxins from Staphylococcus aureus, θ-toxin from Clostridium perfringens, listeriolysins from Listeria monocytogenes and others.[1] These toxins all of which can be considered as hemolysins disrupt eucaryotic membranes by different modes of action, which are only partially understood. Whereas some cytolysins act as enzymes, like the staphylococcal β-toxin which is a sphingomyelinase,[2] others like the "SH-activated cytotoxins" including streptolysin O, C. perfringens θ-toxin, cereolysin (Bacillus cereus) and listeriolysin disrupt eucaryotic membranes by a non-enzymatic mode of action, using probably cholesterol as receptor.[3]

Hemolytic strains are also found in Gram-negative bacteria, especially in Escherichia coli, Proteus morganii and Pseudomonas aeruginosa. The significance of these hemolysins in pathogenesis, however, is still controversial and based on more circumstantial evidence.[4-6] Whereas only a small percentage of E. coli strains isolated from the intestines of healthy individuals or patients suffering from acute diarrhoea are hemolytic, E. coli strains with the capability of producing hemolysin are frequently occurring in extra-intestinal infections. E. coli strains are frequently causing urinary tract infections and a high percentage of these E. coli strains are hemolytic as already observed by Dudgeon in 1921 and reconfirmed by several other groups (Table 1). There is still some debate whether or not there is a correspondence between faecal *E. coli* strains and *E. coli* strains found in urinary tract infections. It appears, however, that certain *E. coli* O-serotypes, especially O4 and O6 are more frequently encountered in urinary tract infections than others and again most O4 and O6 *E. coli* isolates are hemolytic.[6] This seems to support the suggestion that these two serotypes may be especially pathogenic for the urinary tract and that hemolysin may be a special virulence factor. Hemolytic *E. coli* strains are also frequently occurring in other extra-intestinal infections, such as peritonitis, appendicitis or bacteremias.[6,7] No correlation, on the other hand, has been found between hemolysin and enterotoxin production and there is no evidence for an association of hemolysin production and colonization factors, such as CFAI or II.[8,9] Transmissible plasmids have been found to determine hemolysin production in many faecal hemolytic *E. coli* strains from human and animal sources. On the contrary, most hemolytic *E. coli* strains from extra-intestinal infections do not seem to carry plasmids connected to hemolysin production. A large number of Hly-plasmids have been isolated and characterized.[10-14] Their molecular weights range from 40 to 93 x 10^6 daltons; they are transmissible and most of them belong to rather rare incompatibility groups, such as *inc*I2, *inc*FIII, IV and VI (Table 2). There is circumstantial evidence that the Hly determinant may move between various plasmids residing in the same bacterial cell and, as shown later, there are Hly plasmids which share only the hemolysin determinant as common sequence.

Table 1. Frequency of Occurrence of Hemolytic E. coli

Origin	No. Hemolytic/ No. Tested	%	Reference
Stool	8/100	7.3	DeBoy et al.(1980)
Blood	7/ 14	50.0	"
Urine	4/ 20	35.0	"
Misc. Wounds	8/ 23	34.8	"
Blood	18/ 51	35.0	Minshew et al.(1978)
Urine	29/ 59	49.0	"
Sputum	2/ 5	40.0	"
Miscellaneous	16/ 27	59.0	"
Stool			
EEC	0/ 9	0	"
Normal	1/ 20	5.0	"
Urine	26/59	44.4	our data
Stool	2/39	5.0	"

Plasmid-determined hemolysin of E. coli is secreted apparently through both membranes since most of it appears in the mid-logarithmic growth phase in the supernatant from where it can be isolated as a protein with a molecular weight of about 60,000 daltons. In addition, internal active hemolysin is found which can be chased into the extracellular pool[15] suggesting that it represents hemolysin en route to secretion.

By mutagenizing hemolytic E. coli cells with nitrosoguanidine, we obtained two types of hemolysis-negative mutants, those which do not synthesize any active hemolysin and those which still produce active internal hemolysin that is not secreted. Similar mutants were obtained by transposon mutagenesis with the ampicillin transposon Tn3. These mutations have been mapped on the Hly plasmid pHly152.[16] Tn3 insertions leading to a complete loss of hemolysin activity map within a region of about 3500 bp (Fig. 1), whereas insertions causing a defect of the extracellular transport of hemolysin map immediately to the right in a region of about 1500 bp (Fig. 1). Recombinant plasmids with either EcoRI-F or HindIII-E inserted into pACYC184 are able to complement hemolysin-negative Tn3 mutants with Tn3 insertions located in the first 500 bp of the 5000 bp region. Both of these restriction fragments cover the left part of the hemolysis region (Fig. 1). Tn3 mutants with impaired transport functions for hemolysin, all of which carry the Tn3 insertions in the right 1500 bp region covered by EcoRI-G, can be complemented to full extracellular hemolysin production by a recombinant DNA carrying this fragment. The other hemolysin-negative mutants with Tn3 insertions in the middle 3000 bp part of the hemolysis region are complemented by recombinant DNA carrying a Bam-Sal fragment, which includes a large part of the whole hemolysis region. Cloning of this part of the hemolysis determinant proved to be difficult and was only possible with the aid of the vector plasmid p31 (J. Hedgpeth, personal communication) which allows the insertion of the Bam-Sal fragment into a site of very

Table 2. Plasmids from Hemolytic E. coli Strains

Plasmid	M.W. ($x10^6$ dalton)	Inc Group	Source	Reference
pHly152	40	I2	Mouse	Goebel et al.(1974)
pHly167	40	I2	Pig	"
pHly20	42	I2	Pig	"
pHly-P212	ND	FVI	Pig	Monti-Bragadin (1975)
MIP240	ND	FIII	Human	LeMinor et al.(1976)
MIP241	ND	I2	Human	"
pSU316	48	FIII/IV	Human	DelaCruz et al.(1980)
pSU5	93	Iα/I2	Pig	"
pSU105	77	FVI	Pig	"
pSU233	60	?	Pig	"

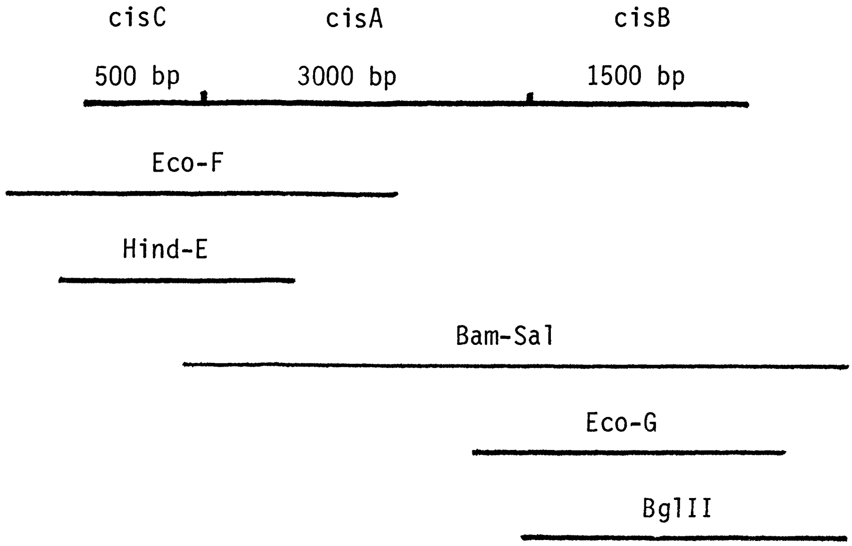

Fig. 1. Schematic presentation of the hemolysis region which consists of three cistrons, C, A and B. These cistrons are defined by Tn3 insertions leading to different hemolysis-negative mutants and their complementation be recombinant plasmids carrying the restriction fragments listed below.

low transcription activity (Fig. 2). Increased gene expression of this part of the hemolysis region is lethal to the cell as demonstrated by the following experiment. A *Bgl*II fragment carrying the λcI_{857} gene together with the left (P_L) and the right (P_R) promoters of phage λ was inserted in front of the *Bam*-*Sal* fragment (Fig. 3) of the recombinant DNA p31-2, thus allowing an induced transcription of the genetic information of the inserted *Bam*-*Sal* fragment at elevated temperature (42°C). Whereas *E. coli* cells carrying this new recombinant plasmid (p31-2cI) grow normally at 30-35°C, no growth occurs anymore upon a shift of the temperature to 42°C. The rate of survivors after 1 hr treatment of these cells at 42°C is less than one in 10^5 cells. The removal of the right part of the *Bam*-*Sal* fragment by deleting the *Bgl*II fragment of p31-2cI (Fig. 3) does not eliminate the killing activity at 42°C, indicating that the region between the *Bam* and the *Bgl*II site is responsible for the lethal effect. Whereas neither p31-2cI nor p31-2cI $BglII_{del}$ determine extracellular hemolysin (intracellular hemolysin activity is, however, observed in *E. coli* cells harboring these plasmids), extracellular hemolysin is secreted when cells carrying the plasmid p31-2cI are complemented with recombinant DNAs having either *Eco*RI-F or *Hin*dIII-E inserted into pACYC184. Cells carrying this combination

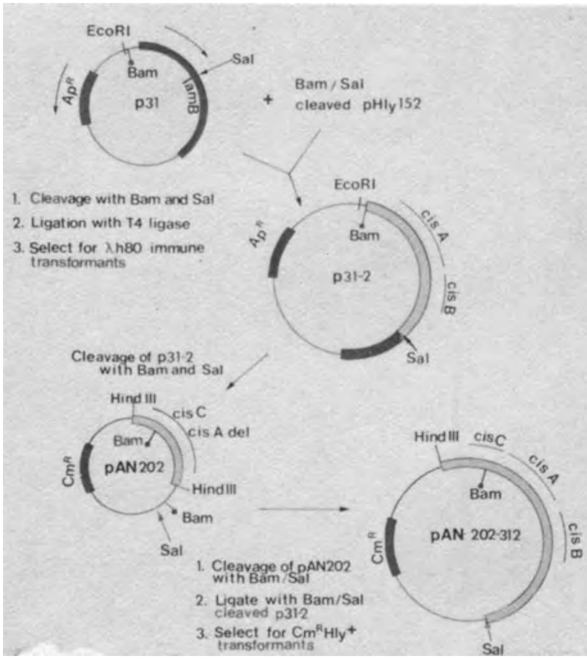

Fig. 2. Construction of recombinant plasmids carrying either cisA and cisB (p31-2) or the whole hemolysis determinant, i.e. cisC, cisA and cisB (pAN202-312).

of plasmids are still killed at 42°C. Under these conditions a large amount of extracellular hemolysin is produced.

The complementation data described suggest that the hemolysis region consists of three cistrons (Fig. 1), which are determining synthesis and transport of hemolysin. Recently we succeeded in the construction of a recombinant plasmid, which carries the whole hemolysis determinant inserted into pACYC184 (Fig. 3). In spite of the high copy number of this plasmid, pAN202-312, cells harboring it synthesize and secrete roughly the same amount of external and internal hemolysin as cells carrying the single copy wild-type plasmid pHly152. This may indicate a rather tight control of the expression of the hemolysis determinant, which seems to occur from a single promoter transcribing all three cistrons from left to right (i.e. cisC → cisB).

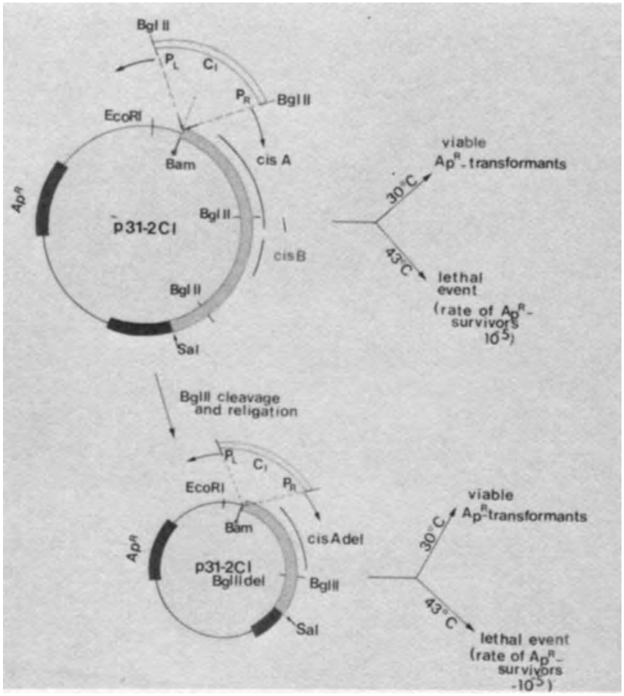

Fig. 3. Construction of recombinant plasmids which carry the promoter P_R and P_L together with the cI gene of phage λ in front of cisA.

Hemolysis is not a very specific reaction and it is conceivable that different extracellular proteins may be causing the hemolytic phenotype in hemolytic E. coli strains. There are reports on different hemolysins in E. coli.[4] We therefore carried out hybridization studies with the cloned hemolysis determinant of plasmid pHly152 and various Hly plasmids of faecal hemolytic E. coli strains and the chromosomal DNA of hemolytic E. coli strains from extra-intestinal infections. All plasmids tested hybridize well with radioactive DNA probes carrying cisC, A or B of pHly152, indicating that these plasmid-inherited hemolysin determinants are alike if not identical. There are, however, remarkable differences in the overall hybridization between the standard Hly plasmid pHly152 and the other Hly plasmids (Table 3). Four Hly plasmids all of which belong to the incompatibility group incI2 share sequence homologies extending far beyond the hemolysis determinant. Others have only little sequence homologies besides the common hemolysis determinant (Table 3). There appears to be a rather defined right end of the hemolysis determinant (at the cisB side) in all Hly plasmids, whereas the left end (at the cisC side) varies to a much larger extent in these plasmids.[17]

Chromosomally determined hemolysin from extra-intestinal E. coli isolates has similar biochemical properties as the plasmid-determined hemolysin from faecal strains. Besides, similar hemolysis-negative mutants can be isolated from these hemolytic E. coli strains, i.e. those that produce no active hemolysin and those that produce internal hemolysin only. This suggests that the chromosomal Hly determinant has a similar genetic complexity as the plasmid Hly determinant. Hybridization of chromosomal DNA from three such hemolytic strains with the plasmid Hly determinant is considerably lower than that of total DNA from plasmid-determined hemolytic strains with the same probe. A more detailed hybridization study indicates that a fragment carrying cisB of plasmid-encoded Hly determinant shows strong hybridization, one with cisC still shows some hybridization, but a fragment with cisA shows little or no hybridization with DNA of hemolytic E. coli possessing chromosomal Hly determinants. The hybridization data are supported by the recent observation that the chromosomal Hly determinant can complement plasmid-coded cisC and cisB but not cisA mutants. Thus it appears that plasmid- and chromosome-inherited Hly determinants may share two common cistrons (cisB and cisC), but cisA which seems to code for the hemolysin protein itself is entirely different in both determinants suggesting possibly different functions of these two types of hemolysins.

Table 3. Hybridization of different Hly Plasmids with the Hly-Determinant of pHly152

Plasmid	Hybridization with cisC	cisA	cisB	Hybridization Outside of the Hly Determinant
pHly167 incI2	+	+	+	100%
pHly20 incI2	+	+	+	>90%
pHly124 incI2	+	+	+	~70%
pSU5 incI2/Iα	+	+	+	~50%
pSU233 —	+	+	+	~15%
pSU105 incFVI	+	+	+	~ 8%
pSU316 incFIII/IV	+	+	+	< 5%

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CHROMOSOMAL AND PLASMID-MEDIATED TRANSFER OF CLINDAMYCIN RESISTANCE IN BACTEROIDES FRAGILIS

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ABSTRACT

The characteristics of the clindamycin-erythromycin (clin^r) resistance transfer factor from Bacteroides fragilis TMP 10 are presented. Transfer ability and the determinant for clin^r are found on a 15.6 kilobase plasmid named pBFTM 10. Recent clindamycin and tetracycline resistant strains of Bacteroides fragilis have been isolated in Chicago. The Chicago tet^r isolate, TMP 230, transfers both clin^r and tet^r, but appears to be plasmid free when tested by standard methods. Homology between the clin^r transfer factor pBFTM 10 and the chromosome of the TMP 230 could be demonstrated by the Southern hybridization technique. The location of the clin^r determinant on the chromosome and mode of transfer are under invistigation.

Anaerobic bacteria are prominent members of the normal flora of man; in the colon anaerobic organisms including Bacteroides, Clostridia and non-sporing, gram-positive bacilli outnumber facultative bacteria such as E. coli and Streptococcus fecaelis by about 1000:1 (1). Over the past 20-25 years anaerobic bacteria have been increasingly recognized as important pathogens in human suppurative infections (2). Bacteroides fragilis emerges as the most important anaerobic bacterium in abdominal, surgical and gynecological infections because it most frequently invades the bloodstream in this setting. Most B. fragilis strains are resistant to intermediate levels of penicillin G and cephalosporins; they are uniformly resistant to the aminoglycoside antibiotics, and in the 1960's it was noted that there was the emergence of widespread resistance to tetracycline (3,4). This latter resistance was important because tetracycline was the agent of choice in treating infections involving B. fragilis in the 1950's. More recently there have been reports of the increasing incidence of high-level penicillin resistance and scattered reports of resistance to chloramphenicol, clindamycin, cefoxitin, and metronidazole (5-9). Clindamycin resistance is important because this drug has currently been the prime agent for treating bacteroides infections.

Because of the widespread resistance in Bacteroides fragilis and closely related species, numerous attempts have been made to transfer the penicillin or tetracycline resistance (tet^r) determinants both within B. fragilis and from B. fragilis to E. coli (10,11). Until the late 1970's the only documented successful transfer of tetracycline resistance was from B. fragilis to E. coli by an undescribed mechanism by Mancini and Behme (12). There is one report of the transfer of ampicillin resistance from E. coli to B. fragilis and a fusobacterium, but the ampicillin resistance was unstable (13). Transformation of E. coli to ampicillin resistance was reported with DNA from B. fragilis; however, the plasmid used to transform could not be visualized in the E. coli (15). In 1979, three laboratories concurrently reported the transfer of clindamycin resistance determinants within the genus Bacteroides, Privitera et al. at the Pasteur Institute, Welch and Macrina in Richmond, Virginia, and our own studies (15,16,17).

Investigations at the Pasteur Institute disclosed the transfer of both clindamycin ($clin^r$) and tetracycline resistance (tet^r) from a strain of B. fragilis isolated in France to another B. fragilis strain. They showed that erythromycin and streptogramin resistance were transferred with the $clin^r$, and these resistances were spontaneously curable. Further work by the French group demonstrated that transfer of tet^r in B. fragilis could be induced to a higher frequency by pretreatment of the donor culture with subinhibitory levels of tetracycline (18). Welch and Macrina working with the isolate from the Pasteur Institute demonstrated that the transfer of clindamycin, erythromycin and streptogramin resistance was associated with a 27 megadalton plasmid (16). Our laboratory was working with a different strain of Bacteroides fragilis, isolated in California, that was highly resistant to clindamycin and erythromycin and possessed a different plasmid associated with the transfer of the clindamycin resistance. This paper describes the characterization of our clindamycin resistance transfer factor.

Standard anaerobic techniques in an anaerobic glovebox were employed, and the matings were carried out utilizing Nalgene filters (17). DNA was analyzed by agarose gel electrophoresis, and cells were lysed by a number of different procedures. DNA-DNA hybridization studies were carried out by a modification of the Southern technique (19,20,21,22,23).

Table 1. Characteristics of the Donor and Recipient Strains of B. fragilis[a]

Organisms	Phenotypic Characteristics
DONORS	
B. fragilis TMP 10	$clin^r, tet^r, rif^s, nal^s, phage^r$
B. fragilis TMP 230	$clin^r, tet^r, rif^s. nal^s, phage^s$
RECIPIENTS	
B. fragilis TM 2000	$clin^s, tet^r, rif^r, nal^s, phage^s$
B. fragilis TM 4000	$clin^s, tet^s, rif^r, nal^s, phage^s$
B. fragilis TM 4500	$clin^s, tet^s, rif^r, nal^r, phage^s$
B. fragilis JC 101	$clin^s, tet^s, rif^r, nal^s, phage^s, his^-, arg^-$
B. thetaiotaomicron TM 5000	$clin^s, tet^r, rif^s, nal^s, phage^r, rham^+, ara^+$

[a] Abbreviations: clin-clindamycin-erythromycin, tet-tetracycline, rif-rifampicin, nal-nalidixic acid, phage-phage susceptibility, arg^-,his^- requires arginine or histidine to grow on minimal medium, $rham^+$ ara^+ - grows on minimal medium with rhamnose or arabinose as only carbon source.

Bacteroides fragilis TMP 10 was mated with B. fragilis TM 2000 for $clin^r$-rif^r isolates, and a low number of transicpients were obtained which were confirmed as transcipients by their phage patterns. Transcipient strains were tested for retransfer of $clin^r$ to Bacteroides thetaiotaomicron. $Clin^r$, ara^+ isolates were confirmed by checking for $rham^+$. In all instances erythromycin resistance was transferred with clindamycin resistance. Thus, these studies plus the studies by the French group and the Richmond Virginia group show that there is intra-and interspecies transfer of resistance determinants within the genus Bacteroides.

Analysis of the extrachromosomal DNA in a number of our transcipients is shown in Figure 1 (17). In lane 1 is the original recipient, TM 2000, and in lanes 7 & 8 are the original donor, TMP 10. In lane 2 is a strain which was originally isolated as $clin^r$ but subsequently was found to have spontaneously lost $clin^r$; it posseses a 2.8 kb plasmid shown in two molecular forms (covalently closed circle and open circle). In lane 3 is a strain which has retained its $clin^r$, and the only additional plasmid is the high molecular weight one. Thus, the minimal requirements for the transfer of the $clin^r$ that we originally described were the presence of these two plasmids (17).

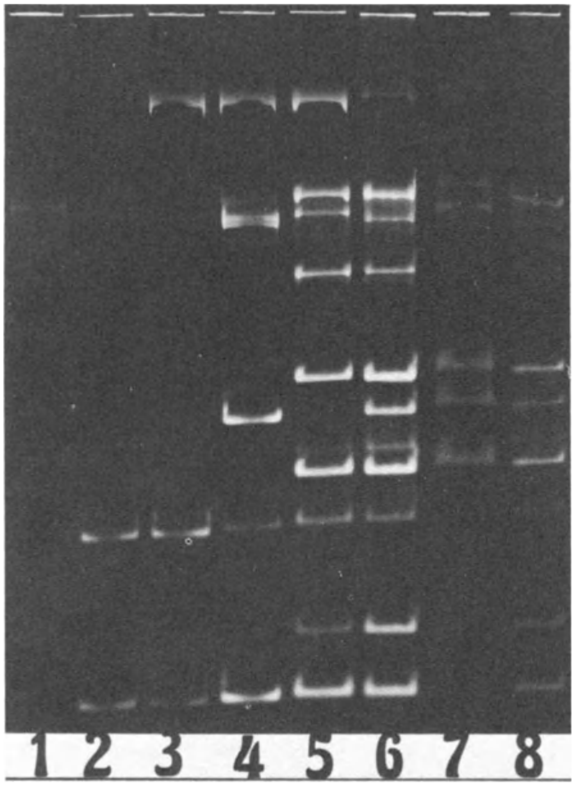

Figure 1. Agarose gel analysis of DNA in parental and transcipient strains of Bacteroides. Each gel slot contained 5-25μl of DNA purified by the CsCl-EtEr (cesium chloride-ethidium bromide) method (lanes 2-8) or phenol-extracted cleared lysates (lane 1). Samples were obtained from: (1) Bacteroides fragilis strain TM2000; (2) B. fragilis TM2010; (3) B. fragilis TM2006; (4) B. fragilis TM2008; (5) B. fragilis TM2002; (6) B. fragilis TM2001; (7) B. fragilis TMP10, preparation A; (8) B. fragilis TMP10, preparation B (obtained on a different day from preparation A).

At this point, Privitera published his studies on the inducibility of tetracycline resistance transfer in B. fragilis (18). This prompted us to re-examine our strains since the original donor TMP 10 and recipient TM 2000 were known to be tet^r. Utilizing a plasmid-free recipient strain TM 4000 kindly supplied by Dr. Sebald from the Pasteur Institute we studied the transfer of both $clin^r$ and tet^r from TMP 10 to a nalidixic acid resistant (nal^r) derivative of the Sebald strain called TM 4500. The original donor was mated under 3 conditions, uninduced, induced with tetracycline, and induced with clindamycin (Table 2).

Table 2. Effect of Antibiotic Pretreatment on Resistance Transfer Frequency from *B. fragilis* TMP 10 to *B. fragilis* TM 4500

Condition	(Mating Frequency per donor input) Tet Transfer*	Clin Transfer**
Untreated	10^{-7}	10^{-8}
Clindamycin	10^{-5}	10^{-6}
Tetracycline	10^{-3}	10^{-4}

*Primary selection on tet rif plates, secondary character phage^{s} nal^{r}
**Primary selection on clin rif plates

In the uninduced mating there was low level transfer of both clin^{r} and tet^{r}; however, with tetracycline or clindamycin induction there was a 2-4 log increase in the frequency of transfer. The transfer of tet^{r} was not associated with a detectable extrachromosomal element (25).

In order to properly characterize our clindamycin resistance transfer factor it was necessary to isolate it in a plasmid-free tetracycline susceptible background. *Bacteroides fragilis* TMP 10 was mated with strain TM 4000, selecting for clin^{r}-rif^{r} and checking for tet^{s} and phage^{s}. Several tet^{s}-clin^{r} transconjugants were isolated, and they could retransfer clin^{r} to a TM 4500.

Several isolates were analyzed for extra chromosomal DNA; some strains contained one plasmid. The plasmid DNA from strain TM 4003 was isolated and designated pBFTM 10. pBFTM 10 measures 15.6 kb when compared to pBR322 by electron microscopy. This plasmid was also sized by specific restriction endonucleases including Eco Rl, Hind III and Pvu II. A preliminary restriction map of pBFTM 10 is illustrated (Figure 2). A spontaneous clindamycin susceptible derivative of TM 4003 possesses a plasmid with a 4.5 kb deletion of pBFTM 10. For this reason, the 4.5 kb fragment of pBFTM 10 is believed to carry clin^{r}.

Close cell-to-cell contact is required for transfer. The process is resistant to DNAase treatment of the donor and recipient cells, and supernatants of chloroform-treated donor cells do not transfer. Finally, there is no increased frequency of transfer by pretreatment with clindamycin when the donor and recipient are in a tetracycline susceptible background. Despite the small size we feel that pBFTM 10 is a self-transferable plasmid, but we cannot rule out the possible requirement for host chromosomal functions to affect the transfer. It has been used to mobilize penicillin/ampicellin resistance from another strain of *B. fragilis* (26).

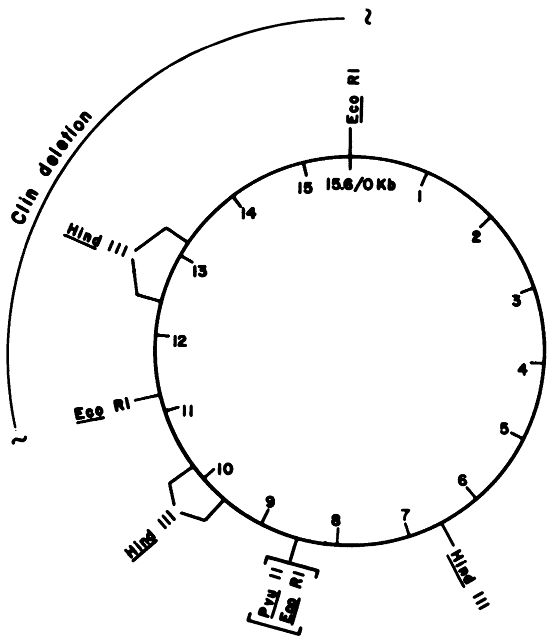

Figure 2. Preliminary Restriction Map of pBFTM 10.

The clindamycin transfer factor, pBFTM 10, can also be used as a probe to investigate other $clin^r$ bacteroides, and was employed as such to study strains from a recent outbreak of $clin^r$ bacteroides on the surgical wards of the University of Illinois Hospital in Chicago.

B. fragilis TMP 230, a $clin^r$ and tet^r isolate from Chicago which is known to be rif^s and nal^s, was mated with a laboratory derivative of strain TM 4000 called JC 101 ($clin^s$,tet^s,rif^r,nal^s, arg^-,his^-) using filter mating techniques with and without tetracycline induction of the donor. There was no transfer of tet^r or $clin^r$ without tetracycline induction. However, with tetracycline induction transfer of both tet^r and $clin^r$ occurred. The frequency of tetracycline transfer was 5 X 10^{-4} and clindamycin 1 X 10^{-5}. All clindamycin resistant isolates were also tetracycline resistant while one-third of the tetracycline resistant transconjugants were clindamycin resistant.

Analysis of the donor and transcipient strains for extrachromosomal DNA failed to reveal the existence of closed circular molecules. Based on the assumption that the clindamycin resistant determinant in strain TMP 230 may be related to the previously described clindamycin transfer factor pBFTM 10, an Eco Rl digest of the chromosome of TMP 230 was probed with nick-translated ^{32}P DNA from pBFTM 10 utilizing a modification of the Southern hybridization technique (23); the uncleaved chromosome of TMP 230 possesses some homology with pBFTM 10, and this homology has been localized in 3 Eco Rl fragments of the chromosome.

TMP 230 was mated with JC 101 and the chromosome of 2 transconjugants were probed with ^{32}P-labelled pBFTM 10 as described above. The homology with pBFTM 10 was strongest in the donor but was clearly evident in both the transcipients. These data indicate that there was transfer of clindamycin resistance determinants similar to that found on pBFTM 10 from the chromosome of strain TMP 230 to the chromosome of the recipient JC 101 by a yet undisclosed mechanism. Probing of Eco Rl and Pvu II digests of the TMP 230 and its transcipients reveals that there are different amounts of DNA being acquired in the transcipients and that the sites of insertion in each transconjugant are changed from the donor. This supports our belief that the DNA coding the resistance is inserted into the chromosome rather than residing on a large plasmid which was not detected. The mechanism of chromosomal transfer is not presently understood. It may be similar to the "conjugal transposon"-mediated tetracycline resistance transfer in _Streptococcus fecaelis_ as reported by Clewell and his associates (27).

In conclusion, we have presented data from our laboratory showing that clindamycin resistance transfer in our strain of _Bacteroides fragilis_ TMP 10 is associated with a 15.6 kilobase plasmid called pBFTM 10. This clindamycin transfer factor can be used as a prob for the location of clindamcyin resistance determinants in other resistant bacteroides. Clindamycin transfer may be independent or associated with a tetracycline transfer element located in the chromosome.

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CAMPYLOBACTER JEJUNI: CHARACTERISTIC FEATURES OF THE ORGANISM AND IDENTIFICATION OF TRANSMISSIBLE PLASMIDS IN TETRACYCLINE-RESISTANT CLINICAL ISOLATES

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INTRODUCTION

In recent years Campylobacter jejuni has been recognized throughout the world as a common cause of bacterial diarrhea[1,2]. The organism is microaerophilic, and therefore requires special conditions for selection and growth. Methods developed by Butzler and colleagues[3,4] in Belgium and Skirrow[5] in the United Kingdom have enabled many microbiological laboratories to isolate this organism from stools and have led to its recognition as a significant enteric pathogen. The relative frequencies of organisms causing gastroenteritis isolated during 1978 and 1979 at the Hospital for Sick Children, Toronto are shown in Table 1. Among pediatric patients reporting with diarrhea, C. jejuni was isolated almost as often as non-typhoidal salmonella. Other enteric pathogens were isolated much less frequently. The most common clinical features of campylobacter enteritis are diarrhea, often accompanied by blood in the stools, and abdominal pain[2]. Several excellent review articles have been published recently, dealing with both the disease and the causative organism[1,2,6,7,8]. The reader is referred to them for more detailed information. In this article, I will describe the significant features of campylobacters, including their morphology, growth requirements and resistance to antibiotics. I will then discuss recent work on transmissible plasmids that mediate tetracycline resistance in C. jejuni, a preliminary report of which has been published[9].

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Table 1. Relative Frequency of Different Enteric Pathogens Isolated at The Hospital for Sick Children, Toronto[a]

Pathogen	Number of Cases 1978	1979
Campylobacter jejuni	103	100
Salmonella spp. (non-typhoidal)	129	105
Salmonella typhi	6	6
Shigella spp.	22	15
Yersinia enterocolitica	12	17
Enteropathogenic Escherichia coli serotypes	57	50

[a]Cases of bacterial diarrhea, inpatients and outpatients seen during 1978 and 1979. (M.A. Karmali and P.C. Fleming, unpublished data.)

CHARACTERISTIC FEATURES OF CAMPYLOBACTERS

The Genus Campylobacter

The organisms in the genus Campylobacter are oxidase-positive, microaerophilic, gram-negative bacilli. They are divided into two groups based on their ability to produce catalase[1]. Three species, Campylobacter jejuni, C. fetus subsp. fetus, and C. fetus subsp. venerealis belong to the catalase-positive group. Two other species, C. sputorum and C. bubulus, are catalase-negative[1]. Studies of plasmids are limited to work on strains of C. jejuni, although one strain of C. fetus subsp. fetus (ATCC 27374) has been used as a recipient in interspecies matings. The catalase-negative campylobacters are not pathogenic for humans, and since nothing is known of their plasmid content, they will not be discussed further. Campylobacter fetus subsp. fetus causes abortion in cattle and sheep, but it is occasionally implicated in some human infections. In contrast, C. fetus subsp. venerealis is associated with sterility in cattle but is not thought to be pathogenic for humans.

Campylobacters because of their morphology were once included in the genus Vibrio, however studies of the guanine plus cytosine (G + C) content of DNA from members of the two genera indicate that they are unrelated[10]. DNA from strains of Campylobacter fetus subsp. fetus and C. fetus subsp. venerealis has a G + C content of 34.4%; DNA from strains of C. jejuni has a G + C content of 32.7%. The genus Vibrio, however, comprises bacteria which contain DNA with a G + C content of 40 to 53%[10].

Morphology of Campylobacters

Campylobacters are spirally curved rods which have a single polar flagellum at one or sometimes both ends of the cell. When examined by phase-contrast microscopy, the organism is motile with a characteristic corkscrew-like motion. Karmali et al. have shown that catalase-positive campylobacters may be differentiated morphologically[11]. Campylobacter jejuni is a small rod with tightly coiled spirals, which have a wave-length of 1.12 μm and an amplitude of 0.48 μm. The spirals of Campylobacter fetus subsp. fetus are intermediate in size with a mean wave-length of 1.8 μm and an amplitude of 0.55 μm. Of the three species, C. fetus subsp. venerealis form the largest spirals, with a mean wave-length of 2.43 μm and an amplitude of 0.73 μm.

Growth Conditions

The catalase-positive campylobacters are microaerophilic, growing best in an atmosphere in which the oxygen tension is reduced to 5-7%. Such an atmosphere may be achieved by evacuating two thirds of the air from an anaerobic jar (without catalyst) and replacing the evacuated air with carbon dioxide or a mixture of carbon dioxide and nitrogen[1,2,11]. Commercial gas generating systems (BBL, Oxoid) are also available.

Differential Features of Campylobacters

Table 2 shows characteristic features of C. jejuni and C. fetus subsp. fetus. These characteristics are important in the choice of temperatures for plasmid transfer, in the choice of antibiotic for counter-selection, and to allow differentiation of C. jejuni donors and C. fetus subsp. fetus recipients after plasmid transfer.

Table 2. Characteristics for the Differentiation of Campylobacter jejuni and Campylobacter fetus subspecies fetus

	Growth at			Resistance to	Resistance to
	25°C	37°C	42°C	nalidixic acid[a]	cephalothin[a]
C. jejuni	-	+	++	S	R
C. fetus subsp. fetus	+	+	-	R	S

[a]S, susceptible: zone of inhibition surrounding disc containing 30 μg of nalidixic acid or cephalothin; R, resistant: no zone of inhibition.

EPIDEMIOLOGY AND PATHOGENESIS OF C. JEJUNI

The epidemiology of campylobacter enteritis is not well understood. The organism is a pathogen or commensal in a wide range of animal species, including cattle, sheep, pigs, poultry, dogs, cats and wild birds[2,6], consequently there is a large natural reservoir. Ingestion of contaminated water[12] and unpasteurized milk[6,13,14] has been implicated in some human infections. Serotyping schemes are currently being developed[3,13,14,16] as one approach to understanding the epidemiology of campylobacter enteritis.

Little is known about the pathogenesis of C. jejuni. Studies by Butzler and Skirrow using chick embryos and 8-day-old chicks indicate that pathogenesis depends on the direct invasive ability of the organism although a few strains also produced a thermostable enterotoxin[1]. The presence of blood in the stools from patients with campylobacter enteritis also suggests an invasive process. Pathogenicity studies have been hampered by the lack of a suitable experimental model. Recently, however, Ruiz-Palacios et al.[17] reported that the three-day-old chick may constitute a suitable animal model for campylobacter enteritis.

ANTIBIOTIC RESISTANCE IN C. JEJUNI

Although campylobacter enteritis is usually self-limiting, antibiotics may be indicated for treatment of the more severe cases. Erythromycin, tetracycline, and the nitrofuran derivative, furazolidone, have been recommended[18]. Approximately 1% of C. jejuni strains isolated at The Hospital for Sick Children, Toronto, in 1978 and 1979 were resistant to erythromycin[19]. This result is similar to the report of 0.5% erythromycin-resistant strains in the United Kingdom[20]. Higher levels of erythromycin resistance were observed in Belgium[18] and Sweden[21] where 9% of C. jejuni strains were erythromycin-resistant. In Toronto, studies are in progress to determine if erythromicin resistance in C. jejuni is plasmid-mediated.

In the Toronto study[19], all C. jejuni isolates were susceptible to nitrofurantoin with a minimal inhibitory concentration (MIC) of 2μg/ml. About 20% of strains were resistant to 4μg/ml of tetracycline; 12% showed high level resistance to tetracycline (MIC $\geq$ 64μg/ml). The significant number of tetracycline-resistant strains of C. jejuni in Toronto contrasts with the lower percentage of European isolates resistant to this antibiotic. In Belgium, Vanhoof et al.[18,22] showed that about 5 to 8% of clinical isolates were tetracycline-resistant. In Sweden, however, Walder[23] found that all clinical isolates of C. jejuni were susceptible to tetracycline. In North America, owing to the high incidence of tetracycline-resistant C. jejuni, tetracycline may be of limited use in the treatment of campylobacter enteritis.

ANTIBIOTIC RESISTANCE TRANSFER AND PLASMIDS IN C. JEJUNI

The relatively recent recognition of C. jejuni as a pathogen and the special conditions required for growth of the organism have delayed the study of plasmids in this genus. The first report of plasmids in C. jejuni was that of Austin and Trust[24], who found that approximately 19% of strains from various geographic locations contained plasmids. The molecular weights of these plasmids varied from 5.0 to 77×10^6. Only one isolate harboured more than one plasmid. Some of the strains of C. jejuni studied were resistant to antibiotics, but no direct correlation was made between antibiotic resistance and plasmid carriage[24].

Intraspecies Transfer of Tetracycline Resistance

At the Hospital for Sick Children, Toronto, clinical isolates of C. jejuni with MIC of tetracycline $\geq$ 64μgm/ml were studied. Tetracycline resistance was shown to be plasmid-mediated and transmissible within the genus Campylobacter[9]. A clinical isolate of C. jejuni, MK22, which had a tetracycline MIC of 128μg/ml, was used as the donor strain. The recipient strain, SD2, was a spontaneous nalidixic acid-resistant mutant of C. jejuni (MIC of tetracycline $\leq$4μg/ml and of nalidixic acid = 256μg/ml). Tetracycline resistance transfer was performed using broth and filter mating methods. For both procedures, the strains were grown in a special medium devoid of blood (M.A. Karmali and P.C. Fleming, unpublished data). It contained per litre: Tryptone soya broth (Oxoid) 10g, special peptone (Oxoid) 5g, yeast extract (Oxoid) 5g, hemin 0.01g, TRIS (BDH) 0.75g, and sodium pyruvate 10g. Dithiothreitol (0.15g per litre) was added to the broth as a reducing agent and the pH adjusted to 7.2 by addition of hydrochloric acid. All solid media contained 3% agar to prevent swarming, which is a characteristic feature of C. jejuni[11].

Liquid matings were prepared by adding 0.5 ml of the donor culture to 1.0 ml of recipient culture and 1.0 ml of fresh broth. The mating mixtures were incubated at 42°C for 48 hours, then aliquots from the mating mixtures were diluted in 0.05M sodium phosphate buffer pH7.2. The C. jejuni transconjugants were selected on Diagnostic Sensitivity Testing agar (DST, Oxoid) containing 5% lysed horse blood, 50μg/ml nalidixic acid and 16μg/ml tetracyline. Filter matings were prepared by adding 0.5 ml of the donor culture to 1.0 ml of the recipient culture. The mating mixtures were collected on nitrocellulose filters (Millipore HA type, pore size 0.22 μm). The filters were placed on agar plates, containing the special medium plus 3% agar and incubated under reduced oxygen tension (7%) at 42°C for 48 hours. The filters were removed from plates, placed in tubes containing 1.0 ml of sterile phsphate buffer, and cells were separated from the filter by vortexing the tubes. Dilutions were made of the cell suspensions and the transconjugants were selected as described above.

The tetracycline resistance determinant was transferred from C. jejuni strain MK22 to strain SD2. In broth matings, the frequency of transfer was $2.4x10^{-6}$ transconjugants per recipient in a 48 hour mating period at 42°C. For filter matings, the frequency of transfer was $5.0x10^{-4}$; an increase in the transfer frequency of about two hundred-fold. No significant difference in the transfer frequency of tetracycline resistance was noted when the temperature of the mating mixtures was reduced from 42°C to 37°C.

Interspecies Transfer of Tetracycline Resistance

The higher transfer frequency observed for the filter-mating procedure indicated that a solid surface facilitates intraspecies transfer of the C. jejuni tetracycline resistance determinant. As the filter-mating method is somewhat tedious, we used a plate-mating procedure for the interspecies transfer studies.

Interspecies transfer was demonstrated with two donor strains of C. jejuni, MK22, described above, and MK175, a clinical isolate resistant to both tetracycline (MIC = 64μg/ml) and ampicillin (MIC = 128μg/ml). The recipient strain was C. fetus subsp. fetus ATCC 27374 (CIP 5396)[10], which is naturally resistant to nalidixic acid (MIC = 256μg/ml). The strains were grown on Columbia base agar (Gibco) containing 7% defibrinated horse blood (BA) and incubated for 48 hours, at 42°C for C. jejuni and at 37°C for C. fetus subsp. fetus. The cells from the plates were suspended in 0.05M sodium phosphate buffer at $1x10^{9}$ cells/ml. Aliquots of 0.15 ml of donor and recipient cell suspensions were mixed together and spread over BA plates. The plates were incubated at 37°C for 48 hours. To select for C. fetus subsp. fetus transconjugants, cells were washed off the plates, diluted and spread on DST agar containing 5% lysed horse blood, 75μg/ml nalidixic acid and 16μg/ml tetracycline.

Interspecies transfer of tetracycline resistance from clinical isolates of C. jejuni to C. fetus subsp. fetus ATCC 27374 is shown in Table 3. The tetracycline resistance determinant transferred equally well to both the C. fetus subsp. fetus and C. jejuni recipients. Attempts were also made to transfer tetracycline resistance to Escherichia coli. Three strains were used: E. coli K12, J53-1 (pro met gyrA), NM148 ($Kr^{-}Km^{+}$gyrA), a restriction deficient mutant of E. coli K12, and the E. coli C strain RG176[25] which does not possess the K restriction system. None of the three E. coli strains was able to act as a recipient.

Mechanism of Transfer of C. jejuni Tetracycline Resistance Determinants

Cell-free filtrates of the donor strains of C. jejuni MK22 and MK175 could not promote the transfer of tetracycline resistance

Table 3. Interspecies Transfer of Tetracycline Resistance from C. jejuni to C. fetus subsp. fetus[a]

Resistance pattern of C. jejuni isolate	Resistance determinant transferred[b]	Plasmid	Transfer frequency[c]
Tc	Tc	pMAK22	2.7×10^{-4}
ApTc	Tc	pMAK175	9.1×10^{-6}

[a]Tetracycline resistance determinants were transferred from C. jejuni clinical isolates to C. fetus subsp. fetus ATCC 27374 in a 48 hour mating at 37°C.

[b]Ap, Ampicillin; Tc, tetracycline.

[c]Calculated as transconjugants per recipient.

determinants to strains of C. jejuni SD2 or C. fetus subsp. fetus ATCC 27374 ($< 1 \times 10^{-8}$ transconjugants per recipient after a 48 hour mating period). These results indicate that the transfer process was not mediated by bacteriophage transduction. The addition of DNase, at 100μg/ml, to the agar used in the plate-mating experiments, also did not affect plasmid transfer frequencies. DNA transformation therefore would not appear to be involved in the transfer process. It appears most likely that transfer of C. jejuni tetracycline R determinants involves a plasmid-mediated conjugative process.

Isolation of C. jejuni Plasmid DNA

Physical isolation of plasmid DNA was performed using a modification of the method of Meyers et al.[26]. The DNA samples were analyzed immediately after isolation by agarose gel electrophoresis as described by Taylor and Levine[27]. Plasmid-enriched DNA fractions were prepared from a total of four tetracycline-resistant clinical isolates of C. jejuni isolated in Toronto. Tetracycline resistance determinants could be transferred from all these strains to C. jejuni SD2 and to C. fetus subsp. fetus ATCC 27374. Similar extracts made from the recipient strains C. jejuni SD2 and C. fetus subsp. fetus ATCC 27374 were plasmid-free. In contrast, all of the above tetacycline-resistant campylobacters harboured a plasmid with a molecular weight of approximately 38×10^6. A plasmid of the same molecular weight was observed in tetracycline-resistant transconjugants of C. jejuni and C. fetus subsp. fetus.

Other Reports of Plasmids in Tetracycline Resistant C. jejuni

Goldstein and Acar have observed transfer of tetracycline resistance between strains of C. jejuni, although transfer to E. coli was not achieved (F.W. Goldstein and J.F. Acar, personal communication). Cohen and Falkow noted the presence of a plasmid with a molecular weight of about $38x10^6$ in a tetracycline resistant clinical isolate of C. jejuni (M.L.Cohen and S.Falkow, personal communication).

Ampicillin Resistance in C. jejuni

Recent studies in Toronto have indicated that ampicillin resistance, observed in approximately 15% of clinical isolates of C. jejuni, is associated with β-lactamase production by these strains (Karmali, D'Amico and Fleming, in preparation). MK175 is one such strain. Transfer of tetracycline resistance and the concomitant transfer of a $38x10^6$ plasmid were observed in this strain (Table 3), however, ampicillin resistance was not cotransferred with tetracycline resistance, and is presumably not located on the same plasmid. As no other plasmid was visualized in DNA prepared from MK175, it is likely that ampicillin resistance in this strain is of chromosomal origin.

CONCLUSIONS

Transmissible plasmids encoding tetracycline resistance were identified in four clinical isolates of C. jejuni. The plasmids had molecular weights of $38x10^6$. All four clinical isolates originated in Toronto, and it is possible that the plasmids are related and may have had a common source.

Transfer of plasmids in Campylobacter was facilitated on solid surfaces. A similar preference for a solid surface for mating has been noted for plasmids of some incompatibility groups in Enterobacteriaceae[28]. Our experiments indicate that DNA transformation and bacteriophage-mediated transduction are not involved in transfer of tetracycline resistance from C. jejuni. The process probably involves conjugation, via cell to cell contact.

Intraspecies transfer in C. jejuni was demonstrated, as well as interspecies transfer from C. jejuni to C. fetus subsp. fetus. The ability of C. fetus subsp. venerealis to act as a recipient, is currently being tested. Attempts to transfer campylobacter plasmids to E. coli were unsuccessful. Analogous host range limitations have been reported for other gram-negative organisms. Plasmids of the P2 incompatibility group from Pseudomonas aeruginosa could not be transferred to E. coli[29]. Apparently it is also difficult to transfer plasmids from Bacteroides fragilis to E. coli[30]. The reason for the failure of plasmids from one species to transfer to and/or replicate in a different species is, as yet, undetermined.

The studies reported here demonstrate that antibiotic-resistant campylobacters, like many pathogenic bacteria, may harbour resistance plasmids. Further work is required to determine both the source of these plasmids and the origin of their resistance determinants.

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STUDIES ON DRUG RESISTANCE TRANSPOSONS IN *HAEMOPHILUS INFLUENZAE* R PLASMIDS

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INTRODUCTION

The emergence of R plasmids in *H. influenzae* is of great clinical concern, since *H. influenzae* causes serious infections including meningitis, epiglottitis, pneumonia, and otitis media. Resistance to ampicillin (Ap), as well as to tetracycline (Tc) was shown to be plasmid linked (1, 2). The conjugative Haemophilus R plasmids that have been described are closely related to each other and have most of their base sequences in common independent of their geographical origins and their antibiotic resistance markers (3, 4). This paper examines whether these R factors arose as a result of the transposition of different resistance genes on to closely related indigenous *H. influenzae* plasmids or whether closely related R factors from the same incompatibility group with different resistance genes have now infected *H. influenzae* strains throughout the world.

MATERIALS AND METHODS

Bacterial strains, media, isolation of plasmid DNA, conjugation, DNA-DNA duplex studies and electron microscope DNA heteroduplex and homoduplex analysis have been recently described (5).

RESULTS

Relationship between the H. influenzae R plasmids

The *H. influenzae* R plasmids which were shown to be self-transmissible have similar molecular properties. The molecular weights of these R plasmids are between 30 to 40 Mdal and they have most of their base sequences in common as was shown by the analysis of plasmid DNA homo- and heteroduplexes using the single-strand specific endonuclease S1 (Table 1).

Table 1: Relationship between *H. influenzae* R plasmids

R plasmid	Resistance marker	Molecular weight (Mdal)	% DNA sequence homology with pKRE5367 (Ap^r)
pKRE5367	Ap^r	30	100
pFR16017	Tc^r	33	71
pHK539	Tc^r, Ap^r	36	80
pR1234	Tc^r, Cm^r	35	63
pHI706	Tc^r, Cm^r, Ap^r	38	81

Characterization of the resistance genes by molecular DNA-DNA hybridization using plasmids with known transposons as molecular probes

The presence of the ampicillin transposon Tn3 in the ampicillin resistance specifying plasmids was demonstrated by molecular hybridization studies using the 5.5 Mdal plasmid RSF1030 (6) as a molecular probe. The DNA-DNA duplexes between the ^{3}H-labeled RSF1030 DNA and the ampicillin resistance specifying plasmids were analysed using the single-strand-specific endonuclease, S1, of Aspergillus oryzae (7). Plasmid RSF1030, which contains the whole Tn3 representing 58 % of its total DNA, had 49 % to 53 % of its base sequence in common with the ampicillin resistance specifying *H. influenzae* plasmids (Table 2).

As a molecular probe for the detection of the tetracycline resistance genes pKT007 (8) and a lambda phage containing Tn10 (9) were used, which contains the whole DNA sequence of Tn10. The homology ranged between 12 %

and 17 % indicating, that the tetracycline resistance inducing H. influenzae R plasmids contain the transposable element Tn10 (Table 2).

Table 2: Hybridization between ^{3}H-labeled RSF1030 (Tn3), pKT007 (Tn10) and phage λ::Tn10 DNA and whole cell DNA from H. influenzae strains

Source of unlabeled DNA	DNA sequence homology with ^{3}H-labeled plasmid or phage DNA (%)		
	RSF1030 (Ap^r)	pKT007 (Tc^r)	λ::Tn10 (Tc^r)
pKRE5367 (Ap^r)	51	0	0
pFR16017 (Tc^r)	0	28	17
pHK539 (Tc^r, Ap^r)	53	21	12

The presence of Tn9 in the R plasmids coding for combined tetracycline and chloramphenicol resistance could not be demonstrated as clear as the presence of Tn10 since only a small part of the lambda phage used as a molecular probe represented the DNA sequence of Tn9 (10). The presence of Tn9 was mainly indicated by analysis of homo- and heteroduplexes in the electron microscope.

Characterization of DNA sequences in the electron microscope specifying tetracycline, ampicillin and chloramphenicol resistance

Heteroduplex molecules between the different H. influenzae R plasmids were studied in the electron microscope. The plasmids specifying for ampicillin resistance were heteroduplexed with those specifying for tetracycline resistance. The heteroduplex molecules showed an insertion loop with the characteristics of Tn3 (6) and an insertion of a structure characteristic for Tn10 (9).

The H. influenzae R plasmids coding for combined resistances, however, showed unexpected insertions.

The plasmids coding for combined ampicillin and tetracycline resistance had the Tn3 integrated in one of the inverted repeats of Tn10 close to its end toward the Tc resistance specifying genes. The plasmid coding for combined tetracycline and chloramphenicol resistance carried a translocatable DNA segment composed of Tn10

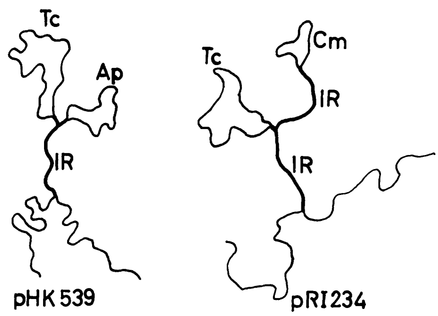


Fig. 1. Diagram of self-annealed pHK539 (Tc^r, Ap^r) molecule shows the Tn3 (Ap^r) inserted in one of the inverted repeats (IR) of Tn10 (Tc^r) and the self-annealed pRI234 (Tc^r, Cm^r) shows the Tn9 (Cm^r) on long inverted repeats inserted in one of the inverted repeats of Tn10. Electron micrographs of formamide-spread single stranded plasmid DNA.

containing an insertion of the chloramphenicol resistance transposon Tn9 (11). Tn9 was found to be inserted into one of the components of the Tn10 inverted repetitions and is itself flanked on both sides by long inverted repetitions. The localisation of the integration site of Tn9 in one of the inverted repeats of Tn10 was similar to that of Tn3 in Tn10 (Fig. 1).

The R plasmids coding for tripleresistance against tetracycline, chloramphenicol and ampicillin showed the same combined structure out of Tn10 and Tn9 as was found in the doubly resistant R plasmids (Tc^r, Cm^r). In addition the Tn3 integrated independently of the combined transposon at a different site of the plasmid core.

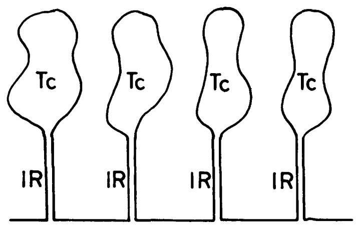
Transposition of Tn3

A 3.2 ± 0.2 Mdal DNA sequence of pKRE5367 as well as of pHK539 was transposed into the 5.5 Mdal plasmid RSF1010 (Su^r, Sm^r) (6) by conjugative transfer of the H. influenzae R plasmids to *E. coli* C600 carrying RSF1010 and selecting for ampicillin resistant clones. The resulting 8.7 ± 0.2 Mdal hybrid plasmid was shown to be RSF1010::Tn3 by analysis with restriction enzyme EcoR1 and electron microscope heteroduplex studies.

Multiple integration of drug resistance transposons

The *H. influenzae* R plasmids with the transposon Tn10 showed after prolonged growth in medium containing tetracycline one, two, three, four or even five copies of Tn10 integrated in the same plasmid. The minimum inhibitory concentration against tetracycline increased from 20 µg to 30 µg of tetracycline ml^{-1}. The molecular size of the DNA sequence between the integration sites was found to be similar in all molecules studied (Fig. 2).

The *H. influenzae* R plasmids with the tetracycline-chloramphenicol resistance transposon was integrated two or three times in the plasmids after their growth in medium containing tetracycline. The presence of multiple copies of the transposon correlated with higher minimum inhibitory concentrations against tetracycline as well as against chloramphenicol. The MICs had risen from 10 to 30 µg of tetracycline ml^{-1} and from 10 to 40 µg of chloramphenicol ml^{-1}. The intervening DNA segments between the integration sites had the same length as those found between the Tn10 copies found in the monoresistant strains (Fig. 2).



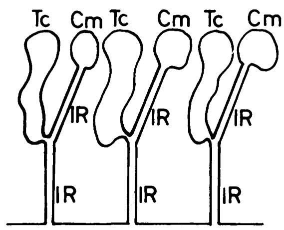


Fig. 2. Schematic drawing of the multiple integration of Tn10 (Tc^r) in pFR16017 (Tc^r); 1 to 5 copies of Tn10 were found to be integrated in the same plasmid core in self-annealed molecules (top); one, two or three copies of the Tn10-Tn9 (Tc^r, Cm^r) transposons were found to be integrated in pRI234 (bottom). The intervening DNA sequences between the integration sites had the same molecular size in pFR16017 as well as in pRI234; IR: inverted repeats.

In vitro creation of multiresistant H. influenzae R plasmids

Doubly resistant H. influenzae strains were obtained by conjugational transfer of H. influenzae R plasmids carrying Tn3 and H. influenzae R plasmids carrying Tn10 within the H. influenzae isolates. R plasmids were obtained which contained Tn3 and Tn10 integrated at different sites in the same plasmid core and only in one out of ten molecules examined in the electron microscope the Tn3 was integrated in one of the inverted repeats of Tn10 as it was found in the doubly resistant natural isolates.

The Tn10 of pFR16017 as well as Tn3 of pKRE5367 was found to be integrated in the same plasmid core with the combined Tn10 - Tn9 transposon of pHK539 after conjugation of the different strains. However, Tn3 was never found linked to the combined Tn10 - Tn9 transposon.

Isolation of an indigenous H. influenzae plasmid

Fresh isolates of H. influenzae and H. parainfluenzae were examined by agarose gel electrophoresis for the presence of extrachromosomal DNA. Among 699 isolates only one H. influenzae type B isolate was found with the expected indigenous plasmid (pW266). Its molecular size was 27 Mdal and it had 82 % base homology with pKRE5367. This plasmid, which does not carry any detectable drug resistance markers, could not be regularely demonstrated by agarose gel electrophoresis and it seems possible that the chromosomal integration of pW266 is a frequent event (12).

DISCUSSION

The H. influenzae R plasmids examined are not replicate isolates of one plasmid clone. They not only have different molecular weights and different resistance markers, but also differ slightly in the base sequences of their plasmid cores. The presence of Tn3 in the ampicillin resistance specifying H. influenzae R plasmids was indicated by base sequence homology with the plasmid RSF1030 containing the Tn3 and the ampicillin resistance specifying DNA sequence was transposed from the H. influenzae plasmid pKRE5367 and pHK539 on to the E. coli plasmid RSF 1010. The genes for tetracycline resistance were shown to be Tn10 by molecular hybridization studies with a lambda phage containing Tn10.

It was shown by electron microscopy that the H. influenzae P plasmids coding for combined tetracycline and ampicillin resistance or combined tetracycline and chloramphenicol resistance carried Tn3 or Tn9 integrated in one of the inverted repeats of Tn10. The passage of the Tc plasmids in medium containing tetracycline regularely resulted in the multiple integration of the Tn10 or of the combined Tc-Cm transposon into the same plasmid core. The multiple integration of the transposable elements was paralleled by an increase of the resistance against Tc in the case of pFR16017 and against Tc and Cm simultaneously in the case of pHK539.

The similarity between the cores of the H. influenzae R plasmids examined and the identity of the drug resistance transposons with those found in the enterobacteriaceae is compatible with the hypothesis that the H. influenzae R plasmids arose as a result of the transposition of different drug resistance transposons on to closely related indigenous H. influenzae plasmids. This idea is further supported by the isolation of an indigenous plasmid which had the expected molecular size and had almost all of its base sequences in common with the H. influenzae R plasmids. Furthermore, the in vitro creation of multiresistant H. influenzae plasmids by conjugation of monoresistant and doubly resistant strains revealed plasmids which were indistinguishable from the multiresistant clinical isolates. An alternative hypothesis is that closely related R factors from the same incompatibility group have now infected H. influenzae strains throughout the world. Such an incompatibility group, however, has not been detected yet.

ACKNOWLEDGMENTS

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PLASMIDS IN STREPTOCOCCI: A REVIEW

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INTRODUCTION

The genus Streptococcus contains several clinically, industrially and ecologically important species, and many relevant properties resemble, phenotypically, similar traits known to be associated with plasmids among members of other bacterial genera. Studies on the contributions of plasmids to the virulence and metabolic versatility of the streptococci have, however, only recently begun. The purpose of this review is two-fold: 1) to highlight some of the major developments in the field of streptococcal plasmid biology over the past eight years, and 2) to present data relevant to areas of current activity in the field.

HISTORY OF STREPTOCOCCAL PLASMID BIOLOGY

The first report of plasmids in streptococci appeared in 1972 with the demonstration by Courvalin et al.[1] of plasmid-linked tetracycline and erythromycin resistance in a strain of Streptococcus faecalis. In 1974, Jacob and Hobbs[2] provided the first conclusive evidence for conjugal transfer of plasmid-borne multiple antibiotic resistance in S. faecalis, and in 1975 Jacob et al.[3] described a transmissible hemolysin plasmid in the same strain. These reports stimulated several groups to become actively engaged in streptococcal plasmid research, but, with few exceptions[4,5,6,7], work prior to 1976 was done with strains of S. faecalis. This was primarily due to difficulties in lysing other streptococcal species, especially those obtained from natural sources. In 1976 Chassy[8] reported that growth of streptococci in a rich medium supplemented with threonine, which inhibits cell-wall cross linking, yielded cells which could be rendered susceptible to detergent lysis following treatment with lysozyme. Using this approach, and adapting existing plasmid enrichment techniques originally used for the isolation of plasmids from gram-negative bacteria, we[9], as well as others[10], were able to isolate and visualize directly on agarose gels, plasmids from virtually any species of Streptococcus, and quite often from less than 10 ml of culture.

Another initial obstacle to the study of plasmids among the streptococci was the unavailability of plasmid transfer systems in this genus. All three of the major bacterial genetic transfer systems, transduction, transformation and conjugation are now available. Plasmids have been transferred by transduction among Lancefield groups A and G[11], and between strains of group N[12] streptococci. Two strains have been used extensively for interspecies transfer of plasmids by transformation, the group H Challis strain of S. sanguis[13,14,15,16,17] and a group F isolate[18]. More recently pneumococci have also been used for this purpose[19]. All three of these strains exhibit physiological competence. So far, all attempts to render other streptococcal species competent for transformation by artificial means have failed. Two types of "conjugation" systems have been described in the streptococci. The CIA, or pheromone-enhanced mating system, which appears to be confined to group D streptococci, is discussed in the paper by Clewell (this volume). The second type of plasmid transfer requiring cell-to-cell contact is facilitated by bringing donor and recipient cells together on a membrane filter. This system has been used for intra- and interspecies transfer of a number of transmissible plasmids[15,16,17,20,21,22,23].

With the development of plasmid transfer systems, and improved plasmid isolation techniques, the role of extrachromosomal elements in a variety of streptococcal functions has now been established. Plasmid-mediated single and multiple antibiotic resistance has been reported in most species of streptococci which are isolated from human or animal sources[1,2,7,14,15,16,17,20,22,24,25]. Efstathiou and McKay[26] have described a role of plasmids in the resistance of S. lactis strains to inorganic ions such as silver, copper, chromate, arsenite and arsenate. Many clinical isolates of S. faecalis are β-hemolytic on horse and human blood, but not sheep blood[27]. These hemolysins are often linked, if not identical, to bacteriocins on plasmids which are usually transmissible[3,27,28]. Two transposons, one carrying resistance to erythromycin[29] and the other tetracycline[30], have been identified in a strain of S. faecalis. We have recently obtained preliminary results suggesting the presence of a kanamycin-streptomycin resistance transposable element in a different strain of S. faecalis (D. J. LeBlanc and L. N. Lee, unpublished observations). Plasmids may also play an important role in the metabolism of the group N streptococci, as discussed below.

PLASMID-MEDIATED METABOLIC ACTIVITIES AMONG THE GROUP N STREPTOCOCCI

The ability of the group N, or dairy, streptococci to catabolize or produce a variety of organic compounds appears to be plasmid-mediated. Plasmids have been implicated in the fermentation of lactose[31,32], galactose[33], or sucrose[34], glucose-mannose[34] and D-xylose[34]; in the utilization of citrate[35] and uric acid (J. A. Breznac, personal communication); and in the production of proteinases[31] and the small peptide antibiotic, nisin[34], in at least some strains of the Lancefield group N streptococci. With the exception of a few lactose plasmids, which have been shown to be transmissible[36,37,38], and some lactose and proteinase plasmids which have been transferred by transduction[12,39], all plasmid-associated traits in this group of streptococci have been identified by curing experiments.

We are currently using a new approach to learn more about the group N metabolic plasmids. We have chosen the lactose pathway, which is quite different from the corresponding pathway in enteric bacteria, for our initial studies. Among streptococci lactose enters the cell as a phosphorylated molecule by the activity of a phosphoenolpyruvate-dependent phosphotransferase system[40], or PTS. The lactose phosphate is then cleaved, by a phospho-β-galactosidase[41], or P-β-gal, to glucose and galactose-6-phosphate, which are further catabolized by glucose- and galactose-specific pathways. In collaboration with E. J. St. Martin we have recently employed the Challis transformation system to obtain preliminary evidence suggesting that the structural genes for the two specific lactose pathway enzymes are carried by the lactose plasmid in a strain of *S. lactis*. These results are summarized in Table 1. When *S. lactis* strain DL11 was grown at the expense of lactose it possessed PTS activity for both galactose and lactose in a ratio of 0.3 to 1[33]. Using an activity stain for P-β-gal (E. J. St. Martin, L. N. Lee and D. J. LeBlanc, manuscript in preparation), on a polyacrylamide gel containing total cell protein from strain DL11, we obtained a molecular weight estimate for P-β-gal of 40,000. In contrast, a lactose-grown wild-type culture of the Challis strain had a galactose to lactose PTS ratio of only 0.05 to 1, and its P-β-gal had a molecular weight of 52,000. We isolated a lac-negative mutant of the Challis strain, lac-8, which was missing P-β-gal activity, but retained lactose PTS. This mutant was transformed by plasmid DNA from the *S. lactis* strain, and selected for ability to grow on lactose. These transformants exhibited the same galactose to lactose PTS ratios as the Challis strain, but now possessed a P-β-gal activity with the same molecular weight as that of the donor strain. A second mutant, lac-83, missing both lactose PTS and P-β-gal, was isolated from the lac-8 mutant of Challis. When this strain was transformed by plasmid DNA from strain DL11, it had PTS and P-β-gal activities with the properties of the donor *S. lactis* strain.

Plasmid DNA could not be isolated from the Challis transformants, nor were they curable for lactose fermentation. We tentatively interpret this to mean that structural genes on the lactose plasmid from S. lactis have integrated into the Challis chromosome to restore functions required for lactose metabolism. We are currently preparing labeled probes of whole lactose plasmid DNA, as well as restriction fragments, to locate the appropriate sequences on the plasmid, and to determine if, in fact, these sequences are integrated into the Challis chromosome. Similar approaches are also being used to examine other plasmid-mediated metabolic functions of the group N streptococci.

ANTIBIOTIC RESISTANCE

The streptococci, as with all other bacterial genera inhabiting human and animal niches, have become increasingly resistant to antibiotics. Several of these resistance traits have been shown to be plasmid-mediated in at least some streptococcal isolates. Plasmid-associated resistance to aminoglycosides and to chloramphenicol is mediated by antibiotic modifying enzymes which resemble in activity, but are clearly different from, their counterparts in gram-negative bacteria[24,25,42].

The vast majority of studies on antibiotic resistance plasmids in the streptococci have centered on resistance to erythromycin[7,13,14,15,16,18,20,21,22,23,29]. Streptococcal and staphylococcal plasmids mediating resistance to this antibiotic are referred to as MLS plasmids because the mechanism of resistance, N^6-dimethylation of

Table 1. Characterization of lactose$^+$ transformants of S. sanguis by plasmid DNA from S. lactis

Strain	Gal/Lac PTS[a] Activity Ratio	P-β-Gal[b]
S. lactis DL11	0.30	40,000
S. sanguis (Challis)	0.05	52,000
S. sanguis lac-8	+	-
pDL1 X S. sanguis lac-8	0.07	40,000
S. sanguis lac-83	-	-
pDL1 X S. sanguis lac-83	0.29	40,000

[a]See reference 33 for assay procedure
[b]Assay procedure in (E. J. St. Martin, L. N. Lee, and D.J. LeBlanc, manuscript in preparation).

adenine in 23S ribosomal RNA, also results in resistance to other macrolides, to lincosamides and to streptogramin B type antibiotics[43, 44]. This mechanism is common among streptococci[44], *Staphylococcus aureus*[43] and the producer of erythromycin, *Streptomyces erythreus*[45]. MLS plasmids isolated from several streptococci[16,44,46,47], as well as staphylococci[44], all share at least some common sequences with each other. With the exception of a few large MLS plasmids in strains of *S. faecalis*[2,20], with transmissibility apparently limited to this species, and one small non-transmissible plasmid isolated from a strain of *S. sanguis*[14], the vast majority of MLS plasmids in streptococci have a very narrow molecular weight range, between 15 and 20 million, and a very broad range of hosts to which they may be transferred by filter matings[15,16,20,21,22,23]. Not only can these plasmids be conjugally transferred among virtually all species of streptococci, but at least three intergeneric crosses have been successfully attempted[48,49] (O. E. Landman et al., Abstracts of the Annual Meeting of the American Society for Microbiology, p. 114, 1980). One of these MLS plasmids, pAMB1[50], often becomes partially deleted following transfer to a new host. These deletions, which range from approximately 2.3 to 18.8 kilobases, from a molecule originally 25.7 kilobases in size, often result in the loss of transmissibility. We have collected several of these deleted molecules and are attempting to locate on a restriction map of the original plasmid, and enumerate, plasmid functions required for conjugation.

UNUSUAL PROPERTIES OF TETRACYCLINE RESISTANCE DETERMINANTS

Among gram-negative bacteria of clinical origin, tetracycline resistance has almost always been associated with a plasmid[51]. However, Burdett[17] has reported that among 30 tetracycline resistant group B streptococcal isolates, 27 could not be shown to harbor tetracycline resistance plasmids. Similarly, in a recent study of tetracycline resistant streptococci obtained from the human oral cavity, we were able to isolate plasmid DNA from only 23 of 121 strains examined[52]. Yet, we have observed transfer, on membrane filters, of resistance from 14 out of 50 of these isolates to a strain of *S. faecalis*. Only 4 of the strains with transmissible tetracycline resistance had detectable plasmids (D. J. LeBlanc and L. N. Lee, unpublished observations).

Recent reports from two different laboratories appear to offer possible explanations for these results. Guild and associates[19] have demonstrated chromosomal linkage, by transformation, of a chloramphenicol and a tetracycline resistance determinant in a strain of *S. pneumoniae*. These investigators subsequently observed conjugal transfer of these determinants by filter matings to a recipient strain of the same species[53]. No plasmid DNA could be isolated from the donor or from transconjugant isolates. Franke and Clewell[30] recently reported the conjugal transfer of a tetracycline resistance determinant located on a transposon between strains of S. faecalis, in the absence of a plasmid DNA. The authors suggest that the transposon may be a plasmid-like (or phage-like) element that lacks replicative autonomy while retaining specific information for transfer. We, in collaboration with J. A. Donkersloot, have been studying a transmissible tetracycline resistance determinant in a porcine isolate of S. mutans, which exhibits a somewhat different type of apparent plasmid-less transfer. S. mutans strain DL5 is resistant to high levels of streptomycin, MLS antibiotics and tetracycline. Extensive searches for plasmids in this strain, using several different methods, have all proven negative. Although MLS and streptomycin resistance could not be transferred by this strain, tetracycline resistance was, as shown in Table 2. When the JH2-2 strain of S. faecalis[2] was used as the recipient transfer occurred almost equally as well in broth matings as on filters. A recombination-deficient strain of S. faecalis, UV202[54], was also a good recipient. However, with a S. mutans strain, DR0001/1[21], as the recipient the transfer frequency was almost two orders of magnitude lower and occurred only on filters. One of these transconjugants, strain DL43, transferred tetracycline resistance to strain JH2-2, but at a low frequency and only on filters. Resistance was not transmissible from any of the S. faecalis transconjugants. As shown in Table 3, we have observed plasmid DNA in some transconjugant isolates. The S. mutans transconjugant, strain DL43, which can transfer tetracycline resistance, occasionally yielded a band in agarose gels with a migration rate consistent with a molecular weight of approximately 19 million. The S. faecalis transconjugants, which do not appear to transfer the resistance determinant, consistently yielded a plasmid with a molec-

Table 2. Transmissibility of a Streptococcus mutans tetracycline resistance determinant

Donor Strain	Recipient Strain	Conjugation[a] Frequency
S. mutans DL5	S. faecalis JH2-2	2×10^{-6} (F)[b]
		5×10^{-7} (B)
	S. faecalis UV202	1×10^{-6} (F)
	S. mutans DR0001/1	1×10^{-8} (F)
S. mutans DL43	S. faecalis JH2-2	1×10^{-8} (F)

[a]per input donor colony forming units
[b](F) or (B) refer to filter or broth matings

Table 3. Properties of transconjugant isolates receiving tetracycline resistance from S. mutans DL5

Strain	Derivation	Plasmid (Mdal)	Curing Frequency[a]
DL5	S. mutans donor	-	< 0.3%
DL43	DL5 X S. mutans DR0001/1	19?	< 0.3
DL40	DL5 X S. faecalis JH2-2	8	4.5
DL178	DL5 X S. faecalis UV202	8	13.5

[a]Curing frequencies were determined after 40 to 50 generations in the absence of tetracycline.

ular weight of 8 million. The covalently closed circular nature of this latter plasmid species has been confirmed by dye buoyant density gradient centrifugation. We have also examined these isolates for spontaneous curing of tetracycline resistance following 40 to 50 generations in the absence of antibiotic. We could not detect curing in either of the S. mutans strains, but the curing frequencies in a JH2-2 transconjugant, strain DL40, and a rec⁻ transconjugant, DL178, were relatively high. All cured strains examined had lost the 8 megadalton plasmid. We cannot yet explain all of these results, but the system resembles, in some respects, those chromosomally integrated conjugative plasmids which appear to be common among antibiotic resistant strains of Haemophilus influenzae[55,56]. We are currently trying to isolate sufficient plasmid DNA from the transconjugants to prepare labeled probes to investigate the possibility of chromosomal integration in the S. mutans strains.

We have recently observed yet another unusual property associated with tetracycline resistance in the streptococci. The JH1 strain of S. faecalis[2,3] harbors two transmissible plasmids, a 50 megadalton species, pJH1, mediating resistance to kanamycin, streptomycin, erythromycin and tetracycline, and a 35 megadalton species, pJH2, coding for hemolysin and bacteriocin production. Each of these plasmids can be transferred to the JH2-2 strain of S. faecalis during broth matings. After conducting such a mating experiment we recently obtained one transconjugant isolate with the properties shown in Table 4. This isolate, strain DL172, was resistant to tetracycline, but did not possess the other resistance traits associated with plasmid pJH1. Whereas the donor strain, JH1, was hemolytic in the presence or absence of tetracycline, strain DL172 was hemolytic only in the presence of tetracycline, and contained a plasmid intermediate in size between pJH1 and pJH2. Initially, we suspected

Table 4. Properties of *S. faecalis* strain JH1, JH2-2 and DL172

Property	JH1	JH2-2	DL172
Resistance to (μg/ml):			
Tetracycline	256	1	128
Kanamycin	10,000	500	500
Streptomycin	10,000	250	250
Erythromycin	1,000	1	1
Lincomycin	500	25	25
Hemolysis on Horse Blood:			
with tetracycline	+	no growth	+
without tetracycline	+	-	-
Plasmids present (Mdal)	50 & 35	none	43

that the tetracycline resistance determinant from plasmid pJH1 had integrated into pJH2. However, when the 43 megadalton plasmid from strain DL172 was purified, labeled with ^{32}P by nicked translation, denatured and incubated with a blot containing pJH1 and pJH2, plus chromosomal DNA from strain JH1, this plasmid hybridized with DNA in the chromosome region and with plasmid pJH2, but not with pJH1. We interpret these results to mean that a second tetracycline resistance determinant, from the chromosome of strain JH1, became integrated into pJH2 and was transferred into strain JH2-2. Regardless of the source of the tetracycline resistance determinant, it would appear that the plasmid-mediated hemolytic activity was affected by the presence of this DNA sequence, probably as a result of the location and orientation of insertion. In support of this conclusion, Clewell and associates[30] have recently described hyper- and non-hemolytic isolates of *S. faecalis* strain DS16, resulting from site-specific insertion of the tetracycline resistance transposon, Tn916, into the hemolysin plasmid, pAD1.

CONCLUSIONS

Although the existence of plasmids in the streptococci was only demonstrated eight years ago, a great deal has been accomplished in the area of streptococcal plasmid biology since then. Plasmids obviously play a significant role in the metabolism of the group N streptococci and in antibiotic resistance and its dissemination among several streptococcal species. Furthermore, the streptococci may be an important link in the dissemination of antibiotic resistance among gram-positive bacteria in general, particularly with regard to MLS resistance. Our ability to answer many existing questions are certainly now enhanced by the development of streptococcal recombinant DNA host-vestor systems recently described by Macrina et al.[57] and by Behnke and Ferretti[58].

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BACTERIAL PATHOGENICITY, AN OVERVIEW

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INTRODUCTION:

The majority of infectious diseases had begun to decline at the turn of the century with the understanding that potentially dangerous microbes could be transmitted by water, food and insects and by the application of sanitation and antisepsis. Since the birth of the modern antibiotic era some 45 years ago the incidence of bacterial disease has declined further and, despite the marvels of R plasmids we heard earlier today, most bacterial infections can be controlled, though usually not prevented, by chemotherapy. Some bacterial infections can be prevented by immunization although except for certain toxins, immunization is generally based on hit or miss whole organism vacccines. The fact is that if we understood more about how particular microorganisms caused infection it might be possible to devise rational means to prevent them.

Moreover, there is still the surprise of 'new' pathogens. The last decade alone has seen the emergence of the toxic shock syndrome (TSS) caused by certain strains of Staphylococcus aureus[1] and pneumonitis caused by Legionella pneumophila[2]. These organisms are, it seems to me, the responses of microorganisms to "civilization". Legionella seems to like water-cooling towers used for air-conditioning and are subsequently spread by aerosols while TSS staphylococci are associated primarily with increased tampon usage by women over the past decade. We might understand something of the selective pressures that have brought these organisms to the fore, we still don't understand why or even how they can cause disease.

In many parts of the world, in fact not far from this meeting hall, microbial diseases considered trivial by wealthy nations exact a heavy toll of mortality and morbidity. Diarrheal disease, leprosy, protozooan diseases, and infection with large parasites are the central problems. Widespread preventive measures are required. It would be easier to formulate these measures if we understand the mechanisms of infection.

In the following sections it is my intent to review general aspects of microbial pathogenicity. I shall not attempt to dwell on bacterial plasmids since the speakers that follow will do that and there have been several excellent reviews of the subject of plasmids and pathogenicity[3,4]. Rather I shall just summarize information that any interested individual might find in a textbook of medical microbiology or infectious disease text[5,6,7,8]. The references are few, the speculations are many. But it is in this way I hope to provide a perspective for the papers to come.

INFECTION AND DISEASE.

Thus far I have used the terms infection and disease interchangeably. It is useful to distinguish between the two terms. Infection is the persistent presence of a microorganism on the surfaces or within the tissue of the human body. The mere presence of the organism in the body however does not lead invariably to clinical illness, disease. In fact the production of disease in humans by most microorganisms is the exception rather than rule. It seems to me that disease occurs more as an abnormal event, an accident if you will, rather than an invariant outcome of infection. Generally speaking most of the time something goes awry with normal host defence mechanisms and tips the host-parasite relationship from a relatively innocuous compromise to a potentially dangerous event.

Hence all of us at one time or another carry pneumococci, meningococci and streptococci without misfortune. But let there be some new factor thrown into the equation, trauma or a toxic insult like alcohol and we may not escape so lightly. Not unexpectedly the disease caused by a particular microorganism depends upon its properties, its pathogenicity. Pathogenicity is a relative term. Virutally any microorganism can cause disease when host defenses are suppressed or compromised. One can visit any burn unit or cancer ward to see the devastation caused by 'ordinary microorganisms'. However the term pathogen is generally employed to refer to an organism that causes disease in 'normal' hosts. Virulence properly refers to the degree of pathogenicity among strains within a given species. It should come as no surprise to the participants of this meeting that the genetic potential for pathogenicity may vary greatly between species of microorganisms. For example only about a dozen of the myriad of E. coli serotypes are regularly found as the causative agents of diarrheal disease.

The Pathogenesis of Infection

a. The initial events.

The inital encounter between a pathogenic microorganism and host involves a variety of factors. As a first step it is clear that sufficient numbers of the organism must be taken in by the human host before infection takes place. The numbers of organisms required to establish infection and disease varies dramatically. Some microbial species (for example Shigella flexneri) are so virulent that only a few hundred viable cells are required to establish clinical disease in a significant number of susceptible persons. In other instances, enteropathogenic E. coli for example, millions of viable cells are required. Since E. coli and Shigella flexneri are so closely related genetically, these differences will be presumably understood in the not too distant future - indeed Dr. Kopecko will speak to this question later on in the proceedings. In any event the offending microorganism must possess the genetic capacity to proliferate within the potential host; obviously the capacity to spread from host-to-host is of equal importance.

After an organism gains entry into a human host, a certain period of time (incubation period) elapses before clinical illness is apparent. It should be apparent also that the incubation period will be dependent upon the multiplication rate of an invading organism. Few microorganisms multiply as well in vivo as they do artificial culture media. Obviously a microorganism which has a doubling time measured in days (for example the tubercle bacillus) will tend to cause a more slowly evolving infection and disease than a microorganism which has a doubling time within a host of a few hours (for example, Salmonella typhimurium). In addition, just as in the laboratory the in vivo pH and oxygen tension must be satisfactory for growth.

We are still pretty much ignorant of the properties of cells after they have grown in vivo; most investigators grow their cultures on plates or in broth. In vivo growth can have an enormous affect on infectivity. It seems clear, as Professor Harry Smith[10] has suggested for many years now, that microbiologists should devise clever techniques to examine the properties of in vivo - grown cells as well as the more conveniently prepared laboratory grown cells of pathogens.

Finally, in analysing the initial encounter between the human and a pathogen it is worthwhile to see that despite differences in the required inoculum size, the capacity of proliferate in vivo etc. virtually all infections can be divided into a relatively few categories:

1. Those in which the organisms have specific mechanisms for attaching to and sometimes penetrating the body surfaces of the human host.

2. Microorganisms introduced into the body of the normal host by a biting arthropod.

3. Organisms which are dependent upon previous tissue damage on severe impairment of host defense mechanisms for invasion to take place.

4. Organisms capable of causing disease through the secretion of toxic substances. Such organisms may not even need to penetrate body surfaces to cause disease.

Few pathogens fall snugly into just one of these categories but, in the simplest possible terms, bacteria cause disease either because they invade tissue, or elaborate toxins.

Bacterial Attributes of Virulence

1. The capacity to attach to the host cellular surface.

May microorganisms have the capacity of specifically adhere to host cells[11]. Indeed this adherence is highly evolved and is often quite specific for a certain host cell type. In the oropharynx, for example, *Streptococcus pyogenes* synthesizes a specific surface protein, the M protein, that permits it to tightly adhere to pharyngeal cells but not to teeth or the epithelial cells of the cheek. Similarly gonococci adhere to microvilli of columnar epithelial cells of the urogenital tract via pili and *Bordetella pertussis* specifically adhere to cilia on epithelial cells of the trachea. We shall hear later on from Jan van Embden concering properties of plasmid-mediated pili of toxigenic *E. coli* that permit adherence to epithelial cells of the small bowel. Thus many successful pathogens possess the capacity to 'stick' to the epithelial cell surface and the spread of the organism is very rapid on epithelial surfaces covered with liquid. These adherence mechanisms point out one fundamental microbial strategy to circumvent host defense mechanisms and to increase their likelihood of establishing a host-parasite relationship.

2. Spread of microorganisms in tissue.

Many infections stay pretty much confined to the epithelial cell surface and the organisms remain as extracellular parasites.

Such organisms may spread through the tissues effectively by mechanical means such as ciliary action, gravity, coughing, sneezing etc. Some degree of subepithelial spread will occur because of the death of host cells from microbial by-products. However, direct spread in the sub-epithelial tissue is often limited because of the connective tissue matrix. Some bacteria are known to elaborate specific enzymes including hyaluronidase that degrades the hayaluronic acid component of the connective tissue matrix and collagenase that breaks down the collagen of connective tissue. Other known bacterial enzymes and other extracellular factors thought to facilitate pathogenicity include coagulase (leading to the clotting of plasma), DNAse (thought to reduce the viscosity of exudates) as well as lecithinase and hemolysins both of which attack cell membranes). Whether one can asign specific roles in pathogenesis to any of these extracellular factors is not clear. Bacteria in general produce a variety of proteases and lipases that would theoretically play a role in bacterial pathogenesis. It is just as likely, however, that these enzymes play a role in bacterial nutrition and metabolism as they do in the infectious process. Certainly they can be considered as accessory determinants of pathogenesis at least in providing for the ease of mechanical spread of microorganisms throughout the tissue.

Most recently, a variety of pathogens that are largely restricted to the mucosal surface including *Streptococcus mitis*, *S. pneumoniae*, *N. meningitidis*, *H. influenzae* and *N. gonorrheae* have been found to elaborate a protease specific for human IgA1[2]. This specificity coupled with the known antibacterial and antiadhesive properties of IgA suggests that this enzyme may play an important role in pathogenesis although this has yet to be proved. Since the enzyme is found within microorganisms readily amenable to genetic study one can expect that it will be possible to assess the importance of this unusual class of enzymes. By the same token some bacteria, notably *Streptococci* and *Staphylococci*, actively bind the Fc portion of IgM, IgA and IgG. This property and antibody-specific proteases may all act to circumvent both specific and non-specific immune mechanisms.

3. *Invasion of epithelial cells*.

Some bacteria regularly penetrate epithelial cells during the course of infection. Precisely how the microorganisms accomplish this is not known. For example, members of the *Shigella* and *Yersinia* group adhere to the host cell surface and enter the cytoplasm often causing a local breakdown in the host cell plasma membrane. This invasive process may be ultimately lethal to the cell. In other cases, for example in *Salmonellosis*, the penetration of the organism may not be fatal to the cell; it is almost as if the microbial cell is "passing through" to deeper tissue layers. This is not simply a passive phenomenon. One can easily isolate mutants of Salmonella, or plasmidless derivatives of Yersinia and Shigella which fail to invade tissue. Yet the precise biochemical mechanisms at play remain the subject to study. Plasmid-mediated determinants that confer invasiveness in Shigella and Yersinia will be discussed later on in this session.

4. Subepithelial invasion.

Once an invading microorganism penetrates the basement membrane barrier it is exposed to important host defense mechanisms. The most important of these is the inflammatory response. The inflammatory response is a subject of considerable complexity. Suffice it to say that the host responds to a microbial insult by a prompt and vigorous change in its microcirculation. The reaction is the same for any part of the body - there is a dilatation of vessels and their permeability increases allowing the influx of serum, immunoglobulins and other proteins. Fibrinogen may be converted to fibrin so that a diffuse network is laid down to retard invading organisms. This is followed by active passage (diapedesis) of leucocytes and other cellular fractions into the insulted tissue. The lymphatics draining the affected area also become dilated, take up the inflammatory fluid and carry it to local lymph nodes where the macrophages lining the node act as filter agents.

It is at the subepithelial level that the battleground between the invading organism and the host take place. The outcome of the infection depends on the capacity of the inflammatory responses, particularly the phagocytes, to handle the invading microorganism and the microorganisms capacity to overcome normal host defense mechanisms. Two microbial factors that will be discussed in some detail at these meetings may come into play here. One of these, serum resistance, will be discussed by Ken Timmis. The other which is a more subtle but highly significant factor is the capacity of infecting microorganisms to sequester the free iron they require from growth from the host which in turn goes to great lengths to bind the iron in an unavailable form. Peter Williams' will describe to us the marvelous way that the Col V plasmid brings this capacity to certain E. coli strains.

a. Anti-phagocytic factors of extracellular parasites.

A number of microorganisms have ways of interfering with phagocytic activity. The best known example is the bacterial capsule although there are other antiphagocytic surface components. For example, the pathogenic success of the pneumococcus and Streptococcus pyogenes are due to their capacity to avoid phagocytosis. Similarly the M protein of Streptococci and the pili of gonococci that provide specific adherence to certain target host cells also appear to be associated with their relative resistance to phagocytosis. In any event, encapsulation or the presence of an antiphagocytic surface is quite common in both gram positive and gram negative microorganism. For most extracellular, parasites (those that cannot survive within phagocytes) the antiphagocytic cell surface is the critical determinant of virulence. If one isolates non-encapsulated mutants of pneumococci or cells that have lost their antiphagocytic surface, the mutant cells are avirulent. If one then isolates reversions or transfers genes conferring encapsulation virulence is regained. I am not aware there have been extensive genetic studies on most antiphagocytic determinants and their specificity, however.

Some microorganisms, like Streptococcus pyogenes, not only resist phagocytosis through the nature of its cell surface but also because it elaborates a phagocytic poison, leukocidan. Similarly some Staphylococci elaborate a substance that induces the granules of white cells to discharge into its own cell cytoplasm; the white cell is thus duped into killing itself rather than the microorganism. Such microbial tactics imply a long-standing evolutionary relationship between host and parasite that would be amenable to genetic study.

b. Antiphagocytic factors of intracellular bacteria.

Some bacteria like the tubercle bacillus, the leprosy bacillus and the cause of undulant fever, Brucella sp. grow in macrophages that phagocytose them. Their success as infectious agents depends on this. It is not clear if these bacteria have specific receptors that interact with the phagocytic surface or not. Clearly if a phagocytosed microorganism is not exposed to the killing and digestive processes of the phagocyte, it can survive and even multiply. This indeed seems to be a factor leading to the survival of the tubercle bacillus within macrophges.

The ways in which microorganisms survive within phagocytes is not clearly understood. In this regard it is survival within macrophages that is crucial. Polymorphs have a short lifespan but macrophages live for comparatively long periods of time. Of course, bacteria in a macrophage are protected against many antibiotics as well as antibody that cannot penetrate the macrophage surface.

6. Bacterial Toxins.

In my mind, bacterial toxins provide one of the more clear cut examples of the polygenic nature of microbial pathogenesis. The bacterial toxins are broadly divided into exotoxins, those that are liberated in the environment from multiplying bacteria and endotoxins, those that are associated with the gram negative cell wall and are released, in large part, upon death of the microorganism.

In some microorganisms the capacity to elaborate an exotoxin is the primary determinant of pathogenicity and the clinical symptoms in a patient can be accounted for by the action of the toxin alone. This is certainly true of botulism, staphylococcal food poisoning, tetanus, diphtheria, cholera and E. coli enterotoxins.

It is important, however, to make a distinction between the pharmocological action of pure exotoxin and the reality of its role in microbial pathogenesis. Undoubtedly in the case of botulism and staphylococcal food poisoning, the ingestion of preformed toxin in food will lead to clear-cut clinical disease; the microorganism need not multiply the host. One can duplicate diphtheria and tetanus, by parenteral toxin infection, and cholera and E. coli diarrhea by directly injecting toxin into ligated loops of bowel. Yet, many animals and man regularly carry toxigenic diphtheria bacilli and Clostridium tetani without apparent harm. Microbial cells which synthesize even large quantities of E. coli enterotoxin but lack the ability to effectively colonize the small intestine are innocuous to animals. Hence the important point to me is not that a purified toxin can cause injury when injected into an animal or a human but rather it is the sum total of toxigenicity coupled with the other determinants of the microorganism that is essential. C. tetani is innocuous if not introduced into tissue with a low redox potential; C. diphtheriae must be able to establish itself in the oropharynx (or a skin lesion), and multiply before the effects of intoxication can be appreciated. The delivery system is as important as the toxin. We still understand very little about the acessory determinants of pathogenicity in "purely" toxigenic pathogens.

At the end of this discussion of the general attributes of bacterial pathogenicity it is useful perhaps to note that rarely are invasiveness and toxigenicity completely separable. Invasive organisms often utilize factors of short-lived or local toxigenicity (a leukocidan, for example) while as noted above, toxigenic bacteria must possess at least some degree of bacterial multiplication and persistance in the tissues.

Plasmids and Pathogenicity

There has been a growing appreciation over the last decade that determinants carried by bacterial plasmids may directly contribute to bacterial pathogenicity. In the papers that follow we shall hear some of the details involving plasmid-mediated determinants of toxigenicity, adherence, serum resistance, invasiveness and iron scavanging. Many of us are fascinated by the fact that most bacterial toxins are associated with plasmids and bacteriophage. Why this should be I have no idea. It can not be accidental that this is so. Is this associated with the transmissibility of the genetic elements or their dispensability? Presumably the tactic that a plasmid-mediated determinant can be lost without affecting viability could be the most important factor. Under many circumstances we can imagine that a determinant of pathogenicity is a deterrent to microbial success. If it can be lost and later regained, it seems the best of both worlds.

Plasmid-mediated determinants of pathogenicity encompass a tantalizing array of elements and I believe we have yet but touched the tip of the iceberg. On the other hand, given the complexity of the steps in the pathogenesis of infection, the many genes that must be at play, the very dispensability of plasmids and the fact that only one or a few genes of pathogenicity are plasmid-mediated makes me a bit cautious in over interpreting their significance. For example we have shown that the heat labile enterotoxin of E. coli seems always to be plasmid-mediated while the closely related gene in V. cholerae is chromosomal[13]. Similarly there are chromosomal iron sequestering systems, determinants of serum resistance, hemolysins and invasive factors. This does not detract from the importance of plasmid-mediated determinants of virulence. Indeed because they can be so readily studied at the genetic and molecular level, they are of considerable importance to better understand pathogenicity. However, my guess is that the importance of plasmids to the pathogenicity of any particular organism lies more in the genetic flexability rather than the precise nature of the pathogenic determinant carried by plasmids.

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CLONING AND EXPRESSION OF THE GENES ENCODING FOR THE ADHESIVE ANTIGENS K88 AND K99

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INTRODUCTION

More than a decade ago, enterotoxigenic E.coli strains were found to be associated with acute diarrhoea in young animals and later such strains were also found to be involved in cases of human diarrhoea. Enterotoxigenic E.coli strains release a heat labile toxin and/or a heat stable toxin which effects the fluid and electrolyte secretion in the intestine by activation of the mucosal enzymes adenyl cyclase and guanyl cyclase, respectively[1,2]. A number of proteinaceous surface antigens of enterotoxigenic E.coli have been identified, that are involved in the colonization of the gut by facilitating the adherence of the microorganism to the intestinal mucosa. Enterotoxins and several of these colonization factors are encoded by plasmids. The significance of organisms that possess plasmid-mediated pathogenic characteristics is that they constitute a genetic pool from which new lines of pathogenic organisms may arise. To the research worker, they represent genetic material that can be added or removed from organisms, thus permitting the construction of new lines which differ only from the parent microorganism by the presence or the absence of one character. Smith and coworkers exploited this idea to elucidate the pathogenesis of E. coli diarrhoea in animals. They showed in an elegant series of experiments that the antigens K88 and K99 promote colonization of the intestine by implanting K88 and K99 plasmids into non-pathogenic strains of E. coli or alternatively by removal of these plasmids from pathogenic strains and subsequently feeding such modified strains to neonates[3,4,5]. The adhesion of K88 to the intestinal mucosa was demonstrated by Jones and Rutter[6]. Vaccination with K88 antigen results in protection of neonatal animals by the antibodies induced in the colostrum[7,8]. The K88 antigen is found on the surface of the E.coli cell as a thin filament of protein. Such filaments, also called pili or fimbriae, have been found among a great variety of bacteria. Among enterotoxigenic E.coli strains, 5 serologically unrelated pili have been found to be associated with adhesion and colonization of the intestine: K88, 987P, K99, CFA I and CFA II[9,10,11]. Each of these adhesive antigens is found among a characteristic set of serotypes of E.coli. E.coli strains producing the antigens K88 and P987 are found in diseased piglets, K99 mainly in calves and lambs and to a lesser extend in pigs. CFA I and CFA II have been associated with human strains. All five adhesive antigens share the following properties: The antigens are high molecular weight structures, composed of identical non-covalently linked polypeptides having molecular weights between 14,000 and 26,000. Each antigen can adhere to a specific set of animal cells, including erythrocytes and epithelial mucosa cells of the intestine; at temperatures of 18-20°C no or very little of the antigen is produced. Furthermore, the capability to produce pili is usually an unstable genetic trait. Although in certain cases this instability is due to the loss of plasmids which encode for the pili, this is not always the case. The protein of the adhesive antigens analysed are rich in hydrophobic amino-acids. Some of the properties of various pili are depicted in table 1. It is presumed that the pili recognize particular receptor structures on the surface of the animal cell. Until now no such receptors for any of the 5 adhesive antigens have been isolated and characterized in biochemical terms, although several attempts have been made[12,13,14]. In order to study the genetic organization and the expression of adhesive antigens we have cloned the genes encoding for K88 and K99.

Table 1. Properties of adhesive antigens of enterotoxigenic E.coli

Antigen	Diameter (nm)	Mol.weight sub unit	Genetic location	Origin
K88	2	25,000	plasmid	piglet
K99	3	18,500	plasmid	calf, lamb, piglet
987P	7	22,000	?	piglet
CFA I	6-9	14,000	plasmid	human
CFA II	7-8	?	plasmid	human

EXPRESSION AND CLONING OF THE K99 GENES

The K99 antigen is of particular interest with regard to its peculiar regulation of expression. Certain serotypes, like O8, O9, and O20, produce considerably less K99 than wild type strains of the serotypes O101[15]. This regulation seems to be a host dependant trait because no difference in the level of K99 expression is observed when K99 plasmids of high and low producing serotypes are transferred to E.coli K12. Furthermore, K99 production is highly dependent on the composition of the growth medium. Because the K99 antigen is usually difficult to detect by agglutination with antiserum after growth on common media, Guinée et al.[16,17] developed a supplemented minimal medium. Isaacson showed that the amount of K99 on the surface of the cell is subject to catabolite repression: glucose repression could be overcome by addition of 0.5 mM cyclic AMP to the medium[18]. More dramatically, however, is the effect of the presence of alanine. When alanine is added to minimal medium at concentrations above 1 mM, the K99 production is less than 3% compared to that after growth in the absence of alanine[19]. By cloning of the K99 genes and introduction of mutations in these genes we hope to get more insight in this regulatory system. Plasmid pRI9901 was used for cloning of the K99 determinant and it originated from E.coli O101:K99 strain B41. The K99 plasmid was transferred conjugally to E.coli K12 in order to separate it from the other 3 plasmids that are also present in B41. The K99 genes were cloned into the Hind III site of pBR322 and subcloned into the Bam HI site of pBR325. Four subclones were obtained which all contained a 4.5 Md BamHI fragment. Three of them expressed K99 and one clone produced very little K99 although the 4.5 Md was undistinguishable from the fragment of the other 3 subclones as analysed by multiply cutting enzymes on agarose gels[20]. Morris et al.[21] found spontaneous K99-negative mutants of B41 and analysis of plasmid DNA of this strain showed no difference with its K99-positive parental strain. Therefore, it seems likely that the expression of the K99 genes can be switched off, without loss of K99 DNA, perhaps by a mechanism analogous to the flagellar phages variation in Salmonella typhimurium[22]. Although the K99 genes were cloned on a multicopy vector, expression of K99 in E.coli K12 was low compared to the production of wild type strains. However, by introduction of the K99 recombinant plasmids into a wild O101 strain, we obtained a strain that produced K99 more than 4-fold, compared to the best wild type producers. This again reinforces the idea that properties of the host play a major role in the expression of K99. By deletion of various regions in the cloned 4.5 Md K99 fragment the approximate location of the structural gene of the K99 subunit could be inferred between the coordinates 4.1 Kb and 5.8 Kb at the physical map as shown in figure 1. It is interesting a region about 1 Md distal from the K99 structural gene is required for K99 expression (as in pRI9915-11), whereas the more proximal region between 3-4 Kb can be deleted without much effect on the level of K99 expression.

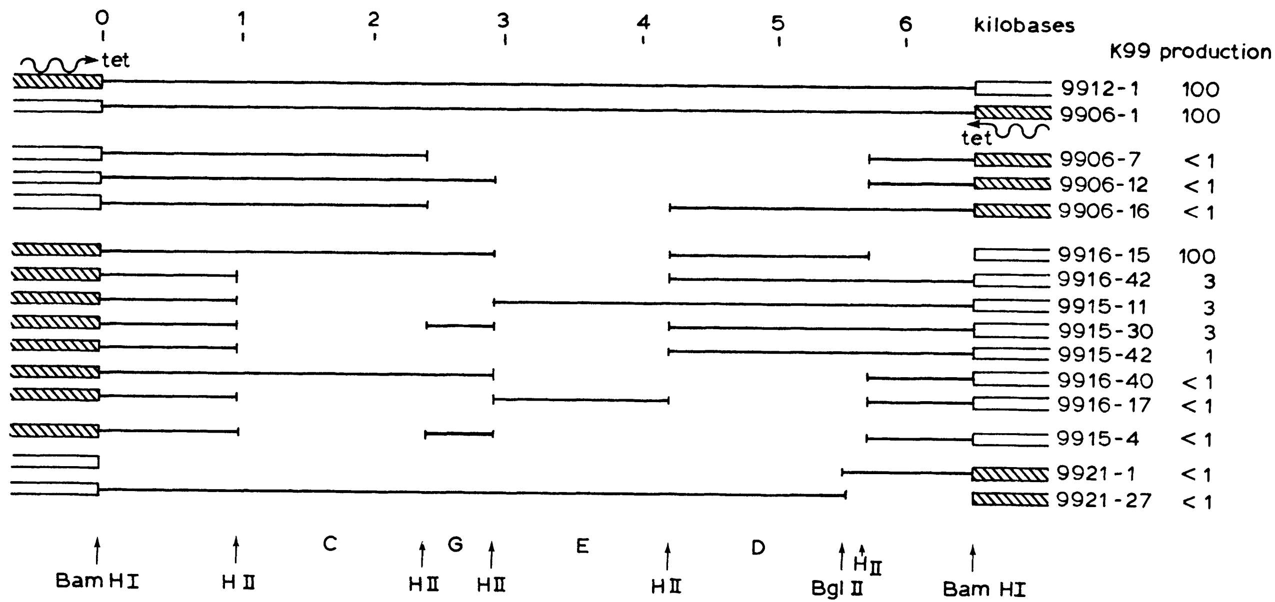

Figure 1. Expression of the K99 antigen by the recombinant plasmids pRI9912-1, pRI9906-1 and various deletion mutants. pRI9906-1 and pRI9912-1 contain the same K99 BamHI DNA fragment, but in different orientation. The direction of transcription of the tetracycline resistance gene of the vector pBR325 is indicated. Deletion mutants were obtained by partial digestion with Hind II (HII) or BamHI plus BglII. The production of K99 in E.coli K12 is measured by an enzyme-linked immunosorbent assay and it is expressed relative to that of pRI9912-1.

CLONING AND EXPRESSION OF THE K88 GENES

Much more is known of the K88 antigen. At least three different antigenic variants exist: K88ab, K88ac and K88ad[23]. Virtually all E.coli isolates that are $K88^+$ have also the ability to ferment the trisaccharide raffinose[24]. Both characters are located on a single plasmid and therefore K88 can easily be transferred conjugally by selection of recipients on raf^+. Shipley et al.[25] found that K88 is generally encoded by plasmids of a molecular weight of about 50 Md and these plasmids showed at least 97% polynucleotide homology. No or only slight differences are observed in the restriction enzyme digest patterns of K88ab, K88ac or K88ad plasmids and the plasmids of all 3 K88 variants contain a 7.7 Md Hind III fragment that encodes for the K88 determinant (25, Meyerink, unpublished). The presence of 2 copies of the IS1 sequence in direct orientation separated by a stretch of about 10 Md of DNA[26] explains the early observations of Let Bak et al.[27] on the dissociation of a 50 Md K88 plasmid into a 40 Md and a 10 Md component. The amino-acid sequence of about 90% of the K88ab subunit is established[28] and no differences between K88ab and K88ac in the sequenced part of the K88 subunit have been found. In contrast, the K88ad polypeptide differs from K88ab at least in 4 amino-acids.

Previously, we reported the cloning of the K88ab determinant. The smallest plasmid obtained that still expressed K88 was pFM205, which is composed of pBR322 and a 4.3 Md piece of K88 DNA[29]. We constructed derivatives of pFM205, having deletions in various regions of pFM205 and the expression was studied in minicells (see figure 2). pFM205 directs the synthesis of 6 non-vector encoded polypeptides in minicells. One of these (26 Kd) is identical to the K88ab subunit, because specific K88 antiserum precipitates this polypeptide as the only one. The K88 surface protein is translocated across the cytoplasmic membrane and therefore one might expect that the mature K88 subunit is a product of proteolytic processing. By inhibition of the processing system with 9.5% ethanol, a 28 Kd K88 polypeptide was found in minicells, which indeed indicates the existence of a signal sequence about 20 amino-acids. This is consistent with preliminary DNA sequence data of the K88 coding region, which indicate that the K88 structural gene has a signal sequence of 22 amino-acids. Deletion of the genes encoding for the 17 Kd and the 81 Kd polypeptides (as in plasmids pFM222 and pFM77, respectively) affects the expression of K88 as an antigen. Extracts of cells carrying pFM222 or pFM77 bind only small amounts of anti-K88 antibodies, although the synthesis of the K88 subunit in minicells does not differ significantly from that in minicells carrying the parentel plasmid pFM205 (figure 2). Furthermore, the antigenic material of these mutants is much more thermolabile compared to wild type K88. Presumably, the 17 Kd and the 81 Kd polypeptides are involved in the assembly of the 26 Kd subunits to complete pili. The function of the other 3 polypeptides (27 Kd, 29 Kd and 30 Kd) which are also synthesized in minicells is presently unknown.

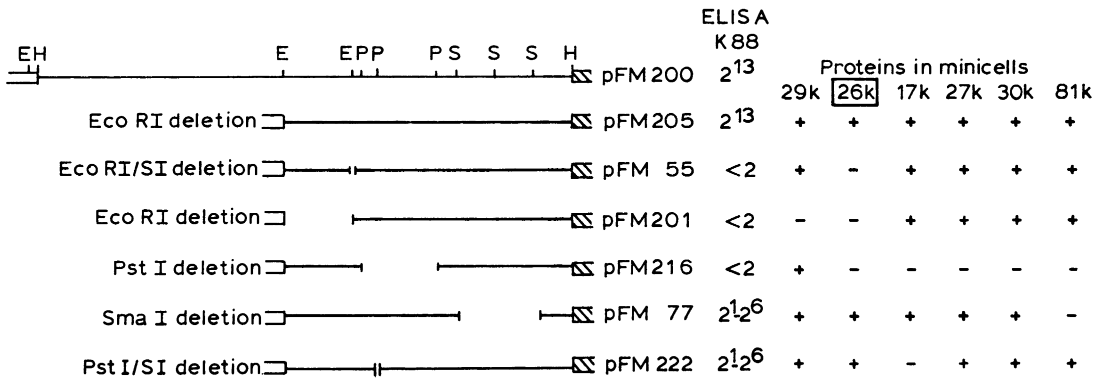

Figure 2. Polypeptide synthesis of the K88 recombinant plasmid pFM205 and deletion mutants in minicells. pFM205 is a deletion mutant of pFM200 obtained by EcoRI treatment and ligation. The level of K88 production was measured in E.coli K12 maxicells. The order of the 6 proteins as written in this figure corresponds to the gene order as derived from these data, except for the 30 K and the 27 K proteins. The mutual gene order of latter proteins is not yet known.

Jones and Rutter[6] found brush borders of pigs from certain litters that were not adhesive for $K88^+$ E. coli. This "non-adhesive" phenotype was inherited as an autosomal recessive characteristic and the experiments showed the "non-adhesive" pigs conferred relative resistance to developing diarrhoea after challenge with $K88^+$ enterotoxigenic E.coli. Recently, Bijlsma (unpublished) extended the study of Jones and Rutter and tested all 3 K88 variants in adhesion tests with brush borders from 42 pigs and piglets obtained from the slaughterhouse. He also found a "non-adhesive" phenotype, type E, to which none of the 3 K88 variants adhered. The brush borders of "adhesive phenotype", however, could be divided into 4 groups: phenotype A was adhesive for all 3 K88 variants, B for K88ab and K88ac, C for K88ab and K88ad and phenotype D only for K88ad (table 2). Preincubation of type A brush borders with an excess of purified K88ad antigen did not interfere with the adhesion of K88ab or K88ac bacteria, whereas K88ab completely blocked the adhesion of bacteria producing K88ac and vice versa.

Table 2. Adhesion of K88ab, K88ac and K88ad E.coli to brush borders of pigs

Brush border phenotype	Adhesion by K88ab	K88ac	K88ad	Number of pigs tested
A	+	+	+	11
B	+	+	-	6
C	+	-	+	11
D	-	-	+	3
E	-	-	-	11
			total	42

These experiments indicate that the antigenic variation of K88 is associated with differences in adhesive properties.

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INVASIVE BACTERIAL PATHOGENS OF THE INTESTINE: SHIGELLA VIRULENCE PLASMIDS AND POTENTIAL VACCINE APPROACHES

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INTRODUCTION

Bacterial diseases of the gastrointestinal tract usually occur by one of three overall mechanisms. The first mechanism, termed "intoxication," occurs by bacterial secretion of an exotoxin that oftentimes is preformed in food prior to ingestion by the host. This process is exemplified by staphylococcal or clostridial food poisoning. In contrast, the remaining two processes require living and multiplying disease agents. In the "enterotoxigenic" mechanism, as discussed elsewhere in this volume, bacteria colonize the small intestine, usually in the jejunum or duodenum. These bacteria multiply on the intestinal surface and elaborate an enterotoxin that stimulates excessive fluid and electrolyte efflux resulting in a watery diarrhea. Enteropathogenic *Escherichia coli* and *Vibrio cholera* serve as typical examples. Finally, a third group of organisms, termed "invasive," actually penetrate the epithelial mucosa of the large intestine. Subsequently, these organisms multiply intracellularly and disseminate within or through the mucosa. This latter mechanism, classically typified by *Shigella* and *Salmonella*, is now thought to be used by invasive strains of *E. coli*, *Yersinia*, and, possibly, *Campylobacter*. In contrast to other invasive bacterial diseases like salmonellosis in which the invading bacteria are disseminated throughout the host, shigellosis is a disease normally confined to the intestinal lining. Whereas toxigenic organisms generally require a large dose of organisms to cause disease, previous studies have shown that as few as ten virulent cells of *Shigella* can cause disease in humans. Thus, these features distinquish the toxigenic from the invasive mechanism of intestinal disease (see reviews[1,2]).

Two common and essential features of invasive bacteria are their ability to penetrate and to multiply within the epithelial cells of the colon[1,2]. Mutants of Shigella strains that fail to penetrate or that penetrate but cannot multiply intracellularly have been isolated. Both types of mutants are avirulent. The process of invasion has thus far been characterized in microscopic, but not biochemical detail. The first visible alteration in the host intestinal epithelium is a localized destruction of the microvilli, the outermost structure of the intestinal lining. The invading bacteria are then engulfed by means of an invagination of the intestinal cell membrane and are contained intracellularly within vaculoes. Subsequently, the microvilli are reestablished and intracellular bacterial multiplication occurs. These bacteria then destroy the vacuole and disseminate to adjacent cells, causing necrosis and resulting in acute inflammation and focal ulceration of the epithelium. The resulting dysentery is characterized by a painful, bloody and mucous diarrhea normally of relatively small volume.

Genetic studies of Shigella flexneri have previously resulted in the conclusion that virulence is multideterminant, with at least two widely separated bacterial chromosomal regions being required for invasion[1,2]. Furthermore, these studies have shown that not only is a smooth lipopolysaccharide bacterial cell surface necessary for intestinal invasion, but also that only certain O-repeat unit polymers are effective in this process; this is true for both shigellae and invasive E. coli. Until recently, plasmids did not appear to play a role in the invasion process or in the virulence of Shigella. Recent evidence amassed over the past three years, however, demonstrates that plasmids of Shigella are involved in the invasion process[3,4,5].

RESULTS

Colonial morphology transition of S. sonnei. Shigellosis is still an important disease worldwide, with approximately 15,000 cases reported in the U.S. during 1980. Of the 4 species of Shigella, S. sonnei is currently responsible for greater than two-thirds of all shigellosis cases in the U.S. and Europe. Because of its importance, this species was chosen as the initial focus of our studies. Unlike the other Shigella species, all S. sonnei strains fall into a single serotype. This serotype is due to a somatic antigen, termed form I, that is required for epithelial cell invasion. Chemical studies have revealed that the from I antigen is the O-side chain[6].

Upon restreaking on agar medium, smooth even-edged form I colonies generate at a relatively high frequency rough uneven-edged colonies, termed form II. Form II colonies appear in different strains at frequencies varying from 1 to 50%. Further study has shown that these rough colonies have irreversibly lost the form I antigen and are always avirulent due to the inability to invade epithelial cells[3,4]. The ability to penetrate epithelial cells can easily be monitored using the guinea pig eye as an assay system[7]. Bacterial strains that can penetrate epithelial cells will elicit a keratoconjunctivitis within 72 hours following inoculation of the guinea pig eye with a bacterial suspension. This assay was used exclusively throughout these studies.

Plasmid analyses of form I and II strains. The high frequency and irreversible nature of the form I to II transition, which always resulted in the loss of virulence, suggested the involvement of a plasmid in this phenomenon. Thus, the plasmid DNA's of various *S. sonnei* strains, obtained from different parts of the world, were examined[3]. Plasmid DNA's of 4 representative isogenic sets of form

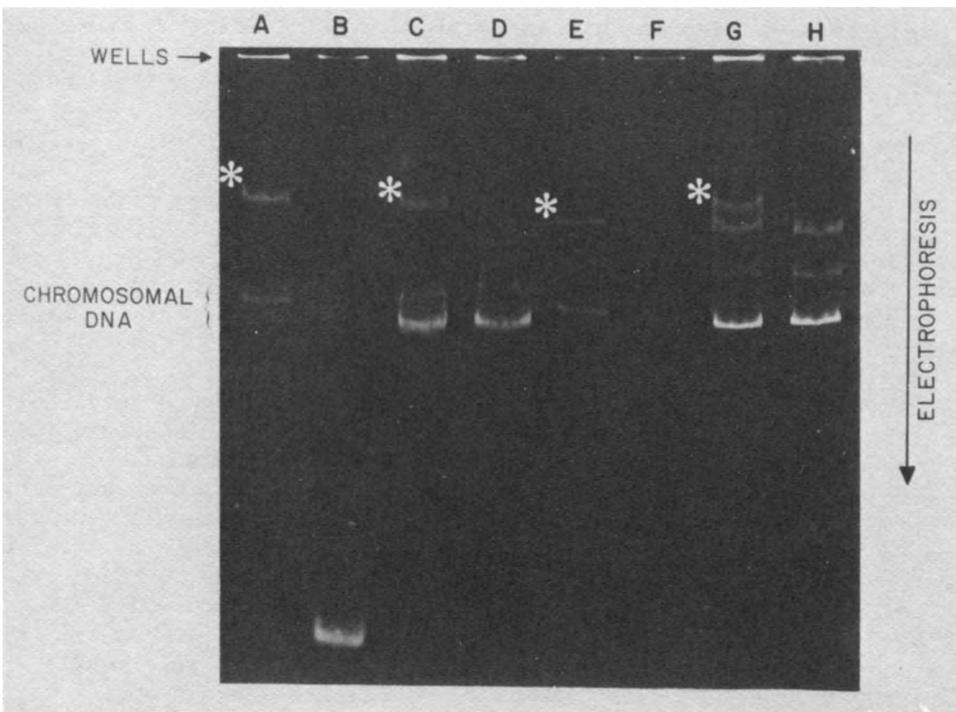

Fig. 1. Agarose gel electrophoretic profiles of circular plasmid DNA obtained from sets of isogenic form I and II *S. sonnei* strains. Plasmid profile of: (A) strain 53G form I; (B) 53G form II; (C) 50E form I; (D) 50E form II; (E) 9774 form I; (F) 9774 form II; (G) MBI form I; and (H) MBI form II. The asterisks mark the large plasmids in the form I strains that are lost in form II derivatives. The gel position expected for fragmented chromosomal DNA is indicated. DNA isolation and gel electrophoresis procedures are described elsewhere[3].

I and form II S. sonnei strains are shown in Fig. 1. Each of the DNA's from the form I strains contained a large plasmid which is estimated, for most strains, to be 120 Mdal in size (Fig. 1A, C, E, G). This large plasmid is missing in all form II derivatives (Fig. 1B, D, F, H). This observation has been independently confirmed[4].

Conjugal transfer studies. Direct proof that this large plasmid is involved in form I antigen synthesis and virulence can only be obtained by reintroduction of this plasmid into a form II recipient cell with concomitant reestablishment of these properties. However, neither the form I antigen nor virulence phenotypes are useful as selective markers to monitor plasmid transfer. Therefore, we attempted to identify any marker of selective value expressed by the form I plasmid. To date, about 175 biochemical and antibiotic resistance characters have been tested for, but we have been unable to detect any other trait encoded on this large plasmid. In addition, the results of further studies indicate that neither bacteriocin production nor iron-chelating systems are encoded by this form I plasmid (Sansonetti, Kopecko, and Formal; submitted for publication). To circumvent this problem, the form I plasmid was phenotypically tagged with the ampicillin resistance transposon, Tn3, or with transposons Tn5 or Tn10. These transposons were introduced into the appropriate strains on a carrier F'_{ts} lac replicon that is temperature sensitive for replication[3]. Strains in which the form I plasmid had been tagged expressed the appropriate transposon-encoded antibiotic resistance; and, this resistance was always lost during the transition to form II cells.

Attempts to detect conjugal self-transfer of these tagged plasmids, using antibiotic selective pressure, were unsuccessful, indicating that these large plasmids are not self-transmissible. However, two systems to mobilize the form I plasmid to recipient cells have been developed. Initially, an F'_{ts} lac::Tn3 plasmid was introduced into an S. sonnei strain carrying a Tn3-tagged form I plasmid. We reasoned that recombination between the Tn3 units on these two plasmids would result in the formation of a composite conjugative plasmid. In fact, form I plasmid transfer was obtained as well as evidence for the composite plasmid species[3]. Using this mobilization system, form I antigen synthesizing ability has been transferred to form II S. sonnei, S. flexneri, E. coli K12, Salmonella typhi and Serratia. These data strongly suggest that this S. sonnei plasmid carries the structural genes for synthesizing the form I antigen.

Although F' lac-mediated transfer of the form I plasmid was achieved, none of these form I transconjugants had reacquired virulence. Further studies, discussed later, have revealed that FI incompatibility (inc) group plasmids inhibit invasiveness. Thus, further attempts were made to mobilize the form I plasmid using various conjugative plasmids of ten different incompatibility groups. Oddly

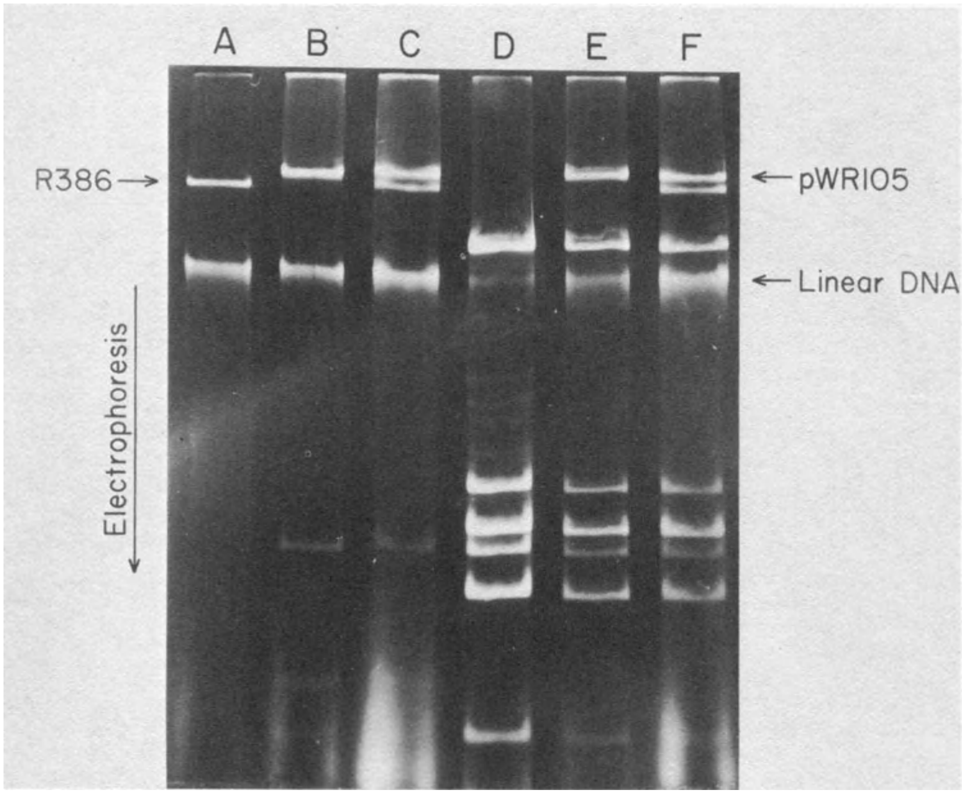

Fig. 2. Mobilization of the form I plasmid by R386. The agarose gel electrophoretic profiles of circular plasmid DNA obtained from donor, recipient, and transconjugant strains: (A) E. coli J53 carrying R386; (B) S. sonnei 482-79 carrying pWR105, a Tn5-tagged form I plasmid; (C) donor 482-79 with pWR105 and R386; (D) recipient form II S. sonnei Rudy; (E) Rudy transconjugant carrying pWR105; (F) Rudy transconjugant carrying pWR105 and R386. Experimental details are described elsewhere (Sansonetti, Kopecko, and Formal, submitted for publication)[3,12].

enough, only the R386 plasmid, of FI inc, was found capable of mobilizing the form I plasmid. The plasmid DNA profiles of donor, recipient, and transconjugant strains from this mobilizing system are shown in Fig. 2. Some transconjugants received only the form I plasmid (Fig. 2E), while others also inherited the R386 plasmid (Fig. 2F). Only transconjugants that did not contain the R386 plasmid were virulent, again verifying the virulence-inhibiting nature of FI inc group plasmids. This mobilizing system has allowed us to establish that form I antigen synthesizing ability and virulence are encoded by the 120 Mdal form I plasmid (Sansonetti, Kopecko, and Formal; submitted for publication).

Incompatibility testing. Next, an attempt was made to identify the inc group of the form I plasmid. Various reference plasmids were conjugally transferred to an *S. sonnei* strain containing a Tn5-tagged form I plasmid. The resulting strains, purified on antibiotic selective media and each carrying the form I plasmid and a reference plasmid, were streaked onto MacConkey lactose agar. The stability of the form I colony type was then monitored. As shown in Table 1, none of the reference plasmids, except R386, significantly affected the normal form I to II transition as compared to the wild-type *S. sonnei* strain. Control studies showed that all of these reference plasmids are stably maintained in the isogenic form II *S. sonnei* derivative strain. Virtually identical results were obtained when two different form I plasmids were tested for incompatibility. Although these experiments are hampered by the natural instability and nonselftransferability of the form I plasmid, these limited data suggest that the form I plasmid is of the FI inc group (Sansonetti, Kopecko, and Formal; submitted for publication).

Table 1. Incompatibility Between The Form I Plasmid Of *S. Sonnei* 482-79 And Other Plasmids

Secondary Plasmid	Incompatibility Group	Colony Phenotype		
		I	I-II	II
		(% of 400 colonies)		
none	-	90.5	8.5	1
R386	FI	52.5	38	9.5
R1	FII	86.5	9.75	3.75
R124	FIV	84	13.5	2.5
R64-11	Iα	92.75	6.25	1
N3	N	91.5	7.5	1
R16	O	81.75	15.5	2.75
RP1	P	89.5	8.0	2.5
S-a	W	96	3	1
RA1	A	82.75	16.5	2.75

Virulence inhibition. As mentioned previously, the F'_{ts} lac plasmid inhibited the virulence of form I-expressing S. sonnei strains. To examine this phenomenon in more detail, plasmids of various incompatibility groups were transferred to several invasive bacteria including S. sonnei, S. flexneri, S. dysenteriae and E. coli. Only a few representative plasmids and the virulence responses of two invasive strains are shown in Table 2, but all invasive strains responded similarly. Only the FI inc group plasmids and plasmid pED830 were observed to inhibit virulence. pED830, constructed in N. Willetts' lab, is a colicin E_1 derivative containing Tn3 and which has inserted into the Tn3 BamH1 site a 45 kilobase (kb) BamH1 fragment containing all of the F plasmid conjugal transfer genes[8]. These data indicate that plasmids of the FI inc group inhibit the ability of invasive organisms to penetrate epithelial cells. Furthermore, the gene(s) responsible for this inhibition is located on the 45 kb BamH1 fragment that carries the conjugal transfer genes of the F plasmid (Sansonetti, Kopecko, and Formal; submitted for publication).

S. flexneri virulence plasmids. S. flexneri is a leading cause

Table 2. Effect Of Different Incompatibility Group Plasmids On The Virulence of Shigella Strains

Plasmid	Incompatibility Group	Virulence: S. sonnei 482-79 I	Virulence: S. flexneri M4243
none	-	+	+
F'_{114ts}lac::Tn3	FI	-	-
F'_{114ts}lac	FI	-	-
pED830	(F tra genes)	-	-
R1	FII	+	+
222	FII	+	+
R124	FIV	+	+
R64	Iα	+	+
N3	N	+	+
R16	O	+	+

Virulence assessed by guinea pig keratoconjunctivitis assay.

of shigellosis in many parts of the world. Initially, representative strains of the six serotypes of S. flexneri were examined for plasmids. Regardless of serotype, all strains were found to contain multiple plasmid species and always contained at least one large plasmid species of approximately 140 Mdal in size (unpublished data). Upon restreaking virulent, smooth S. flexneri colonies on agar medium, granular colonial variants have recently been detected at a frequency of about 0.1% in 4 of the 6 serotypes. No reversion toward the original colonial morphology was observed when these variants were repurified on different media. More importantly, all of these granular derivatives proved to be avirulent. Plasmid DNA profiles of these avirulent granular derivatives were then compared to those of the respective parental strains (Fig. 3). Three of the 4 granular variants have lost the large 140 Mdal plasmid (Fig. 3B, D, H), while in the fourth avirulent variant this plasmid appears to

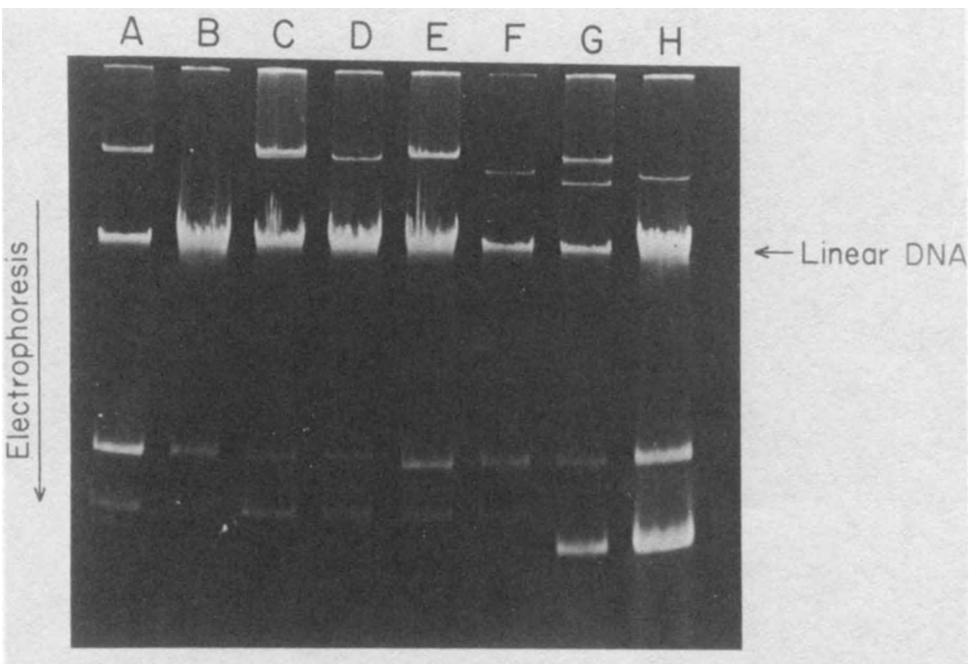

Fig. 3. Agarose gel profiles of plasmid DNA obtained from virulent S. flexneri (wells A,C,E,G) and their respective avirulent derivatives (wells B,D,F,H). (A,B) strain Z, serotype 1b; (C,D) M4243, serotype 2a; (E,F) M90T, serotype 5; (G,H) CCH060, serotype 6. DNA isolation[12] and gel electrophoresis procedures[3] are described elsewhere. The DNA bands in (H) migrated slightly behind the corresponding bands in (G), because the DNA was overloaded in (H) to verify loss of the largest plasmid.

have undergone a deletion (Sansonetti, Kopecko, Washington, and Formal; manuscript in preparation). Although this evidence is not conclusive, these data strongly suggest that plasmid-borne genes are involved in the virulence of S. flexneri. To date, we have been unable to obtain self-transfer of or detect selectable phenotypic properties on these 140 Mdal plasmids. Thus, although these plasmids appear to affect the ability of the bacterial host to penetrate epithelial cells, the exact plasmid-mediated functions involved are undetermined.

Vaccine strain construction. Parenterally administered Shigella vaccines have not been successful, probably because shigellosis is an infection limited to the superficial layer of the colonic mucosa. Therefore, circulatory antibodies do not appear to be protective against shigellosis. On the other hand, attenuated shigellae vaccines administered orally have been effective in protecting against this disease, suggesting that the local intestinal immune response is induced by the living oral vaccine[9]. However, attenuated Shigella vaccines have not been widely used because of difficulties in isolating safe (i.e., nonreverting) and effective strains. Recently, Germanier and Furer[10] have reported on the isolation and characterization of a galactose-epimeraseless (galE) mutant of Salmonella typhi, the typhoid bacillus. This attenuated strain has been tested in more than 15,000 volunteers and has been shown to be a safe and highly effective oral vaccine[11]. We considered the possibility that this strain might be modified so as to be protective also against shigellosis due to S. sonnei. Therefore, the plasmid responsible for S. sonnei form I antigen synthesis was mobilized, as described earlier, into the galE S. typhi strain. The resulting derivative S. typhi was shown to contain the form I plasmid. Furthermore, serological studies demonstrated that this derivative strain expresses not only the somatic antigens of the S. typhi parent, but also the S. sonnei form I antigen. It appeared that this derivative strain would be a good vaccine candidate[5].

Mouse protection tests. To test the effectiveness of this vaccine, preliminary animal tests were conducted. Groups of mice were inoculated with one of several vaccines or with a saline control. Four weeks post-immunization, all mice were challenged with virulent strains of S. typhi or S. sonnei and deaths were recorded 72 hours after challenge (Table 3). Note that the living S. typhi galE typhoid vaccine protected against the homologous challenge strain, but not against the heterologous (i.e., S. sonnei) challenge. Similarly, the living S. sonnei vaccine protected against challenge by S. sonnei, but not by S. typhi. However, the form I galE S. typhi derivative vaccine protected against both challenge organisms[5]. These preliminary studies indicate that the form I-expressing galE S. typhi strain is an effective immunizing agent in mice for protection against S. typhi and S. sonnei. Volunteer studies are currently underway.

Table 3. Protection Of Mice Against S. typhi And S. sonnei Challenge With S. typhi And S. Sonnei Vaccines

Vaccine	Route of Immunization	Challenge Strain*	
		S. typhi Ty2	S. sonnei 53GI
Living S. typhi Ty21a	IP	0/12**	15/15
	SC	4/15	15/15
Living S. typhi-form I 5076-1C	IP	0/13	1/14
	SC	1/16	0/16
Living S. sonnei-53GI	IP	14/16	1/16
	SC	16/16	0/16
AKD*** S. typhi Ty2	IP	2/16	15/16
	SC	1/16	16/16
Saline	IP	10/10	10/10

* Challenges, suspended in 0.5 percent hog gastric mucin, were administered IP.

** Deaths recorded 72 hrs after challenge. / Total

*** Standard acetone-killed and dried typhoid vaccine.

SUMMARY

1. Shigella sonnei contain a 120 Mdal nonconjugative plasmid which appears to be in the FI inc. group. This plasmid codes for the structural determinants of the form I antigen which is thought to be essential for invasiveness. Other virulence properties, excluding iron-chelation, may reside on this plasmid.[3,4] (Sansonetti, Kopecko, Formal; submitted for publication).

2. All six serotypes of S. flexneri contain a large plasmid of approximately 140 Mdal, which also appears to be necessary for epithelial cell penetration. (Sansonetti, Kopecko, Washington, Formal; ms. in prep.).

3. A form I-expressing galE S. typhi vaccine strain has been constructed and has proven to be protective in mice against challenges with both virulent S. sonnei and S. typhi strains.[5]

4. This S. typhi galE strain Ty21a, which has been shown to be a safe and highly effective oral vaccine, should serve as a useful carrier strain for other antigens (e.g., colonization antigens or toxoids) to protect against a variety of different intestinal infections.[5]

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PLASMID-SPECIFIED IRON UPTAKE BY BACTERAEMIC STRAINS OF *ESCHERICHIA COLI*

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INTRODUCTION

Although *Escherichia coli* is a normally harmless major aerobic component of the gut flora of a healthy individual, some strains are invasive, and able to produce extraintestinal infections. *E.coli* has been isolated from urinary tract infections and from cases of neonatal meningitis and bacteraemia. Smith[1] reported that a significant proportion of *E.coli* strains associated with bacteraemia of humans and domestic animals harboured plasmids (ColV) specifying the narrow spectrum antibacterial protein colicin V. Furthermore, Cabello[2] found that many *E.coli* strains isolated from patients with meningitis carried such ColV plasmids. It has been unequivocally shown that possession of a ColV plasmid markedly enhances the virulence of *E.coli* strains in comparison with plasmid-free strains in experimental infections of a number of laboratory animals[1,2,3].

Investigations of the correlation between colicinogenicity and virulence have led to the identification of several ColV plasmid-associated characteristics which may be implicated in pathogenicity. Bacteria carrying a ColV plasmid show an enhanced ability to adhere to intestinal epithelium *in vitro*[4], while colicin V itself, detected in the laboratory by its ability to kill sensitive *E.coli* strains, may act synergistically with endotoxin to increase vascular permeability in the skin[5], and to depress macrophage activity in the peritoneal cavity of infected animals[6]. These may be crucial factors in the initiation of the invasive process. Binns et al[7] have cloned restriction fragments of the prototype plasmid ColV,I-K94 which specify increased resistance of bacteria to the bactericidal effects of antibody and complement in serum. It is difficult to assess the importance of this, however, since the serum resistance of *E.coli* isolates from meningitis was found to be unaffected by elimination of ColV plasmids although lethality in experimental infections was significantly reduced by curing[2].

Another characteristic controlled by ColV plasmids from bacteraemic strains of *E.coli* is the capacity to grow in conditions of iron deprivation. It is this aspect that is considered in detail in this communication.

There is now considerable evidence in the literature that the concentration of free ferric cations in the tissues and fluids of the body is critical to the outcome of the conflict between establishment of a bacterial infection and its suppression by the host animal[8]. Although present in body fluids, iron is predominantly unavailable for microbial growth because it is strongly associated with iron binding proteins (transferrin in serum, lactoferrin in secretions). Inclusion of excess iron in the inoculum in experimental infections enhances bacterial virulence[9]. Moreover, clinical conditions, such as hepatitis, haemolytic anaemia, or haemorrhage due to severe viral infection, which lead naturally to increased levels of free iron in the body fluids are frequently associated with increased susceptibility to, and severity of, bacterial infections[8]. On the other hand, an otherwise healthy body responds to infection in a number of ways to reduce still further the level of free iron, and so deprive invading bacteria of an essential growth requirement[8]. There may be specific reduction of intestinal absorption of exogenous iron, and increased iron flux from body fluids to hepatic storage sites; there may also be increased synthesis of iron binding proteins and their localisation at potential sites of infection. Thus, a bacterial strain which is capable of overcoming such "nutritional immunity"[8] by competing efficiently for iron with the iron binding proteins of the host will be better able to proliferate rapidly after infection and therefore elicit severe disease symptoms.

ColV PLASMIDS AND IRON STRESS

Since iron availability is crucial to the progress of a bacterial infection, the possible involvement of ColV plasmids of bacteraemic *E.coli* strains in iron uptake was investigated (table 1). Iron is normally present in low concentration in defined minimal media as an impurity of the component chemicals. However, addition of purified iron-free human transferrin to minimal medium decreased the growth rate of plasmidless *E.coli* K-12 strain W3110 due to conversion of free iron to a relatively unavailable complexed form. Saturation of iron binding sites of transferrin by excess ferric ions reversed the inhibitory effect. On the other hand, the same concentration of transferrin had no effect on the growth

Table 1. Effect of Transferrin on Bacterial Growth in Defined Minimal Medium

Bacterial strain		Mean generation time (min)	
Designation	Characteristics	-transferrin	+transferrin[a]
W3110	K-12, plasmidless	46	72 (47[b])
LG1327	W3110/ColV-H247	45	45
H247	bacteraemic *E. coli*	34	34
H247V⁻	cured derivative	34	55

[a]Transferrin was added at 250 µg/ml.

[b]$FeCl_3$ at 100 µM was added to growth medium.

rate of the colicinogenic human bacteraemic strain H247. Two observations indicate that the ColV-H247 plasmid has a role in acquiring sufficient iron for growth from the transferrin-complexed state; transferrin significantly inhibited the growth of a cured derivative of strain H247, while conversely a derivative of W3110 to which plasmid ColV-H247 had been transferred by conjugation was unaffected by the presence of transferrin in the growth medium. Identical results were obtained with ColV plasmids from bacteraemic strains of calf (B188), pig (P72) and chicken (F70) origin and with one of the prototype ColV plasmids ColV-K30[10,11].

Growth rate differences of this magnitude account for the observed changes in constitution of mixed cultures of colicinogenic and plasmid free bacteria during growth in conditions of iron deprivation in immunoglobulin-free calf serum[10]. Furthermore, in mixed infections of mice, the minority colicinogenic component of the inoculum was reisolated as the predominant organism from dead animals (table 2). When excess iron was included in the inoculum, however, the relative proportions of the two strains recovered from infected mice were similar to that of the inoculation mixture. Thus, it is clear that ColV plasmids contribute to the ability of the cells that harbour them to sequester iron under conditions of iron stress both *in vivo* and *in vitro*. When iron is freely available the selective advantage of colicinogenicity is abolished.

Table 2. Effect of Iron on the Course of Mixed Infections of Mice

% colicinogenic bacteria in inoculum[a]	Addition of Fe^{3+} [b]	% colicinogenic bacteria recovered[c]
1	-	87
1	+	2
11	-	100
11	+	16

[a]Mixtures of strains H247 and H247V⁻ in the proportions indicated were inoculated I/P into groups of 3 adult white mice.

[b]Ferric ammonium citrate (20 mM).

[c]Peritoneal wash of dead mice.

ColV PLASMID SPECIFIED IRON UPTAKE

A number of routes of entry of iron into cells of enteric bacteria have been described[12]. When the element is present at a high concentration in the growth medium it enters in a passive, non-specific fashion[13]. However, in conditions of iron deficit, the synthesis and excretion of the catechol siderophore enterochelin are induced[14], and the ferric-enterochelin complexes formed in the medium are subsequently actively transported into cells[15]. Alternatively, compounds present in natural environments may be utilised for iron uptake; an example is the fungal siderophore ferrichrome[16].

Mutants of *E.coli* K-12 defective in the synthesis of enterochelin are able to grow either if a high concentration of iron is provided to allow passive entry (as in growth in nutrient medium), or by addition to defined medium of an iron solubilising compound such as sodium citrate which can be actively transported across the cell membrane. The presence of plasmids ColV-H247, ColV-P72, ColV-F70 or ColV-K30 in an enterochelin defective mutant, however, abolishes the growth requirement for citrate[13], indicating the activity of an efficient alternative iron uptake mechanism. This has been demonstrated indirectly by observation of a plasmid-specific sparing of the induction of synthesis of bacterial outer membrane proteins that characteristically occurs when intracellular iron concentrations are reduced[10,11]. Direct confirmation of the operation of the plasmid-specified system comes from measurement of

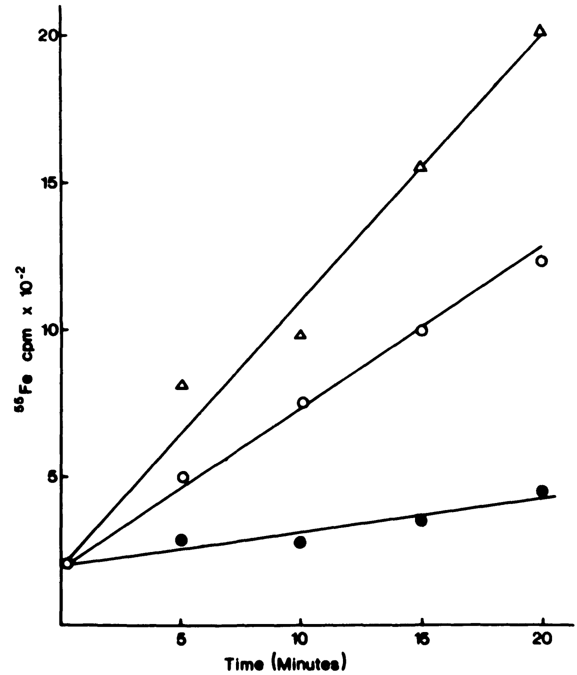

Figure 1. Effect of the presence of a ColV plasmid on bacterial iron uptake. Strains LG1013 (plasmidless, enterochelin producing, O); AN1937 (plasmidless, enterochelin deficient, ●); and LG1315 (AN1937 carrying ColV-K30, Δ) were grown in low iron medium, and the uptake of $^{55}FeCl_3$ into washed, non-growing cells was determined[10,11]

radioactive iron uptake into bacterial strains (fig.1). While the enterochelin deficient mutant AN1937 did not actively take up ^{55}Fe from the medium, a derivative carrying ColV-K30 (strain LG1315, Iu^+) showed more efficient uptake of label than the plasmidless enterochelin producing control strain LG1013.

MECHANISM OF IRON UPTAKE

Like the enterochelin-, citrate- and ferrichrome-mediated routes for the uptake of iron, the ColV plasmid specified system is an active process requiring the *tonB* gene product[10,11]. It involves iron chelation by an inducible hydroxamate siderophore[17]. The observation that plasmid-free strains are not cross-fed by colicinogenic strains in mixed culture and infection suggests either that the plasmid-coded iron chelator is cell bound, or that plasmid-specified products act to transport an extracellular siderophore into the cell. Stuart et al[17] favour the former model on the basis of their finding that hydroxamate compounds were chemically detectable[18] in cell pellets of ColV plasmid-carrying bacteria.

Genetic data, on the other hand, suggest that the plasmid specified siderophore is a cell-free diffusible product[19]. Following mutagenesis and penicillin enrichment of strains LG1315 (enterochelin deficient, carrying ColV-K30), mutants defective in plasmid promoted iron uptake were isolated. All showed reduced virulence in experimental infections of mice. Moreover, they fell into two classes on the basis of cross-feeding tests (table 3), defining two plasmid-specified functions for the uptake of iron. One class (*iuc*) was cross-fed by a strain carrying a wild type plasmid, and is therefore postulated to lack an extracellular diffusible product for which the cross-feeding strain compensates. The other mutant class (*iut*) was not cross-fed by a strain producing extracellular chelator, but was itself able to cross-feed mutants of the *iuc* class; thus it produces normal siderophore, but is defective in some aspect of the transport of siderophore into the cell. The behaviour of these mutants cannot easily be reconciled with a cell-bound mode of action

Table 3. Cross-Feeding Tests[a]

Patch inoculum	Bacterial lawn		
Strain; Characteristics	LG1439[b] *entA*	LG1418 *entA*/ColV-K30*iuc*	LG1419 *entA*/ColV-K30*iut*
LG1315 *entA*/ColV-K30Iu$^+$	-	+	-
LG1418[c] *entA*/ColV-K30*iuc*	-	-	-
LG1419[d] *entA*/ColV-K30*iut*	-	+	-

[a]Lawns of bacteria (10^7 cells/plate) on minimal agar containing α α´-dipyridyl (160 μM) were patch inoculated as indicated. Cross-feeding (+) was observed as a zone of growth of the bacterial lawn around a particular patch; (-) indicates no cross-feeding.

[b]Strain LG1439 is a colicin V insensitive derivative of strain AN1937.

[c]No growth of patch inocula except on the LG1419 lawn.

[d]Poor growth of patch inocula.

of the plasmid-specified system. The general nature of the phenomenon is suggested by the finding that AN1937 derivatives carrying plasmids ColV-H247 or ColV-P72 (from human and porcine bacteraemic strains respectively) were also able to cross-feed *iuc* mutant strain LG1418.

This type of test provides a sensitive quantifiable biological assay for iron chelating activity. There is complete coincidence of elution of biologically determined iron binding activity and chemically determined hydroxamate material of strain LG1315 from both Dowex-1 and Sephadex G-50 columns (fig. 2) indicating that both tests measure the same plasmid characteristic. Furthermore the *iuc* mutant LG1418, deduced from cross-feeding tests to be deficient in chelator synthesis, was found to produce no detectable hydroxamate material, while LG1419, the *iut* mutant defective in transport of iron, produced 10-100 times more chelator (depending on growth phase) than parental strain LG1315 on the basis of both biological and chemical assays.

Furthermore, biological assays have confirmed the previous observation[17] that cell pellets of exponentially growing cultures of strains carrying wild-type ColV plasmids contain iron chelating activity. Cell pellets were washed extensively, sonicated and assayed for ability to promote the growth of *iuc* mutant LG1418 in conditions of iron limitation. Approximately 10% of the total biologically measurable activity produced by strain LG1315 was associated with the cell pellet. Cell-associated activity in sonicates and cell-free activity in culture supernatants eluted identically from Sephadex G-50 columns.

Strain LG1418 is defective in plasmid specified siderophore synthesis but it can grow in conditions of iron deficit if cell-free siderophore is supplied exogenously. In this case also, biological activity was recovered from extensively washed, sonicated cell pellets. These data suggest that the iron chelating material associated with cell pellets was actively involved in iron uptake into growing cells at the time of sampling. That is, it represents the transient association of a diffusible chelator with a membrane receptor rather than the more permanent involvement of siderophore molecules as components of the bacterial membrane as suggested by Stuart et al[17].

SELECTIVE ADVANTAGE OF COLICINOGENICITY

The siderophore elaborated by plasmid ColV-K30 has been identified by field desorption mass spectrometry as aerobactin (A. Bindereif and J.B. Neilands, personal communication). This compound was first purified from a strain of *Aerobacter aerogenes*[20], but has subsequently been found to be synthesised by strains of *Shigella*[21]

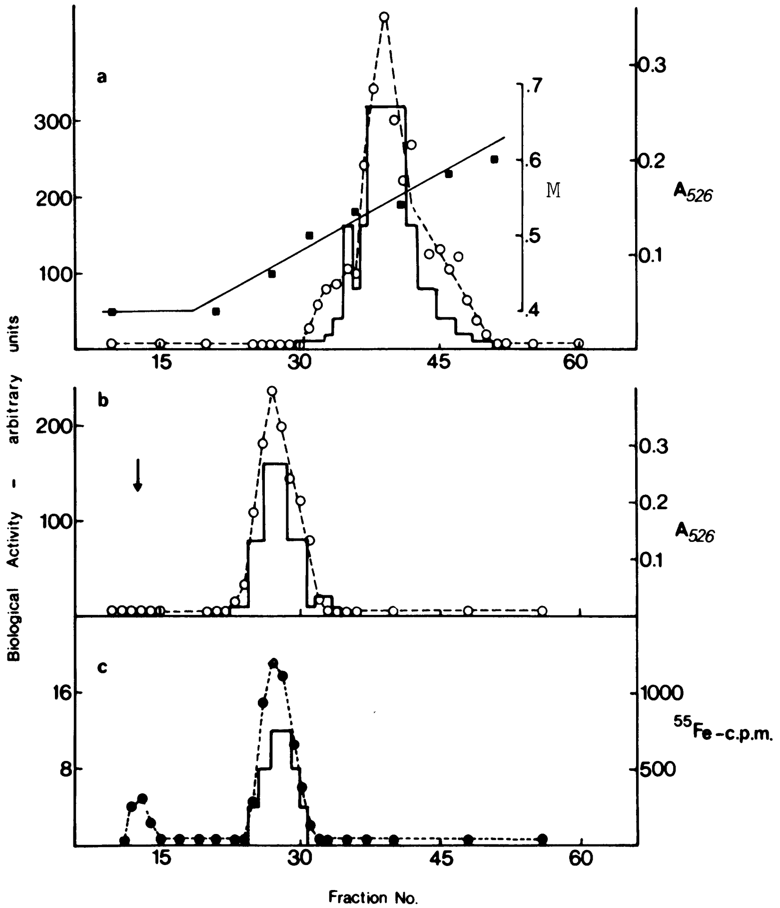

Figure 2. Column chromatography of ColV plasmid specified siderophore. In (a) culture supernatant of strain LG1315 was applied to Dowex-1 and eluted with an ammonium chloride gradient (-■——■-); eluant was tested for biological activity (histogram) and hydroxamate (○---○). In (b) LG1315 culture supernatant material concentrated approximately tenfold by Dowex-1 chromatography was applied to a Sephadex G-50 column (25 cm x 1 cm); eluant was tested for biological activity (histogram) and hydroxamate (○---○). The void volume is marked by the arrow. In (c) the sample was LG1315 culture supernatant to which were added 1 µC $^{55}FeCl_3$ and then excess transferrin to solubilise any non-complexed iron; the mixture was applied to Sephadex G-50 and the column eluant was tested for biological activity (histogram) and ^{55}Fe radioactivity (●-----●). Ferric-transferrin eluted in the void volume. Biological activity is defined as the reciprocal of the highest dilution of a sample which allowed growth of LG1418 in conditions of iron limitation. Hydroxamate compounds were determined colorimetrically (A_{526}) by the method of Csaky[18].

and *Salmonella* (A. Bindereif and J.B. Neilands, personal communication) also. It is not known if aerobactin synthesis in these genera is plasmid mediated, but the observation raises interesting questions about the evolutionary origin of ColV plasmids carried by bacteraemic strains of *E.coli*. Of more immediate interest is the question of why aerobactin, a relatively low affinity iron chelator, should provide a selective advantage to bacterial strains that can also synthesise the high affinity siderophore enterochelin. It should be noted, however, that the synthesis of enterochelin, and its breakdown to release iron within a cell are expensive of metabolic energy[12]. We may speculate, therefore, that in conditions of extreme iron stress the operation of an iron uptake system which requires little energy, albeit a low affinity system, may be crucial to the survival of a bacterial cell.

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SERUM RESISTANCE IN E.COLI

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INTRODUCTION

Pathogenic bacteria that cause generalized infections or meningitis invade the blood stream and are thereby distributed throughout the body. Blood, or serum, contains a number of non-specific (complement, lysozyme, phagocytes, iron-binding proteins, etc.) and specific (antibodies, lymphocytes) agents that alone or in combination lyse, kill or prevent the growth of the majority of bacteria with which they make contact. Abilities to resist, evade or inactivate these host defences constitute major components of the virulence of invasive bacteria. At present little is known about these bacterial properties or their molecular interactions with host defences.

The role of resistance to serum/complement in the virulence of invasive Gram-negative bacteria is indicated, on one hand, by a substantial volume of epidemiological data[1-3] and, on the other, by results obtained with experimental invasive bacterial infections, such as endocarditis, in laboratory animals[4,5]. We have studied resistance to serum in *E.coli* and have found that two cellular components, an outer membrane protein and a polysaccharide capsular antigen, are able to provide bacteria with substantial resistance to serum.

RESULTS

The Plasmid R6-5 Surface Exclusion Gene *traT* Mediates Serum Resistance

Several groups have reported that certain plasmids of Gram-

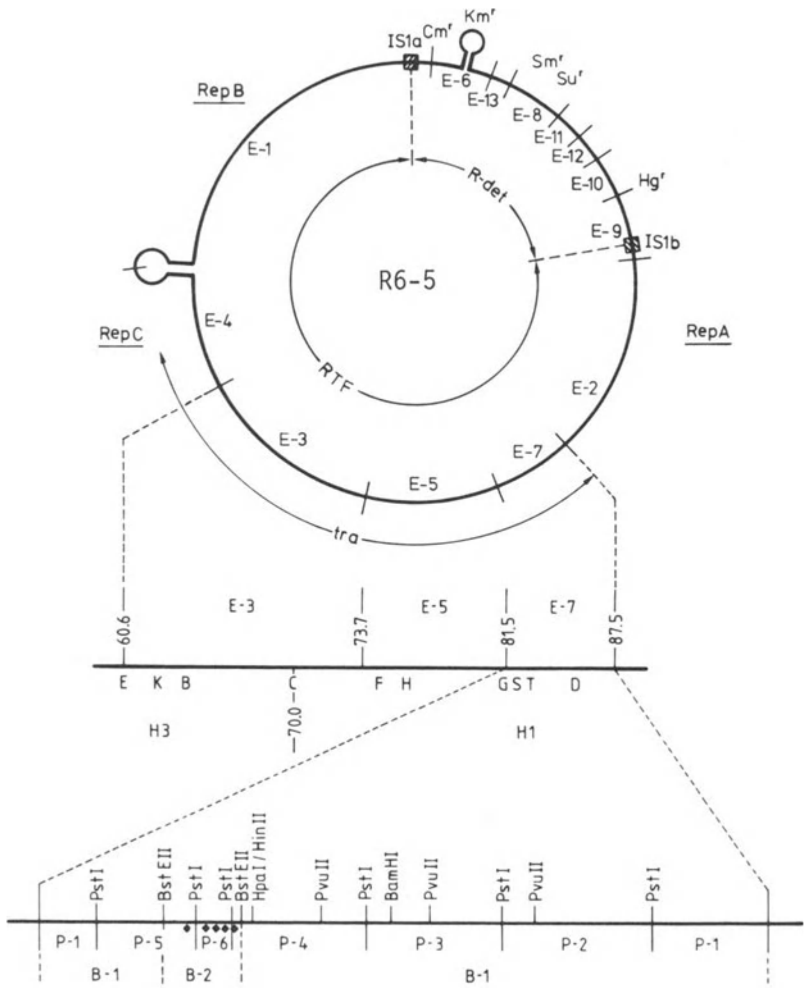

Fig. 1. Physical and genetic map of plasmid R6-5. The R6-5 map (top) is that of Timmis et al.[10]; cross bars indicate EcoRI cleavage sites. The genetic map of the R6-5 tra region (expansion, center) is that of Achtman et al.[11]; the bars above the map indicate EcoRI cleavage sites whereas bars below the map indicate HindIII cleavage sites. The detailed restriction map of the R6-5 EcoRI fragment E-7 (bottom) is that of Moll et al.[12]. E, H, P, and B numbers indicate restriction endonuclease fragments generated by EcoRI, HindIII, PstI, and BstEII, respectively. The PstI and BstEII fragments of the E-7 fragment are numbered according to size as they exist in the pKT107 hybrid plasmid, and not according to size as they

exist in R6-5. The diamond symbols indicate the sites of insertion of Tn3 elements within the B-2 fragment in serum resistant-defective insertion mutant derivatives of the pKT107 plasmid. Abbreviations: Cm^r, Km^r, Sm^r, Su^r, and Hg^r, resistance to chloramphenicol, kanamycin, streptomycin, sulfonamide, and mercury salts, respectively; Rep and IS1, replication functions and insertion sequence 1; RTF, R-det, and tra, resistance transfer factor, resistance determinant, and transfer functions, respectively.

negative bacteria increase the resistance of E.coli strains to serum[6-8]. Plasmid R6-5[9] is a large (100kb), conjugative, multiple antibiotic resistance plasmid (Fig.1) that we have studied extensively and that is closely related to R100, one of the plasmids shown to specify serum resistance[6,8]. Table 1 shows that R6-5 provides a smooth strain of E.coli, E.coli 59rif, with almost complete resistance to serum and significantly elevates the resistance of a highly-sensitive rough strain, E.coli K-12, to low concentrations of serum.

In order to determine the approximate location of the serum resistance determinant on the R6-5 genome, we examined the ability of ColE1 hybrid plasmids carrying EcoRI fragments of R6-5[10] to confer upon E.coli K-12 host bacteria resistance to 3% rabbit serum. Only one type of hybrid plasmid was found to specify a serum resistance function, namely that which carries EcoRI fragment E-7[12]. A detailed restriction endonuclease cleavage map of the E-7 fragment is shown in Figure 1. For ease of subsequent genetic manipulations this fragment was cloned into the pACYC184 vector to form hybrid plasmid pKT107. This hybrid also confers resistance to serum upon E.coli K-12 (Table 1). Three genes, traS, traT and traD, that function in plasmid conjugation are known to be coded by fragment E-7[11]. The traS and traT genes encode proteins that are responsible for surface exclusion, the reduction in ability of bacteria carrying a conjugative plasmid to act as recipients when mated with donors carrying a closely related plasmid, whereas traD specifies a function involved in conjugal DNA transfer from donor to recipient bacteria[13].

Precise localization of the serum resistance gene was accomplished by transposon mutagenesis of the pKT107 plasmid[12]. Transposon Tn3 was introduced into pKT107 by standard procedures and insertion mutant derivatives that no longer specified serum resistance were identified using a recently-developed, colorimetric, rapid screening procedure[14]. Restriction endonuclease cleavage analysis of these mutant plasmids revealed that all Tn3 elements that had inactivated the serum resistance gene of pKT107 were located within a 600 bp BstEII fragment, B-2, although on different

Table 1. Serum resistance levels of bacteria carrying plasmids that encode the *traT* protein or the K1 biosynthesis genes

Percent serum	Percent Survival[a]						
	C600*rif*[b]	C600*rif* (R6-5) *traT*$^+$	C600*rif* (pKT107) *traT*$^+$	59*rif*[c]	59*rif* (R6-5) *traT*$^+$	LE392[d]	LE392 (pKT172) K1$^+$
0	100	100	100	100	100	100	100
1	97	315	286	—	—	107	246
2	12.5	245	263	—	—	0.18	731
3	0.62	192	182	—	—	0.05	1013
6	< 0.001	6.7	9.1	139	333	< 0.001	100.2
10	—	0.03	0.02	5.4	205	—	0.35
20	—	—	—	0.07	124	—	—
50	—	—	—	0.005	74.5	—	—
75	—	—	—	0.005	77.1	—	—

[a] Log phase bacteria were washed with phosphate-buffered saline (PBS), resuspended in PBS, diluted to a concentration of 2×10^7/ml and 0.5 ml of the cell suspension added to 2 ml of PBS containing serum at the indicated concentrations. The cell/serum mixtures were incubated at 37°C for 3 hr before dilution and plating for survivors[14];

[b] a rifampicin-resistant derivative of *E.coli* K-12 C600;

[c] a rifampicin-resistant derivative of *E.coli* 59, a smooth *E.coli* isolated from the feces of a healthy child[12];

[d] *E.coli* K-12 strain used as recipient for λ-packaged cosmid constructions, obtained from J. Collins.

PstI fragments, P-4, P-5 and P-6, that lie within or overlap with the B-2 fragment (Fig.1). These insertion mutations localize the serum resistance gene to a region thought to contain the traT surface exclusion gene.

Definitive identification of the serum resistance gene product was obtained by comparing plasmid-encoded proteins synthesized in minicells containing pKT107 or its serum resistance-negative Tn3 insertion derivatives. As can be seen in Figure 2, the traS, traT and traD gene products were readily detected by polyacrylamide gel electrophoresis of radioactive proteins made by minicells containing the pKT107 serum resistance-positive plasmid, whereas the traT gene product could not be identified among the proteins made by minicells containing the serum resistance-negative insertion mutant plasmids[12]. The traT gene product, a 25,000 dalton protein, is thus responsible for plasmid R6-5-specified serum resistance.

The traT Protein is Located on the Outer Surface of the Outer Membrane

Complement is activated by cell surface structures and it is the cell surface which is the site of action of the membrane attack unit of activated complement. It was therefore anticipated that the traT protein, which mediates resistance to complement killing, would either be localized on the cell surface or excreted into the medium. We have compared the amounts of traT protein in whole cells and in outer membrane preparations of these cells (Triton X-100 insoluble component of the cell envelopes) and have found that the majority of cellular traT protein is localized in the outer membrane (Fig.2). It could be calculated from densitometer tracings of stained polyacrylamide gels of outer membrane proteins that bacteria carrying the pKT107 plasmid contain about 20,000 copies of the traT protein per cell[12].

Outer membrane proteins may be located on the inner or outer surface of the membrane, or may traverse it. In order to determine whether the traT protein is exposed on the outer surface of the outer membrane, we coupled ^{125}I to the surfaces of whole cells using lactoperoxidase and analysed the labeled proteins by polyacrylamide gel electrophoresis, followed by autoradiography[15]. As can be seen in Figure 2, the traT protein is labeled more heavily than outer membrane proteins I and II*, which are larger and present in numbers of copies 5-fold greater than that of the traT protein. This indicates that the traT protein is highly exposed on the outer surface of the outer membrane. Similar findings have been made on the F[15] and R100[16] traT proteins.

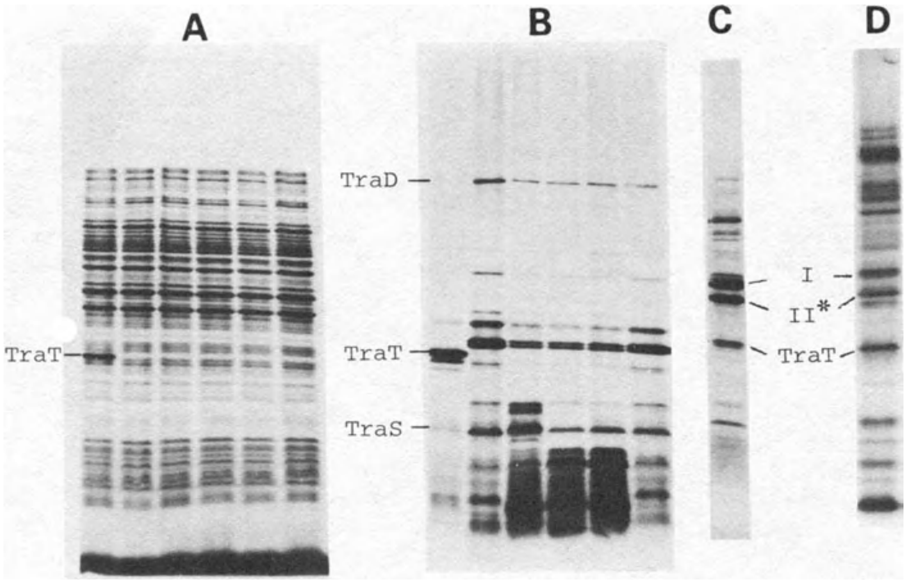

Fig. 2. R6-5-specified serum resistance is mediated by the traT protein which is located on the outer surface of the cell outer membrane. Identification of the serum resistance gene product as the traT protein. (A,B) Analysis of plasmid-encoded proteins synthesized in minicells. Minicells containing pKT107 (a) or its serum sensitive Tn3 insertion derivatives (b-f) were purified, radioactively labeled with [^{35}S]methionine, and analyzed by SDS-polyacrylamide gel electrophoresis[12]. The gel was subsequently stained (A) and autoradiographed (B). (b) pKT116; (c) pKT117; (d) pKT118; (e) pKT119; (f) pKT120. (C) Analysis of outer membrane proteins of plasmid-carrying bacteria. The outer membranes (Triton X-100-insoluble component of the cell envelope) of cells harboring pKT107 were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining[15]. (D) Analysis of proteins exposed on the cell surface. Cells harboring pKT107 were iodinated with ^{125}I in the presence of lactoperoxidase[15] and the total proteins analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

Table 2. Serum resistant bacteria fail to cross protect sensitive bacteria

Bacterial strain	Percent Survival in 3% Serum[a] After 60 min	After 180 min
A. CR34nal[b]	0.67	< 0.001
B. C600rif(pKT107)[c]	282	319
C. CR34nal + C600rif(pKT107)[c]	2.33 237	< 0.001 320

[a]Bacteria were prepared as described in Table 1. At -30 min, C600rif(pKT107) bacteria were added to serum solutions (B and C); at 0 min, CR34nal bacteria were also added (A and C); at + 60 min and + 180 min the bacterial suspensions were diluted and plated on agar containing either nalidixic acid (50 μg/ml, for A and C) and rifampicin (100 μg/ml; B and C);
[b]a nalidixic acid resistant mutant of E.coli K-12 CR34;
[c]present at 3 x the concentration of that of CR34nal.

Functional Aspects of the traT Protein

Although we found no evidence of release of significant amounts of traT protein from bacteria carrying the pKT107 plasmid, the release of small quantities would not have been detected. There are three possibilities regarding the mode of action of the traT protein in serum resistance: (a) inactivation of one or more components of complement in the fluid phase by released traT protein, (b) inactivation by cell-bound traT protein, or (c) prevention of the activation of complement by cell surface structures, or inhibition of the lytic activity of activated complement on the cell surface, due to a traT protein-mediated structural modification of the cell envelope. If the principal mechanism of serum resistance is inactivation of complement in the fluid phase, it should be possible to protect serum sensitive bacteria by preincubating the serum to be used with serum resistant cells. Table 2 shows that this is not the case: preincubation of 3% rabbit serum with serum resistant bacteria (final concentration $6x10^6$/ml) for 30 min did not significantly increase the survival of serum sensitive bacteria (final concentration $2x10^6$/ml) that were subsequently added. This means that the traT protein does not inactivate complement components present in the fluid phase and that it must mediate resistance as an integral component of the bacterial outer membrane (see also ref.17).

As indicated above, the *traT* protein is responsible in part for plasmid surface exclusion. In order to examine the functional relationship between serum resistance and surface exclusion, we have begun to isolate and analyse serum resistance-defective, hydroxylamine-induced point mutant derivatives of the pKT107 plasmid. Twenty-two putative mutant plasmids of this type were initially identified by the rapid screening procedure, which measures bacterial growth in the presence of serum, but only three were subsequently confirmed as serum resistance-defective. The remaining seventeen mutant derivatives all caused substantial over-production of the *traT* protein (up to 200,000 copies per cell)[18] and all resulted in poor growth characteristics of host bacteria (the rapid screening procedure is therefore a useful method for identifying bacterial mutants that exhibit altered regulation of the synthesis of the *traT* protein, and also for mutants with altered regulation of other structural components of the cell and that exhibit poor growth). Two of the three serum resistance-defective plasmid derivatives have been examined: one of them, pKT147, specified normal surface exclusion (exclusion index of pKT107 with R100drd:51) whereas the other, pKT145, exhibited greatly increased surface exclusion (indices with R100drd of 45 and 1032, respectively). This suggests that surface exclusion and serum resistance are independent activities of the *traT* protein, although a change in the activity of *traS* in pKT145 cannot at this time be ruled out.

Outer membranes prepared from bacteria carrying the pKT145 and pKT147 plasmids did not exhibit detectable amounts of a 25,000 dalton protein and we conclude that these mutant plasmids no longer direct the synthesis of the *traT* protein, or that they direct the synthesis of (a) *traT* protein in severely reduced amounts, (b) a protein of altered molecular weight, or (c) a protein that is no longer transported to the outer membrane. In view of the fact that neither mutant plasmid specifies less than the normal level of surface exclusion, alternatives (a) or (b) appear the most plausible, although the isolation and analysis of more mutant plasmids will be required before a firm conclusion can be drawn.

The K1 Capsular Antigen Mediates Bacterial Resistance to Serum

The K1 polysaccharide capsular antigen is an important virulence factor of *E.coli* strains that produce meningitis and septicaemia in neonates[19-21]. Its precise role in bacterial pathogenicity has not thus far been elucidated but it is known to reduce the sensitivity of bacteria to phagocytosis[22] and some epidemiological data indicate that the K1 antigen also provides bacteria with resistance to serum[23], although this latter conclusion has recently been challenged[3,24,25].

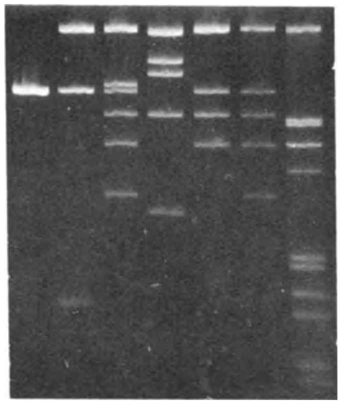

Fig. 3. Cleavage of hybrid plasmids that specify K1 antigen biosynthesis with BamHI endonuclease. Plasmid DNA preparations were obtained, digested with BamHI restriction endonuclease, and the fragments thereby generated analysed by electrophoresis through a 0.8% agarose gel, as previously described[10]. From left to right: pHC79 (cosmid vector), pKT168 ($K1^-$), pKT169 ($K1^+$), pKT170 ($K1^+$), pKT171 ($K1^+$), pKT172 ($K1^+$), λ DNA cleaved with EcoRI and HindIII. pKT170 is from the PstI gene bank; the remaining hybrid plasmids are from the BamHI gene bank.

In order to be able to examine the serum resistance properties of essentially isogenic strains that differ only in their ability to synthesize the K1 capsule, we have cloned the K1 biosynthesis genes in E.coli K-12 strain LE392. This was carried out with the cosmid cloning — λ packaging system[26] using the pHC79 vector[26] and E.coli Bi 7509/41 (O7:K1:H^-) DNA that had been partially cleaved with BamHI or PstI, to produce two gene banks. A number of the clones in these banks were subsequently shown to produce precipitin haloes of specific antigen-antibody complexes, when grown on agar plates containing meningococcus B antiserum (the meningococcus B polysaccharide is identical to the K1 polysaccharide[17]), and hence to synthesize K1 antigen. Plasmid DNA was prepared from four representative $K1^+$ clones, three from the BamHI gene bank and one from the PstI gene bank, and from one $K1^-$ clone and analysed by BamHI endonuclease cleavage (Fig.3). Comparison of the digest patterns of the three hybrid plasmids from the BamHI gene bank indicated that they possess three common BamHI fragments, having sizes of approximately 20, 5.3 and 4.3 kb, which is consistent with the fact that they all specify biosynthesis of the K1 antigen.

Comparison of the serum resistance properties of the LE392 strain of E.coli K-12 and its $K1^+$ derivatives LE392 (pKT172) provided unequivocal evidence that the K1 capsular antigen provides bacteria with substantial protection against serum killing (Table 1). A similar conclusion has been arrived at by comparison of the serum resistance of $K1^+$ wild strains of E.coli and $K1^-$ mutant derivatives thereof[21,27].

DISCUSSION

At least two bacterial components, the R6-5 plasmid-determined traT outer membrane protein and the K1 polysaccharide capsular antigen, mediate resistance to complement killing; as anticipated, both are components of the cell surface.

The R6-5 traT protein is a 25,000 dalton polypeptide that is present in about 20,000 copies in plasmid pKT107-containing bacteria and that is highly exposed on the outer surface of the outer membrane. It provides resistance to complement not by inactivating complement components in the fluid phase but by modifying cell surface structure to prevent one or more steps in complement activation or action. It has been suggested that the traT proteins of the F and R100 plasmids, which are similar to that of R6-5 and which also mediate serum resistance (A. Moll, unpublished data), exist as multimeric aggregates in the outer membrane[15,16]. If this is also the case for the R6-5 traT protein, it is unlikely that it can be randomly distributed in the membrane and at the same time block all of the approximately 30,000 complement binding sites on the cell surface. Indeed, recent data show that there is little difference in the binding of complement components up to C8 to serum resistant and serum sensitive bacteria (ref. 17; Binns et al, this volume; D. Bitter-Suermann, personal communication). Taken together, these results suggest that the traT protein is localized at specific sites in the outer membrane, presumably sites of complement attack (adhesion zones between inner and outer membrane[28] ?), and that it functions either by inhibiting the binding of the terminal complement component C9 to form the membrane attack unit or, more likely, by inhibiting the action of the membrane attack unit.

The R6-5-type of serum resistance does not appear to be uncommon. ColV, a plasmid that is found in a high proportion of invasive strains of E.coli[21,29,30], has been shown to increase bacterial virulence[29,31] and to provide resistance to serum[31] via an outer membrane protein (Binns et al, this volume). Moreover, there appears to be a high degree of correlation between virulence, serum resistance, and the presence of an outer membrane protein in gonococcus[32].

On the other hand, capsules are also common attributes of invasive bacteria and these would appear to provide resistance to serum by a distinct, almost certainly less specific, mechanism that probably involves the shielding of cell surface structures which are ordinarily responsible for activating complement[33]. The fact that invasive strains of E.coli, Haemophilus, etc. frequently contain plasmids of the ColV and R6-5 type and synthesize capsules suggests that both types of serum resistance factor, outer membrane protein and capsule, may be important for bacterial virulence.

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ANTIBIOTIC RESISTANCE - A SURVEY

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The study of antibiotic resistance determinants is an active area of investigation that covers many aspects of plasmid biology. Thus, there is interest in, not only the biochemical mechanism by which the determinants express their resistance, but also in the distribution, origins and dissemination of resistance mechanisms. The problem of dissemination is particularly interesting since antibiotic resistance provides a convenient marker for the investigation of transposable elements. Parenthically, it should be added that plasmid-encoded resistance determinants are key components of all cloning vectors used in recombinant-DNA experimentation and "amp" and "tet" have become almost bywords in the field!

Although this brief review will focus on resistance to clinically useful antimicrobial agents, it should be remembered that R-plasmids may encode resistance to a wide variety of agents that are toxic to bacteria such as bacteriophages, bacteriocins, heavy metals, ionising radiation, serum components, detergents and other environmental poisons ; determinants exist (probably) that protect bacteria against toxic agents which have not yet been recognised. R-plasmids are the ultimate prophylactic agents (1).

In the past few years, studies of R-plasmid encoded antibiotic resistances have identified four distinct biochemical mechanisms that may be involved. These are listed in Table 1 together with some representative examples ; there are still some forms of antibiotic resistance that remain incompletely characterized in biochemical terms (for example, tetracycline, and certain forms of aminocyclitol and chloramphenicol resistance). In addition, resis-

Table I

Mechanisms of R-plasmid encoded antibiotic resistance*

Mechanism	Examples
Enzymatic detoxification	β-lactams, chloramphenicol, aminocyclitols, pristinamicin
Alteration of target site	erythromycin-lincosamide
Altered uptake or retention by cell	tetracycline
By-pass sensitive step with drug-insensitive enzyme	sulphonamides, trimethoprim

* There are other resistance mechanisms known that are due to mutation and are not R-plasmid determined. In addition, there are classes of R-plasmid resistance (to chloramphenicol, aminocyclitols) that are uncharacterized biochemically.

tance to some antibiotics may involve a combination of biochemical mechanisms as exemplified by the aminocyclitols ; these agents are detoxified inside the resistant organism which has the effect of preventing strong binding to their target site (the ribosome) necessary for uptake and maintenance of the drug inside the cell. The overall result is that uptake of aminocyclitols into R^+ cells is drastically reduced (2).

It is well-nigh redundant to discuss the appearance of new forms (allomers) of resistance mechanisms that appear almost routinely with the continued selective pressure of antibiotic use in human health and agricultural applications. The appearance of β-lactamases with different substrate ranges and their spread to different species and genera of bacteria is well-known and is cause for concern now that penicillin-type resistance has been characterized in Neisseria, Hemophilus (3), and other important pathogenic genera. The presence of multiple-drug resistance in these organisms will certainly complicate therapy and in some instances the efficacy of the more advanced cephalosporins is threatened. Although penicillin-resistance in Streptococcus is not of the β-lactamase type its emergence has been a portent of other natural mechanisms of resistance to the β-lactams (4). A similar situation has been encountered in the case of the aminocyclitols and many mechanisms of resistance have (and continue to be) identified in Gram-negative and Gram-positive pathogens. The situation *vis a vis* aminocyclitols is more complex than that of resistance to β-lactams and other antibiotics since a variety of different aminocyclitol modifying enzymes exist in multiple allomeric forms with different substrate ranges (see Table II). More than one form of enzyme has been identified with respect to modification of the 6', 3', 2', 3 and 2" positions of aminocyclitols. For example in the case of modification at the 2"-OH group an allomeric form of adenylyltransferase has been reported recently, with a substrate range that includes the third-generation aminocyclitol amikacin (5) ; this drug was thought to be inert to resistance modification of this type (Fig. 1). The dissemination of such a resistance mechanism into other genera of Gram-negative bacteria could have serious consequences for aminocyclitol therapy of nosocomial infections. It is relevant at this point to voice some concern over the likelihood that large quantities of antibiotics may be used soon in industrial fermentations employing recombinant plasmids ; if "amp" or "tet" are used to maintain the plasmids involved it will be necessary to take steps to remove these agents before disposal of spent medium. As alternatives, the maintenance of plasmids in their hosts during industrial fermentations by other selective or genetically conditional methods should be investigated.

The mechanics of dissemination of R-plasmids and drug resistance is a complex problem ; many R-plasmids are non-conjugative (especially in Pseudomonas and Gram-positive organisms) and the mechanism of resistance spread in such genera in nature is not understood ; clearly transduction and transformation are mechanisms that could operate. However, it is apparent that even extremely rare events such as Staphylococcal-Streptococcal (6) conjugal exchange could lead to the establishment of a new group of resistance mechanisms in a hitherto "virgin" organism. Detailed nucleic acid and protein homology studies should throw some light on this question and positive support for Staphylococcal-Streptococcal exchange comes from the demonstration that the

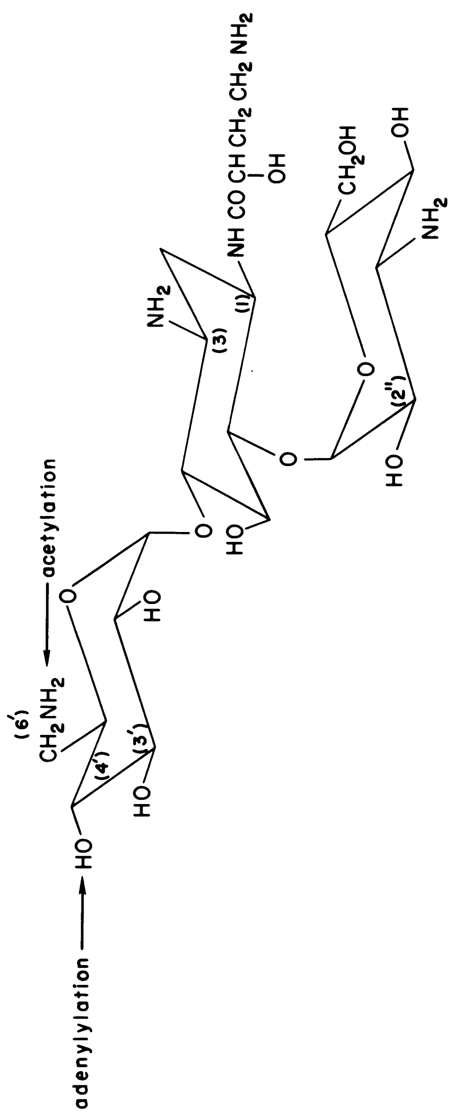
Table II

Aminocyclitol-modifying enzymes

Enzyme	Typical substrates[+]
6'-acetyltransferase (AAC-6')	Kanamycin, tobramycin, amikacin, sisomicin, neomycin.
2'-acetyltransferase (AAC-2')	gentamicins
3-acetyltransferase (AAC-3)	gentamicins, tobramycin, kanamycin, neomycin, fortimicin
4'-adenylyltransferase (AAD-4')	tobramycin, amikacin, kanamycin, neomycin
2"-adenylyltransferase (AAD-2")	gentamicin, tobramycin, kanamycin
3"-adenylyltransferase (AAD-3")	streptomycin, spectinomycin
6-adenylyltransferase (AAD-6)	streptomycin
3'-phosphotransferase (APH-3')	neomycin, kanamycin
3"-phosphotransferase (APH-3")	streptomycin
2"-phosphotransferase (APH-2")	gentamicins, kanamycin
5"-phosphotransferase (APH-5")	ribostamycin, lividomycin
6-phosphotransferase (APH-6)	streptomycin

[+]These vary with the isozymic form of the enzyme.

macrolide-lincosamide resistance determinants in Staphylococcus and Streptococcus are homologous both in biochemical function and sequence (7).



AMIKACIN

Figure I

With regard to the dissemination of resistance between replicons, transposable drug resistance provides a satisfactory mechanism for this exchange (8) and at present most, if not all drug resistance genes can be demonstrated to transpose either singly or in groups (Table III). The case of gentamicin resistance is interesting ; until recently transposition of these resistance genes, in spite of the widespread use of the drug and occurence of resistant strains, had not been recognized. However, several examples of gentamicin resistance transposition have now been identified and the reason for difficulty in detection is probably due to the fact that the gentamicin resistance transposons are large elements in which the gene for gentamicin resistance is associated with at least 3 or 4 other resistance determinants ; their size > 10 Md prevented detection by transposition onto bacteriophage lambda which could not accomodate such a large insert, even though λ is normally a convenient receptor in transposition assays (9). Studies

Table III

Transposable drug resistances

β-lactams
chloramphenicol
streptomycin-spectinomycin
trimethoprim
erythromycin-lincomycin
gentamicin-tobramycin
sulphonamide
tetracycline
fosfonomycin

of gentamicin resistance transposition from plasmid to plasmid (by the use of incompatibility) have demonstrated the widespread nature of transposable elements encoding gentamicin resistance (10, 11). More recently in studies with a number of clinical isolates using bacteriophage P1 as a receptor for transposons, it has been demonstrated that a large proportion of gentamicin resistance determinants is capable of transduction either by transposition or cointegration (12). To date most of the transposable resistance elements that have been studied are from Gram-negative organisms, and substantial information has been obtained with respect to their structure and function. Only one Gram-positive transposable element has been relatively well characterized, an erythromycin resistance element (Tn551) that, interestingly, has short inverted repeat sequences that share some homology with Tn3, the β-lactamase transposon of Gram-negative bacteria (13). In addition, there is strong circumstantial evidence that the aminocyclitol resistance genes of Staphylococci are transposable since the genes are associated with invertible DNA structures of wide distribution and similar structure (14). However, definite proof of transposition is lacking. It is worth noting that, with respect to the intrageneric exchange of resistance mechanisms, most if not all Gram-positive resistance genes have been found to be expressed in Gram-negative hosts, although the reverse has not been demonstrated.

No survey of R-plasmid encoded resistance can be complete without some mention of recent studies on the sequences of the resistance genes and their regulatory elements. The complete DNA sequences of a β-lactamase (15), chloramphenicol acetytransferase (16, 17) two aminocyclitol phosphotransferases (18, 19) and erythromycin ribosomal RNA methylase (20, 21), as well as partial sequences for other genes are available. Of particular interest is the regulatory region of the erythromycin resistance gene ; sequence studies have led to the proposal that induction of the expression of this gene (erythromycin resistance) can be explained by antibiotic inhibition or slowing of ribosome movement over the leader sequence to allow the formation of a new mRNA conformation that exposes the initiator sequence to start translation. This attentuation mechanism bears a strong resemblance to those proposed for the regulation of tryptophan, histidine, and threonine biosynthesis. Sequences of the regulatory regions of point mutants to constitutivity support the model since the single base changes of the mutants are in sites that expose the initiator and allow continuous translation (20, 22). It will be of interest to see if the same situation obtains in Streptomyces erythreus, the erythromycin producing strain.

With regard to the regulation of other resistance determinants, much less is known. The Gram-negative chloramphenicol acetyltransferase (23) is a constitutive enzyme and is (apparently) regulated by catabolite repression only. The Gram-positive chloramphenicol acetyltransferase is inducible and it will be of interest to see how this gene is regulated - will it be like the attenuator-controlled erythromycin resistance of Staph aureus, or will it involve classical repressor-operator interactions ? In what way will chloramphenicol act as inducer ? There has been much nice work on the regulation of tetracycline resistance which functions through a typical repressor-operator interaction (24). It will be intriguing to know how the tetracycline actually interacts with the repressor ; will the same inducible mechanism obtain for the tetracycline resistance of Gram-positive R-plasmids ? The sequence of the aminocyclitol phosphotransferase 3'-I of Tn903 presents several interesting features, the gene is expressed in several eukaryotic organisms and the probable regulatory region has certain "eukaryotic" features (e.g. a Hogness box) that may well be associated with its capacity to be expressed well in a eukaryotic cytoplasm (25). Studies of the regulation of other resistance genes are eagerly awaited, since they may offer additional surprises concerning regulatory mechanisms.

To conclude this brief review, mention must be made of the ubiquity of antibiotic resistance mechanism in the microbial population of nature. In a variety of microorganisms other than clinical isolates, antibiotic resistance mechanisms have been characterized that are biochemically identical to those found in R-plasmid harboring organisms. In addition resistance mechanisms to new or rarely used antibiotics have been found in antibiotic producing organisms (Table IV). The detection of these mechanisms may be of predictive value in the design and modification of antibiotics in the future ; biochemical mechanisms of resistance to a given antibiotic may be very limited. The strong biochemical homology between resistance of clinical isolates and producers has been used as the basis of a hypothesis that R-plasmid-encoded resistance mechanisms may have evolved from antibiotic-producing organisms in nature. The failure to detect any nucleic acid homologies between the various determinants rules out the possibility of any direct (and recent) gene transfer between the different types of organisms (26). However, it will be interesting to see if DNA or protein sequence studies indicate any active site homologies. In the one case where two members of an allozymic group have been compared directly at the sequence level there is no evidence of any similarity in DNA or protein sequence (except that they contain the same bases and amino acids !) (Table V).

Table IV

Naturally-occurring (non R-plasmid) antibiotic resistance mechanisms

Resistance mechanism	Source
β-lactamase	Actinomycetes, Bacillus
chloramphenicol acetyltransferase	Actinomyces
erythromycin ribosome methylase	Actinomycetes, Bacillus
aminocyclitol acetyltransferases	Actinomycetes
aminocyclitol phosphotransferases	Actinomycetes, Bacillus
aminocyclitol adenylyltransferases	Bacillus
thiostrepton ribosome methylase	Actinomyces
viomycin phosphotransferase	Actinomycetes
hygromycin phosphotransferase	Actinomycetes

Table V

Comparison of	APH(3')-I	APH(3')-II
M.W.	27k	23-25k
Amino acid residues	~280	~200 (sequence incomplete)
Base composition	44 % GC	63 % GC
K_m neomycin	2 uM	4 uM
Amino acid composition	high in asp arg~lys	asp~glu arg>lys

This would tend to indicate that, at least in the case of these two aminoglycoside-3'-phosphotransferases, evolution occurred independently from two different and entirely unrelated sources. Will we be able to find the sources, or possible replicons on the evolutionary route to these genes (enzymes) as they now exist ? Since some R-plasmid resistance determinants are (unlike Gram-negative chromosomal genes) expressed in Gram-positive cytoplasm, one might argue that the determinants are not typical Gram-negative genes even though they reside in such hosts.

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REGULATION OF PLASMID SPECIFIED MLS-RESISTANCE IN *BACILLUS SUBTILIS* BY CONFORMATIONAL ALTERATION OF RNA STRUCTURE

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INTRODUCTION

Resistance to the macrolide-lincosamide-streptogramin B (MLS) group of antibiotics, often mediated by plasmids, is widespread among clinically isolated strains of *Staphylococcus* and *Streptococcus* (1-5). The mechanism of resistance to these inhibitors of protein synthesis has been elucidated by B. Weisblum and his colleagues (6-8). MLS-resistance is associated with the presence of additional methyl groups (as N^6,N^6-dimethyl adenine) on 23S rRNA. This modification reduces the ribosomal affinity for the MLS antibiotics. In many cases exposure to a subinhibitory concentration of erythromycin (Em), results in induction of resistance to elevated levels of antibiotic. Although only Em and a few closely related macrolides like oleandomycin (Om) act as inducers, cultures exposed to these drugs acquire resistance to the entire range of MLS antibiotics. We will deal in this report with the MLS resistance specified by the 3.5 kb plasmid pE194. This entity was isolated from *Staphylococcus aureus* (9) and then transferred to *Bacillus subtilis* (10). All of our work has been carried out in the latter organism.

Certain features of induced resistance deserve emphasis. The regulatory system must possess some means of avoiding what appears to be an intrinsic dilemma: how to induce increased synthesis of a protein (ribosomal methylase - see below), by exposure to an inhibitor of protein synthesis (Em). This contradiction might be resolved kinetically or spatially. For instance, the Em-sensing

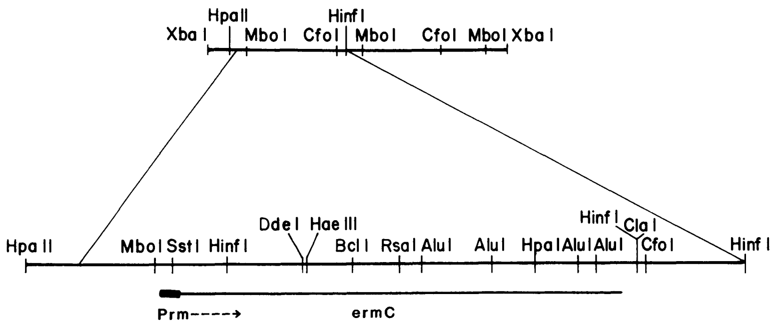

Fig. 1. Restriction endonuclease cleavage site map of pE194. The plasmid is shown linearized at its single XbaI site. The segment containing the ermC gene is shown in expanded form and the position of the ermC determinant is indicated by a horizontal bar. The location of the ermC promoter (Prm) and the direction of transcription are also shown.

sites for induction may be separated physically from the ribosomes which must translate the ribosomal methylase. Also, since resistance is dependent on the specific methylation of rRNA, we can predict that an economic regulatory system might sense both the intracellular concentration of Em and the existing extent of rRNA methylation. Such a dual requirement for Em and unmethylated ribosomes as positive effectors for methylase induction would generate a feedback loop, since induced methylase will decrease the concentration of unmethylated ribosomes. Such a system would be capable of maintaining a steady state level of enzyme just sufficient to methylate newly synthesized ribosomes.

The ermC gene and its product.

The inducible MLS-resistance specified by pE194 is encoded by the ermC gene. This determinant specifies a 29,000 dalton polypeptide, the synthesis of which has been shown to be induced by Em (11). Cloning of restriction fragments and deletion analysis have defined the location of the ermC gene on the pE194 physical map (12,13). The gene is located between the single SstI and ClaI sites (Fig. 1). These studies have also served to confirm the essential role of the 29 K protein in MLS resistance. The direction of transcription and the location of the ermC promoter have been established by RNA polymerase binding studies, deletion analysis, transcription mapping and by DNA sequencing (12,13). The promoter is near the single SstI site and transcription of ermC proceeds from left to right on the map (Fig. 1). Recently, we have purified to homogeneity an inducible ribosomal methylase specified by pE194 (unpublished).

This enzyme co-electrophoreses with the 29 K protein on SDS-polyacrylamide gels. The methylase requires S-adenosyl methionine and methylates only "free" 50S ribosomes *in vitro*.

Induction is Posttranscriptional.

Using the *B. subtilis* minicell system (11,14), we have established that induction of the *ermC* gene product is mediated posttranscriptionally (15). Enhanced synthesis of the 29 K protein occurs in response to an inducing (27nM) concentration of Em, even after transcription is arrested by the addition of either rifampicin or streptolydigin. This enhancement is specific; synthesis of the other four known pE194 polypeptides is unaffected by this concentration of Em. Tylosin (Ty), a non-inducing macrolide antibiotic, does not stimulate synthesis of the 29 K protein. These experiments also reveal that Em (and not Ty) specifically lengthens the functional half-life of the 29 K protein transcript, since the synthetic capacity of the minicells for this protein decays very slowly in the presence of both rifampicin and Em. Although we suspect that stabilization of *ermC* mRNA by Em is a secondary consequence of enhanced translation, it may very well be an important factor contributing to the all-over induction of ribosomal methylase.

Induction Requires a Ribosomal Em-Binding Site.

Minicells pre-incubated in the presence of Em cannot be induced when the drug is washed away and then added back (15). Instead, methylase synthesis continues at the basal (uninduced) rate. This is easily explained in terms of the feedback loop postulated above, since pre-induced cells contain methylated ribosomes. The non-inducibility of pre-induced cells is consistent with the notion that a ribosomal Em-binding site is required for induction. In support we cite two more observations. First, all Em-sensitive *ermC* mutants studied to date, are hyper-inducible for the mutant protein (15). This strongly indicates that a feedback mechanism is operative during induction of the normal methylase and is consistent with the hypothesis that an unmethylated ribosomal site is required for induction. Second, we can perturb the Em-binding site by introducing *ole-1*, a chromosomal mutation which alters ribosomal protein L17 and results in a low-level resistance to Em (G. Williams and I. Smith, pers. commun.; 16). Ribosomes isolated from *ole-1* cultures bind Em poorly *in vitro* (15). Minicells from an *ole-1* strain carrying pE194 cannot be induced to synthesize the ribosomal methylase at an elevated rate by the usual inducing concentrations of Em. Once again we are led to the conclusion that a ribosomal Em binding site is required for induction. If an Em-ribosome complex must form in order for induction to occur, then the system has an effective way to meter the intracellular concentrations of both Em and unmethylated

(Em-sensitive) ribosomes as postulated above. These properties of the system seem eminently consistent with our conclusion that induction is regulated posttranscriptionally.

Regulatory Mutants.

Selection of colonies capable of growth on Ty in the absence of Em, permits ready isolation of constitutive (tyc) mutants (10). These plasmid mutations result in elevated levels of methylase in the absence of induction (11; unpublished). The map location and the nature of the tyc mutations strongly support the posttranscriptional model of methylase regulation. Out of 21 spontaneous tyc mutants which we have studied, 12 contained plasmids of larger molecular weight than the pE194 parent. Restriction endonuclease mapping of the "extra" DNA present in these molecules revealed that this material (ranging in size from about 100 to 600 base pairs) was inserted between the single SstI and HaeIII sites of pE194 (Fig. 1). Several other tyc mutations which do not appear to result in larger plasmids, were mapped by marker rescue (17) and found to be located near the HaeIII site (unpublished). Thus, the tyc mutations are located at the promoter proximal end of ermC. If these mutations exert their effects posttranscriptionally, as does Em-induction, we would expect that the mutations might be located within the transcribed portion of ermC. In this respect they would be unlike operator-promoter mutants which are themselves usually not transcribed. This expectation was confirmed for two mutants, tyc-16 and tyc-9 which result from insertions of about 100 and 600 base pairs respectively. The sizes of the tyc-16, tyc-9 and wild type ermC transcripts were measured in a blotting-hybridization experiment. The ermC transcript, normally about 0.97 kb in size, was enlarged by about 0.1 and 0.6 kb in the mutants (13). Thus the tyc mutations most likely act posttranscriptionally as does Em-induction. Two tyc mutants have been sequenced and will be described below.

Structure of the ermC Gene.

The ermC gene has been entirely sequenced (13). A portion of the sequence, corresponding to the promoter proximal region is reproduced in Fig. 2. Transcription-mapping has confirmed that transcription initiates near the tandem A residues at positions 196-197 (13). Centered about 10 bases upstream from this position is a TATAAT sequence which is a typical prokaryotic consensus "-10 sequence" (18). The sequence contains a single open reading extending from an ATG codon at 337 to a TAA termination codon at position 1069 (not shown). This is sufficient to encode a protein of 244 amino acids with molecular weight 28,947, in agreement with the known molecular weight of the ribosomal methylase. Thus the sequence

```
TCGTAATTAAGTCGTTAAACCGTGTGCTCTACGACCAAAACTATAAAACCTTTAAGAACTTTCTTTTTTTACAAGAAAAAAGAAATTAGATAAATCTCT   99

CATATCTTTTATTCAATAATCGCATCCGATTGCAGTATAAATTTAACGATCACTCATCATGTTCATATTTATCAGAGCTCGTGCTATAATTATACTAAT   198
                                               MboI           -35            SstI      -10

                    MetGlyIluPheSerIluPheValIluSerThrValHisTyrGlnProAsnLysLys
TTTATAAGGAGGAAAAAAATATGGGCATTTTTAGTATTTTTGTAATCAGCACAGTTCATTATCAACCAAACAAAAAATAAGTGGTTATAATGAATCGTTA   297
      S.D.1                                                                            HinfI

                                        MetAsnGluLysAsnIluLysHisSerGlnAsnPheIluThrSerLysHisAsnIluAsp
ATAAGCAAAATTCATATAACCAAATTAAAGAGGGTTATAATGAACGAGAAAAATATAAAACACAGTCAAAACTTTATTACTTCAAAACATAATATAGAT   396
                              S.D.2

LysIluMetThrAsnIluArgLeuAsnGluHisAspAsnIluPheGluIluGlySerGlyLysGlyHisPheThrLeuGluLeuValLysArgCysAsn
AAAATAATGACAAATATAAGATTAAATGAACATGATAATATCTTTGAAATCGGCTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAAT   495
                                                     DdeI    HaeIII
```

Fig. 2. The promoter proximal portion of the ermC sequence (13) is presented. The coding strand is shown. The promoter is located near the SstI cleavage site and the inferrred RNA polymerase binding and recognition elements are denoted as "-10" and "-35." The probable transcriptional initiation point at 196-197 and the direction of transcription are marked by an arrow. The inferred ribosomal binding sites are indicated as "S.D.1 and S.D.2" and probable translational start codons are shown by wavy underlining. The stop codon for the 19 amino acid peptide is denoted by double underlining.

defines a 141 base leader sequence, between positions 197 and 337. Within this leader are several noteworth features summarized in Fig. 2 and 3. SD1 and SD2 (Shine-Dalgarno sequences (19) represent probable ribosomal binding sites, possessing 7 and 9 base complementarities with the terminal 3' sequences of bacillus 16S rRNA (20-22). Correctly situated downstream from SD1 and SD2 are ATG codons. The first potentially initiates translation of a 19 amino acid polypeptide. The second almost certainly initiates methylase synthesis. In addition to these features, the leader region contains 6 complementary segments (I-VI) which permit folding into several possible stem-loop structures (Fig. 3). Within two of these potential structures (A & B), the ATG codon for methylase synthesis and part of SD2 are buried within a base-paired region. In structure C, SD2 and its associated ATG codon are unpaired. In all of these possible structures, SD1 and its ATG codon are exposed and therefore available for interaction with a ribosome.

Fig. 3 also indicates the locations of the tyc-1 and tyc-16 mutations (13). tyc-1 substitutes A for C at position 317. tyc-16 inserts an extra 109 base pairs between positions 321-322. The

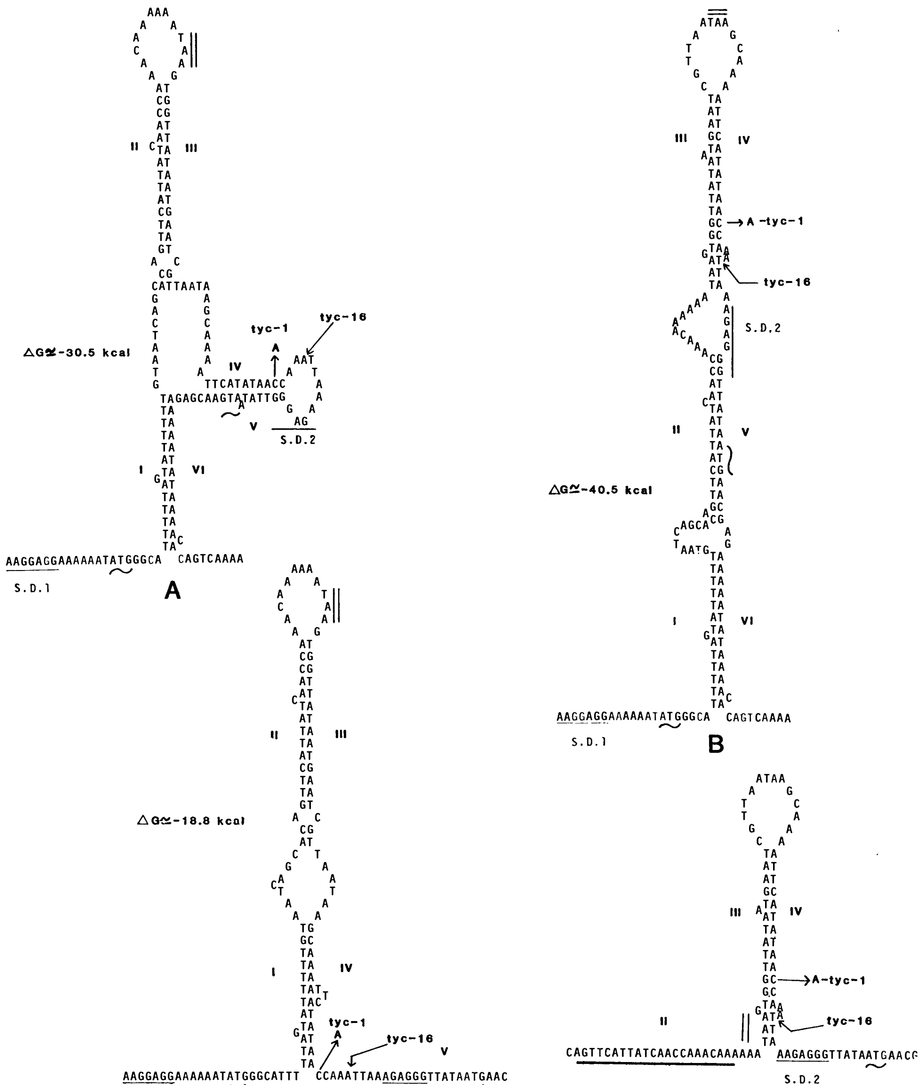

Fig. 3. Hypothetical hairpin structures for the 5' end of ermC mRNA. The sequences are written with thymine residues to facilitate comparison with Fig. 2. The locations of the tyc-1 base change and the tyc-16 insertions are indicated. Calculated energies of structures A and B (inactive) and C (active) are given (31,32). The inferred ribosomal binding sites and start and stop codons are denoted as in Fig. 2. The heavy bar on structure D is meant to suggest the approximate location of a ribosome, stalled in the presence of Em.

inserted DNA is a direct tandem duplication of the 109 residues immediately downstream from the point of insertion.

We will now suggest a model for the regulation of ribosomal methylase synthesis, which is based on the features of the ermC system described above.

Translational Attenuation.

Several of the features of the ermC system are reminiscent of the attenuation mechanisms proposed for regulation of a number of amino acid biosynthetic operons (23-28). These features include the presence of a leader with extensive potential secondary structure, the possible translation of a short polypeptide within the leader sequence and a requirement for a ribosome as a positive effector. We propose that ribosomal methylase synthesis is regulated by an attenuation-like mechanism, but entirely on the translational level. Other, less attractive alternatives have been discussed (13). The present model suggest that mRNA folding determines the rate of methylase synthesis and that this folding can be influenced by tyc mutations as well as by Em-induction. The transcriptional attenuation models (23-28) hold that depletion of an amino acyl tRNA species causes stalling of a ribosome at a sensitive site during leader-peptide translation. This in turn induces isomerization of the nascent mRNA, which removes a structural feature required for the termination of transcription. Consequently, transcription of the structural genes can occur. Our model of translational attenuation suggests that Em causes ribosome stalling during translation of the 19 amino acid peptide which results in refolding of the already completed transcript into an active configuration for methylase synthesis. This mechanism is consistent with a large body of work which points to the importance of mRNA secondary structure in regulating translation by masking or exposing initiation sequences (29,30).

Some Specific Features of ermC Regulation.

In minicells and in whole cells a basal level of methylase is produced without induction (11 and unpublished). The basal synthesis corresponds to about 5% of the induced level. This can be explained by the spontaneous refolding of inactive structures (A and B) into an active form e.g. C. (Fig. 3). The calculated energies of these structures (31,32) suggest that they are stable enough to exist in the cell. Considerable uncertainties are associated with these calculations however, making quantitative assertions about the equilibrium ratios of active and inactive forms very dangerous. Nevertheless, A, B and C should be in equilibrium with one another, with the concentrations of A and B being higher than that of C. The tyc-1 mutation elevates the basal level 2-4 fold (unpublished). This mutation increases the calculated energy of A from -30.5 to -22.5 Kcal/mol and that of B from -40.5 to -31.6. On the other hand, by permitting the formation of several additional base pairs, the stability of the tyc-1 mutant form of C is, if anything, slightly increased (from -18.8 to -19.4 Kcal/mol). Thus an increase in the concentration of C would be expected to occur, leading to an increased basal synthesis, as observed. tyc-16 permits an entirely new structure to form, since complementary segments V and VI are duplicated. The inserted DNA should form structures identical to A and B, but with the "original" V and VI segments located downstream, in an exposed configuration and contiguous with the remaining protion of the ermC coding sequence. This arrangement should result in enhanced basal synthesis as observed.

The translational attenuation model of induction presupposes that a ribosome can bind at SD1 and initiate translation of the 19 amino acid peptide. Inhibition of this ribosome by a bound molecule of Em, will result in stalling within segments I or II. Ribosomal stalling, we suggest, induces isomerization of structure A or B to generate D, exposing SD2 and the methylase start codon. We will first consider this model in the light of what is known about the mode of Em action. This antibiotic appears to slow the movement of ribosomes on mRNA (33). Em binds to "free" ribosomes and to polysomes, from which peptidyl tRNA has been removed. It binds poorly to native polysomes (33,34). Em (and Om) inhibit neither initiation complex formation, nor formation of the first peptide bond (35,36). These macrolides seem to inhibit translocation or transpeptidization only when the nascent peptidyl tRNA has grown to some (unknown) chain length (37). In addition to size, the chemical nature of the polymerized amino acid residues is an important determinant of Em sensitivity, with bulky and hydrophillic amino acids contributing to enhanced susceptibility (38). Other MLS antibiotics, such as Ty, can inhibit formation of the first peptide bond following initiation (36). We would expect then, that Em will bind to a 50S ribosome before or immediately following initiation of 19 amino acid peptide synthesis. When the peptidyl tRNA reaches a critical length and when the appropriate amino acid substituents are polymerized, the ribosome will stall. In Escherichia coli, initiating ribosomes protect 30-35 bases in mRNA from the action of RNAse A (39). Thus it is plausible to suggest that ribosome stalling within segments I or II will sequester segment II leading to the freeing of segment III (Fig. 3). This will result in formation of structure D directly from B. Freeing of III within structure A will also increase the concentration of D, since the III-IV structure is more stable (-15.0 Kcal/mol) than IV-V (-11.7 Kcal/mol). This model can also accomodate the failure of Ty and other MLS antibiotics to act as inducers of ermC expression. Em and Om may induce because they permit the nascent peptidyl tRNA to elongate somewhat before ribosome stalling occurs. Ty would be expected to prevent formation of the first peptide bond (fmet-gly) thus possibly stalling the ribosome in a position which does not induce isomerization to an active structure. Whether a given MLS antibiotic acts as an inducer should therefore depend on the details of molecular structure in a given system. A dramatic feature of the transcriptional attenuation system is the presence of several tandem repeat amino acid codons which comprise a site of extraordinary sensitivity to depletion of a specific amino acyl tRNA (23-28). It is tempting to speculate that the 19 amino acid peptide is likewise a specialized sensor of Em. In fact, polylysine synthesis seems particularly sensitive to the action of Em (40). Perhaps the HisTyrGlnProAsnLysLys sequence at the C-terminus of the peptide comprises a string of bulky hydrophillic residues for sensing the presence of Em (38).

The translational attenuation model surmounts the dilemma posed initially, since it separates the Em-sensing device from the ribosomes which translate the methylase structural sequence. Since induction can occur at low ($\sim$20nM) Em levels and since ribosomes are half-saturated in vitro by Em at about 1μM (15) most of the ribosomes which bind and initiate at the newly exposed SD2 sequence will be uninhibited. Once translation of methylase begins, increased Em concentrations will have no effect, since Em does not bind to polysomes (33,34). Slow colony formation can occur when uninduced cells carrying pE194 are seeded on plates containing as much as 8mM Em (unpublished). Since a basal level of methylase exists in this system, some methylated ribosomes presumably are also present prior to induction. Em will cause non-methylated ribosomes to stall within the coding sequence for the 19 amino acid peptide, thus exposing SD2 by isomerization of the mRNA. If a rare methylated ribosome then attaches and initiates, methylase will be synthesized. Thus, the ingridients are present for a slow exponential escape from inhibition by high concentrations of Em, a bacteriostatic agent.

Some Features of Posttranscriptional Regulation.

RNA polymerase (41-43), gene 32 protein of T4 (44), ribosomal proteins (41,42,45-48) and proteins of RNA bacteriophage (30) all seem to be regulated on the level of translation and all are proteins capable of interaction with nucleic acid. The ribosomal methylase of pE194 clearly fits into this category. The former systems also seem to be regulated autogenously, by direct binding of each protein to specialized secondary structures at the 5' end of mRNA. Methylase induction is at least formally autogenous, although the feedback loop is probably mediated via rRNA methylation. Direct autogenous regulation of methylase synthesis by binding of the protein to mRNA has not been excluded and it is tempting to speculate by analogy, that such direct autorepression operates.

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CONTROL AND DNA STRUCTURE OF THE *ampC* β-LACTAMASE GENE OF *ESCHERICHIA COLI*

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INTRODUCTION

Escherichia coli K-12 is coding for a β-lactamase which hydrolyzes the β-lactam ring of both cephalosporins and penicillins including ampicillin. Its structural gene, *ampC*, has been mapped to 93.8 min on the *E. coli* chromosome (Burman et al., 1973; Grundström et al., 1980). The level of *ampC* β-lactamase is stric - ly proportional to the gene dosage, and to the ampicillin resistance (Normark et al., 1977). These features enabled us to directly select for ColEl *ampC* hybrid clones within the collection of ColEl hybrids prepared by Clarke and Carbon (Clarke and Carbon, 1976; Edlund et al., 1979). One ColEl *ampC* hybrid plasmid was physically mapped and the location of *ampC* within this plasmid was deduced by subcloning (Grundström et al., 1980). We could thereby demonstrate that the *ampC* gene was present on a 1,370 bp DNA segment. By selecting for various degrees of ampicillin resistance a number of *E. coli* mutants have been isolated that hyperproduce the *ampC* β-lactamase due to mutations in *ampA*, a control sequence region for *ampC* (Grundström et al., 1980).

In this paper we report the complete nucleotide sequence for the *ampC* operon. We also show that *ampA* contains both a promotor and an attenuator. Mutations in both types of control sequences may cause elevated production of *ampC* β-lactamase. The relative synthesis of *ampC* β-lactamase increases with growth rate (Jaurin and Normark, 1979). We present data suggesting that the growth rate dependent regulation of *ampC* is due to antitermination of transcription at the *ampC* attenuator.

RESULTS AND DISCUSSION

Nucleotide sequence of the ampC gene

The entire ampC gene with flanking sequences was DNA sequenced using the procedure of Maxam and Gilbert (Fig. 1). To localize the beginning of the coding region for ampC, the order of the twelve N-terminal amino acids of purified ampC β-lactamase was determined by Edman degradation. A complete correspondence was found with the codons stretching from base +117 to base +152. The nearest translation start sequence appeared nineteen codons before the N-terminal amino acid, alanine, of the purified enzyme. This means that the primary translational product of ampC carries a nineteen amino acid long N-terminal extension. This extension has all the structural features of a signal peptide.

The ampC β-lactamase consists of 358 amino acids and has a molecular weight of 39,600. It is therefore considerably larger than previously sequenced β-lactamases (Ambler, 1979). The four previously sequenced β-lactamases all show a preference for substrates of the penicillin group and are therefore penicillinases, whereas the ampC β-lactamase is a cephalosporinase. The four sequenced penicillinases all show extensive sequence homologies with each other (Ambler, 1979). Short blocks of amino acid residues that show homology between the ampC β-lactamase and the consensus sequence of the four sequenced penicillinases are shown in Fig. 2. Outside the homologous amino acid blocks very little, if any, amino acid sequence homology is found between the ampC β-lactamase and the penicillinases. Only one β-lactamase apart from ampC has been sequenced on DNA level, namely the bla gene of pBR322 (Sutcliffe, 1978). Upon comparing the DNA sequence of these two genes, small sequence homologies were found even in regions outside the blocks coding for homologous amino acid residues. This may indicate that the bla and the ampC genes have evolved from a common ancester gene. An active site peptide fragment of the chromosomally encoded β-lactamase of Pseudomonas aeruginosa has recently been sequenced (S. G. Waley, personal communication). The deduced amino acid sequence showed a significant degree of homology with the region around serine 80 in the ampC β-lactamase. Clearly this suggests that serine 80 is the reactive residue at the active site. The ampC β-lactamase like all four sequenced penicillinases exhibit regions of amino acid sequence homology with some sequenced D-alanine carboxypeptidases (Fig. 2). As these latter groups of enzymes also contain a reactive serine in the active site (Yocum et al., 1979) we speculate that cephalosporinases, penicillinase, and D-alanine carboxypeptidases may have a common evolutionary origin.

The DNA sequences at positions -13 to -8 (-T-A-C-A-A-T-) and -35 to -30 (-T-T-G-T-C-A-) (Fig. 1) show a five out of six basepair homology with the conserved -10 and -35 regions of promotors,

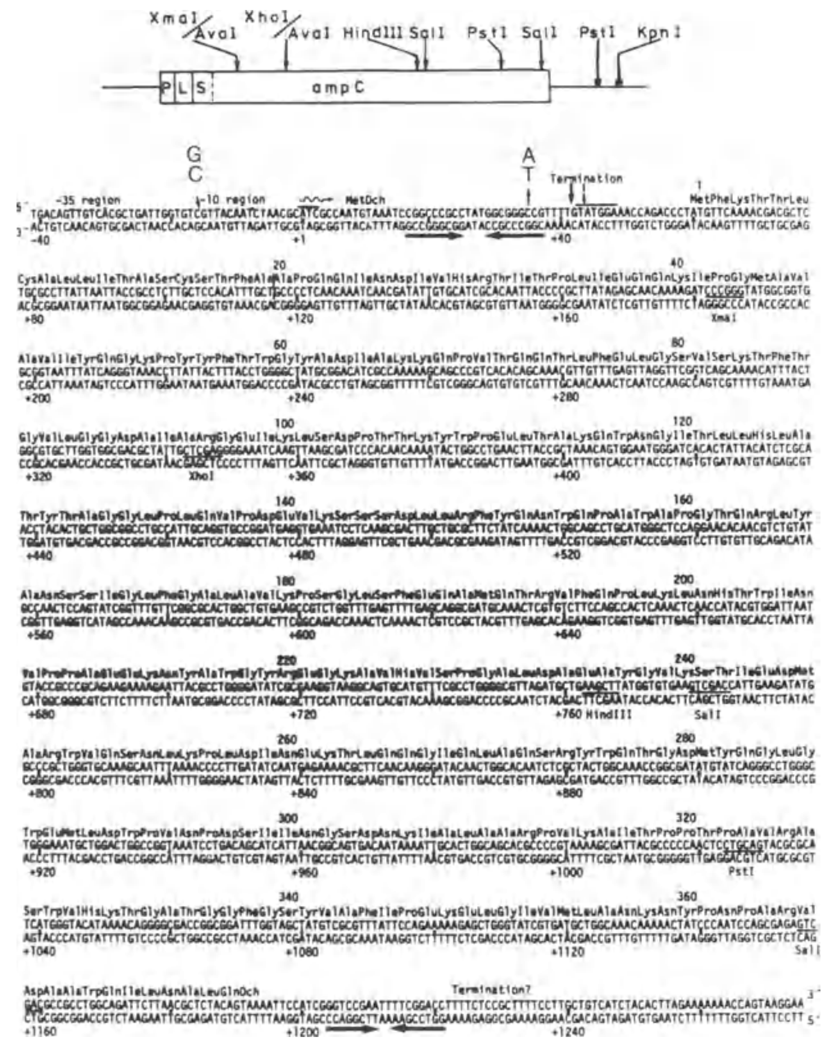

Figure 1. Upper part: Restriction enzyme map for enzymes that cut at most twice in the ampC region. The box displays the location of the ampC gene. P, L, and S indicate promotor region, leader region, and signal peptide, respectively. Lower part: DNA sequence of the ampC gene from Escherichia coli K-12. The three letter abbreviations for the amino acids of the ampC β-lactamase appear directly over their three-base codons and they are numbered (every 20th amino acid) starting from the first methionine. The positions of restriction enzyme sites are marked with a horizontal line between the strands, and the names of the enzymes are written below the strands. The start of transcription is marked by +1 and the wavy arrow. The major and minor termination points of the attenuator are (Continuation next page)

respectively (Siebenlist et al., 1980). By RNA sequencing it was possible to demonstrate that the adenine at position +1 is the first base of the β-lactamase mRNA. The β-lactamase leader-DNA is 59 basepair long. In this leader we find a nine basepair long, exclusively G-C containing, dyad symmetry at nucleotide positions

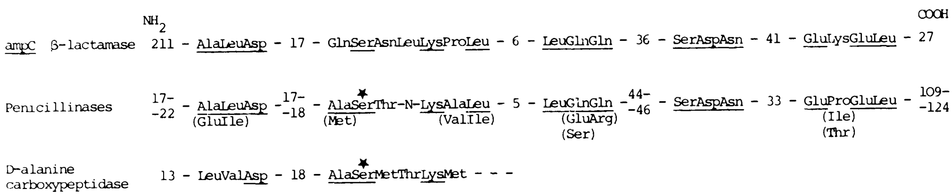

Figure 2: Regions showing amino acid sequence homologies between the ampC β-lactamase, four sequenced penicillinases (Ambler, 1979) and the sequenced N-terminal part of the D-alanine carboxypeptidase of *Bacillus stearothermophilus* (Yocum et al., 1979). The number of amino acids separating the blocks of homology and the termini of the proteins are given. Where the four penicillinases differ from each other, the stretches are indicated as intervals. Within the parenthesis are given the amino acid residues in the case where one of the penicillinases differ from the others. -N- means that four different amino acids are found at that position. Underlined amino acids represent homologous residues found in at least two of the three groups of proteins. The serine residues marked with stars have been shown to bind active site-directed substrate analogous for the D-alanine carboxypeptidase, and the β-lactamase I of *B. cereus*.

(Continuation Fig. 1)
indicated by vertical solid and dashed arrows, respectively. Solid horizontal lines designate possible ribosome binding sites. The regions of dyad symmetry in the attenuator and the possible operon terminator are marked by horizontal arrows. The boundary between the signal peptide and the mature β-lactamase is marked by a vertical dashed line. The -35 and -10 regions of the β-lactamase promotor are indicated. The ampP15G16 G-C insertion and the ampL35A A-T transversion are indicated.

+17 to +25 and +29 to +37. This symmetrical DNA sequence is followed by a stretch of four T residues on the non-coding strand. Thus, this region has all the features of a terminator for transcription. In vitro transcription studies have indeed revealed that about 94% of all initiated transcripts are terminated with base +41 at their 3' end. Thus, the ampC gene was found to be controlled by attenuation of transcription.

The XhoI/KpnI DNA segment that carries most of the structural gene ampC (Fig. 1) was ^{32}P-labelled by nick translation and used as a DNA probe in Southern blotting experiments with restriction endonuclease digests of chromosomal DNA from a number of enterobacterial genera (Fig. 3). The ampC probe hybridized to DNA fragments of the same size in strains of Escherichia coli, Shigella sonnei, Shigella flexneri, Salmonella typhimurium, Serratia marscesens and Klebsiella pneumoniae. Therefore, a region with extensive sequence homologies to that of the ampC gene is present in all tested members of the Enterobacteriacae. It therefore seems likely that the chromosomal β-lactamases within these genera constitute a very related group of enzymes.

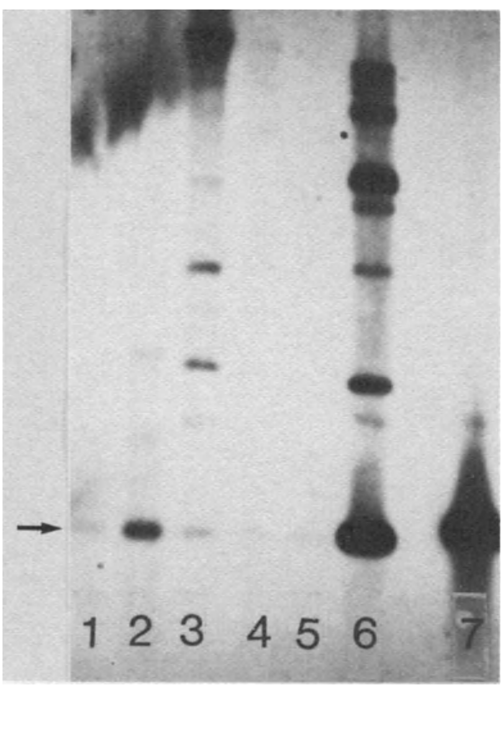

Figure 3: Hybridization of a ^{32}P-labelled XhoI/KpnI ampC probe to XhoI/PvuII digests of enteric DNAs. The XhoI/KpnI ampC probe was 1060 bp large. This fragment was 90 bp larger than the XhoI/PvuII fragment. Lane 1, S. typhimurium lane 2, S. sonnei lane 3, S. flexneri lane 4, K. pneumoniae lane 5, S. marscesens lane 6, E. coli lane 7, ampC probe. The location of the ampC hybridization fragment is indicated by the arrow.

Mutations leading to overproduction of the ampC β-lactamase

i) Promotor mutations

The resistance of *E. coli* K-12 to ampicillin is 1 μg/ml. At an incidence of about 10^{-10} mutants are found with a fifteenfold increase in both resistance to β-lactam antibiotics and production of the *ampC* β-lactamase. One such mutant was isolated several years ago and the mutated control sequence region was denoted *ampA* (Eriksson-Grennberg, 1968). Plasmid derivatives carrying the mutation was isolated, and the *ampA* region was physically mapped, and DNA sequenced. The mutation leading to a fifteenfold increase in β-lactamase production was found to be a G-C insertion between positions -16 and -15 in the *ampC* promotor (Fig. 1). This mutation denoted *amp*P15G16 (previously *ampA1*), is an up-promotor mutation, because *in vitro* transcription of an 1.5 kb SacII-XhoI DNA fragment carrying the mutation resulted in the increased synthesis of both the short leader transcript and a long run off transcript. The *amp*P15G16 mutation increases the distance between the conserved sequences of the *ampC* promotor. The mutation indicates that the sterical arrangement between the two conserved regions is important for efficient initiation of transcription.

ii) Attenuator mutations

From an *E. coli* K-12 strain carrying the up-promotor mutation *amp*P15G16 spontaneous mutants with a further four to tenfold increase in β-lactamase production could be isolated.
These mutants fell into two groups with respect to genetic stability. The group of mutants unstable in a *rec*$^{+}$ background was found to carry multiple tandem repeats of the *ampC* region of the *E. coli* chromosome (Edlund et al., 1979). The genetically stable mutants were each found to contain an additional mutation in the same 370 bp DNA segment as where *amp*P15G16 had been mapped (Grundström et al., 1980).

The nucleotide sequence of the *ampC* control region was established in one such double mutant. In addition to the mutation *amp*P15G16 a transversion from C-G to A-T was found at position +35. This *amp* leader mutation denoted *amp*L35A occurred within the *ampC* attenuator. When a DNA fragment carrying both *amp*P15G16 and *amp*L35A was transcribed *in vitro* no synthesis of the short leader transcript was observed. Instead, the amount of the long run off transcript was further increased. Thus, the *amp*L35A mutation abolishes the terminator located within the *ampC* leader.

In a coupled *in vitro* transcription-translation system the *amp*L35A mutation leads to a fourfold increase in the synthesis of the *ampC* pre- β-lactamase. This corresponds to the fourfold increase in the steady state amount of β-lactamase found *in vivo* at high growth rates. In the *in vitro* transcription system the *amp*L35A mutation increased the transcriptional read-through about sixteenfold (from about 6 per cent to virtually 100 per cent). This suggests that in vivo, and in the coupled transcription-translation system one or several factors decrease the degree of termination at the wild type attenuator.

The relative amount of the ampC β-lactamase increases with growth rate (Jaurin and Normark, 1979). The amount of a majority of the more abundant E. coli proteins exhibit a similar positive correlation with growth rate. This group of proteins includes ribosomal proteins, elongation factors, aminoacyl-tRNA-synthetases as well as the subunits of the RNA polymerase (Pedersen et al., 1978).

The growth rate response of an E. coli strain carrying the ampP15G16 promotor mutation was investigated. The specific amount of ampC β-lactamase increased proportionally with growth rate as in the wild type (Table 1). However, in an E. coli double mutant

Table 1. Relative amount of ampC β-lactamase produced in strains LA51 and TE18 at different growth rates.

LA51[a]		TE18[a]	
k[b]	relative amount[c]	k[b]	relative amount[c]
0.33	1.00	0.26	8.90
0.47	1.25	0.33	8.83
0.95	1.87	0.50	8.65
1.39	2.47	0.97	8.73

[a]The E. coli K-12 strain LA51 carries the ampP15G16 up-promotor mutation. Its derivative TE18 carries in addition the attenuator mutation ampL35A.

[b]Growth rates are expressed as k, the first-order constant, in units of $hour^{-1}$, as calculated from the expression k = ln2/mass doubling time in hours.

[c]The relative amount of ampC β-lactamase produced is based on the relative area obtained from rocket immunoelectrophoresis.

carrying both the ampP15G16 and the ampL35A mutations, the growth rate dependent regulation was abolished. Thus, in this mutant, the level of β-lactamase was constantly high. There was no change in β-lactamase level within a threefold variation in growth rate (Table 1). This suggested that a functional attenuator is required for growth rate dependent regulation. Attenuators have been found within the leader DNA of a number of amino acid biosynthetic operons, e.g. the trp operon (Crawford and Stauffer, 1980).

Unlike these operons, the 41 bases long <u>ampC</u> leader transcript has no coding region for a leader peptide. Moreover, the <u>ampC</u> leader RNA cannot form any alternative secondary structure that would preclude formation of the terminator stem. However, in the <u>ampC</u> leader RNA the first three bases are complementary to a sequence near the 3' end of 16S RNA. At bases 8 to 13 is an initiation codon (AUG) directly followed by an ochre stop codon (UAA). The <u>amp</u> leader RNA has therefore the potential to bind a ribosome

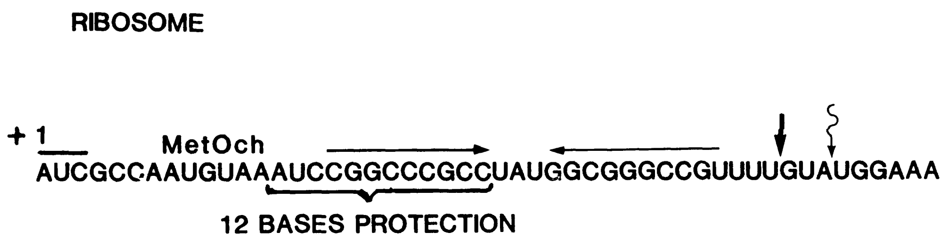

Figure 4: A model for growth rate dependent attenuation of transcription. Hypothetical protection by the ribosome is thought to prevent formation of the termination stem, leading to read-through (see text for Discussion). The stippled area represents a ribosome. Start of transcription is marked by +1. The solid line designate a possible basepairing with the 3' end of 16S RNA. Met and Och stands for initiation codon (Methionine) and stop codon (Ochre), respectively. The termination stem is indicated by the horizontal arrows. The major and minor termination points of the attenuator are displayed by vertical solid and wavy arrows, respectively.

and form an initiation complex. If a ribosome binds to the nascent <u>ampC</u> leader it would preclude the formation of the terminator stem and loop structure and favour transcriptional read-through. The amount of ribosomes per cell mass increases with growth rate. Our current hypothesis is therefore that the concentration of ribosomes is the factor that regulates the degree of anti-termination at the <u>ampC</u> attenuator (Fig. 4).

We have studied the growth rate response of a number of clinical E. coli isolates that hyperproduces the ampC β-lactamase. The response was abolished in one of the isolates but retained in the remaining five isolates tested. This suggets that both promotor and attenuator mutations may be selected for in the in vivo situation.

ACKNOWLEDGEMENTS

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MECHANISMS OF PLASMID-DETERMINED HEAVY METAL RESISTANCES

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INTRODUCTION

Many plasmids of both Gram negative and Gram positive bacteria have genes determining resistances to a wide range of toxic inorganic cations and anions, including ions of mercury (and organomercurials), cadmium (in Gram positives only), arsenic, antimony, bismuth, chromium, silver and tellurium. Three years ago, we reviewed the available information on the physiological and biochemical bases of these resistances, as well as the genetic structures that govern them (Summers and Silver, 1978). Here I will try to summarize both the overall picture and newer findings.

MERCURY AND ORGANOMERCURIAL RESISTANCES

Plasmid-determined resistance to Hg^{2+} and to organomercurials occurs in both *Staphylococcus aureus* (Novick and Roth, 1968) and *Escherichia coli* (Smith, 1967). The frequency of Hg(II) resistance determinants among clinical isolates can be well over 50% (e.g. Nakahara et al., 1977a and b) and among the collection of over 800 plasmids introduced into *E. coli* K-12 in Drs. Datta and Hedges' laboratory, about 25% conferred Hg(II) resistance (Schottel et al., 1974). There are differences in frequencies of these resistances. Although Cd(II)-resistance is found with high frequency in *S. aureus* of both human and animal origin, Hg(II)-resistance is common in human hospital staph but rare or absent in non-hospital human and animal *S. aureus* (Lacey, 1980; Witte et al., 1980).

We have found a small number of resistance patterns for organomercurials among strains with plasmids: (a) In *E. coli* over

90% of the mercury-resistance plasmids confer resistance to the organomercurials merbromin and fluorescein mercuric acetate (FMA) but to no other tested organomercurial (Fig. 1). We called these "narrow spectrum" mercurial-resistance plasmids (Weiss et al., 1978b), since the other 4% "broad spectrum" plasmids additionally conferred resistances to phenylmercuric acetate (PMA) and thimerosal. The plasmids in Pseudomonas aeruginosa also divided into "narrow" and "broad" spectrum with regard to resistance to organomercurials (Clark et al., 1977); however, about 50% of the plasmids tested fell into each class. Furthermore, the "narrow spectrum" Pseudomonas plasmids also conferred resistance to p-hydroxymercuribenzoate (pHMB) and the "broad spectrum" Pseudomonas plasmids showed still additional resistance to methylmercuric and ethylmercuric compounds (Clark et al., 1977; Weiss et al., 1978b). Only a single pattern has been found with S. aureus plasmids (Weiss et al., 1977,1978b), but this pattern is different yet in

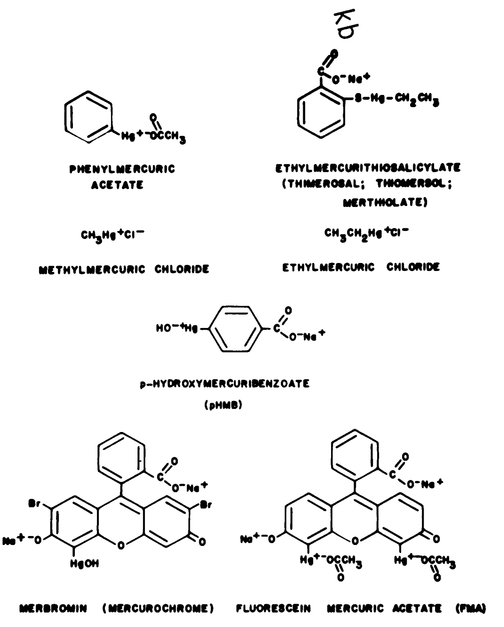

Fig. 1. Structures of the organomercurial compounds.

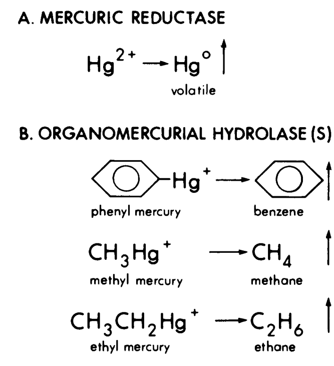

Fig. 2. Enzymatic detoxification of Hg(II) and organomercurials.

that all the S. aureus plasmids conferred resistances to PMA, pHMB and FMA but not to thimerosal or to merbromin. Recently, Hg(II)-resistant plasmid-bearing Bacillus have become available (Timoney et al., 1978; K. Izaki, in press; D. Reanney, personal communication). These plasmids all conferred a pattern of resistance to Hg(II) and organomercurials identical to that in S.

aureus (T.G. Kinscherf, unpublished). Thus plasmids confer resistances to a range of organomercurials, and each type of organism shows only a small number of resistance patterns. To a limited extent these patterns can be correlated with plasmid incompatibility groups (Schottel et al., 1974; Weiss et al., 1978b).

Enzymatic Mechanism of Mercury and Organomercurial Detoxification

Hg^{2+} resistance results from enzymatic detoxification leading to the volatilization of mercury from the growing bacterial culture. This was discovered independently in two laboratories in Japan (Tonomura and Kanzaki, 1969; Furukawa and Tonomura, 1972; Komura et al., 1971; Izaki et al., 1974) and our own (Summers and Silver, 1972). The volatile mercury was shown to be metallic Hg° in each case and the enzyme responsible is the mercuric reductase enzyme.

Several organomercurials were also enzymatically detoxified to volatile compounds. These include methylmercury, ethylmercury, PMA, pHMB and thimerosal (Fig. 2); benzene is produced from PMA, methane from methylmercury and ethane from ethylmercury, and these have been identified by gas chromatography. The enzymes responsible for cleaving the Hg-C bond are organomercurial lyases. In a soil pseudomonad (for which a plasmid has never been demonstrated), Tezuka and Tonomura (1976,1978) were able to separate two small soluble lyase enzymes. Both have molecular weights of about 19,000 and require thiol reagents such as thioglycolate. The two lyases were difficult to separate by chromatographic methods, but when this was accomplished (Tezuka and Tonomura, 1978), it was found that one enzyme cleaved PMA, pHMB and methylmercury, while the other enzyme cleaved only PMA and pHMB. With a plasmid-containing E. coli, there was no evidence for hydrolysis of pHMB (Weiss et al., 1978b) and Schottel (1978) was unable to separate the two lyases. Nevertheless, kinetic analysis indicated that there were two enzymes active toward PMA but only one active toward methyl-and ethylmercury. The E. coli organomercurial lyases appeared to have a somewhat greater molecular weight, but otherwise the general properties of the enzymes from the soil pseudomonad and E. coli were rather similar.

Mercuric reductase has been studied in greater detail both with plasmid-bearing E. coli (Izaki et al., 1974; Schottel, 1978) and with the soil pseudomonad (Furukawa and Tonomura, 1972). The intact enzyme has a molecular weight of about 180,000 and consists of three identical subunits (Schottel, 1978), each containing an FAD molecule. The enzyme is strictly NADPH-dependent and one NADPH is oxidized per Hg(II) reduced (Schottel, 1978).

Antibodies have been prepared against purified mercuric reductases coded by two different plasmids in E. coli (Kinscherf, in preparation). All reductases tested (obtained from different Gram negative sources) reacted with these antibodies as shown by inhibition of enzyme activity and formation of precipitin bands on double-diffusion gels. The enzymes divided into two major subclasses, based on only partial immunological identity. The prototype enzyme of the first class is coded by transposon Tn501, the first well-studied mercuric resistance transposon (Bennett et al., 1978). This enzymological class also includes mercuric reductases governed by a variety of plasmids found in clinical isolates of enteric bacteria and P. aeruginosa, in marine pseudomonads, and in Pseudomonas putida (the MER plasmid). The MER plasmid harbors a transposon, Tn1861, which appears indistinguishable (Friello and Chakrabarty, 1980) from Tn501 (Bennett et al., 1978) which originated in a clinical P. aeruginosa isolate. That is one strong conclusion from studies of plasmid-determined mercuric resistance: the same system appears widely in clinical isolates and in bacteria from other environments. The second immunological subgroup of the Gram negative mercuric reductases has as its prototype the enzyme coded by plasmid R100, one of the earliest and most thoroughly studied of the antibiotic resistance plasmids. It is with this plasmid that the genetic structure of the mercuric resistance operon was recently studied (Foster et al., 1979; Nakahara et al., 1979). This subgroup also includes enzymes from plasmids of a wide variety of incompatability groups and also the enzyme determined by a second characterized Pseudomonas mercury transposon, Tn502 (Kinscherf, in preparation; Stanisich, in preparation). Although all of the mercuric reductases from Gram negative bacteria were immunologically related, the antibodies prepared against the two classes of Gram negative enzymes did not cross react with mercuric reductases from S. aureus strains and marine and soil Bacilli. These enzymes from Gram positive sources showed similar masses and functional requirements to those from E. coli (Weiss et al., 1977), but they are immunologically distinct from the Gram negative enzymes.

To summarize briefly the current understanding of plasmid-determined mercuric and organomercurial resistances: (a) These occur widely in both Gram positive and Gram negative species and are the best understood of all plasmid-coded heavy metal resistances. (b) Resistance is due to enzymatic detoxification of the mercurials to volatile compounds of lesser toxicity that escape from the growth media. (c) The enzymes responsible (mercuric reductases and organomercurial lyases) have been purified and studied in vitro.

CADMIUM RESISTANCE IN S. AUREUS

Plasmid-determined cadmium resistance has been found only in S. aureus (Novick and Roth, 1968). In some clinical collections, Cd(II) resistance is the most common of the S. aureus plasmid resistances, exceeding in frequency both mercury and penicillin resistances (Nakahara et al., 1977a). Gram negative cells without plasmids are just as resistant to Cd(II) as are staph cells with plasmids (Nakahara et al., 1977b), probably because of relatively reduced Cd(II) uptake by the cells (Silver, unpublished).

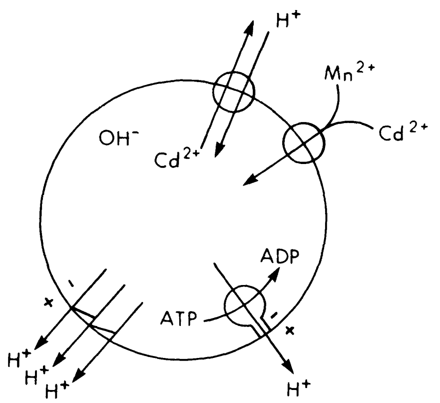

Fig. 3. Model for Cd^{2+} uptake and efflux systems (from Tynecka et al., 1981).

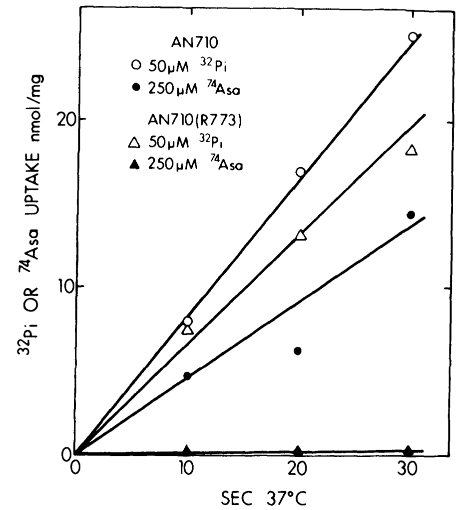

Fig. 4. Arsenate and phosphate uptake by sensitive and resistant E. coli (from Silver et al., 1981).

The mechanism of Cd(II) resistance is a constitutive block on the accumulation of Cd(II) by the resistant cells (Chopra, 1970, 1975; Tynecka et al., 1975). This was initially considered a direct permeability block (Chopra, 1975), but it was later found that Cd(II) enters S. aureus cells as an alternative substrate for the cellular Mn(II) transport system (Weiss et al., 1978a; Silver, 1978). Resistance prevented Cd(II) accumulation through this transport system. Most recently, it has been shown that the lowered accumulation is due to a plasmid-coded efflux system that rapidly excretes Cd(II) rather than a direct effect on the uptake process itself (Tynecka et al., 1981). Fig. 3 shows the current model of Cd(II) resistance including the shared Mn(II)/Cd(II) uptake system found in both sensitive and in resistant S. aureus cells and the Cd(II)/H^+ exchange system that functions only in resistant cells (Tynecka et al., 1981). Studies with membrane vesicles (R.D. Perry, in preparation) support this picture by showing identical kinetic parameters for Cd(II) and Mn(II) transport in right-side out vesicles from sensitive and from resistant cells. Unfortunately, the type of inside-out vesicle studies used by McMurry et al. (1980) to demonstrate a similar efflux transport system for tetracycline in plasmid containing E. coli have not succeeded in S. aureus.

ARSENATE, ARSENITE AND ANTIMONY(III) RESISTANCES

Arsenic and antimony resistances are governed by the same S. aureus plasmids that code for other heavy metal resistances (Novick and Roth, 1968). The first arsenic-resistance plasmid in E. coli was found by Hedges and Baumberg (1973) and more recently many similar plasmids have been isolated (Smith, 1978). The first detailed report of arsenate, arsenite and antimony(III) resistances is, however, still in press (Silver et al., 1981). I will summarize here the basic findings of that paper.

Arsenate, arsenite and antimony(III) resistances are coded for by an inducible operon-like system in both S. aureus and E. coli (Silver et al., 1981). All three ions induce all three resistances. Genetic studies with S. aureus plasmids demonstrate that the gene for arsenate resistance is different from but closely linked to the gene for arsenite resistance, which in turn may not be the same as that for antimony(III) resistance (Novick et al., 1979). Bi(III) is a gratuitous inducer of this system with E. coli plasmid R773, which does not confer Bi(III) resistance (Leahy and Silver, unpublished).

The mechanism of arsenate resistance is a reduced accumulation of arsenate by induced resistant cells. Arsenate is normally accumulated via the cellular phosphate transport systems, of which bacterial cells appear to have two (Silver, 1978). Phosphate protects cells from arsenate toxicity, just as high Mn(II) protects sensitive S. aureus from Cd(II) toxicity (R.D. Perry, unpublished). The separateness of arsenate and arsenite resistances was shown by the finding that phosphate did not protect against arsenite (Silver et al., 1981). The presence of the resistance plasmid does not alter the kinetic parameters of the cellular phosphate transport systems, not even the K_i for arsenate as a competitive inhibitor of phosphate transport. This finding, coupled with direct evidence for plasmid-governed efflux of arsenate, suggests that the arsenate resistance system will be an efflux transport system (Silver et al., 1981), similar to that described above for Cd(II).

We do not know the mechanism(s) of arsenite or of antimony resistances. Arsenicals and antimonial compounds are toxic by virtue of inhibiting thiol-containing enzymes (e.g. Albert, 1973). Some dithiol reagents such as BAL (British anti-Lewisite) protect against arsenicals and antimonials. We have experimentally eliminated two possible hypotheses for arsenite and antimony resistances proposed in our earlier review (Summers and Silver, 1978). Arsenite is not oxidized to the less toxic arsenate by plasmid-bearing E. coli or S. aureus (Silver et al., 1981). Growing resistant cells do not excrete soluble thiol compounds into the medium to bind arsenite and antimony, since pre-growth of resistant cells in medium containing these toxic ions does not allow subsequent growth of sensitive or of uninduced resistant cells (Silver et al., 1981). We are left only with untested hypotheses of an alteration in uptake or a change in a key intracellular target.

SILVER RESISTANCE

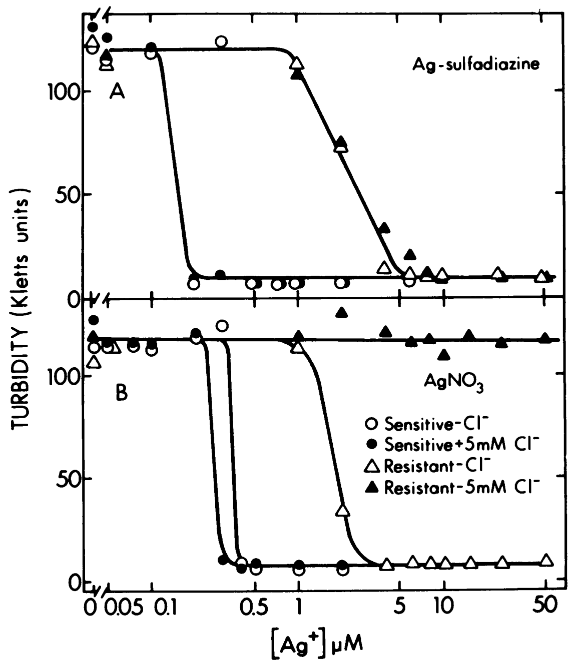

Fig. 5. Resistance of E. coli strains J62 (sensitive) or J62 (pSC35) (resistant) to Ag sulfadiazine and $AgNO_3$ in low and high Cl^- (Silver and Leahy, unpublished).

Silver-resistance plasmids are among the more recent discoveries of the heavy metal resistance plasmids (McHugh et al., 1975; Annear et al., 1976; Summers et al., 1978; Bridges et al., 1979). These resistance plasmids have been found (not surprisingly) following the widespread use of silver salts as topical treatments for extensive burns (e.g. Fox, 1968). Only one known Ag^+ resistance plasmid was transferrable by conjugation (McHugh et al., 1975). However, R.W. Hedges (personal communication) produced a recombinant between plasmid R1 and a Ag^+ resistance plasmid from Citrobacter (Hendry and Stewart, 1979) and introduced it into an *E. coli* K-12 strain. We have been studying it for much of the last year, since Hedges insisted that "Silver must not ignore silver-resistance," and can report here some progress and a somewhat supported hypothesis for the mechanism of Ag^+-resistance. Silver resistance is constitutive in *E. coli*, like Cd(II) resistance in *S. aureus*, but unlike Hg(II), arsenate-, arsenite-, and antimony-resistances. The plasmid-determined resistance is very great and the ratio of minimum-inhibitory concentrations can be greater than 1000:1 (Fig. 5B). The level of resistance is strongly dependent upon available halide ions; and without Cl^-, there is relatively little difference between the cells with or without a plasmid (Fig. 5B). Br^- and I^- at concentrations far below those required for Cl^- confer resistances on both plasmid-less cells and cells with the plasmid. These results have led to our current hypothesis that both sensitive and resistant cells bind Ag^+ tightly and are killed by effects on cell respiration (Bragg and Rainnie, 1974) and other cell surface functions (Rosenkranz and Carr, 1972; Fox and Modak, 1974). Once bound extracellularly, Ag^+ enters the cells and is found in high speed centrifugal supernatant fluids (unpublished data). Ag^+ precipitates with Cl^-, as the solubility product for AgCl is only 1.6×10^{-10} M at 25°C (and those for AgBr and AgI are significantly lower yet). The hypothesis is that the sensitive cells bind Ag^+ so tightly that they extract it from AgCl, whereas the cells with the resistance plasmid do not compete successfully with Ag-halide precipitates for Ag^+. Because topically applied $AgNO_3$ ointments caused tissue chloride loss, silver sulfadiazine has significantly replaced $AgNO_3$ in clinical practice (Fox, 1968). As seen in Fig. 5A, the $AgNO_3$-resistance plasmid confers resistance as well towards silver sulfadiazine. However, added Cl^- was without effect on the inhibitory concentrations of silver sulfadiazine. This result was expected, since it is known that adding NaCl to solutions of silver sulfadiazine does not cause AgCl precipitates to form. Although many Ag^+-resistant clinical isolates have determinants of sulfadiazine resistance as well, these determinants can be on separate plasmids (Hedges, personal communication). The function of sulfadiazine in topical preparations is not to inhibit bacterial growth directly (the concentrations released are too low; Fox, 1968), but rather to bind silver in a form subject to slow release.

OTHER HEAVY METAL RESISTANCES

There are many other plasmid heavy metal resistances (Summers et al., 1978; Summers and Silver, 1978). Yet, we know nothing today about the mechanisms of resistances to bismuth, boron, cobalt, nickel, tellurium or zinc ions. Chromate resistance in a pseudomonad isolated from river sediment seems to be due to reduction of toxic Cr(VI) to less toxic Cr(III) (Bopp and Ehrlich, Abstract Q111, 1980 A.S.M. Meetings) and this resistance appears to be plasmid determined (Chakrabarty, personal communication). However, caution on this point is needed, since bacteria capable of oxidizing toxic As(III) to less toxic As(V) are also known, but this turned out not to be the mechanism of plasmid-governed resistance (Silver et al., 1981). Hopefully, at future plasmid symposia our understanding of the mechanisms of these heavy metal resistances will be beyond the space limits of such a brief review. We need to start asking why these resistances occur at high frequencies in clinical isolates that have experienced no apparent selection with mercurials, arsenicals, antimonials etc.

ACKNOWLEDGMENTS

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CONJUGATION AND RESISTANCE TRANSFER IN STREPTOCOCCI AND OTHER GRAM POSITIVE SPECIES: PLASMIDS, SEX PHEROMONES AND "CONJUGATIVE TRANSPOSONS" (A REVIEW)

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Until recently, information on the nature of conjugation and related gene transfer in Gram positive bacteria has been relatively scarce. Although conjugation among the actinomycetes has been known for many years,[1,2] fertility plasmids have, so far, been recognized in only a single strain of a single species in this group. Streptomyces coelicolor strain A3(2) harbors two conjugative plasmids, SCP1 and SCP2[3-5]; and whereas SCP2 has been isolated and characterized[5,6], efforts to isolate SCP1 have been unsuccessful. The latter determines the synthesis of the antibiotic methylenomycin[7] and, when integrated into the bacterial chromosome, will mobilize chromosomal segments to SCP1$^-$ strains with almost 100 percent efficiency[8].

It has been only eight years since the phenomenon of plasmid transfer in Gram positive eubacteria was first described[9], and conjugative plasmids have now been identified in a number of species of streptococci, as well as in Clostridium perfringens (Table 1). A report as early as 1964[10] had claimed a high frequency of transfer (2.2 per donor) for a chloramphenicol resistance mutation (presumably on the chromosome) in Streptococcus faecalis. There was no evidence for plasmid involvement, and such a high frequency of chromosomal transfer has not been confirmed. It was not until nine years later that conjugal transfer was again reported--again in S. faecalis. Tomura et al.[11] reported on the transfer of a hemolysin-bacteriocin determinant at relatively high frequency (up to 5.8×10^{-2} per donor). While direct evidence for a plasmid bearing this property was not provided, it is likely that this was the case. [Hemolysin-bacteriocin activity was subsequently shown to be plasmid-borne in a number of other hemolytic (bacteriocinogenic) strains of S.

faecalis[12-16].. Interestingly, these two activities appear to represent one and the same protein[17,18].] About the same time, Jacob and Hobbs[9] presented evidence for conjugal transfer of multiple drug resistance from S. faecalis strain JH1 and were the first to show the direct involvement of plasmid DNA. Strain JH1 actually harbored two conjugative plasmids, a 50 Mdal R-plasmid pJH1, and a 38 Mdal hemolysin-bacteriocin plasmid pJH2[9,13]. In both of the above reports[9,11], transfer occurred in broth in a matter of hours; transfer was DNase resistant, and evidence against transduction was provided. Thus, cell to cell contact seemed a requirement for transfer.

Additional evidence for conjugative plasmids in S. faecalis[12-16, 19-27] as well as in other streptococci[28-37], and even Clostridium perfringens[38, 39], soon followed (see Table 1); and it was shown that, like the case in Gram negative bacteria, nonconjugative plasmids[12, 16, 40] and even chromosomal markers[41,42] could also be mobilized.

Table 1. Gram Positive Species with Naturally Occurring Conjugative Plasmids

Bacteria	References
Actinomycetes	
Streptomyces coelicolor	3-5
Eubacteria	
Streptococcus faecalis (Group D)	9, 12-16, 19-27
Streptococcus pyogenes (Group A)	28-30
Streptococcus agalactiae (Group B)	31-34
Streptococcus lactis (Group N)	35,36
Streptococcus sp. (Group C)	37
Streptococcus sp. (Group G)	37
Clostridium perfringens	38,39

Some attention has been focused recently on pAMβ1, a 17 Mdal conjugative plasmid determining erythromycin resistance. This resistance is representative of the so-called MLS phenotype (i.e., resistance to macrolides, lincosamides and streptogramin B). Originally identified in S. faecalis strain DS5[43], pAMβ1 has been shown to have a broad host range. Its transfer into different species of streptococci was first shown by LeBlanc and co-workers[44], and it now has been shown to establish in nine different species of streptococci[28, 31, 44, 45, 46]. In addition, it has been observed to transfer into Lactobacillus casei[46], Staphylococcus aureus[26, 47] and Bacillus subtilis (O. Landman, personal communication). Interestingly, in S. faecalis, the transferability of pAMβ1 is dramatically inhibited if pAMγ1 or pAD1 is also present in the donor strain; the latter two plasmids remain highly transmissible (Brown and Clewell, unpublished). It is also noteworthy that pAMβ1 has been useful in the construction of streptococcal cloning vehicles[48].

MLS-resistance plasmids resembling pAMβ1 in size (15-20 Mdal) have been identified in S. faecalis[25, 49, 49a], S. pyogenes[29, 30, 50], and S. agalactiae[31-34], as well as in Lancefield groups C and G[37]. One S. pyogenes plasmid, pAC1[30] was found to be more than 90 percent homologous with pAMβ1[51]. [A report[52] showing homology of MLS-resistance determinants in streptococci (including pneumococci) and staphylococci suggests that this determinant has a common origin in Gram positive bacteria.] Malke[28] recently showed that pAC1 (called pDC10535 by him) could be transferred from S. pyogenes to several other species including S. faecalis. In the same report, a rather comprehensive study showed that several MLS-resistance plasmids from different sources transfer (on filter membranes) between strains of streptococcal groups A, B, D and H. Other recent studies demonstrated transfer of drug resistance between strains of groups A, B and D[20, 26, 32], and several MLS-resistance plasmids identified in group B streptococci were transferrable to group B, D, F and H recipients[31]. R-plasmid transfer between S. pneumoniae and streptococcal groups A, B and D[26, 52a] and between Staphyloccus aureus and groups A, B and D[26, 47] has also been reported.

Conjugative systems recently described in group N streptococci[35, 36] involve transfer of the ability to metabolize lactose. Interestingly, variants which donate at high frequency and exhibited an unusual cell-aggregation phenotype were readily generated[35] (L. McKay, personal communication).

SEX PHEROMONES IN STREPTOCOCCUS FAECALIS

In Streptococcus faecalis there appear to be two basic types of conjugative plasmids. There are those such as pAD1, pOB1, pPD1, pJH2, pAMγ1, pAMγ2 and pAMγ3, which transfer at relatively high frequency (10^{-3} to 10^{-1} per donor) in broth[13, 19, 53, 54] (Yagi, Brown, and Clewell, unpublished); and there are those such as pAMγ1, pAC1, pIP501, and pSM15346, which transfer poorly in broth (usually less than 10^{-4}, and in most cases, less than 10^{-6} per donor), but which transfer well (10^{-4} to 10^{-2}) when the matings are carried out on filter membranes[28, 31] (Brown and Clewell, unpublished). The reason for these differences is now becoming clear. Those systems which transfer well in broth, make use of sex pheromones to generate cell to cell contact, whereas those that transfer poorly in broth do not. As illustrated in Fig. 1, recipient strains have been found to excrete soluble, protease-sensitive, heat-stable substances which induce donor cells to become adherent[19, 53, 54]. This induction facilitates the formation of mating aggregates arising from random collisions of these non-motile cells. Since cell-free filtrates of

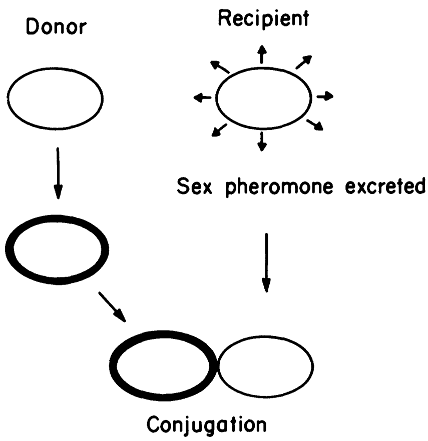

Fig. 1. Expression of sex pheromone by recipient and response by donor containing a conjugative plasmid (see text).

recipients also elicit an aggregation (clumping) response when mixed with donors, this substance has been referred to as "clumping-inducing agent" (CIA). When filtrates of recipients are mixed with donor cells for 20-50 min prior to a short (10 min) mating with recipients, the frequency of plasmid transfer is increased by several orders of magnitude. CIA, therefore, can be viewed as a sex pheromone. The response of donor cells to CIA requires both RNA and protein synthesis, but not DNA synthesis[19]. The acquisition of a conjugative plasmid results in a "shutting off" of endogenous CIA production; and the cell with the newly acquired plasmid becomes responsive to exogenous CIA.

Interestingly, donors harboring different conjugative plasmids respond to different CIAs[53]. A given recipient actually produces multiple pheromones; and the acquisition of a given plasmid shuts off the production of only the related pheromone, while the cell continues to produce other pheromones which can induce other donors with different conjugative plasmids. The pheromones are now identified by relating them to the plasmid originally used to detect them. Thus, cPD1 refers to the CIA to which strains harboring pPD1 respond. Similarly, the other activities are identified as cAMγ1, cOB1, etc.

Studies have now shown that, in addition to an aggregation response, the pheromone induces a function(s) more directly related to plasmid transfer[55]. This was revealed by analyzing isogenic donor-donor matings using derivatives of pAD1 containing two distinguishable transposons (Tn916[42] and Tn917[56, 57]). It was reasoned that if the sole function of the pheromone (cAD1) was to induce aggregation, then once the cells aggregated, transfer should occur equally well in both directions--regardless of which donor was induced with cAD1 prior to mating. It was found, however, that when only one of the donors was induced, transfer occurred only in the direction from the induced to the uninduced strain. If both donors were induced, transfer occurred in both directions. Thus, the pheromone must also induce a "preparation" for plasmid transfer, the nature of which is not known. Conceivably, the pheromone induces a polycistronic operon [perhaps somewhat analogous to the Tra operon of certain conjugative plasmids in Gram negative bacteria (for reviews, see ref. 58, 59)] which, in addition to having determinants related to aggregation, also determines functions related to transfer.

Pheromone activity can be quantitated using a simple microtiter plate system[53]; the highest dilution of filtrate (using serial 2 fold dilutions) that still induces clumping in appropriate responder (donor) cells is taken to represent the pheromone titer. The titer for a given filtrate varies somewhat with the particular responder system; this depends on the conjugative plasmid as well as the host. (Titers typically range from 4-64.) In general, it would seem to be disadvantageous to produce "too much" sex pheromone, or for donors to be "too sensitive"; such behavior would result in donors becoming induced when they are "too far" away from recipients to make contact.

The production of pheromones by recipient cells was found to closely parallel cell growth[53]. In the case of certain plasmid-free strains such as JH2-2 or DR1, CIA activity in filtrates leveled off as cells entered stationary phase. In the case of other strains such as OG1 or ND539, both liquefaciens subspecies, activity in filtrates rapidly disappeared as cells entered stationary phase. It is likely that this decrease is due to degradation by the protease ("gelatinase") produced by these strains (i.e., the liquefaciens subspecies). [A recently derived mutant of OG1, which fails to degrade gelatin, produces CIA activity which does not decrease as the cells enter stationary phase (Yagi, Craig and Clewell, unpublished).]

Recent data have shown that pheromone-induced donor cells have a new antigen on their surface[60]. A highly specific rabbit antiserum prepared against induced pPD1-containing cells (the serum was absorbed with uninduced cells) was found to readily cross-react with different donors (induced) harboring several different conjugative plasmids (pAD1, pOB1, pAMγ1, pAMγ2 and pAMγ3)[60]. The surface material has been referred to as aggregation substance (AS); and, being sensitive to trypsin and pronase, it must be proteinaceous. When submitted to specific immunological staining procedures involving conjugated horse-radish peroxidase and analyzed by electron microscopy, an amorphous surface material (presumably representing AS) could be visualized on the surface of induced, but not uninduced, cells[60]. Pilus-like structures were not seen; however, the possibility that small, difficult to resolve microfimbriae may coat the surface, remains.

AS probably binds to a specific substance, designated binding substance (BS) located on the surface of both recipients and donors. The interaction of AS and BS requires divalent cations and, interestingly, also phosphate ions[60].

Krogstad et al.[61] recently presented electron micrographs of mating mixtures of S. faecalis, showing what appears to be intercellular connections between chains of streptococci in the absence of fimbriae or pili. (While the latter system represented a "high frequency" transfer system, evidence for pheromone involvement was lacking.) Similar "connections" have been observed in pheromone-induced aggregates of cells harboring pPD1[62]; however, preparations of uninduced cells also showed such connections. Thus, in this case at least, it was not clear whether the observed "connections" were an actual reflection of "conjugal contact", or an artifact of the preparation.

The chemical nature of the pheromones is currently being examined. Their sensitivity to proteases [including exopeptidases (R. Craig, unpublished)], as well as heat stability and dialyzability suggests that they are small peptides. [It was originally reported that CIA was sensitive to trypsin[19]. However, subsequent studies have shown that this was probably due to chymotrypsin contamination. Purer preparations of trypsin fail to inactivate cPD1, cAD1, cAMγ1, or cOB1, whereas chymotrypsin inactivates all of these activities (Craig and Clewell, unpublished).] Analyses of cPD1 on molecular sizing columns suggest a molecular weight of less than 1000[54].

Examination of 100 clinical isolates of S. faecalis showed that 34 percent exhibit a CIA response to a filtrate of the plasmid-free strain OG1-10, and 72 percent excreted cPD1[53]. Interestingly, the ability to respond to, as well as produce CIA activities, was significantly more frequent among strains resistant to one or more drugs as compared to drug sensitive strains[53]. Thus, pheromones may contribute to the evolution of drug resistance in this species. A recipient producing numerous sex pheromones would probably be a prime "target" for R-plasmids which confer pheromone responses, or which can be mobilized by such systems. [In the case of pAMγ1, pAMγ2 and pAMγ3, it is worth noting that these plasmids, all having nearly identical molecular weights and previously indistinguishable from each other in their original host (S. faecalis strain DS5[12, 43]), have each been shown to determine responses to different pheromones (Yagi, Brown, Craig and Clewell, unpublished).]

Whereas several of the above-mentioned pheromone-responding plasmid systems (pAD1, pAMγ1, pOB1 and pJH2) determine hemolysin (bacteriocin), this phenotype is not necessarily related to the ability to respond. For example, pPD1, pAMγ2 and pAMγ3 do not determine hemolysin, but confer a pheromone response (Brown, Yagi and Clewell, unpublished). [While it was believed earlier that pPD1 determined a hemolysin[53], this has recently been shown not to be the case. We now know that in the original isolate (strain 39-5), hemolysin is actually determined by a different conjugative plasmid, pPD5, which has a similar molecular weight and which frequently transfers together with pPD1 (Brown, Yagi and Clewell, unpublished). pPD1, however, does determine a bacteriocin activity.] Also, of the 34 clinical isolates mentioned above which exhibited CIA responses, only nine were hemolytic.

A model[53] has been proposed (Fig. 2) to explain the relationship between plasmids, pheromones, and the aggregation phenomenon. The model schematically shows a plasmid-free recipient strain that produces two different pheromones, cA and cB; two isogenic donor strains harboring the conjugative plasmids pA or pB are also shown. All three strains have the chromosomally determined binding substance (BS). Plasmid pA determines the ability to respond to cA; and, at the same time, through an IcA (inhibitor of cA) gene, prevents production of endogenous cA. (Alternatively, an inactivation of cA could be involved). Similarly, plasmid pB allows its host to respond to cB and prevents the production of endogenous cB via gene IcB. The response of the donor cell to the pheromone is depicted as

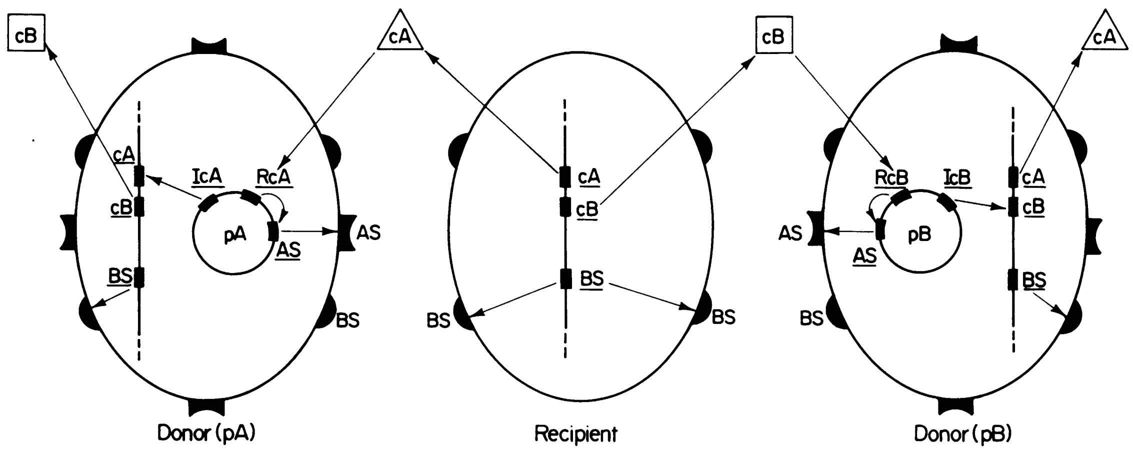

Fig. 2. A model showing various donor and recipient relationships with respect to the synthesis of and response to sex pheromones. cA and cB are the determinants of sex pheromones cA and cB. BS represents the determinant for binding substance (BS) which is located on the cell surface. IcA and IcB are determinants for substances which repress (or inactivate) endogenous cA or cB. RcA and RcB are determinants of regulatory proteins which respond respectively to cA or cB resulting in a "turning on" of the determinant AS which produces aggregation substance (AS) which locates itself on the cell surface. Once a donor has responded to a sex pheromone, AS can now bind to BS which is located on recipients and also donors. Taken from ref. 53.

an interaction (directly or indirectly) of the latter with "responding substance" (repressor or activator?) determined by gene RcA or RcB, which in turn, activates AS synthesis. As, which could be either plasmid (as shown in Fig. 2) or chromosonally determined, locates itself on the cell surface where it can "recognize" BS. (It is clear from the model how induced donors can self aggregate as well as bind to recipients).

The fact that a single recipient strain of S. faecalis may produce numerous sex pheromones specific for different donors seems at first surprising, since it is possible that such cells have never before encountered the "related" plasmids. Conceivably, the pheromones may have other functions in the recipient or represent degradation products of larger proteins. Plasmids might then have evolved in such a way as to take advantage of such molecules to facilitate their dissemination.

CONJUGAL TRANSFER IN THE ABSENCE OF PLASMIDS (CONJUGATIVE TRANSPOSONS?)

When multiply resistant clinical isolates of S. pneumoniae began to appear a few years ago[63, 64], efforts by several research groups to reveal R-plasmids were unsuccessful[65-70] (Brown and Clewell, unpublished). Recently, there have been reports showing that resistance determinants in S. pneumoniae are capable of transfer to recipient strains on membrane filters by a DNase-resistant process[69, 71]. Plasmid-free transfer has been observed in S. faecalis[42, 72], in groups A, B F and G streptococci[73], and certain oral streptococci[74] (D. LeBlanc, personal communication); and there are indications that it may also occur in Clostridium difficile[75].

Shoemaker, Smith and Guild[71] reported that two plasmid-free isolates of S. pneumoniae (BM6001 and N77) could transfer Tc- and Cm-resistance determinants on membrane filters at a frequency of 10^{-6} per donor. Transfer was resistant to DNase; however, transfer of an Em^r chromosomal mutation (ery-2) marker could be eliminated by DNase. The two resistance markers cat and tet had earlier been shown by transformation studies to be closely linked and were shown both physically and genetically to represent insertions (referred to as Ω cat tet) in the bacterial chromosome[67]. In matings, about 90 percent co-transfer of cat and tet was observed; however, while tet could transfer without cat, the reverse did not occur[71]. Also, Cm^r, Tc^s derivatives could be generated by transformation, but they failed to donate cat by conjugation. It was estimated that cat had a size of 4-6 kb, whereas tet was greater than 30 kb[67].

Buu-hoi and Horodniceanu[69] reported that several plasmid-free clinical isolates of S. pneumoniae could transfer resistance traits not only into S. pneumoniae recipients (by filter mating), but also to group B and group D strains. In some of these cases, transfer occurred en bloc as: Tc^r and Cm^r; Tc^r and MLS^r; or Tc^r, Cm^r, MLS^r and Km^r. Similar observations were made in clinical isolates of Group A, B, F and G streptococci[73].

Franke and Clewell have reported that a transferrable Tc-resistance determinant located on the chromosome of S. faecalis strain DS16 is located on a 10 Mdal transposon[42, 72]. Designated Tn916, this element was shown to insert at multiple sites into several different conjugative plasmids at a frequency of about 10^{-6}. Transposition of Tn916 from the chromosome to the conjugative plasmid pAD1 is Rec-independent, as is its ability to transfer in the absence of plasmid DNA (at a frequency of $\sim 10^{-8}$). (Transfer of Tc-resistance was not reduced if either the donor or the recipient was Rec^-.) Transfer involved the entire transposon; after introduction of a conjugative plasmid into transconjugants, typical transposition to plasmid DNA could be detected. Transfer from a plasmid-free donor required cell to cell contact; extensive efforts to implicate transformation or transduction by a variety of means were unsuccessful. [It is noteworthy that S. faecalis has never been transformable (despite exhaustive efforts to obtain transformation), nor have transducing phages ever been reported in this species.]

After transfer from the plasmid-free strain DS16C3, some transconjugants have been found to retransfer Tn916 at an elevated frequency ($\sim 10^{-6}$), about 100-fold higher than "normal"[76]. Interestingly, the transposition frequency from the chromosome to a subsequently introduced pAD1 is also elevated about 100-fold in such strains[76], suggesting a common step for both transfer and transposition.

It has also been shown that after Tn916 transfer, insertion can occur at different sites on the recipient chromosome[76]. This was done in the following way. Insofar as Tn916 has a single Hind III cleavage site, Hind III digests of chromosomal DNA containing Tn916 should give rise to two fragments, X and Y, which constitute the transposon-host DNA junction fragments. With the "Southern blot" hybridization technique, using an EcoR1 fragment of pAD1::Tn916 (there are no EcoR1 sites in Tn916) as a probe, hybridization with the two fragments (X and Y) readily occurs. However, the size of the detectable X and Y fragments varies greatly in chromosomal DNA preparations obtained from different transconjugants (including those from secondary matings), a result which would be expected if Tn916 were located at different sites on the chromosome. (It is noteworthy that this result also is strong evidence against the location of Tn916 on a plasmid which had escaped physical detection.)

It is likely that Tn916 determines (at least in part), functions related to its own transfer; at a size of 10 Mdal there would seem to be room for such genetic information. Thus, transfer could simply represent an elaborate transposition event where the donor and recipient replicons are in different cells. A model generated from an earlier proposal[42, 72] is suggested in Fig. 3, where the transposon is shown to excise and then have the option to: i) re-insert onto the chromosome (perhaps at a different location); ii) insert onto a resident plasmid; or iii) transfer to another cell.

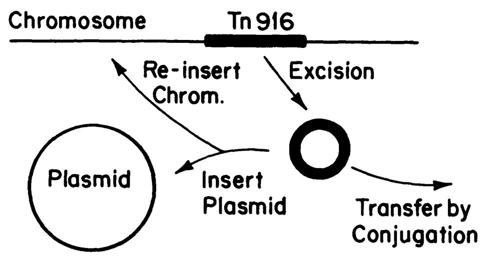

Fig. 3. Model showing Tn916 as a "conjugative transposon" (see text).

After transfer into the recipient, insertion might be facilitated by "zygotic induction" of an "integrase" (the related transposase?). Since transfer probably occurs by a single strand (i.e., plasmid-like) process, a copy of the transposon would remain in the donor and might still be capable of reinserting into host DNA.

In view of the growing evidence in a number of species of streptococci for conjugal transfer in a plasmid-free environment, it will be interesting to see the extent to which these systems represent "conjugative transposons". In this regard, Guild's group (unpublished) has recently shown that Tn916 has homology with the transferrable tetracycline element that they have studied in *S. pneumoniae*[71].

CONCLUSIONS

While many aspects of conjugation in Gram positive bacteria appear similar to the more heavily studied Gram negatives, there are in certain cases characteristics which, so far, appear distinct. Clear evidence for sex pheromones in bacteria other than *S. faecalis* (Gram positive or Gram negative) has not yet been reported, although sex-related chemotactic factors in *E. coli*[77] and *S. typhimurium*[78] have been suggested. Pheromone-related behavior is well known, however, in yeast, fungi and higher organisms[79, 80]. "Conjugative transposons" are also yet to be reported in Gram negative bacteria; however, there are recent indications that such elements may indeed occur in *Bacteroides fragilis*[81, 82].

The use of recently developed cloning systems in *Streptococcus sanguis*[48, 83], and *Bacillus subtilis*[84, 85] should begin to simplify genetic analyses in Gram positive bacteria, and it is likely that rapid progress will be made in revealing the molecular bases of conjugal transfer.

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SITES AND SYSTEMS FOR CONJUGAL DNA TRANSFER IN BACTERIA

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Plasmids isolated from Gram-negative bacteria can be divided into two major groups: large plasmids (>30 kb) that determine conjugation systems, and small plasmids (<10 kb) that do not. However, it was observed many years ago that a representative small non-conjugative plasmid, ColE1, was mobilised with high efficiency if the cell also contained an appropriate conjugative plasmid (Clowes, 1963), and more recent data for other non-conjugative plasmids suggests that this might generally be the case. Indeed, ColE1 contributes not only an "origin of transfer" DNA sequence (*oriT*), but also mobilisation genes that are essential for its own transfer (Inselburg, 1977; Dougan and Sherratt, 1977). Interestingly, about one-third of the total plasmid DNA is devoted to conjugation, both for conjugative plasmids such as F, and for non-conjugative plasmids such as ColE1; this compares to the 5-10 fold smaller proportion required for autonomous replication.

The importance of conjugation to plasmids is underlined by the large percentage of them that encode conjugation systems, by the likelihood that even small non-conjugative plasmids frequently carry an *oriT* and perhaps mobilisation genes, and by the relatively large proportion of the plasmid DNA dedicated to this function. From an evolutionary point of view this importance is not surprising, since as a result plasmid genes are better able to survive. Firstly, conjugation allows plasmids repeatedly to express their "phenotype" genes (which, as in the case of antibiotic resistance, typically confer only a transient advantage) in different hosts in different environments. Secondly, conjugation is essentially a replication process, and allows plasmid genes to replicate faster than host chromosomal genes. This can give rise, for example, to "infectious spread" of a conjugative plasmid through a bacterial population. Transmissible non-conjugative plasmids may have the additional advantages of exploiting several types of conjugation systems (with the corollary of a wider host range) and of their small size being compatible with a high copy number.

In this paper I shall discuss the requirements that must be satisfied for a replicon to be transferred by conjugation, and review the inter-relationships that have been observed between different conjugation systems.

Conjugation systems determined by plasmids belonging to different incompatibility groups

Most, if not all, conjugation systems so far studied are physiologically similar, and two separate though connected components can be recognised: mating pair formation with a potential recipient cell, for which the plasmid-encoded extracellular pilus is essential, and conjugal DNA metabolism that subsequently transfers and replicates the plasmid DNA from its oriT site. For the purposes of this article, the first component will be abbreviated to Mpf and the second to Dtr.

Despite this overall similarity, numerous distinct and non-interacting conjugation systems have been identified. The pilus provides one important means of classifying conjugation systems, since pili differ in their morphology and serology, in the particular varieties of male-specific bacteriophages that they adsorb, and in their abilities to allow conjugation in liquid, as opposed to solid, medium (Table 1; Bradley, 1980; Bradley et al, 1980). A second method is to determine whether non-piliated (Mpf^- Dtr^+ $oriT^+$) mutants of one plasmid can be transferred by the conjugation system of a second (Willetts, 1970 and unpublished data). By these means, the conjugation systems of plasmids falling into different incompatibility groups can be compared: the data presently available suggest that plasmids with similar conjugation systems belong either to a single incompatibility group

Table 1. Pilus types

Plasmid group	Pilus morphology	Pilus diameter (nm)	Isometric RNA phage	Filamentous single-strand DNA phage	Lipid-cont. double-strand DNA phage
I	Flexible	6	-	If1	-
F	Flexible	9	f2, Qβ	f1	-
X	Flexible	9	-	-	-
P	Rigid	8	PRR1	Pf3	PR4
N	Rigid	9.5	-	Ike	PR4
W	Rigid	12	-	-	PR4

(as for IncN, P, W or X plasmids) or to one of a small collection of incompatibility groups (as for the IncF or IncI "complexes"). Furthermore, the lack of complementation between different conjugation systems implies that recognition of a particular oriT sequence by a Dtr system, and of a particular Dtr system by an Mpf system, are both highly specific processes.

Because of the large proportion of plasmid DNA devoted to conjugation, plasmids with similar conjugation systems share a large proportion (40-80%) of DNA homology, while plasmids with different systems do not (<10%). These percentages are for plasmids from the six groups listed above (Falkow et al, 1974). The absence of DNA homology itself provides an indication that the conjugation systems determined by a particular pair of plasmids are likely to be dissimilar.

The F conjugation system

In the case of IncF plasmids, sufficient information is available to provide a substantial genetic and molecular basis for understanding the Mpf and Dtr components of the conjugation system. This information has been reviewed recently by Willetts and Skurray (1980) and will be briefly summarised here.

Approximately 33 kb of DNA is required to determine the F conjugation system, and about 20 conjugation genes have been identified (Fig. 1). Those contributing to the Mpf system are traALEKBVWCUNFHG. The pilin precursor protein is encoded by traA (Minkley et al, 1976), and most of the other genes are required for the (unknown) pathway whereby this is chemically modified and erected into the pilus structure. traG is needed not only for pilus synthesis, but also - together with traN - for stabilisation of mating pairs. As might be expected, the products of all these genes are located in the cell envelope.

Genes necessary for the Dtr system are traMYDIZ. Of these, traYZ probably determine an endonuclease that reversibly nicks one DNA strand at oriT - even in the absence of Mpf (Everett and Willetts, 1980). In response to Mpf, the traMI products may trigger Dtr by displacing the YZ endonuclease from the nicked

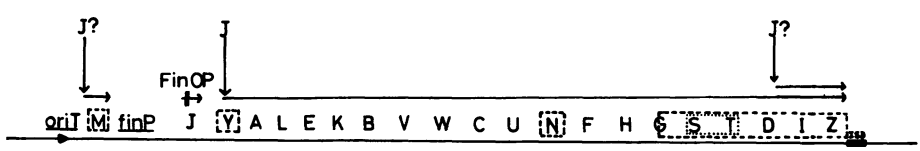

Fig. 1. A map of the F conjugation region. Letters above the line indicate tra genes. Those not required for pilus formation are boxed. The various tra operons and their regulatory systems are indicated.

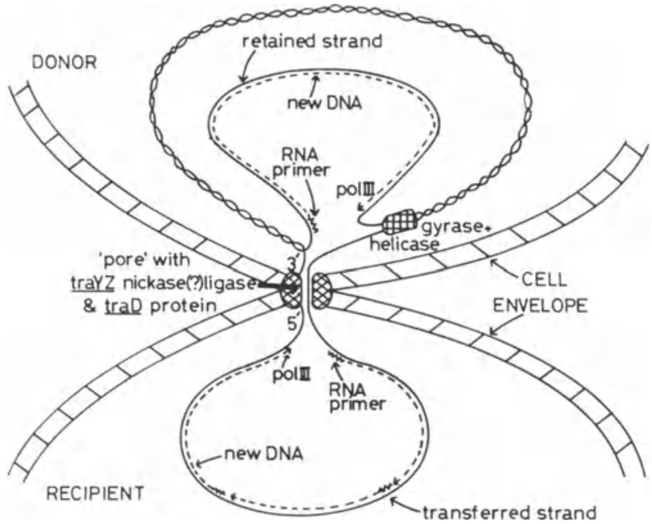

Fig. 2 A model for conjugation

form of the plasmid DNA and allowing the strand separation (to which the traD product may contribute) required for initiation of DNA transfer and synthesis from oriT. Host proteins such as RNA polymerase, DNA polymerase III and gyrase play important roles in the latter two processes. The traZ and traI proteins, together with the host proteins listed, are located in the cytoplasm, whereas the traY and traM components of the nicking and triggering functions and the traD protein are found in the cell envelope, where they perhaps serve to connect the Mpf and Dtr systems, and to locate the oriT sequence strategically at the inner cell surface.

A model for conjugation is illustrated in Fig. 2; one additional feature of this is that the 3' and 5' termini of the transferred strand are both linked to a presumptive membrane-located transfer complex. This would account for the RNA primer requirement for donor conjugal DNA synthesis and for accurate and efficient recA- and tra-independent recircularisation of the transferred strand in the recipient cell.

Specificity of the Dtr systems of F-like plasmids

F-like plasmids synthesise morphologically and serologically similar pili that adsorb (though with varying efficiencies) the same male-specific phages, and their transfer regions are largely homologous. Indeed, all F-like plasmids tested (including R100-1, R1-19, ColV2 and ColVBtrp) allow transfer (via complementation or perhaps successful Mpf-Dtr interaction) of Mpf$^-$ mutants of F. However, F mutants in the Dtr genes traMYIZ are not always transferred by other F-like plasmids, indicating that there are different

Table 2. Complementation of piliated Flac tra mutants

Flac mutation	Complementation (%) by R1-19	R100-1
traM102	0.03	0.1
traY::EDλ4	0.60	1.5
traN548	82	48
traG42	50	61
traD38	73	76
traI41	58	1.0
traΔIZ337	41	3.0

Plasmids with complementation patterns similar to F itself are ColV2, ColB2, ColVBtrp and R124; to R1-19 are R538-1 and ColB4; and to R100-1 are R6 and R136.

allelic forms of these genes. Table 2 presents data to illustrate this point, and lists the three groups into which F-like plasmids can be arranged on this basis. It is the paired traY and traM, and traZ and traI, specificities that suggests that the traYZ endonuclease and the traMI triggering function both act at oriT, within which there may be two domains.

An implication of these observations is that the oriT sequences of the three groups of F-like plasmids differ from each other. This has been verified directly in one case, since pED806, a pBR322 derivative into which the F oriT (but no tra genes) has been cloned, is efficiently transferred by Flac but not by an Ap^s R1-19 variant or by R100-1.

Despite the differences between their Dtr systems, it should be emphasized that the Mpf system of any F-like plasmid will serve to transfer an $Mpf^- Dtr^+$ mutant of any other F-like plasmid, whereas this is not the case for plasmids with unrelated conjugation systems.

Mobilisation of non-conjugative plasmids

Small naturally-occurring non-conjugative plasmids are often transferred efficiently and specifically by one or more types of conjugative plasmid (Table 3). This may prove to be the general rule for all non-conjugative plasmids once sufficient conjugation systems have been tested to allow the appropriate one(s) to be identified. For example, although it has been known for some time that IncI plasmids mobilise non-conjugative IncQ plasmids such as RSF1010 with relatively low efficiency, we have recently found that IncP plasmids mobilise RSF1010 with 100% efficiency in both *E.coli* and *P.aeruginosa* (Willetts and Crowther, 1981). Neither IncN nor IncW plasmids, which like IncP plasmids determine rigid pili that adsorb the male-specific phage PR4, mobilised RSF1010. IncP

Table 3. Mobilisation of non-conjugative plasmids

Conjugative plasmid	Transfer (% of conjugative)					
	Group	ColE1	CloDF13	ColE2	pSC101	RSF1010
R64-11	I	70		80	0.5	6
Flac	F	100	133	<0.01	10^{-5}	10^{-3}
R1-19	F	40	83	<0.01	10^{-3}	10^{-3}
R100-1	F	0.1	93			10^{-3}
R751	P	48			13	100
R388	W	1				10^{-2}

Data are taken from: Reeves and Willetts, 1974; Hardy, 1975; Warren et al, 1978, 1979; Willetts, 1980; and Willetts and Crowther, 1981.

plasmids were also fairly efficient at mobilising pSC101.

The efficiency, specificity and recA-independence of the mobilisation of non-conjugative plasmids indicate that these plasmids contain an oriT sequence from which conjugal transfer can be initiated. This sequence is probably similar to that at which the protein-DNA "relaxation complexes" of ColE1 and some (but not all) other small plasmids are nicked in response to ionic detergents or other stimuli (Clewell and Helinski, 1969). However, caution must be exercised in comparing conjugal DNA metabolism by F, which is relatively well understood, and by ColE1, which is not. Some of the fundamental questions that remain unanswered for ColE1 are whether a pre-existing unique DNA strand is transferred undirectionally, and in what orientation; whether donor conjugal DNA synthesis replaces this strand, and what primes this synthesis; and what the role(s) of the ColE1 mobilisation gene(s) are. Furthermore, despite extensive efforts, we have been unable to identify an F relaxation complex.

The oriT sequence of a non-conjugative plasmid is often, perhaps always, recognised by a Dtr system (which might function in a different way to that of a conjugative plasmid) determined by the plasmid's own "mobilisation" genes (Warren et al, 1978). These genes are essential for mobilisation, indicating that the Dtr system of the conjugative plasmid is of different specificity and therefore probably dispensable. This is indeed the case for mobilisation of ColE1 or CloDF13 by F, since the F traMIZ genes (and the traD gene, for CloDF13 - but not for ColE1) are not required (Table 4; Willetts, 1980). Complementation of Dtr^- mutants of ColE1 by ColK but not by ColE2 (Warren and Sherratt, 1977) shows that different non-conjugative plasmids encode different Dtr systems (and therefore have different oriT sequences), even when they are mobilised by the same conjugative plasmid (R64-11 in this case). Similar

Table 4. Mobilisation by Flac tra mutants

Flac mutant	Function lost	Transfer (%) ColE1	Transfer (%) CloDF13
tra^+	-	27	36
traA	pilus synthesis	0.002	0.002
traG	stabilisation of mating pairs	<0.001	<0.004
traN		0.016	0.014
traM	triggering	21	38
traI		10	32
traIZ	triggering and nicking	18	32
traD	strand separation?	<0.001	51

experiments show that even the 2.3 kb miniplasmid p15A is efficiently mobilised if ColE1 and F are present to provide the necessary Dtr and Mpf systems (Chang and Cohen, 1978), and therefore contains an oriT sequence.

It is clearly essential that the conjugative plasmid's Mpf system should specifically recognise the non-conjugative plasmid's Dtr system (or, possibly, a second DNA sequence other than oriT on the plasmid to be transferred). In either case, the success or failure of this recognition process can account for the ability or otherwise of a non-conjugative plasmid to be mobilised by a particular conjugative plasmid. Insufficient information is available for any clear understanding of the molecular requirements for successful Mpf-Dtr interaction; the data in Table 3 emphasise the complexities of the patterns already observed. In this connection the versatility of ColE1 is of particular note; this plasmid can be efficiently mobilised by IncF (although excluding, curiously, R100-1), IncI and IncP plasmids.

Mobilisation of replicons without an oriT

Replicons that do not carry an oriT sequence, or carry one that is non-functional because of the absence of Dtr or Mpf systems of appropriate specificities, can be conjugally transferred only if they become covalently linked by recombination to a functional oriT sequence. This type of mobilisation is of particular importance for the special case where the mobilised replicon is the bacterial chromosome, since the resultant interchange of genes may accelerate bacterial evolution (Reanney, 1976).

Mobilisation of the E.coli K12 chromosome by F is due mainly to inefficient host-encoded recombination between the short regions of homology provided by insertion sequences present in both replicons (Davidson, 1975). Chromosome mobilisation by autonomous F and Hfr formation are both reduced about 100-fold if the cell is

Table 5. Transpositional mobilisation

Conjugative plasmid	Non-conjugative plasmid	Relevant transposon (source)	
R1-19ΔKm	pSC101	Tn3	(C)
Flac	chromosome::Mu cts	Mu cts	(NC)
Flac	mini-R1	Tn3	(NC)
R388	pMB8::Tn3Δ596	Tn3Δ596 (+RSF1010::Tn3Ap^s)	(NC)
F	pBR322	γδ	(C)
R1-19ΔKm	mini F-Km	Tn3	(C)
R68.45	pBR325ΔAp	IS21	(C)

References (in order) are: Lopecko and Cohen, 1975; Faelen and Toussaint, 1976; Goebel et al, 1977; Gill et al, 1978; Guyer, 1978; Crisona et al, 1980; Willetts et al, 1981.

recombination-deficient (Moody and Hayes, 1972; Cullum and Broda, 1979) or if all the insertion sequences are deleted from F by *in vitro* means (Willetts and Johnson, unpublished data).

Where no such homology exists, mobilisation can be detected as the result of cointegrate formation during transposition of a transposable DNA sequence from the conjugative to the non-conjugative replicon, or *vice versa* (Table 5). This mechanism probably accounts for host chromosome mobilisation by the IncP plasmid variant R68.45, since this contains the highly transposable IS21 (Willetts et al, 1981).

Conclusions

The requirements that must be satisfied for a replicon to be transferred by conjugation are that (a) it must contain (or be covalently linked to) an *oriT* sequence; (b) the cell in which the replicon exists must synthesise a DNA transfer and replication (Dtr) system that specifically recognises this *oriT*; and (c) the cell must also synthesise a system for stable mating pair formation with recipient cells (Mpf) that specifically recognises this Dtr system (or possibly a second specific DNA sequence near to *oriT*). The Dtr and Mpf systems, though often encoded by the replicon itself, can be provided in *trans* by other replicons.

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CONJUGATIVE PILI OF PLASMIDS IN ESCHERICHIA COLI K-12 AND PSEUDOMONAS SPECIES

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SUMMARY

There are three basic morphological forms of conjugative pili for plasmids transferable to *Escherichia coli* K-12: thin flexible, thick flexible, and rigid. Plasmids determining rigid pili transfer at least 2000X more efficiently on a solid surface compared with in a liquid. The majority of such plasmids are naturally derepressed for transfer and pilus synthesis. The following *Pseudomonas* plasmids determine rigid pili: Rms148 (IncP-7), TOL (IncP-9), and R91.5 (IncP-10). Several new plasmid-specific bacteriophages have been found to adsorb to the sides or tips of conjugative pili.

INTRODUCTION

F pili were the first conjugative pili to be identified,[1] and were quickly implicated as organelles of plasmid transfer.[2,3] Conjugative pili have since been found for all incompatibility groups in *Escherichia coli* K-12,[4] and for some in *Pseudomonas* (see below). However, the requirement of pili for conjugation has only been demonstrated in three cases: F pili,[2,3] I pili,[5] and W pili.[6] Conjugative pili are of direct value in plasmid identification and classification when their morphological and serological characteristics are compared. The classification of plasmids by incompatibility (for review see reference 7) correlates well with pilus serotyping on the basis that similar pili (serologically related) are determined by plasmids within an incompatibility group. However, while different incompatibility groups of plasmids usually have unrelated pili, there are a few exceptions: C pili are related to J pili for example.[8] A direct result of studying pilus morphology has been the discovery that certain types of conjugative pili are structurally fragile, and this is linked with poor transfer efficiency in liquids. However, when bacteria carrying these plasmids are mated on a solid surface such as an agar plate, transfer efficiency is dramatically increased.[9] This paper reviews these aspects and describes some new observations on Pseudomonas conjugative pili.

METHODS FOR STUDYING CONJUGATION AND CONJUGATIVE PILI

The standard method for plasmid transfer in the past has been mating in a liquid environment (broth), but the identification of surface mating systems[9] suggests that in many cases a plate mating method would be more appropriate as follows. A nalidixic acid-sensitive donor and a nalidixic acid-resistant recipient are grown in shake culture to an absorbance of 1.0 at 620 nm wavelength, and equal volumes of the cultures are mixed. 0.3 ml of the mixture is spread on a nutrient non-selective plate predried at 37°C for 20 minutes uncovered. The bacterial suspension is allowed to dry on the plate at the appropriate incubation temperature for the plasmid or host strain (5-10 minutes). The plate is then covered and incubated for 55 minutes for mating. The cells are washed off quantitatively with three washes of 1.0 ml of broth using a wire spreader to resuspend them. After adjusting the suspension volume to 3.0 ml, serial dilutions are spread on selective plates (nalidixic acid counterselection to prevent further plate mating) for incubation and the counting of transconjugant colonies. This procedure allows the introduction of mating inhibitors such as inactivated pilus-specific bacteriophages into the mixture on the mating plate. A concurrent comparative liquid mating can be carried out by adding 0.3 ml of the initial mating mixture to 1.0 ml of broth, incubating this for 1 hour, then making the volume up to 3.0 ml and plating serial dilutions as above.

Pili determined by derepressed plasmids can be prepared for electron microscopy by mounting them on specimen grids from a very thick bacterial suspension.[4] For repressed plasmids, the "temporary derepression" method of growth often improves pilus yields. Loopfuls of 6 hour non-selective plate cultures of donor and recipient (both "bald") are spread evenly on a transconjugant-selecting plate after suspending them in a drop of broth. Overnight incubation gives confluent growth which is used for electron microscopy.

MORPHOLOGICAL AND SEROLOGICAL RELATIONSHIPS AMONG CONJUGATIVE PILI

The morphological and serological relationships of conjugative pili correlate well with the existing plasmid classification based on incompatibility.[8] Three basic morphological types of pilus have been identified: thin flexible (Fig. 1), thick flexible (Fig. 2), and rigid (Fig. 3). Thin flexible pili (thickness about 6 nm) are determined by plasmids in the I complex of incompatibility groups, and also IncB and IncK plasmids. Immune electron microscopy has

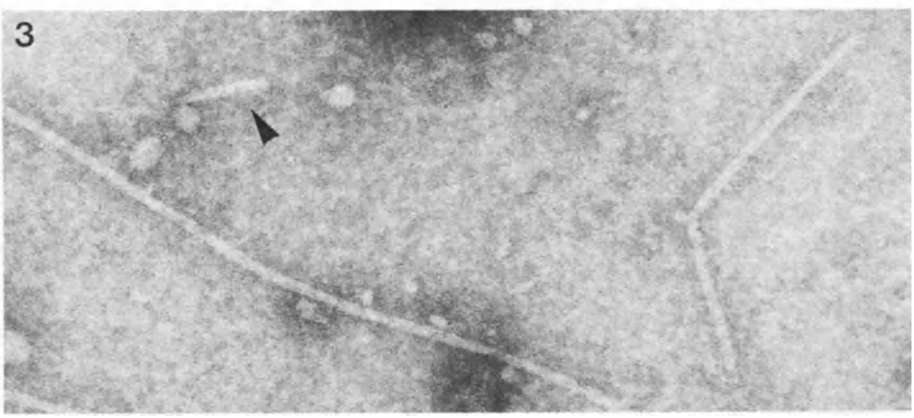


Fig. 1. Thin flexible I_{α} pili from *E. coli* J53(R64*drd*11). Bar for Figs. 1-3, 100 nm.

Fig. 2. Thick flexible H2 pili from *E. coli* JE2571(pIN32).

Fig. 3. Rigid N pili determined by *E. coli* JE2571(N3). Arrow marks a very short pilus, which is common.

revealed two distinct serotypes, the I_α serotype for pili of IncI_α, IncI_γ, IncB, and IncK plasmids, and the I_2 serotype for I_2 and I_δ pili. I_ζ pili reacted with antisera to both I_α and I_2 pili, the first reaction being the stronger. While a relationship between IncB and IncI_α plasmids has been established,[10] the possibility that IncK plasmids belong to the I complex is unexpected. Thick flexible pili (diameter about 9 nm) are determined by plasmids of incompatibility groups C, D, the F complex, H1, H2, J, T, V, X, com9, and the single plasmid (one which forms its own incompatibility "group") $F_o$lac. Of these, C pili were found to be related to J pili, and com9 pili to $F_o$lac pili, the remainder being unrelated. In a recent study (D. E. Bradley, unpublished), it was found that the pili of a transferable plasmid coding for the production of heat-stable enterotoxin (plasmid TP224; strain E7476 in reference 11) were serologically related to $F_o$lac and com9 pili. TP224 was compatible with both $F_o$lac and R71 (com9); it probably forms its own incompatibility "group" (M. McConnell, personal communication).

Rigid pili, which are fragile and easily broken, were determined by plasmids of incompatibility groups M, N, P, U (a tentative new group as yet unpublished), and W, as well as the unclassified plasmid R775 (R. W. Hedges, unpublished). There was no relationship between the pili of any of these groups.

Tests on pili of plasmids within incompatibility groups showed serological identity with two exceptions. pHH1457 (V. Hughes, personal communication), while IncD, did not determine D pili. Its thick flexible pili were serologically unrelated to any others in the morphological group. pDT201 (D. E. Taylor et al., Plasmid, in press), while IncM, determined pili which were serologically related to those of plasmids in the F complex, most strongly to FII pili. It cannot therefore be stated that pili for all plasmids within a given incompatibility group are related, although exceptions are very rare.

The morphological classification of conjugative pili is necessarily somewhat subjective since it depends upon their appearance in the electron microscope, and some inaccuracies could arise. For example, X pili were obviously thick and flexible when obtained from overnight plates.[4] However, R6K, the naturally derepressed prototype IncX plasmid, only determined large numbers of pili during the exponential phase of growth on plates (D. E. Bradley, unpublished), and these appeared much shorter (Fig. 4) and could easily be mistaken for rigid pili.

Another IncX plasmid, R485, illustrates the usefulness of serological techniques for pilus identification. R485 determines very thin pili only 5 nm thick,[12] which are not typical X pili. This, together with its ill-defined incompatibility relationship with R6K, suggested that it might not be truly IncX. However, the

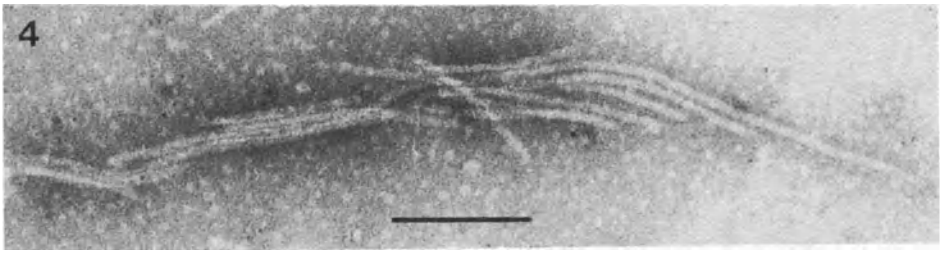


Fig. 4. Aggregate of short thick flexible X pili from a 6 hour plate culture of E. coli JE2571(R6K). Bar marker 100 nm.

temporary derepression growth method revealed that, in addition to the thin pili, thick filaments which labeled with antiserum to X pili were produced (not illustrated). A possible function of the thin pili may be to provide the host organism with the ability to adhere to surfaces, since strains carrying the plasmid adhere very much better to electron microscope specimen support films than those without it.

RELATIONSHIP OF PILUS MORPHOLOGY WITH OPTIMUM MATING ENVIRONMENT

The use of plate mating allows a direct comparison to be made between the transfer efficiencies of a plasmid on a solid surface and in a liquid. By this means, Bradley et al.[9] compared the transfer frequencies on plates with those obtained in broth for representative plasmids from most incompatibility groups. Table 1 aligns the plasmids according to optimum mating environment as indicated by the ratio plate mating frequency/broth mating frequency. A ratio near 1 shows that transfer frequencies were similar in both environments, with higher ratios demonstrating correspondingly greater surface mating efficiencies. It was expected that all plasmids determining thick flexible pili might transfer equally well in both environments, but it can be seen that those in incompatibility groups C, D, T, and X are considerably more efficient on a solid surface than in a liquid. As was expected, all plasmids determining rigid pili transferred very much more efficiently on plates, and in most cases transfer frequencies were at derepressed levels. The broth matings normally used to transfer these plasmids had erroneously suggested that they were naturally repressed.

COMPARISON OF STATE OF PILUS SYNTHESIS (REPRESSED OR DEREPRESSED) WITH PLASMID TRANSFERABILITY

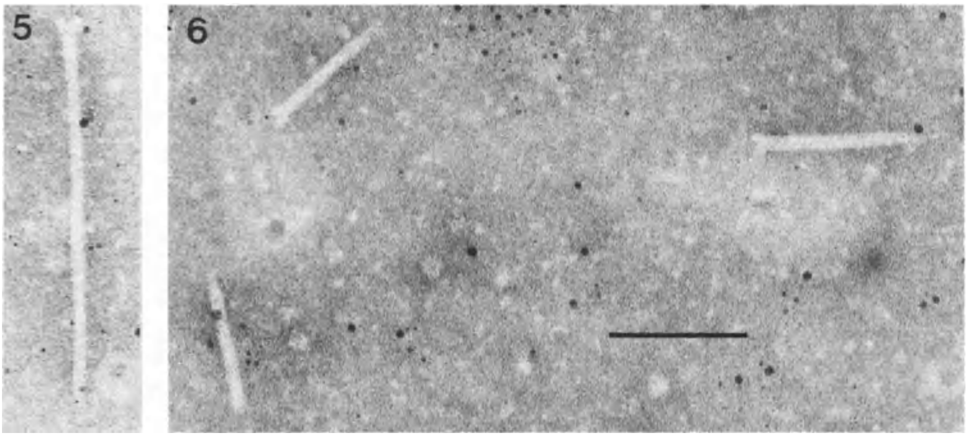
It was possible to ascertain from the number of conjugative pili found in the electron microscope whether or not they were determined constitutively (derepressed synthesis; see reference 8). This could then be correlated with the transfer frequency obtained in the plasmid's optimum mating environment. It might be thought that all

Table 1. Classification of Plasmid Mating Systems Based on Optimum Environment for Conjugal Transfer

Type of mating system[a]	Pilus morphology	Inc group[b]	Representative plasmid	Transfer frequency ratio plate/broth[c]
Universal	Thin flexible	I_α	R64	0.9
		K	pTM559	0.51
	Thick flexible	FII	R100	0.73
		H1	R27	5.5
		J	R391	0.9
		V	R753	0.35
		com9	R71	1.55
Surface preferred	Thick flexible	C	RA1	45
		D	R711b	180
		T	Rts1	265
		X	R6K	250
Surface obligatory	Rigid	M	R446b	16,150
		N	N3	10,200
		P	RP1	2,100
		U	RA3	7,900
		W	Sa	36,450

[a]"Universal", transfer equally good in a liquid or on a solid surface; "surface preferred", transfer significantly better on a solid surface compared with in a liquid; "surface obligatory", transfer fairly low in a liquid and very high (derepressed) on a surface
[b]Single representatives only are included for incompatibility group complexes I, F, and H. IncU is tentative and unpublished (R. W. Hedges, personal communication).
[c]Transfer frequencies on plates divided by frequencies in broth.

plasmids determining conjugative pili constitutively would transfer at derepressed frequencies (>10^{-1} transconjugants/donor/hour). However, representative plasmids from incompatibility groups D (R711b) and T (Rts1) determined pili constitutively but transferred at repressed frequencies. In summary, the following naturally occurring plasmids (as opposed to laboratory derepressed mutants) were repressed for both pilus synthesis and transfer: R64 ($IncI_\alpha$), TP114 ($IncI_2$), and pTM559 (IncK), each of which determined thin flexible pili; RA1 (IncC), R100 (IncFII), R27 (IncH1), R478 (IncH2), R391 (IncJ), R753 (IncV), TP228 (IncX), and R71 (com9), which determined thick flexible pili. Plasmids which determined pili constitutively and were derepressed for transfer were as follows: R6K (IncX), which determined thick flexible pili; R831b (IncM), N3 (IncN), RP1 (IncP), RA3 (IncU), and Sa (IncW), which determined rigid pili. Notably, R6K is a naturally derepressed IncX plasmid, but other IncX plasmids appear to be repressed. Apart from the exceptions already indicated, the IncM plasmid R446b was repressed for pilus synthesis although it transferred at 1.4 X 10^{-1} transconjugants/donor/hour Possibly, like R6K, R446b only determines M pili in large numbers during the exponential phase of bacterial growth. It must be emphasized that, while a plasmid is derepressed in one bacterial species such as E. coli, it may well be repressed in another. Loss of derepression might also occur on transfer between different strains of the same species.



Figs. 5, 6. P-7 pili (rigid) determined by Rms148 derepressed in host P. aeruginosa PAO1150.1. Bar 100nm.

DETERMINATION OF PILI BY SOME PLASMIDS OF PSEUDOMONAS SPECIES

Conjugative pili have been identified for Pseudomonas incompatibility group P-1 only with any degree of certainty. They are rigid and thinner than average.[13] P-7, P-9, and P-10 pili can now be added (D. E. Bradley, unpublished). Pili determined by Rms148 (IncP-7) are short, rigid, and synthesized constitutively by PAO strains of Pseudomonas aeruginosa carrying the plasmid (Figs. 5, 6). TOL (IncP-9) appears to determine two kinds of pilus at a repressed level, one thick and rigid (Fig. 7), and the other thinner and flexible (Fig. 8). However, the latter could be a metabolic product. P-10 pili are determined by R91.5 (R91 derepressed),[14] and are again rigid. They are determined constitutively (Fig. 9). Six of the ten incompatibility groups of Pseudomonas species[14,15] remain to be screened for pili, and all of them except IncP-1 for the surface mating characteristics of their plasmids.

PLASMID-SPECIFIC BACTERIOPHAGES

Plasmid-specific bacteriophages such as fd[16] and R17[17] are useful for identifying plasmids. Also, if plaques are formed, conjugative pilus receptors are determined constitutively. The isolation of five new phages by J. N. Coetzee and colleagues (see F. A. Sirgel et al., J. Gen. Microbiol., in press, for phage C-1) greatly extends their usefulness. From Table 2 (single examples only are included for F-specific phages), it can be seen that phages have now been found for the majority of incompatibility groups, although the host ranges of many are overlapping. All the phages tested adsorb to conjugative pili. They can be divided into two

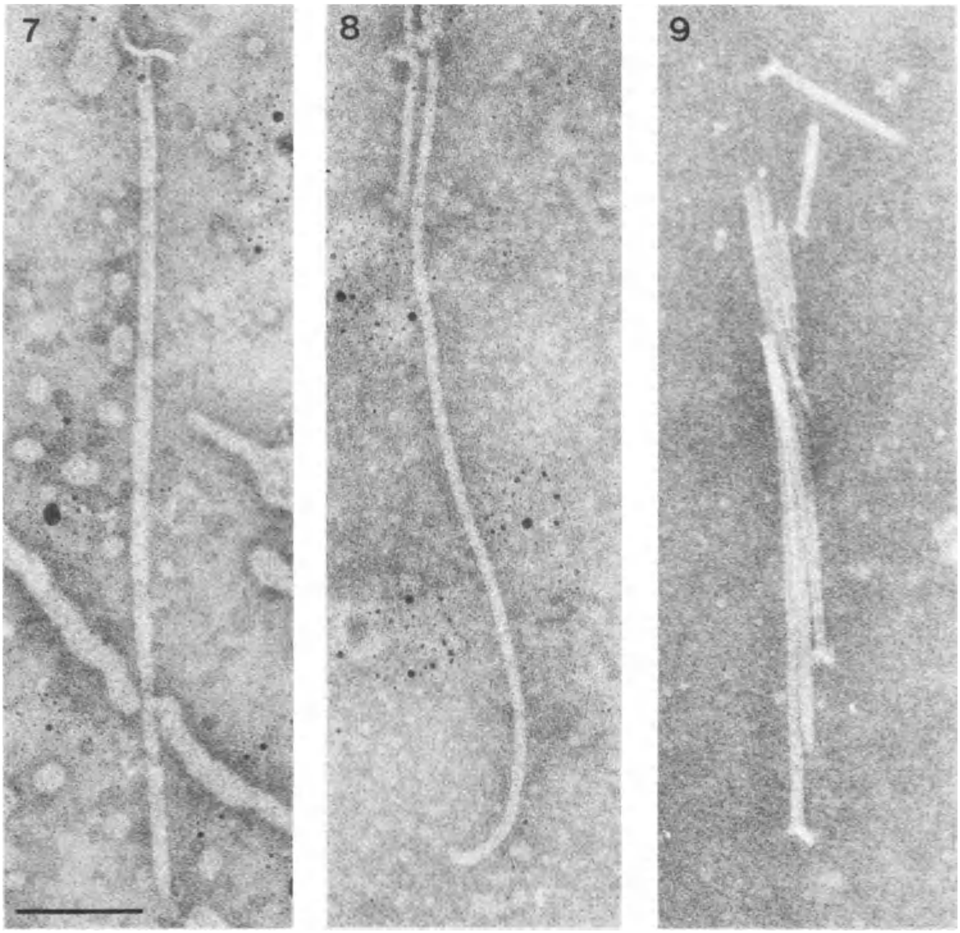


Fig. 7. Rigid TOL pilus from P. putida AC37(TOL). Bar marker for Figs. 7-9, 100 nm.
Fig. 8. Thick flexibile filaments associated with TOL.
Fig. 9. Rigid P-10 pili determined by R91.5.

general classes: RNA phages adsorbing to the sides of pili, and the tip-adsorbing filamentous and tailed types. The first are highly specific, while the tip-adsorbing phages are relatively non-specific.

CONCLUSION

Studies of conjugative pili must clearly be extended. For example, it is desirable to demonstrate unequivocally that all types are required for mating. A new experimental approach to this

Table 2. Plasmid-Specific Bacteriophages

Bacteriophage designation	Type	Inc specificity[a]	Adsorption site (pilus type)
R17	RNA	FI-FIV	Thick flexible
fd	Filamentous	FI-FIV, D, $F_o$lac	Thick flexible
If1, If2, PR64FS	Filamentous	I complex,	Thin flexible
UA6[b], $F_o$lac[c]	RNA	$F_o$lac	Thick flexible
C-1[c]	RNA	C	Thick flexible
C-2[c]	Filamentous	C	Not yet tested
t[c]	RNA	T	Thick flexible
X[c]	Filamentous	X, M, N, U, (W), R775	Thick flexible (X only), rigid
IKe	Filamentous	N, (P),	Rigid
PRD1, PR4	Lipid	N, P, W	Rigid
PRR1	RNA	P	Rigid
Pf3	Filamentous	(P)[d]	Not yet tested
J[c]	Short-tailed	C, D, J	Not yet tested

[a]Plaques formed with most derepressed plasmids. Plasmids in parentheses show multiplication with titer increase test only. Pseudomonas incompatibility groups not included.
[b]Isolated by G. D. Armstrong[19], serologically related to phage $F_o$lac.
[c]Isolated by J. N. Coetzee and colleagues (manuscripts in preparation).
[d]Does not plaque on *Escherichia coli* strains but forms hazy plaques on *Pseudomonas aeruginosa*.

is to block receptor sites on recipient cells by introducing purified pili into mating mixtures. Preliminary experiments with N and P pili have been successful using plate mating.[13] The functional role of pili in conjugation is still not fully understood, although the concept that they attach to recipient cells by their tips, and bring about cell-to-cell contact by retraction, is fairly well supported by experimental evidence.[18] How this model would apply to surface mating systems has not yet been considered. The chemical and physical structures of rigid pili remain to be examined; one would expect them to be different from flexible pili, of which only those of F and $F_o$lac have been extensively studied.[19,20]

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THE PATHWAY OF PLASMID TRANSFORMATION IN PNEUMOCOCCUS

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SUMMARY

Plasmids transform Streptococcus pneumoniae by a process involving low efficiency assembly of replicons from fragments of single strands that have entered the cell separately. Transformation of preexisting replicons is much more efficient. We have cloned the erm gene of pIP501 into pMV158, which so far as we know is the first example of cloning in a pneumococcus host-vector system.

INTRODUCTION

Plasmids have not been found in drug resistant clinical isolates of Streptococcus pneumoniae, which instead carry R determinants inserted into their chromosomes (1, 2). However, a few laboratory strains carry the 2 Md cryptic pDP1 (3), and several R plasmids have been introduced into laboratory strains by conjugation (4, 5) or by transformation (1, 4, 6). We have examined the transformation of pneumococcus by the 3.5 Md tet plasmid pMV158, isolated from a group B streptococcus (7). The results appear useful in thinking about plasmid rearrangements and cloning strategies in streptococci. Here we review work described in three recent papers (8-10) and report the successful cloning of a gene in pneumococcus.

The normal entry pathway for donor DNA in naturally competent pneumococcus, and apparently in S. sanguis and B. subtilis, involves binding and nonspecific cutting of donor duplexes on the cell surface followed by entry of one of the strands of the donor fragments and degradation of the other (11-13). If this is also the major pathway used for plasmid transformation in these gram positive species, it predicts that plasmid replicons have to be assembled inside the recipient cell from fragments of the original donors, as has been shown for transfection by phage DNA in pneumococcus (14).

RESULTS

We first established that transformation by pMV158 appears to share binding and entry steps with chromosomal transformation, in that both required the competent state of the cell surface and a membrane endonuclease needed for the single strand entry pathway, and that both were inhibited to comparable extents by competitor DNA (although a larger plasmid was less inhibited) (8).

We then examined which forms of plasmid DNA were active and characterized their relative contributions to the total transformants observed. In doing so, we paid close attention to the results from B. subtilis, where multimeric forms of very high specific activity were shown to contaminate other fractions and could give misleading results (15, 16). The problem was to know whether activity comigrating with a physically detectable DNA form was due to that form or to another of high specific activity. In particular, in the size range of pMV158, monomer open circles (OC) migrate in 0.5% - 1.0% agarose gels very close to closed circular (CC) dimers (9), and in sucrose gradients dimer OC cosediments with monomer CC (9, 10, 17). A single separation by either method was not sufficient to allow conclusions as to which form contributed the activity, particularly since it was quickly evident that much of the activity was due to dimers or higher

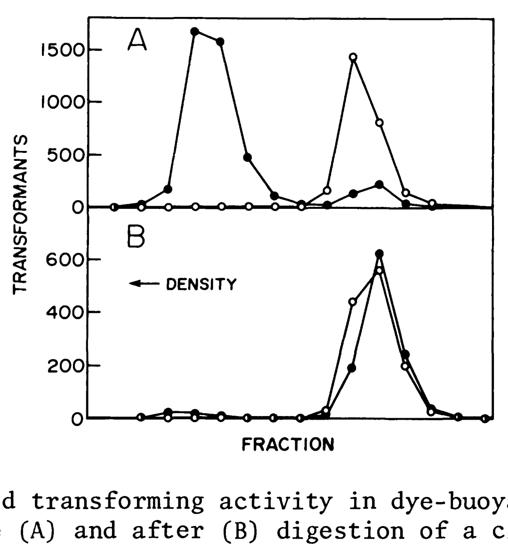

Fig. 1. Plasmid transforming activity in dye-buoyancy gradients before (A) and after (B) digestion of a cleared lysate with S1 nuclease. Filled symbols, pMV158; open, chromosomal reference marker. Twice as much DNA was put into B as into A (from ref. 10).

multimers which were often undetectable by fluorescence of gels stained with ethidium bromide (EtBr). Later work showed that at most 5% of pMV158 DNA was in dimeric forms and that often only a fraction of this was CC. We therefore used combinations of methods to separate and identify which plasmid form contributed a given transforming activity, and then characterized these further with respect to kinetics and relative activities.

Transformants arose from DNA in both the CC and non-CC regions of EtBr-CsCl gradients, with the fraction in each region varying from preparation to preparation (1, 8). On deliberately cutting a cleared lysate by treatment with S1 nuclease, over 99% of the activity disappeared from the CC region, and that in the non-CC region, representing almost all the surviving activity, increased slightly (Fig. 1). Therefore, non-CC forms clearly could transform but had much lower activity per molecule than the CC forms (10).

The critical results came from analysis of the behavior of transforming activity in fractions separated by preparative gel electrophoresis, using automated collection of fractions from a large agarose slab gel, the "Gene Machine" described by Polsky et al. (18). Well resolved peaks of activity were found and the activities in a number of them were examined by various combinations of sedimentation velocity, dye-buoyancy, analytical gel electrophoresis, kinetic response, and sensitivity to S1 nuclease. The profile of a preparation in which almost all activity was in CC forms is shown in Fig. 2. Analytical gels showed that CC monomer coeluted with peak A and that OC monomer was the only plasmid form visible in peak B. However, 98% of the activity in peak B banded as CC in an EtBr-CsCl gradient,

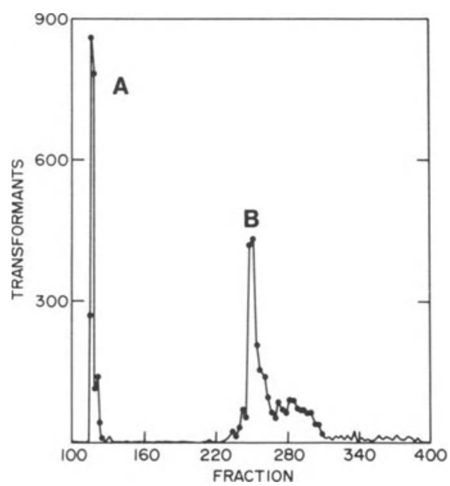

Fig. 2. Preparative electrophoretic fractionation of pMV158 transforming activity (from ref. 9).

and most of it had the sedimentation velocity expected for dimer CC; that in peak A was 100% CC in EtBr-CsCl and had the velocity of monomer CC. On examining kinetics, transformation varied with the square of DNA concentration in peak A; the material in peak B gave linear concentration response (9).

Fig. 3 shows the electrophoretic profile of activity in the preparation of Fig. 1 that had been digested with S1 nuclease before running (10). Essentially all the activity banded as non-CC in dye-buoyancy gradients. Beneath the R_f scale are shown the positions expected for various forms from analytical gels run under similar conditions and, for monomer forms, observed directly in analytical gels of single fractions from this or similar runs (that in Fig. 2 used different conditions and was not directly comparable for R_f).

Fig. 4 shows the sedimentation velocity distributions in the initial cleared lysate before (A) and after (B) S1 treatment and of fractions 51, 68, and 152 from the run of Fig. 3. There was too little activity in fraction 41 to confirm that it was due to monomer linear DNA, but the strong presumption is that it was. The combined results of these runs provided strong evidence that CC, OC, and linear forms of both monomers and dimers were active, but that the non-CC forms were much less active than the CC forms per molecule. A small fraction of the total activity may have come from trimers or higher multimers sedimenting rapidly (Fig. 4A, 4B) and eluting near fractions 80-95 in Fig. 3.

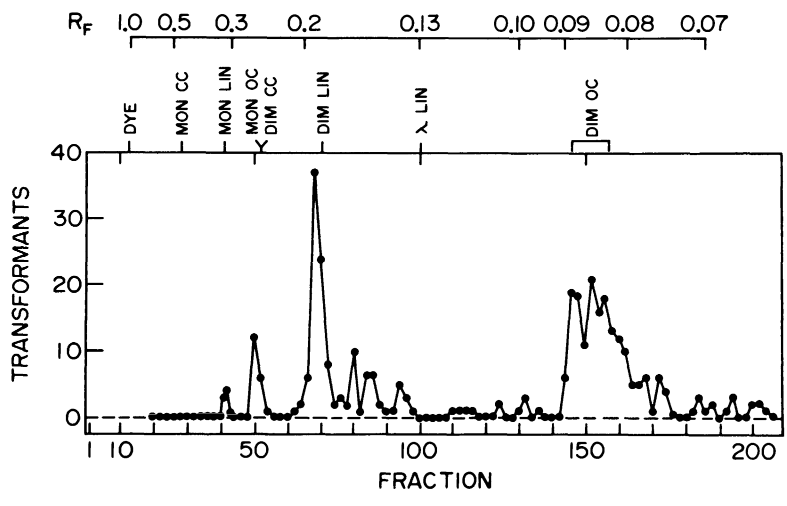

Fig. 3. Preparative electrophoresis of the S1 treated lysate in Fig. 1B (from ref. 10).

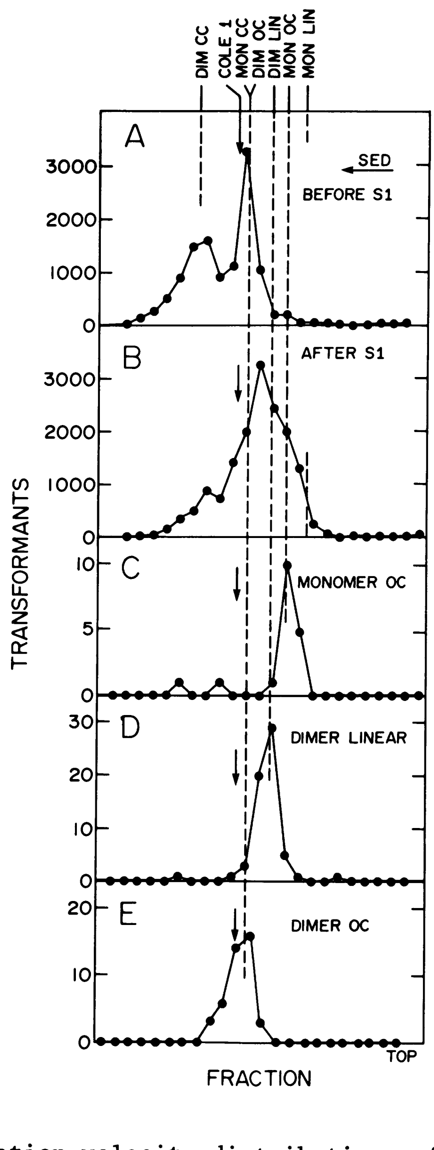

Fig. 4. Sedimentation velocity distributions of pMV158 transforming activity (see text). Positions indicated at the top are predicted from the relations of Clowes (17), relative to the internal ^{3}H-ColE1 standard. Panels C, D, and E show the activities in fractions 51, 68, and 152, respectively, from Fig. 3 (from ref. 10).

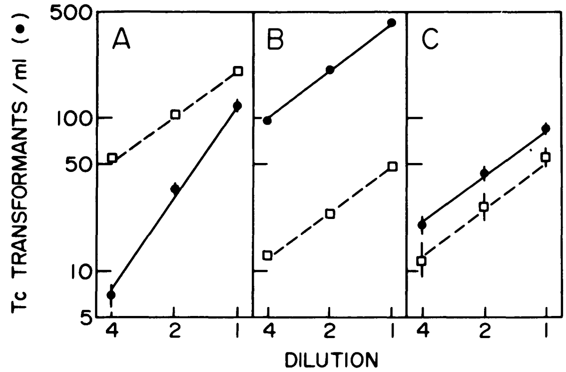

Fig. 5. DNA concentration dependence of transformation for pMV158 (●) and chromosomal marker (□). A, monomer OC (fr. 51); B, dimer linear (fr. 68); C, dimer OC (fr. 152) (ref. 10).

As did CC forms, the monomer OC gave second order kinetics while the dimer forms showed linear responses (Fig. 5). When a cleared lysate was used as donor, the response curve was the sum of a linear component seen at low concentrations and a multi-hit component at higher concentrations (Fig. 6). That is, the fraction of the transformants arising from monomers increased from undetectable at low concentration in the transformation tube to a majority at higher concentrations.

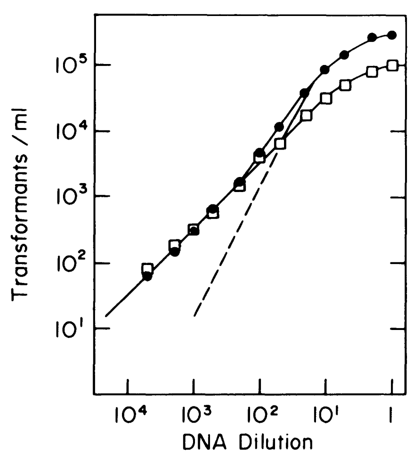

Fig. 6. Dose-response curve for a cleared lysate containing pMV158 (●) and a chromosomal marker (□). It was coincidence that the numbers were the same at high dilution. The dashed line indicates a slope of 2.0 (from ref. 8).

Unique linear products of digestion with either of two restriction enzymes that cut at single sites showed essentially no transforming activity (⩽ 0.1% of that of CC forms). However, a mixture of the two separate digests transformed at a level comparable to that of the mixture of linear monomers produced by S1 nuclease, near 1% of that of an equal weight of CC monomer. In another experiment, monomer CC, dimer CC, and dimer OC forms were first separated electrophoretically and then digested with S1. Surviving activities were 2.6, 2.8, and 72% respectively (10).

MODEL

Fig. 7 summarizes our interpretation of the pathway of plasmid transformation in pneumococcus. We believe that it will prove to be similar in S. sanguis and probably in B. subtilis, although it remains to be established why monomer plasmids are not active in the latter system (15, 16). Quantitative estimates of absolute efficiency imply that less than 1% of the cells that receive the minimum number of strand fragments are in fact transformed. We have suggested that this reflects intracellular degradation of the first strand while it awaits the entry of the second, and that this process may be more extensive in B. subtilis than in pneumococcus (10).

In S. sanguis, Macrina et al. have shown that multimeric forms of pVA736 contribute the majority of the transforming activity and that the monomer CC band cut from gels is active with second order kinetics (19). In contrast, data forcing one to invoke cooperation between donor strands is lacking in B. subtilis. However, based on the similarities of the chromosomal transformation pathways in these

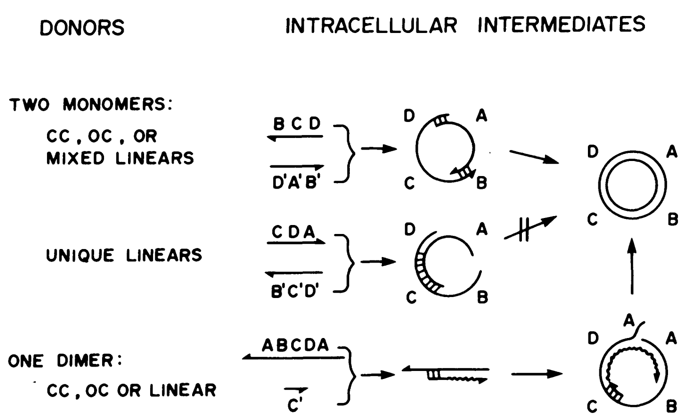

Fig. 7. Intracellular assembly of plasmid replicons from single strand fragments in pneumococcus. See text (from ref. 10).

gram positive species, Dubnau et al. (24) have suggested a model similar to ours for the processing of multimeric plasmid DNA in B. subtilis.

A recent report on transformation of pneumococcus by other plasmids reached some conclusions similar to ours (6). These authors concluded from a single separation by gel electrophoresis that OC monomer was active, whereas we found that activity comigrating with monomer OC was almost entirely due to dimer CC, unless we had first digested the preparation with S1. They found cooperation between restriction digests, as did we. Although our results differ in some quantitative respects, such as relative activities of the various forms, the major qualitative difference is in the kinetics for monomer donors, where they did not recognize the second order response.

IMPLICATIONS

Assembly of replicons from fragments of single strands represents physical recombination, and the low efficiency suggests that the rare successes result from minimal pairings just sufficient to generate a circle carrying intact replication functions. In this situation sequence rearrangements may be expected to occur wherever partial homologies allow them, and those generating smaller replicons should have a selective advantage. We have observed an extensive deletion during transformation of the 20 Mdal pIP501 (4), and Behnke et al. have seen several examples of deletion during transformation of S. sanguis by a derivative of pSM19035 (20).

CLONING IN PNEUMOCOCCUS

The fragmentation on entry and the resulting low overall efficiency of establishing new replicons in the recipient implies that recovering plasmids created by in vitro recombination will be more difficult than if the donor molecule remained intact. However, the efficiency of adding new information to a partially homologous replicon already present is much higher, as in chromosomal transformation and in marker rescue in phage transformation (21). Dubnau's laboratory has explicitly demonstrated this for plasmid transformation in B. subtilis (22, 23). Using this approach, we have cloned the erm (MLS^r) gene from a derivative of pIP501 into pMV158. Digests of pDP4 treated with Hind III and S1 and of pMV158 treated with S1 were treated with T4 ligase and used to transform a recipient carrying pMV158. MLS^r transformants were recovered and lysates of several of these transformed the same recipients again at high efficiencies while transforming plasmid-free cells at much lower efficiencies. The transformants carry plasmids of varying sizes and some of these carry both tet and erm, making them potentially useful for further cloning. So far as we are aware, this is the first successful cloning in a pneumococcus host vector system.

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PLASMIDS OF THE GONOCOCCUS

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There are at least four naturally-occurring plasmids in the gonococcus (Table 1). This paper will review the structure, origins and functions of these plasmids, insofar as known or can be reasonably inferred. Certain hybrid plasmids which have been of particular interest in delineating early steps in entry of DNA into competent gonococci are also discussed.

Table 1. Plasmids of *Neisseria gonorrhoeae*

Plasmid Size	Designation	Mol% G+C	Function	Ref.
2.7	pFA1, pLE2600	50	ND[a]	1-4
3.4	pFA7, pMR0200	41	Pc^r	4,5
4.7	pFA3, pMR0360	41	Pc^r	4,5
7.5[b]	pFA10	ND	Pc^r	5
24.5	pFA2, pLE2450	50	Tra^+	4,6,7
28.0[c]	pFA14	ND	Pc^r, Tra^-	5

[a]Not determined.
[b]Recombinant plasmid pFA3 Ω pFA1, resulting from entry of pFA3 by transformation into a pFA1-containing recipient.
[c]Recombinant plasmid pFA3 Ω pFA2, resulting from entry of pFA3 by transformation into a pFA2-containing recipient.

CRYPTIC PLASMIDS

Most gonococcal isolates contain an approximately 2.7 Mdal cryptic plasmid.[2,3,4,8] The structure of this plasmid is highly conserved, although small deletions or differences in restriction-endonuclease sites have been documented.[9,10] The function(s) of this plasmid have been elusive. There is no evidence that the 2.7 Mdal plasmid (or any other gonococcal plasmid) is involved in control of piliation, iron utilization, or resistance to serum. We have recently observed that strains lacking the 2.7 Mdal plasmid are apparently aberrant in several respects, including their propensity to be highly opaque in their colonial morphology. We have attempted to introduce the native 2.7 Mdal plasmid into plasmidless strains by transformation or conjugation, selecting for entry of a 4.7 Mdal Pc^r plasmid and scoring on agarose gels for coincident entry of the 2.7 Mdal plasmid. None of over 100 Pc^r transformants or transconjugants also acquired the 2.7 Mdal cryptic plasmid. Attempts to cure strains of their cryptic plasmid have also been unsuccessful. Failure to construct isogenic derivatives varying in presence of the 2.7 Mdal plasmid has prevented serious study of its function(s).

PENICILLINASE PLASMIDS

In 1976, strains of gonococci were isolated in the United Kingdom, U.S.A., and in South East Asia which produced a TEM-1 type β-lactamase.[11] The bla gene (penicillinase production) was carried on either a 3.4 or 4.7 Mdal plasmid.[5,11] (Earlier papers considered the sizes of these plasmids as 3.2 and 4.4 Mdal, but these estimates were probably slightly too low.) Isolates of Pc^r gonococci in the U.S.A. and Asia generally contained the 4.7 Mdal plasmid, whereas African and European isolates usually contained the 3.4 Mdal plasmid.[12] Strains of Pc^r gonococci from Asia and the U.S.A. also often contained a 24 Mdal conjugative plasmid, and were either prototrophic or proline-requiring; European-African isolates were frequently arginine-requiring, and rarely contained a 24 Mdal conjugative plasmid.[12] These observations suggested nearly simultaneous origin of two related but epidemiologically distinct clones of Pc^r gonococci in different geographic areas.

Roberts, Elwell, and Falkow showed by DNA-DNA hybridization that gonococcal Pc^r plasmids were closely related to each other and to previously characterized Ap^r plasmids of about 4 Mdal isolated in *Haemophilus influenzae*.[4] The 4.7 and 3.4 Mdal gonococcal Pc^r plasmids have a base content of about 41 mol.% G+C, which is similar to Haemophilus DNA but unlike the approximately 50% mol.% G+C in other gonococcal plasmids and chromosomal DNA.[1,4,6] These data suggested that the gonococcal Pc^r plasmids could have been transferred into gonococci from Haemophilus.

This speculation was strengthened by demonstration of a 4.7 Mdal Haemophilus Pc^r plasmid with HpaII, AluI and BamHI restriction-endonuclease fragment structure identical with the 4.7 Mdal gonococcal Pc^r plasmid. This strain of Haemophilus, which was isolated in 1974 (before the advent of Pc^r gonococci), could conjugally transfer its Pc^r plasmid into gonococci. The Haemophilus Pc^r plasmid was very unstable in a gonococcal host, even in the presence of penicillin or ampicillin.[13] It is also possible, of course, that the similar 4.7 Mdal Pc^r plasmids observed in gonococci and Haemophilus were transferred into each from another unknown source.

Introduction of the gonococcal 4.7 Mdal Pc^r plasmid into an isogenic Pc^s gonococcal recipient by transformation frequently resulted in formation of deleted plasmids, varying in size from 2.3 Mdal to 3.4 Mdal. The most common class of transformation-induced deleted Pc^r plasmid was 3.4 Mdal, which was identical in restriction-endonuclease fragment structure to the naturally-occurring 3.4 Mdal plasmids.[5] Deletions were observed with both 4.7 Mdal Pc^r plasmid transforming DNA prepared from cesium chloride-ethidium bromide density gradients, and with more highly purified preparations from subsequent sucrose gradients. Similar deletions were not observed during serial passage of strains carrying the 4.7 Mdal Pc^r plasmid in vitro, nor after transfer of the same plasmid into gonococci by conjugation or into E. coli by transformation.[5] Thus, it was proposed that entry of plasmids by transformation may produce linear fragments, and that recircularization may have resulted in formation of the 3.4 Mdal Pc^r plasmid from the 4.7 Mdal Pc^r plasmid. This event is perhaps more plausible if one considers the uniform competence for transformation of virtually all naturally-isolated gonococci,[14] and the propensity of gonococci to autolyze and thereby release transforming DNA.

The gonococcal Pc^r plasmids, like the small Haemophilus Pc^r plasmids, contain about 40% of the ampicillin-resistance transposon Tn2 including one of the two terminal inverted repeats.[11,15] This almost certainly means that the gonococcal bla gene cannot undergo transposition into new sites. Hybrid bla plasmids have been observed after transformation into certain recipients, but these are probably the result of classical recombinational events and not transposition (see below).

For several years the prevalence of Pc^r gonococci in the U.S.A. and Europe remained low, although in certain areas of the Far East up to 50% of all gonococci were Pc^r.[12] Very recently, there have been several outbreaks of Pc^r gonococcal infections in the U.S.A. and Europe, which may portend greater problems in the future. The prevalence of Pc^r gonococci in the U.S.A. is apparently still less than 1.0% of all isolates, however, and thus currently recommended regimens for treatment in the U.S.A. have not included routine use of drugs such as spectinomycin or cefoxitin, which are known to be effective for Pc^r gonococcal infections.[16,17]

CONJUGATIVE PLASMIDS

Shortly after the discovery in 1976 of Pc^r gonococci, it was shown that many gonococci could conjugally transfer their Pc^r plasmid to other gonococci, E. coli, or certain other Neisseria such as N. flava.[18] The ability to act as a conjugal donor depended on presence of an approximately 24 Mdal plasmid, which carried no detectable markers for drug, heavy metal, or ultraviolet resistance, but efficiently mobilized itself and also the smaller non-self-transferable Pc^r plasmids into suitable recipients.[6] Transfer was mediated by an Anderson Class II system.[6] Nearly 50% of Pc^r gonococci isolated in the Far East carried a 24 Mdal conjugative plasmid,[12] whereas only 12 of 156 (8%) tested Pc^s gonococci carried a similar plasmid.[6] This suggested that Pc^r plasmids may be conjugally transferred between gonococci in nature.

The structure of a limited number of the 24 Mdal conjugative plasmids has been studied. All had similar (but not identical) restriction digest fragment structures.[6] There were remarkable differences in function, however, when different 24 Mdal plasmids were introduced into a strain carrying the non-self-transferable 4.7 Mdal Pc^r plasmid pFA3. Some 24 Mdal plasmids mobilized the Pc^r plasmid with a frequency of about 1×10^{-3} per donor cell, whereas others did not mobilize it at detectable frequency.[6] In other strains, the same 24 Mdal plasmids were all capable of mobilizing the 4.7 Mdal Pc^r plasmid.[6] Certain deletions of the 4.7 Mdal Pc^r plasmid completely prevented mobilization by the 24 Mdal conjugative plasmid.[5] Thus, efficiency of conjugation was dependent on both plasmid and host strain factors, many of which have not been well characterized.

Recent evidence showed that the gonococcal conjugal system was naturally derepressed, with frequencies of Pc^r plasmid transfer up to 10% per donor CFU in a 90 minute mating on membrane filters.[19] Efficiencies of transfer were often reduced, sometimes by orders of magnitude, in crosses between unrelated gonococcal strains, or between unrelated species (N. flava, E. coli). Maximum frequencies of Pc^r plasmid transfer were only detected when low concentrations of penicillin (about 8-fold greater than the MIC of the recipient strain) were used to select the transconjugants; this was necessary because of the low single-cell resistance of gonococci carrying a Pc^r plasmid.[19] Despite evidence that gonococcal conjugation is derepressed, no sex pili have been observed yet.

The interaction between conjugal donor and recipient cells is poorly understood. Addition of purified lipopolysaccharide isolated from the donor did not reduce frequencies of conjugal transfer of a 4.7 Mdal Pc^r plasmid (unpublished data). Outer membrane protein structure did influence conjugation, however. Gonococci are known to undergo relatively high-frequency bidirectional variation in expression of a series of closely related, heat-modifiable, outer membrane proteins of about 2800 daltons;[19] cells containing these proteins are termed opaque, whereas those without these proteins are transparent. In a series of experiments with isogenic donors and recipients varying in presence of the "opacity proteins", conjugation efficiencies were at least 10-fold higher in transparent x transparent than opaque x opaque crosses.[19] One might have expected the reverse result, since opaque gonococci are much more likely to clump and therefore each opaque CFU contains many more cells. Perhaps the heat-modifiable outer membrane opacity proteins reduce efficiency of mating pair formation. The 24 Mdal plasmid has no effect on outer membrane proteins, and no surface exclusion in conjugation has been observed.[19]

Many laboratories have attempted to demonstrate conjugal transfer of chromosomal genes. Initial results were promising, since recombinants for a variety of chromosomal markers were observed in prolonged filter matings, apparently due to a DNase-resistant transfer mechanism.[20] At least three laboratories have since shown, however, that the apparent initial successes were probably due to transformation which occurred despite initial addition of DNase. No differences in transfer frequencies were observed in isogenic donors which varied only in presence of the 24 Mdal conjugative plasmid.[19,21,22] Norlander et al[21] reported that 24 Mdal plasmids enhanced the transformation-competence of recipient cells, but we were unable to confirm their claim (unpublished data).

HYBRID Pc^r PLASMIDS

When the 4.7 Mdal Pc^r plasmid pFA3 was introduced by transformation into a recipient which contained the 2.7 Mdal cryptic plasmid pFA1 and the 24 Mdal conjugative plasmid pFA2, rare hybrid Pc^r plasmids were formed.[3] One of these, designated pFA10, was about 7.5 Mdal in mass, and has been shown to be a nearly complete cointegrate between pFA1 and pFA3 (unpublished data). Another, designated pFA14, was about 28 Mdal and has been shown to be a recombinant of pFA3 into the conjugative plasmid pFA2 (unpublished data). The insertion into pFA2 rendered it conjugation deficient (Tra^-). We have studied these hybrid plasmids in some detail, because of their markedly enhanced activity in transformation (Table 2).

Table 2. Transformation Frequencies With Native and Hybrid Pc^r Plasmids Into An Isogenic Pc^s Recipient[a]

Plasmid	Mass (Mdal)	Pc^r Transformants Per μg DNA per 10^8 Recipient Cells
pFA3	4.7	10
pFA10[b]	7.5	60,000
pFA14[c]	28.0	140,000

[a]The recipient contained pFA1 and pFA2.
[b]pFA10 is a pFA3 Ω pFA1 hybrid.
[c]pFA14 is a pFA3 Ω pFA2 hybrid.

We recently have completed experiments which provide a rational basis for the markedly increased transformation efficiency of the hybrid Pc^r plasmids pFA10 and pFA14. Two mechanisms are involved: marker rescue, and sequence specific uptake of transforming DNA.

The evidence for marker rescue is straightforward. When the hybrid Pc^r plasmid pFA14 (pFA2 Ω pFA3) was introduced by transformation into competent isogenic recipients which lacked any plasmid, or which contained only the unrelated plasmid pFA1, Pc^r transformants were rare ($\leq 10^{-7}$ per μg plasmid DNA). When Pc^r plasmids were reisolated from the transformants, they were always much smaller than the original 28 Mdal donor plasmid. In contrast, when the pFA2 pFA3 Pc^r hybrid (pFA14) was introduced into the same isogenic recipient, excepting that it now contained the homologous plasmid pFA2, Pc^r transformants were obtained at 1000-fold increased frequency, and each of the tested transformant Pc^r plasmids was of the same size (28 Mdal) and restriction-digest fragment structure as the donor plasmid pFA14 (Table 3). We hypothesized that there probably were endonucleases which linearized the incoming Pc^r plasmid, resulting in rare recircularized Pc^r transformant plasmids of reduced size; however, when the recipient contained a resident replicon homologous with much of the incoming Pc^r plasmid, the linearized Pc^r plasmid was "rescued", possibly by recombination with the resident plasmid. The putative endonucleases were presumably not restriction endonucleases, since the experiments were done entirely with DNA isolated from the single strain used as recipient. (These experiments will be presented in detail elsewhere.)

Table 3. Marker Rescue During Transformation of the Gonococcus With the Hybrid 28 Mdal Pc^r Plasmid pFA14 (pFA3 Ω pFA2)

Recipient Plasmid Content[a]	Pc^r Transformants per μg DNA per 10^8 Recipient Cells	Deleted Plasmids in Transformants/ Total Tested
PFA1	18	13/13[b]
pFA1, pFA2	20,000	0/14

[a]The donor Pc^r plasmid is a stable recombinant between the Pc^r plasmid pFA3 and the native gonococcal conjugative plasmid pFA2. The donor DNA was isolated from the strain used in subsequent transformations; the Pc^s recipients were identical excepting for the addition to one of them (by conjugal transfer) of pFA2.
[b]Pc^r plasmids were isolated from individual transformants and were compared on horizontal agarose gels to the size of the donor plasmid pFA14.

There is a second reason for increased transformation efficiency of the hybrid Pc^r plasmids: acquisition of resident gonococcal DNA containing sequence(s) required for efficient uptake by competent cells. It was shown earlier that non-homologous DNA did not compete effectively against gonococcal DNA during transformation.[23] We have confirmed and extended this evidence for specificity of gonococcal DNA uptake, using the Pc^r hybrid plasmid pFA10. The hybrid Pc^r pFA10 (pFA3 Ω pFA1) has been shown to be 10 to 30-fold more active in transformation of a plasmid-free recipient strain than the naturally-occurring Pc^r plasmid pFA3. Since there was no detectable plasmid DNA in the recipient, marker rescue of the hybrid plasmid by a homologous resident replicon seemed unlikely. (pFA10 is relatively more active in transformation of recipients containing a 2.7 Mdal cryptic plasmid, presumably because of homology with the pFA1 portion of pFA10 - "marker rescue".) Since the experiments were done in a completely isogenic background, differences due to restriction modification also seemed implausible. There was no evidence for increased formation of multimeric forms of the hybrid pFA10; rather, monomeric DNA isolated on sucrose gradients seemed to account for the great majority of transforming activity. Thus, we reasoned that the hybrid pFA10 probably had sequences required for uptake which were not present on the parent plasmid pFA3, and that these sequences were probably on the pFA1 (2.7 Mdal cryptic plasmid) component of pFA10.

The structure of pFA10 is known in considerable detail. It has seven major Msp1 fragments, or which one (M2, 3000 bp) is composed entirely of DNA from the native gonococcal 2.7 Mdal plasmid pFA1. Two other Msp1 fragments contain smaller amounts of pFA1 DNA, plus a majority of DNA from the original Pc^r plasmid pFA3. All other Msp1 fragments contain DNA entirely derived from pFA3. Competent gonococcal cells were briefly exposed to ^{32}P-end-labeled Msp1 fragments of pFA10, followed by digestion with DNase-1 to remove all fragments not taken up. The cells were then carefully washed. DNA extracted from the washed cells was separated on agarose gels, and autoradiography repeatedly demonstrated that the only fragment taken up was the 3000 bp M-2 fragment derived entirely from the native 2.7 Mdal cryptic plasmid pFA1. Thus gonococci, like Haemophilus,[24] recognize specific sequences during DNA uptake. The gonococcal sequences appear to be different from the 11 bp Haemophilus uptake sequence,[25] since Haemophilus DNA did not compete with gonococcal DNA during transformation. (These experiments will be presented in detail elsewhere.)

CONCLUSIONS

The hybrid Pc^r gonococcal plasmids have proven highly useful in better understanding early events in gonococcal transformation. Much remains to be learned however. What is the precise mechanism for marker rescue? What is the nature of the gonococcal DNA sequence required for uptake? What is the receptor for uptake? Why is this receptor apparently inactive in non-piliated non-competent gonococci? Can the hybrid pFA10, which contains nearly all of the ubiquitous 2.7 Mdal cryptic plasmid, be used to identify the functions of the cryptic plasmid? Can future hybrid plasmids be constructed which will effectively mobilize the chromosome in conjugation? Can these or similar plasmids be used as cloning vehicles, so as to understand the basis for many other unresolved problems concerning the biology and pathogenicity of the gonococcus? We believe the answer to many of these questions is affirmative.

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GENETIC ORGANIZATION AND EXPRESSION OF NON-CONJUGATIVE PLASMIDS

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INTRODUCTION

Non-conjugative plasmids are plasmids that are not able to transfer themselves to other cells without the help of a conjugative system provided by the large, so-called conjugative plasmids. Non-conjugative plasmids are small plasmids. Their M.W. generally does not exceed 10 Megadaltons. Furthermore, they are multicopy plasmids; that means that they are usually present to the extend of 10-20 copies per chromosome. As all other small DNA molecules, the non-conjugative plasmids are very attractive for basic research. Over the past ten years studies on plasmids were focused on basic questions dealing with gene-function, gene-organisation, gene-expression, mechanism and control of replication, and plasmid mobilisation. And, ever since it became apparent that plasmids are a very useful tool in genetic engineering also a lot of work has been done on the construction of appropriate vector molecules.

The availability of mutants is of discisive importance in the study on the genetic organisation and gene functions of plasmids. Over the past five years new approaches became available for the construction and isolation of plasmids mutants and for the study of their behaviour. Construction and isolation of plasmid mutants can, in addition to classical methods, be achieved by insertion of transposable elements into plasmid DNA and plasmid deletions/hybrids can be constructed in vitro by using appropriate restriction nucleases. Besides these methods of "site-directed" mutagenesis, new DNA sequencing procedures as well as techniques to study gene expression in vivo (minicells and maxicells) and in vitro (cell-free systems) have become available that allows detailed characterisation of plasmid mutants.

This brief review on the genetic organisation and expression of non-conjugative plasmids, will be focussed mainly on the small bacteriocinogenic E. coli plasmids CloDF13 (originally from E. cloacae DF13) and ColEl, because these plasmids have been studied quite well.

GENETIC MAP OF BACTERIOCINOGENIC PLASMIDS

Glancing at a genetic map of a non-conjugative plasmid, e.g. of CloDF13 (Fig. 1), one can observe, to a certain extend, a clustering of those sites and genes that are functionally related. In the region involved in replication the origin of replication as well as the genetic information essential for the control of replication is located. The adjacent region is involved in bacteriocinogenicity; three genes are located in this region. Another cluster is located at the lefthand side of the map. This region is responsible for the mobilisation of the plasmid, a mobilisation that is regular for the transfer of the non-conjugative plasmid to other cells. In addition to these genes, regions have been located that are involved in the maintenance of the plasmid, the inhibition of the propagation of RNA phages and the transfer of certain other plasmids and the inhibition of the multiplication of DNA phages.

The ColEl and CloDF13 proteins that have been identified both, in vivo (using E. coli minicells) and in vitro are listed in Table 1. CloDF13 encodes at least for 10 polypeptides; the sum of their M.W. comprises about 70% of the coding capacity of CloDF13. In case of ColEl about 13 plasmid encoded polypeptides have been identified. The sum of their M.W, amounts to about 400 KD., which is significantly more than the coding capacity of ColEl. Some of these polypeptides may be breakdown products of other proteins. The functions or presumptive functions of these proteins are also listed in Table 1.

Fig. 2 shows the RNA species produced by CloDF13 in CloDF13 containing minicells. About 15 of these RNA bands are CloDF13 specific: four of them (indicated in Fig.2) have been mapped precisely.

FUNCTIONS SPECIFIED BY CloDF13 AND ColEl

In this section the different plasmid regions as well as their functions will be discussed in more detail. In Fig. 3, the region to the left of gene H is the region involved in replication. Although a description of the mechanisme of vegetative plasmid replication is out of the focus of this paper, it is relevant to mention that these small plasmids are fully dependent for their replication on enzymes specified by the host. The CloDF13 replication starts at the origin (2,8%) and proceeds uniderectional.

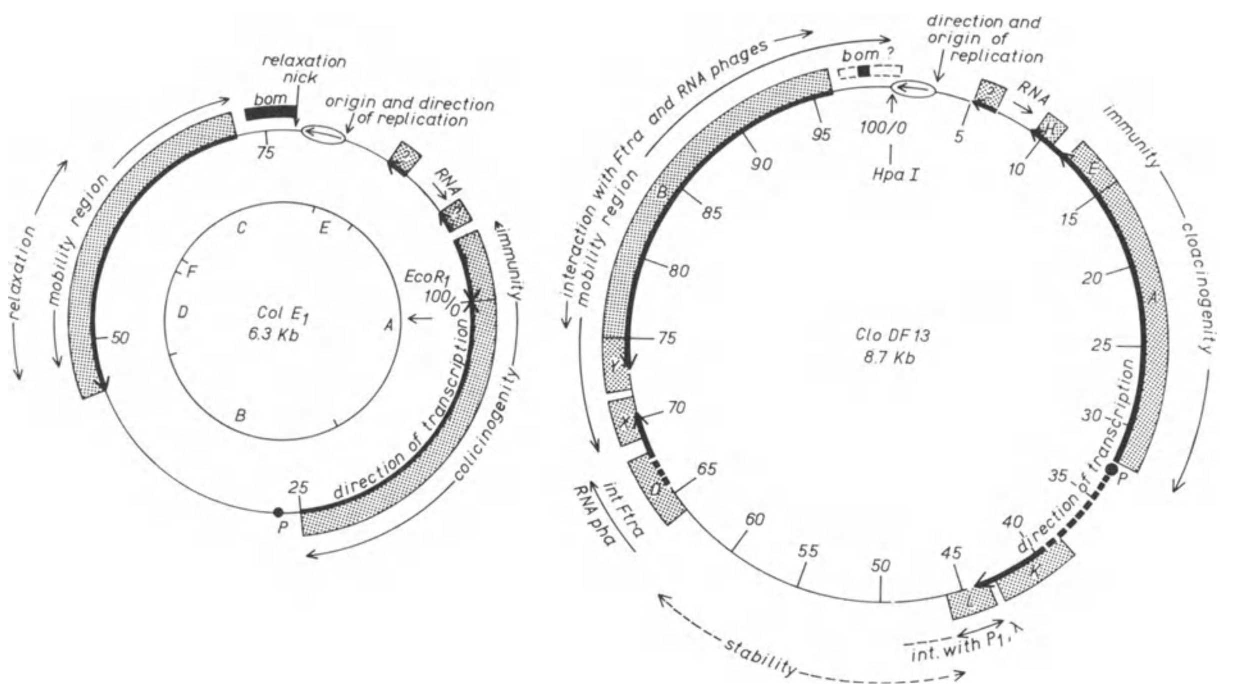

Figure 1. Comparison of the genetic and functional maps of the bacteriocinogenic plasmids ColE1 and CloDF13 [32]. The single Eco R1 site defines the zero point on the ColE1 map whereas the single HpaI site is used as reference point on the Clo DF13 map. Both maps have been orientated in such a way that they can easily be compared. bom indicates the basis of mobilization. For references see text. The inner circle on the ColE1 map represents the HaeII cleavage map.

The sequences upstream the replication origin are required for autonomous replication and may reflect a regulatory role in the initiation of plasmid replication.

What are the mean features of this region? (1) RNA primer for the initiation of DNA replication is synthesized starting from promoter P3. Actually this RNA molecule is a pre-primer, because it is processed into a primer by RNAase H as was shown by Itoh and Tomizawa for ColE1[1]. They showed that the pre-primer of ColE1 is 555 nucleotides long. In case of CloDF13 the length of this pre-primer is 580 nucleotidesas was determined by Stuitje et al.[2] (2) Codon analysis[3] has shown that the pre-primer might code for a basic, arginin-rich protein of about 45 amino acids, both for ColE1 and CloDF13, since an open reading frame is present. However, such a protein has not yet been identified. (3) A small RNA molecule of about 100 nucleotides (RNA-100) is synthesized from the opposite strand of CloDF13[4]. This RNA molecule is therefore complementary to the 5' end of the preprimer RNA. A similar situation exists in case of ColE1[5,6,7,8]. Interestingly, the transcription of the pre-primer is initiated at a position where the RNA-100 is terminated. The crucial question is whether this region has a function in the control of vegetative plasmid replication. In order to tackle that problem, replication control mutants have been isolated in a number of different ways. A few mutants in replication control, so called copy mutants because of an increased copy number, have been studied in detail.

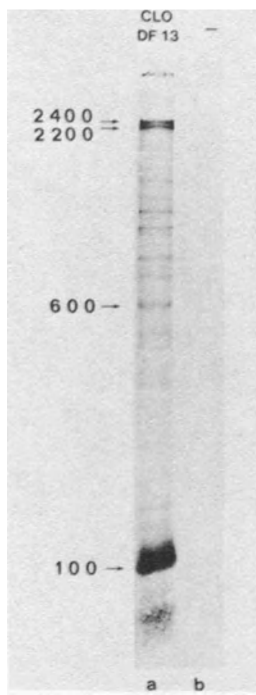

Figure 2.
SDS-urea polyacrylamide gel electrophoresis of ^{3}H-labeled RNA synthesized in minicells harboring the CloDF13 cop3 plasmid (track a) or in plasmidless minicells (track b)[4].

TABLE 1

PROTEINS ENCODED BY COL E1 AND CLO DF 13

Col E1		Clo DF13	
mw x 10^{-3}	Function	mw x 10^{-3}	Function
62	mobility[a]	64	cloacin DF 13[d]
58	colicin E1[b]	62	mobility, RNA phage int.[d]
44	unknown	21	unknown
41	unknown	18	unknown
36	unknown	17	RNA phage int.[d]
33	unknown	12.5	unknown
30	unknown	11	DNA phage int., stability? [d]
27	unknown	10	unknown
17	mobility[a]	8.5	immunity[d,e]
15	mobility[a]	6.5	transport of cloacin
14	immunity[c]		
10	mobility[a]		
6.5	unknown		

[a] The presumptive function of these proteins is based on the fact that the molecular weights of these proteins correspond to those isolated from relaxable DNA[31].

[b] Identification of colicin E1 protein is based on identical molecular weights of labeled and purified colicin protein[37,38,39] immunological crossreactivity[40], and the effect on polypeptide synthesis of mutations affecting colicin activity[37,39].
Possible breakdown products of colicin E1, based on their reaction with colicin E1 antiserum, are omitted from this table.

[c] Identification of this protein is based on effects on polypeptide synthesis of mutations affecting immunity activity[39].

[d] The identification of these proteins is based on the effects on protein synthesis of mutations affecting the activities described[41,27,35,25,26].

[e] The aminoacid sequence of purified immunity protein has been determined as well as the DNA base sequence of the immunity gene[18].

This table is taken from Veltkamp and Stuitje[32].

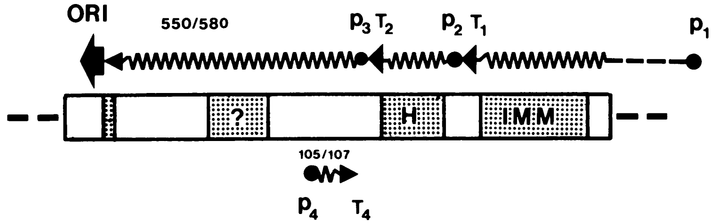

Figure 3. Transcriptional maps of the CloDF13 and ColE1 DNA regions containing the origin of replication (ORI) as well as the genes coding for the bacteriocin and immunity (IMM) proteins. ●—►: direction of transcription. The estimated length of the RNA molecules is given in nucleotides. [?] : CloDF13 and ColE1 homologous sequences that might code for protein. P1 indicates the cloacin promotor; T1 and T2: termination site 1 and 2.

a

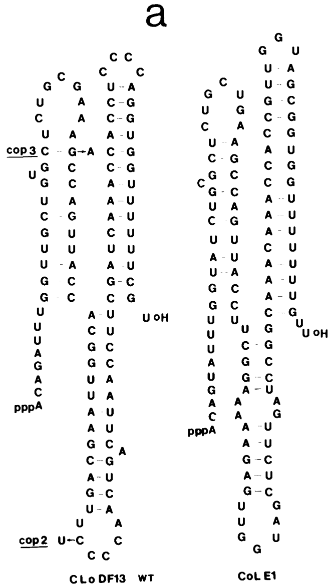

Figure 4A

Possible secondary structure for the 105 and 107 nucleotide RNAs. The CloDF13 copy mutations cop3 (G→A) and cop1 (C→U) are indicated.

b

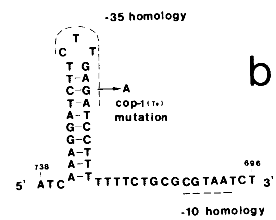

Figure 4B

Possible secondary structure of the DNA region involved in the termination as well as initiation of the down-stream transcription[2,3].
The possible RNA polymerase recognition (-35 homology) and binding site (-10 homology) involved in initiation of primer precursor RNA synthesis are indicated. Downstream transcription proceeds from left to right. The cop1 ts (G→A) is indicated.

For instance two non-conditional CloDF13 copy mutants have been mapped by base sequence analysis[2]. Both mutations cop2, cop3 are located within the region encoding for the RNA-100 suggesting that this RNA molecule modulates the rate of initiation of DNA synthesis in a negative way. However, the situation is complex, because these mutations do alter, at the same time, also the pre-primer RNA. In our laboratory we have also located a conditional copy control mutation cop1-Ts[2], a mutation that causes both, an increase in plasmid copy number and cell death at increased temperature. This ts mutation has been located in the terminator, T2, of the bacteriocin operon[2]. Also, a Col E1 copy mutation has been located by Polisky et al.[10].

Fig. 4A shows the 100 n.RNA molecules of CloDF13 and ColE1. They can be folded in a similar way, although the sequences differ to about 30%[2]. Apparently the secondary structure is very important for the functionning of this RNA[9]. The sequence of the first loop at the 5' end (GCUCUC) of the RNA-100 of CloDF13 is identical to that of ColE1. The sequence of the second and the third loop of CloDF13 (UCCCCA) are identical. These loops sequences are also identical in ColE1 (GUUGGUAGC). However, the latter sequences of CloDF13 differ from those of ColE1. An interesting question is whether these differences might be the reason for the fact that CloDF13 and ColE1 are compatible.

As indicated earlier, the cop1-Ts mutation (G→A transition) is located in the terminator region (T2) of the bacteriocin operon. This terminator region overlaps with the promotor sequence for the synthesis of the pre-primer RNA (Fig. 4B). Therefore, the effect of the cop1-Ts mutation, a temperature inducible plasmid copy number, could be the result of read through of transcription. We postulate that the formation of the pre-primer RNA is regulated in different ways: (1) The synthesis of the pre-primer RNA is negatively controlled by the RNA-100, e.g. by the formation of a RNA-RNA or RNA-DNA hybrid, (2) The synthesis of the pre-primer RNA may be also controlled by transcriptional activities of the bacteriocin operon since promotor P3 overlaps the terminator T2 of the bacteriocin operon (Fig.3) the leftward transcription of this operon might influence the rate of pre-primer synthesis, (3) Additional controls might operate at or around the origin of replication by the formation of the primer and/or start of DNA synthesis.

Adjacent to the replication region, a DNA region is located that is involved in bacteriocinogenicity (Fig.5). This region code for the production of the antibiotic protein cloacine DF13 in case of CloDF13 and colicin E1 in case of ColE1. Both proteins have been purified and characterized.[11,12,13,14]. Cells carrying these plasmids are immune to the lethal effect of their homologous bacteriocines. The genes responsible for this immunity have been located both for ColE1[15,16] and for CloDF13[17,18]. The CloDF13 immunity substance has been purified and characterized in our laboratory as a protein of 85 amino acids[18,19,20]. This protein is able to inactivate the cloacin protein by the formation of an immunity protein-cloacin complex[21]. The cloacin and immunity gene can be induced by e.g. mitomycin C or UV. The mechanism of regulation has not yet been elucidated.

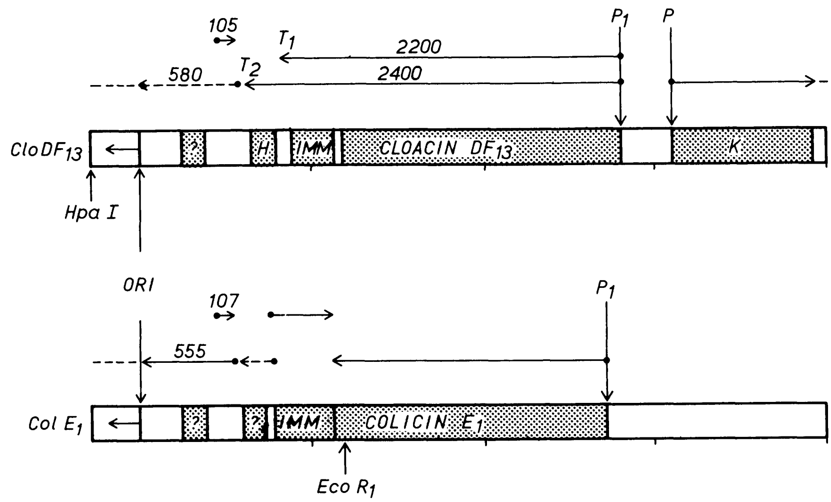

Figure 5. Regulatory sites involved in CloDF13 and ColEl replication. Presumed promotors and terminators are indicated by ● and ◄– respectively. The direction of transcription is indicated by an arrow. The estimated length of the RNA molecules is given in nucleotides.

Recent data show that this CloDF13 region encodes for two classes of RNA molecules; the transcription of these RNA molecule is initiated at the cloacin promotor, located at 32%[4]. The transcription of the first class, consisting of 2200 nucleotides RNA terminates at terminator T1, while the second class, consisting of 2400 nucleotides RNA overlaps the first class and terminates at T2[4]. This latter transcript does not only contain the message for the cloacin and immunity proteins, but it also codes for the third protein, protein H. This latter protein has been identified as a 5800 daltons protein and is localized as an innermembrane protein. The synthesis of this protein largely depends on a functional cloacin promotor (P1). What is the function of protein H? If we raise the level of protein H in the cell by either induction with mitomycin C or by gene dosage effect using a thermosensitive copy mutant, the cells will die and will lyse as well. When gene H is missing e.g. by deletion, the bacterial cells will still die in this experiment, but cells wil not lyse anymore. Protein H is likely involved in the lysis of the cell under these circumstances[23]. The natural function of protein H could be the transport of the cloacin-immunity protein complex through the cell envelope, because H^- cells accumulate this complex inside the cell[23].

With respect to ColE1 the situation seems to be different. The direction of transcription of the immunity gene is the opposite of that of CloDF13[6,24]. That means that in case of ColE1 the genes for colicin, the immunity protein and the hypothetical protein H (gene H is likely present also in ColE1 because of the presence of an open reading frame) are not part of one transcriptional unit.

In Fig. 1, the CloDF13 region next to the bacteriocin operon, two genes (K and L) have been identified[25]. In contrast to the transcription of the bacteriocin operon, the transcription of this region proceeds clockwise[4]. Gene L is involved in an interaction with the development of double standed DNA phages. Although certain transposon insertions in gene K have the same effect as insertions in gene L, it could be demonstrated that this effect is due to a polar effect on gene L. The gene L product inhibits the multiplication of phages like P1, T1, and λ, leading to a reduced burstsize and an altered phage morphology[25]. It is important to note, that plasmids, even small plasmids, can affect the propagation of phages and that, in general, one should be aware of such phenomena in case of phage typing of bacteria.

The neighbouring area (Fig.1) plays a role in the stable maintenance of the plasmid CloDF13. Deletion of this region in a CloDF13 copy mutant gives rise to large multimeric plasmid molecules and finally to loss of the plasmids from the cell[27]. Integration of an ampicillin transposon (Tn901) restores the stability. Probably the Tn901 transposon, like Tn3[23], provides for a system that resolves multimeric molecules into monomeric molecules, a system that the CloDF13 deletion mutant is missing. The stability region has, like the replication control region, a function in incompatibility[23] (Stuitje, unpublished observations).

PLASMID MOBILIZATION

Genetic studies reveal that the transfer of CloDF13 and ColE1 does not entirely depend on the conjugative plasmid, but also on genetic information present in case of non-conjugative plasmids[22,27,28,29]. Three CloDF13 genes (B, X and Y) have been identified that are involved in mobilization of CloDF13[22]. Mutations in either of these genes can be complemented by the wild type CloDF13, but not by the wild type ColE1. The gene products for mobilization are not exchangeable between CloDF13 and ColE1[22]. One type of mutant cannot be complemented at all[27]. The location of such a cis-acting sequence, named bom[30] (basis of mobilization) is interesting because it is very close or may be even identical to the site where the three protein components of the ColE1 relaxation complex[31] are bound and where upon relaxation a single strand nick is produced bij one of these proteins. This nick is considered as one of the steps in the initiation of transfer replication. At the moment none of the three proteins present in the relaxation complex have been identified as one of the gene products of the mobilization genes.

The bom site in CloDF13 and ColE1 is distanced only a few hundred base pairs downstream the origin of regetative replication and this might be significant for the transfer of these plasmids. A model that includes a relationship between the vegetative plasmid replication process and the plasmid mobilization process has been discussed by Veltkamp and Stuitje[33]. A conjugative plasmid, like F. is required for the transfer of non-conjugative plasmids. The question is whether all transfer genes of F are required for the transfer of non-conjugative plasmids. Obviously, the F tra genes, involved in mating pair formation are required, but F tra genes required for the replication and transfer of F itself, like tra M, I, and Z are not required for the transfer of CloDf13 and ColE1[33,34,27]. A difference between these plasmids is that the tra D gene product is required for ColE1 transfer[33], but not for CloDF13 transfer[27]. Apparently, CloDF13 produces its own tra D like product.

One of the mobilization genes, gene B and also gene D, located next to the mobilization region, are responsible for a reduced propagation of male specific RNA phages and for an inhibition of the transfer of F and ColE1[35]. Likely their gene products inhibit the function of the F tra D product[36].

In Fig.1 most of the present knowledge about the genetic constitution of ColE1 and CloDF13 have been summerized. If we compare both bacteriocinogenic plasmids, it is evident that the overall genetic organization is very similar. However, many differences exist, not only at the level of base sequences and transcription patterns but also at the level of the action of the gene products. Probably, this is the reason that certain gene products are not exchangeable between CloDF13 and ColE1. Although during the past 10 years, a reasonable amount of progress have been made with respect to the genetic organization and expression of non-conjugative plasmids, like ColE1 and CloDF13, many questions have still to be answered for a clear comprehension of the molecular biology of non-conjugative plasmids.

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STURCTURE-FUNCTION RELATIONSHIPS IN ESSENTIAL REGIONS FOR PLASMID REPLICATION

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INTRODUCTION

The development of recombinant DNA techniques has made possible the isolation of segments of a plasmid DNA molecule that are essential for plasmid replication and stable maintenance in a bacterial host. The discovery of rapid methods for the determination of the nucleotide sequence of DNA fragments and the development of *in vitro* systems for plasmid DNA replication permit a detailed analysis of the structure-function relationships of these various DNA segments. Such an analysis has been carried out on the essential region for plasmid R6K DNA replication. In addition segments of the replication regions of plasmids R6K and F that are involved in plasmid incompatibility have been isolated and analyzed. A striking feature of the essential region of replication of plasmids R6K, F and the broad host range plasmid RK2 [also studied in our laboratory[1-5]] is the presence of direct repeats of nucleotide sequences. The important role of these direct repeats in both plasmid replication and incompatibility and the major structural features of the essential region for plasmid R6K replication will be considered in this article.

GENERAL PROPERTIES OF PLASMID R6K

The antibiotic resistance plasmid R6K is 38 kb in size and specifies resistance to the antibiotics streptomycin and ampicillin[6]. This multi-copy plasmid (10-15 copies per chromosome equivalent) is a member of incompatibility group X. The positions of three origins of replication, designated α, β and γ, and a unique terminus of replication have been determined on a restriction map of this plasmid (Fig. 1)[7-10]. At least two of the origins, α and β, exhibit in *Escherichia coli* sequential, bi-directional replication toward an asymmetrically located terminus [11,12]. An *in vitro* system has been developed from *E. coli* for the replication of plasmid R6K and its derivatives[13,14]. The frequency of usage of the α, β and γ origins *in vitro* (0.20:0.43:0.37, respectively) differs from that observed *in vivo* where the α origin is used predominantly[9,10].

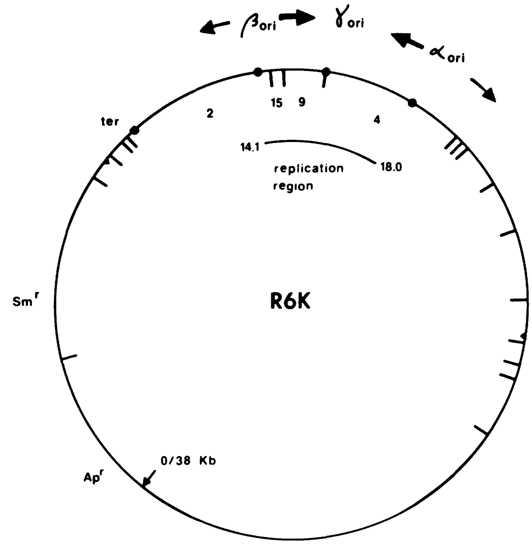


Figure 1. Physical and genetic map of R6K. The arrows indicate the sequential bi-directional mode of replication of replication origins α and β. 2, 15, 9 and 4 refer to specific Hind III fragments. The positions of other Hind III sites, the Bam H1 (↑) site and EcoRI (▼) sites also are indicated. ■ter refers to the terminus of replication.

CONSTRUCTION OF PLASMID R6K DERIVATIVES

A variety of restriction endonucleases have been used to delete regions of R6K non-essential for replication[8,15]. The replication region of plasmid R6K, encompassing all three replication origins, is approximately 4 kb in length and includes Hind III fragments 2, 15, 9 and 4. A number of low molecular weight derivatives of R6K, capable of autonomous replication, have been obtained. We have found that Hind III fragments 15 and 9 and a portion of 4 are common to all of these fragments[15]. This minimal replication region, depicted in Fig. 2, consists of two separate components: the γ origin and a structural gene, designated *pir*, which acts *in trans* to support the replication of the γ origin[16]. Interruption of the junction between Hind III fragments 4 and 9 by insertion of DNA fragments results in inactivation of the γ origin. Similar attempts in our laboratory to derive minimal replicons for the α and β origins have not been successful.

Studies with the *in vitro* R6K replication system identified the π protein as the trans-acting product of the *pir* gene[14]. This 35,000 dalton protein is required for the initiation of R6K replication in a cell-free *E. coli* system[14].

NUCLEOTIDE SEQUENCE OF THE R6K γ-REPLICON

The nucleotide sequence of the entire π gene-γ origin replicon (consisting of 1583 bp) has been determined[17,18]. The γ origin component of this replicon has been delineated by the insertion of the Tn5 transposon into a number of sites in the γ origin region and determination of the effect of the Tn5 insertions on γ origin activity[19].

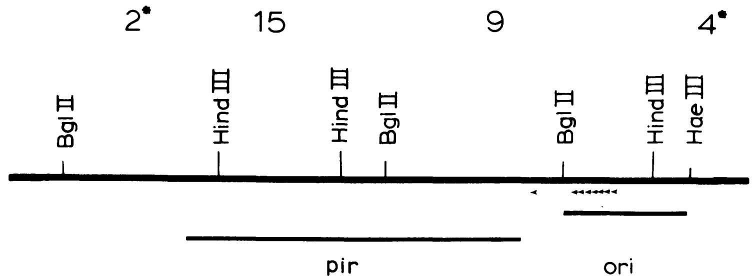


Figure 2. Map of the R6K γ-replicon. The locations of the *pir* gene and γ origin (ori) of replication are indicated. The arrow heads represent the positions of the 22 bp nucleotide sequence repeats.

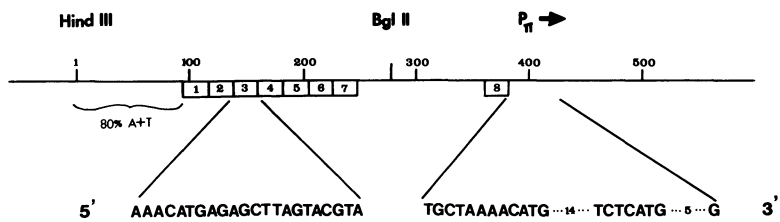


Figure 3. Major features of the nucleotide sequence of the γ origin region of R6K[17,18]. Nucleotide sequences of one of the direct repeats and putative RNA polymerase recognition and binding sites in the promoter region (P_π) of the π gene also are indicated.

The functional γ origin was found to consist of a 260 bp region extending from a short distance to the left of the junction of Hind III fragments 4 and 9 (Fig. 3) to just before the Bgl II site in Hind III fragment 9. The γ origin includes seven tandem 22 bp direct repeats. Removal of three or more of the direct repeats results in loss of origin activity[19]. As indicated in Fig. 3, an eighth direct repeat of 22 bp is located in the putative π gene promoter region. The nucleotide sequence of the R6K γ-replicon contains only one large open reading frame[18] for translation that spans Hind III fragments 2, 15 and 9[18]. This sequence encodes for a polypeptide of 35,000 molecular weight which is in good agreement with the size estimate of the π protein.

ROLE OF THE π PROTEIN IN R6K REPLICATION

Both *in vivo* and *in vitro* evidence have been obtained for the essential role of the π protein in plasmid R6K replication[14,16]. In addition, studies with the *in vitro* system for R6K replication have provided evidence for the requirement for the π protein in the initiation of R6K replication. The analysis of Tn5 transposition mutants and deletions of the γ origin region also has established a role of the 22 bp direct repeats in γ origin activity[19]. If π functions as a regulatory protein, the control of initiation of replication at the γ origin conceivably could involve a relatively simple circuit that consists of the interaction of π as a positive regulatory element with the direct repeats within the γ origin and the autoregulated expression of the *pir* gene (Fig. 4). Autoregulated expression of the *pir* gene would be mediated conceivably by interaction of the π protein with the eighth direct repeat in the putative π promoter region. To test this model several *in vitro* plasmid constructions were carried out to vary the cellular level of π protein, in order to determine the effect of π protein concentration on plasmid copy number[20]. In addition, the effect of the presence of π protein on *pir* gene promoter activity *in vivo* was determined[20].

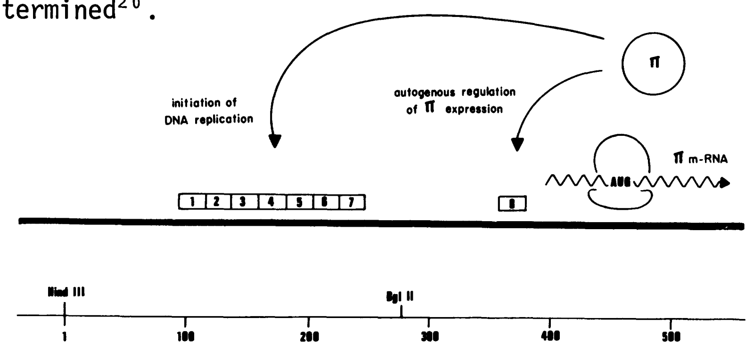


Figure 4. Model for the role of the π protein in the initiation of R6K replication. The numbered boxes indicate the positions of the eight 22 bp direct repeats.

AUTOGENOUS REGULATION OF EXPRESSION OF THE *pir* GENE

If the expression of π is autogenously regulated, then a change in the number of the *pir* genes per cell is not expected to affect the concentration of π protein in that cell. To obtain bacteria carrying different copies of the *pir* gene, isogenic strains of *E. coli* were either made lysogenic with a λ-*pir* hybrid phage[16] or transformed with a ColEI-*pir* recombinant plasmid (maintained at 20-40 copies per chromosome equivalent). The amount of π per cell was monitored by the *in vitro* R6K replication assay using extracts prepared from the λ-*pir* lysogens and the ColEI-*pir* transformants[20]. Similar amounts of π were recovered from both cell types. Thus at least a 20-fold increase in *pir* gene dosage has no significant effect on the concentration of π in the cell. These results are consistent with an autoregulated expression of the *pir* gene.

Nucleotide sequence analysis identified a putative promoter for the expression of the *pir* gene. That the *pir* gene contains its own transcriptional promoter was shown by fusing the putative *pir*-promoter to the *lac* Z gene[20]. Plasmids carrying the *pir*-*lac* fusion allowed expression of β-galactosidase (Fig. 5).

β-Galactosidase Activity in Cells Harboring Operon Fusions

Strain	Plasmids	β-Gal Units	%
MC1000	none	0	0
MC1000	pRK419	0	0
MC1000(λpir)	none	0	0
MC1000	pMC81	41	2
MC1000	pRK776	1364	81
MC1000	pRK776, pRK419	1443	85
MC1000	pRK775	1686	100
MC1000	pRK775, pRK419	763	45
MC1000(λpir)	pRK775	1118	66

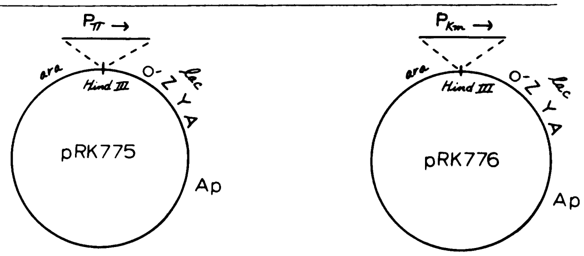
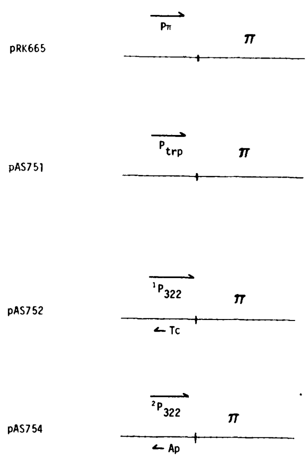


Figure 5. The construction of plasmid pCM81 was described[21]. Plasmids pRK775 and pRK776 are derivatives of pCM81. Plasmid pRK665[20] and pRK419[15] were the sources of the Hind III fragments containing the *pir* and the *kan* promoters, respectively.

When π protein is provided *in trans* by λ-*pir* or plasmid pRK419, a significant reduction in the levels of β-galactosidase expression from the *pir*-*lac* plasmid is observed (Fig. 5). The π protein has no effect on the expression of β-galactosidase from the kanamycin resistance promoter (Fig. 5). These results indicate that the π protein interacts with its own promoter region and thereby regulates its own expression.

π PROTEIN IS NOT THE REGULATORY ELEMENT FOR THE INITIATION OF REPLICATION

A fundamental role assigned to π in the working model (Fig. 4) is its ability to regulate positively the frequency of initiation of R6K DNA replication. This regulatory role of π was tested by placing the expression of π under different promoters and assaying for the effects of different cellular levels of π on the copy number of derivative of the R6K plasmid. We were able to isolate an R6K Hinf I fragment that contains the *pir* gene but is deleted for the region containing the *pir* promoter sequence and the first five nucleotides from its putative translational start signal. This fragment was fused to a tryptophan promoter fragment containing the first seven codons of the N-terminus of TrpE, which provides a promoter sequence, a ribosomal binding site and a translational start signal. The correct reading frame was provided by the introduction of EcoRI linkers between the *pir* and the *Trp* fragments. Cells carrying either one of the four plasmids depicted in Fig. 6 were transformed subsequently with the R6K γ origin plasmid pRK526[16]. By varying conditions for tryptophan expression, the copy number of pRK526 could be determined for varying cellular concentrations of the π protein. Concentration of the π protein was assayed either by following synthesis in minicells or by the *in vitro* R6K replication assay.



		Copy No. γ-ori	S-Met- (minicells)	-activity (in vitro rep.)
pRK665		18	1.0	1.0
pAS751	Repressed	18	1.3	0.057
	Derepressed	17	7.0	N.D.
	TrpR⁻	17	7.0	5.4
pAS752		18	0.1	0.85
pAS754		3	≤0.1	<0.01

Figure 6. The effect of variation of π concentration on the copy number of a γ origin plasmid. All constructs are pBR322 derivatives. pRK665 contains the entire *pir* operon; pAS751, pAS752 and pAS754 carry the π sequences without the *pir* promoter. pAS752 and pAS756 differ in orientation of insertion of the π coding sequence in the EcoRI site of pBR322. pAS754 and pAS751 differ in that the latter also contains the tryptophan promoter fragment.

There is some discrepancy between the results obtained from minicells and the *in vitro* assay (Fig. 6). Nevertheless, regardless of the method for determining π concentration, the results show that varying the concentration of π in the cell over a 70-95 fold range has no effect on the copy number of the R6K γ origin plasmid. Recently this analysis was extended to the entire R6K replicon (includes all three origins of replication) with similar results. These observations argue against a positive regulatory role of π in replication. For the last construction (pAS754), shown in Fig. 6, the level of π was too low to be detected by the methods used and the copy number of pRK526 correspondingly is very low. In addition pRK526 is maintained unstably under non-selective conditions. This result is consistent with a minimal requirement of this essential initiation protein for stable maintenance of the plasmid.

π PROTEIN IS REQUIRED FOR ACTIVITY OF ALL THREE ORIGINS OF REPLICATION

The failure to isolate the most frequently used origins of replication *in vivo* (α and β) even when π is supplied *in trans*, raised the question of whether or not the requirement for π is limited to replication from the γ origin. To answer this question, *in vitro* site specific insertions were carried out, taking advantage of previous information that two Hind III recognition sites span the coding sequence for π. For the insertions a small Hind III fragment of 58 bp that was constructed by dimerization of a 29 bp segment located on pBR322 between the EcoRI and Hind III sites was employed. The EcoRI site provided an easy marker for the mapping of the site of insertion as well as a convenient tool for further genetic rearrangements. Figure 7 summarizes the data from these experiments. In the construction of pAS808 the 58 bp fragment is inserted in the junction of Hind III fragments 9 and 15 which corresponds to the N-terminus of the π protein. This plasmid was found to be unable to replicate in a *pol* A strain of *E. coli* unless a functional π protein is supplied *in trans*. It can be concluded therefore that replication of R6K from any one of the three origins requires the π protein.

Contrary to previous observations (Fig. 7, pRK693/Hin) insertion of this 58 bp fragment in the junction of Hind III fragments 9 and 4 (pAS865 and pAS807) did not abolish the γ origin activity. This unexpected finding may be due to the fact that the 58 bp insert is composed of two tandem inverted repeats and may therefore acquire a structure which would not adversely affect the structure of the γ origin.

THE γ ORIGIN REGION IS REQUIRED *in cis* FOR α AND β ORIGIN ACTIVITY

Plasmid pAS904, a deletion mutant of the R6K replicon that is missing the Hind III fragment 9, was constructed. Hind III fragment

9 contains a major part of the N-terminus of π together with the essential seven 22 bp repeats of the γ origin (Fig. 2). Plasmid pAS904, which contains both the α and the β origins of replication, cannot be maintained in *E. coli* even when π is supplied *in trans*. Thus, replication of R6K from the α or β origins requires the π protein, which may be supplied *in trans*, and a *cis* interaction of the γ origin region with α and β.

It is conceivable that the proposed interaction of the π protein with the direct repeats of the γ origin region activates also the α and β origins either via a transcriptional activation event or by promoting the synthesis of RNA transcripts that subsequently are processed into a functional initiation primer specific for the α and β origins.

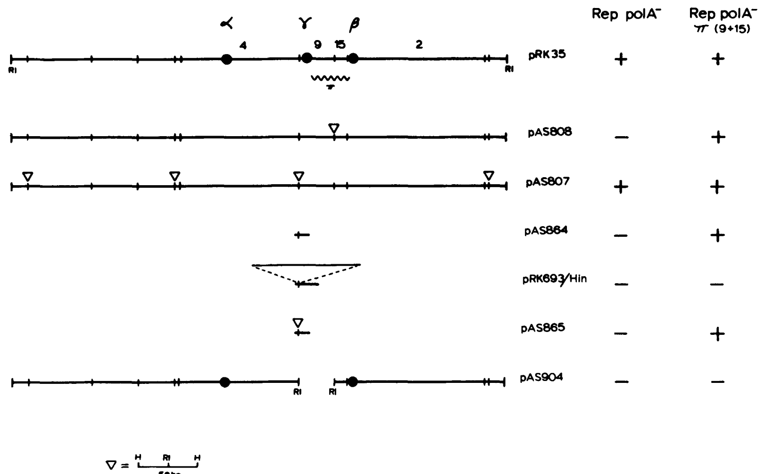


Figure 7. Ability of R6K derivatives to replicate in an *E. coli* *polA* strain. *PolA*⁻ π (9+15) refers to a λ *pir* lysogen of the *polA* strain. Each triangle in the third line represents a different plasmid carrying the 58 bp insert; pAS807 refers to an insert at Hind III junction 9 and 4. Plasmids pAS807, pAS808 and pAS904 are pSF2124 derivatives. pAS864, pAS865 and pRK693 are pBR322 derivatives. The pRK693/Hin represents a group of plasmids carrying individual Hind III fragments from R6K (except Hind III-4) inserted into the Hind III 9/4 junction.

DIRECT REPEATS OF NUCLEOTIDE SEQUENCES FUNCTION IN PLASMID INCOMPATIBILITY

The region of R6K containing the seven 22 bp direct repeats, which is required for replication from the α, β or γ origins, also expresses R6K incompatibility. A segment of R6K containing the seven 22 bp direct repeats, but non-functional as a replicon, was cloned into the normally compatible plasmids pBR322 and pACYC184.

When these two hybrid plasmids were introduced into the same cell, they behaved as an incompatible pair. Moreover, when hybrid plasmids were constructed that carried fewer copies of the 22 bp repeats. the level of incompatibility correspondingly decreased (S. Yang, unpublished observations).

Direct repeats also have been identified in an incompatibility region of the plasmid mini-F, a low molecular weight derivative of the F plasmid[22,23]. The mini-F *inc*C region of about 600 bp (45.8 - 46.4 Kb on F plasmid map) had been cloned on a ColEI replicon and the resulting plasmid pRF7 was shown to express incompatibility with mini-F derivatives (M. Kahn, unpublished results). Mutations[24] have been obtained in the region 45.1 - 46.4 Kb [i.e. *inc*B (45.1 - 45.8 Kb) plus *inc*C], that result in a higher copy number and a loss of incompatibility. The nucleotide sequence of the *inc*C region was analyzed to determine whether this region has the capacity to encode for a repressor protein. The nucleotide sequence[25] revealed a very limited coding capacity; the largest putative polypeptides with an ATG translational start signal are only 4.1 K and 3.4 K. A striking feature of this region, however, is the presence of five 22 bp direct repeats within a 251 bp segment. Fig. 8 summarizes the prominent features of this 453 bp region. To determine whether it is a polypeptide or the direct repeat region that is required for incompatibility, deletions were made in plasmid pRF7. Deletion of the start signal ATG for the 4.1 K polypeptide was obtained by partially digesting pRF7 with MboI; this deletion had no effect on the expression of incompatibility. More extensive deletions were carried out in order to remove the start codon for the putative 3.4 K polypeptide and copies of the 22 bp direct repeats. Plasmid pRF7, linearized with EcoRI, was partially digested with BAL31. The extent of the deletions obtained by this treatment was determined by Dde I restriction enzyme analysis. Deletion derivatives that lack the DdeI site at 129 bp only (type A in Fig. 8) no longer contain the start codon and retain a region that contains three to five repeats. No decrease in the expression of incompatibility was observed with these plasmid derivatives. But type B deletions (Fig. 8) that lack the Dde I sites at both 129 bp and 184 bp exhibited markedly decreased incompatibility. These plasmids retained only two or three of the direct repeats.

These data indicated that the direct repeat region is important for incompatibility. To test directly whether it is the repeats that express incompatibility, we cloned the 58 bp DdeI fragment (129 - 184 bp) containing two 22 bp repeats into pACYC184 that had been linearized by partial DdeI digestion[25]. The 58 bp fragment was inserted into various sites in pACYC184. These hybrid plasmids expressed incompatibility not only with mini-F but also with F'*lac*. When two copies of the fragment were inserted in tandem, expression of incompatibility was considerably stronger. In fact, in this case it was not possible to propogate host cells that contained both the hybrid plasmid and the mini-F under conditions of selection for both plasmids.

While a role of the direct repeats in the expression of F incompatibility is established, the mechanism by which these repeat sequences function in this phenomenon remains to be determined. The sequence is clearly too small to code for a regulatory repressor polypeptide that inhibits the initiation of replication, but the possibility of expression from the direct repeat segment of an RNA molecule that functions in the incompatibility phenomenon is not ruled out.

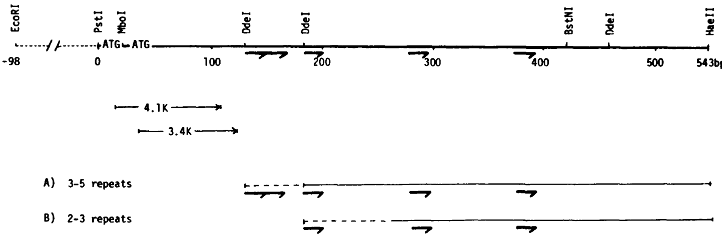


Figure 8. Prominent features of the *inc*C region. The arrows show the location of the 22 bp repeats. The region shown by a dotted line is a ColEI DNA segment. Solid lines in (A) and (B) show the regions that are unambiguously present; dashed lines indicate the portions that may also be present[25].

CONCLUDING REMARKS

Direct nucleotide sequence repeats of 22 bp play a vital role in R6K γ origin activity. The region containing these repeats also is required for functional α and β origin activity. In addition, direct repeats play an important role in the expression of incompatibility by plasmids R6K and mini-F. When a 58 bp segment containing two copies of the 22 bp repeat sequences from the *inc*C region of mini-F is inserted into plasmid pACYC184, which is normally compatible with the F plasmid, the hybrid plasmid is incompatible with mini-F derivatives and the F'*lac* plasmid. Similarly, the insertion of the 22 bp direct repeat region of R6K into the normally compatible plasmids pACYC184 and pBR322 renders these plasmids incompatible. Direct repeats of 17 bp also are a major feature of the replication origin region of plasmid RK2 and play a role in RK2 incompatibility. The biochemical nature of the role of these direct repeats in replication origin activity and incompatibility is unknown. Clearly the repeats can serve as binding sites for plasmid specific proteins involved in the replication and/or plasmid partitioning process. Indirect evidence has been obtained for the binding of the essential π protein to the R6K direct repeats. Alternatively or perhaps additionally, the direct repeats may facilitate association of the plasmid with a replication and/or plasmid partitioning membrane site. Finally, it is possible that RNA transcripts of the direct repeats account for their role in replication and/or incompatibility.

Considerable progress has been made towards an understanding of R6K replication. The nucleotide sequence of the entire R6K λ-origin replicon has been determined. Contained within this 1583 bp sequence is the π protein structural gene and a 260 bp segment that has been identified as the γ origin. No other protein is encoded by this replicon. The π protein is required for the activity of all three R6K origins of replication. Although it is required for the initiation of replication, it is not a regulatory protein. Clearly, however, there are constraints on the replication of plasmid R6K since it is stably maintained at a copy number of 10-15 per chromosome equivalent. The nature of the mechanism of regulation of the R6K copy number remains to be determined.

ACKNOWLEDGEMENT

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CONTROL OF PLASMID REPLICATION AND ITS RELATIONSHIP TO INCOMPATIBILITY

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The mechanisms that determine plasmid copy number and the relationship between control of copy number and incompatibility are controversial topics. Since many people attending this conference do not have a day-to-day interest in them, it has been suggested to me that it would be useful to list the points on which there might be general agreement among those working on these related aspects of plasmid biology.

I propose to do this and then speculate a little about the nature of the control systems involved.

It is hazardous to generalise about plasmids. They are entrepreneurs of the bacterial world and different plasmids will no doubt have found different ways of securing their future. Nevertheless I believe that some generalisations can be made with reasonable confidence. Much of the experimental evidence upon which they are based is referred to in recent reviews by Gustafsson *et al*[1] and by Pritchard[2], and in various contributions to this meeting. It will not be listed exhaustively here.

I. The first point of agreement would be that plasmid replication is controlled at the level of initiation. What this means is that the concentration of plasmid origins is in some way sensed and maintained by regulation of the frequency of plasmid replication. Altering the size of a plasmid by inserting or removing DNA will not affect copy number unless it coincidentally interferes with the functioning of the control mechanism.

In apparent contradiction to this generalisation it has been found that in some cases lengthening a plasmid by insertion of additional DNA results in a compensating reduction in copy number as if it were the total amount of plasmid DNA rather than the number of plasmid copies that is controlled. In all of these exceptions, however, the plasmids being studied were probably copy mutants (*cop*$^-$) in which the wild-type control system was defective. It has been suggested that in such mutants copy number does not rise indefinitely but plateaus when a cellular component involved in DNA chain elongation becomes rate limiting for plasmid replication. Increasing the length of such a plasmid will inevitably lead to a reduction in copy number. A direct test of this hypothesis has recently been made with the plasmid ColE1. Deleting DNA of this plasmid does not affect copy number of *cop*$^+$ derivatives but increases the copy number of *cop*$^-$ mutants[3].

The generalisation is thus only valid for plasmids with a wild-type copy control genotype.

II. The rate of plasmid replication under steady state growth conditions is determined by the concentration of an inhibitor which is plasmid specified and acts in trans on all plasmids of the same incompatibility group (see below). The inhibitor is an RNA species in plasmids as different as ColE1[4] and R1[5] but is a protein in the laboratory construct λdv (see ref. 2).

III. A third generalisation is that if a chimera is made between plasmids with different copy numbers the copy number of the chimera will not be less than that of the component plasmid with the higher copy number. If the control system of the higher copy component functions normally it will passively carry the copy number of the low copy component to the same level. The control system of the latter will sense this elevated copy number and respond by reducing the probability of replication from its origin. This effect has been termed switch-off[2]. The extent of switch-off will depend on the sensitivity of the control system to enforced departure from the copy number it freely determines.

Data conflicting with this generalisation also have been reported. In the most fully analysed case[6] a chimera between ColE1 (average copy number about 20) and RK2 (average copy number about 5) had a copy number of about 5. It was clearly demonstrated, however, that the ColE1 component of the hybrid was incapable of initiating replication and had even lost its capacity to express incompatibility against another ColE1 plasmid. Thus the conflict is only apparent. Since the ColE1 component could be recovered from the cointegrate as a functional replicon[6], its loss of function was probably due to transcriptional read-through from an RK2 promoter across one of the junctions between the two plasmids into the control region of ColE1.

IV. In the case of multicopy plasmids replication occurs at any time during the cell cycle more or less randomly. Whether this generalisation holds for plasmids like F, which have an exceptionally low copy number (less than one plasmid per chromosome) is uncertain because there is an unresolved conflict of evidence[7,8].

V. This generalisation, which follows logically from IV, is that plasmid replication is not correlated in time with (and therefore not coupled to) any identifiable event in the cell cycle or chromosome replication cycle. This generalisation has been shown to be valid for F despite the uncertainty about the timing of replication of this plasmid[9].

VI. In the case of multicopy plasmids the choice of plasmid for replication is approximately random. Thus a plasmid that has recently replicated is as likely to replicate again as one that has not[1].

VII. Incompatibility is the inevitable outcome of a copy control system in which there is a random choice of plasmids for replication and control of the total number of plasmid copies. This would be true even if there were a perfect mitosis-like partitioning of sister plasmids to daughter cells as was pointed out in the Sixties[10]. In cells initially containing an equal number of two phenotypically distinguishable types of the same plasmid, random choice of plasmids for replication would cause the proportions of each type to become distributed randomly in the cell population. Some cells would therefore contain only plasmids of one type. Since this sorting out is irreversible, the whole population will ultimately consist of pure clones containing one or other plasmid type. The severity of incompatibility would be determined by the copy number. Low copy plasmids will show strong incompatibility. High copy plasmids will show weak incompatibility.

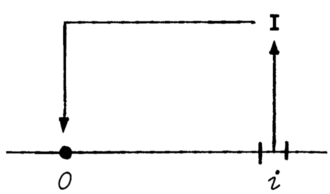
It is not known whether sister plasmids do in fact undergo mitosis-like partitioning of daughter cells. The alternative extreme would be a completely random distribution of plasmids to daughter cells[11]. If there were a random distribution some cells would be born with no plasmid copies and the frequency of this zero class is predictable[11]. For several low copy plasmids the zero class is found to be too small to be consistent with random segregation hence:

VII. There is a partitioning mechanism at least in low copy plasmids which ensures that cells are born with at least one plasmid copy. Plasmid mutants that are unstable (i.e. are lost from a significant proportion of the population at each cell doubling) are well known. Evidence that one class of unstable derivatives of the plasmid R1 is *par*⁻ is given by K. Nordström in his contribution to this meeting.

The nature of the copy number control system is beginning to emerge from molecular genetical analysis and from physiological studies of a number of plasmids. Two observations from physiological studies providing useful insight into the properties of the control system will be mentioned here.

The concentration of all plasmids for which data are available (F, R1, P1, R6K, ColE1) is less at fast growth rates than it is at slow growth rates (e.g. Fig. 1). The shape of the curve differs with different plasmids but the trend is the same suggesting that the relationship is a fundamental property of initiation control systems in plasmids. The simplest relationship is that found for R1 where there appears to be a proportionality between doubling time and plasmid concentration[1]. What this means in the case of R1 is that the number of plasmid replications per minute is a constant independent of plasmid concentration. This has lead Gustafsson and Nordström[12] to suggest that plasmid replication is controlled by a system that determines the frequency of replication without measuring the actual copy number, and that if initiation is controlled by an inhibitor there is no gene dosage effect on its concentration.

Another way of looking at this apparently paradoxical result is to consider the properties of a simple negative feedback loop



in which a plasmid gene *i* produces an inhibitor I which binds to the origin *O* of the plasmid to block initiation. Assume that the probability of initiation at a plasmid origin is the same at all growth rates when the origin is 'open' (i.e. has no inhibitor bound to it). Assume also that $[I]$ >> than K_b the binding constant of inhibitor. Assume, finally, that I is produced constitutively

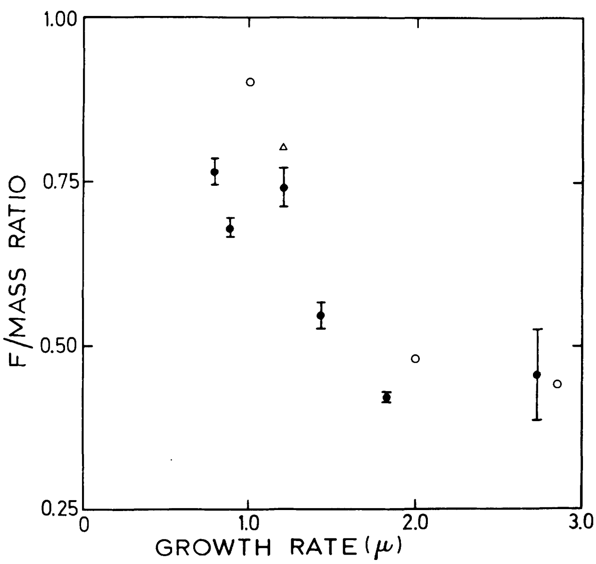


Figure 1. The average concentration of F particles in steady state exponential cultures of *Escherichia coli* at different growth rates. The figure is modified from data presented in reference 9 which gives details of the method of estimating F concentration. One additional assumption made here is that the average concentration of chromosome origins is invariant.

and that the output of I per minute per i gene is proportional to the growth rate. (This relationship has been found for a number of constitutive genes in *E.coli*[13]). Using the first two assumptions:

$$\text{Replications/min/mass} \propto \frac{1}{[\mathrm{I}]} \cdot [O] \quad - \quad - \quad - \quad (1)$$

or

$$= k\frac{1}{[\mathrm{I}]} \cdot [O] \quad - \quad - \quad (2)$$

In other words, the rate of replication in a unit of cell mass will be inversely proportional to the inhibitor concentration and directly proportional to the number of origins available for initiation. If the third assumption is correct then under steady state growth conditions the concentration of I will be proportional to the concentration of plasmid origins since every plasmid has an i gene and partially replicated plasmids with two origins and one i gene can be neglected. So:

$$[\mathrm{I}] \propto [i] = [O] \quad - \quad - \quad - \quad - \quad (3)$$

Therefore substituting for $[O]$ in (2) gives

$$\text{Replications/min/mass} = \mathrm{K}. \quad - \quad - \quad - \quad (4)$$

where K is a constant, and

$$\text{Replications/generation/mass} = \mathrm{K}.\tau \quad - \quad - \quad (5)$$

or

$$[O] = \mathrm{K}.\tau \quad - \quad - \quad (6)$$

From this analysis it can be seen that the apparent constancy of the replication frequency independent of the origin concentration and the inhibitor concentration is due to the fact that they affect the probability of initiation in opposite directions. If the growth rate is raised $[O]$ falls, decreasing the rate of initiation, but $[\mathrm{I}]$ falls proportionately increasing the rate of initiation by the same amount to give no net change of rate.

It is necessary to emphasise that a strict inverse proportionality between plasmid concentration and growth rate is not found for all plasmids (e.g. P1[14] and R6K[15]) indicating that the assumptions underlying equations (4)-(6) are not universally applicable.

The initial 'inhibitor dilution' model[10] proposed that initiation of chromosome and plasmid replication was under the control of a negatively acting inhibitor which was stable. In the same paper an alternative unstable inhibitor model was also considered. It is possible to distinguish between a stable inhibitor and an unstable inhibitor by determining the kinetics with which a plasmid equilibrates at its new concentration following a change of growth rate caused by a nutritional shift. Since the relationship in (3) can only hold during a transition if I has a rapid turnover the rate of replication can only remain constant during a transition if I is unstable. Gustafsson and Nordström[12] have found that equation (4) does hold during a transition indicating that $[I]$ is indeed unstable in R1.

Recent work[4] with the plasmid ColE1 suggests that in this plasmid initiation frequency is determined by a feedback loop with properties very similar to the unstable inhibitor model.

It might also be noted finally that in the case of ColE1 the copy number is not only higher at slow growth rates but also rises during the transition of a culture from exponential growth into stationary phase[3] as equation (6) predicts. The continued replication of ColE1 in the presence of chloramphenicol could also be predicted for a feedback loop of the type described. The fact that in more complex plasmids like F there is little run-on of plasmid replication during stationary phase or inhibition of protein synthesis suggests a more complex control of these plasmids or that other plasmid-coded or cellular products required for replication of these plasmids soon become limiting under these conditions.

R. H. P. especially wishes to acknowledge his appreciation of the many stimulating and challenging discussions he has had over many years with Kurt Nordström.

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STRUCTURE AND FUNCTION OF THE REPLICATION ORIGIN REGION OF THE RESISTANCE FACTORS R100 AND R1

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R1 and R100 are large complex plasmids, approximately 90 kb in size, that code for multiple antibiotic resistance and functions involved in conjugal transfer of plasmid DNA.[1,2] Both R1 and R100 belong to the FII plasmid incompatability group,[3] indicating that the control of DNA replication in these plasmids is similar. Heteroduplex studies have confirmed this relationship by showing that the regions of R1 and R100 that are required for autonomous DNA replication have great sequence homology.[4] This region is about 2.5 kb in length for R100, and, in addition to the replication origin,[5,6,7] encodes at least one function that is required for replication. Part of this 2.5 kb replication region also encodes functions involved in plasmid incompatibility and copy number control.[6] Studies with R1 have led to very similar conclusions.[8]

pSM1 and pTR1 are small, high copy number plasmids that were derived from R100 and R1, respectively.[9,10,11] pSM1 and pTR1 share approximately 2.1 kb of homology, which is within the 2.5 kb replication region. Part of the remainder of the replication region (about 250 bp) is non-homologous in R1 and R100 (Figure 1). We have determined the nucleotide sequence of the entire replication regions of both pSM1 and pTR1.[10,11] Here we will describe our analysis of this replication region with regard to the hypothetical coding frames, regions of possible secondary structure, RNA transcripts, and polypeptides that we have identified. This analysis has allowed us to formulate a model for DNA replication control in large drug resistance plasmids as exemplified by R1 and R100.

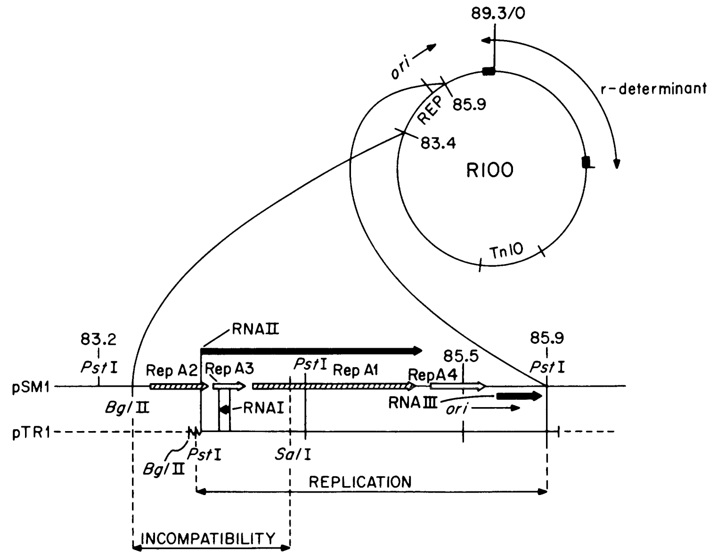

Figure 1. A summary of some physical and genetic properties of plasmids R100, pSM1, and pTR1.. The open, crosshatched, and heavy arrows represent open reading frames predicted from nucleotide sequences, reading frames whose polypeptide products have been identified, and RNA transcripts, respectively.

HYPOTHETICAL CODING FRAMES

Four possible coding frames that are common to both pSM1 and pTR1 have been identified from the nucleotide sequence of the replication regions of these two plasmids. These coding frames, which we have designated RepA1, RepA2, RepA3 and RepA4, are all in the same reading frame and are located within the nucleotide sequence as shown in Figure 1. RepA1 is the longest common coding frame identified and encodes a polypeptide 33,000 daltons in size.[10,11] There are 49 bp changes between the RepA1 coding frame of pSM1 and that of pTR1. However, these changes result in only 8 amino acid substitutions because 39 of the bp changes occur in the third codon position.[10] Because the RepA1 coding frame crosses the junction of two PstI fragments that is required in the original orientation for autonomous plasmid replication,[6,7] it is likely that RepA1 is required for replication.

RepA2 and RepA3 are encoded within the region of R100 and R1 that specifies incompatibility and copy number functions.[6,7] (Figure 1) A part of this region (about 250 bases) is non-homologous

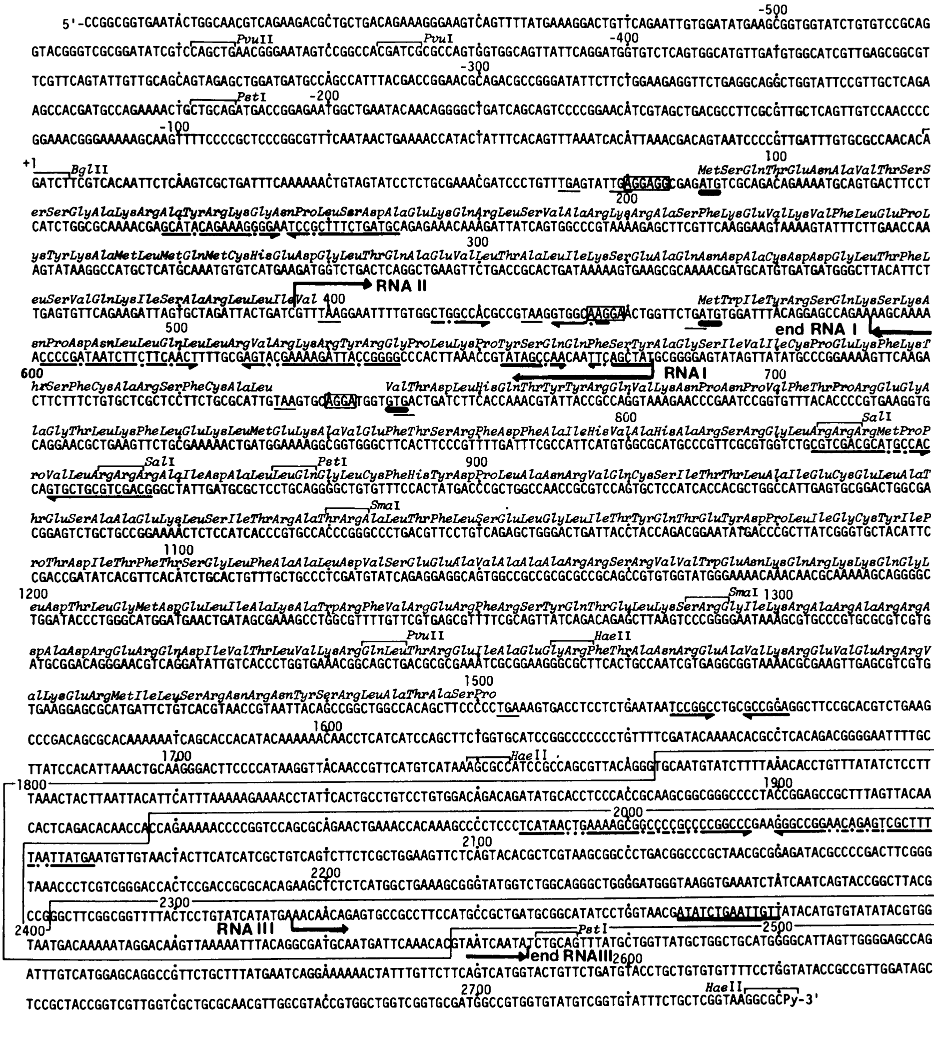

Figure 2. Nucleotide sequence of one strand of the replication region of pSM1. Arrows below the sequence indicate the location of inverted repeat sequences. The sequences within the small boxes preceeding the open reading frames are nucleotides complementary to the 3' end of 16S rRNA. Replication proceeds rightward from the origin region, designated by the large boxes, which indicate one and two standard deviations from the position where the origin has been mapped. Bases corresponding to the 5' and 3' ends of the transcripts RNAI-III are shown by heavy arrows. The sequence in the origin region which is homologous to RNAI is underlined.

between mutually incompatible R100 and R1.[4,10,11] The non-homology lies within the RepA2 coding frame and comprises 83% of the RepA2 sequence so it is unlikely that RepA2 plays a major role in determining incompatibility. In contrast to RepA2, the nucleotide sequences for RepA3 in pSM1 and pTR1 have only two base pair differences, both of which would result in amino acid changes in the RepA3 polypeptide.[10,11] RepA4 is the fourth hypothetical coding region common to pSM1 and pTR1 and encodes a polypeptide approximately 14,000 daltons in size.[10,11,12] The origin of replication is contained within RepA4.[10,11,12] However, because the nucleotide sequence preceeding the RepA4 coding frame does not have the characteristics typical of polypeptide reading frames, we do not believe that RepA4 encodes an actual polypeptide.[10,11]

SECONDARY STRUCTURES AT THE ORIGIN OF REPLICATION

The approximate location of the origin of replication of pSM1 has been determined by electron microscopic analysis,[5] and corresponds to the region between nucleotides 1763 and 2456 allowing for two standard deviations about this point, as shown in Figure 2.[10,11,12] Replication proceeds unidirectionally to the right from this site.[5,12] The origin and mode of replication used by pSM1 is the same as that used by R100-1,[13] indicating that the control of replication of the large plasmid is also present in pSM1, although most of the R100-1 has been deleted in pSM1.[4,9]

Numerous sequences that are either direct or inverted repeats can be found within the nucleotide sequence near the replication

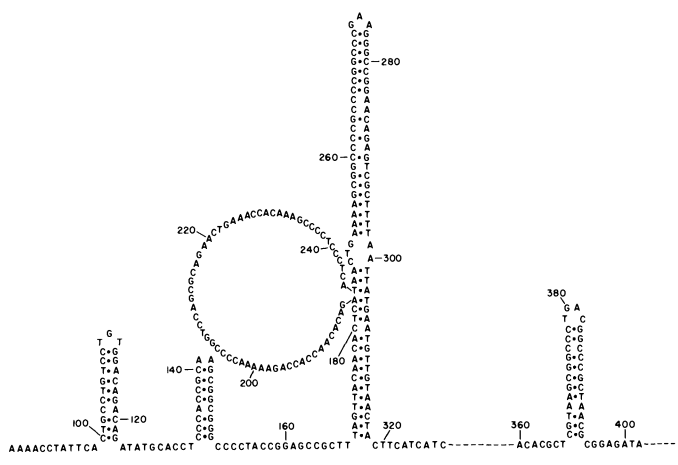

Figure 3. Possible secondary structure at the pSM1 replication origin. Nucleotides 100 to 400 in this Figure correspond to nucleotides 1843 to 2143 in Figure 2.

origin of pSM1 and pTR1.[10,11,12] The stem-loop structures that can be drawn using these repeated sequences is shown in Figure 3. The most striking feature of these structures is that they occur in the region to which the origin has been mapped microscopically. Although there are 12 bp changes in the region of these structures between pSM1 and pTR1, none of these changes affect the base pairing of any of the stem structures. The conservation of this base pairing suggests that these structures are important for DNA replication.[10,12] Complex secondary structures resembling those shown in Figure 3 are also present at the replication origin regions of other organisms such as E. coli,[14] Salmonella typhimurium,[15] and bacteriophage lambda.[16]

RNA TRANSCRIPTS

Three RNA transcripts are produced in vitro when superhelical pSM1 and pTR1 DNA or the appropriate fragment from the replication region of these plasmids are used as substrates. The smallest transcript is 91 nucleotides in length and is designated RNAI. The coding region for RNAI is contained totally within that of RepA3, but RNAI is synthesized in the direction opposite to that of the RepA3 transcript (Figures 1 and 2). Hypothetically, RNAI can form two large, stable secondary structures as shown in Figure 4. There

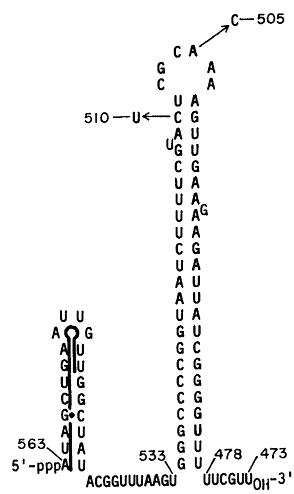

Figure 4. Possible secondary structure of RNAI. The two arrows at the top of the loop indicate the differences in the sequences of RNAI for pSM1 and pTR1. The line within the smaller hairpin shows the region complementary to the pSM1 origin region between nucleotides 2367 and 2380 (See Figure 2).

are only two base changes in RNAI between pSM1 and pTR1, and both changes occur at the top of the larger secondary structure loop (Figure 4). In addition, 13 of the first 14 nucleotides of RNAI are complementary to a sequence at the replication origin region (Figure 2, refs. 11,17).

The second RNA transcript common to both pSM1 and pTR1 is very large and is designated RNAII. This transcript begins 54 base pairs to the 5' side of the initiation codon of RepA3. The location of the 3' end of this transcript is presently not known; however, we believe that RNAII is the mRNA for RepA1 and perhaps also for RepA3. RNAII is synthesized in the direction opposite to that of RNAI and the entire region encoding RNAI is contained within the RNAII coding sequence (Figures 1 and 2).

The third RNA transcript, RNAIII,is synthesized in the same direction as is RNAII from the region previously identified as the replication origin of pSM1 and pTR1. RNAIII is found only when the linear PstI fragment containing the replication origin is used as a template (Figures 1 and 2) and not with superhelical pSM1 and pTR1 DNA. The 14 bp region of complementarity between RNAI and the replication is contained within RNAIII.[17]

POLYPEPTIDES SYNTHESIZED *IN VIVO*

When purified minicells containing pSM1 are incubated with ^{35}S-methionine, thirteen labelled polypeptides are synthesized. These polypeptides range in size from approximately 6,000 to 36,800 daltons, as determined by SDS polyacrylamide gel electrophoresis.[19] The 36,800 dalton polypeptide is the only polypeptide produced by pSM1 that is close to the size predicted for RepA1 (33,000 daltons, ref. 10 and 11). Labeling with different amino acids to identify the 36,800 dalton polypeptide definitively as RepA1 on the basis of amino acid content is not possible because the DNA sequence predicts that RepA1 contains all 20 amino acids.[10,11] Since no other coding region for a polypeptide this large can be found in the nucleotide sequence of the entire pTR1 plasmid and since the 36,800 dalton polypeptide is produced by pTR1 as well as pSM1, we have identified the 36,800 dalton polypeptide as RepA1.[19]

There are three polypeptides produced by pSM1 that are close to the size predicted for RepA2, which is 11,400 daltons (Figure 5, ref. 11). Since the nucleotide sequence predicts that RepA2 should not contain tryptophan (Figure 2 and refs. 10,11), minicells containing pSM1 were labeled with radioisotopes of tryptophan, histidine and proline. Histidine and proline were incorporated in the 12,300 dalton polypeptide, but tryptophan was not.[19] In addition, the 12,300 dalton polypeptide is synthesized by the same PstI fragment of pSM1 that encodes RepA2 and is not produced by pTR1, as predicted

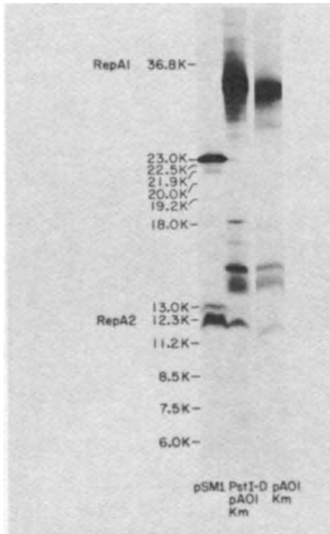

Figure 5. Autoradiogram of Tris-borate polyacrylamide gel (ref. 8). Minicell strains derived from the E. coli K12 strain P678-54 (ref. 22) were purified by the method described by P. Matsumura (personal communication). Plasmids carried by the minicell strains are pSM1, pA01-Km, a derivative of pML2 (ref. 24), and pA01-Km carrying the PstI-D fragment of pSM1 (ref. 19).

by the nucleotide sequence (Figures 1 and 2. refs. 10 and 11). Therefore, we have identified the 12,300 dalton polypeptide as RepA2 because of its size, amino acid content, map location, and lack of production by pTR1.[19]

RepA3 is a hypothetical polypeptide 6,700 daltons in size which the nucleotide sequence predicts should not contain histidine.[10,11] The RepA3 coding regions in pSM1 and pTR1 have only two bp differences and so are highly conserved.[10,11] However, no polypeptide of this size and amino acid content has yet been identified in either pSM1 or pTR1. In addition, no polypeptide close in size to 14,000 daltons (RepA4) has yet been identified. So, it is not clear at this time whether polypeptides are actually made from the RepA3 and RepA4 coding frames.[19]

Recently, a small polypeptide was identified that is produced by the PstI fragment (fragment D) of pSM1 which encodes incompatibility and copy number functions. The size of this polypeptide (approximately 6,000 daltons, Figure 5) is close to that predicted by an extended RNAI transcript. Such an extended transcript actually is synthesized *in vitro* in the presence of glycerol.[17] There does not appear to be another open reading frame in the *Pst*I-D fragment that could code for a polypeptide of this size,[10,11] so we have tentatively identified the 6,000 dalton polypeptide as the product of the extended RNAI transcript.

CONTROL OF DNA REPLICATION

We have used the DNA sites, coding frames, RNA transcripts, and polypeptides identified from our studies of pSM1 and pTR1 to formulate a model for control of DNA replication. We have attempted to emphasize only the most basic processes which might be involved in this control.

From analysis of the replication origin sequences of pSM1 and pTR1, we have identified a sequence that is capable of forming a large secondary structure common to both plasmids and which is similar to structures demonstrated at other replication origins. We assume that the sequence at which this secondary structure occurs, which is within the region previously identified as the replication origin,[10,12] is essential for DNA replication.

One possible model for replication predicts that transcription of RNAIII alters the conformation of this secondary structure. This altered conformation would, in turn, allow an initiation complex, which might include the positive effector polypeptide RepA1, to form at the origin structure. DNA replication could commence after the assembly of the initiation complex. Synthesis of the RNAIII transcript could be regulated by the binding of RNAI at the region of complementarity between the 5' end of RNAI and the sequence of the origin region between nucleotide 2367 and 2380 of the pSM1 nucleotide sequence, as shown in Figure 2. RNAI might function in this way as a repressor molecule to control DNA replication.

However, another level of control of replication could also exist. The bases of RNAI that are changed in pSM1 and pTR1 relative to wild type R1, are found at the top of the large secondary structure in RNAI. These base changes are most likely responsible for relaxation in replication control which results in the high copy number phenotypes of pSM1 and pTR1.[10,11] Since it is unlikely that the top of the stem-loop structure where these changes occur interacts directly with nucleotides at the replication origin, it seems probable that the stem loop structure of RNAI would interact with a polypeptide. RepA3 is a likely candidate for this polypeptide since the RepA3 coding frame is so closely conserved in pSM1 and pTR1, as would be expected of a controlling molecule. RNAI, then, might not itself be a repressor but rather be required for initiation of DNA replication, which would occur in the absence of a repressor.

It is likely that some control is also exerted at the level of synthesis of RNAI and the postulated repressor polypeptide, RepA3. Since the coding regions for RNAI and RepA3 overlap, control of plasmid replication could be linked to cell growth by an attenuator-type mechanism,[20] as described in detail elsewhere.[17]

The model we have proposed here for control of replication of large drug resistance factors bears some resemblance to a model recently described for plasmid ColE1 replication,[21] in that both models involve two RNA transcripts, one of which has nucleotide complementarity with the replication origin.

In summary, we have predicted from analysis of the replication region of pSM1 and pTR1 that three coding frames shared by both plasmids and one coding frame found only in pSM1 are most likely to encode actual polypeptides. To date, we have confirmed the existence of two of these polypeptides. (RepA1 and RepA2). We have also identified three RNA transcripts produced by the replication region of pSM1 and pTR1, one of which has a significant secondary structure. A model for control of DNA replciation that incorporates these features is proposed.

ACKNOWLEDGEMENTS

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PLASMID R1 INCOMPATIBILITY.

CONTRIBUTION FROM THE cop/rep AND FROM THE par SYSTEMS

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INTRODUCTION

Plasmids are normally present in defined copy numbers; these can be expressed as number of plasmid molecules per cell, per protein, per chromosome equivalent, etc., but in the present paper we consequently use the baby cell as the unit. The copy number is determined by the plasmid, by the host, and by physiological conditions. Some plasmids are present in only a few (2-5) copies per cell. Nevertheless, these plasmids are completely stably inherited, loss of plasmid being a very rare event.

LIFE CYCLE OF PLASMIDS

Plasmid Replication and Partitioning

During the cell cycle, a baby cell grows until one generation time (τ) later its volume has been doubled. At that stage, the cell divides. In a plasmid-carrying cell population, the average number of plasmid copies in the cells also doubles during the time τ. At cell division, the plasmid copies are distributed to the daughter cells. Formally, this means that there are two plasmid events during the cell cycle, replication and partitioning (Fig. 1). If the copy number is n per newborn cell, this results in a plasmid cycle $n \rightarrow 2n \rightarrow n$. Let for example n be 2. Assume that one of the two plasmid copies in a newborn cell is mutated in a gene that is not involved in replication or partitioning, which gives rise to two variants of the plasmid, A and B. If the plasmid replication cycle were a mitotic one, the progeny of the cell where the

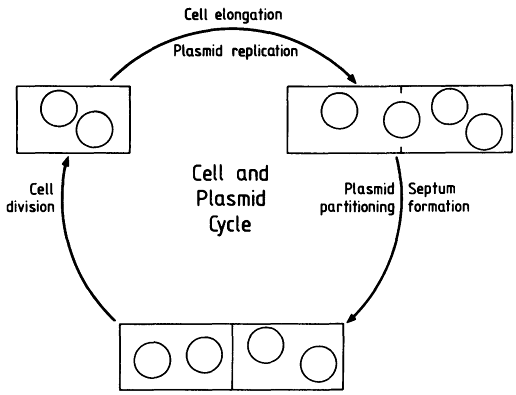

Fig. 1. Life cycles of bacteria and plasmids.

mutation occurred would be heterozygous (heteroplasmid) forever, i.e. would always carry A and B (Fig. 2a). However, heteroplasmid cells are known to segregate into pure A and B lines. What is the mechanism of this segregation? There are several possibilities. In case b (Fig. 2), all plasmid copies are replicated once and only once during the cell cycle, but there is a random assortment of the plasmid copies at cell division. This leads to the appearance of pure A and B lines (the frequency of these events is given within brackets in Fig. 2). Another possibility is that there is randomiza-

Case	Randomization		Pattern		LF
	Rep	Par	Rep	Par	(%)
a	–	–	AB → 2A2B	→ AB	0
b	–	+	AB → 2A2B	→ 2A (1/6) → AB (2/3) → 2B (1/6)	33.3
c	+	–	AB → 2AB → 3AB (1/3) AB → 2AB or A2B → 2A2B (1/3) AB → A2B → A3B (1/3)	3AB → 2A (1/12), A (1/12), 2AB (1/12), AB 2A2B → AB (1/2) A3B → AB, A2B (1/12), B (1/12), 2B (1/12)	33.3
d	+	+	AB → 2AB → 3AB (1/3) AB → 2AB or A2B → 2A2B (1/3) AB → A2B → A3B (1/3)	→ 2A (2/9) → AB (5/9) → 2B (2/9)	44.4

Fig. 2. Effect of randomization during replication and/or partitioning on segregation of plasmids from a heteroplasmid population. A and B denote two genetically marked derivatives of a plasmid; the markers do not affect replication or partitioning. LF is the relative rate of reduction of the heteroplasmid population per generation of growth.

tion during replication but no assortment at partitioning (Fig. 2c). Again, pure A and B lines appear. Finally, randomization may occur at both replication and partitioning (Fig. 2d); in this case the frequency of pure A and B lines is higher than in cases b and c. The appearance of pure lines in a heteroplasmid population, i.e. segregation into heteroplasmid populations is operationally referred to as plasmid incompatibility. The exercise of Fig. 2 demonstrates how plasmid incompatibility is a logical consequence of randomization during replication and/or partitioning of the plasmid (cf. ref. 1).

Randomization Steps in the Plasmid Life Cycle

Randomization during replication has become evident from Meselson-Stahl (density-shift) experiments with many different plasmids[2,3,4]. The data are in agreement with the replication pattern of Figs. 2c-d. That there probably is randomization also at partitioning will be shown below.

Plasmid Incompatibility is a Quantitative Phenotype

Since cases a and b in Fig. 2 are ruled out by the Meselsohn--Stahl experiments, we will treat only cases c and d in more detail. In Fig. 2, we have schematically described the situation at the lowest possible heteroplasmid copy number (*n*=2). However, we have extended the analysis of the effect of plasmid replication and partitioning on incompatibility to higher *n* values (up to *n*=8). This analysis was performed by computer and the following assumptions were used[5]:

1) The partitioning (*par*) mechanism is distinct from the replication (*rep*) and replication control (*cop*) mechanism; a mutation abolishing partitioning does not affect the copy number of the plasmid[6].

2) Selection of plasmid copies for replication is random, i.e. replications occur one at a time and there is a time interval between consecutive replications, thus allowing the newly formed daughter plasmids the same probability as the rest of the plasmids in the cell to be selected for replication[3].

3) Two different replication control systems have been considered:
Model 1. The copy number is always set to 2*n* in all cells before cell division.
Model 2. Irrespective of copy number, exactly *n* copies are synthesized in each plasmid-carrying cell during one cell cycle.

4) A *par* mutation leads to random (binomial) distribution of

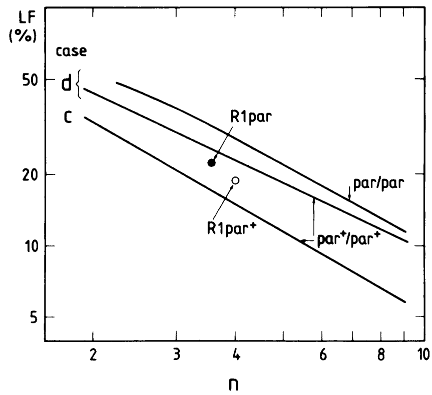

Fig. 3. Theoretical calculation of LF values (see Fig. 2) at different n values according to schemes c and d in Fig. 2. The circles represent experimental values obtained with plasmid R1.

plasmid copies between the daughter cells at cell division.

5) In the par^+ case, plasmid copies are equipartitioned, i.e. 50% of the copies go to each daughter cell at cell division. Two cases were considered in Fig. 2. In case c, the copies of each class (A and B) are equipartitioned. This means that at even numbers the two daughter cells get the same number of copies, whereas at odd

Table 1. Effect of the par System on Incompatibility Theoretical Predictions

Par Function		LF (%)		Distribution of pure lines (%)	
A	B	n = 4	n = 5	A	B
I	I	23	18	50	50
None	None	27	22	50	50
I	None	21	16	62	38[a]
I	II	14	11	50	50
I	III	14	11	50	50
II	III	14	11	50	50

[a]Plasmid-free cells are included in the par group.

numbers one daughter cell gets one more copy than the other. In case d, the plasmid copies are selected randomly for partitioning (random assortment).

The computer analysis gave the following results: (i) The two replication models give virtually identical results. (ii) The rate of reduction of the relative size of the heteroplasmid population (i.e. the degree of plasmid incompatibility) is a function of the plasmid copy number (Fig. 3 and Table 1); this frequency (LF) is reduced by increasing copy number; plotting log(LF) against log(n) gives a straight line. The lowest LF value is obtained in case c, and case d gives a 50-100% higher LF value (the difference increases with the n value). Still slightly higher LF values are obtained in the absence of partitioning system.

PLASMID R1

Plasmid R1 Replication and Partitioning

We have for a long time been interested in control of plasmid R1 replication[7,8], and more recently in partitioning of this plasmid[5,6]. Plasmid R1 is inherited very stably, is present in a low copy number (about 1 per chromosome equivalent or 4 per baby cell when grown in broth). The genetic information for replication, including its control is located on a small part (about 3 kb, kilobases) clustered at the origin of replication (Fig. 4)[8,9]. However, plasmids only consisting of the basic replicon are not stably maintained but are lost in a frequency of 1-1½% per cell generation[6]. This is not due to a replication effect, since the copy number of these miniplasmids is the same as that of the full size plasmid (or 90% of that copy number). This has led to the definition of a region which is concerned with partitioning (par[6], repB[10], or stb[11]). These data show that replication and partitioning are independent processes. Similar data have been reported for plasmids pSC101[12] and P1[13].

The instability (loss rate) of par-deleted R1 replicons is consistent with random partitioning of the plasmids. By using pairs of par^+ or par derivatives of plasmid R1 it was possible to determine the degree of incompatibility (Table 2)[5]. The rate of reduction of the relative size of the heteroplasmid population was slightly higher for par/par than for the par^+/par^+ combination and the data were in agreement with the model of Fig. 2d rather than that of Fig. 2c (Fig. 3). This was the first indication that there is randomization during partitioning as well as during replication.

Table 2. Effect of the par System on Incompatibility
Experimental Results with the Basic Replicon of R1

Par Function		LF	Distribution in Colony (%)[a]			
A	B	(%)	A + B	A	B	None
R1	R1	18	0.1	55	45	0
None	None	22	0.1	20	25	55
R1	None	16	1	95	3	2
R1	F	13	3	94	3	0
R1	pSC101	13	3	91	6	0

[a]Grown in absence of selection.

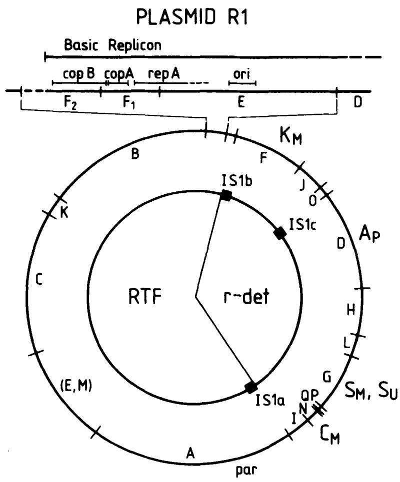

Fig. 4. Genetic and physical map of plasmid R1. Symbols: RTF, resistance transfer factor; r-det, resistance determinant; IS1a, b and c denote the three insertion sequences type 1 located on the r-det; the capital letters inside the circle denote the 17 fragments generated by restriction endonuclease EcoRI; symbols outside the circle are Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; par, partitioning; Sm, streptomycin; Su, sulphonamides; The capital letters D, E, F_1 and F_2 on the linear part denote fragments generated by restriction endonuclease PstI.

Table 3. Stability of Basic Replicons Carrying Heterologous par Functions

Plasmid		Relative Copy Number	Inheritance
Basic Replicon	Par Function		
R1	R1	1.0	Stable
R1	None	0.9	Unstable (1.5%)[a]
R1	pSC101	1.0	Stable
R1	F	1.0	Stable
pSF2124-hybrid	R1	2.0	Stable
pSF2124-hybrid	None	1.8	Unstable (0.3%)[a]

[a]Loss Rate per Cell Generation.

Basic Replicons Carrying par from Other Plasmids

Hybrids between the basic replicon of plasmid R1 with rep(ts) derivatives of either plasmid pSC101 or miniF were stably inherited also at temperatures that were nonpermissive for the rep(ts) plasmids (Table 3). Since plasmids pSC101[12] and miniF[14] both are par$^+$ this suggests that plasmid R1 can use par functions of other plasmids. The copy number of the hybrids was identical to that of R1 at the temperature that did not allow the other moiety to replicate. This adds to the conclusion that partitioning and replication are independent processes.

The ColE1 derivative pSF2124 was used as vector to clone the EcoRI fragment A (cf. Fig. 4), which harbours the par function of plasmid R1. The copy number of plasmid pSF2124 is fairly low and is reduced when large DNA fragments are inserted into the plasmid. This leads to a slightly unstable inheritance (Table 3). However, plasmid pSF2124 carrying the par fragment of plasmid R1 is completely stably inherited[5]. This suggests that plasmid pSF2124 does not carry any par function and that it can use that of plasmid R1.

Incompatibility Effects of the par System[5]

The construction of plasmid pSF2124 carrying the par region of R1 allowed a test of whether the partitioning process affects incompatibility. The result was that plasmid pSF2124 carrying parR1 was incompatible with plasmid R1 (Table 4)[5]. The copy number of either

Table 4. Incompatibility of Unrelated Replicons that have the Same *par* Function.

Plasmid				Relative Copy Number		Plasmid Loss (% in a colony)[a]	
I		II					
Rep	Par	Rep	Par	I	II	I	II
R1	R1	-	-	1.0	-	0	-
pSF2124	R1	-	-	2.0	-	0	-
pSF2124	R1	R1	R1	2.0	1.0	1	21

[a]Grown in absence of selection.

plasmid was not affected by the presence of the other plasmid. The incompatibility was weak and plasmid R1 was being preferentially lost which can be ascribed to the differences in copy number between the two plasmids. Hence, there is (random) assortment during partitioning and this randomization is specific to the *par* system and not to the replicon type.

Incompatibility between R1 Derivatives Carrying Different *par* Functions

The construction of hybrid plasmids consisting of the basic replicon of plasmid R1 and the *par* function of either plasmid F or pSC101 enabled a test of the incompatibility effect of the *par* system in a situation where assortment during partitioning is prohibited (case c, Fig. 2). On the assumption that replication and partitioning are completely independent processes, the rate of reduction of the relative size of the heteroplasmid population (the *LF* value) was calculated (Table 1). The table also contains the expected distribution between the pure lines formed from the heteroplasmid population. We have also included the predicted outcome of an incompatibility test involving a *par*$^+$ and a *par* derivative of the same plasmid.

The corresponding experiments were then performed. As predicted, the rate of reduction of the heteroplasmid population was reduced when the basic replicon of plasmid R1 carried different *par* systems (Table 2). This adds to the conclusion that partitioning involves randomization. However, the homologous pair had a clear advantage over the heterologous one. Similarly, *par*$^+$ had a much

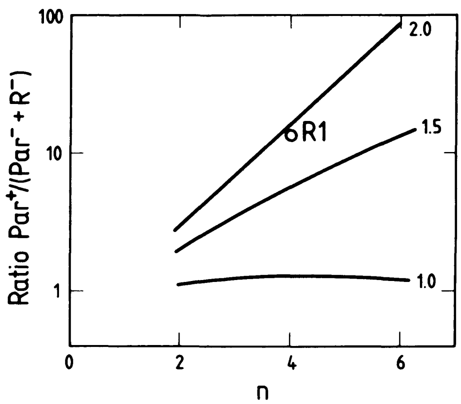

Fig. 5. Effect of different ratios in probability in selection for replication of a par^{+} and a par derivative of a plasmid on the formation of pure lines from a heteroplasmid population according to scheme d in Fig. 2. The lines represent three different ratios as indicated at the lines. The circle is the value found experimentally for plasmid R1.

stronger advantage over the par derivative than predicted. These data suggest that the assumption used in the theoretical calculations are not completely correct. There seems to be a preferential selection of the homologous plasmid during replication. Therefore, we calculated the consequences of the different probabilities for selection of the two plasmids during replication in a heteroplasmid population (Fig. 5). The result was that only a minor bias in selection for replication can explain the results of the incompatibility test shown in Table 2.

OTHER SYSTEMS

It seems to be a general phenomenon that chromosomes and plasmids have partitioning functions that are independent of the replication functions. Recently, this was shown for an eukaryotic system; a minichromosome was constructed from a replicator and the centromer of yeast[15]. This minichromosome behaved as a normal chromosome in mitosis as well as in meiosis. Minichromosomes of *E. coli* (*oriC* plasmids) are unstably maintained but are completely stabilized by the *par* region from plasmid F[16]. The *par* function most likely is identical to *incFI*. Hence, different replicons having the *par* function of plasmid F express a weak incompatibility, exactly as those carrying the *par* function of plasmid R1 (cf. Table 4).

CONCLUSIONS

Plasmid Incompatibility is a Consequence of Replication and Partitioning of Plasmids

As discussed above, randomization either during replication or during partitioning of plasmids leads to segregation of heteroplasmid populations into pure lines, i.e. to plasmid incompatibility. Hence, plasmid incompatibility is a logical consequence of these central events of the life cycle of plasmids[1]. Randomization at one stage (replication or partitioning) is enough to cause incompatibility. It has been shown that many (all) plasmids are selected randomly for replication[2,3,4]. However, randomization also seems to occur at partitioning. This is supported by (i) different replicons having identical *par* functions being incompatible, (ii) the relatively small difference in degree of incompatibility between a *par*$^+$/*par*$^+$ pair and a *par*/*par* pair, and (iii) the fact that the degree of incompatibility is reduced when two derivatives of the same basic replicon carry different *par* functions. Therefore, it seems fair to conclude that there is random selection for replication as well as random assortment during partitioning.

It should be stressed that plasmid incompatibility is a qualitative as well as a quantitative phenomenon. As has been pointed out by Novick and Hoppensteadt[1], plasmid incompatibility is *caused* by randomization during replication and/or partitioning. However, it is only possible to discuss degree of incompatibility in relation to the actual copy number.

In our opinion, plasmid incompatibility is basically (totally) a logical consequence of the properties of the replication and partitioning processes.

Replication and Partitioning are Independent Processes

Partitioning and replication are independent processes, since (i) deletion of the *par* region does not affect the copy number of the plasmid[6], and (ii) plasmids can use different *par* system to get stabilized without any effect on the copy number[5]. However, there seems to be an indirect linkage between these two processes, since a *par*$^+$ plasmid has a great selective advantage over its *par* derivative. Similarly, a basic replicon carrying the homologous *par*$^+$ has a selective advantage over the same replicon with a heterologous *par*$^+$ function. This selective advantage most likely is exerted at the level of selection for replication. Since nothing is known about the mechanism of partitioning the reason for the bias in selection for replication can only be guessed. One possibility is that a replicon with the homologous *par*$^+$ function is kept in the vicinity of the replication site (if there is any) e.g. in the membrane. Similarly, a replicon carrying a heterologous <u>par</u>$^+$ function may be kept further apart from the replication site, i.e. in the vicinity of the replicon site that is normally used by the <u>par</u> function. A decision as to whether these thoughts are correct or not has to await further experimentation.

ACKNOWLEDGMENTS

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COPY NUMBER CONTROL AND INCOMPATIBILITY OF *inc*FII R PLASMIDS

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INTRODUCTION

Bacterial plasmids are stably inherited even when present in low copy number in host cells. Thus, plasmid inheritance must be controlled by a mechanism which ensures that these extrachromosomal elements are replicated during each cell division cycle and that at least one copy is segregated to each daughter cell at division. The molecular nature of the control of plasmid replication and segregation is presently not understood in any detail. Most available data are consistent with the negative control mechanism proposed by Pritchard (1978). This repressor dilution model postulates that an inhibitor or repressor specified by a gene on a replicon interacts with a specific receptor on the DNA to control the frequency of initiation of replication. This model can account for the control of plasmid copy number and also for the inability of two plasmids which share the same replication control mechanism to coexist stably in descendants of the same host cell (incompatibility). Mutations in either the repressor molecule or its binding site could lead to less stringent control so that plasmid copy number would be increased. A number of plasmid copy number mutants have been isolated and many, but not all, have been found to have altered incompatibility properties (Uhlin and Nordstrom, 1975; Miki et al., 1980; Rownd et al., 1980).

R plasmid NR1 (also called R100 and R222) is a 90 kilobase (kb), self-transmissible drug resistance plasmid which belongs to the FII incompatibility group (Rownd and Womble, 1978). The location of the resistance genes and the cleavage sites for several restriction endonucleases (Tanaka et al., 1976; Miki et al., 1978) and the transfer and replication functions on NR1 (Taylor et al., 1977; Taylor and Cohen, 1979; Miki et al., 1980; Rownd et al., 1980) have been determined previously. In this communication we describe more recent experiments on the copy number control (cop) and incompatibility (inc) genes of NR1. A region of less than 2.1 kb is sufficient for autonomous replication and plasmid incompatibility (exclusion) functions. The analysis of deletion mutants and hybrid plasmids formed from NR1 and a copy number mutant of NR1 have shown that the regions coding for copy number control, the ability to exclude an incompatible plasmid, and sensitivity to exclusion by an incompatible plasmid are all located within a 500 base pair (bp) segment located at least 1 kb from the origin of replication. When the inc/cop region of NR1 was cloned adjacent to a lacZ gene which lacks its own promoter, there was a stimulation in the level of synthesis of β-galactosidase which appears to result from transcription from a promoter in the inc/cop region. A greater stimulation was observed when the cloned inc/cop region was from copy number mutants of NR1, indicating an increased level of transcription. This production of β-galactosidase was found to be decreased by the introduction into the cells of plasmids carrying the inc/cop region having the same incompatibility phenotype. Transcription initiation sites have been mapped within the replicator region. A model is proposed in which the inc gene product is postulated to act within the 500 bp segment to repress initiation or extension of an RNA transcript originating within this segment and extending toward the origin of replication.

RESULTS AND DISCUSSION

Cloning of the Replication and Incompatibility Functions of R Plasmid NR1

Restriction fragments of NR1 capable of mediating autonomous replication were identified in sequential cloning experiments using different restriction endonucleases. In the initial experiments a miniplasmid was obtained which used EcoRI fragment B as the replicator (Fig. 1). Smaller miniplasmids were isolated from this derivative which contained two adjacent PstI fragments of size 1.1 and 1.6 kb. The incompatibility (inc) and copy number control (cop) functions have been shown to be located on the PstI 1.1 kb fragment (see below). The origin of replication (ori) is located on the PstI 1.6 kb fragment (Ohtsubo et al., 1977; Rownd et al., 1980). Non-essential Sau3A fragments have been deleted from these derivatives so that the replication region must be less than 2.1 kb. Since this smaller NR1 derivative has the same incompatibility properties as the wild type NR1, this function must lie within the 500 base pairs of the PstI 1.1 kb fragment which remain. In Fig. 1

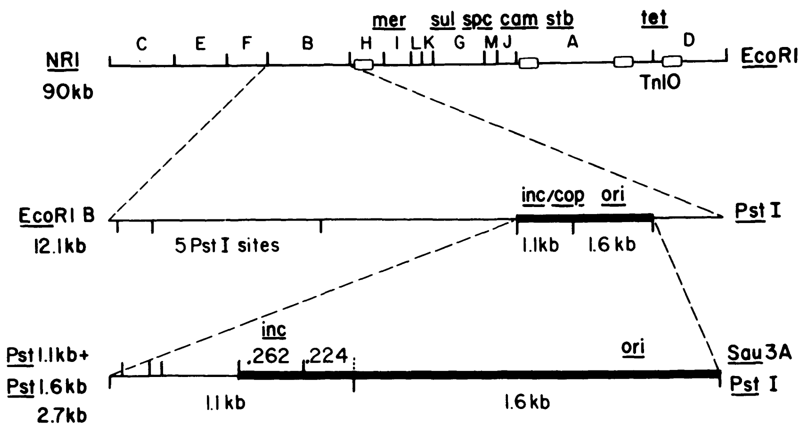


Fig. 1 Subcloning of the replication region of the incFII R plasmid NR1

the thicker lines shown within the restriction fragments represent the plasmid region which was found to be essential for replication in the different cloning experiments.

Incompatibility Properties of Copy Number Mutants Derived from NR1

The PstI 1.1 kb and 1.6 kb fragments from NR1 and from a copy number (cop^-) mutant called pRR12 were cloned separately onto the plasmid vector pBR322. The incompatibility properties of several miniplasmids derived from NR1 and pRR12 and the pBR322 recombinant plasmids have been examined using bacterial transformation. The ability of donor plasmid DNA to exclude a resident plasmid was monitored when there was only selection for the donor plasmid in a transformation experiment (Miki et al., 1980). Plasmids containing the PstI 1.1 kb fragment of NR1 strongly excluded NR1 from the recipient cells, but did not exclude the cop^- mutant pRR12 which therefore must also be an incompatibility (inc^-) mutant (Table 1). This was true irrespective of the copy number of the donor plasmid carrying the PstI 1.1 kb fragment. The pBR322 derivatives carrying only the PstI 1.6 kb fragment which contains the plasmid origin of replication did not exclude either NR1 or pRR12 from the recipient cells. Thus, the incompatibility function of NR1 lies on the PstI 1.1 kb fragment.

The corresponding plasmid derivatives of the cop^- pRR12 did not exclude a resident cop^+ NR1 from the cells which confirms the

Table 1. Incompatibility Properties of NR1 and the Copy Mutant pRR12

Donor Plasmid	Description	Percent of Recipient Cells Retaining Resident Plasmid — Resident Plasmid: NR1	pRR12
pRR104	*Eco*RI B_{NR1} + *kan*	1	100
pRR933	*Pst*I 1.1_{NR1} + *Pst*I 1.6_{NR1} + *cam*	1	100
pRR935	pBR322 + *Pst*I 1.1_{NR1}	0	100
pRR936	pBR322 + *Pst*I 1.6_{NR1}	100	100
pRR114	*Eco*RI B_{cop12} + *kan*	96	96
pRR942	*Pst*I 1.1_{cop12} + *Pst*I 1.6_{cop12} + *cam*	100	100
pRR939	pBR322 + *Pst*I 1.1_{cop12}	100	0
pRR937	pBR322 + *Pst*I 1.6_{cop12}	100	100

Donor DNA was used to transform *E. coli* KP435 (*recA*) harboring either NR1 or its copy mutant pRR12 for drug resistance conferred by the donor plasmid. After transformation, the cells were cultured for 90 minutes in drug-free L broth and appropriate dilutions then spread on nutrient agar plates containing a single drug to which resistance was conferred only by the donor plasmid. Ten individual transformant colonies were suspended in dilution buffer and the suspensions were streaked onto drug-free Penassay agar plates. From each of these streaks 10 single colonies were picked and examined for drug resistance by replica plating (Miki et al., 1980). The donor plasmids were constructed as described previously (Miki et al. 1978;1980). The *kan* fragment is a 6.7 kb *Eco*RI fragment from the *inc*FII R plasmid R6 which confers kanamycin/neomycin resistance. The *cam* fragment is a 2.1 kb *Pst*I fragment from a deletion mutant of NR1 which confers chloramphenicol resistance.

inc$^-$ phentotype of pRR12 previously observed (Table 1). The cop12 plasmid derivatives which were autonomously replicating miniplasmids (pRR114 and pRR942) did not exclude a resident pRR12 from the recipient cells. Presumably a mixture of both donor and resident plasmids can coexist in the host cells as a multicopy pool of plasmids as long as the total plasmid copy number characteristic of the cop12 mutation is not exceeded. However, the pBR322 derivative which carries the PstI 1.1 kb fragment of pRR12 (pRR939) did exclude a resident pRR12 (but not a resident NR1) from the cells. Presumably in this situation the high copy number of the PstI 1.1_{cop12} kb fragment owing to its presence on pBR322 results in the exclusion of the resident pRR12 plasmid. Thus, in addition to increasing the plasmid copy number, the effect of the cop12 mutation is to change the incompatibility properties of the plasmid. pRR12 is not incompatible with the wild type NR1 from which it was derived but is incompatible with itself when there is a high copy number of the inc12 region. Thus, pRR12 is an inc12/cop12 mutant with respect to the inc$^+$/cop$^+$ NR1. It is interesting to note that if NR1 and pRR12 had each been isolated as naturally occurring plasmids, they would have been included in different incompatibility groups.

Similar experiments were carried out with another copy number mutant isolated from NR1 called pRR21. Although it was a cop$^-$ mutant, pRR21 was found to be incompatible with NR1 and compatible with pRR12 (data not shown). In this case the cop$^-$ mutation did not result in a change in the incompatibility properties of the plasmid. Thus, pRR21 is an inc$^+$/cop21 mutant.

Mapping of the Copy Number Control Gene and Incompatibility Receptor Site

Since the PstI 1.1 and 1.6 kb fragments of NR1 and pRR12 have been cloned separately onto pBR322, it was possible to construct "hybrid" recombinant plasmids containing one PstI replicator fragment from NR1 and one from pRR12. The copy number and incompatibility properties of these hybrid plasmids were determined solely by the source of the PstI 1.1 fragment (Table 2). When the hybrid plasmids were used as the resident plasmid in recipient cells in transformation experiments, hybrid plasmids containing the PstI 1.1 kb fragment from NR1 were excluded from cells only by transformation with pBR322 carrying the PstI 1.1 kb fragment from NR1. On the other hand, hybrid plasmids containing the PstI 1.1 kb fragment of pRR12 were excluded from cells only by transformation with pBR322 carrying the PstI 1.1 kb fragment of pRR12. This exclusion specificity was maintained even if both the NR1 and pRR12 hybrid plasmid derivatives were present simultaneously in the recipient cells (data not shown). If incompatibility is due to the interaction of a repressor with a receptor site on a plasmid molecule, these

Table 2. Properties of Hybrid Plasmids Containing PstI 1.1 and 1.6 kb Fragments of NR1 and pRR12

COMMENT \ RESIDENT PLASMID		NR1	pRR949	pRR955	pRR966	pRR957	pRR12
Source of PstI 1.1 fragment		NR1	NR1	NR1	pRR12	pRR12	pRR12
Source of PstI 1.1 fragment		NR1	NR1	pRR12	NR1	pRR12	pRR12
Copy Number		1.0	1.1	1.1	7.7	7.0	3
Percent of Transformants Harboring Resident Plasmids After Transformation by	pBR322 + PstI 1.1 (cop$^+$)	0	0	0	100	100	99
	pBR322 + PstI 1.1 (cop12)	100	41	42	0	0	0
	pBR322	100	84	71	100	100	100

The PstI 1.1 kb and the 1.6 kb replicator fragments from NR1 and its copy mutant pRR12 and the 2.1 kb PstI cam fragment were cloned individually to the vector pBR322 at the PstI site. Appropriate combinations of the DNA of these recombinant plasmids were then mixed, digested with PstI, ligated, and used to transform a polA amber mutant of E. coli (JG112) to chloramphenicol resistance. Using this procedure, recombinant plasmids containing all four possible combinations of the PstI 1.1 and 1.6 kb fragments from NR1 and pRR12 were ligated to the cam fragment. The copy numbers (by assay of chloramphenicol acetyltransferase), ability to exclude a resident NR1 or pRR12 plasmid when used as the source of donor plasmid DNA in a transformation experiment (data not shown), and the ability to be excluded by NR1 or pRR12 when used as a resident plasmid in a transformation experiment were then examined.

results suggest that NR1 and pRR12 differ from one another in both the structural gene for the repressor and its receptor site. Moreover both of these appear to be located on the PstI 1.1 kb fragment at a distance greater than 1 kb from the plasmid origin of replication. Since the copy number and incompatibility properties of pRR12 are both affected, the cop gene may be identical to the inc gene.

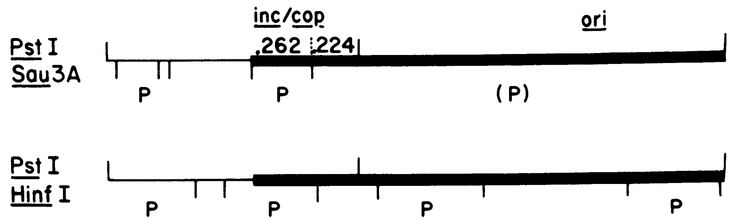
Since a cop^- mutation in the inc/cop region affects the frequency of initiation of replication when joined in cis to the origin of replication, it is possible that initiation is the result of a transcriptional event starting on the PstI 1.1 kb fragment which in some way activates the origin of replication on the PstI 1.6 kb fragment.

Mapping of RNA Polymerase Binding Sites and Transcription Initiation Sites

Using a nitrocellulose filter binding assay (Reznikoff, 1976; Taylor and Burgess, 1979), RNA polymerase binding sites were mapped on the Sau3A and HinfI fragments within the PstI 1.1 kb and 1.6 kb fragments from the replicator region. The thicker lines in Fig. 2 indicate the region essential for replication. When various combinations of nucleoside triphosphates were added to the binding mixture, complexes which were resistant to high salt concentrations were formed between RNA polymerase and the restriction fragments with binding sites. This indicates that these binding sites can serve as sites for the initiation of transcription. There is only one RNA polymerase binding site and transcription initiation site located within the essential replication region within the PstI 1.1 kb fragment. It is possible that this site may represent the promoter for initiation of the transcription which regulates the expression of incompatibility and copy number control.

Construction of Lambda Phages Carrying the inc/cop Region Adjacent to the lacZ gene

Since a region involved in plasmid incompatibility and copy number control is located within a 500 bp segment of the PstI 1.1 kb fragment, it was of interest to examine whether transcription from this region was related to the copy number of a plasmid and whether the level of transcription could be controlled in trans by the presence of the inc/cop region which was cloned on a suitable vector. The inc/cop region from NR1 (inc^+/cop^+) and from the copy number mutants pRR12 (inc^-/cop^-) and pRR21 (inc^+/cop^-) were cloned adjacent to a lacZ gene without its own promoter which was present on the lambda phage λRS205. The location of SalI and EcoRI cleavage sites on λRS205 and the miniplasmids constructed from NR1,



NTP's in BINDING MIXTURE							
A,C	++	–	++	–	+++	–	+
A,G	+++	–	+++	–	++	–	++
A,U,G	+++	–	+++	–	++	–	+++
A,U,G,C	+++	–	+++	–	+++	–	+++
A,C,G	+++	–	+++	–	+++	–	++

Fig. 2 RNA polymerase binding sites and transcription initiation sites on the Sau3A and HinfI restriction fragments within the replicator region. In these experiments RNA polymerase was incubated with restriction enzyme-digested DNA in 0.1 M NaCl. The mixture was then filtered through a nitrocellulose filter and washed with 0.1 M NaCl. Under these conditions restriction fractions which contain RNA polymerase binding sites (designated P) remain bound to the nitrocellulose filter. Filter-bound fragments were subsequently eluted with 1.0 M NaCl or 0.2% SDS. Transcription initiation sites were analyzed by incubating RNA polymerase with restriction enzyme-digested DNA in 0.1 M NaCl in the presence of various combinations of CTP, ATP, GTP, and UTP. The mixture was filtered through a nitrocellulose filter, washed with 1.0 M NaCl, and the filter-bound fragments were eluted with 0.2% SDS. Under both sets of conditions the eluted fragments were analyzed on agarose or polyacrylamide gels to determine the distribution of RNA polymerase binding sites or transcription initiation sites.

pRR12 and pRR21 using PstI cloning were convenient for this purpose as diagrammed in Fig. 3. One of the SalI sites in the mini-plasmids is about 50 base pairs from the PstI site between the PstI 1.1 and 1.6 kb fragments. Lysogens were constructed using the phages that carry the inc/cop region of NR1, of pRR12, or of pRR21. In lysogens the expression of β-galactosidase from the lacZ gene would be under the control of a promoter which in a plasmid would

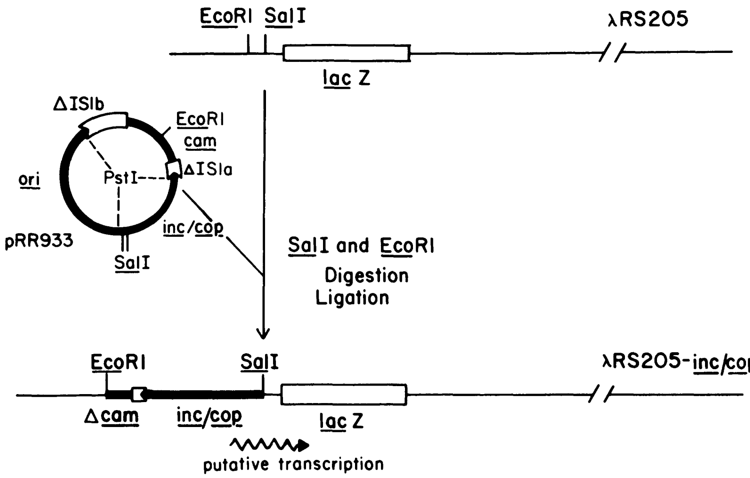


Fig. 3. Construction of lambda phages containing the inc/cop region adjacent to a lacZ gene which lacks its own promoter.

direct transcription from the inc/cop region toward the origin of replication. Lysogenic cells carrying a λ-inc/cop-lacZ phage produced a higher level of β-galactosidase than the control strain harboring a λ-lacZ phage without an inserted promoter (Table 3), presumably due to transcriptional read-through from the inc/cop region. There was a higher level of transcription from the inc/cop region of the copy number mutants pRR12 and pRR21 than from the inc/cop region of the wild type NR1. The introduction of a pBR322 plasmid containing a cloned PstI 1.1 kb fragment of either NR1, of pRR12, or pRR21 into the lysogenic cells reduced the level of expression of β-galactosidase. The decrease was larger when the λ-inc/cop-lacZ phage and the pBR322-inc/cop plasmid both had the same incompatibility phenotype (i.e. both Inc^+ or both Inc^-), irrespective of the copy number phenotype. This indicates that the cloned PstI 1.1 kb fragment encodes a repressor which acts in trans with a specificity which is determined by the incompatibility phenotype to reduce the level of transcription from a promoter in the inc/cop region. This specificity in control of the level of transcription was not as remarkable as that observed in the plasmid exclusion assays (Tables 1 and 2) in which there was little or no interaction between the Inc^+ NR1 and Inc^- pRR12.

Table 3. β-Galactosidase Production by Lysogens of λ-*inc*/*cop*-*lacZ* Phages

Control Strains[a]	β-Galactosidase Units[b]
NK5031 Δ*lacO lacZ*	0
NK5031 Δ*lacO lacZ* (λRS205)	67
NK5031 Δ*lacO lacZ* (λRS205-lacP$^+$)	1420

Plasmid in Lysogen \ Source of *inc/cop* Region	NR1	pRR12	pRR21
None	220	504	1576
pBR322	245	563	1649
pBR322-*inc*$^+$/*cop*$^+$	52	152	232
pBR322-*inc*12/*cop*12	98	91	397
pBR322-*inc*$^+$/*cop*21	63	147	217

[a]The strain used for these tests was NK5031 which contains a *lacO lacZ* deletion. This strain and the phage λRS205 were provided by Dr. K. Bertrand and Dr. W.S. Reznikoff.

[b]Assays were performed as described in Miller (1972).

If the decrease in the β-galactosidase levels in the λ-inc/cop-lacZ lysogens is relevant to the plasmid incompatibility phenomenon, as we propose, this new assay reveals a greater degree of cross reactivity between the incompatibility systems of NR1 and pRR12 than the incompatibility assay used in this laboratory (Table 1).

It seems likely that the incompatibility repressor would also be produced from the plasmid inc/cop region which was cloned into the λ phages. In a lysogen which does not harbor a pBR322 plasmid containing a cloned inc/cop region, the transcription emerging from the inc/cop region in the λ phage which crosses the SalI site would represent the level of transcription determined by the amount of incompatibility repressor which exists in the cell. Since the level of β-galactosidase in the lysogen containing the inc/cop region from the copy number mutants was considerably higher than observed for phages containing the inc/cop region from the wild type NR1, it seems likely that the higher copy numbers of the cop⁻ mutants may be due to increased transcription from the inc/cop region.

In Vitro Transcription from the Replicator Region

Using miniplasmid DNA which contains the PstI 1.1 and 1.6 kb fragments linked to a PstI fragment containing the chloramphenicol acetyltransferase gene in an in vitro transcription system, at least five RNA transcripts have been identified from the two contiguous PstI fragments which form the replicator region of NR1 (data not shown). The largest of the RNA transcripts is greater than 1100 bases in length and hybridizes to both the PstI 1.1 kb (inc/cop) and the PstI 1.6 kb (ori) fragments, indicating that there is transcription across the junction of these two PstI fragments. Preliminary data are available on the mapping of the other RNA transcripts and the determination of their direction of transcription is currently in progress.

Model for Incompatibility and Copy Number Control of incFII Plasmids

Our experiments have shown that the copy number control gene, the structural gene for incompatibility, and the incompatibility receptor site are all located on a 500 base pair region within the PstI 1.1 kb fragment of NR1. This region is located more than 1 kb from the origin of replication. Since a cop⁻ mutation on the PstI 1.1 kb fragment affects the frequency of initiation of replication when joined in cis to the PstI 1.6 kb (ori) fragment from either NR1 or pRR12 but not in trans when cloned on a pBR322 vector, it is possible that the initiation of plasmid replication is the result of a transcriptional event starting on the PstI 1.1 kb fragment which in some way activates the origin on the PstI 1.6 kb

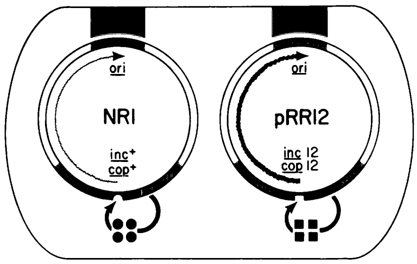


Fig. 4 Schematic illustration of a possible mechanism by which a cop⁻ mutation can affect both copy number control (cop) and incompatibility (inc). These two phenotypes are postulated to result from expression of the same gene (inc/cop) which specifies a diffusible repressor which regulates the frequency of initiation of transcription from a promoter located within or adjacent to the inc/cop gene. The repressor binding site (receptor) is located within the repressor structural gene. The inc^-/cop^- mutation results in an alteration of both the repressor and its receptor site such that the inc^-/cop^- repressor no longer recognizes the inc^+/cop^+ receptor site and the inc^+/cop^+ repressor no longer binds to the inc^-/cop^- receptor site. As a result, the inc^+/cop^+ and the inc^-/cop^- plasmids would be compatible. If the inc^-/cop^- repressor regulates the frequency of transcription less stringently, there would be an increase in the inc^-/cop^- plasmid copy number. Only one copy of the inc^-/cop^- plasmid is shown in this schematic illustration to avoid crowding. The origin of replication is shown attached to a cell surface structure (membrane?) to account for the cis-acting structural feature of the plasmid DNA which must be deleted in order to form a stable plasmid when two fragments containing the origin region are ligated together (Rownd et al., 1980).

fragment as indicated schematically in Fig. 4. According to this interpretation, the frequency of the transcriptional event would be controlled by repressor molecules specified by the inc/cop gene which would determine the frequency of initiation of plasmid replication. The properties of the cop⁻ mutant pRR12 suggest that the cop12 mutation(s) results in an alteration of the repressor and its receptor site simultaneously such that the cop12 repressor no longer recognizes the NR1 receptor (and vice versa), but rather recognizes an altered cop12 receptor and regulates the frequency of transcription less stringently (Fig. 4). As a result, NR1 and pRR12 are compatible with each other and the pRR12 copy number is increased. These findings are consistent with the view that the receptor site may lie within the repressor structural gene such that both are affected simultaneously by the same mutation in the case of pRR12. Although there is relatively little interaction between NR1 and pRR12 in terms of their ability to exclude each other from host cells (incompatibility) (Tables 1 and 2), our experiments on the ability of the cloned inc/cop gene to effect the level of transcription from the promoter in this region indicate that there is still an interaction between the controlling elements of the wild type and mutant plasmids (Table 3).

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REPLICATION AND INCOMPATIBILITY FUNCTIONS IN MINI-F PLASMIDS

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INTRODUCTION

One common approach to building a model for plasmid maintenance is to identify, map and characterize the genes involved in this process. The two major features of maintenance are plasmid replication and partitioning to daughter cells. As depicted elsewhere in this text, the essential components of replication appear to be at least a fixed origin of replication (*ori*), one to two plasmid-specified gene products for replication (*rep*) and a copy number control gene (*cop*) that appears to exert negative control. So too, plasmid F, the classic conjugal plasmid of *Escherichia coli*, appears to fit this general model of replication. However, F may have a more complex genetic organization for this process than other plasmids.

Our approach to characterizing F replication genes has been to clone defined restriction fragments from mini-F plasmids. Plasmid F is a 94.5kb molecule that is cut into 19 fragments by *Eco*RI. One fragment, f5, contains the 40.3 to 49.3kb sequences and the normal maintenance genes of F (Timmis et al., 1975; Lovett and Helinski, 1976). Since F replication is a genetically controlled process, we have also sought to map and clone wild type and mutant copy number control and incompatibility genes. The incompatibility response has been included in our analysis because the pioneering work of Uhlin and Nordström (1975) indicated that copy number control and incompatibility can be different aspects of the same phenomenon.

Normal F maintenance, presumably replication, is inhibited by the drug acridine orange (Hohn and Korn, 1969). We have also examined our mini-F mutants derived from the *Eco*RI f5 fragment for their sensitivity to this drug and have been able to identify a region of F that is essential for a sensitive response to occur. This region does appear to be involved in control of F replication.

The F Replication Region

Figure 1 depicts maps of various mini-F plasmids that we have intentionally constructed or fortuitously isolated. They are all derivatives of pMF21. The constructions and selections have been described previously (Manis and Kline, 1977; Kline and Palchaudhuri, 1980) or are given in the Figure legend. The smallest plasmid found is pBK138-2 which contains just 1.8kb of F DNA between

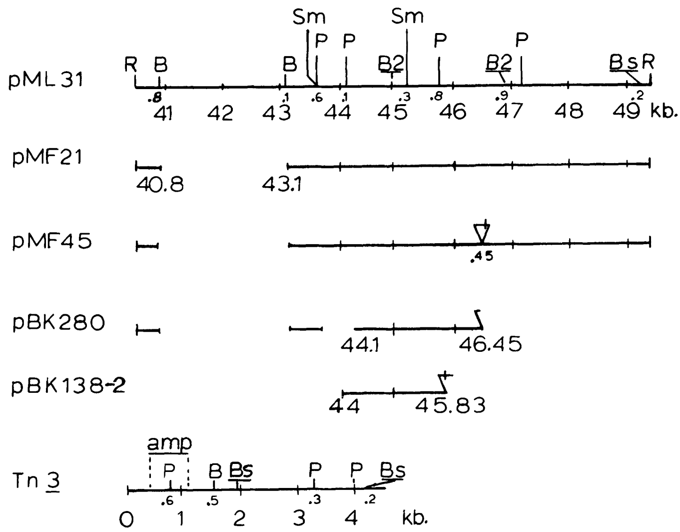

Fig. 1. Construction and characterization of mini-F plasmids. Plasmid pML31 contains the 9kb *Eco*RI f5 fragment 40.3 to 49.3kb. We formed pMF21 from pML31 by *in vitro* deletion of the *Bam*HI 40.8 to 43.1kb sequences (Manis and Kline, 1977). Next, we isolated a pMF21::Tn*3* plasmid, designated pMF45, in which the ampicillin resistance transposon, Tn*3* (∇) is inserted at coordinate 46.45kb (Manis and Kline, 1978; Kline and Palchaudhuri, 1980). Note that the *Bam*HI site in Tn*3* is asymmetrically positioned on the Tn*3* map and is designated by a vertical mark on the inverted triangle. pBK280 was formed from pMF45 by first deleting the sequences between the *Bst*EII Tn*3* 4.25kb coordinate and the *Bst*EII F 49.2kb coordinate and then by deleting the sequences between *Pst*I 43.6 and 44.1kb coordinates. The construction of pBK138-2 was as described by Kline and Palchaudhuri (1980). The restriction sites shown on pML31 and Tn*3* are: (B) *Bam*HI, (B2) *Bgl*II, (Bs) *Bst*EII, (P) *Pst*I, (Sm) *Sma*I and (R) *Eco*RI.

coordinates 44.0 and 45.83±0.03kb. However, control of pBK138-2 replication is not normal since the copy number of this plasmid is elevated about sevenfold and this plasmid is resistant to acridine orange curing. The smallest plasmid found that has a relatively low copy number (between 1.0 to 2.0 times the value of wild type F) and is sensitive to acridine orange is pBK280. This plasmid has the same F sequences as pBK138-2 plus the significant 45.83 to 46.45kb sequences (Seelke et al., in preparation). The other F sequences are without significance to replication control or acridine sensitivity.

Both pBK280 and pBK138-2 contain the origin of replication identified earlier at coordinate 44.4kb by Figurski et al. (1978). Therefore, it is of interest to know if a restriction fragment with the origin at 42.6kb (Eichenlaub et al., 1977) can form a plasmid or can complement *polA*-dependent ColE1 replication. The 42.6kb *ori* is contained on a 40.8 to 43.1kb *Bam*HI fragment. We have tried to make recombinants with this fragment that would form plasmids. For this purpose we used a 45.0 to 46.9kb *Bgl*II fragment with Tn*3* (Ap^r) inserted at 46.45kb or a *Bam*HI fragment containing the 46.45 to 49.3kb sequences as well as *amp* and *kan* genes. In no case were we successful at finding the expected recombinant plasmid. Likewise, Kahn et al. (1979) and Wehlmann and Eichenlaub (1980) have been unsuccessful in making F:ColE1 recombinants that are maintained in *polA* mutants when these recombinants contain the entire *Eco*RI f5 fragment but lack the 44.0 to 45.8kb sequences. From these results we conclude that the 44.0 to 45.8kb region contains *rep* information and that this information is more than just an *ori*.

Incompatibility Loci in Mini-F Plasmids

Results not shown identify the existence of an inc^+ function in pBK138-2. Kahn et al. (1979) have localized this function in the 45.0 to 45.8kb sequences. This function is termed *incB*. A recombinant plasmid containing the *incB* function on a *Pst*I fragment (44.1 to 45.8kb) has been cloned in our lab. This plasmid is designated pBK207 (Figure 2). A different *Pst*I fragment from pMF45 that has an inc^+ function has been cloned in pBR322. This fragment has the 45.8 to 46.45kb F sequences and 0.5kb of Tn*3* sequences. The recombinant plasmid is designated pBK232 (Figure 2) and the *inc* function is designated *incC*. Finally, as we described earlier (Manis and Kline, 1978), there is an *inc* function in the 46.4 to 49.3kb region. This function has been localized more precisely to somewhere within the 47.5 to 49.3kb sequences by making the Finc^+:pSC101 recombinant, pBK163, shown in Figure 2. Originally, we termed this function *incA*, but for reasons discussed elsewhere (Kline and Lane, 1980) the function is now called *incD*. Thus, there are at least three inc^+ functions in the mini-F region of 45.0 to 49.3kb.

An extensive genetic analysis by Tn*3* insertional mutagenesis has shown that the *incC* locus and acridine orange sensitivity locus

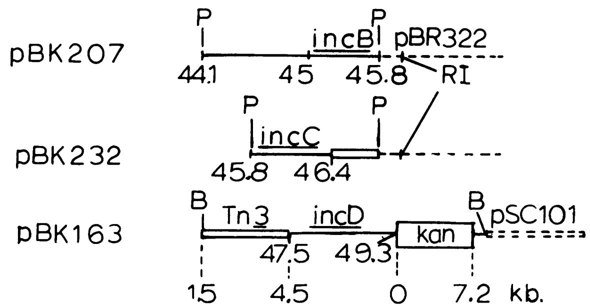

Fig. 2. Identification of F sequences containing *inc*$^+$ genes. The pBK207 and pBK232 plasmids were constructed by cloning the indicated *Pst*I fragments from pMF45 into pBR322 (Bolivar et al., 1977). The pBK163 plasmid was constructed from a pMF21::Tn*3* plasmids (Tn*3* inserted at 45.83kb, Kline and Palchaudhuri, 1980) in which Tn*3* had induced a deletion from 45.83 to 47.5kb. This deletion plasmid is known as pBK103. The *Bam*HI fragment of pBK103 containing the sequences shown above was cloned into the *Bam*HI site of pSC101 to form pBK163. The pBK plasmids 163, 207 and 232 were each shown to be incompatible with an F'*lac* plasmid contained in a *recA* host. The incompatibility test used has been described by Manis and Kline (1978). Restriction enzyme symbols are the same as in Figure 1.

overlap at least within the coordinates 45.83 to 46.35kb (Wechsler and Kline, 1980; Kline, unpublished). Remarkably, not only are *incC* and *aos* functions in pMF21 destroyed by Tn*3* insertions at 45.83 and 46.35kb, but also these insertions cause about a sevenfold copy number increase as well. The results indicate that *incC* is indeed a complex locus. It would be easy to understand sensitivity to acridine if the dye blocked some essential function of *incC*; yet, as the existence of pBK138-2 demonstrates, the *incC* locus is dispensible. This paradoxical behavior is not understood.

Copy Number Control Loci in Mini-F

As described in the preceding paragraph, the *incC* locus is apparently involved in copy number control. Copy number mutants of plasmid pMF45 have also been made by chemical mutagenesis with nitrosoguanidine or ethyl methane sulfonate (Manis and Kline, 1978; Seelke, Kline, Ritts and Trawick, in preparation). Bacteria harboring *cop* mutant plasmids grow readily in the presence of 1.0 mg of ampicillin/ml whereas *cop*$^+$ plasmids do not permit this growth. This is the basis for isolating *cop* mutants.

To map the chemically-induced *cop* mutations, we have made *in vitro* recombinants between *cop*$^+$ and *cop* plasmids and then examined the Cop phenotype of the recombinants. The maps of the recombinants showing a Cop⁻ phenotype (Fig. 3) indicate that the common sequences in all Cop⁻ recombinants are 45.3 to 45.8kb. This analysis has been done for five of the seven independently-generated *cop* mutants we have isolated. The *cop* loci that map within *incB* and *incC*, respectively, are called *copB* and *copC*. Thus far, chemically induced *cop* mutants have always been found to map in *incB* and Tn*3*-induced *cop* mutants to map in *incC* and no mutants isolated as phenotypically Cop⁻ have been found to map in *incD*.

To see if the *incD* locus influences F copy number, we have examined *incD* mutants. A leaky *incD*± mutant of pMF45 has been isolated and characterized. The mutant has the same copy number as pMF45 which is a value of two plasmids per chromosomal equivalent (Kline, 1979). Recently, we have successfully been able to delete *in vitro* the entire 46.4 to 49.25kb region of pMF45 by treating it with *Bst*EII which has only one recognition site in pMF21 at 49.25kb F and two recognition site in the Tn*3* sequences (Fig. 1). The

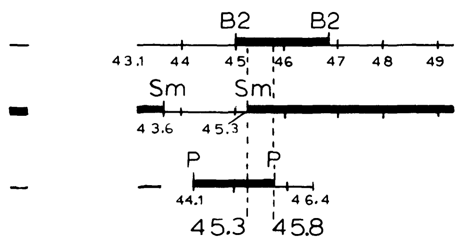

Fig. 3. Location of *cop* mutations in chemically induced Cop⁻ mutants of pMF45. To map the *cop* mutations, first the *Bgl*II (B2) 45.0 to 46.9kb fragment of each *cop* mutant was shown to contain the mutation by *in vitro* recombination of this fragment (▬▬) to the complementary *Bgl*II fragment (——) from the *cop*⁺ pMF45 plasmid and subsequent examination of the copy number of the recombinant. To more precisely localize the *cop* mutation within the 45.0 to 46.9kb region, recombinant plasmids with structures shown above were formed from *Sma*I (Sm) fragments or from *Pst*I (P) fragments, then the copy numbers of the recombinants were examined. Note that the sequence within the 45.0 to 46.9kb region that is common to all *cop*⁻ recombinants is the 45.3 to 45.8kb sequence. Therefore, the *cop* mutation from each of the five pMF45 *cop* mutants examined must map therein. These *cop* mutations are *cop48* and *50* (Manis and Kline, 1978) and *cop211, 213* and *214* (Seelke, Kline, Trawick and Ritts, in preparation).

resulting mini-F plasmid with its *incD* deletion has the same copy number as parental pMF45.

The low copy number and small size (12.4 megadaltons) of pMF45 makes it difficult to measure a small increase in copy number. Therefore, we deleted the *incD* region (46.4 to 49.25kb) from one *copB*-like mutant and three known *copB* mutants. In two of the deletion mutants we found no change and in two others we found a twofold increase over the $copB^{-}incD^{+}$ values; for example, the copy number increased from a value of 20 before deletion to a value of 40 plasmids per chromosomal equivalent after deletion of *incD*. Moreover, we have also made a recombinant of the *copB*-like mutant, *cop44*, that is, we have made *cop44* $incD^{\pm}$ double mutant. The double mutant has twice the *copy* number of the *cop44* parental type. Hence, we suspect that *incD* can influence F copy number although this influence may not be the primary role for *incD*.

When all seven *copB* mutants were subsequently analyzed for the status of their *inc* genes, they were found to be $incB^{+}incC^{+}incD^{+}$. By contrast, the *copC* mutants were found to be $incB^{+}incC^{-}incD^{+}$. These determinations were made by cloning each *inc* gene from each *cop* mutant and testing the clones for incompatibility against F'*lac*.

DISCUSSION

Normal F replication results in a low copy number concentration and is sensitive to the presence of acridine orange. The smallest mini-F isolated by us with these properties has the F replication sequences 44.1 to 46.45kb. There is a smaller F plasmid, pBK138-2, which contains just the 44.0 to 45.83 sequences, but its replication control and sensitivity to drugs are abnormal. Recently, Kahn and Helinski (personal communications) have been successful in making a mini-F plasmid from the 44.1 to 45.8 *Pst*I fragment; but the properties of this plasmid have not been reported. Eichenlaub and Wehlmann (1980) have successfully generated and mapped an amber mutant within the 41.5 to 43.1kb coordinates that is defective in replication. Interestingly, they reported (Eichenlaub and Wehlmann, 1980) that when the 40.8 to 43.1 *Bam*HI fragment is deleted from the amber mutant the resultant plasmid is no longer replication defective. A clear explanation for this is not available, but the observation suggests that it would be premature to conclude from our data that the 44.1 to 46.4kb region contains the sole *rep* determinants. However, this region must play some essential role in replication since no one has been able to show that the 40.3 to 44.1kb region or any part thereof that contains the 42.6 *ori* can function as an independent plasmid (Manis and Kline, 1978; Kahn et al., 1979; Wehlmann and Eichenlaub, 1980). Surprisingly, Wehlmann and Eichenlaub (1980) failed to find any proteins produced by the 44.1 to 45.8kb region.

Kahn et al. (1979) and Kahn and Helinski (personal communication) first cloned *incB* (45.0 to 45.8kb) and *incC* (45.8 to 46.4kb) and recognized them as such. However, Kahn et al. (1979) and Wehlmann and Eichenlaub (1980) missed *incD* for reasons that are unclear; further, they initially confused the *incC* determinant with *incD*. The structure of pBK232 (*incC*) and pBK163 confirms Kahn and Helinski's observation that *incC* maps within 45.8 to 46.4 and establishes that *incC* and *incD* must be separate *inc* determinants.

Aside from the finding that *incB* and *incC* genes overlap *copB* and *copC* genes, respectively, and that *copC* mutations result in loss of the *incC*$^+$ function, there are no other results to indicate the mechanism of incompatibility encoded by these genes. In fact, until we produce point mutations or small deletions in *incC* and find a corresponding increase in copy number, we must entertain the possibility that Tn*3* inactivation of *incC* is inconsequential for copy number control. It might be that Tn*3* insertion has a polar effect on the adjacent *copB* gene and that this effect is responsible for the copy number increases.

One explanation for *incB* and *incC* gene products is that they encode repressors of F replication. If this interpretation is correct, then *copB* mutations have a property of operator mutations in that the *copB* mutants remain *incB*$^+$.

Little is known about the *incD* mutants. At best only a twofold increase in copy number can be seen with *incD* mutants and then it can only be seen with some F plasmids that are in a high copy number state before the *incD* mutation occurs. Clearly *incD* does not have a profound effect on F copy number.

Another clear property of *incD* is that it is not essential for plasmid maintenance; witness the existence of pBK280. Given this observation, it is absolutely intriguing to find that when *incD* is cloned into an unrelated plasmid such as pSC101 the pSC101:F *incD* recombinant can be eliminated by *inc*FI plasmids and vice versa (Kline, 1979). Moreover, IncFI plasmids are completely compatible with pSC101. Thus, we have a situation in which an unrelated, nonessential, *inc* gene can "poison" the normal maintenance of its vector plasmid if the homologous *inc* gene is carried on another unrelated replicon. A very similar finding has been made by Timmis et al. (1979) with an *inc* gene from R6-5 cloned in pBR322. These situations are reminiscent of *incC* being a dispensible gene that can "poison" the normal maintenance of its host plasmid in the presence of acridine orange. Whether or not this is merely a superficial similarity remains to be seen. In any event, the results suggest to us that both *incC* and *incD*, while they apparently make *trans* functioning incompatibility substances, also likely have a *cis* dominant role in maintenance. Put more simply, we feel that when *incC* and *incD* are present in F they are quite important or become essential for F maintenance.

The genetics of F replication and its control intertwined with the phenomenon of incompatibility is a complex subject about which more is probably unknown than is known. One approach to unravelling the complexities is to define the target sites for the *inc/cop* genes via incompatibility tests, mutant analysis and promoter identification, then to analyze promoter expression in the presence of various *inc*$^+$ genes. We are making substantial progress in this analysis, but at present it is incomplete. A summary of this paper is shown in Fig. 4.

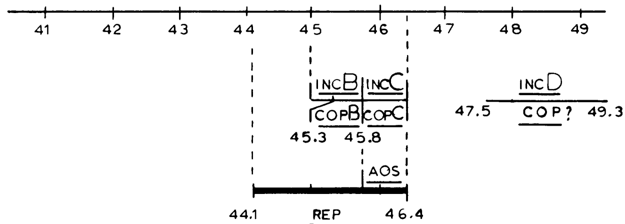

Fig. 4. A map of the known replication, incompatibility, copy number and acridine orange sensitivity genes. Gene symbols are described in the text.

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PLASMID MINI-F ENCODED FUNCTIONS INVOLVED IN REPLICATION AND INCOMPATIBILITY

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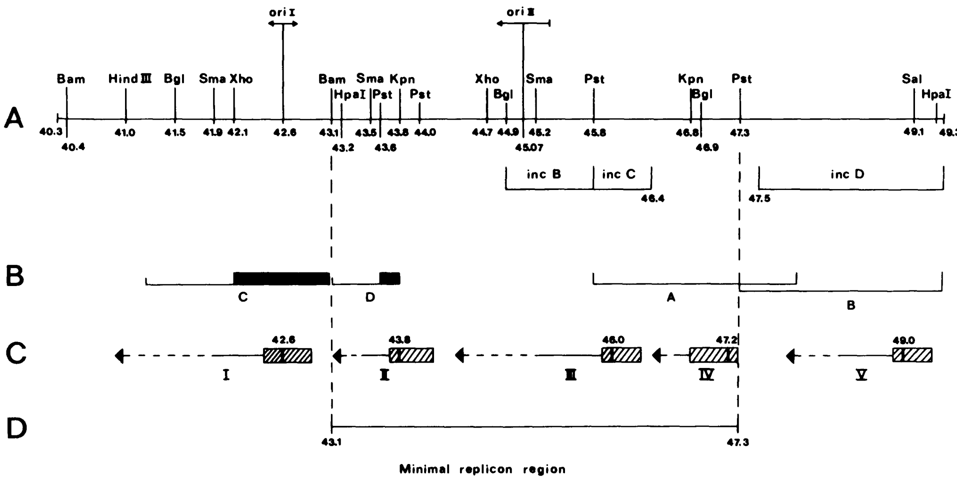
INTRODUCTION

The F factor of Escherichia coli is one of the most extensively studied plasmids. It belongs to the class of plasmids with a stringent mode of replication i.e. F is normally present in a cell in only 1-2 copies per chromosome[1]. In order to maintain the low copy number the replication must be tightly regulated, a notion which predicts that two different F'plasmids should not coexist in a bacterium. This has been experimentally proven and the phenomenon was termed incompatibility[2,3]. Thus incompatibility of two isogenic or related plasmids of the same incompatibility group may result from specific mechanisms engaged in the regulation of replication and partitioning during cell division.

In spite of intensive studies, so far there is no consensus on the nature of the regulatory mechanism controlling the initiation of plasmid DNA replication. Based mainly on studies with F two general models have been proposed which favour either positive or negative control[4,5].

In plasmid R6K an autoregulatory positive control element has been demonstrated[6]. While such a mechanism may function for a plasmid with a copy number of 10-15 per chromosome it is questionable whether a stringent regulation can be achieved by a positive control only. It is conceivable that negative control may be more efficient in stringent replication. Thus negative control, as first postulated by Pritchard et al.[5] has been favoured for the interpretation of copy number control in joined plasmid replicons[7] and Cop^- mutants of R1drd-19[8], although as yet there is no direct evidence for a repressor of replication.

The study of replication and maintenance of large complex plasmid genomes has been greatly facilitated by the recombinant



DNA technology which allowed the in vitro construction of mini-plasmids exhibiting identical replication properties as the parental plasmid. Such a mini-F plasmid, the 9kb EcoRI fragment f5[9,10] derived from F'lac, representing F-coordinates 40.3-49.3F[11,12] has become a useful model system for the study of plasmid replication.

Origins of Replication

Isolation of replicative intermediates of plasmid mini-F'km (pML31) from E. coli P678-54 revealed an unique origin of replication at 42.6F (oriI) with a predominantly bidirectional mode of replication[13]. Surprisingly, this origin located on a BamHI fragment could be deleted without notable effect on replication and incompatibility[14,15]. Replicative intermediates from such a plasmid, pRE25 (mini-FΔBamtrp), linearized with SalI showed an origin of replication at a distance of 30 ±2% of the total length from one of the SalI restriction sites, with an unidirectional mode of replication (R. Eichenlaub, unpublished data;16). This second origin (oriII) was mapped at 44.4F[15]. However, based on more precise coordinates now available for the SalI site (49.1F) and the BamHI sites at 40.4F and 43.1F resulting in a total length of pRE25 of 13.4kb, oriII may rather map at 45.07F ±270 base pairs (Fig. 1A). This revised coordinate is also in better agreement with recent data showing that the region 44.8-45.8F carries sequences resembling the oriC of E. coli (T. Murotsu, pers. communication) and that mini-F plasmids can be obtained deleted from 43.1F to 44.76F (D. Lane, pers. communication).

Fig. 1. Restriction map of plasmid mini-F and map positions of
← mini-F encoded polypeptides and transcripts.
(A) Mini-F restriction map with incompatibility loci incB, incC, and incD according to the nomenclature proposed by B. Kline and D. Lane[17]. Restriction sites in F-coordinates (kb). Bam = BamHI; Bgl = BglII; Sma = SmaI; Xho = XhoI; Pst = PstI; Kpn = KpnI; Sal = SalI.
(B) Map location of mini-F encoded polypeptides, boxed regions in proteins C and D indicate the tentative location of the promoter. This mapping is based on the analysis of proteins obtained in minicells from restriction endonuclease generated deletion derivatives of mini-F: pBR322 hybrids as described by H. Wehlmann and R. Eichenlaub[18].
(C) Map location of *in vitro* transcripts of plasmid mini-F obtained by R-loop analysis. Region of transcription starts (boxed), approximate start points in kb as calculated from the distribution of starts within the boxed region, and direction of transcription (arrow). Length of the arrow corresponds to the longest transcript observed. (From H. Wehlmann and R. Eichenlaub, submitted for publication.)
(D) Minimal replicon region bordered by F-coordinates 43.1F and 47.3F.

Conditional Replication Mutants

Evidence whether plasmid encoded functions are involved in plasmid replication can be obtained by the isolation of conditional replication mutants.

Using *in vitro* mutagenesis with hydroxylamine we isolated mutants of mini-F thermosensitive in replication[19]. Although this already suggested that a mini-F encoded polypeptide is involved in replication further proof for the existance of such a protein may come from the isolation of amber mutants. After *in vitro* mutagenesis of mini-F DNA and using an *E. coli* *supFts* as recipient in transformation (which is su^+ at 28°C but su^- at 42°C) two amber mutants were obtained[20].

A temperature shift from 28°C to 42°C resulted in rapid segregation of the plasmid from the *supFts* host. When plasmid DNA synthesis was followed by the incorporation of tritiated thymidine no label was incorporated into supercoiled mini-F DNA at 42°C[20].

In order to identify the defective polypeptide, the proteins synthesized in su^- minicells of E. coli by wild type mini-F and the amber mutants were compared. SDS-PAGE showed that a polypeptide of 34K was missing in both amber mutants[20]. This documents that replication of mini-F requires a mini-F encoded protein of 34K. Experiments to map the amber mutation on the mini-F genome showed that the am1-mutation maps on the 2.7kb BamHI fragment 40.4-43.1F of mini-F (Fig. 1A,B). Deletion of this fragment which carries oriI was accompanied by the loss of the mutant phenotype. From this observation it was concluded that the mutation am1 is only effecting replication starting at oriI. It appears that in mini-Fam1 the block of replication is not bypassed by initiation of replication at oriII (45.07F). Thus the second replication system is only functioning when oriI together with the gene locus for the 34K protein is deleted.

The other mutant mini-Fam3, however, behaved differently. Upon deletion of the BamHI fragment 40.4-43.1F the amber phenotype was retained indicating that mini-Fam3 carries two amber mutations, effecting both replication systems. Although we have not yet identiied the second defective polypeptide in mini-Fam3 it is suggested that both replication system require mini-F encoded proteins.

Mapping of Polypeptides and Transcripts

Four proteins encoded by plasmid mini-F have been identified in *E. coli* minicells and designated A-protein (44K), B-protein (36K), C-protein (34K), and D-protein (25.3K)[18]. Mapping of the polypeptides relatively to the mini-F genome has been achieved by comparing the protein patterns of mini-F derivatives carrying deletions generated by restriction endonuclease cleavage[18]. The resulting map positions of the four polypeptides are shown in Fig. 1,B.

In order to correlate the corresponding transcripts to the map position of the proteins we analysed R-loops formed with transcripts synthesized *in vitro* (Wehlmann and Eichenlaub, submitted for publication) (Fig. 2). Five different transcription regions can be distinguished (Fig. 2,3). Transcripts I, II, IV, and V originate within the coding region of proteins A, B, C, and D (Fig. 1B,C). Transcript III mapping between coordinates 43.9-45.9F seems not to be translated, since we did not detect a polypeptide in this region. The possible role of this transcript will be discussed later.

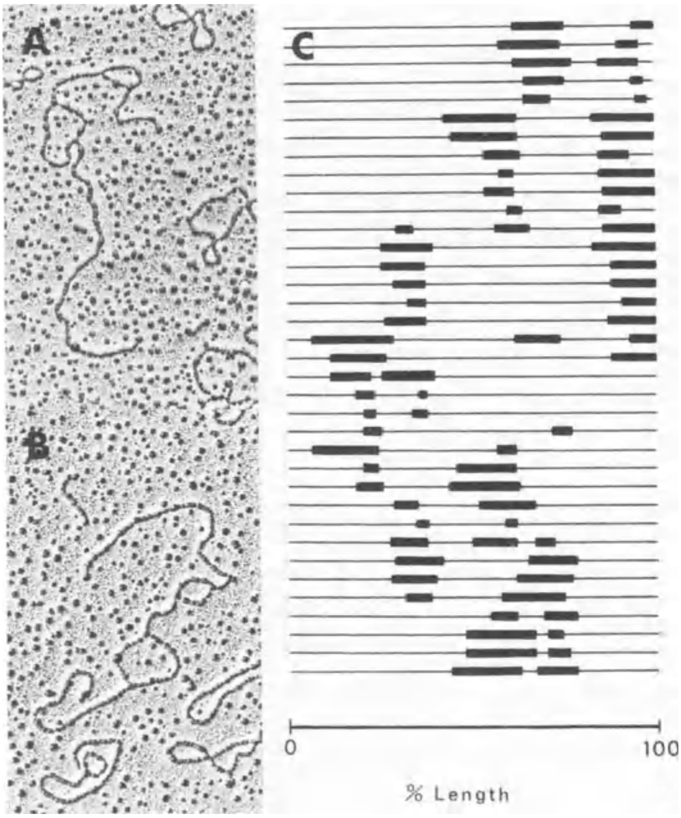


Fig. 2. R-loop molecules of plasmid mini-F and plot. Plasmid pJE401 (mini-F:pBR322)[20] was cleaved by restriction endonuclease EcoRI. After transcription the RNA was hybridized to the template DNA to form R-loop molecules which were analysed in the electron microscope as described by C. Brack[21]. Molecules with more than one R-loop (A and B) were exclusively evaluated. (C) Plot of R-loop molecules of the complete mini-F plasmid (40.3-49.3F) versus percent length. (From H. Wehlmann and R. Eichenlaub, submitted for publication.)

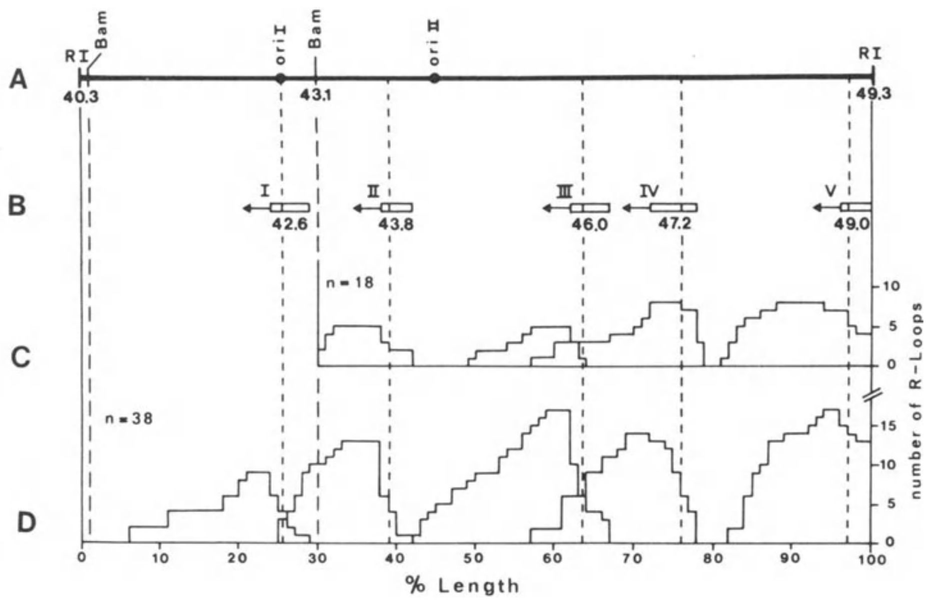


Fig. 3. Cumulative histograms of R-loop molecules.
(A) Map of plasmid mini-F with relevant restriction endonuclease recognition sites and F-coordinates in kb. RI and Bam refer to EcoRI and BamHI, respectively.
(B) Region of transcription starts (boxed), approximate start points in kb as calculated from the distribution of starts within the boxed region, and direction of transcription.
(C) Cumulative histogram of R-loops of mini-F deleted for BamHI fragment 40.4-43.1F.
(D) Cumulative histogram of R-loops of the complete mini-F plasmid (40.3-49.3F).
Symbol n in C and D refers to the number of R-loop molecules evaluated. (From H. Wehlmann and R. Eichenlaub, submitted for publication.)

Complementation of Maintenance Deficient Deletion Derivatives

Although a plasmid reduced in size to a 2.8kb segment with coordinates 44.1-46.9F can be obtained[22], it is observed that deletions are often accompanied by instability of the plasmid. This may be due to the lack of certain polypeptides necessary for a stable maintenance of mini-F. Therefore we tested whether replication deficient mini-F plasmids deleted for restriction endonuclease generated fragments could be established in a bacterium provided that missing functions were supplied through complementation by another mini-F plasmid (Ebbers and Eichenlaub, submitted for publication).

In one such plasmid, pJE1001, the *PstI* fragment 45.7-47.3F was deleted resulting in the loss of the *incC* locus and the 44K A-protein (Fig. 4). This plasmid could only be established in *E. coli* in the presence of a wild type mini-F helper plasmid (pML31), indicating complementation of pJE1001 by the A-protein. Another plasmid, pJE2001, consisting of 44.0-45.7F and the *trpED* genes was constructed (Fig. 4) and was also only successfully established in *E. coli* in the presence of pML31. Since the mini-F segment present in pJE2001 does not encode a polypeptide[18] it appears that all F specific proteins required for replication and maintenance are supplied by the helper plasmid. However, pJE2001 and pJE1001 are still rather unstable, indicating that complementation is either only partially effective or that there is an incompatibility reaction between pML31 and pJE1001 and pJE2001, respectively.

By joining of the segment 44.0-45.7F to the *PstI* fragment 45.7-47.3F a plasmid was obtained (pJE3001) which encodes the oriII, the *incB*, and *incC* loci and the 44K protein (Fig. 4). Plasmid pJE3001 can be introduced into *E. coli* without the requirement for a helper plasmid, but segregation is observed at a rate of about 2 per cent per generation. The segregation indicates that pJE3001 lacks some function for total stability, although it has the A-protein which is required for plasmid maintenance. To test whether the lacking function could be supplied by complementation the stability of pJE3001 was examined in the presence of plasmids pJE421 and pHW30, respectively (Fig. 4). These two plasmids are compatible with other mini-F plasmids, they both lack the 44K protein and pHW30 also lacks the 25.3K protein. It was found that only pJE421 but not pHW30 complemented pJE3001 to total stability. Our interpretation of the complementation experiments is that two trans-acting proteins of 44K and 25.3K are involved in mini-F replication and maintenance.

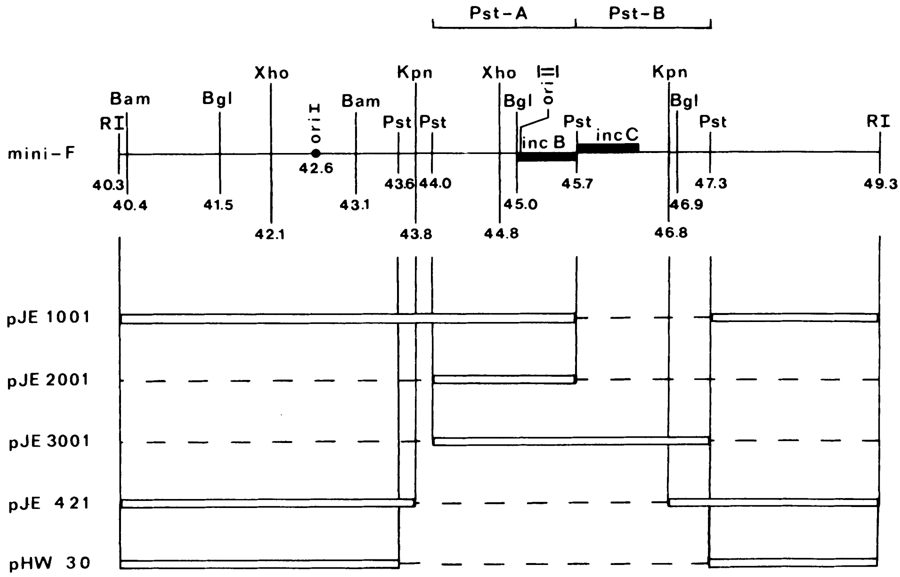


Fig. 4. Restriction map of plasmid mini-F and map of its derivatives pJE1001, pJE2001, pJE3001, pJE421, and pHW30. Recognition sites for relevant restriction endonucleases are indicated with their corresponding F-coordinate in kilobases (kb). RI = EcoRI; Bam = BamHI; Xho = XhoI; Pst = PstI; Kpn = KpnI; Bgl = BglII. Dotted lines represent the deleted part of the mini-F derivative. Plasmids pJE1001, pJE2001, and pJE3001 carry an EcoRI or PstI fragment with the trpED genes as a selective marker. pJE421 and pHW30 represent mini-F: pBR322 hybrids with the mini-F derivative inserted into the EcoRI site of pBR322[18]. (From J. Ebbers and R. Eichenlaub, submitted to publication.)

Minimal Requirements for Plasmid Mini-F Replication and Maintenance

Within the 9kb mini-F genome two origins of replication[13,15], four mini-F encoded proteins[18], five transcripts (Wehlmann and Eichenlaub, submitted for publication), and three incompatibility loci[22,23,24] have been identified. Therefore the question arises, which of these components constitute the minimal replicon region and which function they may have in the replication and maintenance of mini-F. It was shown that the oriI and the B-protein both located within the BamHI fragment 40.4-43.1F can be deleted. The stability and regulated replication of the remaining segment 43.1-49.3F indicates that besides oriII it posseses all control functions required for such a status.

Based on the complementation experiments it becomes apparent that two polypeptides are involved in the maintenance and replication control of mini-F. The D-protein mapping at 43.1-43.8F and the A-protein mapping at 45.9-47.3F. Although the exact function of these proteins is not known we have suggested that the A-protein may act as a negative control element[18]. We further proposed that the D-protein may then play a role as a positive control element in the initiation of replication[18].

The region of 43.1-47.3F carries two adjacent incompatibility loci, incB and incC[22], which can be cloned separately in pBR322. Although, incompatibility is not impaired upon insertions at XhoI (44.8F), BglII (44.9F), and PstI (45.7F) autonomous replication is always abolished (Ebbers and Eichenlaub, submitted for publication; 22), indicating that continuity of the DNA sequence between coordinates 44.8-45.7F is required for replication. Interestingly, this DNA sequence falls into the coding region for transcript III. It is possible that this non-translated RNA may serve as a primer for replication (o-RNA) possibly after being processed by ribonucleases as has been described in another plasmitd system[25,26] or is produced during transcriptional activation of oriII. Another possibility is that transcript III plays a role in the association of F with the folded chromosome of E. coli[27] which seems to be mediated by a rapidly metabolized, untranslated RNA species[28].

The presented data indicate that the minimal region of mini-F required for replication and stable maintenance of the plasmid covers the region 43.1-47.3F (Fig. 1,D). Within this region two polypeptides, two gene loci incB and incC expressing incompatibility[22] and an untranslated RNA species have been identified. At present we can only speculate on the function of these components in F replication and maintenance, however, we exspect that forthcoming experiments employing in vitro studies may eventually answer these questions.

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NUCLEOTIDE SEQUENCE CHANGE IN A COLE1 COPY NUMBER MUTANT

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INTRODUCTION

Despite a great deal of knowledge about the enzymology of DNA replication, the elements that regulate initiation of DNA replication are largely unknown. The definition and ultimate analysis of such elements depends initially on genetic identification of mutations affecting their function. In turn, the genetic analysis requires that the mutant be viable under certain conditions. For complex replicons such as the *E. coli* chromosome, such mutants have not been described. We have studied the multicopy plasmid ColE1 and its derivatives as a model system for the analysis of replication control elements. This plasmid is stably inherited and exists at a characteristic copy number of 10-15 copies per host chromosome. Our approach has been to perturb the control mechanism by isolating plasmid mutants which have altered copy number and then investigating the molecular consequences of the lesion. We have studied a high copy number mutant of the ColE1-derived cloning vehicle pBGP120 (Polisky, Bishop and Gelfand, 1976). The mutant plasmid, pOP1, and its derivatives, such as pOP1A6, comprise about 30% of intracellular DNA, compared to about 5% for the parent, pBGP120 (Gelfand et al., 1978). Previously, we localized the mutation to a 2kb region near the plasmid replication origin and demonstrated that the mutation was recessive, i.e., in cells containing both a copy number mutant and a wild-type plasmid, the copy number of the mutant was lowered to wild-type levels (Shepard, Gelfand and Polisky, 1979). The turn-down of copy number *in trans* was not observed when an unrelated plasmid co-resided with the mutant, suggesting the existence of a specific, plasmid-encoded, negative regulator of replication (Pritchard, 1978).

Here we describe more detailed mapping of the mutation in deletion derivatives of pOP1 by DNA fragment recombination *in vitro*. This approach enabled us to direct DNA sequencing efforts to a small region of the plasmid genome. We have sequenced mutant DNA fragments shown by recombination *in vitro* to contain the overproducer mutation, as well as cognate wild-type fragments. We have found the mutation to be a single GC→TA base-pair transversion in a region of the plasmid genome which encodes two RNA elements synthesized from opposite DNA strands. One of these elements is a small, non-translated RNA, known as RNA1 (Levine and Rupp, 1978). The second element affected is the RNA primer required for initiation of DNA replication *in vitro* (Itoh and Tomizawa, 1980). RNA1 has been reported to be 104 (Levine and Rupp 1978) to 110 (Morita and Oka 1979) nucleotides in length and is transcribed efficiently from supercoiled DNA templates *in vitro*. It is located about 450 nucleotides upstream from the replication origin and transcribed in the direction opposite to that of replication fork movement of ColE1 (Chan, Lebowitz and Bastia 1979; Morita and Oka, 1979). The mutation in pOP1 DNA appears to promote readthrough transcription of RNA1 *in vitro*, generating a series of larger transcripts. We propose that RNA1 may be a negative modulator of ColE1 replication and that the mutation in pOP1 DNA generates larger species of RNA1 which are unable to repress replication initiation. Campbell and Conrad (1979a) have shown previously that an independently isolated copy number mutant of ColE1, pFH118, was generated by insertion of *EcoR1* linkers *in vitro* into the region of the genome encoding RNA1.

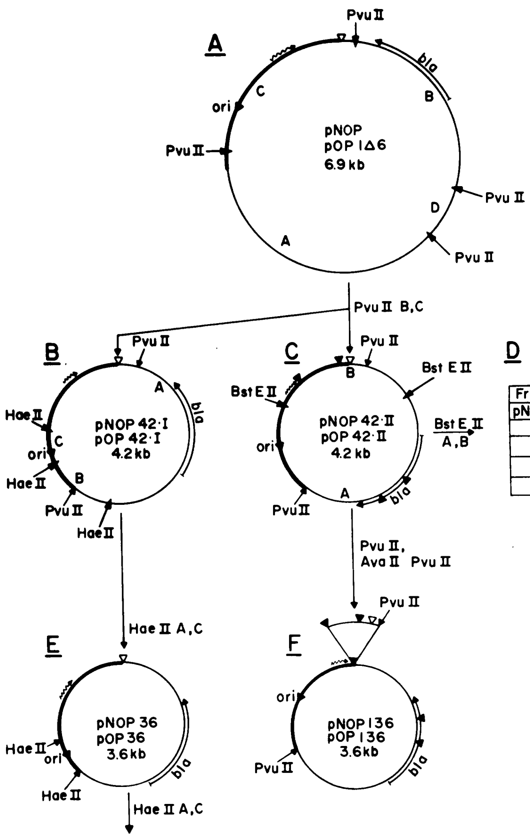
Mapping the Mutation in pOP1Δ6 DNA

Previously, we have shown that the mutation responsible for high copy number in the mutant plasmid pOP1 is located within a 2kb region of the plasmid genome containing the origin of replication and one structural gene, that specifying immunity to colicin E1 (Shepard, Gelfand and Polisky, 1979). This 2kb region is the only ColE1 DNA present in the identically sized, Tn3-containing plasmids pOP1Δ6 and pNOP1 (Fig. 1). These plasmids differ only in that pOP1Δ6 is Cop^- (copy number of 200-300 per chromosome), while pNOP1 is Cop^+ (copy number of 10-15 per chromosome). pNOP1 is a Cop^+ deletion derivative of pBGP120 constructed *in vitro*. pOP1Δ6 is a deletion derivative of pOP1 which arose spontaneously *in vivo* (Gelfand et al., 1978).

To further localize the mutation and minimize the size of the DNA region we had to sequence, we carried out two general types of recombination experiments *in vitro*. In one type, plasmids were constructed with combinations of Cop^+ and Cop^- purified restriction fragments. In the second type, plasmids were constructed with homologous Cop^+ or Cop^- restriction fragments, but deleted for a particular fragment. In these experiments, the Cop phenotype of the resulting plasmids was determined qualitatively both by a plate assay for β-lactamase production and by analysis of cleared lysates by electrophoresis on agarose gels. We screened the Cop phenotype of resident plasmids by adding a chromogenic β-lactamase substrate to colonies on plates (O'Callaghan et al., 1972). Due to gene dosage, more β-lactamase is produced from colonies containing Cop^- plasmids than from colonies containing Cop^+ plasmids. Because β-lactamase is exported from cells, such colonies are easily distinguished by the size of the red halo surrounding them resulting from nitrocefin cleavage.

Our mapping experiments began with the cognate 6.9kb Cop^+ and Cop^- plasmids pNOP1 and pOP1Δ6 (Fig. 1, A). Each of these plasmids contains four PvuII sites, shown schematically in Fig. 1, A. The relevent fragments are PvuIIC, which contains the replication origin, and PvuIIB which contains the gene encoding β-lactamase. The PvuII site immediately downstream from the replication origin lies in ColE1 sequences in the HaeIIB fragment about 200 nucleotides from the ColE1-Tn3 border. We purified the PvuIIB and C fragments from both plasmids, ligated homologous fragments, and transformed them into E. coli strain DG75. Ap^R transformants receiving PvuII B-C from pOP1Δ6 were Cop^-. The resulting new set of cognate Cop^+ and Cop^- plasmids are 4.2kb and designated pNOP42 and pOP42 respectively. These results indicate that the mutation responsible for DNA overproduction does not lie in the 200 bp region between the PvuII site and the ColE1-Tn3 border.

Analysis of colonies containing pNOP42 and pOP42 indicated that both orientations of the PvuIIB and C fragments were obtained. These are designated I and II and are shown schematically in Fig. 1, B and C. Both plasmids have three HaeII sites. The second recombination experiment was designed to map the mutation with respect to the HaeII sites of pOP42, orientation I (Fig. 1, B). In this orientation, the HaeIIA fragment contains the β-lactamase gene and ColE1 sequences between the EcoR1 site of the ColE1 and the HaeIIA/C junction. The HaeIIC fragment contains the origin of replication, and the HaeIIB fragment contains 285 nucleotides of ColE1 DNA downstream from the origin. The three HaeII fragments of pOP42 and pNOP42 were isolated and ligated in the combinations shown in Fig. 1, G. As before, ligated fragments were transformed into DG75 and the Cop phenotype determined. The results of these ligations and transformations are shown in Fig. 1, G. Lines 3 and 4 are homologous reconstructions generating a new set of plasmids with molecular size of 3.6kb and which lack any part of the HaeIIB fragment of ColE1. That these plasmids differ in their Cop phenotype as did their parents means the mutation does not lie in the HaeIIB fragment. These plasmids are



D

Fragment	Source	Phenotype
pNOP 42·II	pOP 42·II	Cop
A	B	–
B	A	+
A,B	–	+
–	A,B	–

G

Fragment	Source	Phenotype
pNOP 36	pOP 36	Cop
A	C	+
C	A	–
A,C	–	+
–	A,C	–

Fig. 1. ← Construction and phenotypic analysis of plasmids used to map the cop mutation in pOP1Δ6 DNA. A, schematic diagram of the cognate Cop^+ and Cop^- plasmids pNOP1 and pOP1Δ6. These plasmids are identical except for the mutation in the copy number control function. Each plasmid has four PvuII sites. The PvuII B fragment carries the β-lactamase gene (bla) while the PvuII C fragment contains the ColE1 replication origin and surrounding sequences. The bold segment in the diagram depicts ColE1-derived sequences while the narrow line shows sequences from the transposon Tn3, and a small segment (about 400bp) derived from the COOH-terminal region of the β-galactosidase gene which was present in pBGP120 (O'Farrell, Polisky, and Gelfand, 1978). B, C, PvuII B and C fragments from pNOP1 and pOP1Δ6 were purified from acrylamide gels, ligated homologously and transformed into DG75 with selection for Ap^R. Four ligated plasmids were generated representing both orientations of PvuII fragments B and C. These plasmids are designated pNOP42·I and pOP42·I representing one orientation, and pNOP42·II and pOP42·II in the opposite orientation. Each plasmid is 4.2 kb and encodes Ap^R. These opposite orientations were exploited to map the cop mutation. Both pNOP42·I and pOP42·I contain three HaeII sites as shown in B. These fragments are designated A, B, and C. Purified HaeII fragments A and C from both pNOP42·I and pOP42·I were ligated in all four possible combinations, transformed into DG75, and the Cop phenotype of the resulting Ap^R transformants determined using the nitrocefin assay. The results are shown in Table G. The ligations resulted in the construction of two plasmids with identical orientation designated pNOP36 and pOP36, shown in part E. Each of these plasmids is 3.6kb, C; plasmids pNOP42·II and pOP42·II contain two BstEII sites. These fragments were isolated and ligated in the four combinations shown in D and the Cop phenotype of Ap^R transformants determined as above. The Cop phenotype of the resulting transformants is shown in D. To demonstrate that ColE1 DNA sequences downstream from RNA1 were irrelevant to the cop mutation, plasmids pNOP136 and pOP136 were constructed from pNOP42·II and pOP42·II (C, F). In these constructions a DNA segment extending from an AvaII site 109bp downstream from the 3'-terminus of RNA1 to a PvuII site in the COOH-terminal region of the β-galactosidase gene (shown as an arc in F) was deleted from both pNOP42·II and pOP42·II. The isolated AvaII-PvuII fragments containing RNA1 and the replication origin from these plasmids were treated with T4 DNA polymerase to convert the AvaII end to a blunt end and these fragments were then ligated to a purified PvuII B

(Continued)

fragment (see 1A) which carries bla. We have previously shown that the Tn3 moiety, from which the PvuII B fragment derives, is devoid of copy number control elements (Gelfand et al., 1978). Consequently, identical results were obtained whether the PvuII B fragment was derived from a Cop^+ or Cop^- plasmid. The ligated fragments were transformed into DG75 and the Cop phenotype determined. These ligations created pNOP136 and pOP136, each 3.6 kb (F). pNOP136 is Cop^+ while pOP136 is Cop^- in DG75.

Δ denotes the EcoR1 sites; ▲, AvaII: short squiggled arrow denotes location of RNA1; ori is the origin of replication with unidirectional fork movement counter-clockwise; bla represents the gene encoding β-lactamase, the arrow denoting the direction of its transcription.

designated pNOP36 and pOP36. Lines 1 and 2 demonstrate that the mutation lies in the HaeIIA fragment of pOP42, since the Cop phenotype of the resulting plasmid depends on the source of the HaeIIA fragment and not on the origin-containing HaeIIC fragment.

The HaeIIA fragment consists of 1014bp of ColE1 DNA and contains three known genetic elements; the colicin immunity gene, the promoter for the primer for DNA replication (Itoh and Tomizawa, 1980), and RNA1. The DNA sequence for the entire region has been determined (Oka et al., 1979). Since several groups have constructed both point and deletion Col^{imm-} derivatives which are not altered in plasmid copy number (see below), it seemed unlikely that the mutation was in the immunity gene (Inselburg, 1977; Itoh and Tomizawa 1980). On the other hand, the region of the genome containing RNA1 has been implicated in replication control (Conrad and Campbell, 1979). In addition to these elements, examination of the sequence in this region (about 400-600bp upstream from the replication origin) has revealed the existence of an open reading frame, potentially capable of encoding a small, very arginine-rich polypeptide. This sequence is highly conserved between ColE1 and CloDF13 (Stuitje et al., 1980).

To distinguish whether the mutation was located in the region containing RNA1 and the primer promoter, or the putative polypeptide, we carried out the DNA fragment switch experiment described schematically in Fig. 1 C, D. This experiment used cognate Cop^+ and Cop^- plasmids pNOP42 and pOP42, orientation II. In this orientation we took advantage of a BstEII site located between the open reading frame and RNA1 to generate two BstEII fragments from both pNOP42 and pOP42. The BstEII site is 85bp from the 5'-terminus of RNA1 (nucleotide 1 in Fig. 3). We

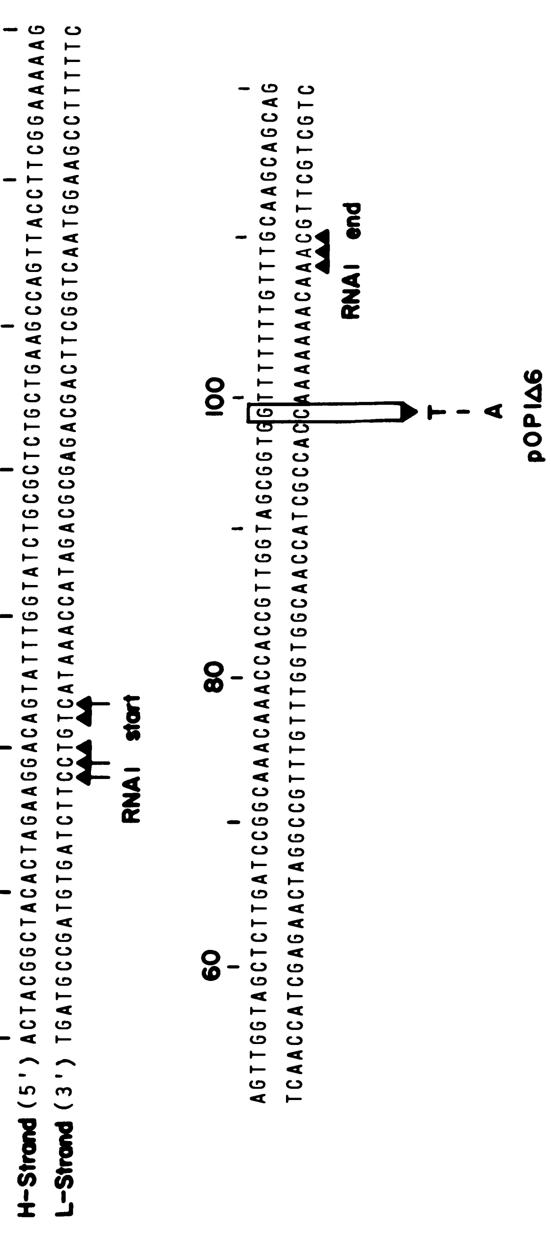

20
20
40
H-Strand (5') ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAG
L-Strand (3') TGATGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTC
RNAI start
60
80
100
AGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGTTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAG
TCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCAACCATCGCCACCAAAAAAACAAACGTTCGTCGTC
RNAI end
T
A
pOPIΔ6

Fig. 2. The DNA sequence in the region of ColE1 encoding RNA1. Triangles denote the A residues reported to be the initiating nucleotides of RNA1 (Chan, Lebowitz, and Bastia, 1979), and the 3' termination region of RNA1. Nucleotide numbers correspond to those of Morita and Oka (1979) for the RNA1 sequence. The mutation in pOP1Δ6 is shown at position 98. The initiating nucleotide of the ColE1 RNA primer has been reported to be the G at position 111 (Itoh and Tomizawa, 1980). Apart from the alteration in the mutant, the nucleotide sequence in the region shown and the region between the BstEII site and the RNA1 start site agrees exactly with that reported by Oka et al. (1979).

purified these fragments and ligated them together in various combinations, transformed DG75, and determined the Cop phenotype of the resulting Ap^R transformants. The ligation combinations and results are shown in Fig. 1, D. The results demonstrate that the Cop phenotype of the plasmids constructed in vitro depends on the source of the BstEIIB fragment, which encodes RNA1 and the primer promoter.

The Nucleotide Sequence Alteration in pOP42 DNA

We determined the nucleotide sequence of the region encoding RNA1 in pNOP42 and pOP42 DNAs. We found a single alteration--a GC→TA transversion located in the structural gene for RNA1 (Fig. 3). In RNA1, this alteration is located in a GC-rich region immediately preceding the uridylate run at the 3'-terminus of the transcript (see Fig. 3).

To demonstrate that the sequence alteration detected in the RNA1 sequence was directly responsible for the Cop phenotype of pOP1Δ6 and its derivatives, it was necessary to establish that the 670bp sequence between the 3'-terminus of RNA1 and the EcoRI site was irrelevant to the Cop^- phenotype (see Fig. 1, C). To show this, we constructed plasmid derivatives of pNOP42II and pOP42II in vitro that were deleted for sequences between an AvaII site 109bp downstream from the 3'-terminus of wild-type RNA1, and the EcoRI site of pNOP42II and pOP42II (Fig. 1, C). This region is known to encode the gene conferring immunity to colicin E1. The construction of these cognate derivatives, called pNOP136 and pOP136, is shown in Fig. 1, C and F. We found that the Cop phenotype of the deletion derivatives depended on the Cop phenotype of the parent plasmid used in the construction (results not shown). In addition, we determined the nucleotide sequence of both pNOP42 and pOP42 DNAs between the AvaII site and the 3'-terminus of RNA1. No sequence changes between the two plasmids were detected in this region (not shown). These results demonstrate that the sequence alteration detected in the RNA1 region of pOP1Δ6 is necessary and sufficient for its Cop$^-$ phenotype.

The role of RNA1 in ColE1 replication has been the subject of considerable speculation. Backman et al. (1978) proposed that RNA1 was a "nomadic primer" which hybridized to the replication origin and served as a primer for elongation by DNA polymerase I. In this model, RNA1 was viewed as a positive element in replication control. Comparison of the nucleotide sequence of RNA1 with the replication origin indicated that RNA1 contains a sequence of 10 nucleotides complementary to the region where the first deoxyribonucleotide is incorporated (Chan, Lebowitz and Bastia, 1979). Moreover, Conrad and Campbell (1979) have detected weak hybridization between RNA1 and DNA fragments containing the replication origin. On the other hand, it is clear that RNA1 is not obligatory for replication since Oka et al. (1979) and others (B.P. unpub. data) have constructed ColE1 deletion mutants lacking the entire template for RNA1 and the primer promoter. The results reported here and those reported previously for the copy number mutant pFH118 (Conrad and Campbell, 1979) implicate RNA1 as a potential element in plasmid copy number control.

How might RNA1 negatively modulate ColE1 replication? Recently, Itoh and Tomizawa (1980) have demonstrated that initiation of ColE1 DNA replication _in vitro_ involves a large RNA molecule which is processed at or near its 3'-terminus by ribonucleaseH to generate a primer approximately 550 nucleotides in length to which deoxyribonucleotides are subsequently added. The involvement of RNaseH in the processing of the primer implies that some portion of the primer remains associated with its template strand after synthesis by RNA polymerase. These results indicate that formation of the primer RNA-DNA hybrid is an obligatory step for successful initiation _in vitro_, since very little replication occurs _in vitro_ if RNaseH is omitted from the reaction (Itoh and Tomizawa, 1980). The addition of RNA1 to the _in vitro_ replication system inhibits formation of the primer (Itoh and Tomizawa, 1980). This inhibition of primer formation by RNA1 could be a result of displacement of the primer RNA-DNA hybrid and reformation of the DNA duplex. With respect to this possibility, it is intriguing that both the ColE1 primer RNA and RNA1 originate from the same template region of ColE1. RNA1 is entirely complementary to the first 100 nucleotides of the 5'-terminal region of the primer. Conceivably, RNA1 could act by formation of an RNA-RNA duplex with the primer leading to displacement of the primer from its template strand. However, this idea seems inconsistent with our observation that a single nucleotide change in RNA1 generating a longer RNA1 species increases the copy number.

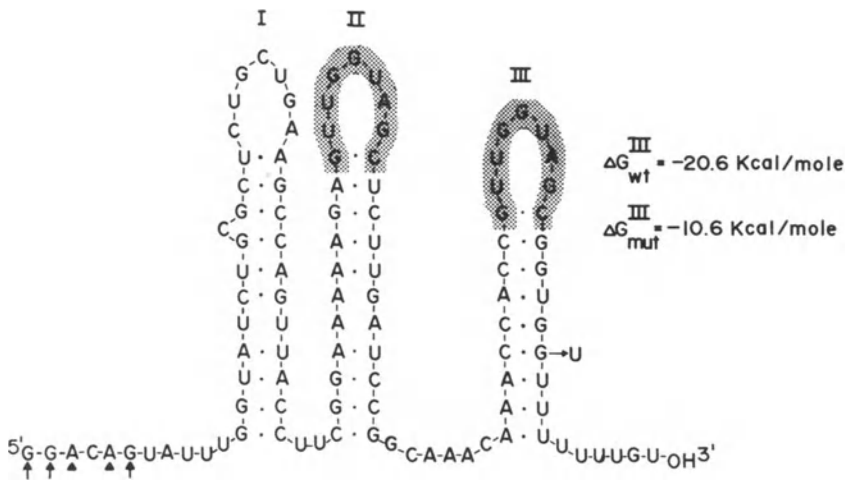


Fig. 3. A model for the secondary structure of RNA1, modified slightly from that proposed by Morita and Oka (1979). Loops II and III are 9bp direct repeats (shaded areas). The arrow denotes the location of the mutation in pOP1Δ6 encoded RNA1. Free energy estimates for the stability of stem III in wild-type and mutant RNA1 are shown. These were determined by the rules proposed by Tinoco et al. (1973). Triangles denote the two A residues from which initiation of RNA1 has been reported to occur (Chan, Lebowitz and Bastia, 1979; Morita and Oka, 1979).

The nucleotide sequence of RNA1 suggests a high degree of secondary structure (Fig. 3). Morita and Oka (1979) have obtained evidence by partial T1 ribonuclease digestion that a substantial degree of secondary structure exists in RNA1 and have proposed a model shown in Fig. 3. Especially striking is the presence of two 9bp direct repeated sequences which comprise loops II and III (Fig. 3). We estimate that the mutation in pOP1Δ6 lowers the stability of stem III from approximately -20.6 kcal/mole to -10.6 kcal/mole. However, at present, we do not know whether the mutation in pOP1Δ6 affects RNA1 function by altering the stability of stem III per se, by the altered secondary structure of the readthrough transcript relative to wild-type RNA1, or by altering the stability of RNA1 itself.

Acknowledgements

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TRANSPOSITION AND REARRANGEMENTS IN PLASMID EVOLUTION

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SUMMARY

Transposable elements participate in two classes of replicative recombination events: (i) full transposition; and (ii) genome rearrangements. Transpositions mobilize DNA sequences internal to the transposable element, while rearrangements also mobilize sequences external to the transposable element. We will illustrate several ways in which rearrangements mediated by IS1 and mutant Tn1 elements lead to the formation of new plasmid replicons. The effect of the Tn1/Tn3 *tnpR*$^+$ gene product leads to dissolution of new replicons formed by Tn1-mediated rearrangements. Replicons formed by IS1-mediated rearrangements are much more stable. This result indicates that IS1 employs a different pathway to full transposition. Thus, there are at least two classes of transposable elements in bacteria which play different roles in plasmid evolution.

INTRODUCTION

Structural rearrangements in plasmids play a key role in the history of bacterial populations. These rearrangements include insertions, deletions, and inversions. They occur spontaneously in nature and can be selected in the laboratory. Frequently, one rearrangement in a previously stable plasmid structure leads to further reorganizations until a new stable molecule has evolved. Many workers have observed such instability followed by stabilizing changes after *in vitro* insertion of DNA fragments into cloning vectors. When studying plasmid rearrangments it is advisable to remember that several recombinational events may have intervened between the first structural change and the final stable molecule.

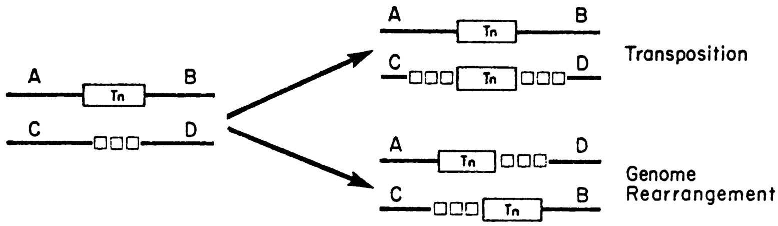

FIGURE 1. Replicative recombination events involving transposable elements (Tn) and target oligonucleotide sequences (boxes).[2]

Transposable elements constitute a group of highly developed agents for inducing plasmid rearrangements. Figure 1 shows two alternative recombinant structures arising from the activity of transposable elements. In full transposition, the element is inserted into the target DNA without otherwise grossly altering the linkage relationships of DNA sequences external to the transposable element. This recombination event is frequently seen with the acquisition of new drug resistance markers by plasmids. In genome rearrangements, the element serves as a kind of recombination sequence to join different regions of the genome and form two "reciprocal" recombinant structures. Such recombination events are responsible for replicon fusions and adjacent deletions (Figure 2).

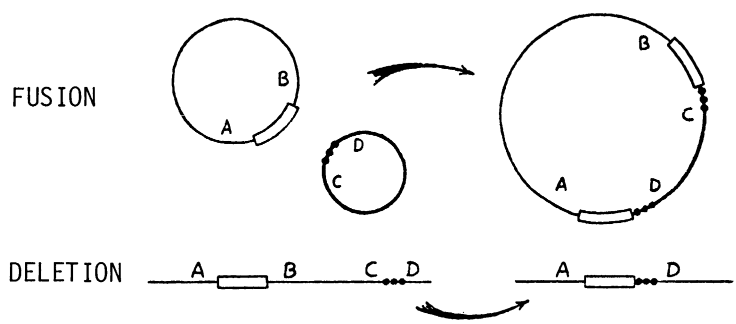

FIGURE 2. Replicon fusion (top) and adjacent deletion (bottom)— two particular examples of genome rearrangement[10].

As other papers in this collection document, replicon fusion plays an important role in conjugal mobilization of some Tra⁻ plasmids and in the formation of new hybrid plasmids. Both full transposition and genome rearrangements involve duplication of two sequences: the transposable element itself, and a short segment of the target DNA[1,2]. Sequence analysis has shown that the target duplication occurs by de novo replication and not by recombination with an homologous oligonucleotide adjacent to the transposable element (data summarized in reference 3). We have similarly shown that transposable element duplication is inherent to the recombination event and does not result from prior theta replication of the donor replicon[4].

Two important questions concern the relationship of full transposition to genome rearrangements: (i) Are the two recombination events related? And, if so, (ii) Is one event a precursor of the other? The answer to the first question is yes. For all elements where it has been studied, mutants defective in transposition are also defective in fusions, deletions, and inversions (see 2, 3 for summaries of the data). In the case of Tn3 and other related transposons, the answer to the second question is also yes because the rearrangement product is clearly a precursor to the full transposition product[2,5] (Heffron et al., Reed, and Schmitt et al. in this volume). Figure 3 illustrates the Tn3 pathway to full tranposition: the rearrangement is catalyzed by the tnpA gene product activity at transposon termini, and the rearrangement products recombine at an internal site, tnpS, in a Rec-independent event catalyzed by the tnpR gene product[6,7] (Heffron et al. and Reed in this volume). Thus, mutants lacking either tnpS or the tnpR gene product yield only rearrangement products in Rec^- cells. When $tnpR^-$ mutant cointegrates (from replicon fusion) encounter tnpR gene product they resolve to yield full transposition products.

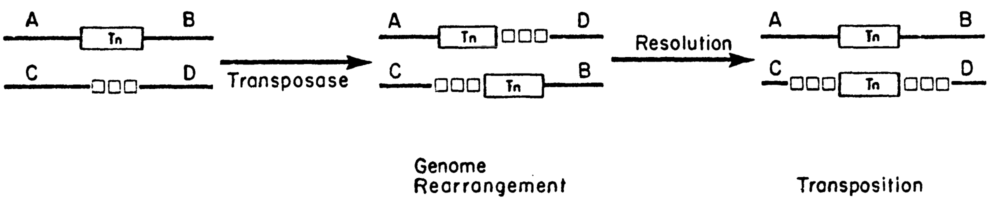

FIGURE 3. Pathway of Tn3 transposition.

Thus, we can discern two classes of transposable elements depending on whether they mediate one or the other class of recombination event. Elements that usually go through full transposition mobilize DNA internal to the transposable element. Elements that usually participate in genome rearrangements also mobilize DNA external to the transposable element. For elements similar to Tn3, the difference between these classes resides in the presence or absence of the second resolution step (Figure 3).

In this paper we concentrate on elements which mediate rearrangements and summarize studies tracing the formation of novel complex plasmid replicons by transposable element-specific recombination events.

CONVERSION OF A BACTERIOPHAGE INTO A PLASMID BY ADJACENT DELETION

The origin of the self-encapsidating plasmid pλCM is an example of adaptively significant genetic change brought about in vivo by a transposable element. In this case, the DNA molecule of temperate bacteriophage λcI857 was converted into a plasmid as a result of natural interactions with the composite transposon Tn9. Tn9 consists of two parallel copies of IS1 bracketing the structural gene for chloramphenicol acetyl transferase: IS1-cat-IS1[8].

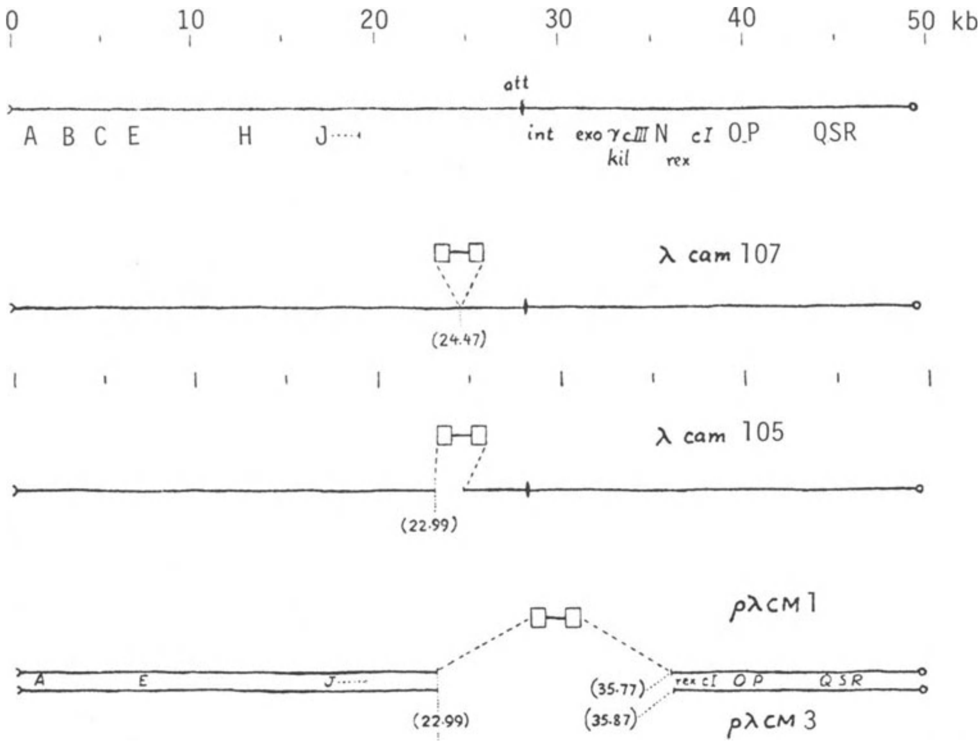

FIGURE 4. Origin of pλCM from λ (top) by Tn9 insertion (λcam107) and successive adjacent deletions (λcam105 and pλCM). Two independent isolates are shown. The coordinates of deletion end-points are given in kilobase positions on the λ map.

The initial insertion of Tn9 into a dispensable region of the λ sequence (Figure 4) produced a plaque-forming chloramphenicol resistant (Cm^r)-transducing phage λ cam107. A short adjacent deletion leftward from Tn9 increased the stability of Cm^r about threefold (λcam105). These phages transduce Cm^r with efficiencies around 10^{-3} per particle, consistent with normal expectations for λ. However, in λcam105 lysates, about one particle in 10^5 is found to transduce about 10^3 time more efficiently than the majority, that is at about 1 transductant/such particle. These particles are sensitive to antiserum, are lower in density than λcam105, impart neither λ immunity nor temperature sensitivity to the transduced cell, and do not form plaques on normal *E. coli* (Muster, MacHattie, Shah and Shapiro, *manuscript in preparation*). However, they can be grown to high titers in the presence of a "helper" λ phage, and helpers incapable of excising or packaging their own DNA can be used to produce pure transducing lysates.

Physical characterization of the particle DNA shows that the increased transduction efficiency results from a second adjacent deletion which removes all λ sequences rightward from Tn9 into the λ*N* gene (Figure 4; Muster et al., *manuscript in preparation*). The rest of the λ sequence from *cI* through DNA synthesis, lysis and structural genes remains intact. Covalently closed circular DNA of the same sequence can be extracted from transductants by standard plasmid DNA extraction methods. The high transducing efficiency is a property of λ phage particles which inject a stably-maintained plasmid DNA into the host cells. This entity has been named pλCM, as a λ-derived chloramphenicol resistance plasmid.

We have used pλCM as a convenient system to study transposition-related events. The plasmid can be synchronously introduced into a population of cells by infection and subsequently recovered free of other cellular DNAs by packaging. The pλCM DNA is 21% shorter than wild-type λ DNA, allowing it to accommodate insertions up to 13 kb and still remain packageable. In the case of composite structures of larger sizes, λ packaging serves as a powerful method to produce and select derivatives that have suffered deletions which reduce them to packageable size.

When pλCM and the defective Tn3-containing plasmid RSF1596[5] are together in the same cell, fusions between the two plasmids can be produced by two different mechanisms (Figure 5). When the system is complemented by a source of *tnpA* gene product, the most frequent fusion event is Tn3 mediated. In this case the novel joint of the fusion product is between direct repeats of the Tn3Δ596 and various points in pλCM (Muster et al., *manuscript in preparation*). The target sites are not entirely random, however: of four structures analyzed, two show the Tn3 inserted at or very close to one end of the Tn9 in pλCM. When the source of *tnpA* gene product is a derepressed *tnpA* (such as that in the Tn1ΔAp carried by RSF103)[5,6], Tn3-promoted fusions with pλCM are frequent: about 2% of the available pλCM molecules become carbenicillin resistant (Cb^r) per cell division cycle. The fusion activity is temperature sensitive, as expected, since *tnpA* gene product is temperature sensitive[9], dropping more than three logs in frequency between 32° and 42°.

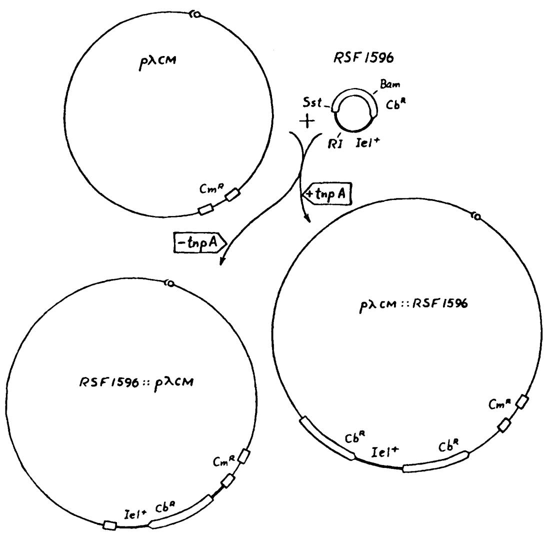

FIGURE 5. Pathways of replicon fusion between pλCM and RSF1596 mediated by IS1 (-tnpA) and Tn3Δ596 (+tnpA).

In the absence of Tn1/3 transposase, pλCM becomes Cb^r at much lower frequencies; between 10^{-7} and 10^{-8} per cell division at 32°. Structural analysis of these Cm^rCb^r molecules shows that fusion is mediated by IS1 activity. The novel joints are between the endpoints of one of the IS1s in Tn9 and various points in RSF1596, and a copy of IS1 is present at each joint between the pλCM and RSF1596 sequences (Figure 5; Muster et al., manuscript in preparation).

The cointegrate replicons produced by the two kinds of fusion events carry two distinct DNA replication systems: that of phage λ, and that of RSF1596 (the ColE1 replicator). The transduction behavior of pλCM::RSF1596 cointegrates demonstrates that both of these replication systems are functional: in contrast to pλCM, whose transduction efficiency drops to about 10^{-4} in the presence of λ repressor under Rec^- Red^- conditions, the pλCM::RSF1596 transduce lysogens as readily as nonlysogens. In contrast to RSF1596, whose ColE1 replicator requires DNA polymerase I, the cointegrates transduce <u>polA</u>$^-$ strains with about the same efficiency as <u>polA</u>$^+$ strains.

INCORPORATION OF A RESISTANCE GENE BY ADJACENT TRANSPOSITION

Tn1ΔAp is a deletion mutant whose genotype is <u>tnpA</u>$^+$, <u>tnpS</u>$^+$, <u>tnpR</u>$^-$ <u>bla</u>$^-$ [5,6,7]. We expect Tn1ΔAp to participate in rearrangements, but not full transposition events in Rec^- cells. The plasmid RSF103 contains Tn1ΔAp inserted into the sulfa resistance (Su^r) gene of RSF1010 adjacent to the streptomycin (Sm^r) gene[5,6] (Figure 6). This[10] plasmid, RSF103, was used to search for "adjacent transposition" of the Sm^r gene mediated by Tn1ΔAp. We envision adjacent transposition to occur by (i) adjacent deletion in RSF103 resulting in excision of a Tn1ΔAp-Sm^r DNA circle followed by its replicon fusion with a second plasmid, or (ii) replicon fusion of RSF103 with a second plasmid followed by an adjacent deletion of RSF1010 material. In either case, the final product should be the Tn1ΔAp-Sm^r-Tn1ΔAp composite "transposon" structure illustrated in Figure 6.

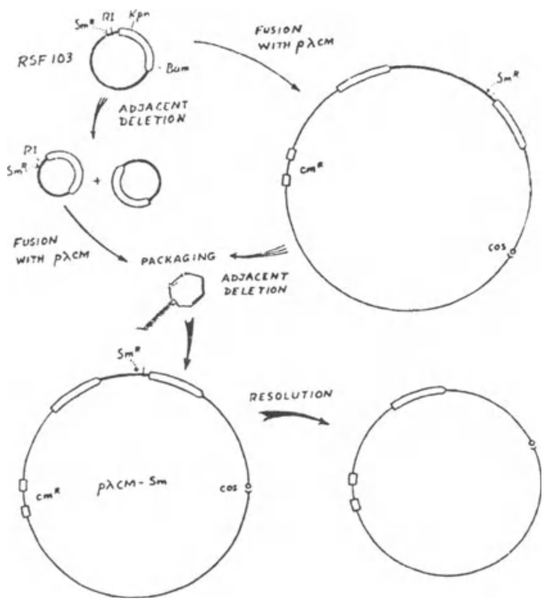

FIGURE 6. Adjacent transposition of Sm^r from RSF103 to pλCM by successive fusion and deletion events.

We used pλCM to infect recA$^-$ strains carrying RSF103 (or an RSF103-R388 cointegrate) and recovered pλCM by superinfection with a red$^-$ cI857 helper phage. These lysates were used to transduce recA$^-$ recipients. Cm^{r}Sm^r transductants were isolated (about 0.1-7% of all Cm^r transductants), and the Sm^r transposons were named Tn417-426 and are here referred to collectively as Tn-Sm. Physical characterization of pλCM::Tn419, pλCM::Tn422, and pλCM::Tn424 revealed the expected Tn1ΔAp-Sm^r-Tn1ΔAp structure inserted in pλCM (Figure 6; Muster et al., manuscript in preparation). pλCM::Tn421 appears to have the Sm^r transposon inserted into one of the IS1 sequences of Tn9. This illustrates a general tendency of transposable elements to insert in other transposable elements.

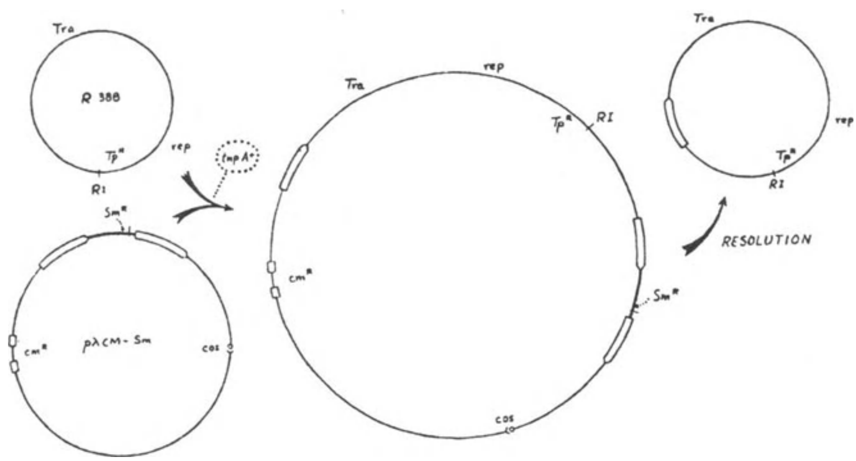

FIGURE 7. Formation and resolution of pλCM::Tn-Sm::R388 cointegrates in λ immune cells.

Since no tnpR gene product is made in Tn417-426, no full transposition is expected in Rec$^-$ hosts. To test this expectation, recA$^-$ λ lysogens carrying the plasmid R388 were infected with lysates containing pλCM::Tn419, 422 and 424. Sm^r transductants were recovered. These transductants should represent pλCm::Tn-Sm insertions into R388 since pλCM cannot replicate in the presence of a λ prophage. Transfer by conjugation of the trimethoprim resistance (Tp^r) of R388 into recA$^-$ recipients resulted in the cotransfer of Cm^r and Sm^r. Physical analysis of plasmid DNA from these exconjugants revealed structures consistent with replicon fusions of pλCM::Tn-Sm and R388 (Figure 7; Muster et al., manuscript in preparation). These plasmids appear stable in recA$^-$ hosts and are free to express λ immunity.

EFFECT OF RESOLUTION ACTIVITY ON Tn1ΔAp CONSTRUCTS

In the presence of *tnpR* resolution function, the pλCM::Tn-Sm structures are expected to resolve to give a single Tn1ΔAp insertion. RP1 and pMB8::Tn3 are plasmids carrying wild type Tn1 or Tn3 where *tnpR*$^+$ is expressed. When these plasmids are introduced into cells carrying pλCM::Tn-Sm or R388::pλCM::Tn-Sm, the expected resolution occurs. In the case of pλCM::Tn-Sm, addition of *TnpR*$^+$ results in the loss of Sm^r, and physical analysis reveals the expected pλCM::Tn1ΔAp structure (Figure 6). Likewise, in the case of R388::pλCM::Tn-Sm, addition of *tnpR*$^+$ results in loss of pλCM and Tn-Sm DNA, and the product recovered was R388::Tn1ΔAp (Figure 7). When pREG118 (*tnpA*$^+$ *tnpS*$^+$ *tnpR*$^-$ *bla*$^+$)[11] is added to strains harboring pλCM::Tn-Sm in a *recA*$^-$ background, no loss of Sm^r is noted. This experiment shows that the resolution is specific for the *tnpR* gene product. Resolution is extremely rapid (>95% complete in 40 minutes at 42^{o}C), and complete (<10^{-4} pλCM::Tn-Sm retained the Sm^r determinant after infection of a *tnpR*$^+$ cell) (Muster et al., *manuscript in preparation*). The observation that the resolution function does not establish an equilibrium between resolved and rearrangement structures is consistent with Reed's observations that only physically linked parallel *tnpS* sites are efficient recombination substrates for *tnpR* gene product (Reed, this volume)

DISCUSSION

We have described several *in vivo* isolations of plasmids/cosmids/phasmids generated by nonhomologous replicative recombination events mediated by transposable elements. Adjacent deletions and/or replicon fusions, not full transpositions, are the bases for these plasmid rearrangements. Our results therefore suggest that rearrangements rather than full transpositions mediate the formation of hybrid replicons.

In the case of Tn1/3 where *tnpR* gene product resolves hybrid molecules rapidly, transposon-bounded DNA such as Tn1ΔAp-Sm^r-Tn1ΔAp is highly unstable and cannot be mobilized. Here, we see that mobilization of internal sequences by full transposition and stable mobilization of external sequences by replicon fusion or adjacent transposition are mutually exclusive depending on the presence or absence of a functional *tnpR* gene.

Some other transposons do not exhibit the resolution-determined distinction between transposition and rearrangements. IS1-flanked sequences are highly stable, even in Rec$^+$ cells, compared to Tn1/3 flanked DNAs[12] (Muster et al., *manuscript in preparation*). Compound transposons such as Tn5 and Tn10 which are flanked by long inverted repeats do not show a high inversion rate even in Rec$^+$ cells (D. Berg, personal communication; N. Kleckner, personal communication).

These differences in character suggested that there must be more than one biochemical pathway for full transposition. It remains to be established whether the pathways to genome rearrangements are similar for these two classes of transposable elements.

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COMPLEMENTATION OF TRANSPOSITION FUNCTIONS ENCODED BY TRANSPOSONS Tn501(Hg^R) AND Tn1721(Tet^R)

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INTRODUCTION

Recent experiments have demonstrated that the two transposons Tn501 and Tn1721, which code for diverse resistance characters, have a continuous sequence of approximately four kilobases (kb) in common (Altenbuchner et al., 1981). This communication contains (i) descriptions of Tn501 and Tn1721 and (ii) an analysis of the four kb-homology region indicating that it encodes functions required for transposition.

THE Hg^R-TRANSPOSON Tn501

The 8.1 kb-transposon Tn501, originally found in *Pseudomonas aeruginosa*, confers inducible resistance to mercuric ions (Hg^R) and organomercurials on host cells (Bennett et al., 1978). The element is flanked by 38 base-pair inverted repeats and generates a five base-pair direct repetition of a recipient sequence at the site of insertion (Brown et al., 1980). Three *Eco*RI restriction sites, two located within the terminal repeats and one at 2.2 kb, divide Tn501 into a large (5.9 kb) and a small (2.2 kb) *Eco*RI fragment (Fig. 1).

The genes responsible for the Hg^R phenotype are probably homologous to the Hg^R determinant of plasmid R100-1 (R. Rownd, pers. communication). This latter has been studied in more detail by Nakahara et al.(1979) and by Foster et al.(1979). It comprises three genes: a regulatory gene (*mer*R), which exerts positive control over two structural genes responsible for the transport (*mer*T) and reduction (*mer*A) of mercuric ions. The minimum length of the

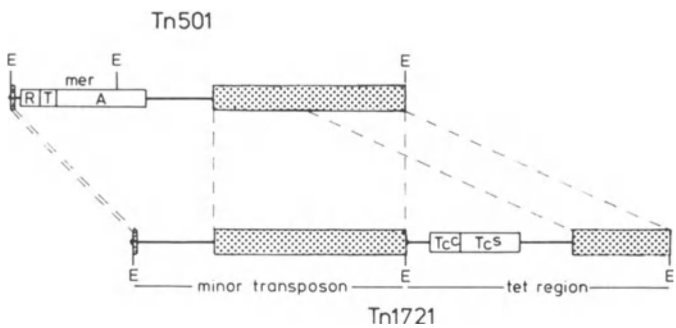

Fig. 1. Diagram of Tn501 and Tn1721 superimposed with respect to the four kb-homology region derived from Fig. 2. Shaded boxes connected by dashed lines indicate portions common to both elements. The location of resistance genes, as described in the text, is shown (mer operon: R=regulation, T=transport, A=reductase; tet genes derived from phenotypes of insertion mutants: Tc^C=constitutive expression of resistance, Tc^S=sensitive to tetracycline). The extent of the minor transposon and the repetitious tet region of Tn1721 and positions of EcoRI sites (E) are marked.

complete region is 2.6 kb; a single EcoRI site is located in merA and inactivation of merA renders cells supersensitive to mercurials (Hg^{SS}). Assuming that the mer operon of R100-1 is homologous to the Tn501-specified system, these results together with the previously established insertion map of Tn501 (Grinsted et al., 1978) locate the mer operon of Tn501 relative to the EcoRI site at 2.2 kb: merR is close to the left-hand inverted repeat (Fig. 1) and transcriptional polarity is from left to right. This assignment is corroborated by the presence of an AUG start codon at 0.2 kb followed by an open reading frame of at least 21 triplets with an "upstream" promoter containing Pribnow and Shine-Dalgarno sequences (Brown et al., 1980 and unpublished observations of these authors). Moreover, cloning of the small EcoRI fragment of Tn501 leads to the Hg^{SS} phenotype indicating, that this fragment contains merT and that cleavage with EcoRI inactivates merA. Since practically all of the small EcoRI fragment codes for Hg^R, transposon-coded transposition functions must reside in the large EcoRI fragment.

THE TET^R-TRANSPOSON Tn1721

Tn1721, originally identified as a constituent of R-plasmid pRSD1 in Escherichia coli, confers inducible tetracycline resistance (Tet^R) on its host (Mattes et al., 1979; Schmitt et al., 1979). The 11 kb transposable element consists of two distinct portions, the "tet region" (5.6 kb), which encodes resistance, and the "minor transposon" (Tn1722; 5.4 kb), which is capable of transposing independently of the rest of Tn1721 (Schmitt et al., 1981). The two portions are defined by three 38 base-pair repeats, two in direct and one in inverted orientation, as shown in Fig. 3. Each repeat contains an EcoRI restriction site. Translocation of Tn1721 leads to five base-pair direct repeats at the site of insertion (Schöffl et al., 1981). The inverted repeats are practically identical to those of Tn501 and are 50% homologous to those of Tn3 (Altenbuchner et al., 1981).

Heteroduplex analysis and Southern hybridisation have shown, that the Tet^R determinants of Tn1721 are homologous to those of RP1 and RP4, respectively (Mendez et al., 1980; Schmitt et al., 1981). Insertion mutagenesis using TnA has been applied to the tet genes of RP1 and mutants sensitive to tetracycline (structural genes) or constitutive for the expression of resistance (repressor gene) have been mapped in an 1.8 kb region (P.M. Bennett, pers. communication). These results and the presence of a single SalI and two SmaI sites in the respective regions permitted location of the tet genes on the map of Tn1721 (Fig. 1). Using a minicell system, we have identified two polypeptides of 34K and 26K, respectively, produced by Tn1721 after induction with tetracycline (K. Schmid, J. Altenbuchner and R. Schmitt, in preparation). Deletions leading to smaller derivatives of 34K are tetracycline sensitive indicating a major role of this species in tetracycline resistance. The function of the 26K polypeptide is still unclear.

The tet genes of Tn1721 are flanked by 1.9 kb direct repetitions, which provide the structural basis for recA-dependent amplification of the tet region (Schmitt et al., 1981). Up to nine tandem repeats of this region have been isolated from rec$^+$ cells. The amplified forms can be stably maintained in a rec$^-$ background. This property of Tn1721 has been used to analyse the relationship between gene dosage and tetracycline resistance of this particular system. The resistance levels conferred by plasmids containing from one to nine copies of the tet region have thus been tested in a recA host. It has been found, that in exponentially growing cells the uninduced level of resistance increases with gene dosage. Moreover, the rate at which maximum resistance is attained upon induction with tetracycline is proportional to the number of tet genes present in a cell. This positive gene dosage effect is a feature distinguishing the Tet^R determinants of Tn1721 and Tn10 (Jorgensen and Reznikoff, 1979; Coleman and Foster, 1981).

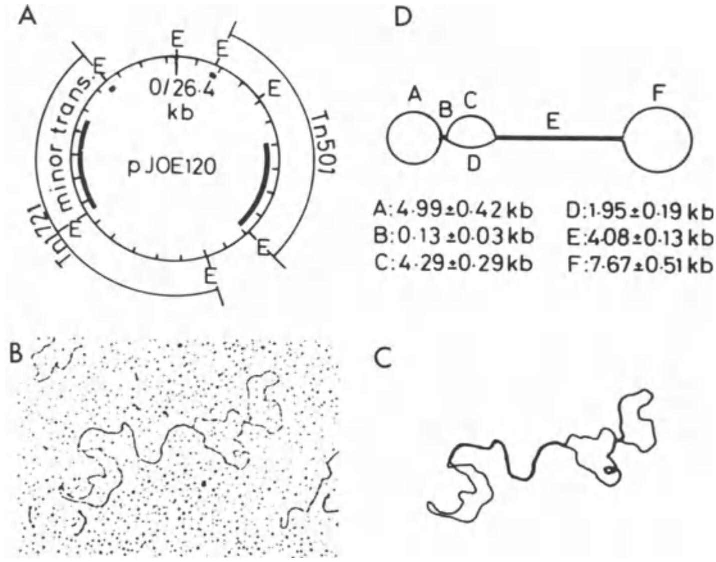

Fig. 2. Heteroduplex analysis of Tn501 and Tn1721. The double-recombinant plasmid pJOE120, which contains the two transposons in inverted orientation, was used for re-annealing experiments. (A) Physical map of pJOE120 showing positions of the transposons and indicating the regions of homology derived from Fig. 2B (E=EcoRI sites). (B) Electron micrograph of re-annealed single-stranded circular molecule of pJOE120 showing two duplexed regions of homology and three single-stranded loops. (C) Tracing of B (—— single-stranded, ▬▬ double-stranded DNA). (D) Diagram of C with assigments of single- and double-stranded regions. Their contour lengths (averaged from 20 independent molecules) are given below ± standard deviations.

Since the minor transposon can transpose independently of the rest of Tn1721 (Schmitt et al., 1981), transposon-encoded transposition function(s) must be part of this segment. The large EcoRI fragment of Tn501 contains the genes that encode the equivalent function(s) of this element (see above). This fragment and the minor transposon have a continuous sequence of about 4 kb in common, as demonstrated by heteroduplex formation within a double-recombinant plasmid containing both elements in opposite orientation (Fig. 2). Thus, the genes responsible for transposition coded for by Tn501 and Tn1721 are closely related suggesting to us the complementation experiments described below.

COMPLEMENTATION BETWEEN Tn501 AND Tn1721

The ampicillin resistance transposon Tn3 is one of the best-studied transposable elements (Heffron and McCarthy, 1979). Genetic analysis and sequence data have revealed, that the transposition of Tn3 requires the integrity of the terminal repeats and at least two transposon-encoded functions, "transposase" (coded for by tnpA) and "resolvase" (coded for by tnpR; Heffron and McCarthy, 1979). Cointegrate structures of the donor and recipient replicons containing directly repeated copies of Tn3 have been identified as intermediates in transposition; their resolution yields the two constituent replicons and requires site-specific recombination at an internal target sequence (the "internal resolution site" of IRS; Shapiro, 1979; Arthur and Sherratt, 1979). Transposition of Tn501 follows a similar sequence of events and the data below show, that there are similar genes coding for the functions required on Tn501 and Tn1721.

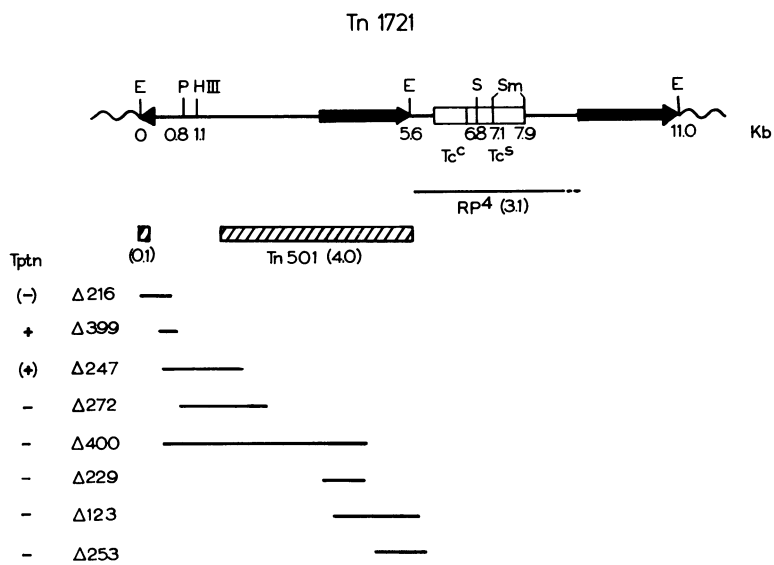

Fig. 3. Diagram of Tn1721 showing terminal and central repeats (arrowheads), extent of internal homology (heavy bars), approximate location of tetracycline resistance genes (Fig. 1) and restriction coordinates (E=EcoRI, HIII=HindIII; P=PstI; S=SalI; Sm=SmaI). Regions of homology with plasmid RP4 (line) and Tn501 (hatched boxes) are shown in kb. A set of deletions was constructed from pJOE105 (a Tn1721 recombinant with a derivative of pBR322; Schmitt et al., 1981). The extent of deletions is shown and their respective transposition profienciencies (Tptn) are indicated (transposition positive:+, reduced:(+), low:(-), negative:-).

Partial HaeII-digestion and subsequent ligation of pJOE105, a derivative of the high-copy vector pBR322 (Schmitt et al., 1981), were used to generate a set of deletions which extend into Tn1721 to various degrees, as diagramed in Fig. 3. The transposition proficiency of these deletions has been tested according to the scheme shown in Fig. 4B. Transposition products were analysed by genetic and restriction mapping. The following results were obtained (also indicated in Fig. 3):

(i) A deletion of the left-hand terminal repeat (Δ216) reduces transposition frequencies about 100-fold. In the ensuing rare transposition events the tet region is translocated alone. This indicates that the 38 base-pair repeats, which flank this region in direct orientation, serve as "secondary substrate" for the initial, transposase-catalysed step.

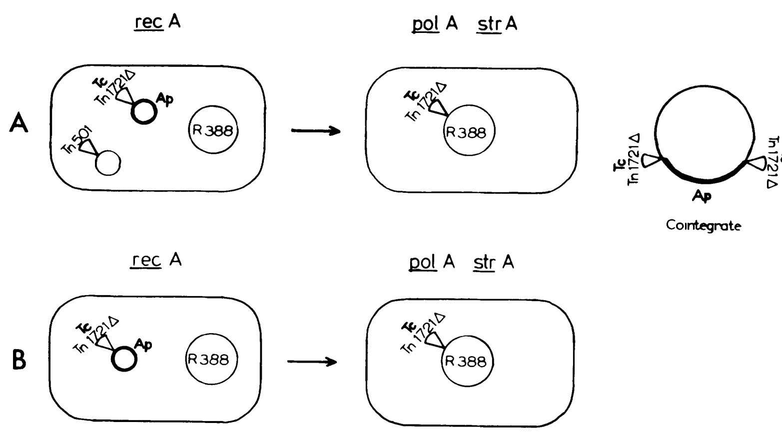

Fig. 4. Diagram of donor and recipient cells used in conjugal crosses to test the transposition proficiencies of various Tn1721(pJOE105) deletions (Tn1721Δ) in the presence (A) and absence (B) of Tn501 (Tc=tetracycline resistance; Ap=ampicillin resistance). The frequency of inter-replicon transposition of Tn1721Δ to R388 was determined in each experiment. Left: recA donor cells containing the conjugative plasmid R388 and transposons Tn501 (part of pACYC184 recombinant) plus various Tn1721Δ (from pJOE105; see Fig. 3) inserted into different compatible, non-conjugative high-copy vectors. Right: Selected exconjugant recipient cell containing R388 with Tn1721Δ inserted (polA mutation prevents the autonomous replication of high-copy vectors transferred as cointegrates). Far right: Structure of a cointegrate shown to be an intermediate of transposition.

Table 1. Complementation of Tn1721-Encoded Transposition Functions by Tn501[a]

Donor[b]	Tn501	Hg^{++}[c]	Transposition Frequency[d]	% Cointegrates[e]
pJOE105	-	-	5×10^{-3}	0 (8)
(wild type	+	-	1×10^{-2}	0
Tn1721)	+	+	3×10^{-2}	0 (12)
	-	-	1.6×10^{-3}	100
Δ247	+	-	4×10^{-3}	98
	+	+	1.5×10^{-2}	100
	-	-	0	-
Δ229	+	-	4×10^{-4}	0 (4)
	+	+	4.6×10^{-2}	0 (2)
	-	-	0	-
Δ272	+	-	3.6×10^{-3}	100
	+	+	3.5×10^{-2}	100
	-	-	0	-
Δ400	+	-	1×10^{-5}	100
	+	+	1.5×10^{-4}	100

[a]Conjugal crosses according to Fig. 4. Overnight cultures (30°) subcultured at 37° were mated during exponential growth for two hours and plated onto selective media.

[b]Plasmid designations according to Fig. 3.

[c]Where indicated, 60 μg/ml of merbromin were added to overnight cultures.

[d]Determined as the quotient of Tet^R transconjugants and total number of trimethoprim resistant transconjugants.

[e]Fraction of Tet^R transconjugants also showing ampicillin resistance (see Fig. 4).

(ii) With the exception of Δ247, deletions extending into the four kb-homology region cause a complete loss of transposition proficiency. Deletion 247, which extends between 0.5 and 0.7 kb into the homology region, reduces transposition frequencies about fourfold and leads to 100% cointegrates.

In a second series of experiments (illustrated in Fig. 4A) the possibility that Tn501 (supplied in trans) complements the deleted transposition function(s) of Tn1721 was tested (Table 1). The following results were obtained:

(i) Transposition frequencies of Tn1721 (pJOE105) and Δ247 are increased two- to threefold in the presence of Tn501. The transposition-deficient deletion mutants Δ229, Δ272, and Δ400 regain proficiency indicating that Tn501 is capable of complementing the deleted Tn1721-specified function(s).

(ii) The high proportion of unresolved cointegrates observed with Δ247, Δ272, and Δ400, but not with Δ229, suggests that the former three derivatives have lost a locus for site-specific recombination (IRS) and/or a gene coding for resolvase (tnpR) or that the resolvase of Tn501 does not complement that of Tn1721. This latter possibility is unlikely (experiments in progress).

It becomes obvious that the diffusible function(s) furnished by Tn501 in complementing Δ229, Δ272, and Δ400, respectively, is analogous to the tnpA product (transposase) of Tn3 (see above). The boundaries of the corresponding gene(s) are located between Δ247 (2.3 kb) and the central inverted repeat of Tn1721 (5.6 kb), with its left-hand terminus located within Δ272 (endpoint coordinate: 3.0 kb). The length of this region thus ranges between 2.6 and 3.3 kb, a size similar to that of the tnpA gene sequence of Tn3 (3 kb; Heffron and McCarthy, 1979).

A three- to 100-fold increase in transposition frequencies was observed, if donor cells were preinduced with mercurials (Table 1). Sherratt and coworkers (Kitts et al., 1981) have shown, that Tn501-specified resolvase requires the presence of Hg^{++}. This two sets of data are consistent with the following model: Hg^{++}-induced stimulation of the transposition functions involves transcriptional read-through from the mer operon into the tnp genes (Fig. 5). This assumes the gene order mer operon - tnpR - tnpA, all with the same polarity (left to right), so that the promoter activated by the merR gene product upon induction with Hg^{++} (Foster et al., 1979) would also be responsible for the transcription of tnp genes. Without induction, the tnpA gene is expressed, but to a much lesser extent (Table 1). It is therefore proposed, that the tnpA gene has a secondary promoter, which becomes apparent when the more efficient promoter of the mer system is inoperative. This would be reminiscent of the *E.coli* tryptophan operon (Jackson and Yanofsky, 1972).

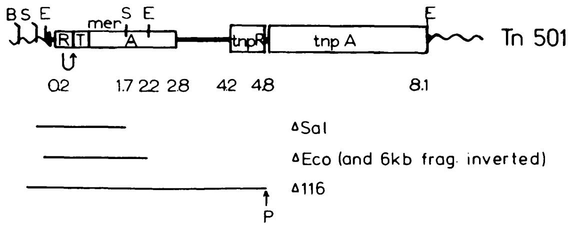

Fig. 5. Diagram of Tn501 with the mer operon located as in Fig. 1 and the transposition genes (tnpR=resolvase, tnpA=transposase) located in the homology region (see text). Relevant restriction sites and coordinates (in kb) are marked (B=BamHI, E=EcoRI, P=PstI, S=SalI). The extent and configuration of three deletions (ΔSal, ΔEco, Δ116) are indicated.

Table 2. Complementation of Δ229 with Deletions of Tn501[a]

Donor	Tn501 Derivative[b]	Transposition Frequency
Δ229	Tn501	4×10^{-4}
	ΔSal	3.3×10^{-3}
	ΔEco	3.5×10^{-4}
	Δ116	2.5×10^{-3}

[a]Test conditions as described in Table 1.

[b]Deletions shown in Fig. 5.

Based on these assumptions, we have tested three deletions that lack the mer control region. These are shown in Fig. 5. The transposition frequencies of Δ229 promoted by these Tn501 deletions are listed in Table 2. Two of these deletions (ΔSal and Δ116) show an unexpected six- to eightfold stimulation of transpositon proficiency compared to the control (Tn501), whereas a third deletion (ΔEco) with an additional inversion of the large EcoRI fragment shows a frequency close to that of the control. In line with the model above, these data are interpreted in terms of an external promoter to the left of Tn501 fused to the tnp genes by deletions ΔSal and Δ116, respectively, but ineffective upon inversion of the fragment containing the tnp genes (ΔEco). A pACYC184::Tn501 recombinant has been used in these experiments, and the tet promoter on pACYC184 (Chang an Cohen, 1978) is in the right relative position to act as transcriptional start.

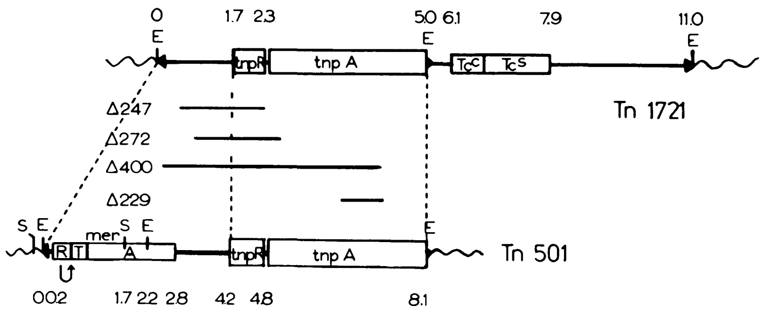

Fig. 6. Diagram showing a comparison of Tn1721 and Tn501. Resistance determinants and genes required for transposition are located as in Figs. 1, 3 and 5, respectively. Regions common to both elements are indicated by dashed lines. Relevant deletions in Tn1721 used to define the location of resolvase (tnpR) and transposase (tnpA) are drawn as in Fig. 3 (see text for details).

A view of Tn501 and Tn1721 in line with the experimental evidence is diagramed in Fig. 6. It shows the two transposable elements superimposed with respect to their homology regions. Genes specifying resolvase (tnpR) and transposase (tnpA) comprise this region, their dimensions being in close agreement with the data published for the corresponding genes specified by Tn3. Unlike Tn3, the tnpR and tnpA genes of Tn501 and Tn1721 are thought to have identical transcriptional polarity. It should be noted, that Tn3 is unable to promote transposition of Tn501 (D.J. Sherratt, pers. communication). Whereas Tn501-specified tnpR is transcribed from an external mer promoter, the tnpR gene of Tn1721 has its own promoter, presumably located outside the homology region. Deletion mapping places the IRS sequence into the left-hand portion of this region, possibly between genes tnpR and tnpA.

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THE STRUCTURE OF TN5

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INTRODUCTION

Tn5 is a transposable genetic element which encodes resistance to aminoglycoside antibiotics such as kanamycin and neomycin. This resistance results from the synthesis of the enzyme neomycin phosphotransferase type II (NPTII, also named aminoglycoside 3'-phosphotransferase-II)(Berg et al., 1978). Its structure is in general similar to other transposons with two inverted repeats 1534 bp long (Auerswald and Schaller, 1980) flanking a unique central region approximately 2700 bp long (Berg et al., 1975; Jorgensen et al., 1979).

Tn5 is a possible model system for studying the genetic control of antibiotic resistance and for examining the transposition process. With this in mind, my laboratory has pursued studies aimed at defining the genetic organization of Tn5. Our experiments (some of which have been described elsewhere; Jorgensen et al., 1979; Rothstein et al., 1980a; Rothstein et al., 1980b; Rothstein and Reznikoff, 1981; Johnson and Reznikoff, manuscript in preparation) were directed at asking the following questions:
(1) How many proteins does Tn5 encode?
(2) Where is the gene for NPTII (subsequently called "neo")?
(3) Where is the neo promoter?
(4) Where is (are) the gene(s) for the diffuseable transposition function(s)?
(5) Where is (are) the transposition function(s) promoter(s)?
(6) Are either of these two sets of promoters regulated and, if so, how?

Although these questions have not been answered in full, the analyses have generated an overall picture of the genetic organization of Tn5 which is schematically presented in Fig. 1. Our approach towards elucidating this structure has been to:

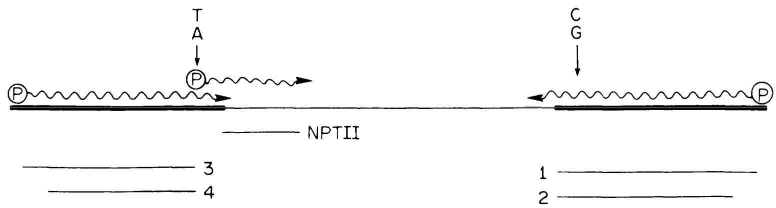

Fig. 1. The Genetic Organization of Tn5. The location of the inverted repeat and neo promoters, the proteins encoded by Tn5 and the single bp mismatch are presented. Protein #1 and possibly protein #2 are required for the transposition process. This figure was first presented in Rothstein et al., 1980b.

(1) Determine the restriction enzyme cleavage map of Tn5.
(2) Knowing this map, use recombinant DNA techniques to derive Tn5 mutations (some of these are shown in Fig. 2).
(3) Analyze the neomycin resistance, transposition and protein coding properties of these mutant DNA's.
(4) Analyze RNA polymerase binding and transcription properties of defined restriction fragments.
(5) Perform specific sequence analyses.
The results of these studies are described below.

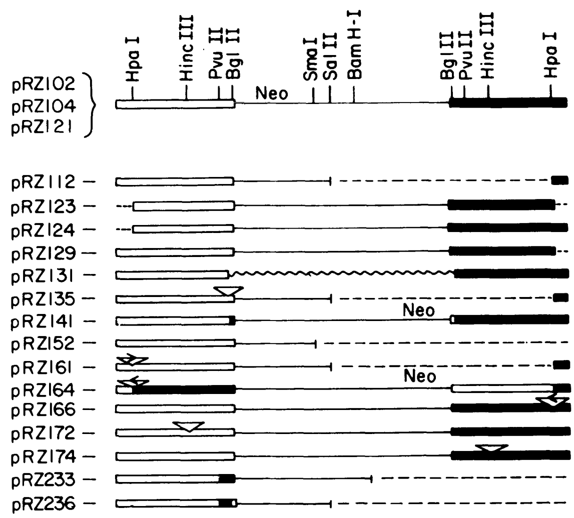

Fig. 2. Tn5 Mutants. The constructions and analyses of these mutants are described in Jorgensen et al. (1979), Rothstein et al. (1980 a & b) and Rothstein and Reznikoff (1981). The symbols are defined as follows: ▭ = "left" inverted repeat DNA, ▬ = "right" inverted repeat DNA, ∿ = substitution, ▽ = insertion (with arrowhead indicating orientation of promoter if present), and ---- = deletion.

RESULTS

I. The NPTII Gene.

The gene for NPTII can be localized to a 960 bp region between the left inverted repeat BglII site and the SmaI site in the unique central region. This was determined by examining the neomycin resistance phenotype and NPTII protein coding properties of various mutant Tn5 DNA molecules (see Jorgensen et al., 1979; Rothstein et al. 1980a & 1980b). The particular mutants of interest for this determination are pRZ112, pRZ135, pRZ152 and pRZ172 described in Fig. 2. The relevant results are presented in Table 1. Plasmids which contain mutations defining the 1275 bp HincIII-Sma I fragment (pRZ112, pRZ152 and pRZ172) encode normal levels of neomycin resistance and the NPTII protein. Plasmid pRZ135 (which carries an insertion in the BglII site) encodes very low but significant levels of both. Its properties can most easily be explained by hypothesizing that the BglII insertion mutation has separated the neo gene from its promoter; a hypothesis which will be verified below. This genetic placement for the NPTII structural gene has been confirmed by the Auerswald-Schaller sequence analysis which localized the NPTII translation initiation codon 34 bp inside from the BglII cut site (Auerswald and Schaller, 1980).

The neo promoter is located between the PvuII and BglII sites in the left inverted repeat and the equivalent sequence from the right inverted repeat can not perform this function when correctly positioned (Rothstein et al., 1980 a & b; Rothstein and Reznikoff, 1981). The results which first suggested that the neo promoter was located within this region were the observations that insertions into the BglII site or deletions up to the BglII site drastically reduced the level of neomycin resistance and the synthesis of the NPTII protein (for example see the results for plasmid pRZ135 in Table 1 which were mentioned above), whereas comparable mutations at the left HincIII site (pRZ172) had no affect. Furthermore, the HincIII-BglII fragment which precedes this cut

Table 1. Neomycin Resistance Levels of Tn5 Mutants.

Plasmid	EOP_{50} (µg/ml neo)
pRZ102	90
pRZ112	75
pRZ135	2
pRZ141	10
pRZ172	130
pRZ236	<1/6 of $Tn5^+$ in same vector

(Results are from Rothstein et al (1980 a & b) and Rothstein and Reznikoff (1981). pRZ236 is carried on a different vehicle and its EOP_{50} is indicated relative to wild type Tn5 in the same vehicle.)

site binds RNA polymerase in a specific, heparin resistant fashion as would be expected for a fragment which contains a promoter (Rothstein et al., 1980a).

The dissimilarity between the two inverted repeats was discovered by analyzing the neomycin resistance properties of mutants such as a BglII inversion mutation (the neomycin resistance level of pRZ141 is drastically reduced, see Table 1), and a construct which substitutes the right PvuII-BglII region for the left PvuII-BglII region (pRZ236 encodes a low level of neomycin resistance). These results not only suggested that the two inverted repeats are different in this region, but also indicated that this was an important target for DNA sequence analysis. A DNA sequence determination of 130 bp containing this region was performed (and was independently confirmed by Auerswald and Schaller, 1980) and a single base pair difference between the two inverted repeats was detected (see Fig. 3). In Fig. 4 a portion of this sequence is compared to the model promoter sequence of Rosenberg and Court (1979). A good match can be achieved if one assumes that the single bp mismatch occurs in the highly conserved "Pribnow Box" region of the promoter.

II. Inverted Repeat Functions.

Each inverted repeat is known to encode two proteins with different N termini but otherwise largely shared sequences although the two repeats differ from each other in that the right inverted repeat proteins extend further at their C termini than the left proteins. As shall be shown below this difference in the C termini has a functional affect (one or both of the right inverted repeat proteins is (are) required for transposition while neither of the left proteins are required for transposition), and is due to the single bp mismatch described above. (Rothstein et al., 1980 a & b; Rothstein and Reznikoff, 1981).

Fig 5 presents an example of several minicell experiments in which the protein coding properties of different Tn5 mutations were examined. These are summarized in Table 2. In the particular

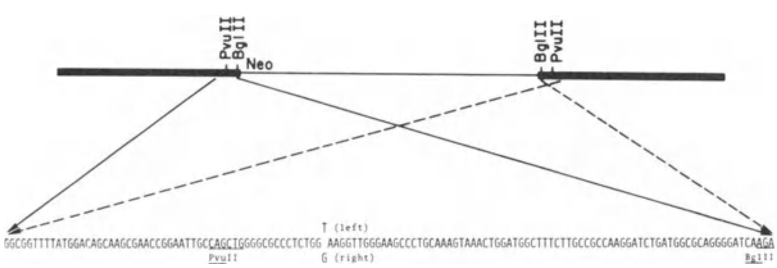

Fig. 3. DNA Sequence of Left and Right Inverted Repeat PvuII-BglII Regions. This 130 bp sequence was determined by both Maxam -Gilbert (1977) and Sanger et al. (1977) protocals as was described in Rothstein and Reznikoff (1981).

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Proposed Neo Promoter

CCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAA
                                |  bp 1442 difference
                                ↓
                                G

Model Promoter Sequence

tt--tgTTGACA-ttt-------atttgtTATAATg---cat------aa
aa   c       cca       g gtg  ag  a    tg       gt
cc           aa        t cc       t
```

Fig. 4. Proposed neo Promoter. Within the sequence described in Fig. 3 is a sequence which resembles the model promoter sequence described by Rosenberg and Court (1979). This model sequence is indicated and its similarities to the proposed neo promoter is shown by underlinings.

experiment shown in Fig. 5, deletions up to the left inverted repeat HpaI site and up to the right HpaI site are compared to the protein coding functions of normal Tn5. This type of experiment reveals that Tn5 encodes 5 proteins; NPTII and proteins 1, 2, 3 and 4. Deletion up to the left HpaI site (or insertions into that site) abolishes synthesis of protein 3 and reduces synthesis of protein 4. This is similar to all other left inverted repeat mutations in that only these two proteins are affected but is different in that the other mutations abolish synthesis of both proteins (see Table 2). The deletion mutation up to the right inverted repeat HpaI site (or insertion into that site) abolishes synthesis of protein 1 and reduces synthesis of protein 2 (other right inverted repeat mutations abolish synthesis of both 1 and 2). The results of this and other similar experiments indicate the coding localization for proteins 1, 2, 3 and 4 shown in Figs 1 and 6, and suggest that their genes are oriented in the following manner. Genes for proteins 1 and 3 have their N terminal coding sites situated between the outside Tn5 edges and the HpaI sites and proteins 2 and 4 have their N terminal coding sites located slightly inside of the HpaI sites.

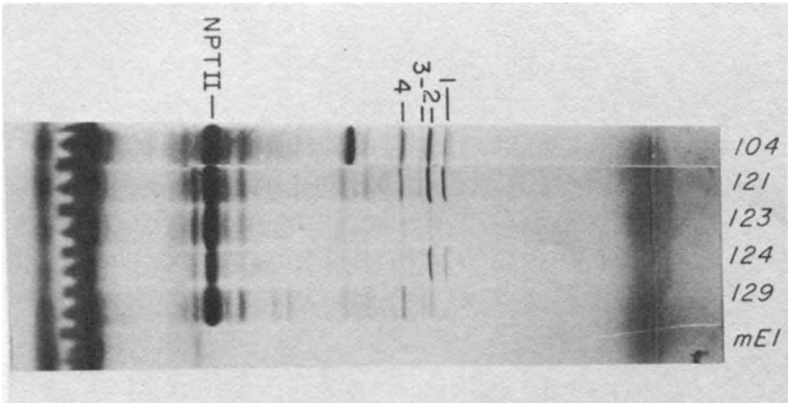

Fig. 5. Proteins Encoded by Tn5 Deletion Strains. Minicells containing the indicated plasmids were labeled with 35-S-methionine and their extracts were analyzed by polyacrylamide gel electrophoresis as described previously (Rothstein et al., 1980). Plasmid mE1 is pVH51.

Table 2. Inverted Repeat Coding Properties.

Plasmid	Tn5 Inverted Repeat Proteins Found 1	2	3	4	% W.T. Transposition
pRZ102	+	+	+	+	100
pRZ112	-	-	+	+	<0.5
pRZ112(SupB)	n.d.	n.d.	n.d.	n.d.	103
pRZ121	+	+	+	+	n.d.
pRZ123	-	<	-	<	n.d.
pRZ124	+	+	-	<	n.d.
pRZ129	-	<	+	+	n.d.
pRZ131	-	-	+	+	n.d.
pRZ164	-	<	+	+	<0.5
pRZ166	-	<	+	+	<0.5
pRZ172	+	+	-	-	27
pRZ174	-	-	+	+	<0.5
pRZ233	+	+	-	-	n.d.

(The minicell protein coding properties (+=normal levels, <=reduced levels, -=non detectable) and transposition frequency data come from Rothstein et al (1980 a & b) and Rothstein and Reznikoff (1981). Transposition data for pRZ131 is available in Berg et al 1980. n.d. means not determined.)

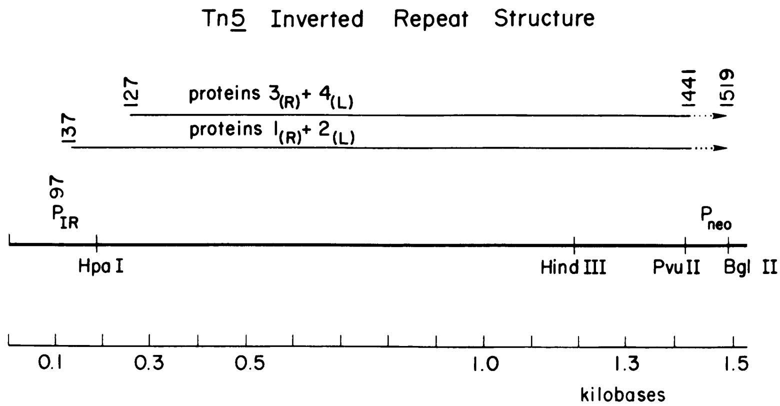

Fig. 6. Structure of Tn5 Inverted Repeats. The locations of the promoters and translation start and stop signals were positioned on the Auerswald-Schaller (1980) sequence using results summarized in the text.

The DNA sequences outside of the HpaI sites are known to contain promoters because fragments from these regions specifically bind RNA polymerase and program transcription which proceeds inward towards the unique region (Rothstein et al., 1980a). From size measurements of full length transcripts made from restriction fragments which carry this region, an RNA start point was approximately located at 95 bp from the end of each inverted repeat. The exact start point has been identified as bp 97 (Johnson and Reznikoff, in preparation) based upon the following types of evidence. The transcript initiates with a 5' pppA residue (transcription requires a high concentration of ATP but not GTP, CTP or UTP and the transcript is labeled with $\gamma^{32}P$ labeled ATP). RNase T1 digestion of $\gamma^{32}P$ ATP labeled mRNA indicates that the first G is at position 8. The oligoribonucleotides synthesized during the abortive initiation process (described in Munson and Reznikoff, 1981) are pppApA, pppApApC and pppApApCpU. The inverted repeat promoter sequence (for both left and right inverted repeat promoters) is shown in Fig 7.

Knowledge of the promoter location allows one to unambiguously select the correct translation start sites for the inverted repeat proteins in the Auerswald-Schaller (1980) sequence. Proteins 1 and 3 must start with the GUG codon corresponding to bp 137-139. This is the only translation initiation codon subsequent to the transcription start site which is in the open reading frame and which is prior to the HpaI cut site. Proteins 2 and 4 must start with the AUG on the other side of the HpaI site at positions bp 257-259. These assignments fit with the apparent molecular weight

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Tn5 Inverted Repeat Promoter Sequence

TCTGACTTCCATGTGACCTCCTAACATGGTAACGTTCATGATAACTTCTGCT
 ......                                    AACUUCUGCU
 CTGACTCTT outer end                       IR mRNA
 CTGTCTCTT inner end

Model Promoter Sequence

tt--tgTTGACA-ttt-------atttgtTATAATg-----cat------aa
aa   c       cca       g  gtg  ag  a      tg        gt
cc           aa        t  cc       t
```

Fig. 7. The Inverted Repeat Promoter. The exact inverted repeat mRNA start point was determined as described elsewhere (Johnson and Reznikoff, in preparation). Also described are the similarities to the Rosenberg and Court (1979) model sequence and to the small inverted repeats found at the ends of each Tn5 inverted repeat.

estimates for the proteins and explain why mutations at the HpaI sites prevent synthesis of proteins 1 and/or 3 but not 2 and 4. A detailed picture of the Tn5 inverted repeat structure is presented in Fig 6.

The protein coding properties of Tn5 derivatives carrying substitutions of the internal BglII fragment (such as pRZ131) suggest that the C termini of proteins 1 and 2 are encoded beyond the right BglII cut sites (this substitution fails to synthesize proteins 1 and 2 instead making fusion peptides) while the C termini of proteins 3 and 4 is before the BglII cut site (Rothstein et al 1980 a & b, Rothstein and Reznikoff, 1981). The observation that plasmid pRZ233 (see Table 2 and Fig 2) encodes proteins 1 and 2 (Rothstein and Reznikoff, 1981) localizes the C terminus of proteins 3 and 4 to the region substituted in this plasmid, the region between the left inverted repeat PvuII and BglII sites.

These conclusions have been confirmed by the DNA sequence analyses (Auerswald and Schaller, 1980; Rothstein and Reznikoff, 1981). The single bp T/A → G/C difference at bp 1442 (see Fig. 3) creates an in phase ochre codon in the left inverted repeat missing on the right where the first in phase nonsense codon is at position 1520-1522 (with the BglII cut site being between bp 1517 and 1518).

The differences in the inverted repeat protein C termini have interesting functional implications. This is apparent from the transposition tests summarized in Table 2. Mutations in the right inverted repeat (pRZ112, pRZ174, pRZ166) prevent transposition while those in the left repeat (pRZ172) lower the level of transporition but do not prevent its occurrence. In these and all other mutants discussed below, the failure to synthesize protein 1 is always correlated with a failure to transpose. This important functional difference between the two inverted repeats can be localized to the same region in which the neo promoter, the difference in C terminal coding capacity and the single bp mismatch are located by virtue of the following types of observations:
(1) Mutations which substitute the left inverted repeat PvuII-BglII region for the right PvuII-BglII region and vice versa always invert the functional polarity of the inverted repeats. For instance plasmid pRZ164 carries a HpaI inversion and a HpaI site insertion in the "new" left inverted repeat. This mutant Tn5 fails to transpose and fails to synthesize protein 1 as would be expected for right inverted repeat HpaI site insertion.
(2) Introduction of some right inverted repeat mutant plasmids (such as pRZ112) into an supB (ochre suppressor) genetic background suppresses the transposition defect (Table 2).

Thus the twenty six additional amino acid residues at the C terminus of protein 1 (or of proteins 1 and 2) must play an essential role in some transposition related function.

DISCUSSION AND CONCLUSION

Our analysis of Tn5 structure has led to a fairly complete picture as is summarized in Fig. 1 and for which details are presented in Figs 4, 6 and 7. In spite of this general understanding, several rather obvious questions are outstanding such as:
(1) How did the overlap of the neo promoter and the left inverted repeat evolve?
(2) What is the enzymatic function of protein #1 in transposition?
(3) Does protein #2 play a role in this process?
(4) Why do left inverted repeat mutations have a partial affect on transposition?

There are also some less obvious questions that have arisen from our studies which I shall describe below.

The studies of Biek and Roth (1980) have suggested that the Tn5 transposition process may be regulated. The sequence of the inverted repeat promoter (Fig 7) suggests a possible mechanism of autoregulation for inverted repeat protein biosynthesis. Doug Berg's laboratory (Berg et al., 1980) has discovered that the right inverted repeat can transpose by itself and that the two ends of the inverted repeat are related by a small inverted repeat (CTGACTCTT...AAGAGACAG) (D. Berg, personal communication; note that this implies that the "true" or "functional" end of the large inverted repeat is at bp 1533 not 1534) with only 1 mismatch. Presumeably this is a sequence which must be recognized by the Tn5 transposase (protein 1 or 1+2). As indicated in Fig 7, the inverted repeat promoter sequence contains a 6 out of 9 bp match with this sequence offering a target site at which the transposase could block the transcription of its own mRNA.

The ochre suppressor studies mentioned above yielded a positive suppression result for most but not all right inverted repeat mutations. Specifically, the transposition defect in pRZ166 was not suppressed. This raises the possibility that the fusion peptide made in pRZ166 acts as a negatively complementing protein or that the production of proteins 1 and 2 must be in the correct ratio for a positive transposition phenotype and this suppressed mutant provides an excess of protein 2.

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TRANSPOSITION OF THE INVERTED REPEATS OF Tn5

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INTRODUCTION

The defining characteristic of bacterial transposons is their ability to move to new loci in the absence of extensive DNA sequence homology. Some, designated IS elements, contain only the genes and sites necessary for their own transposition; other transposons are composites of genes necessary for transposition and genes for auxiliary traits (such as antibiotic resistance or virulence)[1]. Although the mechanism of transposition is unknown, plausible models involving breakage and reunion[2] or replication[3,4] have been proposed.

We are studying kanamycin resistance transposon Tn5 (Fig. 1) because this element transposes at high frequency, exhibits low

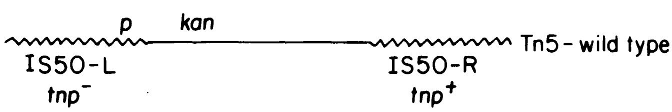

Fig. 1. The functional organization of transposon Tn5. Tn5's inverted repeats are indicated by serrated lines. The gene for transposase (designated tnp^+) is present in Tn5's right repeat (IS50-R). A single base pair change 1442 bp from the outer end of the left repeat renders the left repeat's tnp gene nonfunctional and creates a pro moter (p) used for expression of the kan^r gene[5,10-12].

specificity in the choice of new insertion sites, serves as a good model for analyses of transposition mechanisms, and is a useful molecular genetic research tool[2,5-9]. It is 5700 bp long, contains 1534 bp long inverted repeats[5,10], and is not homologous to any sequences normally present in the chromosome of Escherichia coli K12[13]. A gene whose product is necessary for transposition (transposase) is present in Tn5's right repeat. The left repeat contains a nonfunctional allele of the transposase gene, inactivated by a single base pair change[10-12,14,15].

The mutations Tn5 creates when it inserts into new sites are polar regardless of Tn5's orientation[6]. These insertion mutations revert by excision of Tn5 in $recA^-$ as well as in $recA^+$ cells. Excision of Tn5 is unrelated to transposition, depends on the inverted orientation of Tn5's repeats, but not Tn5's tnp gene, and has been hypothesized to occur by copy errors during DNA synthesis[15,16].

Because of Tn5's structural similarity to several transposons known to contain terminal repeats of IS elements[1] we had hypothesized that each of Tn5's repeats might be transposable[13]. The analyses summarized here confirm that each of Tn5's repeats is an IS element which we have named IS50. Our analyses also suggest functional differences between the two ends of the IS50 element, and spatial constraints on the action of transposase.

RESULTS

We tested three predictions of the hypothesis that each of Tn5's repeats is an IS element: 1. The inverted orientation of Tn5's repeats should not be essential for transposition. 2. Each of Tn5's repeats should transpose from Tn5 to other DNA segments. 3. Tn5 should mediate "inverse transposition".

1. A Tn5 element with direct repeats can transpose.

The Tn5-DR2 element (Fig. 2) was generated to test whether the inverted orientation of the repeats in Tn5-wild type is essential for transposition. We reversed the orientation of Tn5's right repeat in a pBR322::Tn5 plasmid DNA molecule because only the right repeat encodes a functional transposase, and thus might have evolved less than Tn5's left repeat. The plasmid used (Fig. 2) contains Tn5 inserted near position 3,200 bp of pBR322, oriented such that Tn5's right repeat is nearest pBR322's amp^r gene[15,17,18]. Its DNA was digested with BamHI, and the DNA fragments were ligated, and used to transform competent cells to a Amp^r Kan^r Tet^s phenotype. The structure expected (Fig. 2) was verified by restriction endonuclease analysis. Studies reported elsewhere indicate that Tn5-DR2 transposes with a frequency and specificity similar to that of Tn5 wild type[15,17]. Thus a Tn5 derivative with direct repeats can transpose, and the inside end of Tn5's right repeat can join to other DNA sequences, as predicted by the view that these repeats are IS elements.

2. Detection of the IS50 transposon.

The transposition of one of Tn5's repeats to pBR322 would provide a direct demonstration that the repeat is a transposon. Our selection for insertion of IS50 into pBR322 was based on the finding that conjugal transfer of pBR322 mediated by fertility factor F is associated with the insertion of the γ-Δ IS sequence of F into pBR322[19]. We found that FΔ::Tn5, an F derivative lacking all transposons except Tn5, mediated the transfer of pBR322's amp^r tet^r traits at a frequency of about 10^{-10}. Approximately two-thirds of the transfered plasmids encoded kan^r, were 10 kb long, and contained new restriction endonuclease cleavage sites indicative of the insertion of the 5.7 kb Tn5 element into 4.4 kb pBR322. Although most of the remainder contained no detectable insertion, one fourth of them were 5.8 kb in size. Restriction endonuclease analyses of six independent 5.8 kb isolates showed that each contained an insertion of one of Tn5's repeats at a different site in pBR322[20]. We have named this new transposon IS50[15,20].

We constructed λ lysogens of strains carrying each pBR322::IS50 plasmid, induced phage development in these lysogens, and found λ Amp^r Tet^r transducing phage at a frequency of 10^{-8}. Genetic and restriction endonuclease

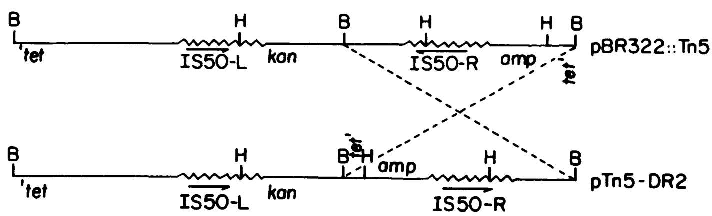

Fig. 2. Construction of a Tn5 derivative with direct repeats by BamHI digestion and ligation of pBR322::Tn5. The plasmid DNA molecule is depicted in linear fashion, arbitrarily cut at the BamHI site (B) in pBR322's tet^r gene[17].

analyses showed that these phages contained insertions of pBR322 bracketed by direct repeats of IS50. DNA sequence analysis of one of the pBR322::IS50 plasmids demonstrated that it contained IS50-R (the Tn5 repeat which encodes transposase)[15,20]. In control experiments in which pBR322 lacking IS50 was used, the frequency of λ transducing phage containing pBR322 sequences was less than 10^{-10}. These results are in accord with findings that the presence of Tn3 and of IS1 in plasmids converts these plasmids to transposons[20,21].

We determined the junction sequences at three sites of IS50 insertion into pBR322, and found the following: (i) The transposed IS50 element extends from nucleotide position 1 to position 1533 in the 1534 bp long sequence[10] of Tn5's inverted repeats; (ii) no pBR322 sequences are deleted by insertion; (iii) 9 bp of pBR322 target sequence are duplicated directly by insertion of IS50; (iv) the ends of IS50 consist of a 8 bp interrupted inverted repeat (Fig. 3)[20].

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5'CTGACTCTTATACACAAGTA·····AGATCTGATCAAGAGACAG
  GACTGAGAATATGTGTTCAT·····TCTAGACTAGTTCTCTGTC5'
  outside end                       inside end
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Fig. 3. The termini of IS50. The 7 bp interrupted inverted repeats are indicated by horizontal lines[20].

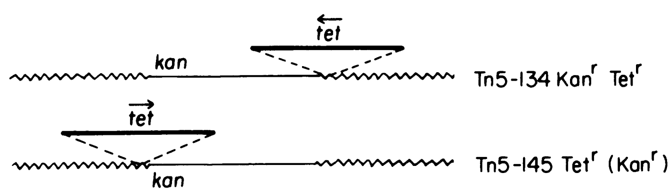

Fig. 4. The sites and orientations of tet^r insertions in Tn5. These tet^r genes are present in a 2700 bp BglII fragment derived from Tn10 and are inserted into BglII sites 1515 bp from the outside ends of Tn5[10]. Although in Tn5-134 the insertion appears to be within the structural gene for transposase (which terminates at position 1519)[10,11,15], the Tn5-134 encoded transposase is active[15,16]. In Tn5-145 the tet^r segment is inserted between the kan^r gene and its promoter, and causes a partially Kan^s phenotype. However, cells carrying Tn5-145 become Kan^r when grown in 1 μg/ml tetracycline, apparently because of induction of transcription in the tet^r segment and readthrough the kan^r gene[15,20].

To determine if Tn5's left repeat could transpose, as predicted from the finding that the IS50 sequence is contained within the left as well as the right repeat, we marked Tn5's repeats by insertion of tet^r genes (Fig. 4), and selected the transposition of tet^r to phage λ; the resulting λ Tet^r phage were scored for Kan^r. Six percent of the Tet^r phage resulted from transpositions involving Tn5-145 (tet^r left repeat), and 22% of the Tet^r phage which resulted from transpositions involving Tn5-134 (tet^r right repeat) were Kan^s. Restriction endonuclease digestions confirmed that Tet^r Kan^s phage resulted from transposition of just the marked IS50 element, and that Tet^r Kan^r phage resulted from transposition of the entire Tn5 element[20]. Thus, although each repeat can transpose as a separate unit, transposition of the entire Tn5 element seems to be preferred.

3. Tn5 mediated inverse transposition.

Inverse transposition in which the inside termini of a transposon's repeats join to new DNA sequences[23] would provide another demonstration that Tn5's repeats are IS elements. We have detected inverse transposition in two ways:

First, we induced λ::Tn5 prophages in cells carrying pBR322 plasmids, and selected Amp^r Tet^r transducing phages. They were Kan^s, and restriction endonuclease anayses showed that they had resulted from the insertion of λ into pBR322 using the inside ends of Tn5's inverted repeats (Fig. 5).

In the reciprocal approach, we induced phage development in a lysogen harboring pBR322::Tn5 as a stable dimer (Fig. 6). Amp^r Tet^r transducing phage were selected, and fell into

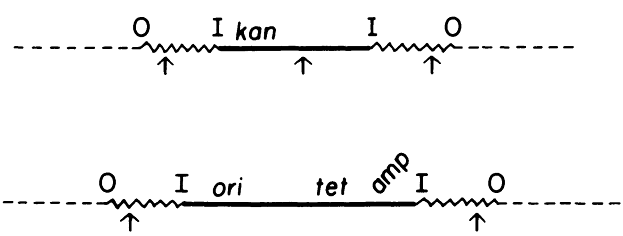

Fig. 5. λ::Tn5, and the product of its insertion into pBR322. Dashed lines represent λ sequences; O and I represent "outside" and "inside" ends respectively of IS50; Vertical arrows indicate XhoI cleavage sites.

three classes: The Kan^s Amp^r Tet^r phage expected following simple inverse transposition comprised approximately 1% of the selected phage. The remaining 99% of Amp^r Tet^r phage were Kan^r, and contained three copies of IS50 in either of two arrangements (Fig. 6).

We used restriction endonuclease digestion to determine if the insertion of pBR322::Tn5 sequences into λ could use IS50-L as well as IS50-R. As shown in Fig. 6, BamHI digestion should generate internal fragments whose size (4450 or 5600 bp) indicates which IS50 element had been used. The results of these digestions showed that in 20 of 21 cases analyzed IS50-R mediated the joining of pBR322::Tn5 sequences to the λ genome; in only one of 21 cases was the insertion mediated by IS50-L (see Fig. 7). Thus, IS50-R appears to transpose much more efficiently than IS50-L.

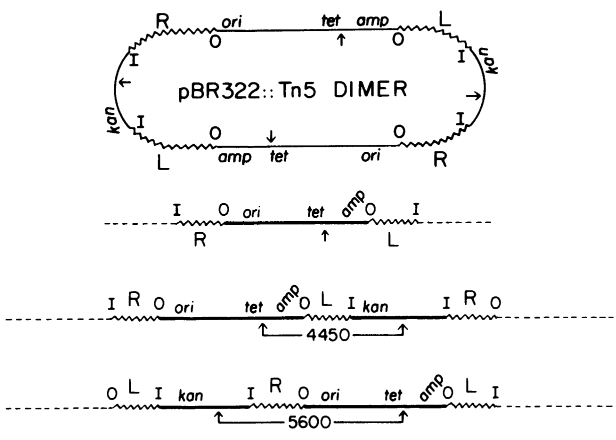

Fig. 6. Structures of the pBR322::Tn5 dimer and of the Amp^r Tet^r and Amp^r Tet^r Kan^r genomes resulting from transposition to phage λ. Solid lines, pBR322 sequences; dashed lines, λ sequences; serrated lines, inverted repeats of Tn5; vertical arrows, BamHI cleavage sites. 4450 and 5600 refer to distances in base pairs between the indicated BamHI cleavage sites[17].

DISCUSSION

Our experiments establish that each of Tn5's long terminal inverted repeats is a transposon which we have named IS50. The ends of IS50 comprise an interrupted 8 bp inverted repeat likely to constitute at least part of the transposase recognition site. Like Tn5[10], IS50 inserts into many sites and generates duplications of 9 bp of target DNA sequences. Pairs of IS50 elements often transpose together, carry with them any intervening genes, and thus are the building blocks of composite transposons such as Tn5.

The transposition of Tn5-DR2, of pBR322 bracketed by direct repeats of IS50-R, and of pBR322::Tn5 sequences containing direct repeats of IS50 all show that composite elements with direct repeats can transpose. Thus, there is no longer reason to suspect[24] that the inverted orientation of Tn5-wild type's repeats might play a specal structural role in transposition.

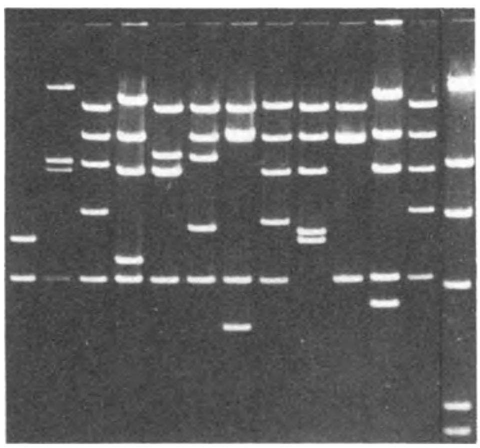

Fig. 7. BamHI digestion of λ Amp^r Tet^r Kan^r phage DNAs generated by pBR322::Tn5 transposition. The left most lane contains BamHI digested pBR322::Tn5. The right most lane contains a HindIII digested λ DNA standard (fragment sizes 22.8, 9.8, 6.4, 4.2, 2.2 and 1.8 kilobases). The λ Amp^r Tet^r Kan^r genomes replicate in cells as plasmids, and were extracted from $recA^-$, immune transductants as described previously[15,17]. The phage genomes in lanes 2-8 and 10-12 resulted from transposition involving IS50-R (Fig. 6, line 3). The phage in lane 9 resulted from transposition involving IS50-L (Fig. 6, line 4).

Although our data show that each of Tn5's repeats can transpose, the data also indicate that the outside end of IS50 (see Fig. 5) is more active than the inside end, and that IS50-R which encodes transposase (see Fig. 1), is more active than IS50-L which does not encode a functional transposase. The strongest indication of inequality of IS50's ends comes from experiments with tet^r insertion elements Tn5-134 and Tn5-145 (see Fig. 4). Even though IS50-R is used in preference to IS50-L, (see Fig. 6 and 7) we found that IS50-R (tet) transposed at only one-third the efficiency of the entire Tn5-134 element. Thus IS50-R's inside end is less active than IS50-L's outside end. The inequality between IS50's two ends can be understood by postulating that the 8 base pairs common to both ends (Fig. 3) constitute a weak transposase recognition site, and that additional sequences unique to the outside end make it a strong recognition site.

The analysis of Amp^r Tet^r Kan^r phages resulting from pBR322::Tn5 transposition showed that IS50-R was used more frequently than IS50-L in transposition (Fig. 6 and 7). Although control experiments to show that preferential use of IS50-R is independent of the position and orientation of Tn5 in pBR322 remain to be completed, we believe that these results indicate that transposase acts preferentially on the DNA segment which encodes it. Independent support for this interpretation comes from findings that the complementation of transposition deficient derivatives of Tn5 by Tn5-wild type is inefficient[25]. Preferential action of transposase on the IS50 element which encodes it can be understood if recognition of IS50's ends is a property of a domain in the amino terminal region of transposase, and if the growing transposase polypeptide generally folds to form this domain prior to completion of transcription and translation of the tnp message. A similar cis action of transposase on the unrelated Tn903 transposon has also been postulated[26]. We suspect that the tendency of IS50's transposase to act on the DNA segment encoding it may have minimized selective pressures to evolve a stronger recognition site at IS50's inside end.

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HOST FUNCTIONS REQUIRED FOR TRANSPOSITION OF Tn5 FROM λ b221 cI857 rex::Tn5

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SUMMARY

By assaying transposition of Tn5 from λ b221 cI857 rex::Tn5 (Berg,1977)(abbreviated as λ::Tn5) in PolA-proficient and deficient cells, both DNA polymerase and 5' to 3' exonuclease activities of DNA polymerase I of *Escherichia coli* K12 have been shown to be required for transposition of Tn5. Such a requirement could not clearly be observed in three other experiments in which the transposon donor replicon had existed in cells before transposition was assayed presumably because a hypothetical repressor-regulated protein encoded by the transposon itself rather than DNA polymerase I became rate-limiting in the overall transposition process. One polA mutant was found among more than 50 transposition-deficient mutants isolated by the λ::Tn5 method. Preliminary experiments also suggested that several host functions related to DNA repair or recombination were involved in determining the frequency of transposition of Tn5.

INTRODUCTION

DNA segments which move to various sites are defined as transposable elements. The insertion sequence,IS, is the most simple and contains no known determinants unrelated to insertion function. The transposon is more complex than IS and contains genes such as antibiotic resistance or toxin determinants in addition to those for transposition or its regulation(see reviews by Kleckner,1977; Starlinger,1980; Calos and Miller,1980). Considering the genetical as well as medical importance of these elements the transposon has extensively been studied. Among many observations reported the following two seem to be the most important. First, DNA sequencing technology has demonstrated that insertion of a transposable element into a new site results in duplication of a 5 to 12-base pair sequence at the target site (Calos et al.,1978; Grindley,1978; Johnsrud et al., 1978; Oka et al.,1978; Schaller,1978; Cohen et al., 1978; Kühn et al.,1979; Ghosal et al.,1979; Habermann et al.,1979). Secondly, transposition accompanies duplication of the transposable element itself, leaving one at the original site(Ljungquist and Bukhari,1977; Bennett et al.,1977; Gill et al.,1978; Klaer et al., 1980). Although the functions encoded by the element itself have been shown necessary for transposition and to be under repression control in several representative transposons(Gill et al.,1979; Chou et al., 1979; Meyer et al., 1979; Rothstein et al., 1980), these two observations suggest that host-encoded DNA repair and replication functions may also play important roles.

In this paper we describe that both DNA polymerase and 5' to 3' exonuclease activities of DNA polymerase I in *E. coli* seem to be important determinants of the frequency of transposition of Tn*5* from λ::Tn*5* to the chromosome. A similar requirement could not be observed in any experiment in which the transposon donor replicon had existed

Table 1. Reduced Transposition Frequencies of Tn*5* from λ::Tn*5* to the Chromosome in DNA Polymerase I Deficient Cells

Strain Code	*polA*	Transposition Frequencies (X 10^{-3})
YC256	+	3.4 ± 1.1
WA5023	*polA1*	0.3 ± 0.1
W3623	+	0.42
HI97	*polA11*	0.051
KS463	+	0.20
RS5064	*polA* ex2 (Ts)	0.004

Stationary cultures of the strains listed were infected with λ::Tn*5*, allowed 15 min at 30C for adsorption followed by 30 min for phenotypic expression and then plated on agar containing kanamycin sulfate at 75 mcg/ml. Incubation of plates was at 30C for 2 days. For the thermosensitive 5' to 3' exonuclease mutant and its parent, growth was at 37C before phage addition to minimize the residual activity.

in cells before transposition event was assayed. The reason will also be discussed why the requirement could be shown only in the former but not in the latter methods to assay the frequency of transposition.

RESULTS

PolA - Mutation Decreases the Frequency of Tn5 Transposition from λ::Tn5 to the Chromosome

Three DNA polymerase I mutants and their respective parents were examined for their ability to produce kanamycin resistant colonies when infected with λ::Tn5. Mutants polA1 and polA11 are defective only in their polymerase with about 1 % residual activity, whereas the mutant polA ex2 (Ts) is thermosensitive with respect to 5' to 3' exonuclease remaining 3 % of the wild type activity at 30 C. For the DNA polymerase mutants, incubation was at 30 C, whereas the 5' to 3' exonuclease mutant was grown at 37 C before the λ::Tn5 infection to minimize the residual activity but subsequently kept at 30 C to prevent thermal induction of the phage due to thermosensitive cI repressor. Under the conditions employed, the frequencies of appearance of kanamycin resistant colonies per viable cell were always lower with polA mutants than with their wild type parents after 2 days incubation (Table 1).

In order to exclude the possibility that the rate with which cells express the kanamycin resistance phenotype is dependent on DNA polymerase I activity, the time of shaking at 30 C for phenotypic expression after λ::Tn5 infection and before selection on kanamycin plates was altered within the range of 0 to 60 min. In all other experiments phenotypic expression was allowed by shaking for 30 min at 30 C after adsorption. As shown in Fig.1A, the number of kanamycin resistant colonies per viable cell was larger in PolA-proficient cells shaken for 30 min than in PolA-deficient cells shaken for 60 min. Thus, such a possibility seems to be unlikely.

During prolonged incubation of kanamycin agar plates the number of kanamycin resistant colonies increased but those derived from PolA-proficient strains were always more than those from PolA-deficient (Fig.1B). As the growth rate of these strains, YC256($polA^+$) and WA5023(polA1) were similar without kanamycin, we believe that comparisons may be made at the same period of incubation time for kanamycin resistant colonies too.

As described by Berg(1977), the majority of these kanamycin resistant colonies was not immune to λ , indicating that they had been formed by transposition of Tn5 to the chromosome. This was also verified by our previous observation of various chromosomal locations of Tn5 among these colonies(Sasakawa and Yoshikawa,1980).

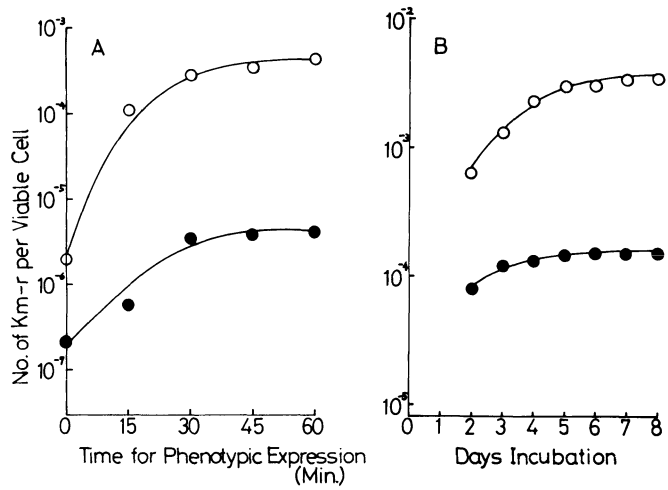

Fig.1. The Effect of the Time for Phenotypic Expression and for Incubation of Selective Agar Plates

Fig.1A; After cells were infected with λ::Tn5, 15 min incubation at 30 C was allowed for adsorption followed by shaking at 30 C for various periods of time to rule out a possibility that PolA-deficiency affects the time required for the phenotypic expression of kanamycin resistance. Fig.1B; Experiments were performed as described in Table 1 but the plates were continued to observe for 8 days. Each circle represents averages of two independent experiments. Open and closed circles are for YC256($polA^+$) and WA5023(polA1),respectively.

To rule out the possibility that λ multiplication rather than transposition is dependent on DNA polymerase I, adsorption rate, relative efficiency of plating, host cell killing and burst size as determined by the one step growth experiment by the use of λ::Tn5 at 30 C were examined and no difference was detected between PolA-proficient and deficient strains in any of these parameters, indicating that phage λ::Tn5 produced progeny at a similar rate in both of them.

When the multiplicity of infection of λ::Tn5 was changed to infect a constant number of bacteria, kanamycin resistant colonies

Table 2. Effect of polA Mutation in Three Established Experimental Systems of Tn5 Transposition

Expt.	Transposon Donor Replicon	Transposon Target Replicon	polA	Frequencies of Tn5 Transposition
I	Established R100-1::Tn5	Exogenously Infected λbb	+	4.6 ± 1.0 (X 10^{-10})
			-	3.3 ± 1.0
	Established R388::Tn5	Exogenously Infected λbb	+	3.9 ± 1.1 (X 10^{-10})
			-	3.1 ± 0.3
II	Prophage λbb::Tn5	Established R100-1	+	1.3 (X 10^{-5})
			-	1.3
III	pSC101::Tn5	R100-1	+	4.1 (X 10^{-6})
			-	6.2
		pMY1011	+	2.3 (X 10^{-6})
			-	1.1

Expt.I; Tn5 transposition from plasmids to exogenously infected phage λ b515 b519 cI857 Sam7(abbreviated as λbb). λbb grown and heat induced in PolA-proficient and deficient strains carrying R100-1::Tn5 or R388::Tn5 were used to transduce C600 to kanamycin resistance. Expt.II; Tn5 transposition from a prophage λbb::Tn5 to R100-1. PolA-proficient and deficient λbb::Tn5 lysogens were infected with R100-1 and the plasmid was transferred by selection with either kanamycin or chloramphenicol. Expt.III; Tn5 transposition from a nonconjugative to conjugative plasmids. To T6 resistant, PolA-proficient and deficient intermediate recipients carrying pMY0019(pSC101::Tn5), R100-1 or pMY1011 was transferred, followed by lysis from without by T6 and subsequent re-transfer by the membrane filter method to the final recipient resistant to rifampicin and T6. Selection was on agar containing both rifampicin and either kanamycin or tetracycline. It was later confirmed that the majority of kanamycin resistant colonies did not show incompatibility phenotype to pSC101::Tn3, indicating that they were unlikely to be formed by mobilization of the nonconjugative plasmid or by co-integration of the donor and the target replicons.

appeared at a constant frequency per phage and always lower in PolA-deficient than in proficient strains.

Unclear Effect of PolA-Deficient Mutation When Examined in Cells Established with the Transposon Donor Replicon

For convenience we describe the following three experiments as established experimental systems because we believe the difference between these and the λ::Tn5 method described above being due to the repressor encoded by the transposon itself, although separate regulatory protein has not yet been identified for Tn5.

The first established experiment(Expt.I, Table 2) was to grow λbb(abbreviation see Table 2) in PolA-proficient and deficient strains carrying R100-1::Tn5 or R388::Tn5 and resulting lysates were used to transduce C600 to kanamycin resistance. There was no clear difference in the frequencies of transposition from preexisting conjugative plasmids to exogenously infected λbb between PolA-proficient and deficient hosts.

The second established experiment(Expt.II, Table 2) was to lysogenize PolA-proficient and deficient cells with λbb::Tn5 to which R100-1 was transferred. The resulting strains were used as the donor to transfer the plasmid to the final recipient. No difference was observed in the frequencies of transposition from the prophage λbb::Tn5 to R100-1 between PolA-proficient and deficient lysogens.

The third established experiment(Expt.III, Table 2) was to transform PolA-proficient and deficient cells with pMY0019(pSC101::Tn5), to which R100-1 or pMY1011(a super-derepressed mutant of an Iγ plasmid R621a(Sasakawa and Yoshikawa,1978) was transferred as the target. After killing the initial donor by lysis from without by T6, the mixture was used as the secondary donor to the final recipient by the membrane filter method. No appreciable difference was observed between PolA-proficient and deficient hosts in the frequencies of transposition from a nonconjugative to a conjugative plasmid.

A Transposition-Deficient Mutant Is Phenotypically Similar to PolA-Deficient Mutants

By a method essentially based on the λ::Tn5 method more than 50 transposition-deficient mutants were isolated and characterized (Uno,Sasakawa and Yoshikawa, manuscript in preparation). One of them was shown to be similar to polA mutants as judged by the phenotypes listed in Table 3.

DISCUSSION

The results reported here seem to indicate that both DNA poly-

Table 3. Characteristics of a Transposition-Deficient Mutant Isolated by the λ::Tn5 Method

Strain Code	Transposition[b] Frequencies	Sensitivity		E.O.P.[c] of λ::Tn5	Transformation[d] of	
		UV	λ red3		pMY1113	pMY0019
C600	1.0	1.0	S	1.0	1.0	1.0
118-3[a]	< 1.2×10^{-3}	10^{-4}	R	0.78	< 10^{-3}	0.41
YC256	1.0	1.0	S		1.0	
WA5023	8.8×10^{-2}	10^{-4}	R		< 10^{-4}	

[a]A mutant 118-3 is one of more than 50 transposition deficient mutants of C600. Results on YC256 and WA5023 were added in the Table for comparison.
[b]Transposition frequencies were calculated based on the experiment as shown in Table 1.
[c]Efficiency of plating of λ::Tn5 relative to the parent as the indicator cells.
[d]pMY1113(Sasakawa and Yoshikawa,1980) is a mini-ColE1, pAO3(Oka et al.,1978) inserted by Tn5. pMY0019 is a Tn5 inserted derivative of pSC101.
All the figures were expressed in relative to the respective parents. S and R represent sensitivty and resistance to the phage indicated.

merase and 5' to 3' exonuclease activities of DNA polymerase I are important determinants of the frequency of Tn5 transposition from λ::Tn5. However, there was no effect of polA mutation in the established experiments where a transposon donor replicon had existed before introduction of the target. The frequency of Tn5 transposition to the chromosome has been known to be 10^{-2} to 10^{-3} and the highest among any method so far reported(Berg,1977). These observations may be explained as follows.

It has been well known that the transposon codes for at least two transposition-related proteins by itself(Gill et al.,1979; Chou et al.,1979;Meyer et al.,1979; Rothstein et al.,1980). The most extensively investigated transposon is Tn3 which codes transposase, another enzyme responsible for site-specific recombination for resolution of the cointegrate composed of the donor and target replicons(personal communication) and an autoregulatory repressor controlling transcription of the transposase operon. Similar regulatory mechanisms have recently been reported for Tn5(Biek and Roth, 1980), Tn10(Beck et al.,1980),Tn1721(this meeting) and an IS-like element, γδ(this meeting). Under established system as defined above, the transposase operon, if any, is repressed and hence the transposase activity itself may be rate-limiting in overall transposition process. This is in accord with the observation, for example, in Tn3 that a mutation within the repressor locus is phenotypically expressed as the actual increase in the frequency of transposition(Gill et al.,1979;Chou et al.,1979).

On the other hand, *polA* mutants ordinarily have some residual activity and no *polA* deletion mutants have so far been isolated (Kornberg,1980). Furthermore, there may be compensation of PolA functions by other related enzymes, although our preliminary results have shown that a *polB* mutation in addition to *polA* exhibits no additional effect on transposition. These factors may result in apparent inability of the *polA* mutation to be rate-limiting in overall transposition process and transposase may still be rate-limiting.If the transposase operon is derepressed phenotypically, as in classical examples of zygotic induction of λ repressor(Jacob and Wollman, 1956) or the lactose operon repressor(Pardee et al.,1959) and high frequency of plasmid transfer in conjugation(Stocker et al.,1963), then the consequence may be the same as a genetically derepressed mutation within the repressor locus of a transposase. Infection of bacteria with an integration-defective λ::Tn5 may result in the phenotypic derepression of the transposase operon although transiently. Under such a condition the transposase activity is no longer rate-limiting in overall transposition process in a *polA* mutant and the effect of the *polA* mutation is now manifested.

What is then the role of DNA polymerase I ? The current models for transposition(Grindley and Sherratt,1978; Shapiro,1979;Arthur and Sherratt,1979) assume DNA repair synthesis for gap filling and DNA replication for duplication of the transposable element itself. In this connection it is interesting that both polymerase and 5' to 3' exonuclease activities of DNA polymerase I seem to be required for transposition of Tn5. In our preliminary experiments several known mutants related to DNA repair or recombination, such as *uvr*, *recB* and *lon*(*capR*) have been shown to be concerned with transposition of Tn5 in the λ::Tn5 system. Furthermore, among more than 50 transposition-deficient mutants isolated we found one *polA* mutant. This supports our view that DNA polymerase I is an important determinant of the frequency of transposition of Tn5. It is also intersting that the majority of these mutants are not thermosensitive in spite of the fact that we isolated them at 30 C. This indicates that many functions coded by the chromosome are involved in transposition but not essential for cell growth.

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PLASMID MOBILIZATION AS A TOOL FOR IN VIVO GENETIC ENGINEERING

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INTRODUCTION

Mutagenesis through the insertion of transposons has proved to be an invaluable technique for mapping the genes of complex plasmids[1]. No selection for a mutant phenotype has to be devised, but a straightforward selection for the antibiotic resistance markers, encoded by the transposon, is sufficient to identify the presence of a mutant plasmid.

One general method[2] for performing this type of mutagenesis uses plasmid conjugation, one of the techniques used originally to identify transposons[3]. Transformation and transduction also are efficient methods for isolating mutant plasmids, but are primarily restricted to small plasmids in Enterobacteriaceae. Conjugal transfer remains the method of choice for large plasmids and for most Gram-negative bacteria. Since all plasmid cloning vector and the majorities of naturally occurring plasmids are autotransferable (*tra*$^-$) or are repressed for transfer, we assessed to possibilities of plasmid mobilization. It is possible to transfer such plasmids to a new host with the aid of some conjugative episomes.

This mediated transfer may take place by any one of three mechanisms[4].

(a) The nonconjugative plasmid may contain an origin of transfer (*ori*T) and an activation site (*bom*) but lack trans-acting functions necessary to activate these genes. These functions can be provided by an appropriate conjugative plasmid.

(b) Both conjugative and nonconjugative plasmids may contain region homologous to each other. Homologous recombination may fuse these plasmids transiently. Both plasmids then transfer as a cointegrate.

(c) When one of the plasmids of the pair contain a transposable element, a *recA*$^{+}$ independent plasmid fusion can occur and the plasmids transfer as a cointegrate. However, upon resolution of the cointegrate, a copy of the transposable element remains in both plasmids.

These three methods of mediating plasmid transfer were used extensively in experiments in which plasmids are transferred between different species of bacteria. The third method was of particular interest, since it provided a general tool for mutagenizing nonconjugative plasmids.

RESULTS

Mobilization of plasmid cloning vectors by transactivation of *oriT* transfers these plasmids without any alteration to their new hosts. This technique has limited application, since one rarely has all necessary complementation functions combined in a single helper plasmid[5].

The *recA*$^{+}$ dependent fusion of a conjugative and a nonconjugative plasmid also allows the original plasmids to be recovered after transfer.

The main advantage of this method is that the sole requirement for cotransfer is a single region of DNA homology between the plasmids. Furthermore, broad host range plasmids with homology to any portion of the cloning vector can be used to transfer this cloning vector to numerous Gram-negative bacteria.

For example, we have recloned sections of the Ti plasmids of *Agrobacterium tumefaciens* in nonconjugative broad host range cloning vehicles derived from the W-type plasmid Sa[6,7]. The transfer of these chimeric plasmids to *Agrobacterium* can be mediated by the N-type plasmids RN3 and R128. These plasmids[8,9] cannot establish themselves in *Agrobacterium*[3] but can be maintained as cointegrates so long as selection for the drug markers of the N-plasmids is applied. RN3 ($Sm^R Su^R Tc^R$) recombined within the streptomycin sulphonamide resistance locus of the Sa derivative (pGV1106) and transferred to *Escherichia coli* with a frequency of 10^{-3} and to *Agrobacterium* with a frequency of 10^{-6}. R128 ($Su^R Tc^R Ap^R$) transfers one tenth as efficient, presumably due to smaller regions of homology. The lower transfer efficiency of pGV1106 and derivatives to *Agrobacterium* might be explained by the instability of the RN3 in *Agrobacterium* and not to inefficient conjugation. In fact, RN3 probably conjugates as efficiently as RP4 since both RN3 and the "suicide plasmid"[10] RP4::Mu::Tn7 introduce the Tn7 transposon at an equal frequency, 10^{-5}. Indeed, RN3 is a preferable "suicide plasmid" for the introduction of transposons into *Agrobacterium* because the entire RN3 is invariably lost whereas portions of RP4::Mu can remain[7].

Transposon-mediated tansfer

The broad host range plasmid RP4 has been widely used to "mobilize" plasmids between different species. A study of the cointegrates between RP4 and a nontransferable plasmid demonstrated that these cointegrates harbored a directly repeated sequence at the junction sites of the two replicons[11]. This repeat had the properties of an insertion sequence and was denoted IS8. Since the cointegrate had the structure of a proposed intermediate in the transposition of an IS-element, we determined whether "mobilization" by RP4 invariably resulted in an insertion of IS8 in the transmitted plasmids. This was indeed the case as was shown by Southern blot analysis : the "mobilization" by RP4 in *Agrobacterium* always involves transposition of IS8.

In order to demonstrate the generality of this phenomenon, we tested the ability to mediate transmission of other P-type plasmids. From the P-type plasmids listed in Table 1, only pUZ8 was unable to transmit pACYC184 between *E. coli* strains. The cotransfer-proficient plasmids listed in Table 1 all contained either IS8 or an other transposable element. The relationship between RP4 and those plasmids, as determined by electron microscopy heteroduplex analysis[12], is shown in Table 2.

Table 1. Transmittance of pACYC184 by several conjugative plasmids in *E. coli*

Conjugative plasmid	Compatibility	*recA* character of donor	Transmission frequency
RP4(KmTcAp)	P	+	1.2×10^{-6}
RP4(KmTcAp)	P	-	1.0×10^{-6}
R934(KmTcApHg)	P	-	10^{-6}
R702(KmTcSmSuHg)	P	-	5.0×10^{-6}
pUZ8(KmTcHg)	P	-	$< 10^{-8}$
pUZ8::Tn7(KmTcHgSmTp)	P	-	$< 10^{-8}$
R483(SmTp)	Iα	-	$< 10^{-8}$
R483::Tn1(SmTpAp)	Iα	-	10^{-6}

Table 2. Electron microscopic heteroduplex analysis of the relationship among several P-type plasmids

Plasmid	Ref.	% RP4 sequences in common	Insertions			
			Size (Md)	Position on RP4 map[17] (Md)	Markers	Remarks and ref.
RP4	1	100%	3.6	3.2 to 5.8	Ap	Tn1 [1]
			1.2	21.5 to 22.7		IS8 [11]
R702	18	83%	10	1.2	Sm Su Hg	Tn1831 [15]
			4.8	7.0	---	---
R934	19	90%	6.0	9.8	Ap Hg [16]	not transposable[16]
			1.2	21.5 to 22.7		IS8[11]
pUZ8	20	83%	2.4	1.2	Hg	not transposable[7]
R1033	21	100%	3.6	3.2 to 5.8	Ap	Tn1[1]
			10.7	8.4	Sm Su Hg Cm Gm	Tn1696[24]
			1.2	21.5 to 22.7	---	IS8[11]
R26	22	100%	4.8	3.2 to 5.8	Ap	Tn1 with insertion
			10.7	8.4	Sm Su Hg Cm Gm	
			1.2	21.5 to 22.7	---	IS8[11]
R938	23	90%	9.4	7.8	Sm Ap	TAβ[23]
			1.2	21.5 to 22.7	---	IS8[11]
			9.2	33.4	Sm Su Hg Cm	---

Table 3. Transposon-mediated transmission of pACYC184-derivatives in *E. coli*

Conjugative plasmid	pACYC184 derivative	Transmission freq./ pUZ8 transfer
pUZ8	pACYC184	< 8.0×10^{-9}
pUZ8	pACYC184::Tn1	5.0×10^{-7}
pUZ8	pACYC184::IS8	1.0×10^{-6}
pUZ8::Tn1	pACYC184	2.0×10^{-6}

The requirement for an insertion element in the process of transmission was systematically examined using the pairs of plasmids listed in Table 3. From these data we may conclude that the transmission of the nonconjugative plasmid is dependent on the presence of a transposable element in either one of the participants. Therefore, every case of plasmid transmittance shown in Table 2 should have resulted in the insertion of a transposable element into pACYC184. This was indeed shown to be the case (Table 4).

Table 4. Identity of transposable elements inserted in pACYC184 after transmission with the indicated conjugative plasmids in *E. coli*

Conjugative plasmid	Transposable element	Frequency
RP4	Tn1	35%
	IS8	65%
R702	Tn1831	100%
R934	IS8	100%
pUZ8::Tn1	Tn1	100%
R483::Tn1	Tn1	100%

RP4 contains Tn1 and IS8, either of which could participate in a transmittance event. In E. coli they are equally effective (Table 4) but in Agrobacterium only IS8 is active. This indicates that the failure of a plasmid to be transmitted does not mean neither of the plasmids contain a transposable element. Tn7, for example, is an efficient transposable element in E. coli, yet it cannot promote transmission in this host (Table 1). A plausible and testable explanation is that resolution of the cointegrates is exceptionally efficient.

Transmission-mediated mutagenesis

It is apparent at this point that this type of plasmid transmission mutates one of the participants.

We have systematically applied this technique for mutagenizing portions of the Agrobacterium Ti plasmid, cloned in pGV1106. In a typical experiment, pGV1106 containing a 15-16 Kb fragment of the pTiC58 Ti plasmid (fragment EcoRI-1)[13] was transmitted by R483::Tn1 at of frequency of 5 x 10^{-6}. Of the 34 transmitted plasmids examined, 26 carried a Tn1 inserted into the Ti fragment and this in at least 16 different locations. These mutated Ti plasmid fragments were subsequently introduced into Agrobacterium by a $recA^+$ dependent mobilization with RN3. Homologous recombination allowed afterwards the exchange of the mutated fragment with the corresponding segment of the Ti plasmid and hence the construction of a new set of mutant Ti plasmids[14].

CONCLUSION

Transmission-mediated mutagenesis has decided advantages over "classical" transposon mutagenesis. For example, it is not possible to select for the insertion of IS sequences in genes that do not have an assayable phenotype. The technique presented here can be used conveniently to provide a large collection of such mutants. The use of the classical technique is primarily limited to the Enterobacteriaceae since it is presently difficult to transform or transduce in most other families. Transmission-mediated mutagenesis has no such limitations. This is important, since it allows the mutagenesis in the host in which the assay for gene expression has to be conducted. Finally, many large plasmids, common in nature, and important to agriculture or industry, have not been analyzed genetically. Transmission-mediated mutagenesis will be the method of choice for mutagenizing these unstudied plasmids.

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PROINSULIN FROM BACTERIA

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One problem we face in the cloning and expression of a small hormone like insulin, is that the normal hormone is made in the pancreas through a series of precursors. Preproinsulin is a molecule some 100 amino acids long that has on its amino terminal end a hydrophobic presequence of 24 amino acids which is cleaved off as that molecule is passed through the cell membrane (1, 2). The resulting fragment, proinsulin, folds up; disulphide bonds form, and then a portion of the peptide chain, the C peptide, is cleaved out between two pairs of basic amino acid residues to produce the final molecule, insulin itself. When we make insulin in bacteria, we can do the final maturation ourselves with a mixture of trypsin and carboxypeptidase B. However, how can we arrange that the amino terminus will be the correct one for insulin rather than bearing some other amino acid or the presequence?

Originally, in collaboration with Villa-Komaroff et al. (3), we synthesized proinsulin attached to a special long precursor. We thought that it would be best to synthesize this molecule not by leaving it inside the bacterial cell but by arranging for it to be secreted to the periplasm. We did that cloning by taking plasmid pBR322, which has a Pst site in the middle of the ampicillin resistance gene, opening it at this Pst site, and inserting into it cDNA for preproinsulin. The ampicillin resistance gene product, the beta-lactamase, is a secreted protein. Three times larger than preproinsulin, it has a presequence of 23 amino acids, that leads to the transport of this protein through the *E. coli* membrane and to the cleavage of this presequence (4, 5; for a review of protein secretion, see ref. 6). The original fusion we had made inserted proinsulin at amino acid 182 in the prepenicillinase molecule, and we could show by antigenic techniques that that combined molecule made in a small amount in the bacterial cell was transported through the membrane and could be recovered from the periplasmic space (3). To examine that transport more closely, we altered this construction to remove the material that separated the presequence from the proinsulin. We made a set of cloning vehicles which enabled us easily to create a series of fusions of proinsulin to the prepenicillinase leader (7).

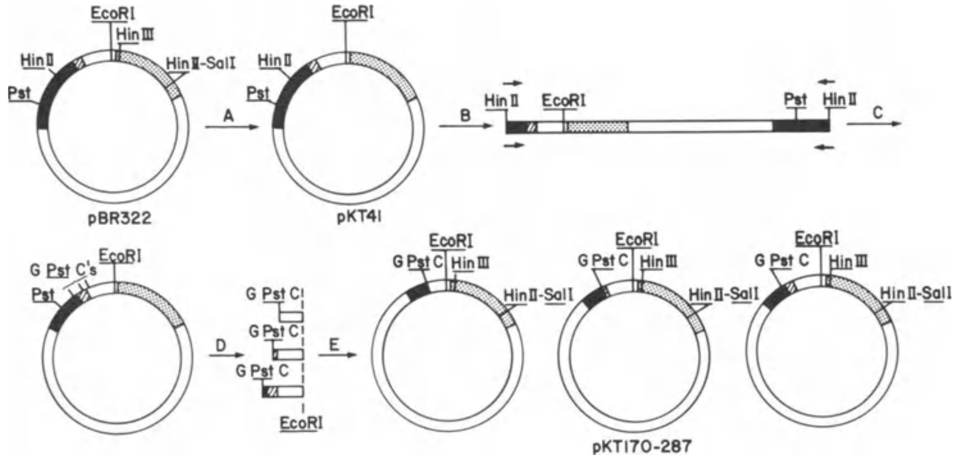


Fig. 1. Scheme by which signal sequence plasmids were constructed. Each step is described in ref. 7. Pst=PstI, HinII=HincII, HinIII=HindIII, G Pst C=an inserted PstI linker, whose sequence is 5'-GCTGCAGC- 3', where CTGCAG defines the PstI restriction site. Certain gene regions are represented as follows: prepenicillinase signal sequence, shaded; mature penicillinase, black; tetracycline resistance, dotted.

Figure 1 shows the procedure. We first removed a few restriction cuts in the tetracycline resistance gene, which could have gotten in our way, by simply mutagenizing the plasmid and selecting for a functional tetracycline gene on a plasmid resistant to the restriction enzyme. We then opened the plasmid at the Hind II cut in the middle of the ampicillin resistance gene, trimmed back the ends with Bal 31 (an enzyme that cuts back on both strands of DNA), inserted a Pst linker and closed the plasmid up again. That produced a shrunken plasmid that still had a tetracycline resistance. If we isolate the Eco R1 to Pst pieces, size them, and combine them with the large Eco R1 to Pst fragment of pBR322, we eventually make a series of plasmids which bear deletions between various points in the leader sequence and the Pst cut. By sequencing we can know what we have. Figure 2 shows this set of plasmids, a set of cloning vehicles with a single Pst cut either right after the leader or inside the leader; enough to have the cut occur in all possible translation reading frames. Similarly, Figure 3 shows we took the cDNA for preproinsulin and trimmed back the end, added a Pst linker, and thus obtained a series of structures in which the hydrophobic leader sequence for preproinsulin has been either extended by a series of glycines (from the original cloning) or shrunk by a series of enzymatic nibblings to provide a set of

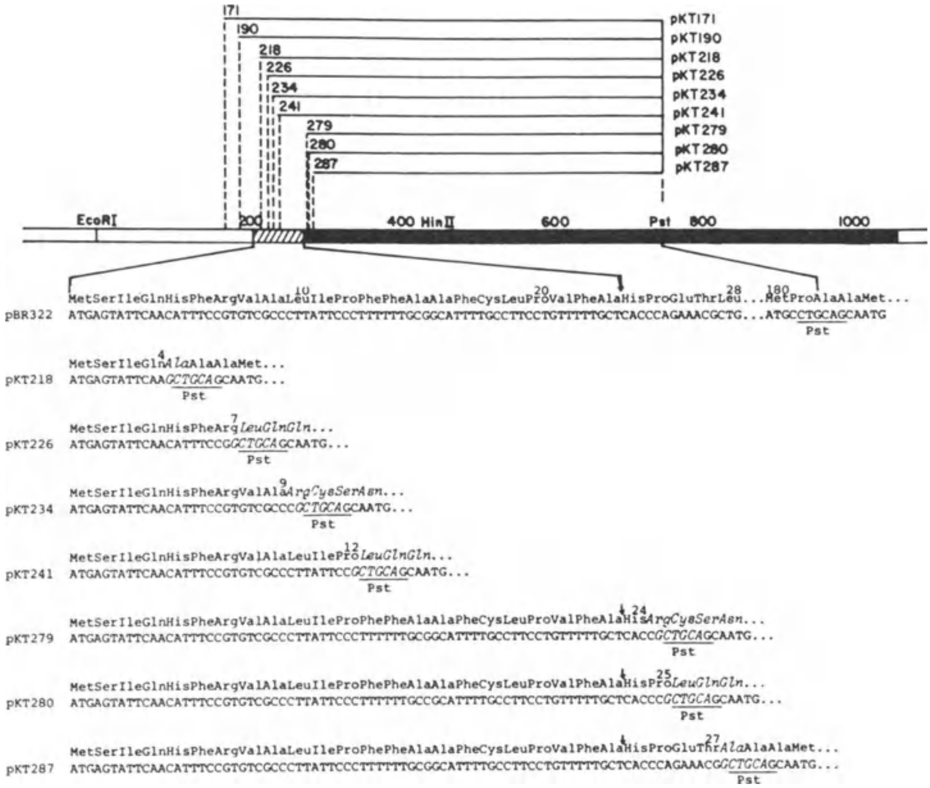


Fig. 2. Deletion map of pBR322 penicillinase gene and sequence of derivative plasmid signal sequence regions (7). DNA regions that encode proteins are represented as follows: penicillinase signal sequence, hatched; mature penicillinase protein, black. The derivatives were deleted from the Pst to the signal sequence coding region and the Pst site (C-T-G-C-A-G) was re-created by insertion of a Pst linker whose sequence is G-C-T-G-C-A-G-C. The bases donated by the linker on that strand are indicated in italics. The last wild-type penicillinase amino acid is indicated by the number of is wild-type position above it. The amino acids encoded by the inserted Pst linker are in italics. The arrows indicate the site of cleavage for maturation of wild-type prepenicillinase to penicillinase.

molecules in which we have either an essentially complete proinsulin hydrophobic leader sequence, a middle-sized, 7 amino acid long hydrophobic leader sequence, or a molecule with no hydrophobic leader sequence at all but just a series of glycines going to the fourth amino acid of proinsulin (8).

For each combination of proinsulin and cloning vector we could

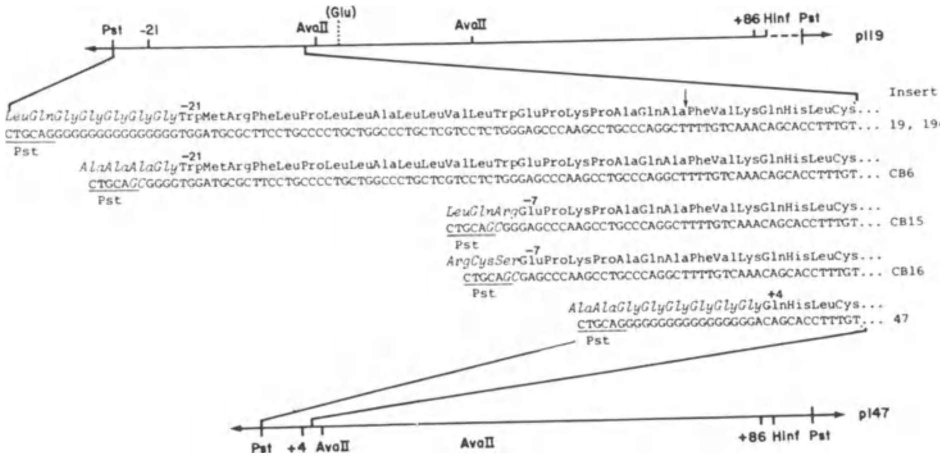


Fig. 3. Restriction map of rat preproinsulin (pI19) and proinsulin (pI47) Pst inserts (1947 is a recombinant between the 19-insert 5' end and the 47-insert 3' end at the first Ava site to remove a mutant glycine encoded in the 19 insert); sequences at the 5' end of these inserts and the digested derivatives of 1947 insert. Bases in the digested 1947 insert sequences in italics have been donated by an inserted Pst linker. The first wild-type amino acid is indicated by the number of its wild-type position above it. Amino acids in italics were created by G-C-tailing during the original isolation of pI19 and pI47 or by the insertion of a Pst linker. Arrows indicate the site of cleavage for maturation of preproinsulin to proinsulin.

measure how much proinsulin was inside the bacterium or in the periplasmic space (defined as a space external to a lysozyme spheroplast) by a standard radioimmune assay, competing labelled proinsulin with cold insulin. We got three characteristic answers: either most of the proinsulin is secreted and only about ten percent is inside, or most is inside the cell, or half is in the cell and half in the periplasmic space (8). Table 1 shows that if there is no hydrophobic leader sequence, we find the molecule inside the bacterium. If we have a full length bacterial leader sequence, the molecule is transported by the bacterial sequence and we find 90% on the outside. We were surprised and delighted, however, that in these cases in which we have very few amino acids from the bacterial sequence but have mainly a eucaryotic signal sequence, again 90% of the molecule has moved to the periplasm. Thus the eucaryotic signal sequence serves in bacteria to transport the preproinsulin throught the membrane to the periplasmic space. The 50% effect molecules we don't understand fully; they do have a complete bacterial sequence, and there is an appreciable amount of transport, however, they have other charges inserted because of the nature of the linker sequence; these charges may interfere with the transport.

Now the general secretion phenomenon is not only that a protein sequence is transported from the cytoplasm to the periplasm but also that, in all but one case, the sequence that does that transporting is at the amino terminus of the protein and is cleaved off of the protein either at the moment of transport or after it. Obviously if we are transporting preproinsulin through the cell membrane with

PENICILLINASE-PREPROINSULIN SIGNAL FUSIONS

pen'ase	MSIQHFRVALIPFFAAFCLPVFA ↓	HPETLVK.......				
i27/+4	MSIQHFRVALIPFFAAFCLPVFA	HPETL	AAGGGGGG		QHLC...	>90%
i25/-21	MSIQHFRVALIPFFAAFCLPVFA	HP	LQGGGGG	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i12/-21	MSIQHFRVALIP		LQGGGGG	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i4/-21	MSIQ		AAAG	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i25/-7	MSIQHFRVALIPFFAAFCLPVFA	HP	LQR	EPKPAQA	FVKQHLC...	50%
i24/-7	MSIQHFRVALIPFFAAFCLPVFA	H	RCS	EPKPAQA	FVKQHLC...	50%
i12/-7	MSIQHFRVALIP		LQR	EPKPAQA	FVKQHLC...	<10%
i9/-7	MSIQHFRVA		RCS	EPKPAQA	FVKQHLC...	<10%
i4/+4	MSIQ		AAGGGGGG		QHLC...	<10%
preproinsulin				MALWMRFLPLLALLVLWEPKPAQA ↓	FVKQHLC...	

Table 1. Each sequence begins at the penicillinase fMet and ends at amino acid 7 of proinsulin (8). Each line represents one continuous sequence which has been grouped to emphasize similarities and differences as follows: first group, penicillinase signal sequence amino acids; second group, matured penicillinase amino acids; third group, amino acids created by the inserted Pst linker (italics) or by poly(G,C) tailing (glycines); fourth group, preproinsulin signal sequence amino acids; fifth group, matured proinsulin amino acids through amino acid 7. The arrows above the prepenicillinase and preproinsulin sequences indicate sites of cleavage for maturation. A, Ala; R, Arg; C, Cys, Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; V, Val.

each of these constructions, the immediate question is are these secretory leader sequences cleaved off the molecule or not? We could try to answer that question by inserting a radioactive label into each of these molecules (in fact we used radioactive sulpher and labelled the methionines and the cystines) and recovering from the periplasm of the labelled cells the radioactive molecule by binding it to antibody and isolating the antibody complex with the staph A protein (10). Figure 4 shows what these first four molecules look like. There are three constructions in which there is a full length preinsulin secretory sequence; each of these produces a protein molecule of the same size (Fig. 4, lanes b-d). The construction with a full length bacterial presequence and a few amino acids of the bacterial protein attached to a proinsulin structure creates a slightly larger molecule (Fig. 4, lane a). The size of these molecules is what one would expect, if they had been correctly processed in the bacteria. However, this is not sufficient evidence to show correct processing; we went to a more explicit experiment. We isolated each of these proteins and, using the sequenator, cut in amino acid after amino acid from the amino terminus to ask where are the labelled cystines (10).

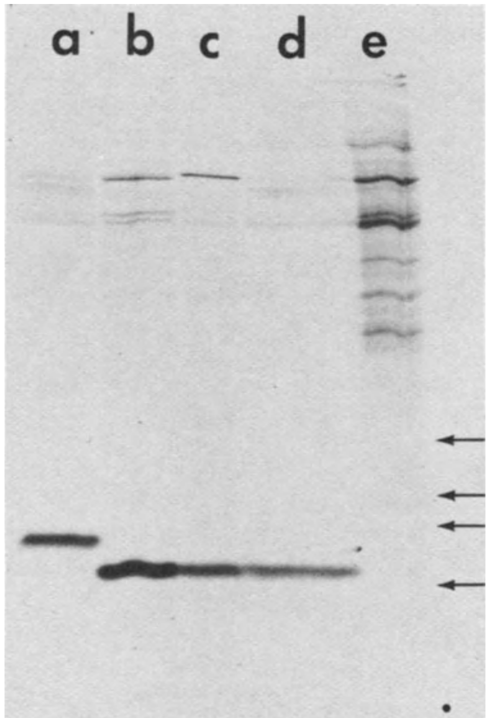
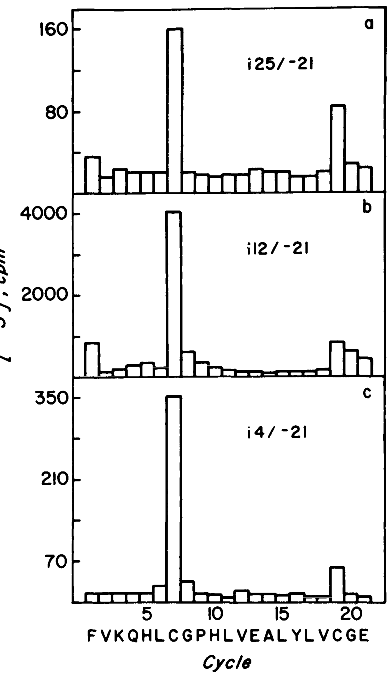


Fig. 4. Immunoprecipitated insulin antigen from E. coli bearing insulin plasmids electrophoresed on a 15% SDS-polyacrylamide gel (10). Lane a, i27/+4; b, i25/-21; c, i12/-21; d, i4/-21; e, PR13 bearing pKT41, a control plasmid without an insulin insert (see table 1). The molecular weight markers (arrows) are: sperm whale myoglobin (17,200), chicken lysozyme (14,400), human Beta2-microglobulin (11,600), and bovine proinsulin (8700). The molecular weight of authentic rat proinsulin is 9100. The dye front is indicated by a dot. The amount of material in each lane corresponds to an input of 0.5 mCi in the labeling. The dry gel was exposed for 12 hours.

Figure 5 (left) shows data for three molecules in which the insulin eucaryotic hydrophobic leader sequence is used for the transport: we find the cleavage is exactly at the beginning of proinsulin. As we sequence along these molecules, label appears only in cystine at position 7 and the cystine at position 19, in all three cases. Not only is the insulin hydrophobic leader sequence, the eucaryotic presequence, being recognized sufficiently well in the bacteria to transport the protein to the periplasm but furthermore the bacterial enzymes recognize the end of that hydrophobic leader sequence, or some property of it, and cleave it off to make a correct proinsulin molecule. Fig. 5 (right) also shows that in the fourth case, with the penicillinase leader, the cleavage occurs at the usual place at the end of the penicillinase sequence.



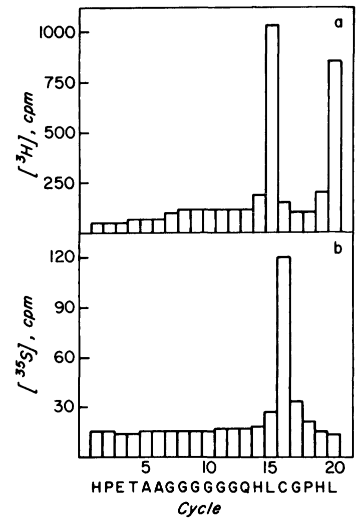


Fig. 5 (left). Location of ^{35}S-containing residues in the amino terminal region of the insulin products of three constructions containing the DNA encoding the preproinsulin signal sequence (10). The antigen was purified from $H_2{}^{35}SO_4$-labeled cells by immunoprecipitation and SDS/polyacrylamide gel electrophoresis and then subjected to automated Edman degradation. The amount of radioactivity released by each cycle of degradation was determined by liquid scintillation counting. The amino-terminal sequence of authentic rat proinsulin is presented for comparison. (A) i25/-21: 20,000 cpm loaded, double-coupled at steps 1, 2 and 10, double-cleaved at step 9, 10% of each cycle analyzed. (B) i12/-21: 150,000 cpm loaded, double-coupled at step 1, 50% of each cycle analyzed. (C) i4/-21: 50,000 cpm loaded, double-coupled at step 1, double-cleaved at step 9, 50% of each fraction analyzed.
(right). Location of the ^{35}S-containing and (^{3}H)leucine residues in the amino-terminal sequence of i27/+4. The insulin antigen was purified from cells labeled with both $H_2{}^{35}SO_4$ and (^{3}H)leucine by immunoprecipitation and SDS/polyacrylamide gel electrophoresis and then subjected to automated Edman degradation. (A) 300,000 cpm loaded; (B) 85,000 cpm loaded. Double-coupling was done at step 1, double-cleaving at steps 2 and 18. The amount of ^{35}S and ^{3}H radioactivity released at each cycle of degradation was determined by liquid scintillation counting, with the crossover into the ^{3}H channel subtracted. Ten percent of

(Continued)

each fraction was analyzed. The amino-terminal sequence of i27/+4 matured at the correct bacterial clipping site is presented for comparison.

The cleavage enzyme must recognize something in the sequence rather than some property of the whole protein. One might have thought that the cleavage enzyme simply recognizes some little tail sticking out from the boundary of the protein and it comes and cleaves that off. That would be a reasonable interpretation of the cleavage of the eukaryotic sequence, because the result, proinsulin, is the mature protein. In the case of penicillinase of course, the cleavage forms the mature protein. But in the case of this particular molecule, there are a few extra amino acids and a string of glycines on the proinsulin; there is no reason for the cleavage to occur at the end of the presequence, unless the sequence at this position dictates the cleavage (10).

We have gone on to pulse label the insulin made in these bacteria. The experiments shown in Figure 4 use material built up over several generations of labelling. If we pulse label, we can see the preproinsulin precursor, the full length precursor synthesized by each of these constructions, as well as the matured product. We see, in a thirty second pulse, the full length molecule, and in several minutes the processing of that molecule. The results of that processing are essentially the ones that we had inferred from the continuous label: that is that the cleavages are either at the end of the penicillinase presequence, in the case in which there is only the penicillinase presequence, or they are at the end of the eukaryotic presequence.

These experiments show that we can make a mature molecule cleaved correctly using the bacterial system to do all the work for us. This does argue that there is a common feature in the eucaryotic and the bacterial systems involving the transport of proteins, which was unsuspected. That feature may be that the transport involves nothing other than the existence of the hydrophobic leader sequence, and its interaction with a membrane, which is a perfectly general structure, rather than the existence highly specific receptors involved in the transport of these proteins. Furthermore, the enzyme that does the cleavage is general. Either the cleavage has something to do again with the shape of the presequence that has to do with the transport, or else, just accidentally, that the enzymes recognize the same sequences.

We originally put the proinsulin molecule into penicillinase, to move it outside the bacterium both because we wanted to study its secretion and also because we expected that the molecule, synthesized within the bacterium, would not be terribly stable. Insulin is a somewhat floppy protein hormone; protein hormones are often subject to proteolytic degradation. We have been able to study the stability problem, using these same strains, by pulse labelling the insulin and asking what happens to the insulin molecule in those bacteria in which it is being rapidly secreted or in those bacteria in which it is not secreted at all and remains in the periplasm. In all the cases in which the molecule remains in the cytoplasm, we recover very much less insulin. If we pulse label, we can follow the degradation of the insulin: in the cytoplasm there is a one minute half-life for insulin; while in the periplasm there is a twenty minute half-life for insulin. In fact, the molecule is very dramatically stablized, protected against proteases in the cell, by being moved through the cell membrane.

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Construction and Properties of Plasmid Vectors Containing the trp Regulatory Region Suitable for Expressing Foreign Genes

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Structure and Regulation of the trp Operon

The E. coli tryptophan (trp) operon consists of a regulatory region followed by five structural genes (trpE through trpA)[1,2]. The trp structural gene products, which are co-ordinately synthesized in equimolar amounts, catalyze the conversion of chorismate to tryptophan[1,3]. Transcription of the operon is repressed by tryptophan by two mechanisms. Tryptophan binds trp repressor (trpR) resulting in an increase in the affinity of the repressor for the trp operator[4]. Since the trp operator sequence overlaps the trp promoter sequence (see Fig. 1) binding of repressor prevents binding of RNA polymerase. Secondly, in the presence of tryptophan about 90% of the RNA polymerase molecules which are able to initiate, terminate transcription about 140 base pairs (bp) from the transcription start (see Fig. 1). The ability of the transcription terminator to function is determined by the level of tryptophanyl tRNA[9].

Methods of Inducing the trp Promoter

Both mechanisms for repressing trp transcription described above can be antagonized by lowering the levels of intracellular tryptophan. The two methods described below for achieving this have the disadvantage that they depend on limiting the availability of intracellular tryptophan which in turn may prevent efficient expression of cellular proteins. However, tryptophan is a rarely used amino acid[10] and substantial amounts of proteins containing tryptophan residues can be synthesized by the mthods described here.[15]

In the first method trp$^-$ cells[11] are grown to stationary

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                                                    -35 region
    Hhal                                             AlulHindll        T
1   GCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTTGACAATTAATCAT
    CGCGGCTGTAGTATTGCCAAGACCGTTTATAAGACTTTACTCGACAACTGTTAATTAGTA

         trp(po)PB
    aql     Hpal     RsaI  mRNA start  trpL SD Taql   MetLysAlaIleP
60  CGAACTAGTTAACTAGTACGCAAGTTCACGTAAAAAGGGTATCGACAATGAAAGCAATTT
    GCTTGATCAATTGATCATGCGTTCAAGTGCATTTTTCCCATAGCTGTTACTTTCGTTAAA
           Hindll

      RsaI              Hhal
    heValLeuLysGlyTrpTrpArgThrSerOP                         Hph
120 TCGTACTGAAAGGTTGGTGGCGCACTTCCTGAAACGGGCAGTGTATTCACCATGCGTAAA
    AGCATGACTTTCCAACCACCGCGTGAAGGACTTTGCCCGTCACATAAGTGGTACGCATTT

                               TT                           trpE SD
180 GCAATCAGATACCCAGCCCGCCTAATGAGCGGGCTTTTTTTTGAACAAAATTAGAGAATA
    CGTTAGTCTATGGGTCGGGCGGATTACTCGCCCGAAAAAAAACTTGTTTTAATCTCTTAT

                              HinflHindlll
       MetGlnThrGlnLysProThr
240 ACAATGCAAACACAAAAACCGACTCAAGCTTACT
    TGTTACGTTTGTGTTTTTGGCTGAGTTCGAATGA
                                  Alu
```

Figure 1. DNA sequence of the trp regulatory region (Ref. 5 and our unpublished results) cloned in ptrpE2-1 (see Figure 3). Regulatory features are in bold type. PB, Pribnow Box[6]; SD, Shine-Dalgarno sequence[7]; TT, transcription terminator[8].

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phase in repressing concentrations of tryptophan (40 µg/ml). They are then diluted 20-fold into medium lacking tryptophan so that tryptophan is present at a nearly derepressing concentration (about 1 µg/ml). After a few hours growth in this medium tryptophan levels will fall and all trp promoters will become fully active. The medium for dilution is usually M9 salts, glucose[12], supplemented with 0.2% casamino acids, which contains no tryptophan but all the other amino acids used in proteins. A trp^- strain is required to ensure that the cells do not synthesize any tryptophan de novo which would result in partial repression of the trp promoters[1]. This method can be readily adapted for use on agar plates. Colonies are initially grown on nitrocellulose filters overlaid on plates containing excess tryptophan and then transferred on the nitrocellulose to plates containing 1 µg/ml tryptophan.

In the second method trp$^+$ cells are grown to stationary phase in a repressing concentration of tryptophan, diluted in medium lacking tryptophan so that tryptophan is present at a derepressing concentration (1 μg/ml) resulting in partial derepression of trp promoters[1]. After a few hours growth the tryptophan analog 3β-indolylacrylic acid (IA) is added so that trp transcription becomes maximal[13,14,15].

Non-regulated maximal expression of the trp promoter can be obtained using a trpR$^-$ strain[16]. This method requires a vector molecule such as ptrpL1 (see Fig. 3) which lacks the trp attenuator[17]. This method has the advantage that cells can be grown in excess tryptophan in complex media; its disadvantage is that expression is constitutive and thus if the over-produced gene product confers a disadvantage on cells synthesizing it, such strains may be unstable or impossible to construct[14].

Levels of trp Mediated Expression Compared with Other Systems

The trp promoter is a relatively powerful E. coli promoter with an efficiency comparable to the phage λ P_L promoter[18,19,20]. Some T-phage promoters are considerably stronger than trp[21] but no vectors containing them are yet available. A comparison of the levels of expression of human interferon and growth hormone from vector systems based on the lac and trp regulatory regions indicates that the fully derepressed trp system is 5-10 times more efficient than lac[22]. It is not known if this is due to higher transcriptional or translational efficiency of trp.

Figure 2 shows the induction kinetics of cells containing the plasmid ptrpED5-1 induced with IA as analyzed by a sodium dodecyl sulfate-polyacrylamide gel stained with coomassie blue[14]. The plasmid contains the trp regulatory region and the first structural gene (trpE). Note that the trpE protein is synthesized in small amounts in the absence of inducer but that 3 hrs after induction the trpE protein represents about 30% of cellular protein. Eucaryotic gene products appear to be synthesized in much smaller amounts[22,23] (unpublished results) but the reason for this is not yet known.

Function of the trp Regulatory Regions on Multicopy Plasmids

The chromosomal trp genes are regulated over approximately a 500-fold range; 50-fold at the operator[24] and 10-fold at the attenuator[25]. This regulation appears to function normally on multicopy plasmids[14,26]. In contrast the lac promoter cannot be regulated on a multicopy plasmid because of insufficient numbers of lac repressor molecules[27]. Recent evidence leads one to expect that trp regulation would be normal on a multicopy plasmid; the trpR gene is autogenously regulated[28] and the trp attenuator is rho-independent and probably depends only on transcription and translation for its function[8]. Thus, none of the

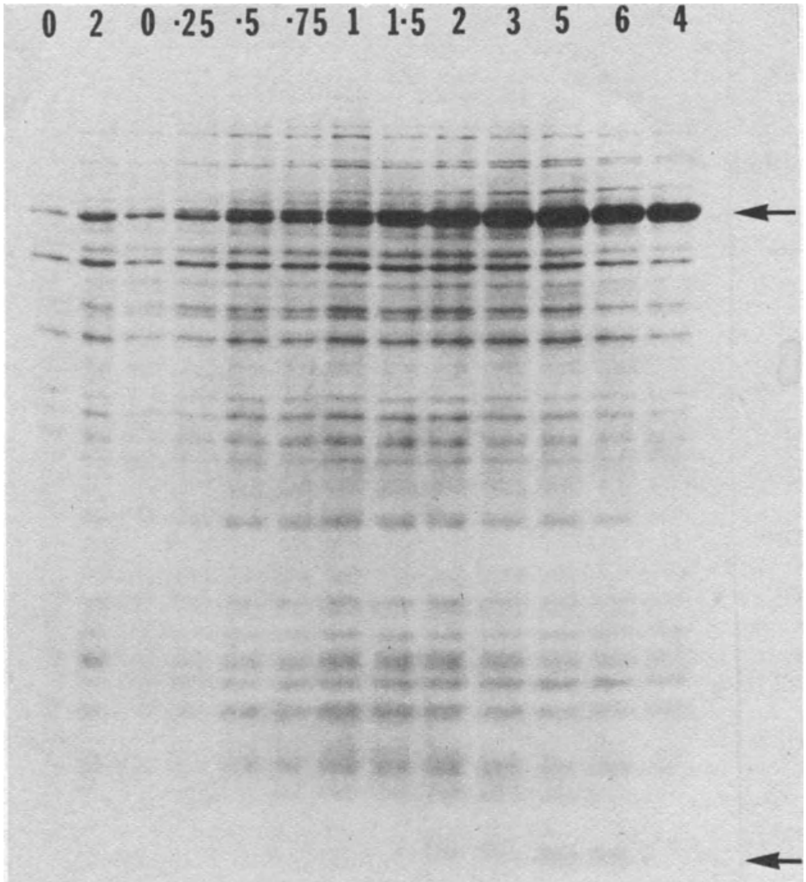

Figure 2. Kinetics and levels of synthesis of trp proteins in cells containig ptrpED5-1[14]. After cells were derepressed for trp transcription with IA, samples were removed at the times indicated above the gel in hrs. A plasmid free culture was similarly induced as a control as shown on the two tracks (0 and 2) on the left of the gel. The SDS-polyacrylamide gel was stained with coomassie blue. The upper arrow indicates the position of the trpE gene product and the lower arrow that of the trpD protein fragment.

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molecules involved in trp regulation should be overtitrated when the trp regulatory region is amplified on a multicopy plasmid.

Construction and Properties of Plasmid ptrpL1

To construct a vector molecule lacking the trp attenuator and suitable for constructing hybrid ribosome binding sites using

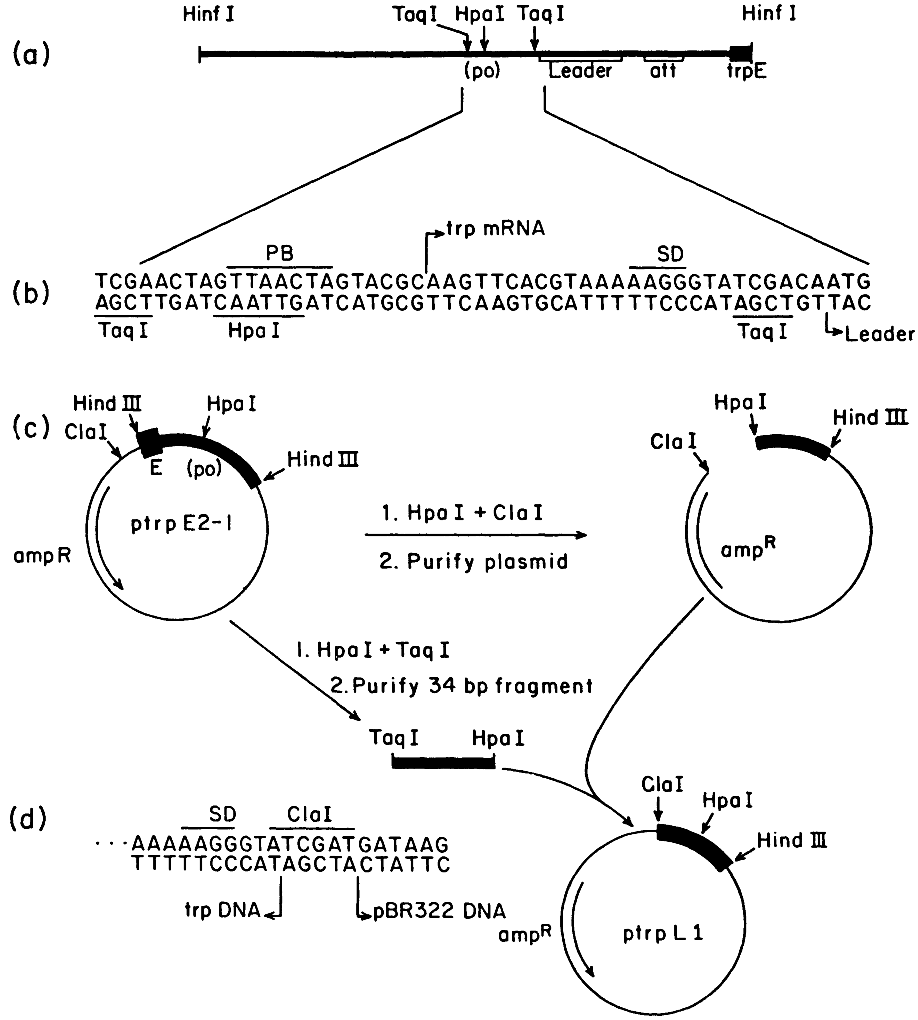

Figure 3. Construction of expression vector ptrpL1[15] (a) partial restriction map of the 492 bp HinFI fragment. The genetic regions promoter-operator, (po); leader region; attenuator, att; and trpE structural gene are shown in their approximate locations[5]. (b) DNA sequence of promoter-operator and proximal portion of the leader region. Regulatory features are as in Figure 1. (c) and (d) construction of ptrpL1 and the DNA sequence at the newly formed ClaI site, respectively.

the initiator codon of eucaryotic cDNAs[15,29] we followed the scheme shown in Figure 3. Initially, plasmid ptrpE2-1 was constructed by cloning a 492 bp Hinfl fragment at the HindIII site of pBR322[30] using HindIII linkers[15,31]. One end of the cloned molecule lies 7 codons from the N-terminus of the trpE gene (see Figure 1). The subsequent steps in the construction depended on the facts that there is a TaqI site, T/CGA, between the region complementary to 16S rRNA[7] (SD) and the trpL initiator codon, that the nucleotide 5' to the TaqI site is a dA residue, and that there is a unique ClaI site, AT/CGAT, in ptrpE2-1. Thus, by ligating the 34 bp HpaI-TaqI fragment to HpaI/ClaI cut ptrpE2-1 a unique ClaI site replaces the TaqI site.

Studies of gene fusions between trpL and the trpE[32] or trpC[33] genes indicate that the trpL ribosome binding site is equally efficient at initiating translation as those of the trp structural genes. Construction of hybrid ribosome binding sites with human interferons[22], human growth hormone[23], and hepatitis B virus (HBV) core antigen[15] show that such vectors are suitable for expression of foreign genes.

Strains containing ptrpL1 overproduce β-lactamase such that when trp transcription towards this gene is fully derepressed about 20% of cellular protein is β-lactamase[15]. The insertion of HBV surface antigen at the PstI site of ptrpL1 using the dG/dC tailing procedure has shown that this fused β-lactamase/foreign gene construct can be expressed at a high level in E. coli[15]. It should be noted that the levels of β-lactamase produced by ptrpE2-1 and ptrpL1 containing cells after induction with IA are the same (unpublished results). Furthermore, the trpL and trpE ribosome binding sites appear to be of comparable efficiency. Therefore, there should be no difference in levels of expression between constructs using ptrpL1 or derivatives of ptrpE2-1[31] (unpublished results). Thus, while there are advantages in using ptrpL1 (see Methods of Induction), for the expression of potentially deleterious proteins it may be better to use vectors based on ptrpE2-1, which have a 10-fold lower basal level of expression.

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ISOLATION AND ANALYSIS OF A COSMID HYBRID CONTAINING THE HUMAN GENOMIC INTERFERON GENE, HuIFNβ1

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INTRODUCTION

Human fibroblast interferon HuIFNβ has an anti-viral activity and can also stimulate natural killer cell action against neoplastic cells[1 2 3]. The IFNβ-gene belongs to a rare class of eukaryotic genes for which the immediate induction of transcription in response to certain inducers such as poly I:poly C has been demonstrated[4 5 6]. Recent findings indicate that two IFNß mRNAs exist which are at the most only distantly related, but are co-ordinately induced in human fibroblasts[7]. It therefore seemed of great interest to isolate the chromosomal region for the IFNß1 gene so as to study the structure of the transcription unit, the possible adjacent transcription units, and the later application of this information to the production of interferon ß in eukaryotic cells.

The initial impetus to isolate an IFNß cDNA clone came primarily from the wish to produce an IFNß-producing E.coli strain, as has recently been demonstrated by three other groups[8 9 10]. The isolation of a clone containing part of the IFNß1 cDNA and the use of this DNA as a probe for the isolation of a genomic hybrid is described here. Evidence was found that the transcribed region and most of the 3′-tail region of the IFNß gene has no intron.

METHODS

Production of a cDNA Gene Bank

Human fibroblast FS4 cells were cultured and superinduced for the production of interferon according to the method of Raj and Pitha[4], except that a lower polyI: poly C concentration was used. For the extraction of mRNA a number of methods were employed including the polysome isolation method of Palmiter[11], phenol-SDS[7], or guanidine hydrochloride extraction[12]. Clones derived from various batches of DNA were pooled for screening.

mRNA purified by two passages through oligo-dT cellulose columns was used for cDNA synthesis without size fractionation. Reverse transcriptase reactions were carried out as described by Ullrich et al.[13],after denaturation of the mRNA with methyl mercury hydroxide[14]. Terminal transferase reactions were carried out according to Nelson and Brutlag[15]using an excess of terminal transferase, and a 100-fold ratio of dNTP to DNA ends for five minutes at room temperature. Annealing of dC-tailed cDNA to dG-tailed pBR322 was carried out by slow cooling from 65°C to 37°C over a 12 hour period. Starting with 50µg of mRNA some 500ng of appropriately tailed cDNA was obtained. This yielded an initial bank of 600 hybrid colonies. The flow chart for the individual steps is shown in figure 1.

Initial attempts to use pools of plasmid DNA for mRNA enrichment or for hybrid arrest translation experiments in Xenopus oocytes were found to yield erratic results. The screening finally was carried out as shown in the figure 2. The later part of this screening depended very much on the sequence data already available from Taniguchi et al.[16].

Production and Screening of a Genomic Cosmid Gene Bank

The detailed description for the production of the cosmid gene bank will be presented elsewhere (F.Grossveld H.M.Dahl, R.A.Flavell et al., manuscript in preparation). A scheme for the production of the bank is given in figure 2, which essentially follows the protocol and recommendations of Hohn and Collins[17]. The bank consisted of 1.5 x 10^5 colonies, maintained on 15 fifteen cm diameter nitrocellulose filters. Screening of the bank by colony hybridisation was according to Hanahan and Meselson[22], with the following modifications. Filters were boiled before sterilisation. Filters were not dried between consecutive steps of the washing procedure and excess cell debris was wiped off the filter during the wash in 1M Tris, pH 8, 1.5M NaCl. This wiping step was followed by an additional wash in the same buffer.

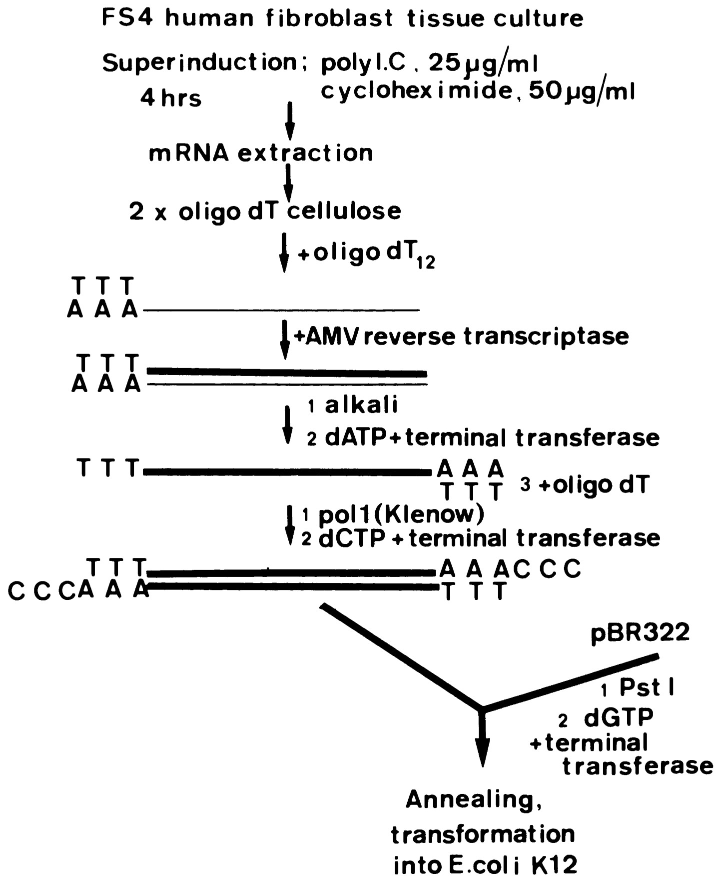

Fig. 1. Scheme for the production of the cDNA gene bank.

cDNA gene bank → 1. Screening by colony hybridisation with cDNA from:

a) polyI:C-induced

or b) non-induced FS4 cell mRNA

2. Restriction maps (PstI, BglII, RsaI)
3. DNA-sequencing

Fig. 2. Scheme for the screening of the cDNA gene bank.

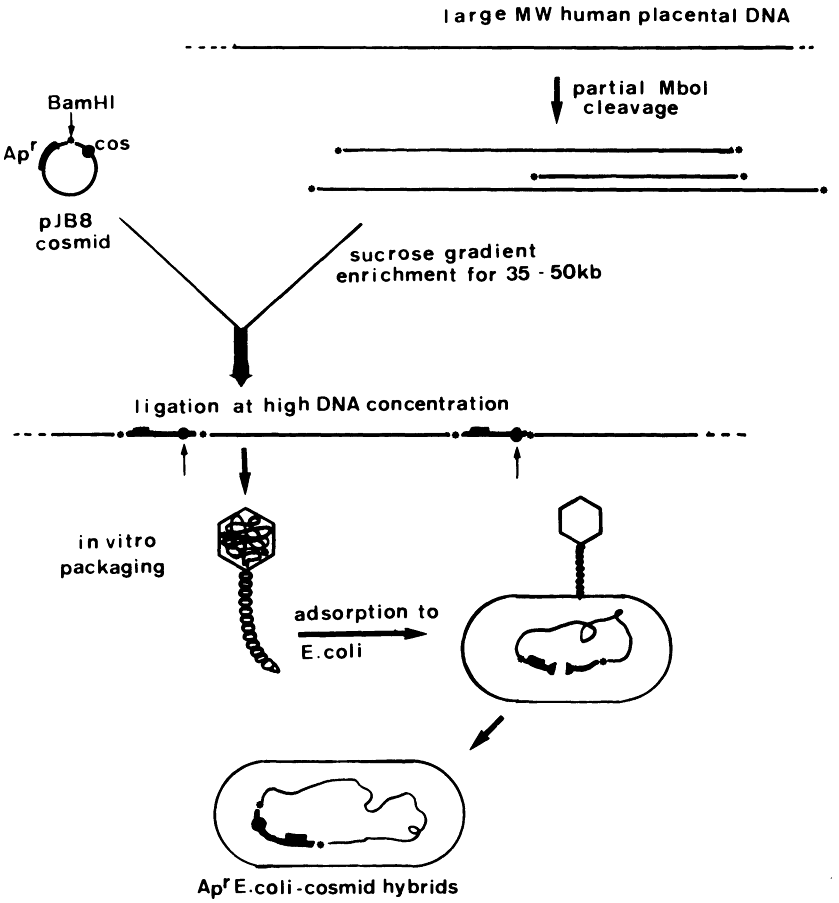

Fig. 3. Scheme for the production of the human genomic cosmid gene bank.

DNA and RNA Blot-Hybridisation

DNA blotting and hybridisation was carried out according to Southern[18]. RNA blotting was carried out as described by Thomas[19], except that the RNA gel-electrophoresis was made in 2% agarose containing 2.2M formaldehyde in the buffer. Nick-tranlation to make ^{32}P-labelled DNA probes was carried out according to Maniatis et al.[20].

RESULTS

Isolation of a Clone Containing IFNß1-DNA

Following the screening of the initial 600 hybrid cDNA clones, as outlined above, a single clone was identified as containing IFNß1 DNA. The structure of this clone is shown in figure 4.

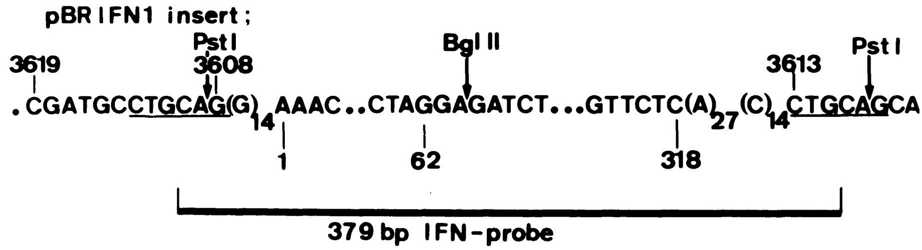

Fig. 4. The structure of the insert in pBRIFN1. The cDNA is inserted at the PstI site of pBR322 by GC-tailing such that two PstI sites are preserved at the boundaries of the insert. The numbering above the sequence indicates pBR322 coordinates and the numbering below, the cDNA homology to IFNß mRNA, homopolymer stretches not included. Bases 1 to 150 and 250 to 318 as well as the homopolymers were sequenced from the PstI and BglII sites using the Maxam and Gilbert method.

By comparison of the sequenced regions with the sequence of Toniguchi et al.[16], bases 1 to 68 were seen to constitute part of the 3'-non-translated tail and 69-318 the C-terminal coding region for IFNß1. The 379 base pair fragment was used as a nick-translated probe to isolate the genomic clone from the cosmid gene bank, and as a probe against mRNA from induced and non-induced cells (figure 5). As can be seen from this "Northern " blot a single band hybridises at 11S in agreement with the observations of Sehgal and Sagar[7]. Moreover, this band is only observed when mRNA from poly I:C induced cells is used (comparison of slots A and B), indicating that no mRNA having homology to this probe is present in non-induced cells.

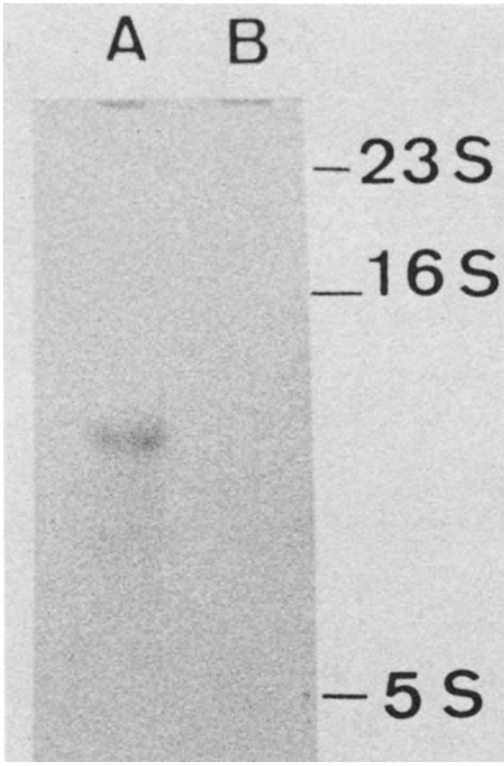

Fig. 5. Hybridisation of an RNA blot with nick-translated 379-fragment (figure 4). A: mRNA from poly I:C induced FS4 cells ; B: mRNA from non-induced cells at 10μg per slot. 3×10^7 c.p.m./μg DNA.

<u>Isolation of a Genomic IFNß1 Clone from the Cosmid bank</u>

The cosmid gene bank was simultaneously screened with three different labelled probes of which only one will be discussed here. One hundred suspected colonies were picked and screened with the individual probes. One colony gave a strong hybridisation with the 379 bp-probe. After two further dilutions and repicking of hybridising colonies, a single colony was isolated and designated pCosIFNß (pCosIF). Using single and double digests of this cosmid DNA with BglII, BamHI, HpaI and HindIII in combination with Southern blot hybridisation with labelled 379 fragment or pBR322 DNA (which designated pJB8 fragments) as shown for example in figure 6, a map of the whole cosmid was constructed with an estimated length of 46.5kb. The pJB8 vector is shown (1.8 to 6.8) as a thick line. Extending the Southern blot analysis with SstI, PstI and

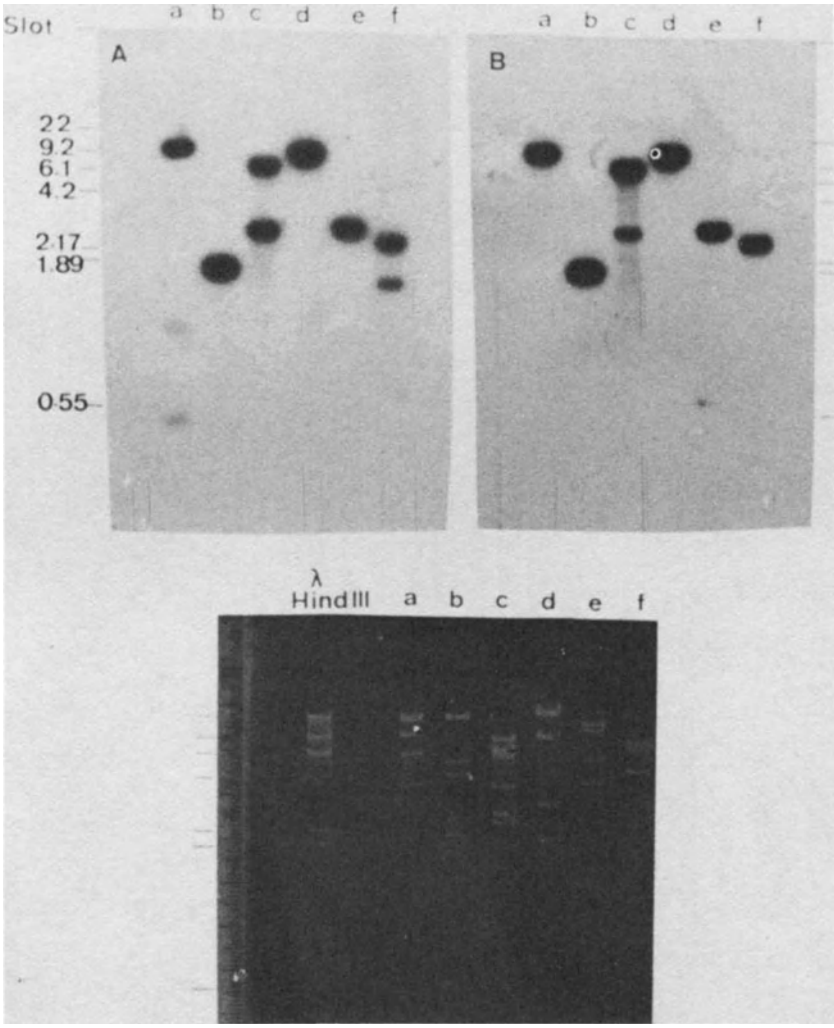

Fig. 6. Restriction mapping of pCosIFNß. A UV-photograph of an agarose gel of the following digests is shown in the lower part of the figure : a,HindIII, b, EcoRI, c, BglII, d,HpaI, e, SstI, f, PstI with λ HindIII fragments as markers. Southern hybridisations are shown to the same scale: A,with the 1.9kb EcoRI fragment, or B, the 379bp fragment as hybridisation probe.

EcoRI a more detailed map of the IFN region was produced. This also gave the orientation of the BglII and PstI sites and hence the orientation of the gene. The genomic clone therefore contains 36kb to the 5' end of the IFNß gene and

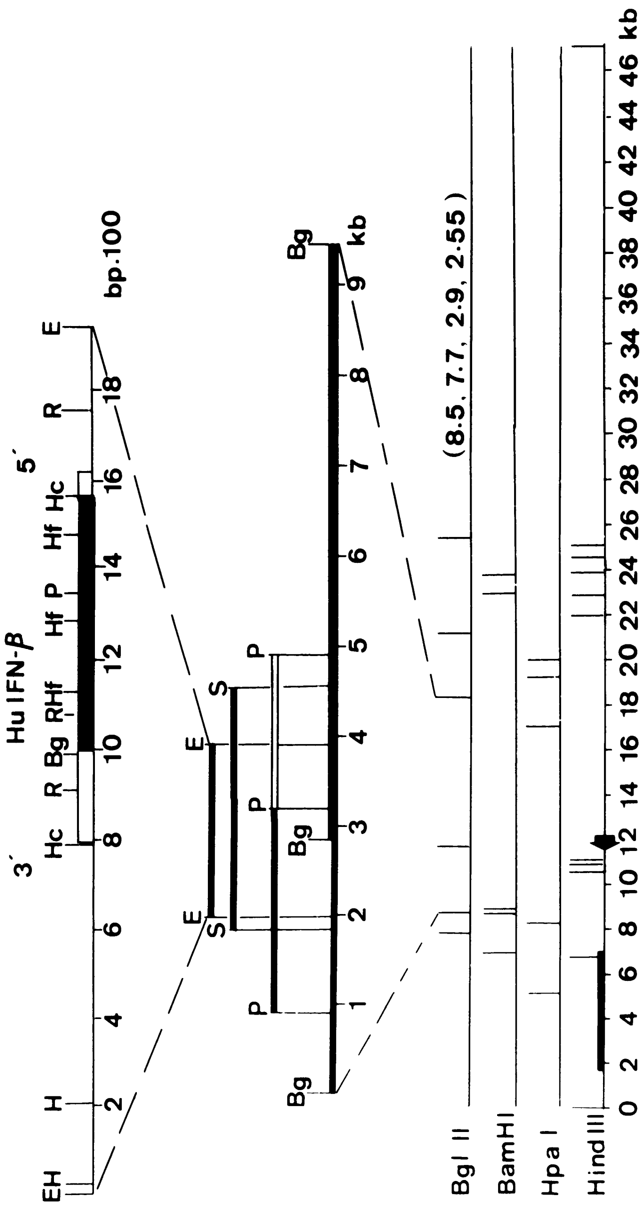

Fig. 7. Restriction map of pCosIFNß (arbitrarily linearised)

5kb to the 3'-end. The 1.9kb EcoRI fragment was isolated preparatively by electroelution and subjected to further detailed mapping as well as being used as a hybridisation probe for Southern blots (figure 6). It is of particular interest that when the EcoRI fragment is used in this manner only one band lights up on the Southern blot, thus indicating the absence of "pseudo-genes" or adjacent duplications of the same gene. Fine mapping with RsaI(R), HinfI(Hf), SstI(S), HindIII(H), BglII(Bg), PstI(P) and HincII(Hc), yielded a map of the 1900bp EcoRI fragment as shown at the top of Fig.7.A box representing the position of the IFNß gene has been positioned according to the fact that: a 179bp RsaI , 167 and 197bp HinfI, and a 570bp BglII fragments are present as predicted from the sequence of the IFNß cDNA [16]. This is interpreted as indicating that no intron exists between the left-most RsaI site and the right-most HincII site of this map, a region which includes all of the translated region and half of the 3'-tail region. Sequencing studies are still in progress. This EcoRI fragment has been subcloned as well as overlapping PstI fragments.

DISCUSSION

The experiments described here are a further demonstration of the use of the cosmid cloning system to isolate very large regions from complex genomes, and in particular is the first application of cosmid cloning of the the human genome.

The IFNß1 gene region was isolated in entirety. It was concluded that the gene was present as a single copy in this hybrid and that no related sequences are present. The translated region and at least half of the 3' non-translated tail region contain no intron, on the basis of restriction mapping.

The presence of some 36kb of DNA 5-prime to the IFNß gene in this clone, make it an interesting subject for studies of gene expression in vivo in eukaryotic cells.

The isolation of at least 8 distinct genomic regions coding for IFNα interferons by Nagata et al.[21] and the publication of the sizes of BglII, EcoRI, HindIII and BamHI fragments allows us to conclude that none of the alpha interferon coding regions are present in pCosIFNß. Nagata et al. also conclude that the IFNα1 gene contains no intron.

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DEVELOPMENT OF BROAD HOST-RANGE PLASMID VECTORS

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INTRODUCTION

The majority of cloning vectors developed so far are based on plasmids or phages with a narrow host-range such as ColEI(pBR322[1]), P15A(pACYC184[2]),pSC101[3] or phage λ[4]. These are limited to *Escherichia coli* or closely related enterobacterial species. For the genetic analysis and manipulation of a wider range of micro-organisms, including those of agricultural, medical, environmental or industrial importance, use has been made of plasmids belonging to the IncP group such as RP4 or RK2. For example RP4 has unique cloning sites for *Eco*RI, *Bam*HI, *Hind*III,*Hpa*I and *Bgl*II[5,6] and has been converted into a *Sal*I cloning vector pRP301[7,8]. From RK2 a two vehicle system: pRK290 plus pRK2013, has been constructed[9]. But such vectors remain relatively large, have a low copy number and are therefore unsuitable for some cloning purposes. For ease of manipulation and high gene dosage of the cloned material we need small, high copy number, broad host-range plasmids.

The non-conjugative plasmids have mostly not been given incompatibility group designations, although several types have been reported to be compatible with one another[10,11]. These are shown in Table 1 although many more groups probably exist. Only one of these groups, as represented by R300B, has been given an Inc designation viz IncQ[12]. This seems to be the only one of these plasmid groups with an extended host range as judged by attempts to mobilize members of these groups from *E.coli* into *Pseudomonas aeruginosa* or *Methylophilus methylotrophus* using RP4 (unpublished observations). We used plasmid mobilization because, in contrast to transformation, it is little affected by restriction systems in the host. IncQ plasmids are very commonly found[10], over a broad geographical and bacterial

Table 1. Incompatibility Groups of Non-conjugative Plasmids

Plasmid	Size (kb)	Phenotype[a]	Inc group	References
P15A	2.0	cryptic	-	2
ColE1-K30	5.3	ColE1$^+$	-	10,11
ColE2-P9	6.9	ColE2$^+$	-	10
ColK-235	6.9	ColK$^+$	-	13
pHH509	8.4	Ap^R	-	11
NTP2,R300B	8.5	SmSu^R	Q	10,11
pSC101	9.3	Tc^R	-	11
R831a	13.8	SmKm^R	-	11

[a]Phenotypic symbols are according to Novick et al[14]. The references give information on incompatibility relationships.

species range[15] and thus represent a particularly successful plasmid type. Members of this group that have been studied in recent years are R300B[15], NTP2[10], RSF1010 (which is identical to NTP2[16]) and R1162[17]. All these plasmids are indistinguishable at present[15,18].

Apart from their broad host-range, the other characteristics that might make IncQ plasmids suitable as cloning vectors are: 1) they are relatively small (the majority of those studied were 8.5kb[15]), 2) they have a relatively high copy number (in *E.coli*, measured as supercoiled DNA, we found 8-12 copies per chromosome[15,11]) and 3) they are non-conjugative but efficiently mobilized by conjugative plasmids of various groups (see below).

In this paper we describe further studies on R300B including the derivation of a restriction map, a genetic map by Tn3 mutagenesis, polypeptide synthesis in mini-cells and the creation of various derivatives useful in cloning.

MATERIALS AND METHODS

Growth media. Growth media were as previously described[19]. For *M.methylotrophus* we used M9 salts medium supplemented with 1% methanol plus $FeCl_3$,10 mg/l.

Plate-mating. Plasmids were generally transferred on solid media: a colony of the recipient strain was spread with a little sterile saline onto half the surface of a selective medium plate and colonies of the donor strains were then streaked in one direction across the plate into the recipient. For transfer into a variety of different (usually prototrophic) species, we used a *thy* donor which can be counterselected on Isosensitest (Oxoid) medium.

Plasmid DNA Isolation. Plasmid DNA was isolated after sarkosyl lysis of cells in ethidium bromide-CsCl gradients in a scaled-up version of the previously described method[15].

DNA Restriction Analysis. Plasmid DNA was restricted and analysed by gel electrophoresis as previously described[7]. Bands were cut out of gels and the DNA isolated by electroelution into dialysis tubing. DNA ligations overnight at 10°C and subsequent transformation were carried out by standard methods.

Analysis of Polypeptides in Minicells. Plasmids were mobilized or transformed into the minicell producing strain χ1411 *trp*EΔ. Minicells were isolated in freeze-thaw generated sucrose gradients[5], radiolabelled, the polypeptides electrophoresed through polyacrylamide gels and autoradiographed approximately as described by Dougan et al[20].

Isolation of Tn3 derivatives of R300B. Cultures of C600 (R1*drd*19/R300B) were grown overnight at 30°C to facilitate transposition[21] and then plated out on media containing 1 mg ampicillin/ml. Clones containing R300B::Tn3 are selected because the increase in copy number of the *bla* gene produced by transposition from the low copy number R1*drd*19 to the high copy number R300B leads to a corresponding increase in ampicillin resistance[22].

RESULTS

The Host-range of IncQ Plasmids

We have previously reported[15] that the host-range of IncQ plasmids includes *E.coli*, *Salmonella typhimurium*, *S.senftenberg*, *S.dublin*, *Proteus mirabilis*, *P.morganii*, *Providencia sp.* and *Pseudomonas aeruginosa*. Since then they have also been reported to replicate stably in *Ps. phaseolicola*[23] *Rhizobium meliloti*[24], *Rhodopseudomonas sp*[25] and *Acinetobacter calcoaceticus*[26]. Experiments in this laboratory have shown that R300B or its derivatives can also stably inhabit *Methylophilus methylotrophus*, *Alcaligenes eutrophus*, *Ps.putida*, *Klebsiella aerogenes* and *Serratia marcescens*. We have used conjugative plasmids of the following groups to mobilize IncQ plasmids into some of the above species:- IncFI: F104[15]; IncFII: R1*drd*19, JR72; IncIα: R144*drd*3[12], R483, JR66a; IncN: N3T; IncP: RP4, R751, R702; IncW: R7K. Not all of these plasmids are effective in all crosses except for RP4. This host-range list is not complete, merely those tested so far. However we have not yet found a Gram negative species that IncQ plasmids do not inhabit.

A Restriction Map of R300B

The construction of broad host-range cloning vectors from R300B depends upon knowing its restriction and genetic maps. A simple map of R300B was published recently[7]. Figure 1 shows our present restriction map. Although the *Eco*R1 and *Hpa*I sites are not distinguishable by restriction analysis we have shown that deletion of DNA to the left of the *Eco*R1 site (as in the construction of pGSS8, see below) does not remove the *Hpa*I site. A genetic map of RSF1010 relative to the *Eco*RI has been produced in Falkow's laboratory[16,18]. We have superimposed it onto our map in the orientation shown because removal of the 0.8kb *Pst*I fragment from R300B led to loss of sulphonamide resistance[7] whereas the cloning of random *Sst*I-cut fragments of *M.methylophilus* chromosomal DNA into the *Sst*I site always led to loss of streptomycin resistance.

In addition to the sites shown in Figure 1 there are also several sites on R300B susceptible to *Bgl*I[27], *Hae*II, *Hae*III, *Hinf*I[27] and *Sau*3A and a single site for *Sst*II[28]. But we found no cleavage sites for *Bam*HI, *Bgl*II, *Cla*I, *Hind*III, *Kpn*I, *Pvu*I, *Sal*I, *Sma*I, *Xba*I, *Xho*I, or *Xma*I. A similar map has been published for RSF1010[29] and two simple maps for R1162 are also consistent with it[17,30].

A Genetic Map of R300B

We have begun a genetic analysis of R300B using Tn3 mutagenesis, Figure 2 shows some of the Tn3 insertions we have mapped using restriction endonucleases. These data confirm the hypothesis first proposed by Heffron et al[18,31] that the two genes giving sulphonamide (*sul*) and streptomycin (*aphC*) resistance are in a single operon with *sul* proximal and *aphC* distal to the promoter : thus, pLT108 has lost

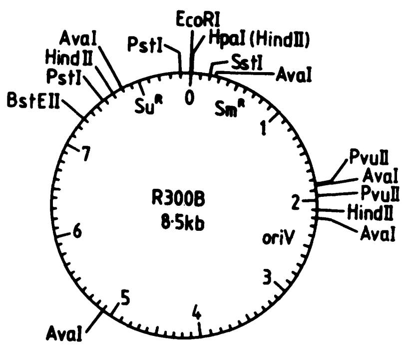

Fig 1. A restriction map of R300B

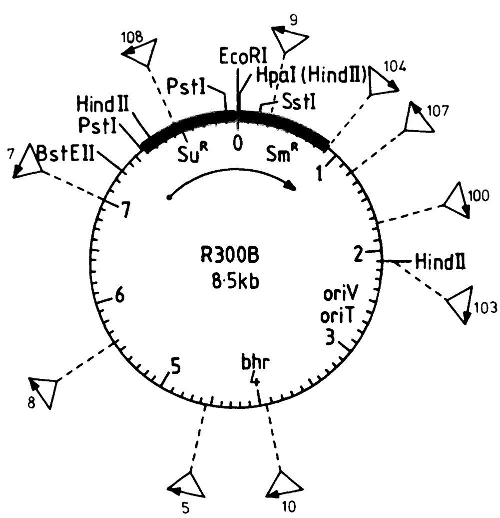

Fig.2. A Tn3 insertion map of R300B. The arrow heads on the Tn3 symbols are at the *bla* end and show the direction of transcription out of the transposon[31] when Tn3 is in its normal repressed (*tnp*R+) state[32]. The arrow within the circle shows the proposed start and direction of transcription of the operon giving drug resistances.

both Su^R and Sm^R whereas pLT9 has lost only Sm^R. The promoter is presumably to the left of the *Pst*I site at 7.6 kb since, as noted above, removal of the *Pst*I fragment does not lead to loss of Sm^R, although the level of resistance is somewhat reduced.

Insertions of Tn3 at around 4kb affect the broad host-range *(bhr)* properties of R300B. Such plasmids are still mobilizable into *E.coli* but not into *M.methylotrophus*. We do not know whether this is an effect on their transfer into, or maintenance in, the latter species. We also include in Figure 2 the approximate site of the presumed transfer origin (*ori*T). Nordheim and Timmis[33] have shown that the relaxation nick site of RSF1010, presumed to be *ori*T, is close to, but not at, the site of the replication origin (*ori*V).

Transcription of the *sul aph*C Operon

We have examined the polypeptides expressed by R300B and some of its Tn3 derivatives in minicells. As *sul* and *aph*C are in the same operon, their expressed polypeptides are likely to be present in about equal amounts. In Figure 3 it can be seen that band E is reduced to approximately half intensity by the Tn3 insertion of pLT9 (Sm^S),showing that the eliminated polypeptide (the amino-glycoside 3" phosphotransferase) bands at this position and suggesting that the remaining polypeptide in band E is the dihydropteroate synthase (*sul*). Dougan et al[34] have also deduced the identity of the former polypeptide. These polypeptides have a molecular weight of a little under 30,000 which require a coding capacity of just less than 900 base pairs each. The *sul* and *aph*C genes have therefore been drawn as this size on Fig.2. Their positions come from various considerations: (i) the Tn3 insertion in pLT104 does not affect the level of Sm^R, (ii) genes in an operon are normally adjacent and (iii) cleavage at the *Eco*RI site of R300B separated but did not inactivate the genes giving Su^R and Sm^R in the construction of pGSS8 and 9 from pGSS6 (next section). The *Eco*RI and probably the *Hpa*I sites therefore appear to be cloning sites within a transcription unit that would not give rise to fused proteins. This *sul aph*C operon appears to be highly expressed from a comparison of band E with the β-lactamase bands F and G.

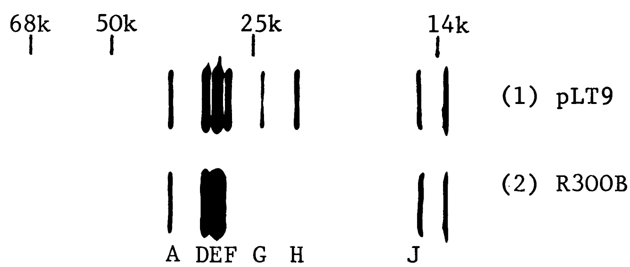

Fig.3. Autoradiogram of ^{35}S-labelled polypeptides from minicells containing (1) pLT9 (see Fig 2) and (2) R300B. Molecular weight markers are indicated above.

Construction of new Cloning Vectors from R300B

R300B has few sites for the restriction enzymes normally used for cloning. We therefore generated new derivatives by introducing genes from other plasmids. As pBR322 has been sequenced and is well understood, we used it as a source. By *Hae*II partial cleavage of both R300B and pBR322, followed by ligation, we generated a series of cointegrate plasmids such as pGSS6 (Figure 4). In this figure we have designated the origin of pBR322 as *ori*E and that of R300B as *ori*Q. Replication from *ori*E is dependent on *pol*A^{+}[35] whereas from *ori*Q it is not[36]. We have used this difference to distinguish between the two types of origin in the cointegrates and the subsequent cleavage products. We next cleaved pGSS6 and similar cointegrates with *Eco*RI and self-ligated the two fragments formed. From pGSS6, the ApSu^{R} plasmid (pGSS9) produced was found to be *pol*A^{+} dependent and not mobilizable into eg *M.methylotrophus* whereas the TcSm^{R} plasmid (pGSS8) shown in Figure 4 is *pol*A^{+} independent and has the broad host-range of the parental R300B. It also has the *Cla*I, *Hind*III, *Bam*HI and *Sal*I cloning sites in the gene conferring Tc^{R}. Transcription of the gene conferring Sm^{R} is however dependent on a backward reading promoter near the beginning of the *tet* gene. Cloning into the *Cla*I or *Hind*III sites can therefore cause loss of both markers.

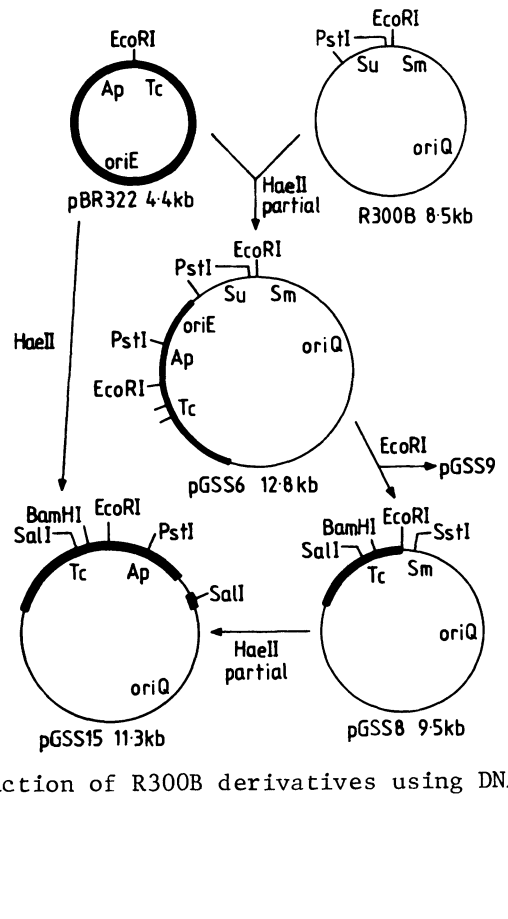

Fig. 4 Construction of R300B derivatives using DNA from pBR322.

Because of this, we decided to introduce the *bla* gene, which has its own promoter, from pBR322. *Hae*II cleavage of pBR322 and pGSS8 followed by ligation and transformation into a *pol*A host led to the recovery of pGSS15. In this plasmid the *bla tet* boundary of pBR322 has been reconstructed but the plasmid has the replication and host-range properties of R300B. (Unfortunately, an extra *Hae*II fragment carrying the *Sal*I site was also introduced. We are at present attempting to remove it).

Another series of vectors was made by addition rather than substitution of genes: these retain the strong *sul aph*C promoter of R300B. We restricted pACYC184[2] and pMK20[38] plasmid DNAs to completion with *Hae*II, ran them on a gel and then electroeluted the 1.3kb band from the former and the 1.5kb band from the latter. These contain the genes conferring Cm^R and Km^R respectively from the two plasmids as shown in Figure 5. These were separately ligated to partially *Hae*II cut R300B DNA eluted from a gel at the whole linear plasmid (8.5kb) position and transformed into *E.coli* selecting for Cm^R or Km^R clones. Few of the Cm^R clones proved to contain R300B::Cm^R plasmids like the example pTB86 in Figure 5: the majority had plasmids consisting of

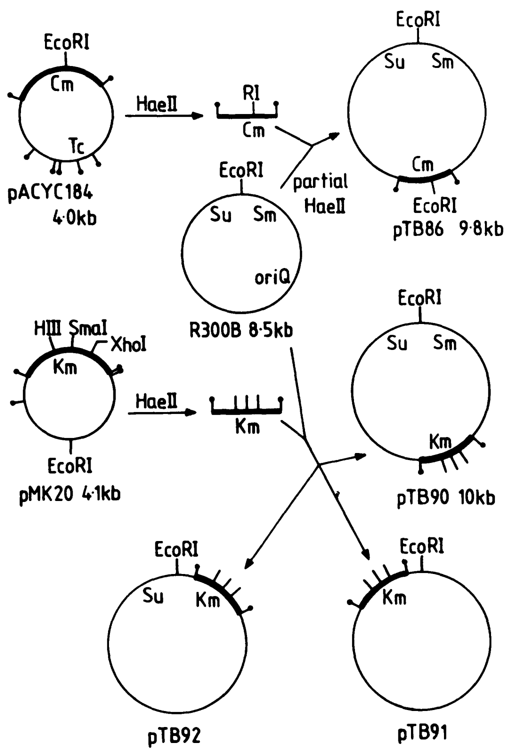

Fig.5. Construction of the Cm^R and Km^R derivatives of R300B. The *Hae*II sites are marked thus ↑.

the two largest, adjacent *Hae*II fragments of pACYC184 which can form a replicon[39]. The original *Eco*RI site needs to be removed from pTB86 and its sisters in order to use the one in the gene conferring Cm^R as an insertional inactivation site.

The Km^R clones were found to contain R300B::Km^R plasmids. Of 78 examined so far, 62 were $SuSmKm^R$ like pTB90, 6 were Km^R only, like pTB91 and 10 were $SuKm^R$ like pTB92. The latter two classes are consistent with the model that R300B has a single *sul aph*C operon with *sul* being proximal to the promoter and suggest that the inserted *Hae*II fragment blocks transcription in either orientation. Each of these plasmids has gained a *Hind*III, *Sma*I and *Xho*I cloning site. R300B has about 20 *Hae*II sites so we would expect insertion of the Km^R fragment at several of these sites causing other changes in phenotype apart from the loss of $SuSm^R$ or Sm^R already noted. We have therefore tested these 62 clones for such changes. Two have a reduced and one an increased, plasmid copy number (as determined by the Km and Sm resistance levels), 13 are non-mobilizable and 10 have a reduced host-range. We are mapping the inserts in these plasmids at present to complement our Tn3 mutagenesis mapping.

Transposon Derivatives of R300B

Another way of introducing cloning sites into plasmids is by using transposons. Such derivatives can then be further adapted by excising specific segments from them using pre-existing and newly-introduced restriction sites (as we have done for the RP4::Tn7 system[5,7]). This is another reason for our isolating the R300B::Tn3 derivatives described above. A Tn5 derivative of R300B (pTB70) has been used by us recently to clone gdh^+ from *E.coli* into a glutamate synthase mutant of *M.methylotrophus*[8]. By the consequent switch in the pathway for ammonium assimilation this has led to a significant improvement in the efficiency of conversion of methanol to single-cell protein by this organism. We have also isolated a Tn1771[40] derivative of R300B. The *tet* region of Tn1771 (and the indistinguishable Tn1721) can be amplified to give multiple tandem repeats[41]. Genes cloned into this portion will therefore be similarly amplified. Furthermore genes cloned into suitable sites on transposon derivatives of R300B can be transposed into the chromosomes of a wide range of organisms.

DISCUSSION

We have described the genetic structure and properties of R300B and the construction from it of some broad host-range cloning vectors which are proving to be very useful in our cloning systems. Bagdasarian et al[29] have also generated IncQ vectors, with *Bgl*II and *Xba*I insertional inactivation cloning sites. Meyer and Shapiro[37] and Bagdasarian et al[29] have reported the construction of cointegrates between a ColE1 and an IncQ plasmid using the *Eco*RI or *Pst*I sites respectively, but these plasmids do not stably inhabit *Pseudomonas*, or if selected, delete part of the ColE1[37]. (Gautier and Bonewald[30] also made such cointegrates via *Eco*RI but they do not report any reduction in host-range). It seems there may be a region of ColE1 that is inimical to broad host-range maintenance. If so, it must have been disrupted in the construction of pGSS6, as this plasmid (and its derivatives pGSS8 and 15) do not suffer this handicap: they are stable in at least *E. coli*, *P. aeruginosa*, *M. methylotrophus* and *A. eutrophus*.

There is some confusion in the literature about whether or not IncQ plasmids require polymeraseI for replication. Our data confirm the painstaking data of Grindley and Kelley[36] that they do not. Gautier and Bonewald[30] however, have drawn the opposite conclusion. This may be due to the slight instability of these plasmids in some *pol*A mutants[36].

There is clearly plenty of scope for the further development of these broad host-range cloning vectors. We do not know at present whether they can be reduced in size without loss of valuable functions. But we can introduce or select stronger promoters and put in cloning sites suitably down-stream from ribosome binding sites with perhaps a secretion leader sequence between. We also hope that our genetic analysis of R300B will lead to an understanding of how this fascinating plasmid functions.

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THE SURVIVAL OF EK1 AND EK2 SYSTEMS IN SEWAGE TREATMENT PLANT MODELS

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PREFACE

In March 1977 the National Academy of Sciences (USA) convened a Forum on Research with Recombinant DNA. It was clear to participants that this potentially was an opportunity to affect national science policy. In trying to assess the benefits and risks inherent in recombinant DNA technology, some argued the risks were not different than any in the microbiology laboratory; others warned that such research was the first step towards the manipulation of human genetics, that it could contaminate the biosphere irrevocably.

The decision to support work determining the potential for survival of EK1 and EK2 hosts and vectors in sewage treatment processes (as well as the vectors' capacity to be transmitted to secondary hosts indigenous to sewage) must be understood in this 1977 context rather than in the triumphant editorial in Science Recombinant DNA Revisited (1).

What was proposed in these studies was (a) to monitor the survival of EK1 and EK2 hosts and vectors and (b) to monitor the transmission of vectors to secondary hosts during sewage treatment. The importance of risk assessment was underlined by such reports as the interbacterial transfer of inter-*E. coli*-*Drosophilia melanogaster* recombinant plasmids (2) and the mathematical analysis of the probability of establishing these or other chimeric plasmids in natural populations of bacteria (3). Work subsequent to that reported here has resulted in a technique for expressing eukaryotic genes in bacteria (4) and the converse, the expression of a bacterial gene in mammalian cells (5). In addition, Peden et al. (6) have used the plasmid pBR322 to clone Simian Virus 40 in *E. coli*.

Levy et al. (7) have attempted a preliminary assessment of the probability of survival of an *E. coli* host-vector system in mouse and human intestines. In their report, no recoveries of *E. coli* K12, strain χ1776 were made from mice or human subjects 24 hours after ingestion. However, where the same strain bearing plasmid pBR322 was fed, recoveries were made for four days ($6/10^6$ ingested). The non-disabled *E. coli* K12 strain χ1666 (with or without pBR322) survived in 10^4 greater number and was recovered for six days. No evidence for intestinal colonization was obtained, nor was there any evidence for plasmid transfer to indigenous aerobic fecal bacteria.

Abelson (8) discussed the risk problem in terms of the inadvertent creation of a pathogen. The example he used was a "worst case" model in which *E. coli* carrying polyoma DNA would be found to induce tumors in mice. Less dramatic, but equally important examples of recombination among *E. coli* plasmids were cited (9-11). Striking in this context were the results of Gyles and his co-workers (12) who reported that genes for drug resistance are spread in nature not only by being part of an R factor but also by becoming incorporated into other plasmids. Almost at the same time, Williams (13) reported on self-transmissable plasmid transfer in the human alimentary tract. He found that in the absence of selective pressure, transfer of col V plasmids to indigenous fecal coliforms occurred in the human intestine after the ingestion of *E. coli* K12. These results are not completely consistent with the recent report of Levy et al. (7) discussed above.

In the U.S., municipalities generally use either primary settling coupled with lagooning or secondary biological treatment before disinfection and discharge to surface waters or irrigant ponds. The primary and secondary sludges may be dewatered and buried or incinerated or -- occasionally -- used as soil emendation agents with or without further treatment. (Alternatively these sludges may be digested anaerobically, and then dewatered, etc.)

Large cities, where land for lagooning is less available and far more costly, are likely to use some form of activated sludge treatment by which solid human organic wastes are solubilized. A significant by-product of this microbial degradation process is a large mass of biologically-generated secondary sludge. This often is subjected to anaerobic digestion at elevated temperatures with the production of methane as a potentially useful product. The stabilized product of such anaerobic digestion is then dewatered and/or incinerated, buried in sanitary landfills, or used as a soil emendation agent.

In small communities with inexpensive available land, treatment plants may use only primary settling followed by more or less prolonged lagooning prior to releasing the primary effluent (with or without disinfection) to receiving surface waters or land to be irrigated.

Foster and Engelbrecht (14) have noted the high level of removal of enteric viruses by biological secondary treatment. This is consistent with Schaub and Sagik's report (15) of the high efficiency of association of such viruses with clay particulates and colloidally-suspended organics. Removal was not synonomous with inactivation and such adsorbed viruses were still infectious in cell culture and in animals. K.R. Ranganathan and his colleagues (16) demonstrated in bench-scale models that viruses were protected by occlusion in the secondary sludge biomass. Moore et al. (17) in their analysis of a 10 mgd contact stabilization treatment plant found that well over 90% of enteric viruses entering the plant were concentrated into the mixed liquor suspended solids. This observation was confirmed in studies of virus distribution in other activated sludge treatment plants. Further studies by Moore (see Sagik ref. 18) and by Sanders et al. (19) showed the relative longevity in anaerobic digesters of sludge-associated viruses as compared to free viruses.

Analyses of wastewater grab samples for possible pathogenic bacteria were performed by Sagik et al. (20). Among the organisms isolated, enumerated, and identified were several which could serve as potential secondary hosts for plasmid vehicles.

MODEL TREATMENT PLANT RESULTS

The Treatment Plant. The wastewater treatment system used in this study was a bench-scale model incorporating all of the widely-used conventional treatment modalities (see Figure 1). As designed and operated, the system was fed with approximately 55 liters of raw sewage daily. The central treatment train was an activated sludge system which utilized primary and secondary (activated sludge) unit processes. There were three additional unit processes ancillary to this system: lagooning of both primary and secondary effluents and anaerobic digestion of wasted sludges. For simplicity, the unit processes are not described here, but details may be found in Eckenfelder's text (21) and in Sagik and Sorber (22).

During the course of this study, the unit processes comprising the model treatment plant were operated within the usual limits of loading and generally functioned as efficiently as do the field installations being simulated. Operational characteristics of the model treatment plant for the first six operational studies compared well with data typical of full-scale field installations (22).

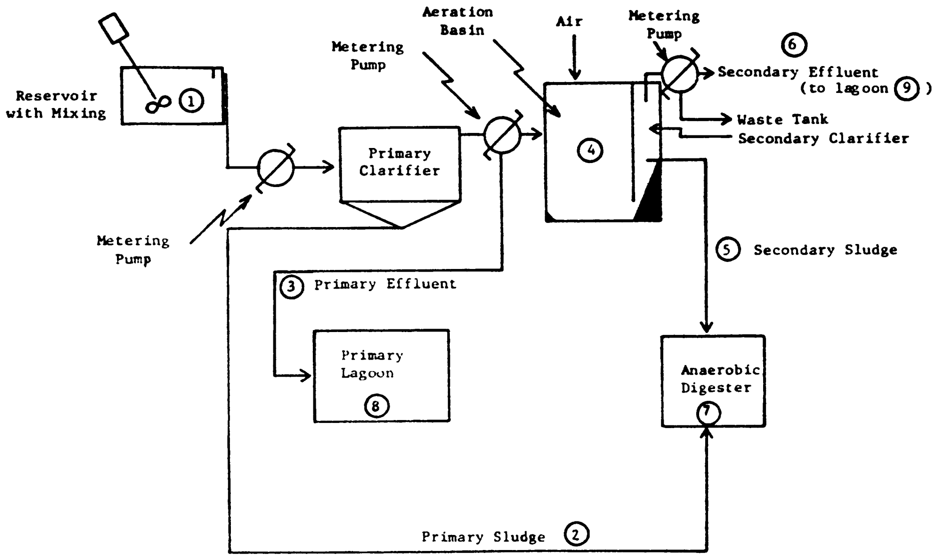

Figure 1. Schematic of Model Wastewater Treatment Facility and Sampling Points

Organism Survival. To provide a basis for the interpretation of the survival of EK1 and EK2 hosts within the treatment model, a series of operational studies were undertaken using a genetically marked sewage isolate, *E. coli* GF 215. Raw wastewater was seeded at a level of approximately 5 x 10^7 cfu/ml with sampling continued over 120 hours. The survival of E. coli GF 215 in the wastewater reservoir (0-48 hr) and in the primary and secondary lagoons (48-120 hr) is summarized in Table 1. Similar results were obtained using two marked prototypes of parental *E. coli* K12. The results obtained all show a high degree of correlation (r^2) between microbial inactivation and time. The k value (decay constant) of about 2 observed for parental *E. coli* K12 GF 29 was quite similar to that demonstrated for indigenous *E. coli* GF 215. The fate of these three prototypic *E. coli* strains during anaerobic digestion at 37°C is summarized graphically in Figure 2.

TABLE 1 Correlation Coefficients and Decay Constants for Survival of an Indigenous *E. coli* (GF215)

Unit	mean BOD_5 (ml/l)	r^2	k
Raw Wastewater Reservoir	140	.95	2.2
Primary Lagoon	21	.89	1.9
Secondary Lagoon	<10	.85	.96

Of the EK2 hosts, strain Dp50supF demonstrated survival analogous to parental E. coli strains while E.coli χ1776 was inactivated more rapidly in raw wastewater and primary effluent with a maximum decay constant of 3.6. The effectiveness of anaerobic digestion of sludges can be inferred by the rapid disappearance of E. coli χ1776 to a level of nondetectability within 20 hours (5 $\log_{10}$ loss). The survival of E. coli Dp50supF in an anaerobic digester was closer to parental K12 strains, with a 90% reduction evident after 20 hours.

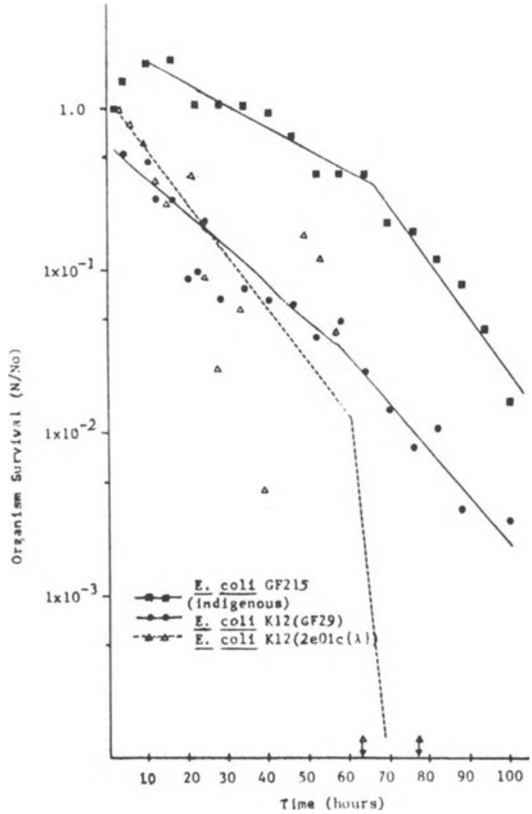

Figure 2. Inactivation of Prototypic and Paremtal E. coli strains during Anaerobic Digestion

Two plasmid-bearing hosts also were evaluated as part of this study. Survival data for E. coli χ2656, carrying pBR322, and E. coli GF2174, carrying pBR325, are given in Table 2. It is seen that E. coli GF2174 was more labile than either E. coli χ2656 or total coliforms in raw sewage. Similarly, strain GF2174 was inactivated rapidly during anaerobic digestion with only sporadic recovery of viable organisms 20 hours after introducing seeded-sludges into the digester. E. coli χ2656(pBR322) was more stable in this unit process, with an observed 90% reduction within 30 hours. From these results it is not evident that the presence of a plasmid within a host cell confers any unique survival capabilities to an organism.

The only cloning vector used during this study was the lambda phage, Charon 4A. Its correlation coefficient is near 1.0, as is that of indigenous E. coli, indicating a linear relationship between organism decay and time. As in previous studies the decay constant (k) for total coliforms was about 1.5. The Charon 4A phage displayed a similar decay constant in both lagoons. In raw wastewater, however, the vector disappeared much more rapidly as evidenced by a k value exceeding 5. A plausible explanation for this extreme discrepancy may be viral adsorption to particulate matter present in raw sewage at a greater concentration than in lagooned effluents.

TABLE 2 Correlation Coefficients and Decay Constants for Survival of Total Coliform and Plasmid-Bearing EK2 Hosts

Unit	mean BOD_5 (mg/l)	Total Coliforms r^2	Total Coliforms k	E. coli 2656(pBR322) r^2	E. coli 2656(pBR322) k
Raw Wastewater Reservoir	280	.60	.38	.89	1.2
Primary Lagoon	28	.89	1.4	.91	1.9
Secondary Lagoon	2	.71	.79	.93	1.5

Unit	mean BOD_5 (mg/l)	Total Coliforms r^2	Total Coliforms k	E. coli GF2174(pBR325) r^2	E. coli GF2174(pBR325) k
Raw Wastewater Reservoir	400	.83	.62	.90	2.7
Primary Lagoon	26	.85	1.8	.89	1.9
Secondary Lagoon	6	.24	.70	.26	.67

Organism Removal. The treatment effectiveness of the primary and secondary unit processes were evaluated using BOD_5 and TSS values. The reduction of organisms within either unit was viewed in relation to the removal of the physical/chemical parameters. For purposes of this presentation, secondary treatment was handled as an independent unit process. Mean removals were calculated using only positive values; that is, when effluent values were lower than influent values. This approach was used in order to compensate for treatment system upsets. The small model plant operated during this study had very little buffer capacity when compared to the field treatment plants which seldom lose their ability to affect some degree of wastewater treatment.

Studies using genetically identifiable indigenous *E. coli* K12 GF 215 and parental *E. coli* K12 GF29 demonstrated similar reductions of 20% to 30% as a result of primary treatment and 95% to 99.9% removal as a result of secondary treatment. Comparable results were obtained with EK2 hosts during primary and secondary treatment. The removal of both *E. coli* Dp50supF and *E. coli* χ1776 was compared to the behavior of indigenous bacteria as measured by total coliform organisms and similar results were obtained.

Results in Table 3 demonstrate the behavior of the lambda phage, Charon 4A, within the treatment train. While bacterial levels in primary effluent are decreased, very little effective removal of the Charon phage occurred during primary treatment. However, phage removal from sewage during secondary treatment was quite effective.

Overall, the removal of EK1 and EK2 hosts and the Charon phage during the process of conventional sewage treatment paralleled the behavior of indigenous wastewater bacteria. Not unexpectedly, more variability was observed during primary treatment. This unit process essentially represents passive settling of particular matter that has a higher specific gravity than water and is too large to remain in suspension due to convection. Indigenous bacteria may be associated with solids to a variable extent depending upon their source (fecal material or other) and the degree of solids dispersion due to turbulence in transmission lines and, in the case of this study, mixing within the wastewater reservoir. Test organisms were added to the wastewater as a suspension and initially were unassociated with particulates. Even so, organism removal occurred during primary treatment and was confirmed by the detection of viable seed bacteria and phage within the primary sludge.

TABLE 3 Treatment Effectiveness for the Removal of Charon 4A Phage

	Primary Treatment (% removal)				Secondary Treatment (% removal)			
Time (days)	BOD_5	TSS	Total Coliform	Charon 4A	BOD_5	TSS	Total Coliform	Charon 4A
0	55	64	91	-	53	29	89	-
1.0	50	66	36	-165	70	50	98	97
2.0^{+}	78	66	67	41	70	9	77	93
5.0	38	55	**	**	64	39	96	α
mean$^{\beta}$	50	62	53	41	69	46	90	96

α organism concentration below detection levels
β calculated from positive removals
* not analyzed
** not calculated after cessation of seeding
\+ organism seeding discontinued at 48 hr., fresh sewage placed in reservoir
++ not calculated after cessation of seeding

Secondary treatment of wastewater by activated sludge processes involves the active development of bacterial floc utilizing the soluble organics in sewage as a nutrient source. Organisms entering the aeration basin become entrapped within the mixed liquor suspended solids (MLSS). This association with MLSS does not immediately lead to organism inactivation. For secondary treatment to achieve effective organism removals, therefore, separation of the liquid and solid phases (MLSS) must be achieved within the secondary clarifier. Because of this, the correspondence between TSS removal and organism removal is more evident during secondary treatment.

As with the primary treatment process, the biomass generated by secondary treatment carries viable test organisms. Seventy-two hours after the cessation of seeding the raw wastewater reservoir, relatively high levels of selected host bacteria were still being recovered from secondary sludge in these studies. The accumulation of viable organisms in sewage sludges and their persistence in this mileau reiterate the need for adequate sludge handling prior to terminal disposal of these solids. Efficient high-rate anaerobic sludge digestion can provide a useful buffer for this purpose.

Mass Balance of Test Organisms With the Primary Clarifier. In an attempt to ascertain the potential for EK1 and EK2 host colonization within the central treatment plant model, a limited mass balance approach was used. The primary treatment process was assumed to be the ideal unit to study, in that the organisms were subjected to relatively quiescent conditions with maximum organic load (nutrient source). The data documenting influent and effluents (sludge and primary effluent) of the primary clarifier were readily available.

A mass balance ration (MBR) relates the level of test organisms transferred out of the primary clarifier to the number of test organisms entering the primary clarifier over a 12 hour period:

$$\text{MBR} = \frac{\Sigma(E + S + Cap)}{\Sigma(I + Cip)}$$

where: E = total cfu leaving the clarifier in primary effluent

S = total cfu in primary sludge wasted from the clarifier

I = total cfu entering the clarifier in influent wastewater

Cap = average cfu in primary clarifier

Cip = instantaneous cfu in primary clarifier at the end of the preceding 12 hour period.

If organism colonization (as evidenced by growth) occurred, the MBR value should be significantly greater than unity.

As evidenced by MBR values of approximately one, no significant colonization of the primary clarifier could be documented for any of the *E. coli* hosts tested.

Plasmid Transfer Studies. Laboratory studies using pure cultures of test organisms were conducted to ascertain the most favorable conditions under which the transfer of plasmid DNA might occur. Based on the results of these controlled laboratory studies, it was expected that the transfer of either pBR322 or pBR325 to indigenous organisms in sewage would be quite low. Addition of a mobilizer strain such as *E. coli* χ1784 would be expected to increase the frequency of plasmid transfer to a more readily detectable level.

Initial testing was conducted by mixing equal volumes of *E. coli* χ2656 with raw sewage or primary sludge. Stationary cultures were held at 37°C and sampled at times 0, 5, and 25 hours. A rapid disappearance of χ2656 was observed along with an increase of 3 - 4 $\log_{10}$/24 hr of indigenous organisms showing resistance to tetracycline (12.5 μg/ml) and carbenicillin (500 μg/ml). Subsequent experiments demonstrated that this increased antibiotic resistance of sewage bacteria was attributable solely to the test conditions promoting growth of this population. No transfer of pBR322 could be demonstrated in this system.

Plasmid transfer of pBR325 from *E. coli* GF2174 to indigenous sewage bacteria was evaluated in both the presence and absence of the mobilizer strain, *E. coli* 1784. Representative results from such an experimental series are shown in Table 4. In the absence of either donor or mobilizer strains, levels of indigenous wastewater bacteria resistance to tetracycline (12.5 μg/ml), carbenicillin (100 μg/ml) and chloramphenicol (25 μg/ml) increased by a factor of 4.7 at 24 hours. When *E. coli* GF2174 (pBR325) was added to wastewater, the level of this resistant indigenous population was observed to increase 8.1 over the same time interval. Interestingly, with both donor and mobilizer *E. coli* strains present in the test system, a 25-fold increase in the level of antibiotic resistant indigenous wastewater bacteria was measured. Such observations are suggestive of plasmid transfer.

SUMMARY AND CONCLUSIONS

We proposed in 1978 to monitor (a) the survival of EK1 and EK2 hosts and vectors and (b) the transmission of such vectors to secondary hosts during sewage treatment. In order to do (a) meaningfully, we carried out comparative studies with indigenous bacteria modified so as to permit their selective observation against the background of unaltered microbiota.

These studies have shown a good linear relationship between organism decay and time in the raw wastewater reservoir, and in the primary and secondary lagoons for both the strain derived from indigenous flora and that derived from a non-debilitated *E. coli* K12. During anaerobic digestion, the indigenous strain showed greater stability than did the K12 (1 $\log_{10}$ reduction in 70 hours for the former vs 30 hours for the latter). The EK2 host *E. coli* Dp50supF showed survival characteristics similar to the non-debilitated K12 derived strain. *E. coli* χ1776 was inactivated far more rapidly in raw wastewater and primary effluent and could not be recovered from anaerobic digestors by 20 hours after seeding. In contrast, Dp50supF suffered only a one $\log_{10}$ reduction in 20 hours (being similar to the non-debilitated parental *E. coli* K12 strain). There is no evidence that the presence of a plasmid within the host cell confers any differential survival.

TABLE 4

Frequency of pBR325 Plasmid Transfer to Indegenous Wastewater Bacteria

		E. coli GF2174		*E. coli* χ1784		Tet^R $Carb^R$ Cm^R Wastewater Bacteria	
Flask	Sampling Time (hr)	cfu/ml	N/No	cfu/ml	N/No	cfu/ml	N/No
A	0	None	-	None	-	5.5×10^6	1.0
	0.5					6.5×10^6	1.2
	1.5					5.5×10^6	1.0
	24					2.6×10^7	4.7
B	0	5.5×10^8	1.0	None	-	8.0×10^6	1.0
	0.5	6.0×10^8	1.1			6.5×10^6	0.8
	1.5	6.5×10^8	1.2			6.0×10^6	0.8
	24	3.5×10^8	0.6			6.5×10^7	8.1
C	0	5.7×10^8	1.0	1.6×10^{10}	1.0	8.3×10^6	1.0
	0.5	7.5×10^8	1.3	1.8×10^{10}	1.1	8.3×10^6	1.0
	1.5	8.3×10^8	1.5	2.0×10^{10}	1.3	7.4×10^6	0.9
	24	6.0×10^8	1.1	1.6×10^{10}	1.0	2.1×10^8	25

The lambda phage Charon 4A was studied as one example of a cloning vector. With the exceptions of raw wastewater and the anaerobic digestor, the data indicate a linear relationship between organism decay and time (correlation coefficient equal to 1.0). In raw wastewater, however, disappearance of this phage was very rapid (with $K \geq 5$). In contrast, recovery of Charon 4A was excellent in anaerobic digestors with about a one $\log_{10}$ reduction in 40 hours. These data suggest the importance of continuing studies on plasmid transfer to indigenous flora.

In laboratory studies using E. coli χ2656 (pBR322) as a donor strain and other strain χ1784 (R - $\overline{100^{+}}$ drd) - derepressed, for transfer - or F 101/C600 as mobilizer strain, conditions for transmission of the plasmid to strain χ1997 were examined. In untreated wastewater, in the absence of either donor or mobilizer strains, indigenous organisms resistant to tetracycline (12.5 μg/ml), carbenicillin (100 μg/ml) and chloramphenicol (25 μg/ml) increased by a factor of 4.7 in 24 hours. With the addition of E. coli GF2174 (pBR325), the recovery of such multiply resistant possibly indigenous organisms increased 8.1-fold in the same time. With both the donor and mobilizer E. coli strains present in the raw wastewater, there was a 25-fold increase in the level of multiply resistant organisms recovered. This observation is consistent with plasmid transfer during initial contact with indigenous flora, with the recipients then replicating in the next 24 hours of monitoring.

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cos PLASMID IN BACILLUS SUBTILIS

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INTRODUCTION

The genome of temperate phage λ is a linear duplex DNA molecule with single stranded termini. The single ends contain complementary base sequences allowing λ DNA to circularize *in vitro*. Treatment of such hydrogen bonded circles with DNA ligase produces a covalently-closed circle. *In vivo* λ DNA replicates via the rolling circle model (1) and the viral DNA is packaged into phage heads as linear molecules. The cohesive ends of λ DNA appears to serve as initiation sites for packaging the λ genome (2). Insertion of the cohesive ends (*cos*) of λ DNA into a plasmid, by recombinant DNA technology, confers on the resulting *cos* plasmid susceptibility to packaging by λ *in vivo* or *in vitro*, if the size of the plasmid approximates that of the viral genome (3). Insertion of *cos* into a small plasmid, such as pBR322, generates a *cos* plasmid that can only be packaged into mature phage particles if additional DNA is cloned into the small *cos* plasmid to increase its molecular size. Thus, λ *cos* plasmids or cosmids serve as excellent vectors for cloning rather large DNA segments (3).

Two relatively well studied *Bacillus subtilis* temperate phages, Ø105 and SPO2 (4), were chosen to determine if the cohesive ends could be cloned on a plasmid in *B. subtilis*, and the biological properties of such a chimera.

CLONING RESTRICTION FRAGMENTS OF Ø105 and SPO2 DNA IN *B. subtilis*.

We previously reported that the *B. pumilus* temperate phage Ø75 was capable of transduction of plasmid pPL10 (5). In contrast, Ø75 did not mediate transduction of many other plasmids including pUB110 and pCM194. Subsequent studies demonstrated that the Ø75 genome shared extensive homology with pPL10 (R. Taylor, unpublished). Thus we tested whether insertion of segments of the genomes of Ø105 and SPO2 into plasmids would render the resulting chimeras susceptible to transduction by the phage whose DNA was cloned into the plasmid. The resulting method, called transductional cloning, allows one to directly select for plasmids containing phage DNA inserts (6). In practise, phage DNA (e.g., Ø105) is digested with a chosen restriction endonuclease such as EcoR1, which generates cohesive termini. A vector plasmid such as pUB110 which specifies neomycin-resistance (Neo^R) and has a single EcoR1 sensitive site, is similarly digested and ligated with the phage DNA fragments (7). The mixture is used to transform a B. subtilis 168 derivative that is lysogenic for Ø105 [BR151 (Ø105)]. Neo^R transformants are selected in liquid culture, and the cells are treated with mitomycin C to induce the Ø105 prophage. The resulting Ø105 lysate is used to transduce BR151 (Ø105) to Neo^R. Each transductant contains a pUB110 derivative with a phage DNA insert (6).

Cloning EcoR1 fragments of SPO2 DNA into pUB110 with selection by the transductional cloning procedure allowed recovery of only a single type of chimera designated pPL1010. pPL1010 (4.6 Md) consists of the 1.6 Md segment of SPO2 DNA corresponding to the cos region of the phage genome previously identified by Yoneda et al (8) joined to pUB110 (3 Md).

BIOLOGICAL PROPERTIES OF pPL1010

pPL1010 is not detectably transduced by Ø105 whereas the plasmid is transduced by SPO2 at a frequency of 1 transductant per 100 PFU. This frequency is about 100-fold greater than that of other chimeras constructed (6) and is presumably the reason for pPL1010 being the only detected product of cloning EcoR1 SPO2 DNA fragments. Hybridization of nick translated pUB110 to Southern blots of undigested DNA from SPO2 (pPL1010) transducing particles subjected to agarose gel electrophoresis, demonstrated that pPL1010 was carried by the phage in a form whose molecular weight approximated that of the SPO2 genome (6). Accordingly, pPL1010 was thought to be carried by transducing particles either as a multimer or as a recombinant between plasmid and the SPO2 genome. If the plasmid were carried as a multimer then the transducing activity of SPO2 (pPL1010) lysates should be more resistant to ultraviolet irradiation than if the plasmid were carried as a single copy per phage particle due to complementation and/or recombination among the plasmid subunits in the multimer. As shown in Table 1, SPO2 transduction of pPL1010 is more resistant to ultraviolet irradiation than is inactivation of PFU when the recipient is recombination-proficient. This apparent resistance to inactivation is lost when

Table 1. Effect of ultraviolet irradiation on transduction by SPO2 (pPL1010)

Time of irradiation	PFU	Neo^R Transductants Recipient recE$^+$	recE$^-$
0 min	100%($1.6x10^8$PFU/ml)	100%($1.6x10^6$/ml)	100%($1.4x10^6$/ml)
1	60%	98%	30%
2	11%	94%	9%
4	2%	88%	0.9%
5	0.4%	72%	0.4%

the transduction recipient is recombination-deficient (Table 1).

pPL1010 has a buoyant density of 1.699 whereas SPO2 DNA has a buoyant density of 1.702. If the plasmid were carried as a multimer, then the transducing particles should have a reduced buoyant density relative to SPO2 infectious particles. In fact, nearly complete resolution of transducing particles from infectious particles can be achieved in a CsCl equilibrium gradient (Fig 1). DNA isolated from such enriched transducing particles and centrifuged to equilibrium in CsCl (using an analytical ultracentrifuge) contained predominately the 1.699 DNA species (pPL1010) and a second species of 1.702 (SPO2 DNA). Thus, the plasmid isolated from enriched transducing particles retained its characteristic buoyant density, which would not be the case if the plasmid were carried as a recombinant with the SPO2 genome.

RESTRICTION ENZYME ANALYSIS OF DNA FROM ENRICHED TRANSDUCING PARTICLES

pPL1010 and SPO2 DNA can be distinguished by their sensitivity to BamHl and Sst-1 restriction endonucleases (9). BamHl cuts pPL1010 once but does not cut SPO2 DNA, and Sst-1 digests SPO2 DNA into seven fragments but does not cut pPL1010. Hybridization of nick translated pUB110 to Southern blots of electrophoretically separated products resulting from Sst-1 digestion of DNA from enriched transducing particles demonstrated homology only with DNA migrating at the approximate position of intact SPO2 DNA (9). Thus, pPL1010 is likely carried as a multimer. Substituting BamHl for Sst-1 resulted in an autoradiogram demonstrating that pUB110 hybridized predominently to 4.6, 3.3, and 1.3 Md linear digest products (9). These data suggest that pPL1010 is carried by SPO2 as a linear multimer with the 3.3 and 1.3 Md linears representing the BamHl ends of the multimer.

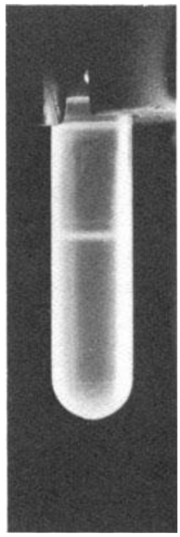

Fig. 1. An SPO2 (pPL1010) lysate centrifuged to equilibrium in CsCl. Upper band contains approximately 10^{10} tranducing particles and 10^{10} PFU, while the lower (main) band contains approximately 10^{12} PFU and 10^{9} transducing particles.

DISCUSSION

The evidence presented indicates that pPL1010 is carried by SPO2 transducing particles as a linear multimer. A diagram of the proposed structure of pPL1010 as isolated from SPO2 transducing particles is shown in Figure 2. The number of plasmid monomers in the multimer (probably seven) is inferred from the molecular weight of pPL1010 (4.6 Md) and the molecular weight of the SPO2 genome (approx 31 Md; ref 9).

Transducing particles carrying a plasmid containing the cohesive ends of λ DNA harbor the plasmid as a monomeric linear (10). The ends of the linear contain the cohesive ends of λ. A plausable explanation for the origin of the multimeric linear form of pPL1010 detected in SPO2 particles requires pPL1010 to replicate according to the rolling circle model (1). It is not essential that the normal mode of replication for pPL1010 follow the rolling circle model; this replication mechanism could be induced by infection of a cell carrying pPL1010 by SPO2. The product of this mode of replication is a linear concatamer from which head full pieces, starting and finishing with cos can be packaged by SPO2. The key features of the pPL1010 multimer that are consistent with its origin from such a replication mechanism include the similarity in molecular weight of the plasmid multimer and the SPO2 genome, the

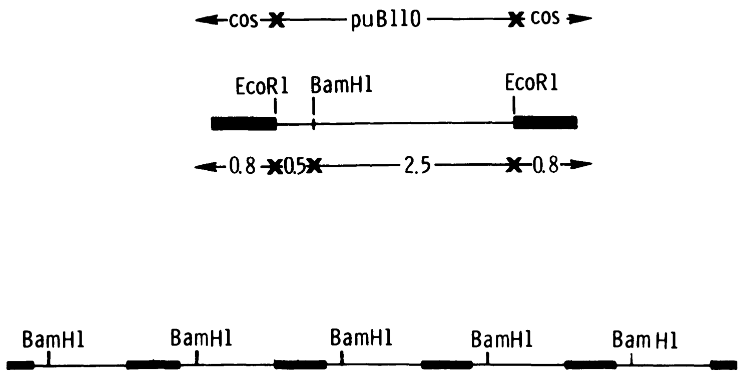

Fig. 2. Diagram of pPL1010 (opened at the cohesive ends) and the proposed structure of the multimeric linear carried by SPO2 transducing particles. The multimer is shown as a pentamer for diagrammatic purposes only. The multimer is more likely a heptamer since its molecular weight approximates that of the SPO2 genome.

organization of the subunits in the multimer in the same polarity, and the 3.3 and 1.3 Md BamH1 generated ends of the multimer which suggest the ends contain the cohesive ends of SPO2.

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A MUTATIONAL AND TRANSCRIPTIONAL ANALYSIS OF A TUMOR INDUCING PLASMID OF AGROBACTERIUM TUMEFACIENS

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INTRODUCTION

The large tumor inducing (Ti) plasmids (Zaenen et al., 1974) of *Agrobacterium tumefaciens* are the causitive agents of gall tumors on dicotylendonous plants. The plant cell transformation is brought about by the stable integration of a portion of the bacterial Ti-plasmid into plant nuclear DNA (Chilton et al., 1978a; Thomashow et al., 1980a; Thomashow et al., 1980b; Lemmers et al., 1981; Chilton et al., 1980; Yadav et al., 1980). Transformed plant cells are characterized by the following properties: the ability to grow in azenic culture without an exogenous supply of the plant hormones auxin and cytokinin (Braun, 1958) and the synthesis of unusual amino acids called opines (Petit et al., 1968; Menage and Morel, 1964; Goldman et al., 1968; Goldman et al., 1969; Fermin and Fenwick, 1978). The transferred plasmid DNA (T-DNA) is transcribed, (Drummond et al., 1978; Yang et al., 1979; Gelvin et al., 1981; Ledeboer, 1978; Gurley et al., 1979) influences the levels of plant hormones, and directs the synthesis opines (Bomhoff et al., 1976; Montoya et al., 1977; Kemp et al., 1979; Hack and Kemp, 1980; Guyon et al., 1980) in transformed plant cells. Thus, crown gall tumorigenesis is a model system for the study of the mechanism by which a bacterial plasmid transforms a eukaryotic cell causing a neoplastic disease.

While the T-DNA plays an essential role in crown gall induction and maintenance, it only comprises 8-20% of the Ti-plasmid. Koekman and coworkers (1979) found that only 40% of the Ti-plasmid could be deleted before the bacteria could no longer produce tumors. Thus, other areas of the plasmid must encode functions necessary for tumor formation. Avirulent mutants have been generated by insertion of the transposons Tn7 (Hernalsteens et al., 1978) and Tn904 or Tn1821 (Ooms et al., 1980) into the Ti-plasmid of Agrobacterium tumefaciens. These studies were only concerned with Ti-plasmid borne insertions, while gene products necessary for tumorigenesis might be encoded by the chromosome or by other plasmids present in some virulent strains. In this study we have utilized the transposon Tn5 to mutagenize the entire bacterial genome. We then selected mutants with altered virulence, host range and ability to catabolize opines; properties known to be coded by the plasmid (Bomhoff et al., 1976; Montoya et al., 1977; Guyon et al., 1980; Thomashow et al., 1980c). We have mapped these insertions to specific restriction fragments of the Ti-plasmid or to the chromosome. In addition we have also investigated the transcription of the Ti-plasmid during different growth regima and will correlate the information from the transcriptional studies with the mutant data.

RESULTS AND DISCUSSION

Isolation and Mapping of Transposon Induced Mutants. Berg (1973) has shown that mutations due to Tn5 insertion result from direct gene inactivation or polarity effects and usually result in complete loss of gene function. We used the vehicle pJB4J1 to deliver the Tn5 transposon to Agrobacterium. This plasmid has been shown to be unstable in Rhizobium (Beringer et al., 1978) and Agrobacterium (Garfinkel and Nester, 1980). The kanamycin resistant gentamycin sensitive transconjugants represent Tn5 transpositions into the Ti-plasmid, the chromosome or other cryptic plasmids. Insertions into loci that code for functions necessary for tumor induction or maintenance will yield strains that are avirulent. We screened 8,900 kanamycin resistant transconjugants for virulence by inoculating wounds on leaves of Kalanchoe diagremontiana and 40 mutants were identified with altered virulence properties. All transconjugants were also screened for the utilization of octopine as a sole source of nitrogen on bromthymol blue indicator plates (Hooykaas et al., 1979). Seven mutants were isolated which failed to utilize octopine.

Ti-plasmid was isolated from the 40 avirulent and 7 octopine non-utilizing mutnats. These preparations were cleaved with the restriction endonucleases KpnI, HpaI, and SmaI and subjected to electrophoresis on 0.7% horizontal agarose slab gels. The gels were then stained with ethidium bromide, visualized with a short wavelength ultra violet light, and photographed. The restriction endonuclease KpnI does not cut the Tn5 transposon, while SmaI cuts it once near the center and HpaI cuts it twice in the inverted repeats approximately 0.2 Md from the end of the transposon. Therefore, in KpnI digests of the plasmid the fragment in which the transposon is located will increase in molecular weight by 3.5 Md, the size of Tn5, and will exhibit an altered mobility upon electro-

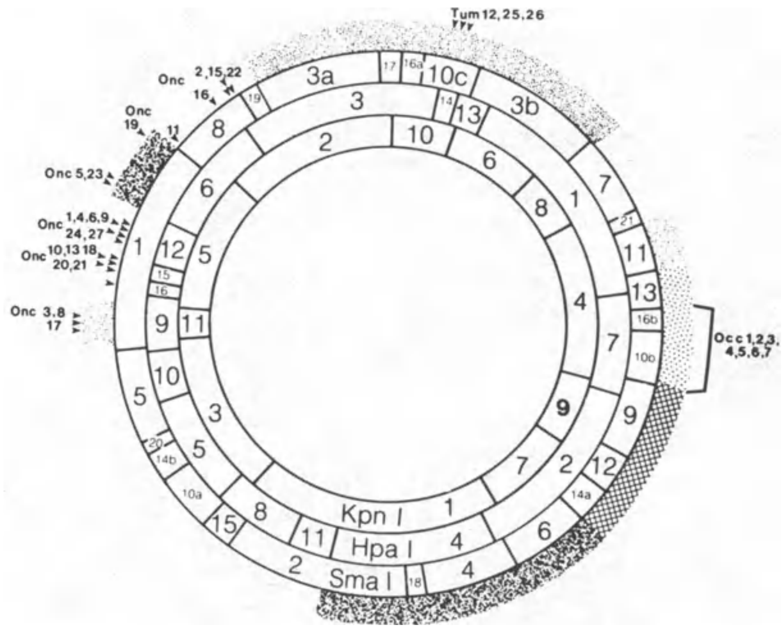

Fig. 1. Restriction endonuclease map of the pTi$B_6$806 plasmid (Chilton et al., 1978b; Ooms et al., 1980) showing locations of Tn5 transposition insertions and regions of transcription in the bacterium. [light stipple] Regions showing low levels of homologous transcripts. [dense stipple] Regions showing high levels of homologous transcripts. [dotted] Regions showing homologous transcripts inducible by octopine. [cross-hatched] Regions showing homologous transcripts inducible by agropine. Onc - mutants with altered virulence properties. Tum - mutants which give rise to tumors with altered morphologies. Occ - mutants which are unable to catabolize octopine.

phoresis. In SmaI digest the original fragment in which Tn5 has transposed will disappear and two new fragments will appear. Three new fragments will appear in the HpaI digest. Any ambiguities presented by multiple fragments of similar molecular weights in any one digest can be resolved in this way and the Tn5 insertions can be located on the map of the octopine plasmid established by Chilton et al. (1978b) and extended by Ooms et al. (1980) (see Figure 1).

Twenty-five of the 40 mutants affected in virulence had Tn5 located on the Ti-plasmid. Twenty-one of these mutants were completely avirulent on the four plants tested and mapped in a region bordered by HpaI fragment 9 on one end and SmaI fragment 8 on the other end. (See Table 2 and Figure 1 for details.) The other four mutants which had Tn5 on the Ti-plasmid exhibited altered tumor morphologies. Two of these mutants mapped on HpaI fragment 6, while the other two mapped in the region of DNA transferred to the plant cell (T-DNA)(Figure 1). The two mutants which mapped in HpaI fragment 6 produced tumors on Kalanchöe leaves and stems and on tobacco stems which proliferated abundant roots from the tumors, while tumors on sunflower, tomato and carrot slices were normal. The two T-DNA mutants mapped in two different loci in HpaI fragment 14 and exhibited a lack of root proliferation on Kalanchoe stems and production of shoots from the unorganized callus on tobacco stems (Figure 1, Table 1). One additional insertion mutant was identified on the T-DNA fragment HpaI-14. However, the plasmid DNA from this mutant did not exhibit the expected cleavage patterns when cut with HpaI, KpnI or SmaI and demonstrated no homology between the Ti-plasmid and Tn5. Therefore, the 1.0 Md insertion into HpaI fragment 14 may have originated from an Agrobacterium insertion sequence. This mutant also gave rise to a tumor with altered tumor morphology. On Kalanchöe stems tumors developed massive root proliferation from the center of the callus in contrast to the parental strain which only produced roots from the periphery of the callus. In addition tumors produced on carrot slices and tobacco stems also developed a proliferation root from the callus, while the parental strain exhibited no root proliferation. All of the plasmids which gave rise to tumors with altered morphologies were transformed into a plasmidless strain. The transformants retained all of the characteristics of the original mutant.

Twelve of the avirulent mutants did not map on the Ti-plasmid when these plasmids were transformed into a plasmidless strain selecting for octopine catabolism, all twelve preparations gave transformants which were kanamycin sensitive and virulent. These data confirm that the Tn5 was not located on the plasmid. Plasmid DNA preparations were made from these strains by the procedure of Casse et al. (1978) and were then subjected to electrophoresis on 0.7% vertical agarose gels. Under these conditions the Ti-plasmid, cryptic plasmid and chromosomsal DNAs migrate to different locations in the gel. The DNAs were then transferred to nitrocellulose by the procedure of Southern (1975). The nitrocellulose transfers were then hybridized with a radiolabeled Tn5 probe, washed and autoradiographed. The resulting autoradiograph demonstrated homolgoy between the Tn5 probe and only the linear chromosomal DNA. No homology was detected to the cryptic or Ti-plasmids, even after extended periods of time for exposure of the autoradiograph. Thus, the insertions in these twelve strains must be in chromosomal genes that encode virulence functions. Seven of these mutants which were avirulent on Kalanchöe were virulent on sunflower, with two of the seven also being virulent on tomato. The other five chromosoaml

Table 1. Virulence of *Agrobacterium tumefaciens* strains harboring mutated Ti-plasmids

Strain	Position of insertion			Virulence						Ocotpine[a] production
	HpaI	KpnI	SmaI	Klanchoe Stems	Klanchoe Leaves	Carrot	Sun-flower	Tomato	Tobacco	
Controls										
A114	-	-	-	-[a]	-	-	-	-	-	Negative
A722	-	-	-	+[b]	+[c]	+[c]	+[c]	+[c]	+[c]	Positive
A723	-	-	-	+[b]	+[c]	+[c]	+[c]	+[c]	+[c]	Positive
A6NC	-	-	-	+[b]	+[c]	+[c]	+[c]	+[c]	+[c]	Positive
Avirulent										
onc-3,8,17	9	11	1	-	-	-	-	-	NT	NT
onc-14	16	5	1	-	-	-	-	-	NT	NT
onc-10,13,18, 20,21	15	5	1	-	-	-	-	-	NT	NT
onc-1,4,6,9,14,27	12	5	1	-	-	-	-	-	NT	NT
onc-5,23	6	5	1	-	-	-	-	-	NT	NT
onc-11	6	5	8	-	-	-	-	-	NT	NT
onc-2,15	3	2	8	-	-	-	-	-	NT	NT
onc-28	ND	ND	ND	-	-	-	-	-	NT	NT
onc-29	ND	ND	ND							
Tumor morphology										
onc-16	6	2	8	+[e]	+[f]	+[c]	+[c]	+/-[h]	+[e]	Positive
onc-19	6	5	1	+[e]	+[f]	+[c]	+[c]	+/-	+[e]	Positive
tum-12	14	10	10c	+[g]	+[g]	+/-	+[c]	+/-	+[i]	Positive[j]
tum-25	14	10	10c	+[f]	-	+[f]	+[c]	+/-	+[f]	Positive
tum-26	14	10	10c	+	+	+/-	+/-	+/-	+	Positive[j]

[a] Avirulent. [b] Unorganized callus overgrowth with roots proliferating from the callus periphery. [c] Unorganized callus overgrowth. [d] NT, not tested; ND, not determined. [e] Abnormal callus with roots proliferating from the entire callus. [f] Unorganized callus with roots proliferating from the callus overgrowth. [g] Necrotic callus overgrowth. [h] Unorganized callus, but smaller in size than tumors induced by the parent strain. [i] Unorganized callus with shoots proliferating from the entire callus. [j] Unorganized callus produced octopine, but shoots did not produce detectable levels of octopine.

mutants were avirulent on all plants tested. The parental strains are virulent on the plants tested. Therefore we can conclude that some chromosomal mutations may influence the host range of the bacterium.

Two additional mutants were isolated which were avirulent on all plants tested. The restriction endonuclease patterns of these plasmids appeared identical to the parental plasmid and the Tn5 probe hybridized to the linear DNA. Thus, the Tn5 insertion in these strains is in the chromosome. However, when these plasmids were transformed into a plasmidless strain selecting for octopine catabolism the resulting transformants retained the avirulent characteristics of the mutant and were kanomycin sensitive. Thus, there must be an alteration in the plasmid that cannot be detected at the level of restiction endonuclease digestion pattern.

Seven mutants were isolated that could not utilize octopine as a sole source of nitrogen. These Tn5 insertions were located in HapI fragment 7 and KpnI fragment 4. This map position is consistant with the location of the octopine catabolism genes which had previously been roughly mapped by deletion mutants (Koekman et al., 1979). These octopine non-utilizing mutants were plated on minimal media with octopine a the sole carbon and nitrogen source. Octopine-utilizing revertants arose at a frequency of $5x10^{-8}$. The revertants were kanomycin sensitive, indicating that the Tn5 excision repaired the gene function and that Tn5 was lost from the cell. All of the octopine non-utilizing mutants were virulent on all plants tested.

<u>Transcriptional analysis of the Ti-plasmid</u>. In order to gain some insight into the pattern of gene expression of the Ti-plasmid, the bacteria were analyzed for transcripts originating from various regions of the Ti-plasmid. When the steady state population of RNAs were examined by production of ^{32}P-labeled complementary DNA and subsequent hybridization to nitrocellulose transfers of the Ti-plasmid two areas are delineated which have abundant RNA populations present in cells grown both in minimal and rich medium. The larger area extends from HpaI fragment 4 to SmaI fragment 6. To date no genetic loci have been mapped in this region. The other area homologous to abundant messanger RNAs is located within SmaI fragment 1 and HpaI fragment 6. Garfinkel and Nester (1980) have located insertion mutants within this region which are avirulent or demonstrate an altered tumor morphology, suggesting that these transcripts may have a function in the process of tumorigenicity. Lower levels of RNAs were reproducibly detected that were homologous to three other regions of the Ti-plasmid. The first region is SmaI fragment 11, to which no genetic loci have been mapped. The second region is KpnI fragment 11 and HpaI fragment 9. Garfinkel and Nester (1980) have isolated avirulent mutants which map in this fragment. The final region of homology to low levels of RNAs ex-

tends from SmaI fragment 3a to SmaI fragment 3b. This region encompasses the DNA that is transferred to the plant upon initiation of a tumor and is found to be transcribed in the plant tumor. Insertion mutants within this region have given rise to tumors with altered morphologies (Ooms et al., 1980; Garfinkel and Nester, 1980). Additional studies are being carried out and it will be very interesting to see if the messenger RNA transcripts found in the bacterium are the same as those found in the tumor.

Klapwijk and Schilperoort (1979) have shown that three and possibly four genes are concerned with the conversion of octopine to pyruvic acid and arginine. Two other operons also appear to be under the coordinate control of octopine. These are the degradation of arginine for utilization as a carbon source (Ellis et al., 1979) and the transfer of the Ti-plasmid to other strains by conjugation (Klapwijk et al., 1978; Petit and Tempe, 1978). Thus, one would expect to see a difference in the messenger RNA populations isolated from cells grown on minimal medium in the presence and absence of octopine. Gelvin et al. (1981) have shown that the region from SmaI fragment 13 to SmaI fragment 10b is heavily transcribed both in cells grown in the presence of octopine and in constitutive octopine utilizing strains while this region has very low levels of transcription when the bacteria are grown in minimal media without octopine (Figure 1). This data also indirectly confirms the map position of the genes concerned with octopine degradation as mapped by deletion mutants (Koekman et al., 1979) and transposition insertions (Garfinkel and Nester, 1980).

Fermin and Fenwick (1978) have shown that octopine utilizing strains can also catabolize another tumor specific metabolite called agropine. Tempe and co-workers (1980) have suggested that this inducible degradation involves at least three genes. The first is a permease which permits agropine to be taken up by the cell and the other two would degrade the agropine by first opening the cyclic structure and then cleaving the noncyclic compound to metabolites that could be used as a carbon source by the bacteria. Thus, one would expect to detect the transcription of such an operon when the messenger RNA populations of induced and noninduced bacteria are compared. Gelvin and co-workers (1981) have shown that ^{32}P-labeled complementary DNA made to RNA isolated from agropine induced bacteria hybridized to a region extending from SmaI fragment 9 to SmaI fragment 14a (Figure 1). This area of the plasmid showed no detectable level of transcription in cells grown in the absence of agropine. Thus the genes concerned with agropine degradation most likely map within the region encompassed by SmaI fragments 9 and 14a and KpnI fragments 7 and 9.

Very low levels of transcripts were occasionally detected in the remaining areas of the plasmid. Thus, we cannot exclude the possibility that all regions of the plasmid are transcribed. Identification of messages and fine structure mapping would be required before silent regions of the plasmid might be identified. Identification of transcripts from regions where genetic loci have not yet been identified suggest that there is a great deal to be established before we have characterized the Ti-plasmid and understand the mechanisms of tumor induction and maintenance.

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TRANSFER, MAINTENANCE AND EXPRESSION OF GENES INTRODUCED INTO PLANT CELLS VIA THE TI PLASMID

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INTRODUCTION

The capacity of a microorganism to establish itself successfully in a particular ecological niche often seems to depend upon the activities of a very small number of genes that are absent in competiting species. This additional DNA is frequently part of a plasmid that allows its host to metabolize rarely exploited carbon or nitrogen sources. Because of the presence of such genes, these plasmids have been called degradative or catabolic plasmids[1]. We believe that the Ti plasmids of *Agrobacterium tumefaciens* form a special class of catabolic plasmids[2,3]. In addition to encoding for proteins that catabolize several common amino acids[4,5] and some polyphenols[5], these plasmids also carry genes whose products catabolize compounds calles opines. Opines are unusual amino acids, such as nopaline[6], octopine[6] or agropine[7] and phosphorylated sugars, such as the agrocinopines[8]. These opines have only been found in plant cells transformed by Ti plasmids into crown gall tumor cells. By inducing crown gall tumors, *Agrobacterium tumefaciens* forces a plant to synthesize compounds which only the same virulent strains can use.

The Ti plasmids isolated from different strains can be grouped into three major classes named after a characteristic opine produced in the tumor cells. Consequently, these plasmids are called nopaline, octopine or agropine Ti plasmids[9]. Agropine is also synthesized in the hairy root tissue, a tumor induced by Agrobacterium rhizogenes[10]. The plasmids from the hairy root strains however, show only limited homology with the A. tumefaciens plasmids[11].

Upon tumor induction, a segment of the Ti plasmid becomes stably integrated in the plant chromosomes. This segment, called the T-DNA, encodes for the functions responsible for the biosynthesis of the opines and for the maintenance of the transformed phenotype of the tumor cells. The extent of the T-DNA has been determined by Southern blot analysis. The T-DNA of the nopaline plasmids was found reproducibly to be 23 Kb[12,13,14]. The octopine T-DNA, in contrast, was about 15 Kb long, although the extent of the right border of this T-DNA varied significantly in some cases[15,16]. Genomic cloning of crown gall DNA allowed the identification of the T-DNA border sequences and proved that this DNA was inserted into plant DNA[17,18]. The sum of available evidence suggests that the "ends" of the T-DNA are involved in the integration event. The exact number of T-DNA copies varied from tissue to tissue and in some tumors was five or more. Some of these copies were arranged as interspersed tandem repeats[17]. This amplification may have occurred after the initial integration event as a result of unequal crossing over between flanking sequences. The latter, indeed, were in all cases investigated, repetitive DNA. In simple terms, Agrobacterium changes its environment by selective gene transfer to plants. Perhaps feats of genetic engineering are not as infrequent as commonly thought.

The physical organization of both the octopine[19,20,21] and nopaline[21] plasmids has been established. This has allowed the construction of a genetic and functional map of these plasmids. Mutations have been isolated by transposon insertion mutagenesis [23,24,19,18] and by deletion formation[23,25].

The most important conclusion from this work was that the non-T-DNA part of the Ti plasmid contains extensive regions essential for tumor induction. These oncogenic (Onc) regions seem to be conserved among nopaline and octopine plasmids[26]. Some of the DNA segments possibly may encode for functions essential for the transfer of the T-DNA into the plant nucleus. Others might interfere with the balance of growth factors (plant hormones) of the infected tissue and therefore be essential to the initial stimulation of cell proliferation. We have indeed shown that exogenous auxin can restore the tumor-inducing capacity of some mutant strains, while exogenous cytokinins inhibit tumor formation[5].

A second conclusion was that no unconditional ONC$^-$ mutations in the T-DNA were found which were not the result of extensive deletions. Small deletions or insertions have allowed identification of T-DNA regions responsible for opine synthesis and host specificity. Mutations in the latter regions permit tumors to form on some plant species (e.g. Kalanchoë) but not on others (e.g. tobacco). Remarkably, large portions of the T-DNA can be disrupted by insertion sequences or deletions without visibly affecting tumor formation or opine production. Recent efforts have concentrated on making a more detailed analysis of this area.

RESULTS

The transposon insertion mutagenesis of Ti plasmids allowed a rudimentary localization of some relevant loci[23,24]. Due to their rather high site or regional specificity, transposons cannot be expected to integrate in all the genes of the T-DNA. For this reason, we have begun an extensive program of site specific mutagenesis of cloned segments of the T-DNA.

Construction of a mutant Ti plasmid by in vitro mutagenesis of cloned T-DNA fragments

In an initial phase, well-defined insertions and deletions were constructed in cloned T-DNA fragments using identified restriction sites as endpoints. The alterations were then introduced in the corresponding Ti plasmid by *in vivo* recombination. To accomplish this, a cloning vector containing the mutated T-DNA fragment was transmitted from an *E. coli* host into an *Agrobacterium* harboring a transfer constitutive Ti plasmid. Two consecutive conjugations, the first one followed by selection for markers of the cloning vector and the second one followed by screening or selection for the loss of these markers, readily allowed the isolation of the required mutant Ti plasmid. The first conjugation gives rise to cells in which the vector plasmid has been inserted in the Ti plasmid by a single cross-over between the cloned T-DNA segment and the corresponding segment in the Ti plasmid. This event is easily selected since it occurs with a frequency between 10^{-3} and 10^{-6}, depending on the length of the fragments involved (respectively 16 Kb and 2 Kb). The resulting plasmids carrying a segment in duplicate are relatively unstable since the inserted vector DNA can be lost upon crossover in the manipulated T-DNA segment. We found that this occurs with a frequency of 1 to 0.01 %, depending upon the length of the fragments (respectively 8 and 1 Kb). The second conjugation produces strains harboring the desired Ti plasmid recombinant. The definitive proof of the structure of the isolated plasmid is obtained by Southern blot analysis of digests of the total bacterial DNA, using a cloned segment of the mutated T-DNA as probe [27].

In a first set of experiments, DNA segments isolated from the antibiotic resistance determinants of R factors were introduced either as a simple insertion in a restriction site or as a substitution insertion, replacing a restriction fragment. Once this type of mutant Ti plasmid was obtained it could be used for subsequent exchanges. When this exchange employed a T-DNA fragment containing an insert of a cloned eukaryotic gene, a Ti-plasmid was obtained which could transfer the new gene into plants.

Similarly, a well-defined T-DNA mutation could be introduced by exchanging the insert for a homologous Ti fragment harboring a deletion spanning the site of the insert. By using two cloned fragments derived from the borders of the T-DNA, or from outside the T-DNA, it is possible to construct Ti plasmids which lack most or all of the T-DNA.

Construction of mutated Ti plasmids using in vivo mutagenesis of cloned T-DNA fragments

In vivo mutagenesis is basically analogous to the *in vitro* technique, instead of using recombinant DNA technology to constructing an insertion, this second procedure inserts a copy of a movable element into the cloned T-DNA fragment when the T-DNA fragment is mobilized from one bacterium to another[28]. One practical advantage of the *in vivo* method is that many independent insertions can be isolated through a single replica conjugation. Following mutagenesis, the mutated T-DNA fragment is recombined into the Ti plasmid by double cross-over, following the procedure described in the previous section. Through this *in vivo* approach to mutagenesis, Tn*1* has been inserted into several different sites in the T-DNA. As was previously demonstrated for the 15 Kb long Tn*7*[29], the transposon can be co-transferred to a plant without any apparent rearrangements as part of the T-DNA[29]. This proves that the T-DNA can serve as a vector to introduce foreign genes into plants.

Stability of the inserted T-DNA

The crown gall tissues induced by nopaline Ti-plasmids have a tendency to redifferentiate into shoots. For this reason, this kind of tumor is called teratoma tissue. These shoots can be grafted to new plants where they grown into reasonably well developed tobacco plants. Southern blot analysis has shown that the T-DNA was conserved in the different tissues of the regenerated plants[14,30]. In marked contrast, in those rare cases where fertile flowers formed, T-DNA was absent both from cultures derived from anthers and from the F_1 generation.

This loss of the T-DNA after meiosis could seriously limit the use of the T-DNA as a cloning vector in plants. Studies of various mutants of the T-DNA have indicated a particularly attractive method of overcoming this difficulty. Some mutated T-DNAs induce tumors that proliferate into either roots or shoots. Frequently, these shoots do not contain any opines and in this way resemble shoots formed from some "genetic tumors". At the same time, shoots do arise which produce opines and therefore presumably contain intact portions of T-DNA. Therefore, by inserting foreign DNA into a suitable site, it should be possible to mutagenize the T-DNA in such a way as to ensure that the foreign DNA will become part of a new plant. One such example is particularly noteworthy. In this case, Tn7 was inserted into the EcoRI-32 fragment of the T-DNA of an octopine plasmid (pGV2100) [24]. The tumors of this mutant, unlike normal octopine tumors, gave rise to shoots which are able to form roots. Intact plantlets could be separated from this mass that grew well in isolation and were found to contain octopine. One shoot is particular developed into a fully grown, flowering plant. Both the pollen and ova of this tobacco plant were fertile and, after selfing, provided seeds for further analysis. These seeds germinated into normal-looking plants of which 75% contained octopine and 25% did not. In addition to this 3:1 segregation, we found the progeny of a cross between the regenerated plant and a wild type plant segregated 1:1. Finally, 50% of the haploid plantlets obtained from anther cultures derived from the mother plant contained octopine and 50% did not. The results indicate that the T-DNA segregates as a Mendelian trait and consequently, that the T-DNA is present as a single locus on one of the chromosomes.

Expression of the T-DNA

Several transcription studies of the T-DNA[32,33,34] have been published. Our results with both the octopine and the nopaline plasmids indicate that all of the T-DNA is transcribed but that some segments, particularly those situated at the ends of the T-DNA, are transcribed most actively[35,36]. Roughly, the same pattern was found when T-DNA was hybridized to nuclear or polysomal RNA. Interestingly, different regions of the T-DNA were transcribed in stationary phase tumor cells than in actively dividing ones. This may be the first evidence that some genes of the T-DNA are transcriptionally regulated in the plant host. In plants the transcription the T-DNA sequences is completely inhibited by 0.7 μg/ml α-amanitin as if it was dependent upon RNA polymerase II[36].

As expected from genetic studies[23,24], portions of the

Ti plasmid are transcribed in *Agrobacterium*. This was shown by hybridizing total, *in vivo*-labelled RNA, isolated from bacteria, to restriction fragments covering the whole Ti plasmid. The portions of the plasmid coding for proteins which catabolize opines are preferentially transcribed. On the other hand, the T-DNA is expressed only very weakly. From these results, it would appear that the Ti plasmid is organized into discrete blocks of prokaryotic and eukaryotic genes.

Expression of a prokaryotic gene in a plant

One of the more important questions that must be answered before the Ti plasmid is used to produce new kinds of plants, is whether the DNA of one species is readily expressed in the cells of another. With this in mind, several experiments were performed to determine whether the bacterial DNA of Tn*7* is expressed in a eukaryotic host. In these experiments, RNA was extracted from tumors induced by a Ti plasmid with Tn*7* inserted in the right border of the T-DNA, in the nopaline synthase locus. Nuclear transcripts were found which correspond to the entire Tn*7* genome [29]. At least some of these transcripts were found also in the polyA fraction of the polysomal RNA. Significantly, Tn*7* transcripts in polysomes lacked detectable poly A sequences. This may indicate that the RNAs terminate within Tn*7*, perhaps at prokaryotic termination sequences, and not at the end of an adjacent gene. Although it is still not certain that any Tn*7* gene is translated in plants, these results indicate that plant enzymes may be able to produce some messenger-like RNAs from foreign genes, transport them out of the nucleus, and incorporate these molecules into polysomes.

CONCLUSION

The Ti plasmids present some intriguing questions to plasmidologists. For example, is the T-DNA segment derived from eukaryotic DNA that became integrated into a prokaryotic host ? This could account for the presence of an uninterrupted block of genes that are transcribed only by polymerase II of plants and that can control plant growth.

A second question is, how is the T-DNA transferred to the nucleus ? Virtually nothing is known about early steps in the infection of the plant. The Ti plasmid (or some portion of it) may enter the cell as a naked molecule. In this event, infection may be similar to bacterial conjugation, and may even employ the same origin of transfer of the Ti plasmid. This model still provides no explanation for how the DNA can reach the nucleus safely. Perhaps it is worthwhile to reassess the evidence against the uptake of whole *Agrobacterium* cells by plants.

Finally it is necessary to determine how the T-DNA integrates into the host chromosome. At this time, it is not possible to provide a specific model. The T-DNA may have the properties of a movable element. The T-DNA in nopaline-producing tumors, at least, appears to integrate quite precisely. However, no transposition of the whole T-DNA has been observed in a bacterial background. It should be possible to detect transposition of the T-DNA in plants by cloning from tumors the ends of T-DNAs that have been tagged with antibiotic resistance markers. Such markers provide a method of selecting clones containing the sites of T-DNA integration. Analysis of these sites might clarify how the T-DNA is incorporated into chromosomes and whether the integrated form can jump to a new location.

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RHIZOBIUM PLASMIDS: THEIR ROLE IN THE NODULATION OF LEGUMES

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Many important crop plants, such as soybeans, groundnuts, beans, peas, clover and alfalfa are legumes, and hence the symbiotic nitrogen fixing relationship between *Rhizobium* and the roots of legumes is of major agronomic importance. The cost of nitrogenous fertilizer is closely linked to the cost of oil, so it is not surprising that there is an increasing interest in a biological process that allows some crop plants to grow without the application of nitrogen fertilizer.

The symbiosis is also noteworthy purely as a problem in developmental biology because it involves biochemical and morphological differentiation in both partners. The infection process has been reviewed by Newcomb (1976). Typically, penetration by the bacteria begins at the tip of root hairs. An infection thread is formed within the root hair by invagination of the plant cell wall. The bacteria multiply inside this thread. As it grows into the cortex unknown signals induce localised plant cell proliferation ahead of the zone of infection. As the nodule develops the infection threads continue to penetrate the host cells and the bacteria near the tips are pinched off, surrounded by plant membrane and liberated into the cytoplasm. These forms are known as bacteroids; owing to the loss of much of the bacterial cell wall they are pleiomorphic and much larger than free-living *Rhizobium*. The bacteroids synthesise nitrogenase and the ammonia that is produced is exported to the plant cytoplasm where it is assimilated and from which it is transported to the rest of the plant.

Although the precise morphology of root nodules varies between different legumes, in all cases they are organised structures with a defined meristem and a well developed vascular system.

A feature of the symbiotic interaction is its specificity: different legume species are nodulated by different Rhizobium strains and the host-range of the bacteria is used to define Rhizobium species. Thus R. leguminosarum, R. phaseoli and R. trifolii nodulates peas, Phaseolus beans and clover respectively.

It is reasonable to suppose that during the course of nodule development a number of genes in both partners have to be expressed in a co-ordinate manner. Although we know little or nothing of the precise control and function of any 'symbiotic' genes, it is apparent that genes determining nodulation, nitrogen-fixing ability and host-range are plasmid-borne in at least some Rhizobium species. In this paper we shall describe the evidence that has led to this conclusion.

Isolation of Rhizobium Plasmids

Since Nuti et al. (1977) first demonstrated, in R. trifolii and R. leguminosarum, the presence of plasmids of > 100 Md it has become clear that such plasmids are widespread in other species also (Casse et al., 1979; Gross et al., 1979; Beynon et al., 1980). A single strain may contain several large plasmids of different molecular weights but the number and sizes may vary between strains of the same species. In a small survey of strains of R. leguminosarum the number of plasmid bands per strain seen on agarose gels ranged from two to seven but there was no plasmid of the same size present in all strains (Beringer et al., 1980). Thus there does not appear to be a 'pea nodulation plasmid' of uniform molecular weight. The situation may be different in strains of R. meliloti; in this species there is a plasmid of c. 350 Md in many strains of diverse geographical origin (J. Dénarié, personal communication). There is strong evidence that this plasmid determines nodulation and nitrogen-fixing functions (see below).

Transcription of Plasmids

A clear demonstration of the importance of plasmids in the nodulation process comes from the observations by Krol et al. (1980) that RNA isolated from bacteroids of pea root nodules hybridised extensively to *R. leguminosarum* plasmid DNA whereas there was no detectable hybridisation between plasmids and RNA obtained from cells grown *in vitro*. Thus, between the free-living state and the bacteroid form, there appears to be a major shift in the pattern of transcription of plasmid-linked genes.

Location of nif genes

The genes that specify the components of the nitrogenase complex (*nif* genes) are normally expressed only within the root nodule bacteroids: hence the *nif* genes are an obvious example of what we describe as 'symbiotic genes'. In some *Rhizobium* species *nif* genes have now been shown to be plasmid-linked. The demonstration of this depends on the fact that two of the *nif* genes (*nif D* and *nif H*) which specify the structural components of nitrogenase are highly conserved among nitrogen-fixing bacteria so that cloned *nif* DNA from *Klebsiella pneumoniae* can hybridise with *nif* DNA from a wide variety of nitrogen-fixing bacteria (Ruvkun & Ausubel, 1980).

Nuti et al. (1979) found that the plasmid pSA30, which contained the *nif K*, *D* and *H* genes of *K. pneumoniae*, specifically hybridised to plasmid DNA of *R. leguminosarum*. By transferring plasmids which had been separated on agarose gels to nitrocellulose filters ("Southern blotting") and probing with labelled pSA30 we have identified specific '*nif*' plasmids in several strains of *R. leguminosarum* and *R. phaseoli* (see below).

It is clear that this hybridisation with pSA30 is not due to some spurious homology. In some elegant studies (Ruvkun & Ausubel, 1981; Ditta et al., 1981) it was found that non-fixing mutants of *R. meliloti* could be isolated by insertion of the transposon Tn5 into DNA that hybridised with pSA30.

Other plasmid determined symbiotic functions

Other symbiotic genes, less well defined than those specifying nitrogenase, have been shown to be plasmid-linked. In the discussion that follows we shall refer to 'Nod$^-$' or 'Fix$^-$' mutants, meaning respectively that the defective strains induce no detectable nodules or that nodules are formed which fail to fix nitrogen. Presumably these classes will become sub-divided as we know

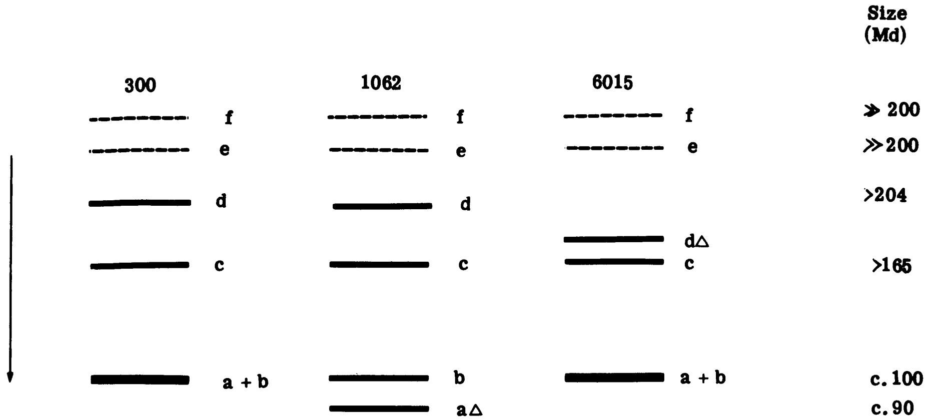

Fig. 1. Representation of agarose gels of R. leguminosarum strain 300 and two derivative strains. Plasmids were isolated and the gels were run according to the method of Hirsch et al. (1980). The dotted lines indicate that these two bands were seen only in some preparations.

more of the biochemical and genetic detail of symbiosis but at present such analyses are not available.

Most of the work to be described involves the study of plasmids in strains of R. leguminosarum and R. phaseoli. In the former species we have concentrated on the genetically well characterised strain 300 (Beringer et al., 1978b) and derivatives of this strain into which plasmids were introduced from other R. leguminosarum field isolates.

Strain 300 yields five plasmid bands following electrophoresis on agarose gels (see Figure 1), the two largest being seen only in some preparations (Hirsch et al., 1980). The fastest migrating band (a + b in Figure 1) actually comprises two co-migrating plasmids which in strain 1062 were resolved by a deletion of c. 10 Md in the a plasmid.

Of the six plasmids in strain 300, only the one corresponding to band d in Figure 1 has been shown to be required for nodulation and nitrogen fixation on peas (Hirsch et al., 1980). Following UV treatment, we have isolated a Nod$^-$ derivative of strain 300 and it can be seen that in this strain (6015) there has been a substantial deletion of the d plasmid. This deletion appears not only to have removed genes essential for pea nodulation; at least some of the

R. leguminosarum nif genes are also absent from this strain. Following Southern blotting of gels containing the plasmids of strains 300 and 6015, pSA30 (the plasmid containing the K. pneumoniae nif genes) hybridised to band d of strain 300 but there was no detectable hybridisation to the d△ plasmid of strain 6015 (unpublished observations). Similarly, when pSA30 was used as a probe against total DNA from strain 300 which had been digested with endonuclease EcoRI, a 1.5 Md fragment was found to hybridise but there was no homology between pSA30 and any EcoRI fragment derived from strain 6015.

The plasmid d is apparently not self-transmissible. Derivatives of strain 300 in which the transposon Tn5 has been inserted into this plasmid fail to transfer kanamycin resistance (specified by Tn5) to other strains of R. leguminosarum (frequency $< 10^{-9}$).

A. Kondorosi (personal communication) and J. Dénarié (personal communication) have found that in R. meliloti a single deletion in a large (c. 350 Md) plasmid can lead to the loss of nod and nif genes. It is interesting that genes governing such different steps as the early stages of nodule induction and of nitrogenase synthesis should be closely linked on single plasmids in at least two Rhizobium species.

Of the other plasmids in strain 300 we know very little: indeed the plasmids corresponding to bands c, e and f determine no known phenotype. We have inserted Tn5 into both the b and the a△ plasmids in derivatives of strain 1062 using the method of Tn5 mutagenesis described by Beringer et al. (1978a). These plasmids are both transmissible at low frequencies (c. 10^{-6} and 10^{-7} respectively) to other R. leguminosarum strains. We have isolated derivatives in which either the a△ or the b plasmid is missing and in both cases the strain nodulates and fixes nitrogen normally, indicating that neither is required for symbiotic proficiency.

Transmissible plasmids with symbiotic functions

In addition to the plasmids of R. leguminosarum strain 300 we have identified a number of plasmids originating in other R. leguminosarum field isolates which can be transferred by conjugation into strain 300 and have shown in some cases that such conjugative plasmids determine symbiotic functions.

pRL1JI. This plasmid has a molecular weight of c. 130 x 10^6 (Hirsch et al., 1980) and was identified initially by the fact that the field isolate (strain 248) of *R. leguminosarum* in which it was detected made a bacteriocin whose production could be transferred at high frequencies (c. 10^{-2}) to non-producing strains such as strain 300 (Hirsch, 1979). Our interest in this plasmid was stimulated by the finding that when it, or a derivative containing Tn5, was transferred to the Nod^- Nif^- strain 6015 (see above) all the transconjugants induced nitrogen-fixing nodules on peas (Johnston et al., 1978). This plasmid can also suppress a number of chemically induced Fix^- mutants of *R. leguminosarum* strain 300 (Brewin et al., 1980a) and several Nod^- and Fix^- derivatives of pRL1JI have been isolated following Tn5 insertion into the plasmid (Buchanan-Wollaston et al., 1980; C-S. Ma, personal communication).

A Tn5-marked derivative of pRL1JI has also been transferred to strains of *R. phaseoli* and *R. trifolii*. The transconjugants gain the ability to nodulate and fix nitrogen on peas and they retain their ability to nodulate their normal hosts, although the nodulation both on peas and on clover or *Phaseolus* is later than when these hosts are inoculated with the normal homologous species (Johnston et al., 1978).

Some properties of the transconjugants of strain 1233 of *R. phaseoli* have been examined by Beynon et al. (1980). As will be seen, another plasmid-linked character relevant to the understanding of these interspecific transconjugants is the production of melanin. For reasons that are not understood, strains of *R. phaseoli* but not of *R. leguminosarum* or *R. trifolii* make melanin following prolonged growth on rich medium.

Following the transfer of pRL1JI to strain 1233 the transconjugants contained three plasmids, the smallest corresponding to pRL1JI plus the two larger plasmids of strain 1233 (see track 1 in Fig. 2). These transconjugants were stable in culture and could still make melanin. As mentioned above, peas inoculated with these transconjugants nodulated later (by about one week) than when *R. leguminosarum* strains were used. The great majority (c. 95%) of bacteria isolated from nodules induced by the strain 1233 pRL1JI transconjugants differed in three ways from the original transconjugants: (a) they could no longer make melanin; (b) they nodulated and fixed nitrogen on peas as well as did strains of *R. leguminosarum* but failed to nodulate *Phaseolus* beans;

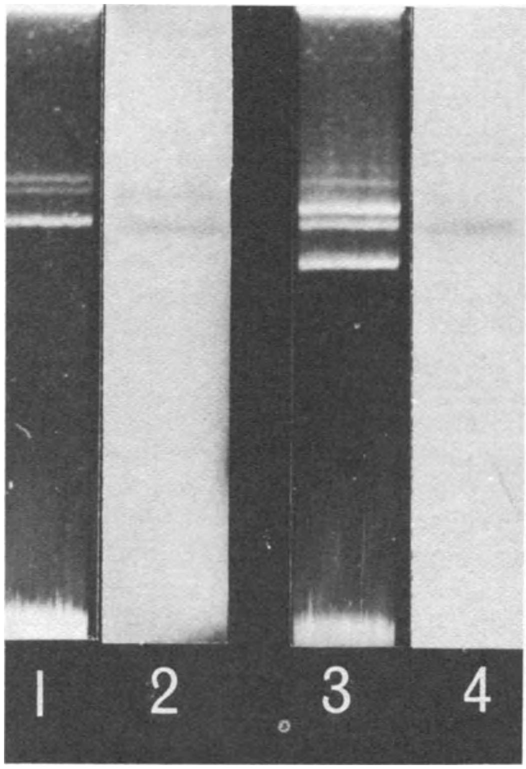

Fig. 2. Isolation of plasmids from R. phaseoli containing pRL1JI and demonstration of plasmid-linked nif genes. Plasmids were isolated according to the method of Hirsch et al. (1980). Transfer of DNA from gels to filters was essentially as described by Wahl et al. (1979). Track 1. Agarose gel of R. phaseoli strain 1233 containing pRL1JI. The two larger plasmids are those of strain 1233 - the fastest migrating band corresponds to pRL1JI. Track 3. Gel of R. leguminosarum strain 248. The second smallest plasmid corresponds to pRL1JI (Hirsch et al, 1980). Tracks 2 and 4. Hybridisation of pSA30 to plasmid DNA blotted from the gels in tracks 1 and 3 respectively.

(c) they had lost the smaller of the strain 1233 resident plasmids (termed pRP1JI) but still retained pRL1JI and the larger of the two plasmids of strain 1233.

The fact that following passage through pea nodules there was concomitant loss of bean nodulation ability, melanin production and pRP1JI indicates that both characters are determined by pRP1JI. This has been confirmed by the fact that some spontaneous deletions of pRP1JI in strain 1233 itself result in the loss of melanin production and of Phaseolus nodulation ability (Beynon et al., 1980). To explain why pRP1JI is lost at such high frequency following the passage through pea nodules, it has been proposed that the initially formed strain 1233 pRL1JI transconjugants are in fact unable to nodulate peas because of some uncharacterised inhibitory action specified by pRP1JI which acts on pRL1JI. Only when pRP1JI is lost, as it might be in some bacteria in the rhizosphere, would the pRL1JI-specified ability to nodulate peas be expressed and only these individuals would be able to induce nodules on this host. We know from reconstruction experiments that peas can nodulate if they are inoculated with as few as 10 Nod^+ R. leguminosarum cells even in the presence of 10^8 Nod^- bacteria (Brewin et al., 1980a) so if pRP1JI was lost at frequencies as low as 10^{-6}, nodulation of peas by the transconjugants might still be detected.

In strain 1233, pRP1JI also carried nif genes; in Fig. 2, track 2, radioactively labelled pSA30 can be seen to hybridise both to pRL1JI and to pRP1JI so here is another case where at least some nod and nif genes are on the same Rhizobium plasmid.

pRL5JI. There is some specificity even within 'classical' cross-inoculation groups. For example a primitive pea line called Afghanistan is resistant to nodulation by European strains of R. leguminosarum but can be nodulated by a strain of R. leguminosarum which was isolated in Turkey (Winarno & Lie, 1979). This strain, termed TOM, contains a 160 Md plasmid, pRL5JI, that is transferable at frequencies of c. 10^{-6} to other R. leguminosarum strains. Derivatives of the Nod^- Nif^- strain 6015 (see above) containing pRL5JI are Nod^+ Nif^+ both on Western pea cultivars and on the variety Afghanistan (Brewin et al., 1980b). Thus pRL5JI appears to carry the determinants that confer on strain TOM the ability to nodulate primitive pea lines.

Transfer of genes for an uptake hydrogenase (Hup)

Biological nitrogen fixation is energetically demanding with approximately 18 moles of ATP being consumed for the reduction of 1 mole of N_2. As much as 25% of this energy is not directly involved in the reduction of nitrogen but in a sense is wasted in the reduction by nitrogenase of protons to H_2 (see review by Robson & Postgate, 1980). Some strains of some species of nitrogen-fixing bacteria, including Rhizobium, possess an uptake hydrogenase which can oxidise the H_2 that is liberated and in the process recycle some of the energy that would otherwise have been lost.

Albrecht et al. (1979) isolated Hup^- derivatives from a Hup^+ field isolate of R. japonicum and found that soybeans inoculated with the mutants were smaller (by about 25%) than those inoculated by the Hup^+ parents, suggesting that Hup^+ bacteria are superior and that it would be desirable for any inoculant strain to be Hup^+.

In one Hup^+ field isolate of R. leguminosarum (strain 128C53) the hup genes appear to be on a plasmid termed pRL6JI which also carried nod and nif genes (Brewin et al., 1980c; unpublished observations). This plasmid is not self-transmissible but it can be transferred into Hup^- field isolates of R. leguminosarum after recombination with a transmissible plasmid.

Hirsch (1979) identified two R. leguminosarum transmissible bacteriocinogenic plasmids termed pRL3JI and pRL4JI which were in the same incompatibility group as pRL1JI but which differed from pRL1JI in that they did not appear to carry genes for nodulation or nitrogen-fixing ability (Brewin et al., 1980a). However, they were shown to recombine with the 'symbiotic' plasmid of strain 300 (band d in Fig. 1) and such recombinants could then transfer Nod^+ and Fix^+ at high frequency (Brewin et al., 1980a).

When either pRL3JI or pRL4JI was transferred into the Hup^+ strain 128C53 they recombined with the smaller of the two resident plasmids at high frequency. When this happened such recombinant plasmids could be transferred by conjugation to the Nod^- Nif^- strain 6015. Approximately 70% of the strain 6015 transconjugants could induce nitrogen-fixing nodules on peas and in all cases such nodules contained hydrogenase and liberated less H_2 than did the plants inoculated with Hup^- control strain (Brewin et al., 1980c). We are presently investigating whether inoculation by these construction Hup^+ strains results in enhanced plant growth.

Conclusions

The importance of Rhizobium plasmids in determining several symbiotic functions is now clear. In some cases it is apparent that the genes concerned with rather different steps in the infection process are clustered on one plasmid. However we have virtually no knowledge of the proportion of plasmid DNA which is devoted to symbiotic functions nor do we have any real idea of the contributions of chromosomal genes in the infection process.

Methods are available both for chromosomal and plasmid mapping in Rhizobium (Beringer et al., 1980). As more symbiotically defective mutants are isolated, located, and analysed in detail for the basis of their defects it should be possible to use this information to dissect the various steps that are required for Rhizobium to induce a functioning nitrogen-fixing root nodule.

Armed with such information it may then become feasible to construct rationally strains of Rhizobium that would be of value as inoculants for legume crops.

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METABOLIC PLASMID ORGANIZATION AND DISTRIBUTION

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SUMMARY

Pseudomonas strains carry plasmids under regulation of natural and synthetic organic residues and bear primary roles in mineralization. Aromatic compounds of known oxidation pathways provide convenient models for genetic analyses and for plasmid DNA isolation and structure determination. The alkane and terpene catabolic systems, coded on larger self-fertile plasmids, have provided primary data on gene organization and regulation, as well as plasmid chromosome gene redundancy.

Two aromatic plasmids, NAH7 and SAL1, of about 80 to 90 kb (kilobases), code respectively the conversion of naphthalene and of salicylate to the anaplerotic intermediates, pyruvate and acetaldehyde, plus CO_2 or formate, thus supporting cell growth. The NAH plasmid codes for these two conversions on separate operons, both controlled by salicylate or anthrinilate. Operon 1 codes the conversion of naphthalene to salicylate; operon 2, salicylate via catechol with "meta" (2,3 oxygenase) aromatic ring cleavage. Plasmid DNA isolated from wild type and transposon Tn5 induced insertion mutants was scored for defective loci by enzyme assays in the genomes subjected to gel electrophoresis after restriction digestion. An EcoR1 digest fragment A of 23 kilobases carries the bulk of both operons; Sma1 yields 5 fragments, A of 42 kilobases and B of 18. The latter which lacks the left hand 5+ kilobases of EcoR1 A reveals that the replicon in the *nah*A gene are within this 5 kilobase region. The transcription is from left to right in both operons; an 8 to 10 kilobase segment between the operons carries at least one regulatory locus. The cell plasmid in Sma1 digest yields 5 fragments identical in size to those of

NAH7, plus two smaller, about 3 kb, segments which constitute an insertion in naphthalene operon 1 in the gene AB region. The methods now available for plasmid isolation and DNA analyses, the genetic scoring and cloning, now appear capable of providing, in the near future, a complete structure, organization, and regulation model of the aromatic plasmids in fluorescent *Pseudomonas* species.

INTRODUCTION

The state of metabolic plasmid research in *Pseudomonas* strains can be presented most readily in the space available as examples of work in progress. The relevance of the "metabolic" - to the "resistance" plasmids - procaryote tolerance to therapeutic chemicals - requires additional discussion. Essential references and a suitable working background of *Pseudomonas* biology is provided by the Clarke-Richmond monograph (1) as updated by the recent mini-reviews of Chakrabarty (2) and of Williams (3).

Metabolic plasmid is offered as a more general term than degradative (2) or catabolic (3). It refers to reaction pathways, presumed roles in nature, and methods of phenotypic scoring. It is now well documented that many plasmids coding resistance by antibiotics degrade or modify the active structures by forming less active or inactive derivatives (4).

Genetic exchange among gram negative procaryotes is now generally accepted. While subject to some expression barriers, fluorescent pseudomonads are recognized as a single genetic group (1, 2). The problems of plasmid compatibility (5), inhibition of expression, and the host range, are only partially documented (1-3). Number and variety of fertility factors among the fluorescent pseudomonads, whether scored by growth or as resistant phenotypes remain to be delineated. The total array of plasmids carrying markers for aromatic metabolism and their conformation, aggregate or cointegrate, mode remain unexplored. For many aspects of the fundamental genetics, even among the most studied group, the fluorescent pseudomonads, the data are incomplete; for the nonfluorescent soil-water forms (P. acidovorans-P. testosteroni) genetic problems are virtually undocumented. This, however, should not be difficult as Dagley, Evans, Gibson, and others have provided elegant chemical and enzymatic identities, and Stanier, Doudoroff, and Palleroni have provided taxonomic identity among many of the most-studied strains.

The fluorescent pseudomonad plasmid structure will be illustrated with a self-fertile aromatic plasmid coding naphthalene-salicylate oxidation, e.g., NAH7 and SAL1. The NIC1 plasmid, coding for nicotine-nicotinate oxidation, with or without the fertility factor "T", will be considered briefly. The plasmid isolation procedures, the preliminary maps of restriction and gene organization in some homology studies have been published (6, 7). Primary data are on the NAH/SAL and the TOL plasmids (8-10, 3). The transposon Tn5 has been employed in the study reported here for the elucidation of the gene order including polar effects. The gene loci, regulation and transcription, are presented; the relevance of these data to the aromatic metabolic processes in this genus is offered as a working hypothesis.

RESULTS

Table 1 indicates the principle metabolic plasmids studied so far in the fluorescent pseudomonads. The size of those coding growth phenotypes on alkane, terpene, and aromatic carbon sources range from 50 to > 200 megadaltons (75-300 kilobases, kb). The extent and precision of the data varies widely, primarily as a function of the more recent studies and the extent of commonality in methods used by the more active working groups. The growth data phenotypes are still incomplete in many key instances, pathway intermediates remain to be identified, and the scoring of enzyme lesions and activities are at best rudimentary. In certain cases, gene linkages have been established by transduction and, in others, plasmids accumulated within preferred hosts by transduction or conjugation to auxotrophic recipients. Plasmid DNA has been isolated from both wild type and derived strains in structures deduced from point or transposon-induced mutants with restriction enzyme digestion.

The aromatic plasmid data in Table 1 are perhaps, at this time, the more advanced. See, for example, Chakrabarty (2), Williams (3), Johnston (6), and Farrell (7). This paper provides additional fine structure of gene loci and organization in the NAH7 and SAL1 plasmids. Equivalent data are also included on the heterocyclic nicotine plasmid, NIC, for comparative purposes. The earlier data in Table 1 suffer from defects in 1) multiple bands in agarose gel electrophoresis due to the presence of supercoil, open circle and linear forms, and 2) errors in the size estimation of the larger fragments from restriction digests resulting from underestimates on flat bed agarose gel electrophoretic patterns. Multiple enzyme digests to yield smaller fragments, comparative measurements, among the laboratories working in this area, and elimination of contamination by chromosomal fragments remain to be optimized. The native plasmids are unusually large for optimum analysis by electron micrography although some confirmatory data are available.

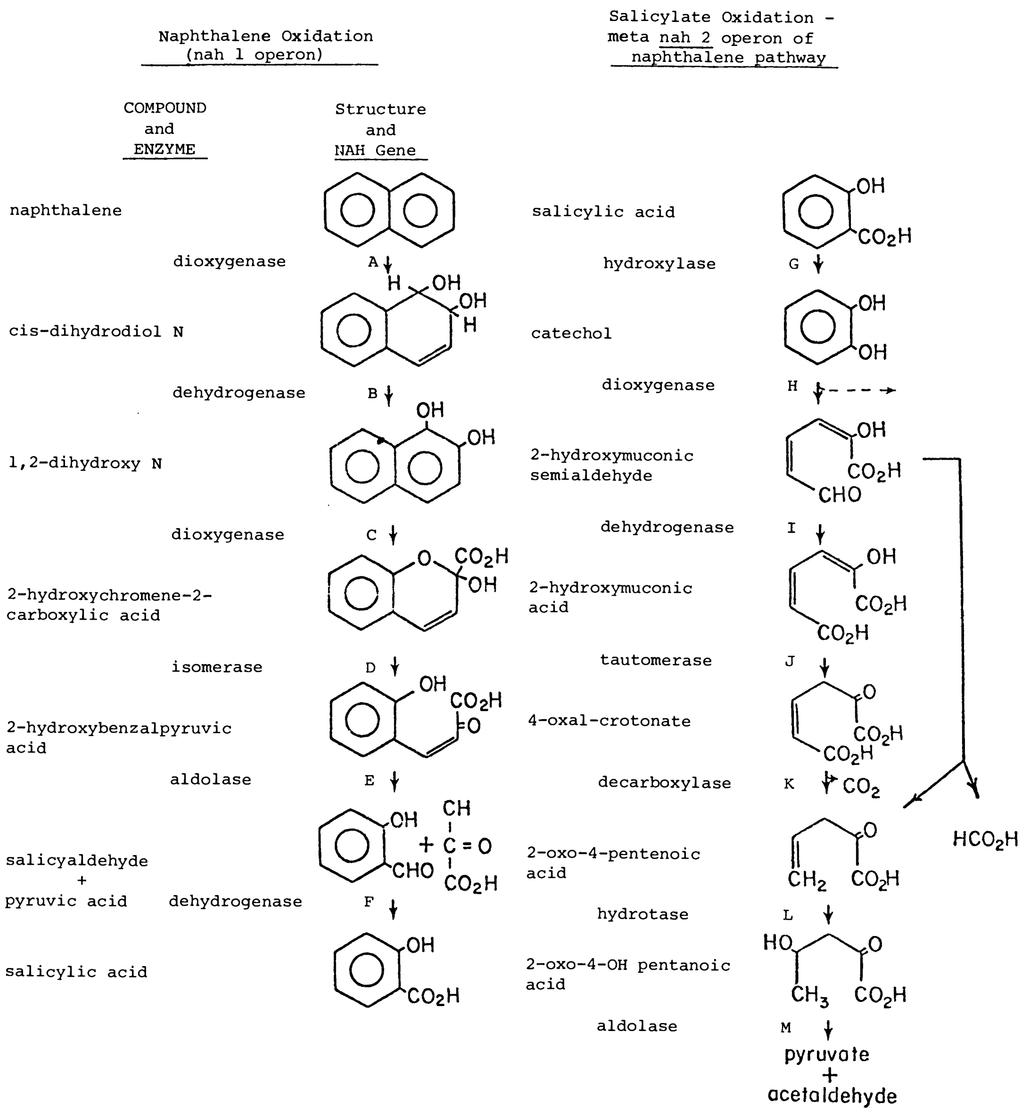
Naphthalene oxidation via salicylate. The bicyclic aromatic hydrocarbon, naphthalene, is oxidized to salicylic acid with the generation of the three-carbon residue, pyruvate, as outlined in the first half of Scheme A. Enzymes and gene designations are indicated. These five genes are controlled as a single replicon. It appears likely that several of the enzymatic transformations require

Table 1. Some *Pseudomonas* Catabolic Plasmids

Phenotype	DNA	Host No.		
		wt		277
	mD			(*trpB615*)
Alkane				
OCT	≥100	6		972
CAM-OCT		1+6		970/977
Terpene				
CAMphor	≥100	1		273
CAMphene	~70	93		
αPN, (pinene)	~70	93		
βPN, "	~70	93		
LINalool	~155	158		
PCYmene	"	"		
Aromatic				
NAH	42	7		1343
"	4,10,42	63		x000
"	-	90		
SAL	45	R1		2100
XYL	-	26,*xy*†		1525
XYL·K	90	AC142		1311
TOL	76	*9,mt2*		2116
TOL*	54	"	AC804	1327*
TOLΔ	39	"	AC803	1328*
TOL*K	108	"	AC797	1318*
TOL·RP4	53		AC810	1329
Heterocyclic				
NIC	44	*25,pcl*		2501

†For strains, we thank P. K. Bhattacharyya, A. Chakrabarty, D. Gibson, and J. Shapiro.

*In *met-1*, PpG1 derivative.



Scheme A. Naphthalene-salicylate oxidation pathway: Enzyme ana gene designations two or more proteins, thus, for example, *nah*A may turn out to be two or three cistrons. Genetic distinction and locus identification have not been completed. Salicylate oxidation to catechol aromatic ring fission and the oxidation of the resulting aldehyde to hydroxy mutanate are under control of a second operon, nah2. Catechol, a first product of salicylate oxygenase, is a primary convergent point of aromatic metabolism. The presence of the plasmid ring fission occurs by the so-called "meta", 2,3 dioxygenase pathway in plasmid-free *P. putida* chromosomal genes and regulation specify ring fission by the "ortho" 1,2 oxygenase. Whether the regulation of these processes is dependent on inducer concentration or other chemical mechanisms in unclear. Catechol and substituted catechols are metabolized by enzymes relatively relaxed in tolerance to alkane and acidic side chains on the aromatic nuclei. The loci, plasmid or chromosome, in the late steps of conversion of salicylate to pyruvate acetaldehyde, i.e., the hydration and retrograde aldol reactions remain to be identified. Present data suggests plasmid loci in operon 2, but the possibility of plasmid chromosome redundancy is not eliminated.

The NAH7 plasmid. A Sma1 Type II restriction digest of the isolated NAH7 DNA produces five fragments. Figure 1 indicates diagrammatically their size, order, and the position of the naphthalene operons 1 and 2. Clearly, fragment B of nearly 18 kb, carries most of the *nah* gene loci. The initiation of operon 1 and the *nah*A loci over to the left in fragment A, ∿ 42 kb, is approximately half of the entire plasmid. Operon 2, for which only three gene positions are shown, is near the right B fragment terminus. Later steps in the pathway may be coded in the adjacent restriction fragment. The gene placements were established with transposon Tn5 insertion mutants by the data summarized in Figure 2. The Tn5, about 5.7 kb (10), carries a Sma1 restriction site, 3.2 kilobases from one terminus, and 2.5 from the other. With the polarity of insertion unknown, an uncertainty of about 0.7 kb remains in determining the insertion locus.

Figure 2 indicates also an EcoR1 fragment A, about 24 kb, with overlap of 5.3 kb to the left of the Sma1 fragment B ∿ 0.5 kb to the right. A BamH1 site, also indicated in this region, also is useful as will be indicated subsequently. The expanded diagram, lower portion of Figure 2, indicates the *nah*A gene loci in the EcoR1 fragment A, presumably also including the replicon. Preliminary data of Gibson (11) indicate for the naphthalene dioxygenase, three protein components, thus multiple cistrons, presumably in the A region. The region of 10 kilobases unmapped between operons 1 and 2, contains at least one regulatory locus.

Table 2 provides enzyme activity data on the wild type strain and representative Tn5 insertion mutants. The levels induced by salicylate, 2.5 mM, are compared to the noninduced levels, i.e., cells grown on sodium glutamate. The insertion mutants show

Table 2. Naphthalene Oxidation Enzyme of NAH7: :Tn5 Mutants

Gene & locus Enzyme	wt 1343	*A*1	*B*11	*C*24	*D*32	*G*66	*I*82
nah	n/i*	nkat -- n moles/min/mg protein					
A dioxygenase	0/2	0	0	.3	.5	1.5	1.5
B dehydrogenase	0/10	0	0	18	6	6	8
C oxygenase	.1/20	.1	.1	0	14	9	13
D isomerase	0/.4	0	0	0	0	<.1	.3
E aldolase	.3/5	.2	.3	.2	.01	3	9
F dehydrogenase	.7/4	.5	.2	.6	4	3	3
G hydroxylase	.06/2	5	2	4	2	0	3
H 2,3-dioxygenase	.2/11	27	8	19	8	.1	6
I dehydrogenase	.05/1	2	1.6	2	1	.04	.05

*n/i = Non or induced 2.5 mM salicylate; $\bar{<}$ sensitivity of assay

Table 3. NAH7 plasmid + Tn5 insertion mutant restriction pattern with SmaI

Gene loci *Nah*	SmaI fragments, kb *A*	*B*	*C*	*D*	*E*	*F*	$\bar{>}$Tn5 (5.7kb)
wt.1343	42.3	17.6	12.7	6.8	3.7	-	-
*A*1	42.7					5.19	+.21
*A*2	44.6					3.72	+.34
*B*11		20.0				3.93	+.63
*C*21		19.5				3.96	+.16
*D*31		14.8				8.84	+.34
*G*67		17.0				6.77	+.47
*I*81		20.2				3.44	+.34

blanks = fragments were wild type size.

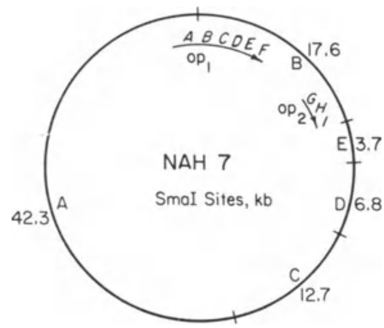


Figure 1. Plasmid NAH7 *nah* operons 1 and 2: Sma1 digest

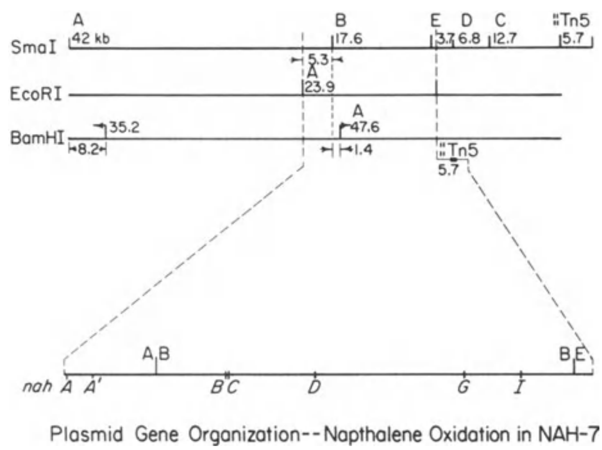


Figure 2. The *nah* gene loci in plasmid NAH7: Restriction maps with Sma1, EcoR1, and BamH1

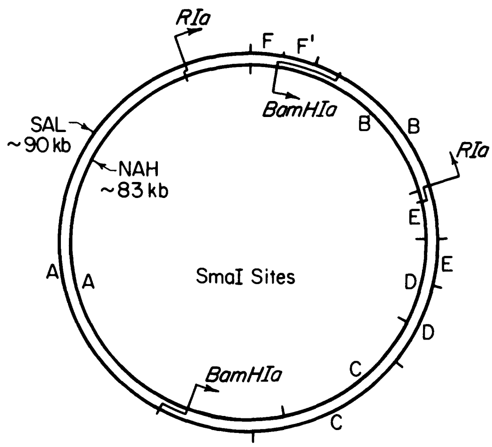


Figure 3. NAH7 *vs* SAL1 plasmid restriction homologies. F and F' insertion segments in the *nah* operon 1 gene AB region

polarity in both operons 1 and 2. The anomolous value of "F" dehydrogenase in the *nah*D mutant remains to be explained. Additional strains conform to the conclusions drawn from these examples. Since the preparation of Table 2, we have synthesized the substrates for later steps in the salicylate oxidation, and identified gene lesions which map to the right of I in an adjacent restriction fragment.

Table 3 presents the electrophoretic data for the Smal digest of wild type and Tn5 insertion mutants upon which the gene positions were assigned.

NAH7 and SAL1 plasmid homology. The NAH7 and SAL1 plasmids are of approximately equal size, 83 and 90 kilobases, respectively. Figure 3 presents superimposed maps of their Smal digest and indicates the loci of EcoR1 and BamH1 cleavage which provides the A, largest, fragment of each.

The Smal digest of the SAL1 plasmid yields five fragments identical in size to those from NAH7 plasmid, and in addition, two smaller fragments, F and F', each of about three kilobases. These arise from an insertion in the SmaI fragment B segment, a position which would coincide with NAH operon 1. This insertion too, produces a polar mutation as occurs in Tn5 insertions. Thus, as one would presume, the reaction pathway from naphthalene to salicylate is inactive. The working hypothesis for homology analyses currently is based on this assumption.

Heteroduplex analyses, by Southern Blot, of the NAH and SAL fragments of SmaI and EcoRl digests, indicate a high degree of homology further supporting the working hypothesis. To date we have been unable to delete the F-F' region from the SAL plasmid nor do we have data indicating whether other insertions or deletions have occurred in this region.

Aromatic plasmids and oxidative pathways. It would appear from the data of Dagley and coworkers (12) and from the molecular genetic data of Farrell (7) that a high degree of homology exists among the TOL, XYL, NAH, and SAL plasmids of the fluorescent pseudomonads. Taken with a relaxed specificity for aromatic derivatives with ring substitutes, with activity in hydroxylation and ring fission, one would seek further evidence of convergence in structure and processes among the aromatic oxidation systems as the molecular genetic studies are refined and the phenotypic and genotypic scoring extended.

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DEGRADATIVE PLASMIDS: TOL AND BEYOND

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INTRODUCTION

The bacteria of soil and water have been presented with major challenges by the chemicals disseminated by man. Although many of these compounds are detoxified, degraded or mineralised, others are more or less recalcitrant. The activities and changes in bacterial populations in these circumstances are of great concern to microbiologists interested in evolution and those seeking solutions to problems of pollution, utilisation of resources and recycling. The question relevant here is how plasmids contribute to these processes.

The degradative plasmids that have emerged (clearly the first of a very large number) are mainly in strains of Pseudomonas, a genus renowned for nutritional versatility. Work in a number of laboratories has centred on the TOL plasmid, since it specifies a well-defined pathway and because it was possible to isolate its DNA. Such work has been facilitated by the cleavage map we have established (Downing et al, 1978; Downing and Broda, 1979). There is now evidence that translocatable elements, re-arrangements of genetic material and transfer between unrelated strains can all contribute to the variation upon which natural selection acts.

THE TOL PLASMID pWWO

The TOL plasmid pWWO (117 kb) encodes 12 enzymes responsible for the degradation of toluene and the m and p xylenes by a pathway that involves meta cleavage of the aromatic ring (Worsey and Williams, 1975). Cells lacking the meta pathway can be isolated after growth on benzoate, which is an intermediate of this pathway and also of the chromosomally-encoded ortho pathway. In cells carrying the genes for both pathways only the meta pathway is expressed. However, in variants that have lost the meta pathway the (more efficient) ortho pathway functions; such cells then overgrow the others. "Benzoate-cured" variants arise either by loss of the whole plasmid or by excision of a specific contiguous 40 kb segment (Bayley et al, 1977). It is believed that the genes specifying the 12 enzymes are organised within two regulons, and are contained within this 40 kb segment (Worsey et al, 1978; Nakazawa et al, 1980; Inouye et al, 1981; Franklin et al, 1981).

THE EXCISION EVENT

We have been studying the excision of the 40 kb segment, exemplified by the formation of Tol plasmid pWWO-8 from pWWO (Table 1). We find that it involves reciprocal recombination between two directly-repeated sequences at its boundaries. These repeats are within the pWWO HindIII restriction fragments HD and HF; a novel fragment present in pWWO-8 is termed Hd. Restriction mapping of cloned fragments of HD, HF and Hd showed that part of Hd is derived from HD and the rest from HF. Heteroduplexes of HD and HF show that there is a direct repeat of 1.4 kb at or near the ends of the excised region. Comparison with the cleavage mapping data shows that the cross-over must occur within this repeat. We are presently seeking to establish whether this repeat is a translocatable element.

HYBRIDS OF pWWO AND RP4

It has been proposed that the formation of hybrids between RP4 and TOL (RP4-Tol plasmids) involves transposition (Jacoby et al, 1978; Chakrabarty et al, 1978) perhaps of the 40 kb moiety. Although the independence of this interaction from homologous recombination has not been tested, some kind of illegitimate recombination is likely since we have been unable to detect any homology between the two parental plasmids. We have tested the transposition model using six independently-isolated RP4-Tol plasmids by examining (1) what RP4 and TOL DNA they carry and (2) the nature of the junction regions. Restriction digests show that all of the six hybrids contain the whole of RP4 (to a level of resolution of 2 kb) as well as TOL DNA. In each case the TOL segment includes the 40 kb segment and extends beyond it in both directions. In four of the plasmids this segment at this level of resolution was the same, as was the region of RP4 that was interrupted (the tetracycline-resistance determi-

Table 1. HindIII restriction fragments of TOL and their fate in various derivatives. The top row gives the co-ordinates of the boundaries of the HindIII fragments given in the next row, according to the kilobase map of Downing and Broda (1979). Asterisks represent the positions and number of minor fragments. The remaining lines show which fragments are present wholly or in part in various derivative strains.

Map co-ordinate (kilobase)	111	15	35	35	39	44	53	62	67	90		99	103	106	111	Amount of TOL DNA (kb)
Fragment	HB	HC	*	HJ	HG	HE	HD	5*	HA	*	HF	HH	HK	HI	*	
Plasmid																
pWWO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	117
pWWO-8 [(a)]	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	77
pWWO-339 [(b)]	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	66
RP4-Tol [(c)]	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	59
pTN2 [(d)]	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	56
pWWO-1211 [(e)]	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	77
pWWO-1001 [(f)]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	117
pWWO-1216 [(g)]	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	98

(a) Plasmid pWWO-8 is the archetypal plasmid lacking the 40 kb segment.
(b) From strain PAW339 (Williams, unpublished). The PR4 Tol plasmids from strains PAW153 (Williams, unpublished), PU21 (RP4-Tol) (Jacoby et al, 1978) and AC810 (Chakrabarty et al, 1978) are similar. The junction of RP4 and TOL DNA is at about 14' on the RP4 map.
(c) From strain AC836 (Chakrabarty, unpublished). The junction of RP4 and TOL DNA is at 16' on the RP4 map.
(d) Nakazawa et al, (1978, 1980). The junction of RP4 and TOL DNA is at about 32' on the RP4 map.
(e) And also the plasmid from strain PAM1 (Williams, personal communication).
(f) There is a 3 kb insert in fragment HD, near the excision site (Williams, personal communication).
(g) There are two 3 kb inserts, in HA and HC (Williams, personal communication).

nant). However, the plasmids from the other two strains had less TOL DNA and different insertion sites on RP4 (Table 1). We conclude that RP4-Tol plasmids do not arise through transposition involving an unique TOL segment.

A benzoate-cured derivative of the plasmid from strain PaW339 was used to study the boundaries of the RP4 and TOL moieties of the hybrid plasmids. A *PstI* fragment including one of the junctions was used as a ^{32}P-labelled probe against *PstI* digests of the hybrid plasmids in strains PU21 (RP4-Tol), *PaW339* and PaW153, and of the parent plasmids RP4 and TOL. With each of the parent plasmids only one fragment hybridises with the probe DNA, as expected. However, with each of the RP4-Tol plasmids there was hybridisation with both junction fragments. This suggests that there is a sequence that is present at both junctions.

We have preliminary data that this sequence is not part of native TOL or RP4. When this same *PstI* fragment is hybridised against digests of chromosomal DNA from several plasmid-free strains of *Pseudomonas putida* (e.g. AC34) several fragments with homology are revealed. When RP4 or parts of TOL that are on this *PstI* fragment are used as probes no such homology is revealed. It is possible that there is an element resident on the chromosome that can translocate to TOL and/or RP4 as a preliminary to the formation of hybrids.

MOVEMENT OF TOL DNA TO THE CHROMOSOME

The DNA of some apparently plasmid-free benzoate-cured strains of *P.putida* mt2 showed homology with pWWO. We have been assessing the extent of this homology by hybridisation, using cloned pWWO fragments as probes. When fragments HD and HF were used against *Hind*III-restricted chromosomal DNA, a single band co-mobilising with HF showed homology with both probes. Further analysis with *Hind*III-*XhoI* double digests showed that this fragment was Hd, the novel fragment produced in the 40 kb excision event. The possibility therefore exists that an excision event similar to that yielding pWWO-8 occurred before, during or after integration.

Not all TOL DNA is present; thus no DNA homologous to the DNA contained in HA, HB, HC, HG, or HI was found. However, both HE and HK had homology with chromosomal *Hind*III fragments of different sizes.

OTHER DERIVATIVES OF pWWO

Reineke and Knackmuss (1979) have studied a strain of *Pseudomonas*, B13, that can utilise 3-chlorobenzoate (3CB) but not 4-chlorobenzoate (4CB). The reason for this inability to degrade 4CB was the specificity of the chlorobenzoate dioxygenase. Since pWWO specifies a benzoate dioxygenase with a wider substrate specificity, they introduced this plasmid into B13 to form B13/TOL strains (e.g. WR211). Such strains were still $4CB^-$, but yielded $3CB^+4CB^+$ derivatives with a frequency of 10^{-3} of the cells plated on selective medium. In such clones (e.g. WR216) expression of the meta pathway is lost. This loss is probably obligatory to avoid the synthesis by this pathway of non-metabolisable products. It is significant that $4CB^+$ strains can be obtained so easily from B13/TOL clones, and also that strain WR216 yields $Mtol^+$ revertants, and that these revertants are always $4CB^-$.

The structure of the plasmids of these strains (Williams and Jeenes, 1981) are interesting. That from strain WR211, pWWO-1211, is identical to pWWO-8. However, whereas the strain carrying pWWO-8 is irreversibily $Mtol^-$, strain WR211 is $Mtol^+$. One explanation is that the 40 kb moiety lacking in both carries all the degradative functions and that in strain WR211 it has become translocated to the chromosome.

Evidence to support this comes from matings of WR211 with the P.putida archetypal strain, which carries no known meta cleavage function or plasmid. A $Mtol^+$ transconjugant carried a plasmid (pWWO-1001) identical to the original TOL plasmid pWWO, except for a 3 kb insertion in HD (very close to the excision site). Its presence may account for the inability of this strain to grow on m-xylene.

The idea that the whole pathway is coded by this 40 kb segment is corroborated also by studies on strain PAM1, a clone of the original pWWO-carrying strain that had been maintained independently for 10 years. This too is $Mtol^+Mxyl^+$ but its plasmid is like pWWO-8. This strain remains $Mtol^+Mxyl^+$ even when the plasmid is eliminated by introducing the incompatible R plasmid pMG18, suggesting again that the 40 kb moiety is carried chromosomally and specifies these phenotypes.

The plasmid in strain WR216 (pWWO-1216) has regained some of the DNA excised in pWWO-1211. This confirms that at least part of this segment can be rescued. It was also noted that there are two novel inserts of 3 kb. A number of independently-isolated WR216-like derivatives of WR211 have similar structures. Whether the remaining TOL DNA is present chromosomally is not yet clear.

A further strain (WRB80) is a $Mtol^+$ derivative of WR216. Its plasmid differs from pWWO-1216 only in the loss of the 3 kb segment in HA, suggesting that this segment contains a structural or regulatory gene involved in the expression of the meta pathway.

The conclusion from the experiments is that there is movement of plasmid material to and from the chromosome that is demonstrable using the methods of molecular genetics and DNA-DNA hybridisation. Indeed, such integration of specific functions in the past may have been the basis of the accretion by Pseudomonas strains of their range of degradative capacities.

FUTURE DIRECTIONS WITH DEGRADATIVE FUNCTIONS

Those working with degradative plasmids have a number of opportunities. These include: (1) pursuing more detailed studies on plasmids that are already well studied, such as TOL and OCT (2) testing whether any of the plasmid/organism combinations that have been devised can actually be developed as effective agents for environmental cleanup (3) establishing whether mutant strains can provide aromatic or other compounds on a scale and with an efficiency that would be attractive to industry (4) establishing the role of plasmids in strains already involved in the degradation of natural and man-made compounds.

An example of the last of these is the work of Salkinoja-Salonen et al (1981) reported briefly in this volume. They implicate plasmids in the degradation by bacteria of soluble aromatic compounds formed in the industrial breakdown of lignin, in the effluent of pulp mills. Lignin is a major component of all plant material. There would therefore be major benefits from developing biological methods for delignification: these include energy saving and therefore cheaper production of cellulose from wood, reduction of pollution downstream from pulp mills, utilisation of straw residues for paper and animal feed, and more efficient use of sugar cane for ethanol production. The building blocks of lignin might also serve as feedstocks for chemical industry.

We do not yet know what the importance of plasmids might be in such systems, or even which are the organisms (e.g. bacteria or fungi) of choice. Nevertheless it is clear that it is a test of Molecular Biology how soon it can provide answers in terms of processes here as well as in the production of fine chemicals such as hormones.

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PLASMIDS IN THE BIODEGRADATION OF CHLORINATED AROMATIC COMPOUNDS

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Over the past several decades, man-made chlorinated aromatic compounds have been released into the environment in massive amounts in the form of herbicides, pesticides, refrigerants, lubricants or simply as industrial or hygienic household products. The presence of chlorine atoms on such molecules renders them toxic for microorganisms, insects and pests, and in some cases for human beings. The effectiveness of such compounds as insecticides or bacteriocidal agents prompted the chemical industry to manufacture varied types of the compounds and use them for enhanced agricultural productivity, various industrial processes and as health and beauty aids. The number of naturally-occurring compounds having carbon-chlorine bonds is very limited, so that microorganisms in nature have a limited capability to act upon all the complex chlorinated compounds synthesized by man[1]. This has resulted in the persistence of these compounds and because such compounds have been widely disseminated in nature, they have created enormous problems of toxic chemical pollution, as exemplified by the episodes in the Love Canal area, the pollution in the James River or the accidental release of extremely toxic dioxins in Seveso, Italy[2]. Although over the years, microorganisms have been reported to slowly biodegrade various chlorinated compounds by co-oxidative metabolism[3], there is still no evidence that pure cultures have acquired the ability to biodegrade highly chlorinated compounds. Reports of pure cultures capable of degrading simple mono- or dichloro compounds are now becoming available[4,5]. The purpose of this short article is to review the genetic basis of the biodegradation of simple chlorinated aromatic compounds such as 4-chlorobiphenyl (pCB) or 3-, 4- or 3,5-dichlorobenzoic acids by pure cultures, and examine the roleof plasmids in extending the range of chlorinated substrates that can be consumed by various bacterial genera.

Metabolism of Simple Chlorinated Aromatic Compounds by Pure Cultures

Although mixed cultures have long been known to slowly biodegrade a variety of chlorinated compounds, there are reports of pure cultures capable of dissimilating simple chlorinated compounds. For example, pCB is known to be metabolized by *Alcaligenes*, *Acinetobacter*, *Klebsiella* etc to 4-chlorobenzoic acid (4Cba)[6,7]. The *Alcaligenes* or the *Acinetobacter* species can also convert di- or trichlorobiphenyls to the respective chlorobenzoic acids. In none of the cases the pure cultures are known to further breakdown the chlorobenzoic acids, which therefore accumulate in the medium. The modes of biodegradation of other chlorinated compounds such as 4-chloro-phenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and (2,4-D) 3-chlorobenzoate (3Cba) have been studied by a number of workers[4,8,9]. Evans et al.[8] have described the characterization of a pseudomonad that could degrade 4-chlorophenoxyacetic acid with the release of chloride ions in the medium. Based on the accumulation of various intermediates and their oxidation by resting cell suspensions, these workers postulated a pathway for the oxidation of 4-chlorophenoxyacetate that involves 4-chlorocatechol, β-chloromuconic acid and maleylacetic acid as intermediates. A similar pathway for the degradation of chlorobenzoates by *Pseudomonas* B13 has been postulated by Schmidt and Knackmuss[10]. In detailed studies on the enzymes involved in the biodegradation of 3-chlorobenzoate, Knackmuss and his co-workers[4,10] have defined the strict specificities of many of these enzymes for chlorinated substrates and have delineated the major parts of the dissimilatory pathways. We have recently demonstrated the ability of a plasmid-containing 3-chlorobenzoate-positive *Pseudomonas* species to utilize maleylacetic acid (Mac)[11]. Some of the mutants, incapable of utilizing 3Cba were also rendered Mac^-. Transductional repair of such mutations to $3Cba^+$ simultaneously rendered them Mac^+, suggesting that Mac is an intermediate of 3Cba degradation by this *Pseudomonas* species. Based on the evidence presented by various workers, a plausible pathway for the biodegradation of 3Cba is presented in Fig. 1.

Plasmids in the Biodegradation of Chlorinated Compounds

Plasmids specifying biodegradation of several chlorinated compounds such as pCB, 2,4-D and 3Cba are now known (Table 1),

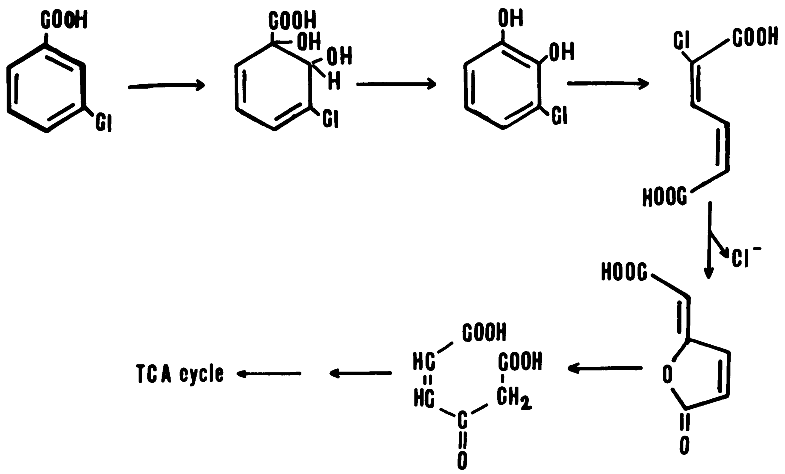

Fig. 1. Proposed pathway for the biodegradation of 3-chlorobenzoate by Pseudomonas species.

While plasmids such as pJP1 and pAC21 are known to encode a partial degradative pathway i.e., allowing conversion of 2,4-D to 2,4-dichlorophenol and 4-chlorobiphenyl to 4-chlorobenzoic acid (4Cba) respectively, pAC25 and pAC27 plasmids encode a complete degradative pathway for the biodegradation of chlorobenzoates with the release of chloride ions in the medium. We have previously reported that pAC25-positive P. putida or P. aeruginosa cells are incapable of utilizing 4Cba[11]. Growth of the pAC25-positive cells in presence of cells harboring the TOL plasmid in a chemostat enriched with 4Cba led to the emergence of cells that could also utilize 4Cba[5]. This observation is analogous to that previously reported by Hartmann et al.[4] that the benzoate oxidase complex induced by 3Cba in Pseudomonas B13 has a stringent substrate specificity so that it does not use 4Cba as a substrate. The presence of the TOL plasmid allows induction of a broad substrate specific benzoate oxidase which can also act on 4Cba with the formation of 4-chlorocatechol. The catechol oxygenase

Table 1. List of plasmids specifying dissimilation of chlorinated compounds

Plasmid	Degradative Pathway	Transmissibility	Size (Mdal)	Reference
pJP1	2,4-Dichloro-phenoxy-acetic acid	Conjugative	58	9
pAC21	4-Chlorobiphenyl	Conjugative	65	7
pAC25	3-Chlorobenzoate	Conjugative	68	11
pAC27	3- and 4-Chloro-benzoate	Conjugative	59	5
pAC29	3-,4- and 3,5-Dichlorobenzoate	N.D.	N.D.	This Manuscript

N.D. - not determined

and subsequent enzymes induced by 3Cba in Pseudomonas B13 can act upon 4-chlorocatechol, leading to its biodegradation. It was therefore anticipated that the pAC25-positive P. putida cells that also acquired the ability to utilize 4Cba due to chemostatic selection in presence of the TOL plasmid would either demonstrate the presence of the TOL plasmid or would have the benzoate oxidase gene(s) recombined with the pAC25 plasmid. Examination of the plasmid profiles of such $4Cba^+$ strains demonstrated the presence of a single chlorobenzoate plasmid with an average molecular size of 59 million daltons (Mdal). This plasmid is termed pAC27. It is also possible to transfer pAC25 and TOL to P. aeruginosa. These two plasmids are normally unstable in the same cell. Growth of the cells with 3Cba also induces the TOL-specified meta pathway, whereby 3-chlorocatechol derived from 3Cba is partly metabolized by the meta pathway. Metabolism of 3Cba by the meta pathway is believed to generate a chlorinated intermediate that is toxic for the cells. TOL and pAC25 are therefore incompatible due to metabolic reasons. It is, however, possible to isolate single colonies of P. aeruginosa capable of utilizing 4Cba from unstable TOL^+ $pAC25^+$ cells. Such $4Cba^+$ colonies are phenotypically Tol^-, but can generate Tol^+ revertants at a frequency of nearly 1×10^{-8}. Isolation of plasmid DNA from

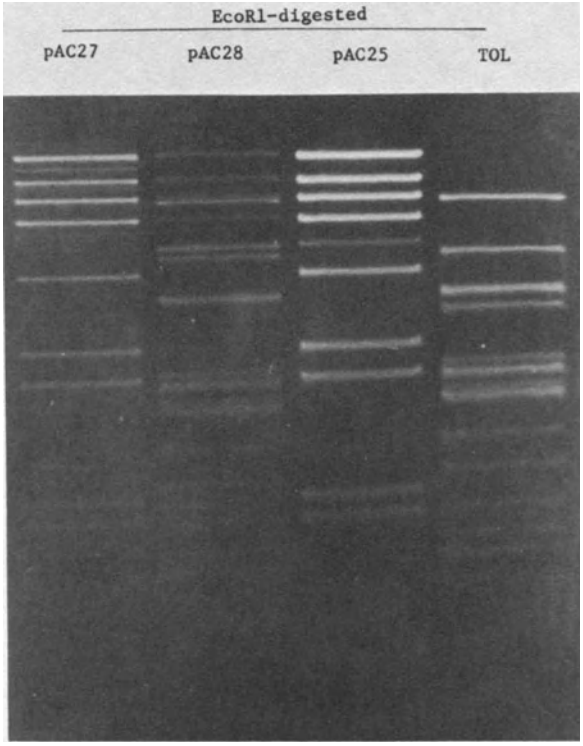

Fig. 2. Agarose gel electrophoretic mobilities of fragments of pAC27, pAC28, pAC25 and TOL plasmids on digestion with EcoRI.

such colonies and subsequent EcoRI digestion demonstrates that both plasmids undergo deletions in order to become compatible in the 4Cba+ cells (Fig. 2). The pAC25 plasmid has a 6.4 Mdal band missing in the EcoRI digest. This modified plasmid is termed pAC28. The fragment missing in pAC28 is different from the fragment missing in pAC27 obtained by chemostatic selection in presence of the TOL plasmid (Fig. 2). The TOL plasmid demonstrates the absence of a 5.6 Mdal fragment in the EcoRI digest of the plasmids isolated from the 4Cba+ P. aeruginosa cells. Since such 4Cba+ cells are normally Tol− but can revert to Tol+ it is clear that the deletion does not span the structural genes involved in toluate metabolism.

Hartmann et al.[4] have also demonstrated that it is possible by continuous enrichment of 4-Cba+ Pseudomonas B13 cells with 3,5-dichlorobenzoate to isolate cells that can utilize 3,5-di-

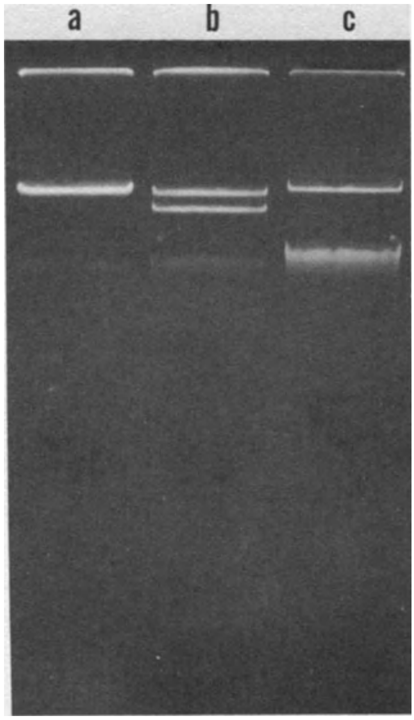

Fig. 3. Agarose gel electrophoretic mobilities of TOL (lane a) plasmid DNA from 3,5-dichlorobenzoate positive cells (lane b) and pAC28 (lane c).

chlorobenzoate. We have grown the 4-Cba$^+$ P. aeruginosa cells in minimal media enriched with 3,5-dichlorobenzoate, and by continuous subculturing in minimal dichlorobenzoate media have isolated cells that can utilize this compound as a sole source of carbon and energy. The profiles of TOL, pAC28 and the plasmid DNA isolated from 3,5-dichlorobenzoate-positive cells are shown in Fig. 3. It is interesting that during selection for the 3,5-dichlorobenzoate character, both the plasmids (pAC28, TOL) appear to undergo further structural rearrangements to generate the plasmid pAC29.

Genetic Homology Between the Chlorobenzoate (pAC25) and Other Degradative Plasmids

In order to determine how much homology pAC25 may have with other hydrocarbon degradative plasmids, we have nick-translated pAC25 DNA and used it as a probe in hybridization experiments with EcoRI restriction fragments of degradative plasmids such as SAL and TOL, and an antibiotic resistance plasmid pAC30 which specifies resistance to tetracycline, carbenicillin and streptomycin. Both SAL and TOL exhibited considerable homology, while 3 out of 12 fragments of pAC30 demonstrated some degree

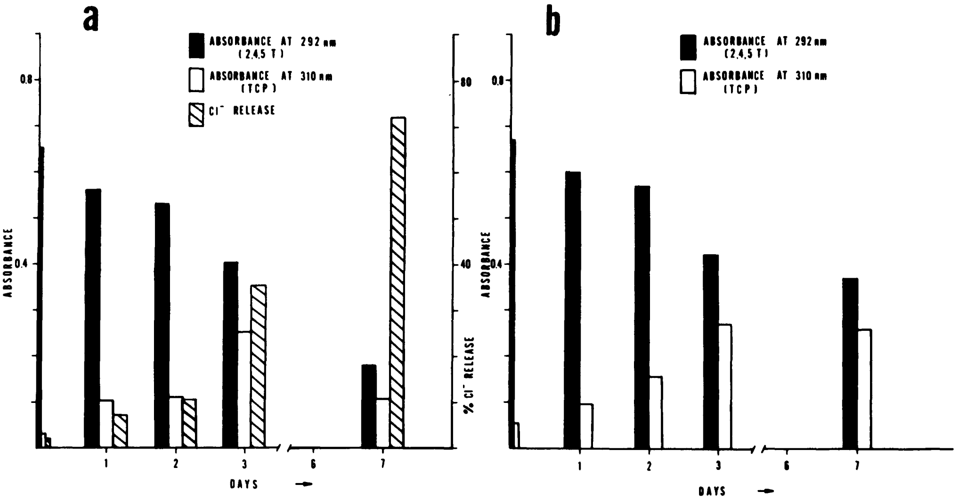

Fig. 4. (a) Growth of mixed cultures with 2,4,5-T as a sole source of carbon with accumulation of 2,4,5-trichlorophenol (TCP) and release of chloride; (b) demonstrates loss of 2,4,5-T and accumulation of TCP during growth of a pure culture isolated from the chemostat. No chloride release was demonstrated in the latter case.

of homology. It is thus clear that a plasmid such as pAC25 may have evolved by recombination of various genetic fragments from plasmids such as TOL and SAL (and to some extent pAC30) specifying biodegradation of analogous non-chlorinated compounds. The homology with pAC30 may be in the region of the replication, maintenance or transfer genes of the plasmids.

Molecular Breeding of Strains for 2,4,5-T Dissimilation

The extensive homology of pAC25 with SAL and TOL, and to a lesser extent with an antibiotic resistance plasmid, appears to indicate that pAC25 may have evolved by recruitment of genes from various plasmids. If it is a general mode of evolution of plasmids, then perhaps it might be possible to allow evolution of degradative plasmids for various toxic chemicals in a continuous culture in the chemostat by supplying a variety of plasmids to microorganisms isolated from toxic waste dump sites. It is known that toxic chemicals such as 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), TCDD (2,3, 7,8-tetra-chlorodibenzo-p-dioxin) etc are very persistent in nature because of their slow breakdown by co-oxidative metabolism[12,13]. It appears that although these compounds occur in minute quantities (usually parts per million; for TCDD parts per billion or less) in nature, they produce severe toxicity symptoms in animals and human beings because of their extreme toxicity. Such low concentrations may pose problems of toxicity for human beings but not for microorganisms and may not be quantitatively enough to serve as a source of carbon and energy. The microorganisms therefore do not appear to have any incentive for evolving plasmids allowing dissimilation of these compounds. In order to determine if microorganisms capable of dissimilating a toxic chemical such as 2,4,5-T can be bred in the laboratory, we have inoculated into a chemostat, soil samples from a number of dump sites and *P. putida* strains harboring a variety of plasmids such as CAM, SAL, TOL, pAC21, pAC25 etc. The chemostat was initially maintained with low concentrations of plasmid substrates such as camphor, toluate, salicylate, chlorobenzoate etc. Gradually the concentrations of plasmid substrates were reduced while that of 2,4,5-T was increased. After about 6 months, the chemostat was run with 2,4,5-T alone (500 μg/ml) as a sole source of carbon. After several weeks with 2,4,5-T as sole carbon source, the medium in the chemostat gradually turned light brown, and an increase in turbidity was visible. Continuous monitoring of the medium demonstrated appreciable loss of 2,4,5-T and release of chloride ions in the reactor medium. This is more clearly seen from the results in Fig. 4a, where an aliquot from the chemostat vessel was inoculated into a minimal 2,4,5-T (500 μg/ml) medium and grown for 7 days. At different intervals, aliquots were taken, diluted and the levels of 2,4,5-T, 2,4,5-trichlorophenol (Tcp) and inorganic chloride were measured. In 7 days, about 72% of the 2,4,5-T was degraded with the release of an equivalent amount of chloride ions. Although initially the Tcp level increased steadily, the level of Tcp fell down considerably after 3 or 4 days. Streaking of the cell suspension on a nutrient agar plate demonstrated the presence of several types of colony morphologies suggesting the presence of a mixed culture. On streaking on a minimal 2,4,5-T plate, single colonies grew slowly within the first 3 days, but stopped growing thereafter. On further examination, they were found to be capable of converting 2,4,5-T to Tcp, but unable to attack Tcp any further (Fig. 4b). No chloride release was observed from 2,4,5-T or Tcp by such cells. The cells were also found to be capable of producing Tcp from 2,4,5-T when grown with glutamate.

Concluding Remarks

The need for the presence of the TOL plasmid in extending the substrate range of the 3Cba degradative plasmid (pAC25) to include 4Cba and 3,5-dichlorobenzoate, and consequent structural changes of the plasmids giving rise to pAC27, pAC28 and pAC29 is an interesting example of the interactions of plasmids in a natural environment for the degradation of novel xenobiotic compounds. The biochemistry of this phenomenon has previously been elucidated by Hartmann et al.[4], and the present study simply delineates the role of plasmids involved in such a process. The emergence of a mixed culture that can continuously be cultivated indefinitely with 2,4,5-T as a sole source of carbon reaffirms the utility of chemostats as a means of selecting specific strains under defined growth conditions, and additionally points out the important roles played by degradative plasmids in the evolution of new genetic functions. It would be interesting to find out if continued growth of the mixed culture with 2,4,5-T will ultimately lead to the emergence of a single culture capable of total degradation of 2,4,5-T, and if such a culture would harbor a 2,4,5-T degradative plasmid. In the event of a positive response for both, plasmid-assisted molecular breeding of strains under chemostatic selective conditions with specific toxic chemicals will become a powerful tool in the application of such strains for practical removal of toxic chemicals from the environment.

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ANTIBIOTIC RESISTANCE OF GRAM NEGATIVE BACTERIA IN MEXICO: RELATIONSHIP TO DRUG CONSUMPTION

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The selection of bacterial strains resistant to antibiotics is closely linked to the usage of antimicrobial agents[1,2,3]. In Japan during 1951, six years after the clinical introduction of sulfanilamide, approximately 80% of the strains of *Shigella* studied were found resistant to it, whereas in 1949 only 10% were resistant. Similarly, increases in the incidence of drug resistant microorganisms have been reported in Great Britain[4], the United States[5], the Netherlands[6] and other countries[1,7]. In addition to the increase in the percentage of strains resistant to individual antibiotics, multiple resistant strains have been isolated with greater frequency as the age of antibiotherapy grows older[4,7,8,9]. In Mexico, Olarte and co-workers[10,11] have reported the incidence of resistance to antibiotics in strains of *Shigella*, *Salmonella* and enteropathogenic *E. coli* and they have noted an increase in the frequency of multiple resistant strains.

We present here the antibiotic resistance patterns of enterotoxigentic *E. coli* strains (Ent ST^+ and Ent LT^+) isolated during 1976-1977 (H. Stieglitz, R. Fonseca, J. Olarte, and Y.M. Kupersztoch-Portnoy, unpublished); the comparison of the antibiotic resistance patterns of strains of *Salmonella* and *Shigella* isolated during 1978-1979 (R. Fonseca, P. Mendoza, S. Garcia, V. Vázquez, and Y.M. Kupersztoch-Portnoy, unpublished); and the antibiotic resistance of *Proteus mirabilis*, indole positive *Proteus*, *E. coli*, and *Salmonella* isolated in the city of Toluca (Mexico) and its relationship to the consumption of antibiotics in Mexico (R. Lara, J. Silva, and Y.M. Kupersztoch-Portnoy, unpublished).

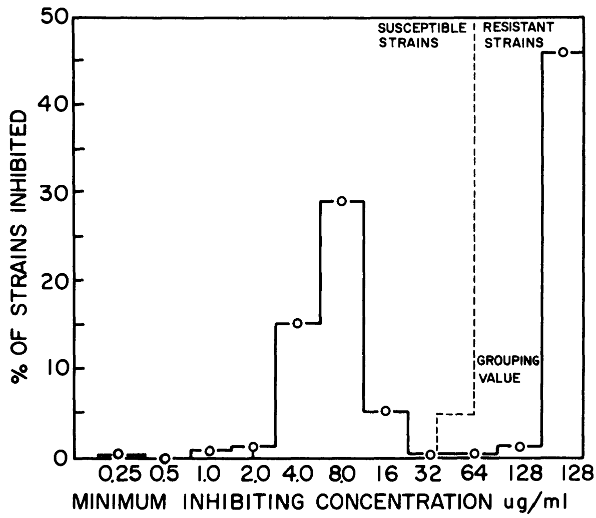

Fig. 1. Discrimination between drug sensitive and drug resistant bacteria. The lowest point between peaks is taken as the grouping value. In all the determinations (30 strains per test) E. coli ATCC10536 and Klebsiella pneumoniae ATCC10031 were included. For Su, Tm, and Tm-X (sulfa-trimethoprim 19:1) Wellcotest Sensitivity Test Agar was used. The data shown are from 252 strains of Salmonella tested for ampicillin susceptibility. As more strains were tested (1419 in total), the most frequent value changed to 4μg/ml.

The determination of the minimal inhibiting concentration (mic) was done by the agar dilution method according to the recommendation of the ICS Report[12]. The criterion we used to distinguish drug sensitive from drug resistant microorganisms groups bacteria from the same species according to the frequency distribution of their mics[13] (Fig. 1). It avoids the classification of a strain as sensitive or resistant considering only preestablished values, regardless of both the statistical fluctuation in a bacterial population and the individual differences in implementation of the same methodology. Table 1 shows a comparison of the values used by

Table 1. Antimicrobial drug resistance in *Salmonella* and *Shigella*

Drug	Grouping criterion: *Salmonella*[1] Mode of Sensitive Strains (µg/ml)	Grouping criterion: *Salmonella*[1] Grouping Value (µg/ml)	Grouping criterion: *Salmonella*[1] % Resistant Strains	Grouping criterion: *Shigella*[2] Mode of Sensitive Strains	Grouping criterion: *Shigella*[2] Grouping Value (µg/ml)	Grouping criterion: *Shigella*[2] % Resistant Strains	ICS criterion: Resistant Strains (µg/ml)	ICS criterion: Resistant Strains *Salmonella*	ICS criterion: Resistant Strains *Shigella*
Ap	4	32	47	4	32	20	32	47	20
Cb	4	128	46	2	64	18	32	46	20
Ce	4	32	40	8	64	2	32	40	5
Cm	8	64	34	8	64	16	25	35	18
Gm	1	16	26	2	16	0.34	6	27	1
Km	2	32	43	4	32	9.5	25	44	12
Nx	4	32	3	1	8	1	32	3	1
Fn	32	256	6	8	256	2	100	18	4
Rif	16	256	1	16	128	0			
Sm	8	64	45	8	32	60	15	67	69
Su	256	512	53	8	32	91	350	63	79
Tc	2	32	31	1	8	71	12	36	71
Tm	0.125	4	1.5	0.125	2	1.5			
Tm-X	2	64	0.35	2	32	0.6	200	0.35	0.5

[1] 1419 independently isolated strains were used for the calculations.
[2] 319 independently isolated strains were used for the calculations.

the grouping method and the ICS criterion to distinguish drug sensitive and drug resistant Salmonella and Shigella, and also the percent of resistant strains with both methods. Even though the values to differentiate drug resistant from drug susceptible microorganisms show differences using the two criteria, the overall percent of resistant strains is similar in the majority of cases. However if we were to take 15µg/ml for streptomycin in the case of Salmonella, we would have had to split a clearly bimodal curve (data not shown) artificially near the mode of the sensitive population; for the clinical distinction of resistant bacteria, the serum concentration of the drug is unquestionably of vital importance, but it is not in the intrinsic properties of a genus or species of bacteria. I feel that the grouping value should be used in antibiotic resistance studies but not in the clinical ones.

Table 2 shows a remarkable difference in the percent of resistant strains isolated in hospitalized patients (no antibiotherapy was given in the hospital before the sample was taken) and from ambulatory patients. These data suggest that before the patient

Table 2. Comparison of the resistance to antimicrobial agents between Salmonella strains isolated in hospitals and in private laboratories.

	Percent of resistant strains	
Antimicrobial agent	Hospitals	Laboratories
Ap	73.95	11.42
Cb	70.86	9.49
Ce	60.98	8.08
Cm	34.25	8.78
Fn	1	0
Gm	42.2	2.63
Km	53.55	8.08
Nx	2	0
Rif	0	0
Sm	63.88	10.19
Su	89.44	95.26
Tc	42.32	16.13
Tm	2.28	2.63
Tm-X	0.38	2.63

50.2% and 26.8% of the Salmonella isolated from hospitals and private laboratories respectively were serotyped as typhimurium.

was referred to the hospital he had had antibiotic treatment and/or that the strains were to begin with resistant (virulent) and the patient had to be hospitalized for proper treatment. The fact that 50.2% of the Salmonella isolated in hospitals were S. typhimurium as compared to 26.8% of the private laboratories favors the latter alternative but does not rule out the former. There is a notoriously high level of Su^r strains in both populations.

Table 3. Multiresistance in strains of Salmonella and Shigella

Type of resistance	Number of strains (%)		Most frequent pattern of resistance	Number of strains with the most frequent pattern (%)	
	Shigella	Salmonella		Shigella	Salmonella
11	0	5(0.72)	ApCbCeCm GmKmFnSm SuTcSi	0	5(100)
10	0	48(6.91)	ApCbCeCmGm KmSmSuTcSi	0	40(83)
9	0	59(8.49)	ApCbCeCmGmKm SmSuSi	0	27(46)
8	0	35(5.04)	ApCbCeCmGmKmSmSu	0	9(27.7)
7	·2(1.035)	45(6.43)	ApCbCmKmSmSuTc	0	13(28.9)
6	5(2.56)	34(4.89)	ApCbCeKmSmSu ApCbCmKmSmSi	0 5(100)	10(29.4) 2(5.88)
5	11(5.64)	34(4.89)	CmKmSmSuTc ApCbKmSmSu	0 3(27.3)	13(38.24) 7(20.6)
4	16(8.2)	56(8.06)	CmSmSuTc ApCbSmSu	2(12.5) 6(37.5)	13(23.2) 4(7.1)
3	23(11.8)	43(6.2)	ApCbKm CmSmSu	0 13(56.5)	13(30) 0
2	71(36.4)	41(5.9)	SmSu	57(80.3)	8(19.5)
1	40(20.5)	83(11.9)	Su	29(72.5)	41(49.4)

Table 3 shows the multiple drug resistance of 697 strains of Salmonella and 195 strains of Shigella (before the collection was completed). It can be seen that 20.5% of the strains of Salmonella were resistant to eight or more antibiotics while no Shigella was found resistant to more than seven antibiotics; it is not clear as to why this difference exists among the two genera. It also shows that the two general do not share the most frequent pattern; i.e. while 13 Salmonella strains were found resistant to ApCbKm (30% of the 43 strains found resistant to three antibiotics), in Shigella, the prevailing group of resistance to three drugs was CmSmSu (56.5%). These results may be taken to indicate different evolutionary patterns in the emergence of multiple drug resistance among the two genera.

We have studied the antibiotic resistance of enterotoxigenic LT^+ and ST^+ E. coli strains isolated during 1976-1977. As seen in Table 4, not one of the 50 strains was susceptible to the 14 antimicrobial agents tested. 2% were resistant to one antibiotic and the rest were multiple drug resistant. It is noticeable that 35 of the 50 enterotoxigenic strains were Tc^r; it is unlikely that the prevention of travelers diarrhea due to enterotoxigenic E. coli will be effective by deoxycycline[18] in a population of bacteria that is 70% resistant to the drug. Thus, on top of the ecological implications of the prophylactic use of antibiotics and the self-limiting nature of diarrheal disease caused by enterotoxigenic E. coli, the high percentage of resistant bacteria should discourage the use of antibiotics in the prevention of the disease.

More detailed studies have been performed on the genetic and physical linkage of antibiotic resistance and Ent ST^+. We have shown that a naturally occurring single plasmid is responsible for Ap^r and Ent ST^+[17] and that the same plasmid is widely distributed in the population studied (H. Stieglitz et al., this volume).

An attempt to correlate the incidence of drug resistant enterobacteria and the national consumption of antibiotics was done in a study in the city of Toluca. From the cultures of each case of diarrhea (500 in total) the prevailing microorganisms and/or well-characterized bacterial pathogens were isolated; not more than one of each genus was isolated from each case. The grouping values were determined for each gera or species and the percentage of resistant strains is shown in Table 5. The national consumption of these drugs[19] (Grunner, personal communication) is indicated in the same table; it includes the human, veterinary, and agricultural consumption. It is clearly seen that the less effective antibiotics are the most widely consumed. Thus, as shown elsewhere[20,21], the appearance of antibiotic resistant strains in bacteria is closely linked to the use of antimicrobial agents.

Table 4. Resistance to antibiotics in enterotoxigenic *Escherichia coli* strains.

LT^+ Strains[1]	Antibiotic resistance	ST^+ Strains[2]	Antibiotic resistance
3IEC-1 and 2	ApCmKmSmSuTc	23IEC-4	ApCbSmSuTc
31IEC-5	ApCeCmKmSmSuTc	*40IEC-2	ApCbSmSuTc
*4IEC-1, 2 and 3	ApCmKmSmSuTc	*40IEC-3	CmSuTc
8IEC-5	CmSmSuTc	*40IEC-4	ApCbSuTc
10IEC-1	ApCbCmKmSmSuTc	*40IEC-5	ApSuTc
16IEC-2	ApCbCeCmKmSuTc	81IEC-3	ApCbSmSuTc
*18IEC-2	CmKmSuTc	*93IEC-3	ApCbSmSuTc
31IEC-5	ApCbCmSuTc	98IEC-1	Cm
67IEC-5	ApCbCmKmSuTc	101IEC-3 and 4	ApCmSmSuTc
78IEC-3	ApCbCmKmSuTc	102IEC-1 and 2	ApCbCmKm
*80IEC-1	ApCbCmKmSmSuTc	*108IEC-2	ApCbKmSmSu
*80IEC-2	ApCbCeSuTc	109IEC-3	SmSu
88IEC-4	ApCbCmKmSuTc	*111IEC-3	ApCbCmKmSu
*96IEC-2	ApCbCmKmSuTc	*113IEC-1	ApCbCmKmSmSuTc
*99IEC-3,4 and 5	ApCbSmSuTc		SuTc
101IEC-1	CmSuTc	*116IEC-2	SmTc
101IEC-2	CmSmSuTc	123IEC-2	ApCbCeTc
*104IEC-2	ApCbCeCmSuTc	123IEC-3	ApCbSmSuTc
104IEC-3	ApCbCeCmSu		
104IEC-4	ApCbCmTc		
110IEC-1	SuTc		
*113IEC-1 and 2	ApCbCeCmKmSmSuTc		
*113IEC-4	ApCbCeCmKmGmSmSuTc		
*113IEC-5	ApCbCmKmSmSuTc		
117IEC-4			

[1] LT enterotoxin was determined initially by the adrenal cell assay as described by Sack[14], and confirmed in the rabbit ileum loop assay[15].

[2] ST enterotoxin was determined initially by the suckling mice assay; positive cultures were then confirmed by the rabbit ileum loop assay[15].

*Patients that had medication before admission to the hospital.

Table 5. Relation between antibiotic consumption and resistant strains

Antibiotic	Mexican consumption 1977 (tons)	Resistant strains (%)*
Tc	134	86.5
Su	111	70.8
Ap	101	63.7
Cb	0.3	55.0
Sm	133**	54.1
Ce	15	36.1
Km	133**	35.6
Cm	85	45.2
Fn	-	19.7
Nx	20	9.6
Rif	5	4.0
Tm	1.33	0.3
Gm	0.55	2.2

*Based on total number of strains studied: 295 *E. coli*, 198 *Proteus mirabilis*, 63 indole positive *Proteus* and 39 *Salmonella*

**Total consumption of Sm, Km, and neomycin.

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PLASMID MEDIATED AMPICILLIN RESISTANCE IN A STRAIN OF HAEMOPHILUS PLEUROPNEUMONIAE ISOLATED FROM SWINE

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INTRODUCTION

Haemophilus pleuropneumonia is a hemolytic, nicotinamide adenine dinucleotide requiring member of the genus Haemophilus. H. pleuropneumoniae produces lobar pneumonia with fibrinous pleuritis in swine. The disease has high morbidity and, when introduced into highly susceptible animals, high mortality. When the microorganism becomes bacteremic, meningitis and arthritis may result. Young, rapidly growing pigs seem to be the most susceptible, though any age pig may contract the disease. The source of H. pleuropneumoniae in the environment is the respiratory tracts of affected as well as recovered asymptomatic pigs (1,2).

Disease produced by H. pleuropneumoniae was first recognized in the 1960s following the independent isolation of the agent from infected tissue in Great Britain, California, Argentina, and Switzerland (1,3-6). Following these initial reports, the agent has been shown to be responsible for diseases in swine herds in Europe, Scandanavia, Australia, and Canada (7-11).

Though infrequently reported, the susceptibility of the isolates obtained from outbreaks occurring through 1970, were found to be susceptible to antimicrobial agents, including penicillin, kanamycin, tetracycline, and chloramphenicol. There is disagreement regarding streptomycin and the sulphonamides, some reports indicate susceptibility others, resistance (4,6,9,10).

In 1980, most of the isolates of H. pleuropneumoniae possessed resistance to streptomycin and the sulphonamides (Libal MC: Personal communication). An isolate from one particular outbreak, involving 100 pound feeder pigs, demonstrated resistance not only to streptomycin and the sulphonamides, but to ampicillin as well. This report presents preliminary data concerning this isolate. For comparative purposes, data concerning an ampicillin susceptible, but streptomycin and sulphonamide resistant isolate obtained during this period of time is also presented.

MATERIALS AND METHODS

Bacterial strains: *Haemophilus pleuropneumoniae* strain SD-1B and SD-2A were isolated from the lungs of swine that had died from porcine pleuropneumonia. *Haemophilus pleuropneumoniae* strain M26 was a plasmid-less strain of serovar 4 obtained from E. L. Biberstein, University of California, Davis, California.

Media and growth conditions: *Haemophilus* cultures were grown in brain heart infusion broth or agar supplimented with 1 µg NAD/ml. All incubations were performed at 37°C in an atmosphere of 10% CO_2 and air.

Susceptibility testing: An agar dilution method was used for all isolates (12).

Preparation of DNA: Bacteria were grown to late logarithmic phase in NAD supplemented brain heart infusion broth. The cells were harvested by centrifugation and lysed by Triton X-100 (13). Covalently closed circular plasmid DNA was purified by isopycnic centrifugation in a cesium chloride-ethidium bromide gradient (14). The final density was adjusted to 1.6199 g/cm^3 (refractive index, 1.3920), and the solution was centrifuged for 48 hours at 15°C and 34,000 rpm in a Beckman type 40 rotor.

DNA bands in the gradients were located with a black-light lamp (UVS-11, Ultraviolet Products, Inc., San Gabriel, CA). Plasmid DNA was removed by puncturing the side of the gradient tube with a 18-gauge needle attached to a syringe.

Transformation: The transformation method was that described by Cohen et al (15). Following transformation, the mixture of cells and DNA was diluted 1:10 in NAD supplemented L-broth and incubated for 6h. When ampicillin resistant transformants were desired, 15 µg of ampicillin per ml were added prior to overnight incubation. Following overnight incubation, transformants were selected by plating on chocholate agar containing ampicillin (15 µg/ml) or streptomycin (25 µg/ml).

Agarose gel electrophoresis: Vertical gel electrophoresis was performed using the method of Meyers et al (16).

Beta-lactamase measurement: Three 100 X 15 mm petri plates containing chocolate agar were flooded with an overnight broth culture of H. pleuropneumoniae. Thirty minutes later, excess culture fluid was removed, and the plates incubated for 18-24h at 37°C under an atmosphere of 10% CO_2 and air. The growth of the organisms on the surface of these plates was removed by gentle washing with 25 mM phosphate buffer, pH 7.0 (3 mls per plate). The cells were pelleted by centrifugation (approximately 10,000 xg, 15 min, 2°C). The pellets were resuspended in 5 ml of 25 mM phosphate buffer, pH 7.0. The cells were sonicated (setting 35 using a microprobe, Biosonik III, Bronwill Scientific, Rochester, NY) three times. The suspension was cooled in ice for 2 min between each sonication. Following sonication, the suspension was spun (20,000 xg, 15 min, 2°C) and the supernatant saved and assayed for β-lactamase activity.

The method used for the determination of β-lactamase activity was the hydroxylamine assay (17). Activity (decrease in substrate in μ moles/min) of the sonicate supernatants against penicillin G was taken as 100.

RESULTS

The susceptibility of the isolates is shown in Table 1.

Agarose gel electrophoresis of DNA isolated following CsCl-ethidium bromide centrifugation is shown in Figure 1, lanes A and B. Two plasmids are seen, one of molecular mass 3.5 MDal, the other 2.3.

Transformation of H. pleuropneumoniae M62 with purified plasmid DNA gave transformants resistant to SmSu and to ApSu (Table 2). Agarose gel electrophoresis of DNA obtained from transformed H. pleuropneumoniae M62 (Figure 1, lanes C through E) shows that the plasmid (pVM105) of molecular mass 3.5 MDal codes for resistance to ApSu, whereas the other of mass 2.3 Mdal (pVM104, pVM106) code for resistance to SmSu.

Table 1 Susceptibility of Haemophilus pleuropneumoniae isolated from swine.

Isolate	Minimal inhibitory concn (μg/ml)								
	Ap	Pc	Tc	Su	Sm	Km	Gm	Cp	Cm
SD-1	32	32	16	256	128	2	0.5	1	0.5
SD-2	<0.25	0.5	8	2048	>128	16	8	<0.25	0.5

Ap = ampicillin; Pc = penicillin G; Tc = tetracycline; Su = sulfadiazine; Sm = streptomycin; Km = kanamycin; Gm = gentamicin; Cp = cephalothin; Cm = chloramphenicol

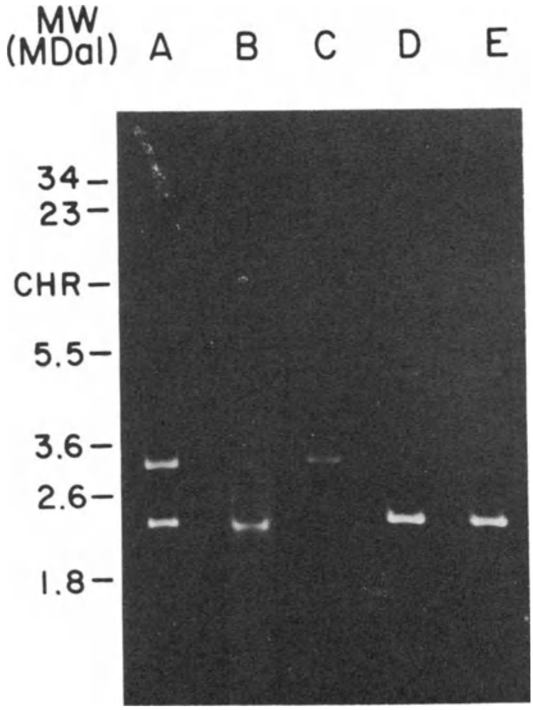

Figure 1. Agarose gel electrophoresis of plasmid DNA obtained from Haemophilus pleuropneumoniae. Lane A: SD-1 (ApSmSu), B: SD-2 (SmSu), C: M62 (pVM105, ApSu), D: M62 (pVM 104; SmSu), E: M62 (pVM 106; SmSu).

Table 2. Susceptibility of transformed Haemophilus pleuropneumoniae strain M62.

Strain of H. pleuropneumoniae	Minimal inhibitory concentration (μg/ml) Ap	Sm	Su
SD-1	32	128	256
SD-2	<0.25	>128	2048
M62(pVM105)	>128	16	>2048
M62(pVM104)	<0.25	>128	>2048
M62(pVM106)	<0.25	>128	>2048
M62	<0.25	8	<100

The activity of sonicate supernatants against various penicillins used as substrates is shown in Table 3. The substrate profile is consistent with a TEM type β-lactamase.

Table 3. Relative rates of hydrolysis of various penicillin substrates by sonicates of *Haemophilus pleuropneumoniae*

Strain of *H. pleuropneumoniae*	Substrate			
	Penicillin G	Ampicillin	Oxacillin	Cephalothin
M62(pVM105)	100*	149	0	0-27
M62(pVM104)	0	-	-	-

*Activity, μ moles/minute, against penicillin G arbitrarily set at 100

DISCUSSION

We have presented evidence that indicates that the genes responsible for resistance to ampicillin, streptomycin, and the sulphonamides in H. pleuropneumoniae strain SD-1 are located on two small molecular weight plasmids, pVM105 (3.5 MDal) coding for ApSu resistance and pVM104 (2.3 MDal) coding for SmSu. The genes responsible for SmSu resistance in H. pleuropneumoniae strain SD-2 appear to be located on the small molecular weight plasmid pVM106 (2.3 MDal).

The somewhat sudden acquisition of these resistance determinants is not surprising. Almost all swine in the midwestern United States are fed feed supplemented with antimicrobials. The most common additive contains a mixture of chlortetracycline (100 gms/ton), sulphonamides (100) and penicillin (50).

The genetic basis for the acquisition of resistance determinants is purely speculative. We hypothesize, as was done in the case of resistance in Neisseria gonorrhoeae and H. influenzae, that SmSu resistance genes were acquired from without, possibly from members of the family Enterobacteriaceae (13,18). These genes became associated with a small resident plasmid already possessed by H. pleuropneumoniae. On the other hand, the plasmid with SmSu resistance markers may have already been in an occasional strain of H. pleuropneumoniae. At first rare, these SmSu resistant strains were selected gradually by the antimicrobics used in animal husbandry.

Ampicillin resistance genes were shown to code for a TEM type β-lactamase. This type of β-lactamase is the most common in members of the family Enterobacteriaceae and is the type seen in H. influenzae and Neisseria gonorrhoeae (13,18,19). The data suggest that Ap resistance may have occurred because of the acquisition of an Ap transposon by a SmSu resistant H. pleuropneumoniae. This element could have inserted into the Sm resistant genes found on the 2.3 MDal plasmid yielding an ApSu phenotype with a higher molecular weight (20,21). An explanation for the disparity of the molecular weight between TnA (3 MDal) and the increase in molecular weight of pVM105 relative to pVM104 is not readily apparent.

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R PLASMIDS IN PATHOGENIC ENTEROBACTERIACEAE FROM CALVES

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INTRODUCTION

Pressure for selection of antibiotic resistant bacteria is greater in intensive calf raising than in any other livestock raising operation. The newborn calf is particularly vulnerable to enteric infections because it is often colostrum deprived, transported long distances without food, mixed with calves from other sources and therefore simultaneously stressed and exposed to a variety of pathogenic strains to which it may have no immunity. The crowded nature of the intensive rearing environment further facilitates spread of infection and favors accelerated feco-oral transfer of enteric pathogens in the group.

Antibiotics are administered in great quantity to minimize the effects of these infections and not surprisingly, have resulted in the emergence of resistant populations of *Salmonella typhimurium* and *Escherichia coli* (1, 2, 3). In at least two instances, clones of *S. typhimurium* selected in this way have spread into the human population (4, 5). Heavy antibiotic usage as described also increases the probability of novel recombinant plasmids. One instance of this was an unusually large *inc* H1 plasmid carrying a lac gene as well as resistance to antibiotics including chloramphenicol that occurred in a *S. typhimurium* strain from calves. This strain caused a mortality of 50% in a group of 320 calves in a veal unit (6). The ability to utilize lactose in the milk replacer apparently allowed the salmonella strain to rapidly attain lethal numbers. The combination of lactose positivity and multiresistance was obviously a formidable problem in respect of therapy, diagnosis and pathogenicity.

Intensive calf raising is therefore a useful model for study of untoward effects of excessive use of antibiotics. In this contribution I shall examine antibiotic resistance in S. typhimurium and enteropathogenic E. coli from calves in N.Y. State in an attempt to elucidate some of the factors that underlie the responses of these two populations of enteric pathogens to heavy antibiotic selection pressure.

Antibiotic Resistance in S. typhimurium from Calves

Since the early 1970's, strains of S. typhimurium from calves in the N. E. United States have exhibited a frequency of resistance to tetracycline, streptomycin and kanamycin that is virtually 100% (Table 1). Only 5% of strains were sensitive to the commonly used antibiotics. Since 1973, when ampicillin was approved for use in food producing animals, resistance to this antibiotic has also increased greatly in frequency (Fig. 1), a situation similar to that observed earlier in strains of S. typhimurium from calves in England in the 1960's (4). In the latter instance spread of resistant strains into the human population was noted. A similar spread has not apparently occurred in N.Y. (2). Although chloramphenicol is not approved for use in food-producing animals in the United States, circumstantial evidence for its use in calves is evident from Table 1 where 5% of calf strains were resistant.

When the frequency of antibiotic resistance in S. typhimurium strains from calves is compared with that of strains from other animals the effect of the greater selection pressure in calves is clearly seen (Table 1). About one third of strains from other animals were antibiotic sensitive and the frequency of resistance to kanamycin, streptomycin and tetracycline, although high, was less than half that present in calves. The high frequency of resistance to ampicillin in horses, dogs and cats probably reflects heavy therapeutic use.

Table 1. Antibiotic Resistances of S. typhimurium and Enteropathogenic E. coli from Calves Compared with the Resistances of S. typhimurium from Other Domestic Animals (N.Y. 1973-78).

	% Resistant		
	Calves		Other Domestic Animals
Antibiotic	S. typhimurium (146)	E. coli (115)	S. typhimurium (153)
Ampicillin	39	80	33
Chloramphenicol	5	31	3
Kanamycin	88	94	41
Streptomycin	97	98	47
Tetracycline	93	97	39
Sensitive	4	0	33

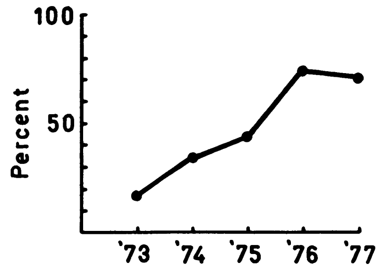

Fig. 1 Increase in frequency of ampicillin resistance in strains of S. typhimurium from N.Y. calves from 1973-77.

Antibiotic Resistance in Enteropathogenic E. coli from Calves

Enteropathogenic E. coli exhibit an even worse situation in respect of the extent of multiple antibiotic resistance than is the case with S. typhimurium. The extent of resistance clearly indicates that antibiotic therapy of colibacillosis in calves in N.Y. is now strongly contraindicated as it can serve only to increase the reservoir of virulent strains without exerting any beneficial effect whatsoever.

The existence of clonal effects is underlined by the finding that 70% of the E. coli studied belonged to 0 groups 8, 9, 20 and 101. In contrast to the situation in enteropathogenic strains the frequency of resistance in nonpathogenic E. coli strains from healthy calves has been shown to be less (7), a reflection of less severe selective pressure from sub-therapeutic use of antibiotics for prophylaxis and growth promotion.

Characteristics of R Plasmids in S. typhimurium and Enteropathogenic E. coli

Conjugation experiments at 28^{o} and 37^{o} revealed that resistance in S. typhimurium and E. coli in this study were transferable in 91% and 70% of strains respectively. The reason for the larger proportion of nontransferable plasmids in E. coli is not known. In 74% of strains of S. typhimurium that transferred, the plasmids involved were shown to be inc H2 (8). Only one inc H2 plasmid was found in the collection of E. coli strains. Other major differences in distribution of plasmid incompatibility groups between the 2 enteric species were also detected (Table 2). Most of the antibiotic resistance in E. coli was carried on inc Iα or FII plasmids in contrast to S. typhimurium where similar resistance patterns were carried on inc H2 or, to a lesser degree on a nontypeable 73 Md plasmid (10% of strains). A 5.5 Md plasmid carrying ampicillin resistance was present in 19% of S. typhimurium strains but in only 2% of E. coli strains. This

Table 2. Occurrence and Characteristics of Resistance Plasmids from Calves (N.Y. 1973-78).

Inc Group or Size	Source: S. typhimurium (140) %	R Type(s)	Enteropathogenic E. coli+ (115) %	R type(s)
H2	74	ApKmSmTc KmSmTc	1	KmSmTc
Iα	0		32	ApKmSmTc KmSmTc
FII	7	Tc ApKmTc	47	ApKmSmTc KmSmTc
5.5 Md	19	Ap	2	Ap
73.0 Md++	10	KmSmTc	Not known	-

+84 strains belonged to "0" groups 8, 9, 20 and 101.

++Compatible with inc FI, FII, FIV, Iα, M and N plasmids.

plasmid is similar to the prototype ampicillin plasmid described by Anderson in S. typhimurium from English calves in the 1960's (9) and later observed in other enteric bacteria elsewhere (10). The transfer factor of this plasmid has been shown to be inc Iα (9). Although not identified in this study, it is probable that its presence would have increased the actual number of inc Iα plasmids shown in Table 2 for S. typhimurium.

The results outlined above clearly indicate that the two enteric pathogens in the calf harbour rather different sets of R factor plasmids. This is surprising since the calves from which the isolates were obtained were from the same general area of N.Y. and were collected over the same time period (1973-78). Antibiotic selection pressures must also have been similar because colibacillosis and salmonellosis are diseases seen early in the calf's life.

It seems clear that the host bacterium is a critical factor in the epidemiology of R factor plasmids in the calf and that the relationship of each pathogenic enteric species with the normal background enteric flora of the intestine and of the animals' environment must be of considerable importance in the eventual dominance of certain R plasmids in enteric pathogens. This seems to be the case in respect of inc H2 plasmids whose thermosensitivity of transfer (Figure 2) implies transfer outside the host. In vitro experiments with feces from 2 four week old calves revealed that a typical inc H2 plasmid (pJT4) did not transfer at 37^o but was transferred between E. coli strains at a frequency of 10^{-3} at 30^o. S. typhimurium strains must therefore acquire their inc H2 plasmids in the calf's environment. The original source of these plasmids for this transfer is unknown. They have been observed in Serratia (11), Citrobacter

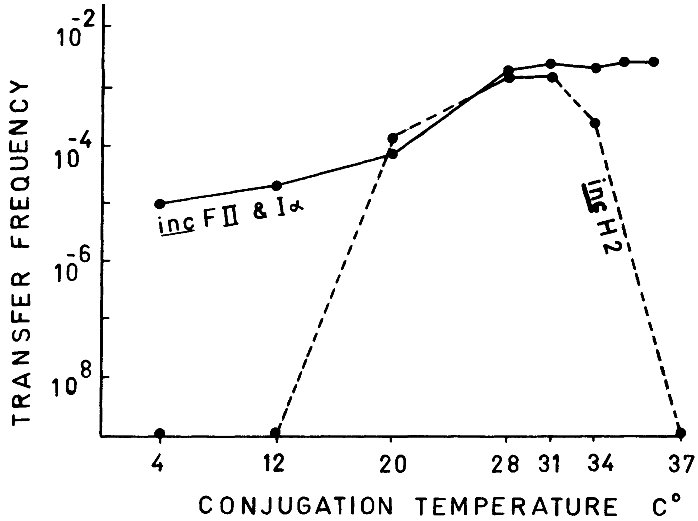

Fig. 2. Effect of temperature of mating mixture on transfer of inc H2, FII and Iα plasmids.

and Klebsiella sp.but are uncommon in E. coli (12) with the possible exception of E. coli strains isolated from calves recently infected with S. typhimurium that already possessed inc H2 plasmids (13).

Non-Transfer of inc H2 Plasmids in the Intestine of the Calf

The failure of typical inc H2 plasmids to transfer at 37° in calf feces suggested that they did not transfer in the intestine. Some recent work (Timoney and Linton, 1980 Unpublished Data) has provided direct experimental confirmation of this. Three attempts to demonstrate in vivo transfer in groups of 4 calves were made using E. coli strains of O groups 21, 45 and 69 as donors of inc H2 plasmids. These plasmids were obtained from E. coli isolated from calves that survived an outbreak of S. typhimurium (Phage type 193) infection in S. W. England and were similar to the epidemic inc H2 plasmid in calves in Britain that has been recently described by Threlfall et al. (5) and by Rowe in this volume. Recipient E. coli used in the in vivo transfer attempt were smooth Nal^R mutants of the donor E. coli. Both donor and recipient E. coli were excellent colonizers of the calf intestine and were selected because of their dominance in the intestine of the calves from which they were originally isolated. Transfer of the inc H2 plasmids was not detected following oral (stomach tube) administration when calves were muzzled and denied oral contact with their environment. This was the case even when tetracycline or chloramphenicol was administered to exert selection pressure for transconjugants the day after donor and recipient E. coli were given. Evidence of transfer of non-thermosensitive R factors was obtained under these conditions and suggested that conditions permissive of R factor transfer did exist in the intestine at the time of the experiment. However, transfer of the inc H2 plasmids to a variety of E. coli "O" types was detected at a low frequency ($1x10^{-6}$) in experiments when the calves were not muzzled or after the muzzles had been removed.

An attempt to demonstrate in vivo mobilization of an inc H2 plasmid in the intestine by a coexisting Class 2 complex consisting of a 5.5 Md plasmid coding for ampicillin resistance and an inc Iα transfer factor was also unsuccessful.

Effects of inc H2 Plasmids on Virulence and Colonization

The epidemic distribution of inc H2 plasmids in S. typhimurium from calves as seen in N.Y., in Britain (5) and previously in man in the Iberian Peninsula suggests that they may confer advantages additional to antibiotic resistance. Williams Smith et al. (12) were unable to show an effect of inc H1 or H2 plasmids on virulence or intestinal colonization ability of S. typhimurium for chickens. Similar experiments comparing mouse virulence of twenty inc H2 + and eleven inc H2 - strains of S. typhimurium from N.Y. calves have failed to show that inc H2 plasmids increase virulence (Figure 3). This result was perhaps to be expected because the effects of inc H2 plasmids are more likely to be expressed in the intestine than elsewhere in the body. Accordingly, three experiments to compare the colonization or persistence in the calf intestine of E. coli strains (021, 045 and 069) with and without inc H2 plasmids were run. The results of 2 of these experiments are shown in Figure 4. Both inc H2 + E. coli and its Nal^R counterpart cured of the plasmid by incubation at 43° (14) were administered by stomach tube in equal quantities ($3x10^{11}$ CFU) and counts of both strains compared in the calf's feces for the following 3 weeks. A consistent finding in respect of all three E. coli serotypes was that the E. coli with the inc H2 plasmid persisted to a progressively greater extent than its cured counterpart. The difference became most apparent at between 7 to 10 days. By 20 days the inc H2 + organisms were still present in substantial numbers ($\simeq 10^7$ CFU/g feces) whereas counts of the identical

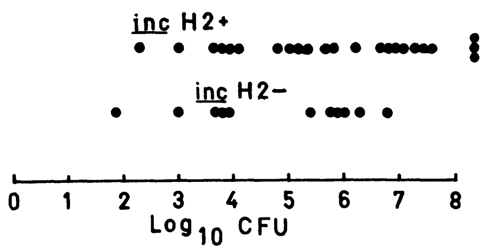

Fig. 3. Comparison of the virulence for mice (i/P LD_{50}) of strains of S. typhimurium with and without inc H2 plasmids. Three strains carrying inc H2 plasmids were avirulent.

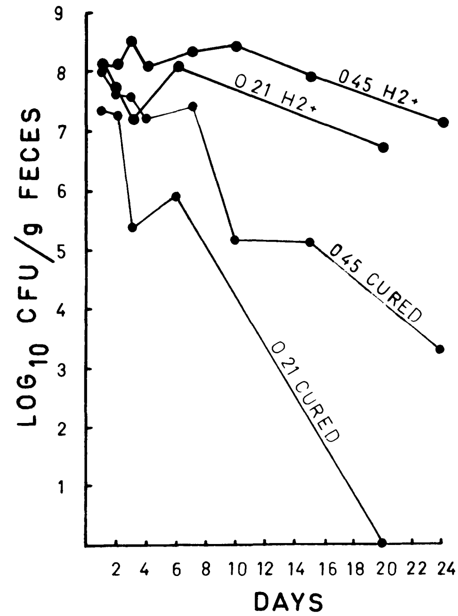

Fig. 4. Effect of the presence of an inc H2 plasmid on the persistence of E. coli strains (021 and 045) in the intestine of the calf. Each point represents the mean of counts from four calves.

but cured organisms were zero to 3 x 10^3/g feces. Thus a marked effect on persistence was evident. This appears to be quite different to the colonization effect of the K88 antigen of E. coli where differences of up to 1000 X in counts of $K88^+$ and $K88^-$ strains in pig intestinal contents occur 24 hours after oral administration. The relatively accelerated disappearance of the cured strains was not caused by antibiotics because the calves were not receiving antibiotics of any kind. As well, antibiotic sensitive E. coli were detected at a frequency of 10^7 CFU/g feces throughout the experiments.

These results indicate that inc H2 plasmids carry genes which augment the host bacterium's ability to maintain itself in the calf intestine. The accelerated decrease of the cured strains at 7 to 10 days suggests the possibility that the intestinal immune response may be involved and that inc $H2^+$ strains are in some way able to counteract host antibody or are not initially as antigenic because of differences in cell surface chemistry or structure. In any event, increased persistence in the intestine could be contributing to the epidemic character of infection by S. typhimurium strains carrying inc H2 plasmids as in the case of the Type 204/193 epidemic in Britain. The greater numbers of inc H2 + strains produced in the intestine over a longer time span greatly increases the probability that such clones will be maintained and passed on to other susceptible hosts, an effect that is independent of antibiotic usage.

SUMMARY

Antibiotic usage in intensive calf raising in N.Y. has resulted in populations of S. typhimurium and enteropathogenic E. coli that were almost completely resistant to a range of commonly used antibiotics. These resistances were mainly encoded on inc H2 plasmids in S. typhimurium and on inc FII or Iα plasmids in enteropathogenic E. coli. This suggests that E. coli and S. typhimurium share different reservoirs.

Inc H2 plasmids do not transfer in the intestine of the calf but transfer well in voided feces at 30° suggesting that multiresistant S. typhimurium clones derive their inc H2 plasmids by conjugation with other bacteria in the calf's environment. The presence of these plasmids in E. coli confers on the host organism an enhanced ability to persist in the calf's intestine beyond 7 to 10 days, a property which could be an important factor in the clonal character of inc H2 positive S. typhimurium infections in the calf.

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EFFECTS OF ANTIBIOTICS IN ANIMAL FEED ON THE ANTIBIOTIC RESISTANCE OF THE GRAM POSITIVE BACTERIAL FLORA OF ANIMALS AND MAN

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INTRODUCTION

The use of antibiotics in the raising of farm animals has become an area of considerable controversy in recent years. There have been numerous allegations that this practice, particularly the incorporation of subtherapeutic levels of antibiotics into feed for purposes of growth promotion, is contributing to the increasing incidence of drug resistance in bacterial pathogens that infect man. It has been suggested that the selective pressure exerted upon the bacterial flora of animals by these antibiotics gives rise to large populations of resistant microorganisms. The organisms are then postulated to enter the human population, either through agricultural and meat processing workers, or via the food chain, as a result of contamination of meat products. Once in contact with man, the resistant bacteria could presumably cause disease directly, or transfer their resistance to organisms more pathogenic for humans. Although there is documentation of specific instances of human disease caused by resistant organisms from the farm, the extent to which this occurs has been difficult to assess. Representatives of the drug and animal raising industries have argued that therapeutic use of antibiotics in the treatment of human diseases has a much larger effect on the human resistance problem than agricultural use of antimicrobials. In fact, proponents of both points of view have used the same experimental data to support their particular position on several occasions. Virtually all of the research done in this area has been focused on the gram negative bacteria, particularly the members of the *Enterobacteriaceae*.

Our laboratories are engaged in studies of the plasmids of gram positive bacteria, especially staphylococci and streptococci. Although there has been considerable interest in plasmid-determined drug resistance in human isolates of these genera, there is a surprising dearth of knowledge about the resistance and plasmid properties of the gram positive bacteria found in animals. Examples of several basic questions to be considered in assessing the effects of antibiotic use in animals on human drug resistance include the following:

1) Are antibiotic resistance plasmids prevalent in the bacterial flora of normal animals on the farm and/or animals being treated for diseases, and if so, are these plasmids similar to those found in bacteria isolated from human infections?

2) Does the feeding of an antibiotic to farm animals affect the resistance of the bacterial flora of the animals, their caretakers, and the environment?

3) Do resistant and/or pathogenic microorganisms from animals enter the human food chain in significant numbers?

While numerous studies on gram negative bacteria have been directed toward these questions, virtually no data on gram positive organisms is available in this regard. In this communication, we will summarize the results to date, of studies begun in our laboratories during the past year. We feel that these initial findings suggest that the effects of antibiotics in animal feed on gram positive drug resistance are very relevant to these questions and should be strongly considered in making decisions regarding future use of these substances.

RESULTS AND DISCUSSION

The U. S. Food and Drug Administration has been collecting representative bacterial isolates from farm animals throughout the country. We have examined 100 isolates each of staphylococci and fecal streptococci for plasmid content and antibiotic resistance profiles. We have found that virtually all of these strains are resistant to at least one antibiotic, and the vast majority carry multiple resistance. Table 1 shows the resistance and plasmid profiles of 20 animal streptococci which are typical of what was found with the streptococcal isolates. Multiple resistance and multiple plasmid species were commonly observed, including a number of fairly large plasmids. Similar findings were obtained when human strains isolated from infections of New York Hospital patients were examined (data not shown here). We are presently attempting to identify specific macrolide resistance plasmids from the two groups of strains, so that their sequence homology may be determined. When a similar analysis was carried out on staphylococci from human

Table 1. Properties and Plasmids of Animal Streptococci

Strain Number	Resistances	Plasmid Sizes (Mdal)
1	Tc, Pn, Km	4.7; 1.6
2	-	47.8; 2.0
3	Tc, Em, Ne, Km, Sm	35.4; 2.6; 1.0; .8; .4
4	Tc, Em, Sm	22.9
5	Tc, Em, Sm	22.3; 17.3; 9.5; 6.7; 3.2; 1.0; .5
6	Tc, Em	-
7	-	27.5
8	Tc, Ne	53.7; 41.6; 33.4; 25.1; 9.3; 8.3; 5.7; 5.0; 2.4; 2.2
9	Tc	35.0
10	Tc	-
11	Tc	4.3
12	Tc, Km	16.9
13	Tc, Em, Km, Sm	2.1
14	Tc, Km	-
15	Tc	37.1; 29.5
16	Tc, Em, Sm	39.8; 1.3; .95
17	Tc, Km, Sm	-
18	Em, Km, Sm	45.7; 35.4; 2.5; 2.4
19	Tc, Em	.8
20	Tc, Km, Sm	-

Antibiotic resistance was determined by disc diffusion method and plasmid content was determined by agarose gel electrophoresis.

infections and farm animals, multiple resistances and multiple plasmids were also observed, but most of the plasmids were relatively small in size, many having a molecular weight less than 5×10^6. In the case of the staphylococci, several macrolide resistance plasmids have been identified (Table 2) and the restriction enzyme digests of a few of these isolates have been compared. In Table 3, it can be seen that there is considerable similarity in the banding pattern of human and animal plasmids, suggesting a common evolutionary origin. As shown in Table 4, multiresistant streptococci are also readily isolated from animals in a veterinary hospital and many of these isolates transfer their resistance to a recipient strain of human origin. Taken together, the data obtained from examination of gram positive bacteria from humans and animals reveal that drug resistance and plasmids are prevalent in both populations. Although the comparison of the sequence homology of specific plasmids from these isolates has not been completed, there is already evidence for common evolutionary origins of animal and human macrolide resistance plasmids.

Table 2. Molecular Weight of Macrolide Resistance Determinants in Staphylococci

Strain or Plasmid Number	Source	Location of Determinant	M.W.x10^{-6}	Transposon
RN1550	Human	Chromosome	5000	Tn554 (4.3 Mdal)
pr258	Human	Plasmid	18.4	Tn551 (3.4 Mdal)
pe194	Human	Plasmid	2.4	-
pSA1104	Human	Plasmid	1.45	-
pSA1105	Human	Plasmid	1.40	-
pEB111	Human	Plasmid	1.58	-
EB116	Human	Plasmid	*1.75,0.85	-
pEB201	Animal	Plasmid	2.4	-
pEB102	Animal	Plasmid	2.7	-
pEB100	Animal	Plasmid	2.7	-
pEB203	Animal	Plasmid	2.8	-
pEB97	Animal	Plasmid	2.8	-
pEB88	Animal	Plasmid	2.8	-
pEB90	Animal	Plasmid	2.9	-
pEB214	Animal	Plasmid	3.15	-

*Plasmid screening of Em^r transductants always revealed two plasmid DNA species - = uncharacterized

Table 3. Molecular Sizes of Restriction Fragments of MLS Plasmids from Humans and Animal Staphylococci

HincII Fragments	pE194	pEB97	pEB102
A	1.0	1.35	1.15
B	0.83	1.0	1.08
C	0.7	0.74	0.74
D	0.62	0.635	0.615
E	0.38	0.39	0.395
F	0.245	0.25	0.25
G	0.205	0.205	0.205
	3.98Kb	4.58Kb	4.43Kb

Table 4. Multi-Resistant Fecal Streptococci from Animals at the Cornell Veterinary Hospital

Species	Strain Number	Resistances
Canine	1	Pen, Amp, Tet, Kan, Str
"	2	Tet, Neo, Str. Chl, Kan*
Bovine (Adult)	3	Amp, Chl, Kan, Pen, Str, Tet, Neo*
"	4	Chl, Tet, Neo*
Bovine (Calf)	5	Tet, Chl, Str, Kan*
Equine	6	Tet, Chl, Str, Kan, Neo*
"	7	Tet, Chl, Str, Kan, Neo*
Porcine	8	Str, Kan, Tet, Amp
Avian	9	Pen, Amp, Chl, Kan, Tet
"	10	Kan, Str, Tet, Neo

*Indicates that strain could transfer Tet resistance to a human recipient strain, JH2-2.

A second problem which we have addressed in our research is the effect of incorporation of the antibiotic tylosin, in the feed of pigs on the macrolide resistance of their gram positive microflora. A major obstacle in this work was the lack of availability of animals carrying low levels of indigenous drug-resistant bacteria. After an extensive search, we succeeded in obtaining 16 piglets having less than 20% macrolide resistant staphylococci and streptococci in their normal flora. We constructed pens on open land which had not been previously used for raising animals, and carried out the study outlined in Table 5. As can be seen in Figure 1, the resistance of the tylosin-fed pigs' gram positive flora was relatively low during the baseline period. The fecal streptococci in these animals became almost 100% macrolide-resistant within a few days of the addition of tylosin to their feed, whereas the staphylococcal populations appeared to be reduced initially, followed by establishment of a resistant population within a few weeks. In contract, there was considerable fluctuation in the percentage of resistant organisms in the control group. During the baseline sampling period, there was a steady rise in the percentage of resistant fecal streptococci in these pigs at a time when both groups were being fed the same, non-tylosin containing ration. We feel that this rise may have been due to the presence, in the herd, of a fortuitously resistant strain which was very proficient at colonizing the pigs' intestinal tracts. This strain may have become predominant during this time and was eventually displaced by sensitive strains, resulting in the decrease in resistance seen during the middle portions of the experiment. Near the end of the study, there was a second rise in

Table 5. Pig Raising Studies

2 groups of piglets (8 per group)
Group A - fed 100 g/ton tylosin in feed
Group B - no tylosin

Culture biweekly - rectal swabs — CNA-horse blood agar
skin and nasal swabs — mannitol-salts agar

Culturing procedure:

1) Suspend material from swab in 10 ml of sterile saline + 0.05 μg/ml Em. Incubate at 37°.
2) Make serial dilutions and plate on media described above + 10 μg/ml Em.

Caretakers and environmental samples are also periodically cultured and feed is tested for antibiotic residues. We tested erythromycin resistance and preincubated our samples as described above because the most common macrolide resistance phenotype in gram positive bacteria is the so-called "MLS" phenotype.[1] CNA agar selects for gram positive bacteria, whereas mannitol-salts agar selects for staphylococci.

[1] Weisblum, et al. J. Bacteriol. 138:990-998 (1979).

resistance of the streptococci and staphylococci. Based on the results of assays for tylosin residues (carried out in the laboratory of Dr. S. Katz, Rutgers University) in the various batches of feed used in the study, we believe that this increase in resistance was due to inadvertant contamination of two batches of feed (see Figure 1) with approximately 20 g/ton of tylosin. This unfortunate incident actually illustrates a very serious problem which we encountered in this work. Namely, that it is surprisingly difficult to obtain "clean" feed (and animals) to do this sort of project, even in university experimental animal raising facilities, due to the prevalence of antibiotics in the environment. In spite of these problems, the percentages of resistant organisms in the control group never reached the levels observed in the tylosin-fed pigs, nor did we see the abrupt rise in resistance in the controls that was evident in the tylosin group. It is our feeling that these data do show that tylosin feeding does cause an increase in macrolide resistance of the gram positive bacteria of pigs. Analysis of resistance and plasmid profiles of strains isolated from the pigs during the experiment (which is currently being carried out) should reveal effects on multiple resistance and plasmid content of the bacterial flora. Even though tylosin is not used in human medicine, its agricultural use would appear to result in an increase in resistance to drugs useful in human medicine, such as erythromycin.

We also cultured the caretakers of the two groups of pigs, and

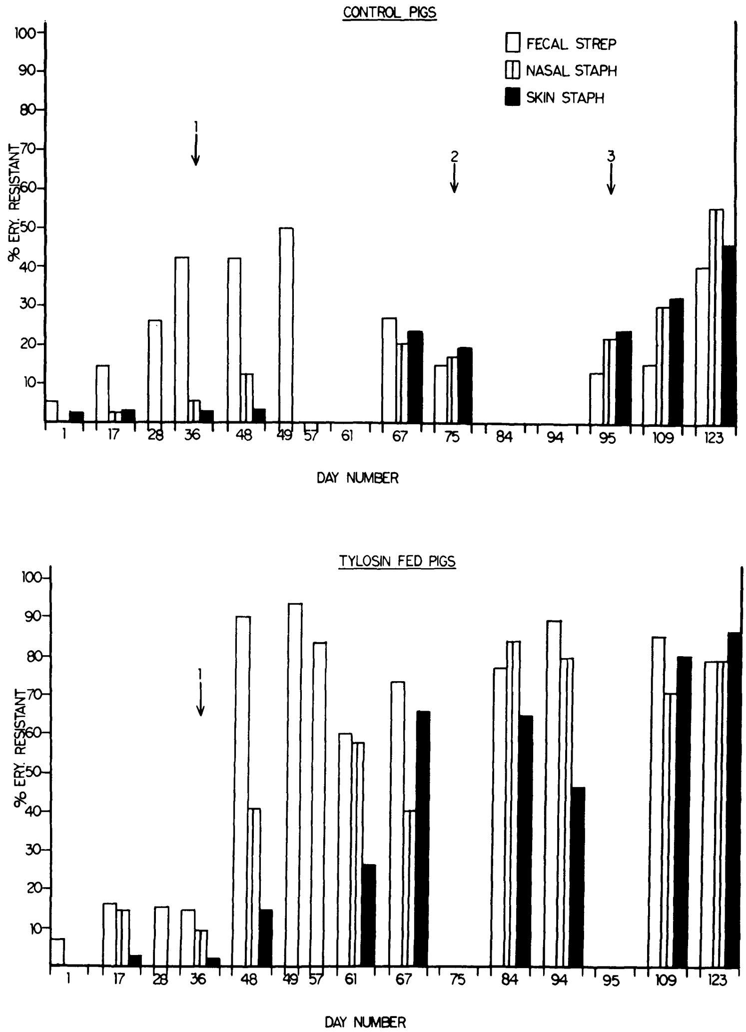

Fig. 1 The percentages of resistant organisms at various samplings are plotted in this figure. Arrow #1 indicates the time tylosin feeding was initiated. Arrows 2 and 3 indicate the time period when inadvertantly (tylosin) contaminated feed was given to the control group. The numbers are cumulative averages of all the pigs in the two groups. The data for each pig is based on the average of four plates.

we found that the fecal streptococci of the control and experimental caretakers both increased from less than 5% erythromycin resistant to about 15%. The resistance of the nasal staphylococci of the control caretaker increased from 2% to 9%, whereas the percentage of resistant organisms of the tylosin group's caretaker increased from less than 1% to 20% at the end of the study. Plasmid analysis of erythromycin resistant strains from the caretaker revealed that some staphylococci from the tylosin-fed pigs had similar plasmid profiles to organisms isolated from their caretaker. While these data are suggestive of some colonization of the caretaker by organisms from the animals, we feel that more frequent sampling of the caretakers, as well as using larger numbers of experimental animals (to increase the exposure of the caretakers to the animals and feed) would be necessary to conclusively determine the extent to which this colonization actually occurs.

While the evidence discussed above indicates that there is a production of resistant organisms in farm animals, the next question that arises is whether these organisms reach the human population via the food chain in significant numbers to facilitate a direct interaction with man and his bacterial flora. We have recently begun to culture the gram positive bacteria present on cuts of meat which might reach the consumer. Table 6 shows that fairly large numbers of staphylococci and streptococci contaminated cuts of meat from our tylosin fed pigs at the time of processing. We strongly suspect that these organisms were from the animals because the butchering was done under very rigorous hygenic conditions, and the bacteria isolated were mostly macrolide-resistant. Culturing of pork portions which have been refrigerated for several days, to simulate conditions on meat market shelves, indicates that significant growth of streptococci and staphylococci occurs under these circumstances. We have also cultured chicken meat purchased from various supermarkets and found it to be invariably contaminated with streptococci, as illustrated in Table 7. Strains we have isolated from this source

Table 6. Gram Positive Organisms Isolated from Meat Samples

Cut	Total staph	Em-resistant staph	Total strep	Em-resistant strep
Chops (pig 4)	6.1×10^3	4.4×10^3	1.1×10^3	1.0×10^3
Ham (pig 4)	1.5×10^3	3.0×10^3	1.0×10^3	1.0×10^3
Chops (pig 1)	1.6×10^4	7.0×10^3	1.0×10^2	1.0×10^2
Sausage (pig 1)	4.1×10^4	5.0×10^4	1.5×10^3	1.0×10^3

Meat samples from tylosin fed pigs at the time of packaging. The numbers represent the number of organisms isolated (on Erythromycin containing, and drug-free plates) from swabs rubbed vigorously on cuts of meat.

Table 7. Drug-Resistant Streptococci from Supermarket Chickens

cfu/mlon:	Drug-free plates	Tet plates	Ery plates
Chicken 5	4.2×10^4	6.1×10^3	5.3×10^3
Chicken 6	3.2×10^5	2.6×10^5	2.5×10^2

Fluid was aseptically removed from a package containing a whole fryer or roasting chicken and plated on CNA agar plus the antibiotics indicated above. Under these conditions about 60-80% of the colonies which grew were streptococci.

included β-hemolytic group L streptococci (whose identity was confirmed by Dr. R. Facklam of the Center for Disease Control) and group D streptococci which transferred drug resistance to a human recipient strain. There is certainly indication from these results that gram positive bacteria from farm animals do reach the human food chain in significant numbers, representing a significant reservoir of pathogens and resistance genes.

Although much of our data is preliminary, we feel that the use of antibiotics in animal raising does markedly effect the gram positive bacteria of animals, and there appears to be considerable potential for these organisms to interact with humans and their bacterial flora. We hope to confirm and extend these observations in the near future, and it is our belief that the effects we have observed with the gram positive bacteria should be taken into account in considering any future changes in the use of antibiotics in animal raising.

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MULTIPLY-RESISTANT CLONES OF SALMONELLA TYPHIMURIUM IN BRITAIN: EPIDEMIOLOGICAL AND LABORATORY ASPECTS

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In Britain, salmonellosis is the most important cause of food-poisoning and Salmonella typhimurium causes about 25 per cent of all human salmonella infections each year. Poultry and cattle are the main sources of human infections with this serotype and phage typing studies have demonstrated that in general the poultry-associated phage types are almost exclusively drug-sensitive whereas the majority of cattle-associated types are resistant to antimicrobial drugs. This reflects the use of antibiotics in the different animal species.

Salmonellosis in cattle can be a severe disease, particularly amongst calves and is an important economic factor in the cattle-rearing industry. Antibiotics are used extensively for therapy and prophylaxis in cattle although their use for growth promotion has been prohibited since 1971. In contrast, salmonellosis is not an economically important disease in poultry and the use of antibiotics is comparatively insignificant.

The effects of the legislation resulting from the recommendations of the Swann Committee[1] are difficult to quantify. Prior to 1963, about three per cent of S. typhimurium from cattle were drug-resistant but the incidence of resistance increased dramatically between 1963 and 1969 following the appearance and epidemic spread of a multiresistant clone of phage type 29 in calves. The peak was reached in 1965 when 73 per cent of all isolations of S. typhimurium from cattle were caused by this strain[2]. By the time the Swann recommendations were implemented, isolations of type 29 were at a low level and the strain has subsequently disappeared from bovine animals in Britain. Thus although the appearance and spread of this particular clone contributed to the enactment of the Swann legislation, its disappearance was related to other factors.

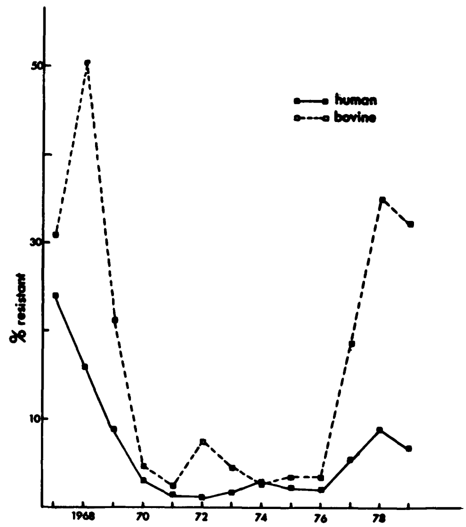

Fig. 1. Multiple drug resistance in S.typhimurium in Britain

Fig. 1 shows the percentage of multiresistant S.typhimurium from cattle and humans in Britain from 1968 to 1979. The early peak corresponds to the period when type 29 was predominant. It is tempting to speculate that the low proportion of multiresistant strains between 1971 and 1976 (about eight per cent of bovine and two per cent of human isolations) was a direct result of the Swann legislation, but this cannot be proved and there was little diminution in the overall use of antibiotics in animal husbandry during this period.

The increase in the proportions of multiresistant S.typhimurium in cattle and humans since 1977 has followed the sequential acquisition of resistance plasmids in the bovine host by one strain of S.typhimurium, type 204 and the resultant epidemic dissemination of resistant clones (Table 1). Because of the influence of the drug-sensitive poultry types, the overall increase in multiresistance in S.typhimurium from humans has been not as pronounced as may be expected from that observed in cattle.

Type 204 resistant to sulphonamides and tetracyclines (R-type SuT) was identified in calves in Britain in 1974. The strain spread in cattle and entered the human food chain. Sulphonamides and tetracycline resistance were encoded by independent plasmids and the tetracyclines resistance plasmid was found to be type-determining. Thus the probable progenitor of type 204 was a strain of phage type 49, a phage type common in cattle before 1974.

Table 1. Appearance of type 204 and related strains

Strain	R-type	Date
Type 204	SuT	1974 January
	CSSuT	1977 June
Type 193	ACKSSuT	1977 December
Type 204c	CSSuTTm	1979 March
	ACKSSuTTm	1979 October
	SSuTTm	1980 July

In June 1977 a strain of type 204 resistant to chloramphenicol, streptomycin, sulphonamides and tetracyclines (R-type CSSuT) appeared in calves and spread epidemically. Genetic studies showed that a type 204 strain of R-type SuT had acquired a compatibility group H_2 plasmid coding for the complete resistance spectrum. Human infections were subsequently identified[3].

In December 1977 a new strain appeared spread epidemically in calves in Britain. This strain was assigned to phage type 193, and was resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracyclines (R-type ACKSSuT). Genetic and molecular investigations demonstrated that this strain had been derived from type 204, R-type CSSuT, following the acquisition of a group I_1 plasmid specifying resistance to ampicillin, kanamycin and streptomycin. This plasmid also coded for restriction of S.typhimurium typing phages and thereby converted type 204 R-type CSSuT to type 193, R-type ACKSSuT[4,5]. During 1978 and 1979 these multiresistant strains of types 204 and 193 spread to Europe following the export of infected calves from Britain[6].

In March 1979 a strain of S.typhimurium resistant to chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim (CSSuTTm) was identified in calves. This strain was designated type 204c because of its derivation from type 204 of R-type CSSuT by a complex process involving loss of typing phage restriction from the type-determining tetracycline resistance plasmid present in all type 204 strains, acquisition of a trimethoprim transposon by the H_2 resistance factor and acquisition of a temperate bacteriophage, the presence of which converted type 49 to the new type, type 204c[7]. This strain became established in calves during 1979 and in due course acquired further resistance plasmids coding for ampicillin and kanamycin resistance. By

December 1979 the predominant R-type in type 204c isolations was that of ACKSSuTTm. All type 204c isolations have been resistant to trimethoprim and the appearance of type 204c coincided with an intensive promotional campaign in Britain to encourage the use of trimethoprim for the treatment and prophylaxis of bovine salmonellosis.

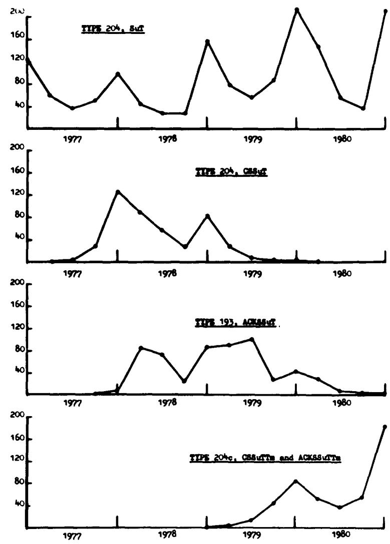

Fig. 2. S.typhimurium types 204, 193 and 204c in cattle, 1977-1980

The clone of type 204 of R-type CSSuT had become the predominant strain in cattle by late 1977 and has subsequently disappeared. Type 193 peaked in 1978/1979 but the frequency of isolations of this clone was reduced in 1980. Type 204c, which appeared in 1979, became the predominant multiresistant clone in cattle during 1980 (Fig. 2). In contrast, strains of type 204 of R-type SuT have been regularly isolated throughout this period. The initial rise in prevalence of these multiresistant clones, followed by their subsequent decline may be related to changes in selective pressure brought about by the successive use of different antimicrobials in animal husbandry in attempts to combat the increasing spectrum of resistance in these strains.

As with all strains of S.typhimurium, there was a range of symptoms in calves infected with the multiresistant strains. However the disease appeared unusually virulent; many reports mentioned severe scouring frequently accompanied by septicaemia and mortality was high - up to 50 per cent in some calf herds. The proportion of infections in cattle caused by these multiresistant clones has increased from 14.7 per cent in 1977 to 27.4 per cent and 24.2 per cent in 1979 and 1980 (Table 2) and economic losses have been considerable. In terms of the actual number of animals infected, it is important to realise that the strains referred to this laboratory represent only a proportion of infections in cattle since in many instances only representative strains are referred from an outbreak.

Table 2. S.typhimurium types 204, 193 and 204c from cattle, 1977-1980

Year	Strains received	Per cent type 204		Per cent type 193	Per cent type 204c
		SuT	CSSuT		
1977	1194	20.0	14.1	0.6	0
1978	1790	14.2	13.6	15.8	0
1979	1732	26.4	2.3	16.4	8.7
1980	1585*	28.8	0	3.4	20.9

*Provisional figures

Source: Strains referred to DEP

Since 1977, 457 human infections with the multiresistant clones of types 204, 193 and 204c have been recognised. In addition a further 48 patients have been infected with a related multiresistant strain of type 204a, R-type CKSSuT which appeared in 1980 (Table 3). In the majority of instances the symptoms were those of mild to moderate enteritis but severe diarrhoea which persisted for several weeks was reported. Enteritis was reported as the cause of death of two patients. The strain spread extra-intestinally in ten patients and one child died of septicaemia in a family outbreak of type 193 of R-type ACKSSuT. When these multiresistant clones spread extra-intestinally, the clinicians choice of drug for therapy is obviously extremely limited.

The therapeutic and prophylactic use of antibiotics in cattle has directly contributed to the appearance of these multiresistant clones but the importance of drug resistance in their epidemic spread cannot be quantified. However there is no doubt that the prophylactic use of antibiotics in animal husbandry has provided selective pressure which has allowed the strain to persist and become disseminated in calf herds. It is noteworthy that epidemic spread occurred subsequent to the acquisition of resistance plasmids.

Table 3. Human infections with multiresistant S. typhimurium types 204, 193, 204c and 204a

Year	Type 204 CSSuT	Type 193 ACKSSuT	Type 204c CSSuTTm and ACKSSuTTm	Type 204a CKSSuT	Total
1977	37	4	0	0	41
1978	51	89	0	0	140
1979	15	94	20	0	129
1980	0	29	118	48	195
	103	216	138	48	505

Source: Strains referred to DEP

Although reports indicated that infections in calves were unusually severe, as yet there is no evidence that the multi-resistant strains have increased virulence. However the use of antibiotics to which the strains were resistant may have aggravated the disease by suppressing competition by sensitive bacteria in the bowel and certainly infected animals have not responded to treatment.

The dissemination of these clones was facilitated by the extensive movements of young animals due to the distribution practices of the calf-dealing trade and cross-infection in dealers' premises was undoubtedly a major contributory factor. In Britain, existing legislation - the Zoonoses Order[8] - permits movement control restrictions on infected animals and effective use of this legislation would help limit the spread of infection. However it is essential that any measures to prevent cross-infection and the dissemination of infected stock be combined with a more judicious use of antibiotics for therapy and prophylaxis in cattle.

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R PLASMIDS FROM *S. typhi* AND *S. typhimurium* STRAINS ISOLATED IN MEXICO CITY HOSPITALS

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S. typhi strains harboring R plasmids are a common finding in Mexico City hospitals. The predominant R plasmids share the following properties: they belong to the same incompatibility group (H_1), have a molecular weight of 135 Mdal, carry a temperature sensitive transfer system, and code for the resistance to Cm, Sm, Su, Hg and Tc. The general organization of the resistance genes resembles that of R100, since homogenic derivatives which have lost the r-determinants, Tn*10* or all the resistance genes can be isolated *in vitro* by several methods. Furthermore, naturally occurring R plasmids deleted for the r-determinants or Tn*10* have been found, although at low frequency. Complementation experiments indicated that the *ts* transfer system of the mexican plasmids is not related to that of F*lac*, R1-19 or Col Ib*drd*.

Although the resistance patterns of *S. typhimurium* are more complex than those of *S. typhi*, it has been possible to isolate R plasmids which are indistinguishable from those described above. This is epidemiologically important since *S. typhimurium* strains may be one of the sources of R plasmids in Mexico. The use of antibiotics in animal feedening programs and the lack of appropiate sanitary conditions may play an important role in the distribution of R plasmids.

Furthermore, most of the *S. typhimurium* strains are resistant to Ap, the second antibiotic of choice in the treatment of typhoid in Mexico. The resistance to this antibiotic is encoded by a small conjugative plasmid of 17 Mdal.

A PLASMID-MEDIATED SURFACE ANTIGEN OF THE CLINICALLY ISOLATED *ESCHERICHIA COLI* STRAINS

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A plasmid-mediated surface antigen of naturally occurring *Escherichia coli* strains were detected. This antigen was classified into L type (heat-labile K) antigens but suggested not to be pilus antigen, because we found no pilus on the surface of common pili-free *E. coli* C strains carrying these plasmids, and because these strains did not hemagglutinate any red blood cells. Incidences of this antigen-forming strains were high in the strains isolated from feces and respiratory tract secretions. Plasmid DNAs from these strains were different in their molecular sizes, but they had a common size band after digestion by EcoRI endonuclease, suggesting that they had the similar or same origen(s).

Distributions of the strains which had the surface antigen common to the *Klebsiella* strain

Strains	No. of strains isolated from (%) feces	urine	pus	resp.sec#	others	total
examined*	271	268	141	88	58	826
K*	20(7)	3(1)	2(1)	5(6)	1(1)	31(4)
plasmid*	4	1	0	1	0	6

\# resp.sec.; respiratory tract secretion.

* examined ; Numbers of strains examined.

K ; Numbers of strains which had the surface antigen common to *Klebsiella pneumoniae*.

plasmid ; Numbers of strains which had plasmid mediating this antigen. Transfer of these plasmids to *E. coli* C which is agglutinated by all commercially available anti-sera against K antigens of *Vibrio parahemolyticus* but not agglutinated by any of the commercially available anti-sera against OK antigens of enteropathogenic *E. coli* removes or covers antigens for *V. parahemolyticus* groups I, II, III, VII and VIII and adds antigens agglutinated by anti-sera against enteropathogenic *E. coli* K60, K62, K69, K74 and Kxl.

RESISTANT AND BIOACTIVE *ESCHERICHIA COLI* STRAINS FROM CLINICAL MATERIALS AND THEIR PLASMIDS

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We surveyed tha antibiotic resistant, adhesive, enterotoxigenic, hemolytic, colicin producing, cell invasive, capsule forming, special surface antigen producing and actively iron incorporating strains in the *Escherichia coli* strains from various clinical materials of five general hospitals in different districts in Tokyo area. Some of the bioactive strains were isolated in different frequencies from various organs but in similar frequencies from every hospitals. The differences of distributions of the strains carrying these various activities suggesting that human cell specific adhesins and hemolysins gave advantages to the strains to reside in respiratory tract, that capsules helped the strains to survive in pustular foci, and that the surface antigens common to enteropathogenic bacteria helped the strains to grow in intestine and respiratory tract. Antibiotic resistances, colicin productions and active incorporations of iron could give advantages to grow over other bacteria but gave no preferences to the special organs. Enterotoxigenic and cell invasive strains were found to be very rare in the clinically isolated strains in general hospitals. Conjogative R plasmids were detected in average 40% of the resistant strains by direct drug selection. Conjugative plasmids mediating all other bioactive characters were examined by their mobilizations of a non-conjugative R plasmid. But, we detected only a few colicinogenic plasmids and plasmids which mediated a surface antigen common to *Klebsiella* by the mobilization test.

GENETICAL BASES OF MICROCIN CLASSIFICATION

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Microcins are low molecular weight antibiotic substances produced and excreted by Enterobacteriaceae. About 25% of hospitalized patients harbour microcinogenic strains in the intestinal tract.

With the purpose to study the biological diversity of microcins, 26 wild type E.coli microcinogenic strains have been classified by cross-activity spectra into four groups. Microcin producing E.coli transconjugants were obtained from several strains of each group in order to applie cross-immunity criteria excluding tolerance or resistance of wild strains.

E. coli transconjugants of each group II,III or IV showed internal cross-immunity and are susceptible to the activities of the other groups. Group I contains two different microcin activities which can be separated by conjugation or transformation, one of them presenting cross-immunity with group IV.

Groups I and IV activities are associated with the presence of a 3.7 Md plasmid. Transconjugants with group II activity-immunity group presents a single 48 Md plasmid of very similar restriction pattern in all the studied strains. A physical map of this plasmid including the location of the microcin immunity region by cloning is presented.

GENETIC, MOLECULAR, AND BIOCHEMICAL CHARACTERIZATION OF PLASMID-MEDIATED ATYPICAL UTILIZATION OF CITRATE BY ESCHERICHIA COLI

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Although Escherichia coli strains normally do not utilize citrate, citrate utilizing (Cit$^+$) variants of otherwise normal E. coli strains have been detected at a low frequency in animal and human isolates. We have examined citrate utilization in the atypical E. coli variant strain V414 isolated from a diseased human. Plasmid-mediated citrate assimilation was suspected because citrate-nonutilizing derivatives of the Cit$^+$ strain appeared spontaneously at a high frequency. In conjugation experiments with plasmid-free E. coli K12 recipients, we found the atypical Cit$^+$ character to be part of a self-transmissible plasmid which also conferred resistance to tetracycline and chloramphenical. Purified plasmid DNAs from the original Cit$^+$ host or K12 Cit$^+$ transconjugants were examined by agarose gel electrophoresis and electron microscopy. Both strain V414 and the transconjugants contained a 130 megadalton conjugative plasmid which is transferable upon selection for citrate utilization or the antibiotic resistances. The cit$^+$ determinant was cloned from the 130 megadalton plasmid into the Pstl site of pBR325. Several independent Cit$^+$ recombinant plasmids were examined and found to contain essentially identical cloned Pstl fragments of approximately 9 kilobases in size. Although E. coli cells are normally unable to transport exogenous citrate, they do possess the enzymes necessary to catabolize it intracellularly. Metabolic studies of cells containing the Cit$^+$ plasmid indicate, however, that intact citrate is not incorporated directly into whole cells, but is metabolized at the cell surface before uptake and assimilation by the cell. Further studies show that this plasmid does not enhance the ability of an enterochelin-deficient E. coli Cit$^+$ transconjugant to grow in the absence of iron, thus demonstrating that citrate utilization does not involve iron uptake.

ANTIBIOTIC RESISTANCE IN *VIBRIO CHOLERAE* 01 AND ITS PUBLIC HEALTH SIGNIFICANCE

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Clinical studies have firmly established the value of tetracycline and of a number of other antimicrobial drugs as adjuncts to rehydration therapy in the treatment of cholera; they have also been shown to be effective in reducing the transmission of infection, provided their use is limited to close contacts of patients. Sporadic isolations of drug-resistant strains have been reported from time to time in different areas since the early sixties, but the resistance has generally been found to be unstable. Although in a few strains it was found to be stable and encoded by a group C plasmid, it is only in the recent years that such strains have become a cause of concern to public health authorities. Since 1977, outbreaks of cholera caused by resistant strains have been reported from Tanzania and Bangladesh. In the former, the incidence of tetracycline-resistant organisms increased from 0% in November 1977 to 76% in March 1978, during which period about 1788 kg of tetracycline were reported to have been used for mass prophylaxis and treatment of cases. During 1977/78, 67% of the isolates were resistant to tetracycline when 4436 kg of tetracycline were consumed. Thereafter, use of tetracycline for mass prophylaxis was restricted and during 1979/80 only 4.8% of strains were tetracycline resistant when 1028 kg of tetracycline were used.

In Bangladesh, isolation of resistant strains increased from 5% in the first month of the epidemic to 13%, 28%, and 36% in subsequent months and then gradually declined. This increase and decrease in drug-resistance could not be ascribed to any unusual increase or decrease in the drug consumption. The R-types in Bangladesh (ApKmSmSpTcSuTm, ApKmTcSuTm, ApTcSuTm) differed from those in Tanzania (ApKmSmTcCmSu, ApKmSu) in that they did not include resistance to chloramphenicol, but in both countries the plasmid responsible belonged to incompatibility group C. An understanding of the factors involved in the acquisition and loss of resistance in V. cholerae is important for ensuring a rational use of antimicrobials.

CHARACTERISATION OF THE TETRACYCLINE RESISTANCE REGION OF THE INCP PLASMID RP1.

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Preliminary investigations (1,2) indicated that the tetracycline resistance gene(s) carried on RP1 are located approximately 14 kb from the *Eco*R1 site of the plasmid. Plasmid pUB307 is a deletion derivative of RP1 which has lost the resident TnA of RP1 but carries the *tet*-gene(s) intact. Tn802 (TnA) and Tn501 (TnM) insertion mutants of pUB307 have been used to map more precisely the *tet* region of RP1. The structural gene(s) comprise a nucleotide sequence of about 1.3 kb. Adjacent to this and proximal to the *Eco*R1 site of the plasmid is a region of about 500 bp which encodes a repressor. A fragment of about 2.2 kb which carries the entire 1.8 - 1.9 kb *tet* region of RP1 has been cloned into pSF2124. The new plasmid, pUB1246, confers inducible tetracycline resistance at a level approximately twice that conferred by RP1. The *tet* resistance determinant of RP1 (which is indistinguishable from RP4) is homologous with the prototype *tet*A of pIP7, a finding consistent with that of Mendez et al (3).

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REGULATION OF TRANSPOSON Tn10 TETRACYCLINE RESISTANCE

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The maximal expression of Tn10 tetracycline resistance is induced by exposure to low concentrations of the drug itself. Induction appears to involve inactivation of a Tn10 encoded repressor protein that acts negatively to control the rate of transcription of the resistance function(s). We have analyzed the genetic organization of the resistance region by constructing *in vitro* recombinant plasmids that carry different segments of Tn10 DNA. The structural gene for the repressor is within a 695 base pair Hind II restriction fragment situated adjacent to the promoter for the resistance function(s). The DNA sequence of this region, in conjunction with mutational analyses, predicts the amino acid sequence of a 23,500 dalton repressor protein. Several lines of evidence indicate that the structural genes for the repressor and the resistance function(s) are transcribed in opposite directions from functionally overlapping promoters. Fusion of either the repressor promoter or the resistance promoter to an otherwise promoterless *lacZ* gene places *lacZ* under the control of the *tet* repressor *in vivo*. Repression of *lacZ* in these gene fusion strains is overcome by low concentrations of tetracycline. Plasmids that carry the repressor gene direct the synthesis of a 23,000 dalton protein in minicells, and the synthesis of this protein is induced by tetracycline. We conclude that the repressor is autogenously regulated--it negatively regulates transcription of its own structural gene as well as regulating transcription of the resistance gene(s). *In vitro* studies employing purified RNA polymerase and various restriction fragments as DNA templates indicate that the transcription initiation sites for the repressor and resistance promoters are only 15-20 base pairs apart. The DNA sequence of the regulatory region suggests a model in which transcription of the repressor and the resistance function(s) is controlled simultaneously by repressor binding to a common operator sites.

PLASMIDS AND PHAGES AND COMPLEMENT RESISTANCE

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Plasmid-specified resistance to complement is well documented; R100, R6-5 and ColV.I-K94 have been studied extensively (see article by Dr. K. Timmis in this volume). Resistance conferred by temperate phages is less well understood.

Results using "Southern blots" and specific antibody against the *tra*T protein of R100 indicate that the *iss* gene of ColV.I-K94 and the *tra*T gene of R100 are distinct. However the levels of resistance conferred to a range of serums by each gene, cloned into the plasmid vector pBR322, are remarkably similar. Resistance in both cases is to the classical and the alternative pathway of complement action.

The consumption of C8 by cells with and without the plasmid genes which had been treated with R8 (complement from which C8 had been removed using a C8 specific antibody column) were identical. This result indicates that the gene products which confer resistance do so at the level of C8 action or C9 binding or action, i.e. the gene products inpair the formation and/or structure of the terminal complex.

*tra*T-containing cells remain resistant to complement after pretreatment with antibody to either *tra*T or to *E. coli*, indicating that the *tra*T protein may have a "passive" structural role rather than an "active" function in complement resistance.

E. coli J6-2 lysogenic for λ is approximately four-fold more resistant to a range of serums than the non-lysogenic strain. Two major genes, *c*I and *rex* are expressed in prophage λ. Complement resistance conferred by the prophage does not involve the *rex* gene as demonstrated by studies with *rex* mutants. The role of the *c*I gene is unclear. A *c*I clone producing high levels of *c*I repressor confers increased sensitivity to complement. Further studies to determine the genetic basis of the complement resistance by λ are in progress.

POSSIBLE VIRULENCE DETERMINANTS IN YERSINIA PSEUDOTUBERCULOSIS

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It is known that certain strains of Yersinia pseudotuberculosis (Y.p.) are highly virulent for birds, rodents and other animals. When given orally these strains cause lethal infection in Swiss albino mice (1). It was found that one of these virulent strains of Y.p. (strain III) carried a plasmid showing a molecular weight of about 60 Kb. A plasmid free derivative of this strain was incapable to cause a lethal infection in mice when given orally. These results clearly indicate that the virulence of Y.p. is associated with a plasmid. This is also confirmed by results obtained by others (1). Several temperature effects of Y.p. may also be correlated to the presence of a plasmid. When a growing plasmid containing strain (Y.p. III), was shifted from growth at 26°C to 37°C a number of differences in the protein profile of the sarcosyl insoluble membrane fraction was found. At least one protein showing a molecular weight of about 100 000 Mdal was induced by this temperature shift. This protein was correlated with the presence of plasmid, as a plasmid free derivative of Y.p. III lacked this protein in the corresponding membrane fraction. In addition we were unable to detect any differences in the rate of synthesis of this 100 K protein correlated to the presence of either Ca^{2+} or Mg^{2+} ions in the growth medium after the temperature shift. We have shown that strains of Y.p. are virlent for guinea pigs when injected intraperitionally and that this virulence is correlated to the capability of these strains of adhere to HeLa cells. However, this HeLa cell attachment is not associated with a plasmid, as we found strains of Y.p. lacking plasmid but still maintaining the capacity to adhere to HeLa cells. This HeLa cell adherence of Y.p. was found to be mannose insensitive but temperature dependent. When strains of Y.p. was grown at 26°C they adhered in a high degree to HeLa cells in sharp contrast to cells grown at 37°C which showed a very low capacity to bind to HeLa cells. We were unable to detect pili on the bacterial cell surface, indicating that the Y.p. adherance to HeLa cells is not mediated by pili. By allowing a total cell extract of sonicated Y.p. to react with HeLa cells prior to the addition of intact Y.p. we were able to block the specific attachment between the bacteria and HeLa cells. The adherance was markedly decresed after addition of sonicated cell extract. Furthermore, by using the same strategy it was shown that the sarcosyl insoluble membrane fraction contained maximum blocking capacity. These results indicate that the ligand mediating the adherance between Y.p. and HeLa cells can be recovered in the sarcosyl insoluble membrane fraction.

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INFLUENCE OF HOST CELL METABOLISM ON EXPRESSION OF FERTILITY OF F-LIKE R PLASMIDS

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INTRODUCTION:

The natural habitat of enteric bacteria is largely anaerobic. Anaerobic growth of an *E. coli* K12 host did not affect replication or drug resistance of 45 R plasmids studied, whereas transfer was strongly reduced (by 10^2 to 10^4-fold) for F-like plasmids but not for I or N plasmids. The conjugative process *per se* was not impaired by anaerobiosis. Instead, this condition appeared to increase repression of the *tra* operon of F-like plasmids and augmented their inhibition of F factor fertility (*fin*). Thus, anaerobic "superrepression" of plasmid fertility was active also in *trans*. (L.G.Burman; J.Bacteriol. 123:265, 1975 and 131:69, 1977.)

RESULTS:

The response of the F-like R plasmid R1 to anaerobiosis can be mimicked by aerobic growth of the host in the presence of high concentrations of glucose (0.5-2%), which is known to induce a metabolic state similar to anaerobiosis, i.e. increased glycolysis and repression of TCA cycle enzymes. This glucose effect occurred only in yeast extract based media, was not alleviated by cyclic AMP and was seen in all *Enterobacteriaceae* spp. investigated. Other sugars, glycolytic intermediates and end products tested had no effect on *tra* control except for pyruvate which decreased R1 fertility by 100-fold. A possible clue to the effect of host cell metabolism on *tra* control was suggested by experiments with NaF. This glycolysis inhibitor alleviated the glucose effect but augmented that of pyruvate. However, the intermediate implicated, phosphoenol pyruvate (PEP, see Fig.), could not be assessed *in vivo* since it was not taken up by *E. coli* cells.

DISCUSSION:

It seems unlikely that O_2 tension *per se* influences the control of fertility of F-like plasmids. In situations when the PEP pool is large (anaerobiosis, high glucose, pyruvate + NaF) repression of *tra* is much stronger than during low PEP (aerobiosis, high glucose + NaF). Therefore, one hypothetical interpretation of the findings is that phosphorylation of a soluble control element using PEP as donor is involved in the expression of the *tra* operon of F-like plasmids.

enolase
Fig. Glycolysis. Glucose .. <——> PEP <——> pyruvate -- TCA cycle
↑
NaF

TRANSFERABLE DRUG RESISTANCE IN *BACTEROIDES FRAGILIS*: IN VITRO AND IN VIVO OBSERVATIONS

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Plasmid-mediated transferable drug resistance (tetracycline, clindamycin) has been described among strains of *Bacteroides fragilis*. As beta-lactamase production by bacteroides is known to be common, we sought to determine if transferable resistance to beta-lactam drugs occured among strains and if it could be ascribed to transfer of extrachromosomal elements. Transfer of drug resistance among these strains of bacteroides was also examined in an experimental subcutaneous abscess model, to ascertain if resistance transfer occurs at infected sites.

The findings indicate that transferable beta-lactam (penicillin, ampicillin, cephalothin, cephamandole) resistance occurs between strain TMP 16 and a suitable recipient. Localization of the beta-lactam resistance determinant has not been established. In addition, we have detected, in the experimental abscess model, transferable clindamycin and tetracycline resistance between TMP 10 and TM 4500. A 10 megadalton plasmid encoding clindamycin resistance and originating in TMP 10 is seen in clindamycin resistant progeny.

E. COLI K1 PATHOGENICITY

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The work of the Cooperative Neonatal Meningitis Study demonstrated that 81% of the *E. coli* strains isolated from the spinal fluid of sick neonates have the K1 capsular antigen. Animal studies showed that *E. coli* K1 strains were more virulent than non K1 *E. coli* and that antibodies against K1 antigen were protective, confirming the role of K1 antigen as a virulence factor. The fact that not all the neonates colonized with *E. coli* K1 develop disease, that the rates of colonization among neonates fluctuates widely and that there are variations in the LD50 of different *E. coli* K1 strains for the mice led us to think that these strains are not a homogeneous population and that other bacterial factors may be involved in their ability to colonize and produced disease. These factors could be the same ones that have been associated with the ability of *E. coli* to invade, i.e. harboring of ColV plasmids, hemolysin production and the capacity to hemagglutinate.

To investigate the heterogeneity of *E. coli* K1 strains regarding these properties we tested several *E. coli* K1 strains isolated from stools, blood and spinal fluid for production of colicin V, hemolysin and ability to agglutinate human red blood cells. We found that *E. coli* K1 isolates carry these traits with high frequency regardless of the site of isolation. To further prove the relevance of these characters and K1 antigen to *E. coli* K1 pathogenicity isogenic strains were isolated and their LD50 for mice were determined. The results showed that the K1 antigen is essential for pathogenicity and that this basal pathogenicity can be increased by the presence of the ColV and hemolysin plasmids. Additional experiments indicated that the presence of K1 antigen but not that of ColV protect the bacteria from the action of complement and phagocytosis. The isolation, by transposition, of colicin negative ColV plasmids allowed us to demonstrate that the increase of pathogenicity is not mediated by colicin production. Preliminary results suggest that the ColV plasmid confers a selective advantage to *E. coli* K1 in an iron poor environment (see P. Williams this volume).

The newborn rat model is now being used to test the importance of these factors in colonization and ability to invade (see Clancy and Savage, this volume). The cloning of the K1 antigen genes and the ColV plasmid DNA has been achieved and will facilitate progress in further understanding of their biology and their relationship to disease formation.

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ANTIBIOTIC RESISTANCE IN STAPHYLOCCI ISOLATED IN DUBLIN HOSPITALS

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A large number of isolates of Staphylococcus aureus from nosocomial infections and the hospital environment were characterised by phage typing and plasmid analysis. The strains were isolated in a group of eight hospitals over a four year period. Isolates which were resistant to gentamicin and several other antibiotics including penicillin, tetracycline, erythromycin and methicillin belonged to four main 'phage types'. The gentamicin resistant strains (GMRSA) were widespread in the hospitals and were responsible for cases of serious infection including 34 cases of septicaemia. The Table shows the periods when the different 'phage types' were present in the hospitals.

Plasmid screening of more than 200 out of a total of some 2,000 GMRSA isolates revealed a conserved plasmid profile. Restriction analysis, transformation and transduction studies allowed antibiotic resistance markers to be assigned to particular plasmids. All strains harboured a 21Md penicillinase plasmid. Type 85 and 77 strains harboured a 3.0Md tetracycline resistance plasmid whereas type 90 and 6/47/54/84/85 strains harboured a 24Md tetracycline resistance plasmid. GMRSA contained acetyl and phosphotransferase aminoglycoside inactivating activity. However gentamicin, amikacin, erythromycin and methicillin resistance seemed to be encoded on the host chromosome. Thus a small number of related strains were responsible for a large number of nosocomial infections.

TABLE

Phage type	Period of Isolation of Strains
77	1977 - 1979
85	1978 - 1979
6/47/54/84/85	1980 - present day
90	1979 - present day

PLASMIDS AND DELTA-ENDOTOXIN PRODUCTION IN BACILLUS THURINGIENSIS

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Five strains of *Bacillus thuringiensis* that produce crystalline δ-endotoxin were used as parental strains to isolate acrystalliferous (Cry^-) mutants: HD-2 (*B. thuringiensis* var. *thuringiensis*, flagellar serotype 1); HD-1 and HD-73 (both var. *kurstaki*, serotype 3ab); HD-4 (var. *alesti*, serotype 3a), and HD-8 (var. *galleriae*, serotype 5ab). The parental strains contain complex plasmid arrays ranging from 4 to 11 plasmids per strain, with sizes from 1.4 to 150 Md. The plasmid patterns of both Cry^- and Cry^+ variants were analyzed and compared to the parental strains using a modified Eckhardt lysate-electrophoresis method.

Most Cry^- mutants derived from strain HD-2 exhibited a distinctive colony morphology which facilitated their isolation. Loss of crystal production was associated with loss of a 75-Md plasmid. A 50-Md plasmid of strain HD-73 was lost in the Cry^- mutants. Crystal production in strain HD-4 appeared to be associated with a plasmid about 105 Md in size; in strain HD-1, a smaller plasmid (29 Md in size) seemed to be involved. In strain HD-8, a large plasmid (∿130 Md in size) was implicated in crystal production. Direct bioassay of several mutant strains confirmed the loss of δ-endotoxin activity in the acrystalliferous isolates.

The evidence supports the notion of a relationship between specific extrachromosomal DNA elements and δ-endotoxin production in *B. thuringiensis*, and suggests that in each strain only a single plasmid is involved, although the size of the implicated plasmid varies from one strain to another.

CONJUGAL TRANSFER OF PLASMID-ASSOCIATED LACTOSE METABOLISM IN *LACTOBACILLUS CASEI* subsp. *CASEI*.

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Many strains of *Lactobacillus casei* subsp. *casei* lose the ability to ferment lactose when cultured in the presence of plasmid curing agents such as acriflavin. The curing is accompanied by the loss of distinct plasmids ranging in size from 17.5 to 36 Mdalton (Mdal) depending on the strain studied. Analysis of these lactose plasmids with several restriction endonucleases revealed no fragments of identical size, however, the possible presence of a homologous sequence of DNA associated with lactose metabolism has not been evaluated. In order to assess possible mechanisms for the widespread distribution of lactose plasmids in *L. casei*, a number of Lac^+ and Lac^- strains were crossed by a filter pad mating technique. For example:

DONOR: *L. casei* 4646 Lac^+, Rif^s, $Ribitol^-$, white smooth colonial morphology
RECIPIENT: *L. casei* 64H Lac^- (cured of 23 Mdal lactose plasmid) Rif^r, $Ribitol^+$, glassy mucoid colonial morphology

Cells (10^8 of donor and recipient) were mixed onto a Millipore filter pad, incubated for 18 hr at 37° on glucose-LCM-agar and then transferred to lactose-rifampin-LCM-agar for 3-5 days at 37°. Typically, 100-200 glassy mucoid, Lac^+, $Ribitol^+$, Rif^r transconjugant colonies were observed. Spontaneous reversion to Lac^+ has not been observed in *L. casei* 64H Lac^-, nor has spontaneous acquisition of $Ribitol^+$ been observed with *L. casei* ATCC 4646. The latter strain spontaneously becomes Rif^r at a frequency of $<1/10^7$ cells, but colonies resulting from this mutation were easily distinguished from *L. casei* 64H. Plasmid isolation, followed by agarose gel electrophoresis, revealed that transconjugants always contained a plasmid which was identical in size to that found in the donor (36 Mdal). Some isolates also contained one, or both, of the small cryptic plasmids found in the donor; perhaps indicative of a conjugal "mobilization". While purified plasmid DNA from ATCC 4646 would not transform 64H Lac^- under these conditions; experiments incorporating DNAse into the agar were not performed. These results indicate that the plasmid-determined ability to ferment lactose is transmissible among *L. casei* strains by a "conjugation-like" process. To our knowledge, no naturally occuring system of conjugation, transformation or transduction has been described previously in the genus *Lactobacillus*.

DIFFERENCES IN RECOMBINATION BETWEEN TWO TRANSPOSON SEQUENCES ORIENTED AS DIRECT OR INDIRECT REPEATS IN recA OR $recA^+$ HOSTS

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When intramolecular transposition occurs to produce a second copy of the transposon, Tn3 or Tn2660, this copy is invariably oriented inversely to the resident transposon, and in some cases, the plasmid DNA sequence between the two transposons has undergone an inversion. We have recently determined that the orientation of the DNA sequence between these two inverse repeat transposons is stable (less than 2% change after 60 generations of growth), irrespective of whether inversion of the plasmid DNA occurred during transposition, and irrespective of whether growth is observed in a recA or $recA^+$ host. This stability has been observed in two plasmids differing in their replication control.

In contrast, the DNA sequence between two direct repeats of the same transposon (in the one host tested) appears to be highly unstable. We draw this conclusion from experiments attempting to couple two plasmids *in vitro*, each with a Tn2660 transposon, each with a mutually-compatible replication control and with different antibiotic resistance markers, followed by selection of transformants in a $recA^-$ host. Whereas, these two plasmids can be coupled with the Tn2660 sequences as an inverse repeat, no composite plasmids with the transposons as a direct repeat can be isolated, but two recombinant plasmids, consistent with recombination between the two transposons are isolated with a high frequency. There thus appears to be a marked difference in the frequency of recombination in recA or $recA^+$ cells between two transposons in a plasmid, depending upon whether they are oriented as a direct or an indirect repeat.

Tn 10 ENCODED PROTEINS THAT MEDIATE TETRACYCLINE RESISTANCE IN *E. COLI*

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Other workers have shown that transposon 10 probably codes for 3 proteins (of molecular weights 36K, 25K and 13-15K) which are involved in tetracycline resistance. The function of these proteins is unknown but studies on their location in whole cells may clarify their roles. Immuno-precipitation demonstrated the 25K protein in the outer membrane. Expression of Tn10-mediated resistance was defective in certain outer membrane mutants suggesting that the 25K protein is involved in resistance. The 36K protein (p I about 6.4) was resolved by two dimensional electrophoresis and its content in the inner membrane was correlated with reduced drug uptake. The 25K protein was not resolved by standard 2D-electrophoresis suggesting that it is basic (pI>7). The 25K and 36K envelope associated proteins probably contribute to decreased antibiotic uptake. The location of the third polypeptide (13-15K) is presently unknown, but might be ribosomal.

ANOTHER COLICIN V PHENOTYPE: ADHESION *IN VITRO* OF *ESCHERICHIA COLI* TO MOUSE INTESTINAL EPITHELIUM

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ABSTRACT

Two assays were designed with which isogenic laboratory strains of *E. coli* K12 with and without ColV plasmids were compared for their ability to adhere *in vitro* to mouse intestinal epithelium. In both assays discs of intestinal tissue were exposed to bacteria. In the first, discs were homogenized and the numbers of viable bacteria adherent to them were estimated from colony counts of plates inoculated with dilutions of the homogenates. In the second, bacteria were labeled with ^{14}C-aspartic acid; the number of adherent cells per disc was estimated by liquid scintillation spectrometry. Data from each assay were compared by analysis of variance. In both assays, strains bearing the ColV plasmid adhered in two to three-fold greater numbers than isogenic strains without the plasmid. These differences were highly significant statistically. A non-colicinogenic strain free of the ColV plasmid was selected by treatment of a ColV strain with Sodium Dodecyl Sulfate (SDS). In the radioisotopic assay, the ColV strain associated with the epithelium in significantly greater numbers than the cured derivative. A ColV strain was created by conjugation; in the radioisotopic assay this strain bound to epithelium in significantly greater numbers than the recipient strain without the plasmid. The original ColV strain, when negatively-stained and examined by electron microscopy, had pili that adsorbed male-specific bacteriophage while its isogenic variant without ColV did not. Some such properties, coded by the plasmid, may increase the virulence of the bacteria.

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EXPRESSION OF TN10 ENCODED TETRACYCLINE

RESISTANCE IS REDUCED IN MULTIPLE COPIES

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Plasmid pNK133 carries the tet genes of Tn10 inserted in a multicopy vector derived from pBR322. The Tc^r level was 10-fold lower than determined by a chromosomal element. Minicells harbouring pNK133 failed to synthesize the 36K tet protein. Most deletions and Tn5 in tet on pNK133 caused Tc^s mutations which also prevented expression of high level Tc^r from chromosomal Tn10 present in the same cell. Only those insertions in the promoter-proximal 90-130bp of a 1275bp HindII fragment known to carry the tet structural genes did not reduce the single copy Tc^r level.

A gene-fusion system resulting in constitutive expression of β-galactosidase from a tet promoter was used to assay tet repressor. Multicopy plasmids encoding tet repressor reduced the basal (uninduced) level of β-galactosidase by 17-fold, whereas single copy tet repression was 2-fold. The tet::Tn5 mutants defective in the trans-dominant multicopy effect still made normal amounts of repressor. This shows that overproduction of repressor was not responsible for the multicopy effect.

In conclusion, the trans-acting multicopy tet effect was inactivated only by Tn5 insertions located in the first 90-130bp of the tet structural gene, possibly in the coding region for the amino-terminus of a tet protein. We postulate that a regulatory mechanism in addition to repressor control of induction exists which prevents attempts to overproduce the tet protein.

NATIONAL INSTITUTES OF HEALTH PROGRAMS IN ANTIBIOTIC RESISTANCE AND RECOMBINANT DNA

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These two programs are supported by the Molecular Microbiology and Parasitology Branch in the Microbiology and Infectious Diseases Program of the National Institute of Allergy and Infectious Diseases.

The first of these is Mechanisms of Resistance to Antimicrobial Agents whose principal goal is to elucidate fundamental biological mechanisms involved in the development of microbial drug resistance and to increase our basic understanding of this phenomenon. More specific goals involve investigations of the origin, development, evolution, expression and mechanisms of drug resistance in a variety of specific microorganisms. Of particular interest to the program are the Enterobacteriaceae, *Pseudomonas*, *Neisseria*, staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Research of special interest to this program is included in one or more of the following categories: (1) genetic and structural studies of R factors and related plasmids; (2) origin, development, and evolution of drug resistance in microorganisms; (3) replication and conjugal transfer of plasmids; (4) biochemistry and genetics of plasmid-determined functions, especially resistance to antimicrobial agents; (5) correlated epidemiological and microbiological studies of naturally-occurring plasmids with special reference to R factors. The branch currently has approximately 3.7 million dollars invested in this program.

The branch has also supported the Stanford Plasmid Reference Center for 4 years. This serves as the sole collection and coordination center of its type in the United States, and, as such, is an important establishment that is very useful to workers in this rapidly expanding area of research.

The second program, Recombinant DNA, had its origins in the first. Our most important goal in this program is the utilization of the recombinant DNA technology to provide us with a greater knowledge of the molecular basis of pathogenicity. This information may lead to improved prevention, diagnosis, and treatment of infectious diseases.

Another goal is the production of a variety of biologically useful substances through the construction of bacterial cells containing functional DNA of animal origin. Currently, Institute-supported scientists are working to clone the interferon gene.

An equally important goal is the identification, assessment, and elimination of any and all potential biohazards encountered in the exploitation of this technology. Currently the branch invests 3.3 million dollars in this program.

DNA SEQUENCE OF THE ST_{A2} ENTEROTOXIN GENE FROM AN E. COLI STRAIN OF HUMAN ORIGIN

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The DNA sequence of the ST_{A2} gene from CRL25090 (see Harford et al : this meeting) is presented and compared to the DNA sequence derived by So and McCarthy (PNAS 1980 ; 77 : 4011) for the ST_{A1} gene from Tn1681. The genes are similar in having a conserved promoter region and an open reading frame of 72 amino acids including a 19 amino acid putative signal sequence. However there are 27 % base mismatch and 38 % amino acid differences between the two coding sequences. This explains the lack of homology between the two genes in stringent DNA-DNA hybridizations. The C-terminal region is highly conserved in the two genes including 6 half cysteine residues. In the case of ST_{A1} this region corresponds exactly to the amino acid composition found by Staples et al (J. Biol. Chem. 1980 ; 225 : 4716) for a purified ST_A toxin from a human E. coli isolate. It appears that the primary gene product undergoes extensive processing during the release of mature toxin from the cell.

GENETIC ANALYSIS OF CONJUGATION IN *STREPTOCOCCUS FAECALIS*

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pCF-10 is a 35 megadalton plasmid which was identified in a human clinical isolate of *S. faecalis*. A series of conjugation and curing experiments has revealed that this conjugative plasmid determines tetracycline resistance and also carries genes which enable its host cell to elicit a clumping response and high frequency of transfer when exposed to bacterial sex pheromones (CIAs). This plasmid is the first naturally occurring R-factor identified which carries genes for CIA response. We have been using pCF-10 to begin genetic analysis of streptococcal conjugation. In the course of attempting to cure pCF-10, we obtained tetracycline sensitive variants which still carried pCF-10. Some of these plasmids appear to carry small deletions and are also affected in their CIA response. These plasmids may be very useful in physical analysis of the transfer region of the plasmid. A second type of variant of pCF-10 has been identified by looking at rare transconjugants obtained after short matings in the absence of CIA. This variant plasmid transfers at higher frequencies than wild-type pCF-10 in the absence of CIA, and cells carrying it spontaneously clump in liquid culture. Further genetic and physical characterization of these plasmids should help to better define the conjugal transfer process.

TANDEM DUPLICATIONS OF THE *ampC* GENE OF *ESCHERICHIA COLI* K-12

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The *ampC* gene at 93.8 min on the *E. coli* K-12 linkage map codes for a β-lactamase. By selection for ampicillin resistance mutants have been isolated that carry multiple tandem *ampC* repeats[1]. The size and end points for ten independent amp duplications were determined by direct cleavage of chromosomal DNA with relevant restriction endonucleases. The amp duplications were all between 9 and 18 kilobasepairs in size. The end points for seven of these duplications were accurately determined and found to be essentially randomly distributed. By reciprocal recombination between a ColE1-*ampC* hybrid plasmid and the chromosome of an *amp* amplified mutant, a plasmid derivative was isolated carrying multiple copies of a 9.8 kb *amp* repeat. The nucleotide sequence of the novel joint created by the duplication was compared to the sequences of the two DNA segments that participated in the formation of this novel joint. The fusion had occurred within a 12 bp perfect homology with the sequence 5'-CAACACCACGCG-3'. It is suggested that tandem *ampC* duplications are the result of unequal *recA* dependent crossing overs between short homologous sequences of any composition. *E. coli* strains carrying about 10 tandem *ampC* repeats were virtually stable in a *recA* background. In contrast, a plasmid carrying five 9.5 kb repeats was found to segregate these repeats as covalently closed circular (ccc) DNA molecules in a recA background. This provides evidence for intramolecular *recA* independent recombinations in plasmids carrying repetitive DNA.

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R67: A NATURALLY OCCURRING R PLASMID ENCODING TWO DISTINCT TRIMETHOPRIM-RESISTANT DIHYDROFOLATE REDUCTASES

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The mechanism of plasmid-associated trimethoprim (Tp) resistance involves the synthesis of novel dihydrofolate reductases (DHFRs) which are highly resistant to Tp. R plasmid-encoded DHFRs can be arbitrarily divided into two broad classes (type I and II) based on different levels of sensitivity to Tp and related antifolate compounds. Hence, type I DHFRs have 50% inhibitory concentrations in the micromolar range whereas type II enzymes are inhibited by millimolar amounts of trimethoprim. Representative enzymes of each class appear to differ antigenically as well as in subunit structure. Plasmid R67 is a multiply antibiotic resistant plasmid originally isolated from a citrobacter sp. We previously cloned a DNA segment encoding a type II DHFR from R67 and characterized this enzyme in *E. coli* minicells. This R67 reductase was shown to consist of 4 identical 8,444 molecular weight subunits and to be antigenically unrelated to the type I DHFR harbored by transposon 7 (Tn*7*). In cloning experiments using purified R67 DNA and pSC101 DNA a small number of transformants (4% of the total) had an ampicillin-resistant, trimethoprim-resistant, tetracycline-sensitive phenotype. These transformants harbored plasmids 2-5 X 10^6 daltons in mass. Unexpectedly, these chimeric plasmids directed the synthesis of an 18,000 molecular weight polypeptide in *E. coli* minicells (the type I DHFR harbored by Tn*7* has a subunit molecular weight of 18,000). Furthermore, the reductases harbored by three independently isolated derivative plasmids appeared to be type I - like on the basis of inhibition kinetics, pH activity profile, stability studies and lack of antigenic reactivity with anti-type II (R67) antibody. Chimeric plasmids of this description were never isolated when pBR322 DNA was substituted for pSC101 DNA or when either cloning vehicle was omitted from the reaction mixture. EcoR1-digested R67 was probed, using the Southern blotting technique, with ^{32}P-labeled DNA segments containing a type I or a type II gene sequence. Different EcoR1 digestion fragments showed homology with either the type I or the type II probe. Therefore, both gene sequences appear to be present in this plasmid. Hence, plasmid R67 appears to harbor the genes for two distinct trimethoprim-resistant dihydrofolate reductases. The evolutionary implications of this finding are intriguing but, as yet, are unclear.

BEHAVIOR OF ANTIBIOTIC RESISTANT PLASMIDS OF Staphylococcus Aureus STRAINS.

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Thirteen multirresistant strains of S.aureus coagulase positive, isolated from different lesions were used. The strains were maintained at 4°on slants of soja-tripticaseine agar added with antibiotics. The propagation, segregation and curing experiments were carried out in soja-tripticaseine agar and broth.

The strains have different patterns of resistance to aminoglycosides They were resistant, to five, four and two antibiotics. The strains were considerated resistant if they grew on medium with concentration of antibiotics higher than the maximal concentration found in blood after a therapeutic dose.

In order to determine if the aminoglycoside resistance markers were in chromosomal or in plasmid DNA, the genetic material of the resistant strains was isolated and separated by agarose gel electrophoresis. The data showed that strains have at least one and some of them more than one plasmid.

Experiments of spontaneous lost of these markers were conducted by incubation of the strains 4 h and 18 h in TSA without selective pressure. The markers are lost together in a characteristic frequency for each strain, except in R13, R14 and R5 in which the percentage of lost for amikacin marker was higher than that of - the other markers, indicating the location of this marker in a different plasmid.

Ethidium bromide (EtBr) and sodium dodecyl sulphate (SDS) effect on the resistance patterns was the last parameter analized. Six strains were treated and only two were cured. With strain R13 the curing of the amikacin marker was less than that of the other markers, suggesting again its location in a different plasmid.

Strain	Resistance pattern	% segregation		% curing		No Electrophoretic bands
		3h	18 h	EtBr	SDS	
R1	K,G,T,S,A	63	78	-	-	2
R2	K,G,T,S	-	-	18	-	-
R5	K,G,T,S,A	5	97	-	-	1
R8	T,S	45	19	0	0	-
R10	T,S	4	15	-	-	-
R11	K,G,T,S,A	0	28	0	0	3
R12	K,G,T,S,A	5	84	0	0	3
R13	K,G,T,S,A	0	68	28	18	3
R14	K,G,T,S	-	-	-	-	1

K=Kanamycin,G=Gentamicin,T=Tobramycin,S=Sisomycin, A=Amikacin

MULTIPLE KIL GENES OF THE BROAD HOST RANGE PLASMID RK2

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The broad host range capability of IncP plasmids very likely involves plasmid-specified functions. We are examining the IncP plasmid RK2[1] for genes involved in host-plasmid interactions. Our results show that three separate regions of RK2 contain genes whose expression can apparently kill an E. coli host cell. Each "kil" gene (kil I, II, III) has a corresponding "kor" ("kil-override") gene (korI, II, III) to prevent cell death.

The three kor genes have been cloned. Since the kor functions act in trans, the Kor$^+$ strains allowed cloning of the kil genes. None of the kor genes is close to the kil gene it controls. KorI and korII map together in the 50-56.4 kb region of the plasmid, but deletions of korII suggest that these are separate genes. Kil I and kil III map in regions known to be non-essential for RK2 replication in E. coli. Kil II is near a replication gene, trfA,[2] but mutations of kil II show that these are different genes.

The kor genes are also non-essential, unless kil genes are present. Previous work[2] indicates that at least two separate genes (trfA and trfB) code for the trans-acting functions[3] essential for RK2 in E. coli. Our studies show that trfA alone is sufficient for replication at ori. The trfB region is only needed to control a non-essential kil-like gene (possibly kil II) that maps next to trfA.

Four different IncP plasmids (R906, R995, pUZ8, R751) were tested for korI- and korII-like genes, and all four were found to have both. This predicts that kil I and kil II are also present on these plasmids. If the kil and kor genes are truly conserved among IncP plasmids, they are likely to have a significant role in the proliferation of these plasmids, perhaps in hosts other than E. coli.

The existence of kil genes on promiscuous plasmids rich in antibiotic resistance genes suggests a novel approach for the control of organisms carrying these plasmids. An understanding of the regulation of kil genes may lead to antibiotics that will induce suicide by these bacteria specifically.

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ISOLATION AND IDENTIFICATION OF A DNA FRAGMENT OF Rts1 PLASMID RESPONSIBLE FOR TEMPERATURE SENSITIVE GROWTH OF HOST BACTERIA

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A kanamycin (KM) resistance factor, Rts1, causes inhibition of growth of host bacteria if grown from high density (10^6/ml) at 42° C (temperature sensitive growth effect, tsg$^+$), and replicates without forming covalently closed circular (ccc) DNA. On the other hand, at this temperature, this plasmid is eliminated from cultures if cells were grown overnight from low density inoculum (10^3/ml). To isolate the genetic region responsible for tsg$^+$, we utilized digests of pAK8, a spontaneous smaller derivative of Rts1 which retains all the characteristics of Rts1 except for the phenotype of T_4 phage growth restriction. Electrophoresis of restriction enzyme digests of Rts1 and pAK8 DNA demonstrated overall sequence homology between these two plasmids. pAK8 DNA provided a better source of tsg$^+$ regions because the molecular weight of pAK8 is 83 Mdal, while that of Rts1 is 126 Mdal. Digests of pAK8 were rejoined with T_4 ligase and used to transform *E. coli* 20S0. Transformants selected for KM resistance were found to contain Rts1 mini-plasmids expressing tsg$^+$ or the instability phenotype. In a second experiment, digests of pAK8 were inserted into the cloning vehicle pBR322. Restriction enzyme analysis of these pBR322 derivatives and Rts1 mini-plasmids allowed the identification of BAM HI fragments essential for replication (18.6 Mdal), KM resistance (14.1 Mdal), and the tsg$^+$ phenotype (8.0 Mdal). Alkaline sucrose gradient analysis of tsg$^+$and tsg$^-$ mini-plasmids demonstrated that the presence of the 8 Mdal Bam HI fragment also correlated with thermosensitive inhibition of ccc DNA formation. It was observed that many pBR322 derivatives with Rts1 inserts became unstable and were rapidly eliminated from host cells, suggesting that Rts1 contains elements which adversely affect plasmid stability. This effect works only in *cis* since a co-existing second plasmid replicated normally. One mini-plasmid synthesized by ligation of Sal I digests of pAK8 was KM^r and tsg$^+$; since the total molecular weight of this plasmid was around 5 Mdal, the region(s) influencing tsg would be relatively small and therefore may not consist of multiple genes. Most Rts1 mini-plasmids expressed T group incompatibility, identical to Rts1, except for the Sal I mini-plasmid. This may suggest that one Rts1 replication region is separate from the T-incompatibility gene. Analysis of the phenotypes of various mini-plasmids led us to conclude that the elimination gene (the Rts1 gene causing instability) is distinct from tsg$^+$. These studies suggested that regions influencing tsg$^+$ instability, and replication appear to be independently controlled genes. (U.S.P.H.S. GM-12053).

MOLECULAR AND FUNCTIONAL ANALYSIS OF THE TOL PLASMID pWWO

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Soil bacteria are able to utilize or transform an enormous range of natural and synthetic organic molecules. They are therefore of great value as vehicles for environmental protection and have virtually unlimited potential for recycling and regenerating valuable aromatic compounds.

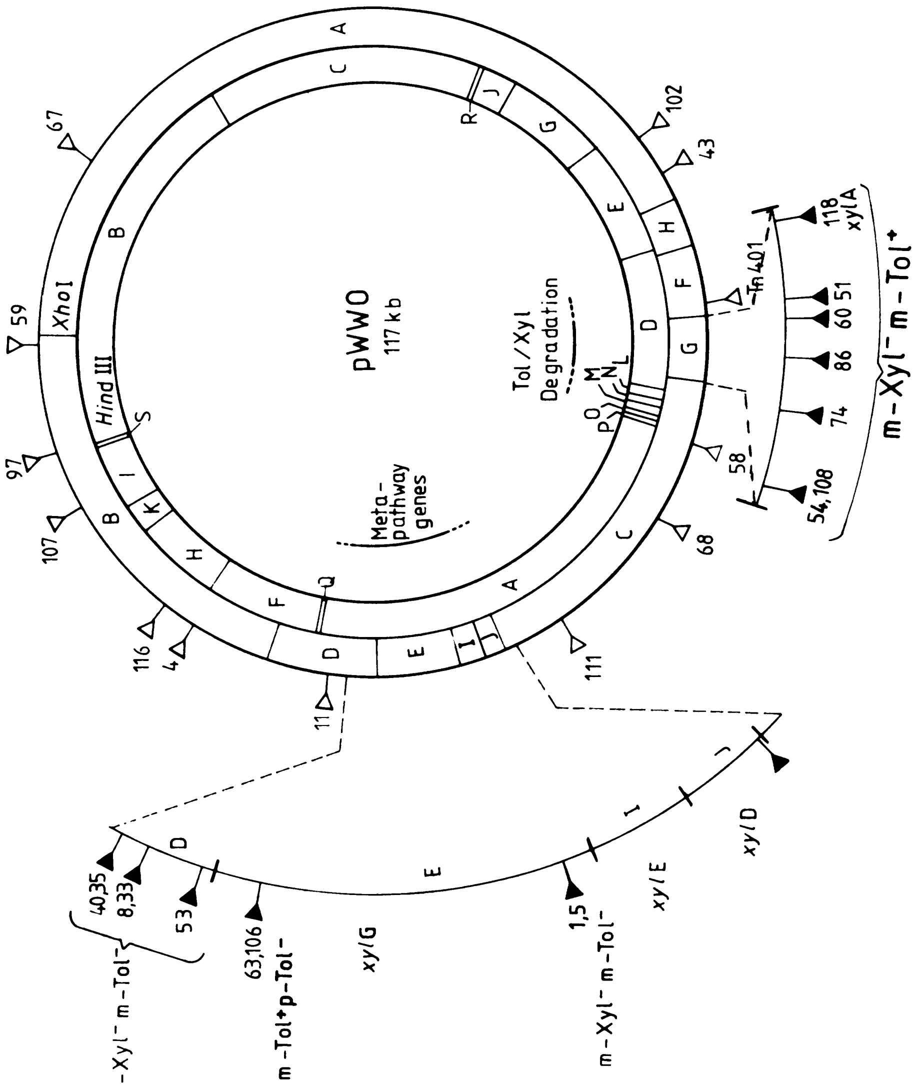
Many of these pathways are known to be plasmid coded. We have made a detailed molecular analysis of one such plasmid, the TOL plasmid pWWO from Pseudomonas putida mt-2. This plasmid codes for the utilization of the hydrocarbons toluene, m- and p-xylene together with their alcohol, aldehyde and carboxylic acid derivatives via a meta-ring cleavage pathway. The analysis was made by Tn5 transposon mutagenesis and gene cloning in a system specially developed for soil bacteria. The gene cloning system consists of a number of vectors derived from the broad host range plasmid RSF1010 and strains of Pseudomonas aeruginosa and P.putida which are restriction deficient and can be transformed at high frequency.

The Tn5 insertions in pWWO were mapped by restriction endonuclease analysis and characterized phenotypically by studying their substrate utilization patterns. XhoI, SstI and HindIII generated fragments of pWWO were cloned and characterized by enzyme assay and complementation analysis. Based on this we have constructed a functional map of the TOL plasmid pWWO (Figure 1).

This reveals that the genes encoding the degradative enzymes map in two separate regions of the plasmid. One of the groups consists of the genes encoding the meta-ring fission enzymes, this is probably of evolutionary significance as the same enzymes are found in quite different degradative pathways. This suggests that there is a high degree of conservation of this DNA sequence. The cloned fragments encoding these enzymes are of great value in further investigation of the degradative pathway regulation and provide a basis for construction of novel degradative pathways with relaxed substrate specificity.

FIGURE 1

Functional map of pWWO. Filled triangles indicate Tn5 insertions that inactivate all or part of the xylene/toluene pathway, whereas open triangles indicate insertions that have no influence on the catabolic functions.



BIOCHEMICAL STUDIES ON THE ANTIGENIC DETERMINANTS OF CFA/I PILI

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Enterotoxigenic E. coli H10407 (078:H11) produces CFA/I, a colonization factor antigen found on a plasmid (60 megadaltons) which also encodes the heat stable (ST) toxin and is mobilized by a second smaller plasmid encoding the heat labile (LT) toxin[1]. The CFA/I virulence factor was demonstrated to be pili which mediate adherence to human epithelial tissue of the upper small intestine as well as mannose-resistant hemagglutination of erythrocytes.

The CFA/I pilus is composed of a repeating protein subunit of molecular weight 14,200 which contains no sugar or phosphate. The amino acid composition (43% hydrophobic amino acids) and N-terminal valine residue agree with the findings of P. Klemm[2]. The circular dichroism spectrum for CFA/I pili shows that the amount of helix in the protein is 11% while there is approximately twice as much β-form. Preliminary fiber diffraction patterns indicate a 70 Å periodicity along the axis of the pilus filament/fiber. No values for the number of subunits/turn nor radial density distribution have been determined.

The pilin subunit was purified by gel filtration on a Sephadex G200 column in the presence of 1% SDS, followed by precipitation with acetone. The CFA/I monomer was digested with trypsin (E/S = 1/50) to yield 10 peptides (11 peptides expected). Four of these peptides were fairly large (15 - 47 amino acids) while the other 6 peptides were small (<3 amino acids). Using a competitive ELISA assay whereby a given amount of antibody was pretreated with a known amount of protein or peptide for 0.5 h at 37°C followed by 12-16 h at 4°C, the relative antigenicity of the pilin monomer and tryptic peptides relative to whole pili was determined. While tryptic digestion of the pilin monomer completely destroyed its antigenicity, digestion of whole pili decreased its antigenicity only slightly (21%). No conformational or compositional change in the pili could be detected by electron microscopy, circular dichroism or peptide mapping. The N-terminal peptide (MW4550) and the C-terminal peptide (MW1646) were found to compete with whole pili in the ELISA assay indicating the presence of antigenic determinants in these two peptides.

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THE NATURE OF THE FOSFOMYCIN RESISTANCE DETERMINANT FOUND IN PLASMIDS

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Fosfomycin (1,2,epoxy propyl phosphonic acid) is a cell-wall active antibiotic produced by some *Streptomyces* strains. Chromosomal mutants resistant to fosfomycin are easily found in nature, and this resistance is due to the lack of transport of the drug into the cell.

Recently we have described the finding of plasmids coding for resistance to fosfomycin in clinical isolates of *Serratia marcescens*. The fosfomycin resistance determinant from one of these plasmids, pOU 900, could be mobilized into the plasmid ColE1. The resultant plasmid was designated pSU912. It codes for colicin E1 production and immunity in adition to fosfomycin resistance. The size of the plasmid pSU912 was found to be 11.8 Mdal. This result implied the adition of a 7.6 Mdal fragment of DNA to the plasmid ColE1.

Using the plasmid pSU912 as donor we could observe the traslocation of the fosfomycin resistance determinant into the plasmid RP4 using a system composed of an *E.coli* recA containing both plasmids (pSU912 and RP4) as the donor and an *E.coli* polAts strain as recipient at the restrictive temperature.

On this way we isolated the plasmids pSU920 and pSU923 which showed a size of 43 Mdal. and carried the resistance to fosfomycin in adition to the other markers of the plasmid RP4. Restriction analysis of these two plasmids showed that they carried a 7.6 Mdal. DNA insertion, located at different sites. This result confirmed the existance of a DNA fragment of 7.6 Mdal. in size, capable to move from replicon to replicon independently of the recA host function. It was designated Tn2921.

In order to locate more precisely the DNA region responsible of the fosfomycin resistance we attempted the cloning of this region into the plasmid vector pBR322. Plasmids pSU912 and pBR322 were cleaved with the restriction enzyme PstI and ligated. The ligation mix was used to transform competent *E.coli* C600 cells. Several plasmids conferring to the host the Ap^s, Tc^r, Fo^r phenotype were analised and all of them showed the presence of a 3.45 Mdal. DNA fragment generated by the enzyme PstI.

MIC determination showed that the fosfomycin resistance level was not affected by the plasmid copy number.

TN*916*: A CONJUGATIVE TRANSPOSON IN *STREPTOCOCCUS FAECALIS*?

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Streptococcus faecalis strain DS16 harbors a hemolysin-determining conjugative plasmid pAD1 (35 Mdal) and a non-conjugative multiple drug resistance plasmid pAD2 (15 Mdal). A chromosome-borne tetracycline resistance determinant is located on a 10 Mdal transposon designated Tn*916*. Transposition to pAD1 occurs at a frequency of $\sim 10^{-6}$. A derivative of DS16 cured of both pAD1 and pAD2 (i.e., strain DS16C3) is capable of transferring Tn*916* at low frequency ($\sim 10^{-8}$) to plasmid-free recipients (JH2-2) in "filter-matings" by a Rec-independent, DNase-resistant process resembling conjugation [J. Bacteriol. Vol. 145: 494 (1981)]. When examined using the Southern hybridization method, Tn*916* was found to be inserted into different sites in different transconjugants. This was demonstrated by probing *Hin*dIII-digested chromosomal DNA with a ^{32}P-labeled *Eco*R1 restriction fragment of pAD1::Tn*916* containing the entire transposon. Insofar as Tn*916* has a single *Hin*dIII site, two transposon-host junction fragments are easily resolved. The size of these two fragments varied greatly in different transconjugants.

Certain Tc-resistant transconjugants of JH2-2, such as CG110, are able to donate Tc-resistance at 100-fold elevated frequencies ($\sim 10^{-6}$). Experiments which measure the frequency of Tn*916* transposition from the chromosome to a newly introduced pAD1 indicate that for CG110, an increased ($\sim$100-fold) frequency of transposition is also exhibited. Southern hybridization experiments that probed host-transposon junction fragments in *Hin*dIII-digested chromosomal DNA isolated from successive cultures of CG110 that had originated from a single colony revealed that Tn*916* readily moves from one site to another during growth.

It would appear then that a common step is involved in both transposition and conjugal transfer of Tn*916*. The conjugal transfer of Tn*916* may, thus, represent a complex transposition event in which the transposon is excised from the donor chromosome, transferred by a conjugation-like event, and inserted into the recipient chromosome.

EFFECT OF NALIDIXIC ACID AND NOVOBIOCIN ON pBR322 GENETIC EXPRESSION IN *Escherichia coli* MINICELLS

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The *E. coli* enzyme DNA gyrase catalyzes the introduction of superhelical turns into closed, circular, double-stranded DNA in an ATP-dependent reaction. Gyrase has been shown to be involved in a number of cellular processes such as supercoiling of the chromosome; DNA replication, transcription, and repair; λ integrative recombination; and general recombination. Gyrase has also been involved in the selectivity of gene expression.

The purpose of this work was to determine and to compare the effects of two different gyrase inhibitors (nalidixic acid and novobiocin) on gene expression of the well-studied small plasmid pBR322 in *E. coli* minicells.

Quantitative estimates of the synthesis of pBR322-coded polypeptides in novobiocin-treated minicells showed that, compared to control levels, the synthesis of a polypeptide of molecular weight of 34,000 (the tetracycline resistance protein) was reduced to 10-16% while that of a polypeptide of 30,800 (the β -lactamase precursor) was increased to as much as 200%. Nalidixic acid affected the synthesis of pBR322-coded polypeptides in a manner similar to that of novobiocin, although to a lesser extent.

The results suggest that the gyrase inhibitors modifie the interaction of RNA polymerase with some promoters either by decreasing the supercoiling density of plasmid DNA or by changing the gyrase association constant at some specific DNA sites.

STRUCTURAL AND GENETIC ANALYSIS OF PLASMIDS OF AMINOGLYCOSIDE RESISTANT STAPHYLOCOCCUS AUREUS

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Staphylococci resistant to aminoglycoside antibiotics were first reported in 1975. Resistant strains usually contain plasmids and frequently express multiple aminoglycoside modifying enzymes in addition to other antibiotic resistances.

United States and Canadian clinical isolates of *Staphylococcus aureus*, resistant to the aminoglycoside antibiotic amikacin, were studied with respect to antibiotic resistances and plasmid content. All isolates contained large (∿ 30,000bp) plasmids and express multiple aminoglycoside modifying enzymes including phosphotransferase (3') and/or (2"), adenylyltransferase (4') and/or (2") and acetyltransferase (6'). These enzymes mediate resistance to high levels of amikacin, gentamicin, kanamycin, tobramycin and sisomycin. In addition, all isolates studied were resistant to penicillin and the inorganic ions cadmium +2, lead+2, arsenate and mercury+2. The strains could be divided into two groups on the basis of their sensitivity to ertyhromycin and trimethoprim/sulfamethoxazole.

Restriction endonuclease analysis of isolated plasmid DNA revealed that the erythromycin resistant strains possess a series of similar plasmids which are related to the *S. aureus* penicillinase plasmid I524; a different interrelated series of plasmids is present in the erythromycin sensitive strains. The variation between plasmids in each related series is apparently due to the insertion/deletion of specific DNA sequences. The location and size of each insertion was confirmed by electron microscopic examination of heteroduplex pairs of linearized plasmids from each plasmid group. One insertion which occured in the erythromycin resistant proup of plasmids appears as a stem-and-loop structure in electromicrographs. Analysis of deleted and recombinant plasmids suggests that this insertion encodes the kanamycin modifying enzyme adenylyltransferase (4') and that this gene is present in all plasmids studied and in the small *S. aureus* kanamycin resistance plasmid pUB110.

The stem-and-loop structure, similar to the one reported here, is a common feature of the antibiotic resistance determinants which have been observed to transpose. The data presented here suggests that the kanamycin resistance determinant may transpose and could be involved in the recent spread of aminoglycoside resistant *Staphylococcus aureus*.

CHROMOSOMAL LOCATION OF CONJUGATIVE R DETERMINANTS IN STRAIN BM4200 OF *STREPTOCOCCUS PNEUMONIAE*

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BM4200 is a multiply resistant pneumococcus that transfers a *cat tet erm aphA* block by conjugation.[1,2] No plasmid can be detected in either BM4200 or the transconjugants. However, because conjugative transfer of chromosomal elements in gram positive eubacteria was unprecedented when found by Shoemaker et al.,[3] it is essential to have other evidence that the genes are in the chromosome. Our approach was to use transformation to examine the physical nature of the DNA particles carrying the genes and to ask whether the results resembled plasmid or chromosomal transformation. For BM6001 (*cat tet*) and derivatives, *cat* cosedimented with chromosomal DNA and was linked to *tet* and a chromosomal gene. The genes in BM4200 will transform laboratory strains; *cat* goes into wild type readily and all the genes transform a strain that carries *tet* from BM6001 in its chromosome.

In lysates of BM4200 each transforming activity cosedimented with the chromosomal DNA both when the lysate contained very large DNA and after it had been sheared to a mean size near 6 Md. The shear had only a small effect on the level of activity but shifted its velocity distribution greatly. These results imply that the genes were carried initially on very large DNA particles but could transform almost as well from much smaller fragments. Because they differ strongly from those for plasmid transformation or phage DNA transfection, these results exclude the hypothesis that the transformants arose by formation of new replicons in the recipients.

An alternative might be that the R determinants were on a very large plasmid in the donor but transformed by inserting into the normal genome of the recipient. *If so, the result is* that the determinants are inserted into the normal genome of the transformants, which also transfer them by either conjugation or transformation with properties not distinguishable from those of the original donor. One is forced to the conclusion that inserted R determinants can transfer from one chromosome to another by a process that looks like conjugation. The absence of detectable plasmid DNA is consistent with the chromosomal location but is not the basis for reaching this conclusion. The conjugative plasmid pIP501 has no influence on the transfers when it is deliberately added to the cells.

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CLONING OF TWO DISTINCT BUT RELATED ST ENTEROTOXIN GENES FROM PORCINE AND HUMAN STRAINS OF E. COLI

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Many strains of enterotoxigenic *E. coli* excrete a low molecular weight, heat stable toxin (ST_A) into the culture medium. We have cloned an ST_A gene from a human enteropathogenic strain, CRL25090, as a 1.0×10^6 d Pstl fragment inserted on pBR322. Evidence from restriction endonuclease mapping, DNA-DNA hybridization under stringent conditions and absence of *IS1* sequence homology shows that the gene differs from the *Tn1681* ST transposon described by So et al (Nature 1979 ; 277 : 453). Nevertheless the gene products are similar since both toxins are active in the baby mouse test and antisera directed against porcine ST_A neutralize the CRL25090 toxin. We propose to name the *Tn1681* ST_A gene product ST_{A1} and the CRL25090 gene product ST_{A2}.

RELATION OF ENTEROTOXIN PLASMIDS TO KINDS OF ENTEROTOXIN PRODUCED AND THE PATHOGENICITY OF ST-PRODUCING *ESCHERICHIA COLI* FROM PIGS AND CATTLE

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Bovine enterotoxigenic *Escherichia coli* (ETEC) have several features in common with those porcine ETEC referred to as "porcine class 2 ETEC". These similarities include production of heat-stable enterotoxin (ST) and possession of the O antigens 8, 9, 20 or 101 as well as the K99 antigen. This study compared these two groups of ETEC for their toxin production and for their plasmid content with particular emphasis on the enterotoxin plasmids. Four strains behaved differently with respect to their ability to cause fluid secretion in suckling mice, 1-week-old piglets and 6-week-old piglets; one was assayable in the 1-week-old piglet and suckling mice, the second in 1-week and 6-week-old piglets and suckling mice, the third in 1-week and 6-week-old piglets but not in suckling mice and the fourth assayable only in the 1-week-old piglet.* Two of the strains under investigation appear to carry plasmid-linked genes for antibiotic resistance and heat-stable enterotoxin activity. A total of 12 strains from 5 serogroups were examined for the presence of extrachromosomal genetic elements by a modified cleared lysate procedure and agarose gel electrophoresis.

*EFFECTS OF PORCINE CLASS 2 ENTEROTOXIGENIC *E. COLI* ON SUCKLING MICE AND LIGATED ILEAL LOOPS OF 1-WEEK-OLD AND 6-WEEK-OLD PIGLETS

		PIGLETS		SUCKLING MICE
Strain	Serogroup	1 Week (V/L)	6 Weeks (V/L)	3 Days (GW/BW)
0329-A	09:K103	0.9±0.4 (+)	0 ± 0 (-)	0.137±0.015(+)
P16	09:K103	1.0±0.1 (+)	0 ± 0 (-)	0.062±0.001(-)
P16M	09:K103	1.8±0.4 (+)	2.7±0.6 (+)	0.129±0.009(+)
G53	020:K?	0.9±0.2 (+)	2.0±0.4 (+)	0.062±0.003(-)

V/L Ratio of volume to length. The mean ± standard error of the mean for four trials.
GW/BW Ratio of Gut Weight to Body Weight. The mean ± standard error of the mean for four trials.
In parentheses: + = positive; - = negative.

GENETICS OF F PLASMID SEGREGATION INTO *E. COLI* MINICELLS

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F plasmid segregates poorly, if at all, into *Escherichia coli* minicells. Studies using mini-F plasmids constructed from the 40.3 - 49.3F (F kilobase coordinates) *Eco*R1 fragment of F plasmid--which includes three *inc* loci: *inc*B, 45.0 - 45.8F; *inc*C, 45.8 - 46.4F; and *inc*D, 47.5 - 49.3F--have shown that these F loci affect segregation of the plasmids into minicells.

The minimum amount of F DNA required for autonomous plasmid replication reported to date is 44.0 - 45.8F, which includes *inc*B. Four such *inc*B$^+$ plasmids segregated into minicells. Addition of *inc*C$^+$ or *inc*D$^+$ loci, or both, to these plasmids resulted in little, or no, segregation. Thus either *inc*C or *inc*D present with *inc*B$^+$ inhibited segregation. Some understanding of this interaction emerged from studies of two plasmids which had retained the *inc*B$^+$ *inc*C$^+$ *inc*D$^+$ phenotype but had copy number mutations (Cop$^-$) mapping in *inc*B. One plasmid segregated, the other did not. This result demonstrated that a site in *inc*B, apart from incompatibility, was involved in minicell segregation. Eleven of thirteen mini-F plasmids studied are Cop$^-$ with copy numbers increased up to fourteen-fold over the wild-type copy number of 1-2. Seven Cop$^-$ plasmids segregated into minicells. Thus there appears to be no direct relationship between copy number and the ability to segregate into minicells.

We have proposed that the ability of a plasmid to segregate into minicells reflects an association of the plasmid with a septation site, e.g., polar sites in the minicell strain (1). This would be one means of assuring proper partitioning into daughter cells at cell division. Another means, proposed for F (2), would be by association of the plasmid with the chromosome. These plasmids would not be expected to segregate into minicells, where chromosomal DNA is not found. There was no detectable difference in inheritance during cell growth of segregating and non-segregating mini-F plasmids after $>$ 100 cell divisions. This result showed that segregation of the mini-F plasmids into minicells did not affect stable inheritance; however, the mechanism of partitioning could affect the ability of a plasmid to segregate into minicells.

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STABLE RNA MOLECULES ENCODED BY THE RESISTANCE PLASMID R1

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Some resistance plasmids code for stable RNAs of various size classes.[1,2] The biological role of these RNA molecules is unknown.

Genes coding for stable RNA species could be identified on a 7.7 kb (5.1 Mdal) EcoRI fragment and on a 3.6 kb DNA piece (situated on the edge of a 17.7 kb (11.7 Mdal) EcoRI fragment), belonging to the RTF-region of the resistance plasmid R1. The 7.7 kb piece, when analyzed by the Southern-hybridization technique, bound exclusively a 4S RNA while the second fragment proved to be complementary to 5S, 9S and still larger RNA molecules. Northern blots showed that the most prominent RNA species encoded by R1 belong to a large size class, measuring about 335 nucleotides. From this we conclude that the primary gene product is a large RNA which subsequently is cleaved to give the small RNA species mentioned above.

An increased amount of the 335 nucleotide long RNA and, in addition, still larger RNA molecules were found to be present in *E. coli* cells harboring the derepressed plasmid R1*drd*19. RNA of plasmid-less bacteria showed no hybridization to R1-DNA.

Map location and difference in RNA composition in the derepressed state indicate an involvement of the R-factor specific RNAs in the conjugational transfer process.

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HIGH-LEVEL, PLASMID-BORNE BESISTANCE TO AMINOGLYCOSIDE ANTIBIOTICS IN GROUP D STREPTOCOCCI

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Group D streptococci are etiological agents of bacterial endocarditis. Four S. faecalis, 10 S. faecium and 7 S. bovis strains isolated from blood and urine cultures carried genetic markers for high-level resistance to aminoglycosides (streptomycin: Sm, kanamycin: Km, gentamicin: Gm) and tetracycline (Tc), chloramphenicol (Cm) and macrolides (MLS-type resistance). Mating experiments were carried out on membrane filters. Recipient strains were JH2-2 (S. faecalis) and BM132 (group B Streptococcus). Molecular weight (MW) of plasmid DNAs (isolated by dye-buoyant centrifugation) was calculated by agarose gel electrophoresis.

All S. faecalis strains transferred by conjugation their resistance to aminoglycosides into JH2-2 at a high (10^{-2}) or low (10^{-5}) frequency. Resistance to Gm and Km was carried by R plasmids of 44×10^6. Six S. faecium strains transferred their resistance markers into JH2-2 at a low frequency (10^{-8}). Resistance to Sm and Km alone was carried by R plasmids of 15×10^6 or 16×10^6. Resistance to Sm and Km linked to MLS, Cm and Tc was carried by R plasmids of 20×10^6, 24×10^6 or 25×10^6. Each of these plasmids had identical MW with one of the plasmids found in the donor strain. Two S. bovis strains transferred their resistance markers en bloc (Tc,MLS,Sm,Km) at a low frequency (10^{-7}) into BM132 or at a high frequency (10^{-4}) into JH2-2 and BM132 . When low, no plasmid DNA was found in both wild-type and transconjugant strains, suggesting that resistance markers are chromosome-borne. When high, plasmid DNA of 38×10^6 was found in transconjugants.

High-level aminoglycoside resistance is plasmid-borne in group D streptococci and the MW of the plasmids varied from 15×10^6 to 44×10^6. The relationships between all these plasmids are under study in our laboratory.

THE MER OPERON: POLYPEPTIDES AND A PROMOTER

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Four polypeptides are synthesized in response to $HgCl_2$ induction of minicells carrying the cloned mer operon of the plasmid NR1. The molecular weights of these polypeptides are: 69,000 daltons,15,000 daltons, 14,000 daltons, and 10,000 daltons. Antibody to the purified mercuric ion reductase reacts with the largest polypeptide. An additional inducible polypeptide of 65,000 daltons can occasionally be seen. Since this polypeptide also reacts with antibody to purified reductase, we believe it is the proteolytically degraded form of the enzyme observed by Schottel. Data on polypeptides synthesized by cloned sub-fragments of the operon suggest that the bulk of the reductase resides in the EcoRI-H fragment of NR1.

Hydroxylamine-generated mutants of the operon demonstrate sensitive, super-sensitive, and temperature-sensitive phenotypes. There are two classes of sensitive mutant: one class has no reductase activity and no inducible polypeptides; the other class has very high levels (both uninduced and induced) of the enzyme and all four polypeptides. The supersensitive mutants have no detectable reductase activity; only one can be seen to form an altered reductase polypeptide but all have pleiotropic alterations in the smaller polypeptides. All temperature sensitive mutations isolated are altered in regulation rather than in reductase activity.

Using EcoRI* we have cloned into the "promoter-cloning" vehicle, pHB1, a $HgCl_2$-responsive promoter from the purified EcoRI-H fragment of NR1. This promoter requires a functional mer regulatory element in trans and the level of tetracycline resistance provided is directly proportional to the $HgCl_2$ concentration (at sub-toxic levels). The single HincII site in the 200 bp fragment carrying this putative mer promoter corresponds to a site in the EcoRI-H fragment approximately 380 bp from the "right" end of IS1b. Since this distance would be sufficient to determine a polypeptide of 14,000 daltons, and since genetic evidence suggests that the operon immediately abuts the end of IS1b, we believe that this is the promoter for one of the smaller, inducible mer polypeptides.

PROVIDENCIA PLASMIDS

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Providencia commonly cause nosocomial infections, usually of the urinary tract. Both P. stuartii and P. rettgeri readily become resistant to multiple antibiotics. At our medical center over the past four years, Providencia have often been aminoglycoside resistant. Aminoglycoside-resistant (Gm^r or Tm^r) P. rettgeri and P. stuartii (56 strains from 53 patients) were speciated by API biotype: 29 were from the Charleston VAMC, 11 from Columbia, S.C. V.A., 9 from Medical University Hospital (MUH), 1 from Walter Reed Army Medical Center, and 7 from the Center for Disease Control, Atlanta, Georgia. All strains were tested by Bauer-Kirby disc methods to 13 antimicrobials. Cleared lysates were subjected to agarose gel electrophoresis for detection of plasmid DNA. Conjugal transfer of plasmid containing strains was attempted using as recipients P. mirabilis F-67 (Rif^r) and E. coli C (Rif^r). Transformation of purified plasmid DNA from one strain of P. rettgeri was performed into E. coli C (Rif^r). Bristol Laboratories, Syracuse, New York, performed determination of aminoglycoside modifying enzymes for one strain. Of 56 different strains 18 were P. rettgeri, 38 P. stuartii (14 urease$^+$). The percent of susceptibility to various antimicrobial agents was as follows: Tet (0%), Col (2%), Tm (2%), Gm (5%), Cr (5%), Ch (11%), Tmp-Smz (14%), Su (17%), Ap (20%), Km (43%), Cb (46%), Nal (54%), and An (54%). Of the 56 strains, 43% contained one or more plasmids. The most common plasmid pattern was a 29-3.1 Md pair seen in 16 strains which were obtained from Charleston (VAMC and MUH) Columbia VA. Other plasmid sizes ranged from 2.9 to 115 Md. Conjugation of 13 different strains resulted in transfer of at least 1 marker in 9 strains. Ap^r and Cb^r were the most commonly transferred. Gm^r, Tm^r, Km^r transfer was seen only once, then associated with a 105 Md plasmid. Ap^r and Cb^r transconjugants contained the 29 Md plasmid and in one case also coded for urease. Transformation of purified plasmid containing the 29 and 3.1 Md plasmids demonstrated that the 29 Md plasmid coded for Ap^r and Cb^r and that the 3.1 Md plasmid coded for Cr^r. Aminoglycoside 2'-N-acetyltransferase (AAC-2') was elaborated by one organism containing the 29-3.1 Md pair, suggesting a chromosomal locus for production of this enzyme. We conclude that various size plasmids in multi-resistant Providencia were present in South Carolina and other geographic locations. Amikacin and carbenicillin were the most active agents in this group of Providencia. In these strains, aminoglycoside resistance was usually non-plasmid mediated; moreover, the presence of AAC-2' in Providencia may be chromosomally mediated. In addition, in some Providencia, we found urease$^+$ to be plasmid mediated and this may explain the variability of urease production within the genus Providencia.

A RAPID MINI-SCREEN PROCEDURE FOR THE DETECTION AND ISOLATION OF SMALL AND LARGE PLASMIDS

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High molecular weight plasmids of various gram-negative bacteria can be resolved within 3 hr. Cells from a single colony or from 3 ml broth cultures are suspended in E buffer (40 mM Tris-acetate, pH 7.9, 2 mM Na_2EDTA) and lysed by adding 2 volumes of 3% Na dodecyl sulfate (SDS in 50 mM Tris-OH, pH 12.6). The mixture is incubated for 20 min at 50-95°C (depending on the bacterium) and then briefly emulsified with an equal volume of a distilled unbuffered phenol/chloroform mixture (1:1, vol/vol). The mixture is centrifuged for 10 min, 8000 g. A 20-50 µl sample of the aqueous phase is used directly for electrophoresis in 0.7% agarose gel (in E buffer), 1.5 hr at 12 V/cm with water cooling. The plasmid DNA is visualized by soaking the gel in a solution of ethidium bromide (0.5 µg EB/ml) for 30 min and then placed over a short wave ultraviolet light source. For preparative isolation, the phenol/choroform is removed by dialysis. The plasmid preparation can then be used directly for transformation, nick translated as probes, ligated and restricted. Plasmids with molecular masses ranging from 2.2 to 350 mdal have been clearly resolved and readily isolated. The procedure is particularly useful for rapid screening of *E. coli* harboring recombinant plasmids. Single colonies can be analyzed for recombinant plasmids as follows: A single colony is resuspended in 100 µl of 3% SDS in 0.05 M Tris-OH, pH 12.6. The suspension is thoroughly mixed and heated at 50°C for 20 min. The lysate is then extracted (briefly by shaking) with an equal volume of unbuffered phenol/chloroform (1:1; vol/vol) and centrifuged at 10,000 rpm for 5 min. A 25 µl sample of the upper aqueous phase is directly placed in sample wells containing electrophoresis buffer in 1% agarose gel in E buffer. Electrophoresis is carried out at 12-15 volts/cm for 1.5 hr with water cooling. Plasmid can be isolated from the aqueous phase by first extracting the phenol/chloroform residue with ether and then precipitating the plasmid with two volumes of -20°C ethanol. The precipitate is dried with nitrogen and is redisolved in distilled water.

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A MODEL FOR THE MECHANISM OF RESISTANCE TO AMINOCYCLITOLS

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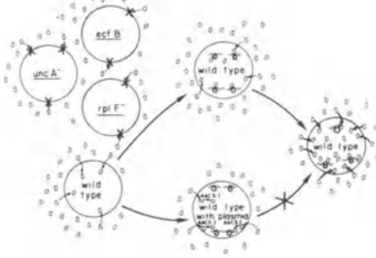
A clinically isolated plasmid, pJR89, confers resistance to aminocyclitol antibiotics. It encodes an enzyme, aminocyclitol acetyltransferase (3)-I, which both modifies the aminocyclitol molecules and diminishes the amount of drug accumulated by the bacteria. Both functions are important for the expression of the resistance phenotype.

This study addresses the mechanism by which the enzyme, (AAC 3-I,) diminishes cellular accumulation of drug. One possible mechanism invokes a stable enzyme-substrate complex, which blocks uptake of additional drug molecules. If this mechanism were correct, one would expect a good substrate for the enzyme to protect cells against the effects of poor substrates. However, this appears not to be the case. This was shown in two ways: 1) gentamicin (a good substrate) did not lessen the inhibition of protein synthesis, in vivo, by tobramycin (a poor substrate); 2) sisomycin, (a good substrate) did not diminish the amount or rate of netilmicin (a poor substrate) accumulation. Good substrates did not merely fail to protect cells against poorer substrates; in fact, when both were present in the growth media together, they exerted a synergistic antibiotic effect. Poor substrates also enabled the better substrates to overcome the block to transport.

The mechanism of enzyme-mediated resistance could have involved either modification of all intracellular drug or just of a critical portion thereof. Therefore, the complement of intracellular drug was assayed radioenzymatically to determine the ratio of modified to non-modified forms. One hundred percent of the intracellular aminocyclitol was found to be modified in resistant cells.

On the basis of these data the following model was proposed. Initially, there is a slow, energy-dependent phase of uptake. This corresponds to the drug crossing the cell membrane. Intracellular drug then binds to ribosomes. Ribosome-binding triggers the second, faster phase of uptake. In resistant bacteria, the first, slow phase of uptake occurs. However, the AAC 3-I modification rate is sufficient to modify all drug that gains access to the cytoplasm. Therefore, active drug cannot bind to ribosomes, and the faster phase of uptake does not occur.



MOLECULAR CHARACTERISATION OF THE K88 MEDIATED ADHESION SYSTEM OF PORCINE ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC)

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By using DNA cloning techniques, Tn5 transposon mutagenesis and the *E. coli* minicell system we have identified and mapped four cistrons and their corresponding polypeptides which are involved in expression and assembly of the K88 fimbriae of procine ETEC. The cistrons are arranged in at least two operons which are located adjacent to each other and are transcribed in the same direction. The operons have been cloned separately onto different plasmids. Operon I encodes 70,000d, 29,000d and 17,000d polypeptides in that order. Operon II encodes the 23,500d K88 fimbriae subunit and is normally expressed at high levels. Cells harbouring Operon II alone do not express levels of the 23,000d polypeptide which can be detected using our assay systems whereas cells harbouring Operon I and Operon II on separate plasmids express near normal levels of the 23,000d K88 subunit.

Tn5 insertions in *adhA* (70,000d) express reduced levels of the 23,000d K88 subunit which is found in culture supernatents and not on the cell surface. Inserts in *adhB* (29,000d) produce reduced levels of the 23,500d K88 subunit, are phenotypically Adh^+ (bind *in vitro* to pig intestinal epithial cells) but are $MRHA^-$ (failed to agglutinate red blood cells in the presence of D-mannose). Inserts in *adhC* (17,000d) do not express detectable levels of the 23,500d K88 antigen from Operon II. Inserts in *adhD* (23,500d) are $K88^-$, Adh^-, $MRHA^-$. The *adhD* product is the K88 fimbrial subunit. The results suggest that the *adhA* product is part of a basal structure attaching the K88 fimbriae to the cell membrane. The *adhC* product may be a positive regulator controlling expression of *adhD*.

MULTIPLE ANTIBIOTIC RESISTANCE AMONG GRAM-NEGATIVE BACTERIA ISOLATED IN KARACHI

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The incidence and extent of multiple antibiotic resistance among gram-negative bacteria, isolated from various sources in Karachi, was investigated. The bacterial strains were isolated from clinical specimens, food, milk, water and sewage and were screened against the commonly-used antibiotics: ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), neomycin (Nm), streptomycin (Sm) and tetracycline (Tc). Of the total 518 bacterial strains screened, 446 (86%) were resistant to one or more antibiotics. Among the clinical bacteria, the incidence was particularly high (93%), reflecting an indiscriminate use of antibiotics in chemotherapy. The most common resistance pattern among *Escherichia coli* was ApCmSmTc, followed by CmSmTc and ApCmKmNmSmTc. Pseudomonads were found to exhibit resistance to all or most of the antibiotics. Other gram-negative bacteria, including *Salmonella*, *Shigella*, *Aerobacter* and *Proteus* showed resistance to one or more antibiotics in different combinations. Seven R plasmids, R62, R63, R64, R65, R66, R67 and R68 isolated from resistant bacteria, were studied for their genetic behaviour.

EPIDEMIC SPREAD OF AN R PLASMID TO VARIOUS BACTERIA IN A HOSPITAL

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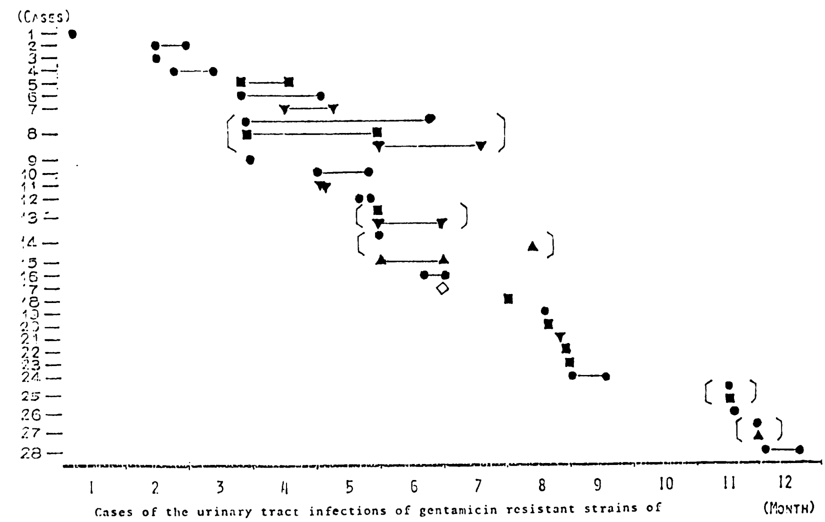
Incidences of gentamicin resistant strains were very high in the enteric bacteria isolated in the urology wards in our hospital. Because of their antibiotic resistance patterns, we studied for their R plasmids. Majority of these strains were found to have conjugative R plasmids. Because of the similar resistance patterns of these R plasmids, we tried to classified these R plasmids into incompatibility groups. We have two independent wards in the urology department. Almost all R plasmids detected in the strains in one ward were found to be A-C group and most of the R plasmids in the other ward were not identified despite of the similar resistance markers, although detailed study for aminiglycoside antibiotic inactivating enzymes of these R plasmids suggested some differences of these two groups. Thus, it was found that only a certain R plasmid had been disseminated in various enteric bacteria in the different patients and that this dissemination had been limited in a certain ward.



Cases of the urinary tract infections of gentamicin resistant strains of inpatients of 7th floor ward and their R plasmids

○ = C.freundii, □ = E.cloacae, ▽ = Proteus spp., △ = K.pneumoniae, ◇ = S.marcescens; Black symbols = A-C complex group; White symbols = other than I_2, A-C, M, W, M_1.

MODULAR CONSTRUCTION OF R PLASMIDS *IN VIVO* : TRANSLOCATION EVENTS IN *SALMONELLA ORDONEZ*.

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Salmonella ordonez strain BM2000 encodes ApCmKmSpSuTc resistances and production of colicin Ib (Cib). The Km and Cib characters are carried by a 97 kb IncI1 plasmid (pIP565). In addition to the Km and Cib traits all or part of the other resistances (ApCmSpSuTc) can be transferred by conjugation from *S. ordonez* to *E. coli* where all the acquired characters are borne by an IncI1 plasmid designated complete or partial composite plasmid respectively. This suggests that in BM2000 the ApCmSpSuTc R determinants are encoded by a DNA sequence able to translocate, en bloc or in part, from a donor replicon to pIP565. DNA from pIP565 and composite plasmids, and total DNA from *S. ordonez* BM2000 have been studied by agarose and polyacrylamide gel electrophoresis following digestion with *EcoRI*, *BamHI*, or *SalI*, and by Southern hybridization.These comparative analyses enable us : a) to show that, in each case, acquisition by pIP565 of all or part of the resistances is due to the insertion of a single DNA fragment into the receptor plasmid ; b) to detect two types of composite plasmids with regard to the specificity of insertion into pIP565 and the mapping of the inserts ; c) to demonstrate that the ApCmSpSuTc R determinants are integrated into *S. ordonez* chromosomal DNA ; d) to map the endonuclease-generated DNA fragments of the translocatable sequence whether integrated into BM2000 chromosome or into pIP565.

The results obtained are compatible with the existence of two distinct molecular mechanisms : a site specific recombination between two of the four directly repeated IS-like sequences present in *S. ordonez* chromosome leading to the circularisation of all or part of the ApCmSpSuTc R determinants followed by 1) either a second site specific recombination with the copy of the IS-like sequence of pIP565 (Type I composite plasmids), 2) or transposition of precise groups of characters in various sites of pIP565 (Type II).

RELATIVE COLONIZATION POTENTIALS OF *E. COLI* K-12 AND HUMAN FECAL STRAINS IN STREPTOMYCIN-TREATED MICE

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Relative colonization potentials of *E. coli* K-12 strains and human fecal isolates were assessed in a competitive streptomycin-treated mouse system. Mice pretreated and subsequently maintained on streptomycin were simultaneously fed two strains of streptomycin-resistant *E. coli* and the level of each strain was monitored for 10-14 days. Neither *E. coli* K-12 nor human fecal isolates were able to colonize mice which had not been treated with streptomycin. When a single strain of *E. coli* K-12 or a fecal isolate was fed to streptomycin-treated mice, all strains colonized at approximately equal levels (10^8 organisms/g feces). Experiments involving the competition of one fecal strain against another fecal strain also resulted in approximately equal levels of colonization (10^8 organisms of each strain/g feces). When a fecal strain was competed with any *E. coli* K-12 strain, both strains colonized the large intestine, however, the level of *E. coli* K-12 was always 100-1000 fold less than that of the human fecal strain.

In two instances genetic alterations were demonstrated to have an affect on colonization potential. In the first instance, two fecal isolates (F-18 & F-56), each cured of a single plasmid, demonstrated decreased colonization potential relative to the plasmid containing parent strain. In the second instance, a number of rifampicin-resistant mutants of both K-12 and fecal strains were isolated and assayed for colonizing ability relative to the parent strain. Interestingly, each rifampicin-resistant mutant which contained one or no plasmids demonstrated a decreased colonizing capacity, while those strains containing multiple plasmids failed to show a decreased colonizing capacity relative to the rifampicin-sensitive parent strain. Together these data indicate that both chromosomal and plasmid genes can influence the colonization potential of *E. coli*. Further characterization of these genes and the products they code for should contribute to our basic understanding of the colonization process.
Supported by NIH Grant AI 16370.

THE PLASMID REFERENCE CENTER (PRC)

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The PRC serves as a central research resource facility for the acquisition, maintenance, and distribution of important, prototype plasmid cultures, and has been in operation for just over three years. The collection comprises plasmids carried by enteric bacteria, especially *Staphylococcus aureus* prototype plasmids, reflecting historically earlier research developments. New plasmids in other species have since been discovered and will gradually be added to the collection. In addition, some plasmids reported since the CSH compilation, including useful cloning vectors from the laboratories of S.N. Cohen and H. Boyer, have been deposited and are available.

The collection currently consists of over 800 members, but a catalog is not available. The CSH compilation serves as a basic catalog. If any plasmid listed or donated to the collection is requested, it will be shipped; if not available, it will be sought for the collection and sent when received. Certain plasmid bearing strains are assembled for distribution as kits. Those currently available include:

(1) an *E coli Inc* tester kit.
(2) Size Standards kit for agarose gel determinations.
(3) A kit of representative colicinogenic strains.
(4) Metabolic representatives: (β-lactamase-coding plasmids and *Tc* subtypes).
(5) *Tn*1 to *Tn*10 standards kit.

Investigators are encouraged to deposit prototype plasmid strains, representatives of new *Inc* classes, new plasmid derivatives and mutants. The PRC will then assign a PRC catalog number, maintain, store, and distribute these cultures. Requestors of plasmid strains are encouraged to report to the PRC new information from their investigations of the plasmids provided.

The proposal for the uniform nomenclature for bacterial plasmids will be used. Symbols for newly discovered plasmid attributes may be registered with the PRC for inclusion in future compilation. To avoid duplications of plasmid names, a registry of plasmid prefix designations is maintained at the PRC. Over 200 permutations of the proposed code, pXY prefix, are still available. A registry of *Tn* allocations (*Plasmid* 2: 466, 1979) is also maintained.

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UNDERSTANDING THE INVIRONMENTAL EFFECTS OF APPLIED GENETICS

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The Applied Genetics program evolving at EPA is an effort to prepare a foundation for understanding the impact of increased production and application of Applied Genetics products. To this end, we have instituted a contractual effort to survey the industry, an in-house research effort to estimate the probability of genetic exchange in sewage, and an extramural grant effort to consider the probability of escape from containment and to develop a model which will permit estimation of the probability of survival and persistence of novel genomes under a variety of environmental conditions. In addition, we are examining the regulatory and legal bases under which EPA operates to determine appropriateness as a basis for action. It must be stressed that there have been no adverse effects or any real evidence of significant potential hazards. However, questions relating to large scale (factors of 10,000) processes and deliberate or accidental release have simply not been encountered nor adequately explored.

Specifically, the program will attempt to:
(1) Scope The Industry: Estimate the types and quantities of products anticipated as well as production methods involved. (Contract effort, Battelle (Columbus) and Teknekron to be completed by April, 1981). Battelle will concentrate on Agricultural aspects of Applied Genetics research while Teknekron will survey the potential industrial applications and impacts;
(2) Assess Potential Effects: Develop a model which will permit the evaluation of the potential effects of application and accidental escape of these products on public health, welfare and environmental problems (Grant Program, University of Rhode Island/Tufts University, Carnegie Mellon/Naval Bioscience Laboratory, Cornell University). The URI/Tufts study involves construction of a plasmid which can be traced after it has been introduced to a model sewage plant to simulate accidental release. Evidence of transfer to sewage microflora and percent survival during treatment will be sought. The Carnegie Mellon/Naval Bioscience Laboratory study will estimate the probability of escape of organisms from large scale equipment. A model will be developed (using fault tree analyses) and verified both in laboratory trials using large scale equipment and on site. The Cornell Study will attempt to define and quantitate parameters in colonization of a new niche by microorganisms. An attempt will be made to develop a model relating colonization to physiological or biochemical characteristics of microorganisms.
(3) Evaluate Existing Regulations: Explore the regulatory and legal mechanisms available to achieve the desired result (in-house); and
(4) Investigate Beneficial Components of the Applied Genetics Industry (e.g., reduction in hazardous wastes, enhancement of clean-up capability) which should be encouraged (University of Illinois). This study will attempt to categorize needs in terms of susceptibility to techniques developed as a result of research in this area.

A MINI-Ti PLASMID OF AGROBACTERIUM TUMEFACIENS AS A VECTOR FOR THE INSERTION OF FOREIGN GENES INTO HIGHER PLANT CELLS

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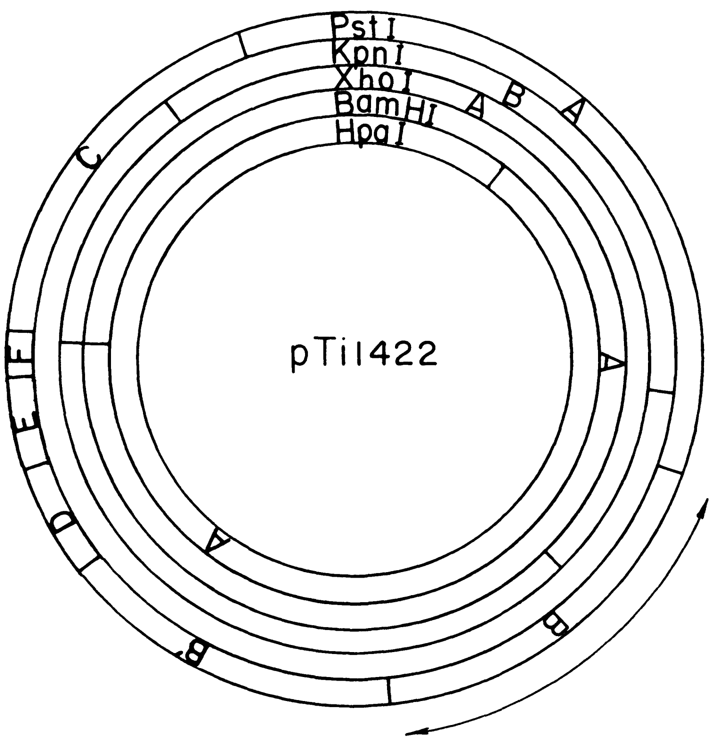
It has been long thought that oncogenic and virulence properties are conferred by large Ti plasmids in *Agrobacterium* strains. However, we predicted that Ti plasmids of much smaller sizes might exist in nature because only small segments of these Ti plasmids carry the genetic information necessary for crown gall oncogenesis. We report here the confirmation of this prediction by the discovery of an *Agrobacterium* strain that harbors a Ti plasmid one-fifth the size of its larger 120 mdal counterpart. The mini-plasmid, pTi1422, has a molecular mass of 28.7 mdal and is harbored in strain 1D1422 that was originally isolated from a tumor on a grapevine. Unlike many *Agrobacterium* strains, strain 1D1422 does not harbor any cryptic plasmids. It is oncogenic on a number of different hosts. Crown gall tumor formation is best expressed on carrot discs and is markedly delayed on woody hosts. Strain 1D1422 best fits the biotype-1 group of *Agrobacterium* strains similar to octopine and nopaline strains. However, strain 1D1422 is unable to utilize or produce octopine or nopaline and preliminary tests indicate that it produces two unidentified acidic opines. A small deletion of 2-3 kilobases in the mini-Ti plasmid is sufficient to cause complete loss of oncogenicity.

Unlike the usual large Ti plasmids, pTi1422 possesses single sites for restriction endonuclease BamHI and HpaI (Fig. 1). Thus, the whole intact mini-Ti plasmid has been cloned in *E. coli* HB101. Also proteins coded by cloned fragments of pTi1422 DNA were synthesized in P678-54 mini-cells except in the region designated in Figure 1. This region and the rest of the mini-Ti plasmid DNA have no apparent sequence homologies with large Ti plasmids such as in strains 15955 and C58 as judged by reciprocal Southern blot hybridizations. This is supported by the fact that the mini-Ti plasmid is compatible with the Ti plasmids of 15955 and C58 when they were either inserted in strain 1D1422 or when pTi1422 was transferred with pTiACH5 or pTiC58 into Ti plasmid-free avirulent strain NT1 by cotransformation.

These studies, therefore, clearly show that Ti plasmids substantially smaller than 120 mdal exist in nature. Although the mini-Ti plasmid has no detectable sequence homologies with large octopine and nopaline Ti plasmids, it nevertheless confers oncogenic properties on *Agrobacterium*. This suggests that T-DNA sequences can be quite distinct and argues against the notion of a common DNA among all Ti plasmids. Of primary significance is the presence of single sites for two restriction enzymes, which permits the insertion of foreign DNA such as the β-lactamase gene into these sites. Hybrid plasmids carrying this gene have already been propagated and expressed in E. coli and such hybrid plasmids can be readily transferred into plants. pTi1422 therefore has a number of advantages over its larger counterparts: a) the essential genes necessary for oncogenesis and virulence make up a considerable portion of the mini-Ti plasmid; b) with fewer restriction endonuclease sites, genes conferring functional properties can be easily located on a physical map and therefore isolated as cloned fragments; c) the whole plasmid can be genetically manipulated in E. coli; d) single and double restriction sites already exist on the mini-Ti plasmid. This will permit us to reconstruct the plasmid into an useful vector that can be used directly (direct insertion into plant protoplasts) and indirectly (insertion mediated by Agrobacterium).

This work was supported in part by CA-11526.



RECOMBINANT DNA SYSTEM IN *STREPTOCOCCUS SANGUIS*

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We have previously described the construction of plasmids that may be used as vehicles in a streptococcal molecular cloning system (Macrina, et al. J. Bacteriol 143, 1425-1435 [1980]). Extended characterization of two such plasmids has revealed additional useful information. pVA380-1 is a 2.8 Mdal, multicopy plasmid originally isolated in *S. ferus*. It is a phenotypically silent plasmid but may be used to clone directly selectable markers (e.g., resistance determinants) using *Eco* RI, *Ava* I, *Hind* III or *Hpa* II. The *Eco* RI, *Ava* I and *Hind* III sites may be used in any paired combination to insert DNA into this vector. A derivative (designated pVA736; 5 Mdal) of pVA380-1 bearing an erythromycin-resistance (Em^r) determinant has been constructed for cloning non-selectable DNA sequences. Passenger DNA may be inserted into pVA736 using *Hind* III, *Eco* RI, *Kpn* I, *Ava* I or *Hpa* II restriction enzymes. The pVA380-1 plasmid has been used to study the organization of a 20 Mdal conjugative R plasmid, pIP501, originally isolated in *S. agalactiae*. Using molecular cloning methods, the chloramphenicol resistance (Cm^r) determinant of pIP501 was found to reside on the 4.1 Mdal *Hind* III A fragment while the Em^r determinant was located on the 3.0 Mdal *Hind* III B fragment. The *Hind* III A and B fragments were contiguous on pIP501. A 2.3 Mdal *Hpa* II - *Ava* I fragment bearing the Em^r determinant of pIP501 was replaced with a 2.3 Mdal *Hpa* II - *Ava* I fragment derived from pVA380-1. Unlike pIP501, the resultant 20 Mdal plasmid, pVA797, (bearing only Cm^r), did not display segregation from cells grown at 42°C, indicating that the pVA380-1 portion of pVA797 was governing its replication. In addition, pVA797 was unable to promote its own conjugative transfer suggesting that a structural or regulatory gene(s) for transfer proficiency resides near the Em^r determinant.

The use of pVA736 to clone chromosomal gene sequences from cariogenic *S. mutans* into *S. sanguis* is being assessed. Transformation of *S. sanguis* with purified monomeric pVA736 forms was found to be a second order process, whereas multimeric plasmid forms have been inferred to transform with one-hit kinetics. "Shotgun" cloning of *S. mutans* chromosomal fragments into *S. sanguis* resulted in the recovery of chimeras that had suffered deletions. We attributed this to the negligible amounts of monomeric or perfect oligomeric chimeras formed during ligation which are presumably needed for effective transformation. We are currently attempting to solve this problem by adapting the "helper plasmid" method of Gryczan, et al. (Molec. Gen. Genetics 177, 459-467 [1980]) to the *S. sanguis* cloning system. (Supported by USPHS Grant DE 04224).

ECOLOGY OF ANTIBIOTIC AND HEAVY METAL RESISTANCE IN NATURE

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We have studied the frequency of antibiotic and heavy metal resistance in the fecal flora of approximately 1300 samples from populations of patients, laboratory workers, urban and rural dwellers and farm animals. Computerization of the data base has produced statistics on the prevalence of antibiotic and metal sensitivity, and single, multiple and linked antibiotic resistances. Flora was designated resistant if it contained $\geq$ 10% resistant coliforms to any of eight antibiotics: tetracycline (tet), gentamicin (gm), kanamycin (kan), ampicillin (amp), streptomycin (sm), keflin (kef), chloramphenicol and naladixic acid. By this criterion, only 25% of all samples were sensitive to all drugs. High level resistance was defined as flora having $\geq$ 50% resistant coliforms.

In all populations studied, resistance to amp, tet, sm, kef and kan was most common. 25-40% of samples from the hospitalized population were resistant to one or more of these drugs. The frequency of resistance in lab workers was similar, except for a 33-50% decrease in high level resistance to tet and amp. The urban dwellers showed a lower frequency of resistance notably to kan ($\approx$15%) however, tet, amp and sm resistance levels were $\approx$30-35%. High level resistance was also notably less in rural dwellers. These findings indicated an unexpectedly high frequency of resistant fecal organisms in the general population, particularly among those not taking antibiotics. A group of patients on one or more antibiotics showed that multiple resistance to 4-7 drugs was significantly more common than among noningestors.

In 560 fecal samples examined, 15.6% had lead resistance in $\geq$ 10% of the lactose fermenting populations. Other resistance frequencies were mercury (Hg) 14.3%, tellurite (Te) 13.9%, cadmium (Cd) 6.6%, arsenate (As) 5.0%, metaborate (MBO) 3.0%, chromate (Cr) 2.6%, phenylmercuric acetate (PMA) 1.4% and silver 0%. Resistance was unequally distributed in the fecal samples: e.g., many samples were either sensitive ($<$10%), or showed large numbers ($\geq$ 90%) of resistant organisms. This was particularly true for Hg, Pb, Cr and MBO. Lactose nonfermenters were equally or less resistant to all metals. The kinds of high frequency metal resistance (50% resistance) differed in the various populations: in the hospital, 19.5% had high level Hg resistance; Te and Pb were at 12.6% and Cd and MOB at 8.7%. In lab workers, 12.6% of samples were highly resistant to Hg, whereas in rural dwellers the frequency was 6.4%. In this latter group, Pb resistance was at 28.7 compared to 1.5% in the lab group.

UNUSUAL CONJUGAL TRANSFER OF ANTIBIOTIC RESISTANCE IN BACTEROIDES: NON-INVOLVEMENT OF PLASMIDS

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Resistance to tetracycline (Tc^r) and lincosamide antibiotics (clindamycin resistance, Cc^r; erythryomycin resistance, Em^r) was transferred from a strain of Bacteroides fragilis (V503) to a plasmidless strain of B. uniformis (V528) during in vitro filter matings. Resistance transfer was detected at frequencies of 10^{-5} to 10^{-6} drug resistant progeny/input donor cell and was dependent on cell-to-cell contact of donors and recipients. Transfer was insensitive to DNase and was not mediated by chloroform or filter-sterilized donor broth cultures. The Tc^r and Cc^r markers did not segregate away from one another at readily detectable frequencies. Both markers were stable in the V503 donor and in resistant progeny and treatment of V503 cultures with coumermycin or ethidium bromide failed to yield drug-sensitive variants. By standard physical analyses, V503 was found to contain a 3.7 Mdal plasmid (pVA503). Attempts to isolate unusually large plasmids from V503 using the method of Hansen and Olsen (J. Bacteriol 135, 227-238 [1978]) were unsuccessful. Drug resistant transconjugants of V503 x V528 matings usually contained pVA503, but up to 20% of the total transconjugants of such crosses were plasmid free. Filter blot DNA hybridization studies (Southern method) confirmed that V503 was not integrated into the host chromosome of the plasmidless transconjugants. Transconjugants from V503 x V528 matings (with or without pVA503) acted as donors for Cc^r. Tc^r transfer could not be monitored in such secondary crosses due to inherent Tc^r in the recipient strain. Chromosomal determinants for resistance to cefoxitin and rifampicin were not transferred in this system.

To further explore this seemingly plasmidless transfer we exploited a previously characterized conjugative R plasmid (pBF4) from B. fragilis. (Welch, et al. Plasmid 2, 261-268 1979 , and Welch and Macrina, J. Bacteriol 145, in press [1981]). pBF4 is 27 Mdal in size and confers constiutively-expressed Cc^r. ^{32}P labelled pBF4 was used as a probe in hybridizations against filter blotted Hind III cleaved V503 (donor), V528 (recipient) and selected Cc^r/Tc^r progeny from V503 x V528 matings. A single ~4.2 Hind III fragment present in the V503 digest showed homology to pBF4. There was no pBF4-hybridizing material in the V528 recipient. Interestingly, all Cc^r/Tc^r progeny from V503 x V528 matings contained a 4.2 Mdal Hind III fragment that hybridized to pBF4. Our current hypothesis is that the Cc^r, Tc^r and conjugal transfer genes are on a discrete segment of DNA which resides on the host genome rather than a plasmid. (Supported by NSF grant 77-00858)

LAMBDA TRANSDUCING PHAGES CARRYING PLASMID R100 tra GENES

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From the R100::λ cointegrate pEDR101, we have isolated a series of λtra transducing phages which carry R100 tra DNA substituted into the left arm of λ. Five phages have been completely analyzed. The tra genes carried by each phage were assayed in complementation tests with known F'lac tra_{am} plasmids, and this genetic analysis was then correlated with the amount of R100 DNA present in each phage (Fig. 1).

The proteins synthesized from each phage were determined (1) from infections of irradiated λ^- host cells so that all promoters on the phage (both λ and R100) were expressed. Similar infections of irradiated λ-lysogenic cells lead to repression of λ promoters and thus allow detection of any proteins synthesized from R100 promoters. Such experiments led to the unexpected observation that each phage directed the synthesis of one R100 protein (25,800 M.W.), even though the tra promoter located before traY was missing in all but one phage. By analogy with the reported molecular weight of plasmid F traT protein as 25,000, and since one phage encodes only traS and traT, we believe this protein represents R100 traT product. These experiments using the λ lysogen mean that either: (1) traT has its own promoter; or (2) a few $\lambda P_{R'}$ mRNA transcripts are made and traT has a much more efficient translation initiation mechanism than any other gene on these transcripts.

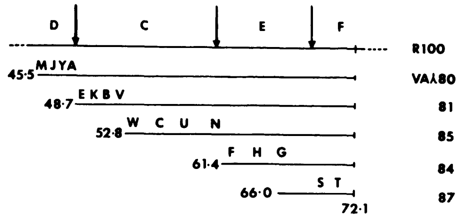

Figure 1. R100 DNA carried by the five λtra phages. Arrows indicate EcoR1 sites. Numbers on the left are kb coordinates for the R100 DNA carried by each phage.

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ACTIVE UPTAKE OF TETRACYCLINE IN MEMBRANE VESICLES OF SENSITIVE *ESCHERICHIA COLI*.

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Tetracycline inhibits protein synthesis in susceptible microbial organisms by interfering with binding of amino-acyl-tRNA to the A site on the ribosome. Since plasmid-borne resistance to this drug involves a decrease in drug uptake, we have studied tetracycline transport in resistant and sensitive *E. coli* cells. Sensitive *E. coli* cells have both an energy-dependent uptake and an energy-independent uptake (1).

There are 3 kinds of active transport in *E. coli*: that requiring ATP; that during which the substrate is modified (e.g. phosphorylated by PEP) and that which depends only upon proton motive force (pmf; an electrochemical gradient of hydrogen ions across the membrane formed by electron transport and/or by ATP hydrolysis specifically by the membrane ATPase).

Because tetracycline uptake in cells was inhibited to the same extent by agents which a) block respiration (cyanide, anaerobiosis), b) inhibit ATP synthesis (arsenate) and c) destroy the proton motive force (2,4-dinitrophenol) (1), it was not clear to which category active tetracycline uptake belonged. Membrane vesicles prepared by osmotic lysis of spheroplasts according to the method of Kaback (2) are free of cytoplasm and endogenous energy sources and serve to distinguish among these possibilities. With such vesicles, addition of electron transport substrates forms a pmf only, so that transport stimulated by such substrates must be pmf-dependent. We have found that such vesicles made from sensitive *E. coli* cells were indeed stimulated by electron transport substrates (D-lactate and phenazine methosulfate plus ascorbate) to concentrate tetracycline 3-5-fold above the level of the drug in the medium. Therefore at least part of the energy-dependent uptake of tetracycline in sensitive cells depends only upon pmf. The pH and Mg^{++} optima were pH 6.9 and 1 mM respectively.

The various inhibitors mentioned above (arsenate, cyanide, dinitrophenol, etc.) presumably all lower pmf to some extent *in vivo*. That they all inhibit tetracycline uptake completely might be explained if this uptake required a relatively high pmf below which no active transport of tetracycline could occur.

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DISTRIBUTION OF PLASMID TYPE β-LACTAMASES IN AMPICILLIN-RESISTANT SALMONELLAE FROM HUMANS AND ANIMALS IN THE UNITED STATES

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The types of β-lactamase produced by 261 non-typhoidal ampicillin-resistant salmonellae, 114 human and 146 animal isolates, was determined by isoelectric focusing of crude sonic extracts. All isolates produced at least one of the types of beta-lactamase known to be plasmid-mediated. The most prevalent type was TEM-1 (n=203), followed by OXA-2 (n=50), OXA-1 (n=3) and PSE-1 (n=2). Three isolates each produced two plasmid-type beta-lactamases, i.e., TEM-1 + PSE-1 and TEM-1 + OXA-1. The PSE-1 β-lactamase, thought previously to be pseudomonas specific, was found in strains of *S. typhimurium*, *S. newport*, and *S. heidelberg*. None of the other 7 known types of plasmid-mediated beta-lactamase were found.

The OXA-2 β-lactamase was far more prevalent in Salmonella than has been reported for any other genera. It was found in 16 (31%) of 52 animal isolates but in only 4 (11%) of 38 human isolates of *S. typhimurium*. In contrast, the prevalence of OXA-2 was high in both animal (60%) and human (79%) isolates of *S. typhimurium, var. copenhagen* (n=29) but low in animal (9%) and human (5%) isolates of other serotypes (n=142).

OXA-2 was found rarely in human isolates of *S. typhimurium, var. copenhagen* from Massachusetts prior to February 1980. It occurred, however, in 8 isolates from different parts of the state over a 2 month period in February and March, suggesting that a previously undetected common source outbreak had occurred. These isolates had an unusual antibiotype to non β-lactam antibiotic characterized by intermediate level tetracycline resistant (mean diameter of zone of inhibition around 30 μg tetracycline disk = 10.5 ± 3.5mm) linked to high level resistance to streptomycin, kanamycin, and sulfonamides. Most animal isolates of OXA-2 containing *S. typhimurium, var. copenhagen* had the same antibiotype indicating that this human outbreak may have been related to an animal source.

MOLECULAR STUDIES OF A COINTEGRATE PLASMID FORMED FROM PLASMID Flac AND $PM10_{LT2}$ IN *Salmonella typhimurium* LT2

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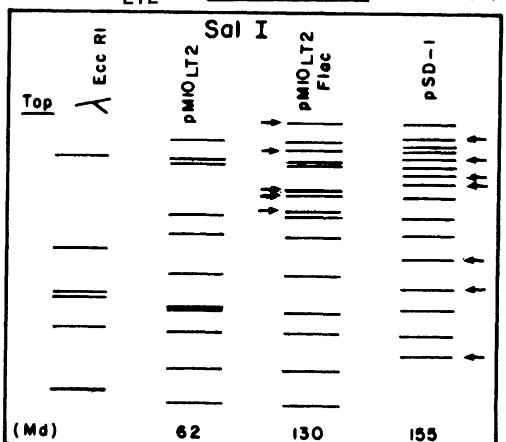
Salmonella typhimurium LT2 strains has been shown to carry a large plasmid of molecular weight 53.5-60 Mdal (1). The plasmid $PM10_{LT2}$, previously named pLT2, appears to be highly stable and compatible with plasmids of F-like incompatibility groups (2).

In *S.typhimurium* dnaC strains plasmid Flac is highly unstable in presence of $PM10_{LT2}$ (3). Stable Lac^+ derivatives of these strains were shown, by alkaline sucrose gradients, to posses a large plasmid (130 Md) which appears to be a cointegrate of Flac and the $PM10_{LT2}$ plasmid (4).

We have further studied the formation of this plasmid (pSD-1) which have shown to be dissociable, in order to understand the mechanism of association/dissociation of the *E.coli* Flac plasmid with the stable resident $PM10_{LT2}$ of *S.typhimurium*.

Using a modified method for high MW extrachromosomal DNA separation we have confirmed the presence of $PM10_{LT2}$ in *S.typhimurium* strains(5)



Analysis of DNA extracts of *S.typhimurium* dnaC $pM10_{LT2}$ Flac strains carrying Lac^+ character relatively stable have shown the presence of a plasmid (pSD-1) of 137 Md Agarose gel diggestion patterns of plasmids pSD-1, $PM10_{LT2}$ and Flac/$PM10_{LT2}$ with restriction enzyme Sal I provide a strong evidence in support of the hypothesis that pSD-1 contains part or all of plasmid $PM10_{LT2}$ and Flac. Digestion of these plasmids using endo nucleases EcoRI or Bam HI also suggest that pSD-1 contains a substantial part of both plasmids.

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ANALYSIS OF F SPECIFIC MEMBRANE PROTEINS

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Elucidation of the pathway which leads to the biosynthesis of F-pili has been complicated by the failure of previous analytical procedures to detect a pool of F-pilin in an unassembled state in the cell. Thus, gene products associated with synthesis of the polypeptide subunit have not been distinguishable from those which participate in its assembly into a pilus structure. We have analyzed 35-S methionine labelled membrane preparations from male and female cells by polyacrylamide gel electrophoresis using slab gels formed in an exponential gradient of 10-16% acrylamide. Under our conditions, an F specific band which co-migrates with purified F-pilin is resolved at an apparent molecular weight of 7,000 daltons. The F-pilin membrane band appears to represent a substantial pool of protein, and contains 4-5% of the total radioactive label in our whole membrane preparations. The polypeptide could be extracted from these preparations with Triton X-100, and was found in the inner membrane fraction of membranes separated on the basis of density. It would appear, therefore, that F-pili are assembled from the inner membrane. traJ mutations, which affect F tra operon transcription, and mutations in traA, the structural gene for F-pilin, resulted in the absence of the F-pilin membrane polypeptide. The F-pilin band was still present, however, in membrane preparations from strains carrying mutations in traL, traE, traB, traV traW, traC, traU, traF, traH or traG, despite the inability of these mutants to elaborate F-pili filaments. This suggests that the products of these genes are concerned with F-pilus assembly and outgrowth rather than F-pilin biosynthesis. Analysis of Hfr deletion mutants, however, indicated that a previously unidentified tra operon activity, located between traF and traH is essential for the appearance of the F-pilin membrane polypeptide. We have named this locus traQ, and suggest that the traQ product is required for processing of the traA product to F-pilin.

Several other F specific polypeptides could also be detected in our membrane preparations. These included a 100,000 dalton polypeptide, identified as a product of traG, a 23,500 dalton polypeptide which co-migrated with traJ product, and a 12,000 dalton polypeptide. Presence of the 12,000 dalton protein is affected by amber mutations in traD.

DETECTION OF ENTEROTOXIGENIC *ESCHERICHIA COLI*

BY DNA COLONY HYBRIDIZATION

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Current methods for the identification of enterotoxigenic *E. coli* involve immunologic or biologic assays for the presence of enterotoxins, and are not entirely suitable for screening large numbers of strains. The object of this study was to test an alternative technique based on the detection of the genes encoding the enterotoxins rather than the toxins themselves.

Genes encoding the heat labile (LT) and heat stable (ST) toxins of *E. coli* have been isolated and characterized by recombinant DNA techniques (1,2,3). Portions of these isolated toxin genes were used as hybridization probes for homologous sequences in strains of *E. coli* isolated from patients with diarrheal disease. Isolated strains of diarrheal stools were inoculated onto nitrocellulose paper (NC) which had been placed on the surface of MacConkey's agar. After incubation at 37°C, the NC was removed from the agar and the resulting colonies lysed *in situ*. The DNA was fixed to the NC and hybridized with radiolabeled gene probes by a modification of the colony hybridization technique of Grunstein and Hogness (4). The strains were also tested for ST and LT production by the infant mouse assay and the CHO cell assay, respectively.

All of 31 strains producing ST+LT or LT-only were detected by the LT probe. The ST probe detected 12 of 17 ST-only strains (70%) and 3 of the 26 ST+LT strains (12%). These results suggest that while LTs produced by different strains of *E. coli* are homologous, there are at least two heterologous STs that are detectable in the infant mouse assay.

This method is suitable for screening very large numbers of strains and detects LT producing strains with complete accuracy. Preliminary data suggest that a probe prepared from one of the ST genes not detected by the ST probe in this study may allow more complete detection of ST-only strains. Isolation and characterization of other genes encoding virulence factors from a variety of organisms will allow a more general application of analagous genetic hybridization techniques to the study of infectious diseases.

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STRUCTURE, REARRANGEMENTS AND MODIFICATION IN THE SMALL UBIQUITOUS PLASMID OF *NEISSERIA GONORRHOEAE*

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About 95% of clinical *Neisseria gonorrhoeae* isolates possess a small 4.2 kilobasepair plasmid. A small unique DNA segment can be deleted from a specific place on this plasmid molecule. The specific deletion was accurately mapped on the plasmid and the DNA spanning the deletion site was sequenced. The DNA sequence of the wild type and of two deletion bearing plasmids revealed that the exact same 54 bp had been lost in the deletion plasmids. One end point of the deletion was flanked by two blocks of 20 bp long sequences that was nearly homologous to two sequence blocks flanking the other end point. The two respective blocks were separated by the sequence -A T C A- and -A G C A-, respectively. In the deleted plasmids one sequence block from each end point was retained. However, unexpectedly the sequence separating these blocks was -G T C G-. A nonequal crossover event between nearly homologous sequence blocks associated with specific base alterations could explain the deletion event. Gonococcal DNA is difficult to cleave by a number of restriction enzymes. We have evidence for that the partial or complete resistance to the restriction endonucleases HaeII (NgoI), HaeIII (NgoII), SacII (NgoIII) and BamHI is due to modification. During the sequencing of the 4.2 kbp gonococcal plasmid we found a HaeIII/NgoII (-G G C C-) site where the internal cytosines are modified. Interestingly, this site is part of both a BgII and a HpaII/MspI site. BgII and MspI will not cleave at this site whereas HpaII will. The results show that: i) HpaII but not MspI can cleave if one of the external cytosines is methylated, ii) BgII cannot cleave if the cytosine at the 3' end of its recognition sequence is modified. Other recognition sequences were also difficult to cleave without any evidence for DNA modification. In some cases these sites on the plasmid were associated with short DNA repeats.

PLASMIDS IN EPIDERMOLYTIC STRAINS OF STAPHYLOCOCCUS AUREUS

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Some strains of *S. aureus* cause blistering conditions of the skin called the Scalded Skin Syndrome. A diffusible exotoxin, epidermolytic toxin (ET) caused the epidermal splitting. Two serological types of ET (types i and ii) have been characterized. Some strains produce type i and ii ET alone, while others produce both serotypes.

We have examined 34 ET-producing strains of *S. aureus* for presence of plasmid DNA. All serotype ii producers carried a 42kb plasmid. Elimination of this plasmid caused simultaneous loss of toxin and bacteriocin production (Bac^+). This suggested that the serotype ii ET genes were linked to Bac^+ on this plasmid. Type i ET was never eliminated and was presumably chromosomally determined. Some serotype i strains also contained a 42kb plasmid but its elimination only resulted in the loss of bacteriocin production. In some strains cadmium resistance was also linked to the 42kb plasmid.

The 42kb plasmids from seven strains expressing different phenotypes were analysed with restriction endonucleases *Eco*R1 and *Hind*III. The plasmids shared 19 of 22 *Hind*III fragments indicating that they are closely related to each other.

REPLICATION, INCOMPATIBILITY AND ACRIDINE ORANGE CURING OF F IN *E. COLI* K12

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In recent years, several investigators reported the conflicting incompatibility behavior of the composite plasmids (Cabello *et al.*, 1976; Ida, 1980; Katz and Palchaudhuri, 1980). In most cases, the multicopy replicon was joined to replicon with low copy number. We studied the incompatibility characteristics of a composite plasmid, pWS1 (the pSC101 plasmid-fragment 5 of F) which can use the replication of either of the two functionally distinct replicons. We aimed to determine the functional replicon of pWS1 by testing the sensitivity to acridine orange (AO). At subinhibitory concentration of AO, the 30% of cells had lost pWS1 even though only one component (f5) was AO sensitive. Presumably, the cells (70%) which escaped the inhibitory effect of AO, were using the pSC101 replicon of pWS1. Under these *in vivo* conditions, we compared the incompatibility of pWS1 against a number of well characterized F' plasmids in *E. coli* K12 hosts ($recA^+$, $recA^-$). The presence of genetically stable transconjugants carrying both plasmids was further confirmed by gel electrophoresis and electron microscopy. These results indicated the weak incompatibility behavior of pWS1 compared to the incompatibility of mini F. In *recA* background, the incompatibility of pWS1 against F's is little reduced. Hence it can be concluded that functional F-replicons show stronger incompatibility properties. As a corollary it was found that the plasmid $ColVtrp^+$ showed partial incompatilbility with F's and was highly compatible with pWS1. Our present data suggest that $ColVtrp^+$ is a double replicon and one of them is F replicon. (See Table on page 2). Incompatibility of pWS1 was further tested by mating the pWS1 containing recipients with Hfr donor. In this case the incompatibility was measured by comparing the number of recombinants from Hfr x MA140 (pWS1) and Hfr x MA140 (pSC138) matings. A few recombinants were formed by the recipients carrying miniF, pSC138,

Infecting Plasmid	Resident Plasmid	Selection for Markers	% of Daughter Colonies Incoming Only	Resident Only	Containing Both
F'Trp+	pWS1(Tc)	Trp+	5	0	95
		Trp+,Tc	1	1	98
F'His+	pWS1(Tc)	His+	48	0	52
		His+,Tc	6	4	90
ColVTrp+	pWS1(Tc)	Trp+	0	0	100
F'His+	Mini F	His+	95	0	5
	(pSC138)	His+,Amp	65	35	0

whereas the F^- or pWS1 carrying recipient cells showed much higher number of recombinants.

The F prime plasmid superinfected into the male recipients (F^+) was converted to the covalently closed, circular duplex form as in the F^- recipients but the subsequent replication of circular duplex of superinfected F was blocked due to incompatibility (Saitoh and Hiraga, 1975). The Hfr donor cells transferred the genes determining inc, rep and pif functions of the integrated F into recipients (male or female) along with early chromosomal markers (Palchaudhuri Ms in prep.). It is conceivable that the mechanism of incompatibility is primarily related to the initiation of replication of the circular duplex F-DNA subsequently followed by its proper distribution into daughter cells. This early step seems to be controlled by proteins, synthesized by the host in limited amount. Our data suggests that the pWS1 which can use pSC101 replicon does not recognize these proteins as efficiently as mini F and thus the F-specific incompatibility is relaxed.

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VIRULENCE FOR MICE OF PLASMID-BEARING EPIDEMIC STRAINS OF SALMONELLA TYPHIMURIUM

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From a sample of 84 multi-resistant strains of *Salmonella typhimurium* obtained from outbreaks in children's hospitals of nine cities of five South American countries we selected at random two to four strains from each outbreak, 30 strains in all. The strains, with transferable drug-resistance, were of high virulence for children as judged by their transmissibility, systemic spread and high fatality rate. The epidemic strains and 18 normal, sensitive strains were tested by i.p. challenge in mice.

The arithmetic mean of the LD_{50} is 1.37×10^7, compared with a mean LD_{50} of 2.65×10^5 for the sensitive strains ($P < 0.0005$). Thirteen of the epidemic strains, highly virulent for children, have an LD_{50} over 10^7 and on the average they are 80 times less virulent than strain CDC 9 kept in the laboratory for over 40 years.

The unexpected results show that apparently the factor(s) which determine virulence for children are not the same as those for mice. It is suggested that the outbreaks are the consequence of the sudden emergence of a strain endowed with high virulence for children and that the genetic material carried by the transfer factor together with the resistance determinants might be held responsible for the increased virulence for children and low virulence for mice. Bacterial virulence depends on a delicate adjustment between the biological characteristics of the pathogen and those of the host. It is not a biological heresy to admit that the loss or acquisition of a metabolic function or structural character may change the pathogenicity in opposite direction for different hosts.

A RAPID METHOD FOR DETERMINING PLASMID INCOMPATIBILITY GROUP

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A rapid method for determining plasmid incompatibility group by determining plasmid size by agarose gel electrophoresis is described.

The method involves checking one plasmid against another to see if they are incompatible. One plasmid is transferred to a strain carrying the other plasmid, selecting only for the incoming plasmid. Transcipients are then checked by agarose gel electrophoresis to see if both plasmids are still present; if the resident plasmid is missing, the two plasmids are clearly incompatible. If both plasmids are still present independently, transcipients are grown up overnight in non-selective medium. Clones are then checked for their plasmid complement: if a plasmid has been lost, the pair of plasmids are incompatible, while if both are present, they are compatible.

To demonstrate the method, two naturally-occurring plasmids (pSE 6 and pSE 16), originally isolated from Salmonella typhimurium, were checked against the plasmid R386 that belong to the incompatibility group FI.

The data indicate that pSE 16 and R386 are incompatible, whereas pSE 6 is compatible with R386. Thus pSE 16 belongs to the incompatibility group FI.

Since this method involves examination of the plasmids themselves, it is much faster that the classical method of incompatibility testing and takesfewer working hours. Futhermore, since no changes in the plasmid DNA need be induced to give unique markers on each plasmid, there is no chance of incorrect classification due to alterations in the inc genes,

GENTAMICIN RESISTANCE IN CLINICAL ISOLATES, "PICKING-UP" OF R-DETERMINANTS ON PHAGE P1

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As a first step towards epidemiological studies on plasmid-encoded gentamicin resistance (gen^r) in gram-negative bacteria from human and animal sources the ability of transposition of the respective r-determinants was investigated. None of the gen^r genes from 48 different R-factors (RF) could be moved onto λ b515 b519 cI857 s7. However, out of 17 tested RF 10 gave rise to high-frequency-transducing (hft) gen^r derivatives of phage P1 cm0 c1ts100.

In the cases studied gen^r was always accompanied by other RF derived antibiotic resistances when "picked-up" by P1. First step (generalized) transduction frequencies of the gen^r phenotype varied between about 10^{-3} and below 10^{-7} per chloramphenicol resistant lysogens formed.Among those hft P1 gen^r derivatives were found within a range from below 5% up to 100% of the heat-inducible gen^r primary transductants.

The analysis of the DNA segments inserted into P1 prophage plasmids revealed the following: (i) their lengths varied within about 15 to 60 kb, but (ii) were constant in independent isolates from a given RF with the same phenotype transduced; (iii) the sites of the insertion into the genome of P1 cm0 varied from isolate to isolate, but (iv) were found to be always within either one of the BglII-2 or BglII-5 fragments (within map units 3 through 35). Further investigation will involve an analysis of their structure and mechanism of movement, and of possible homologies between them.

In conclusion, if the gen^r genes stably integrated into the DNA of phage P1 are within true transposons (which has to be proven), they seem to be mostly part of larger r-determinants together with other resistance genes. Since gentamicin was comparatively late intoduced into clinical use, the resistance conferring genetic material might have been preferentially incorporated into preexsisting selftransmissible elements. Also, the ease with which phage P1 accepts and transduces large fragments of RF's and its broad host range could suggest that this kind of phages might be potent vehicles for the distribution of r-determinants in natural environments.

GENETIC CHARACTERIZATION OF RESISTANCE PLASMID CONTENT IN THREE SALMONELLA SEROTYPE WHICH PRODUCED EPIDEMIC NOSOCOMIAL INFECTIONS.

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Strains of Salmonella (S. enteritidis bioser Java, ser Saintpaul and ser Havana) which showing complex phenotype resistance produced important epidemic nosocomial infections, involving one or more hospitals, were analyzed in regard to their resistance plasmid content. Except for the resistance for nalidixic acid and nitrofurantoin present in some strains, which is a chromosomal type, any other resistance found, was an extrachromosomal type, originated in a polyplasmid state, by the presence of autotransferable and non-autotransferable plasmids. In bioser Java are characterized the autotransferable plasmid H_2 R (Te-Cm) and a new plasmid F-like R (Kn-Nm-Am-Cb-Cr) which is able to propagate the phage fd but not u_2, it does not show incompatibility with any known plasmid F-like but it is incompatible with F-like plasmids that showing similar characteristics have been isolated locally from S. sonnei and S. anteritidis ser Typhimurium. There are others two non-autotransferable plasmids r (Am-Cb) and r (Su-St). In ser Saintpaul coexist two autotransferable plasmid R (Su-Te-Cm-Kn-Nm-Am-Cb-Cr) and R (Am-Cb-Cr) incompatibility groups H_1 and $I_1\alpha$ respectively and r (St) determinant. Ser Havana has an autotransferable plasmid R (Te-Cm-Kn-Nm-Am-Cb-Cr) incompatibility group H_2 and two non-autotransferable plasmids r (Kn-Am-Cb-Cr-Gm) and r (Su-St). In the first two serotypes the polyplasmid state provides for a double mechanism of resistance for ampicillin, carbenicillin and cephalosporins. The whole analysis of the plasmid content allows to deny or to assert the epidemiologic relationship that exists between resistance plasmids, which determine a similar complex phenotypic resistance, and appear in epidemics that happen during certain period of time in a some hospital or different hospitals.

EPIDEMIOLOGY OF ANTIBIOTIC RESISTANCE IN THE BACTEROIDES FRAGILIS GROUP

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Bacteroides fragilis is the most frequently isolated anaerobic organism in human infections. In this species tetracycline (Tc) and macrolide-lincosamide-streptogramins (MLS) resistances have been recently demonstrated to be plasmid-mediated and transferable. In the strains which initially came to our observation, the Tc resistance appeared to be inducible; the transfer ability (tra) of Tc resistance was contemporaneously induced when the bacteria were grown in the presence of subinhibitory concentrations of Tc before mating[1].

In the order to assess the epidemiology of transferable antibiotic resistance in the B. fragilis group, we examined 63 clinical isolates sent for identification to the Anaerobe Reference Center in Paris. The prevalence of Tc and MLS resistant strains was 82,5% and 2% respectively. All Tc resistant strains were studied for inducibility (i) vs. constitutivity(c) for expression and transfer ability of the resistance. 60% of the strains were transfer proficient after induction (tra_i) or constitutively (tra_c); of these, about two thirds were Tc_i tra_i, but $Tc^r{}_c$ tra_c and dissociated phenotypes were also found. Among the strains wich did not transfer the resistance both $Tc^r{}_i$ and $Tc^r{}_c$ phenotypes were observed. MLS resistance is almost constantly associated with Tc resistance. Except one strain which is able to transfer MLS^r independently, this character is either not-transferable or co-transferred with the Tc resistance. In the tra_c strains, the number of all transcipients was increased by Tc induction, but for a given strain the percentage of MLS cotransfer was remarkably stable.

Although different lysis and preparative techniques were employed, we failed to demonstrate any plasmid DNA in most wild-type resistant strains and in their transconjugants.

Experiments performed in gnotoxenic mice colonized with B. fragilis strain 92 $Tc^r{}_i$ tra_i showed that the Tc resistance and transfer ability could be induced in vivo by sub-inhibitory concentrations of the antibiotic. These properties appeared to be quickly lost once the antibiotic was withdrawn; the prolonged administration of higher doses lead however to the isolation of strains constitutive for Tc resistance and transfer ability. The transfer of MLS resistance was observed in vivo in the absence of antibiotic selective pressure.

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IDENTIFICATION OF A BACTERIAL PATHOGENICITY DETERMINANT MODULATED BY PROPHAGE GENES.

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We have previously shown in Klebsiella pneumoniae a special case of lysogenic conversion which seems to be regulated by the expression of immunity to superinfection[1]. We have now studied if the conversion phenomenon, where the prophages FR2 and AP3 cause repression (or masking) of the receptors for the phages P1 and T3-T7 respectively also changed other properties of the converted cells. A possible influence on adherence to human epithelial cells (EC) was studied first. We have found that the non-converted Klebsiellae adhere to EC from intestine (75 bact./EC), oral cavity (180 bact./EC) and urinary tract (75 bact./EC), while the derivatives lysogenic for FR2 did not. Strains cured from AP3 showed the same adherence capability as the non-lysogenic parental strain[2]. The influence of lysogenization by AP3 and FR2 on resistance to phagocytosis by human neutrophils was then studied. It was found that the strains lysogenic for AP3, but not those lysogenic for FR2, were more sensitive to uptake (5 fold) and intracellular killing (8 fold) than the non-lysogneic parental strain. LD_{50} in mice of the non-lysogenic strain sensitive to T3 and T7 was 150 fold lower than that of the strian converted by AP3 to coliphage resistance. The AP3 lysogenic recombinants in which loss of immunity gene was transferred[3] showed the same adherence capability, resistance to phagocytosis and LD_{50} values as the non-lysogenic parental strain. Spontaneous mutants resistant to T3 and T7 showed adherence capability, sensitivity to phagocytosis and pathogenicity for mice similar to those of the AP3 lysogens. AP3-like phages, induced from two non-adhesive Klebsiellae isolated from clinical specimens, were able to convert bacteria to T3-T7 resistance. These lysogens were non-adhesive. Their sensitivity to phagocytosis and pathogenicity for mice were similar to those of the strains lysogenic for AP3.

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SIMULTANEOUS EXPRESSION OF CFA/I AND ST IN 0128 ac SEROTYPES OF *E. COLI* ISOLATED IN SÃO PAULO

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Escherichia coli strains of serotypes 0128 ac: H12 and 0128ac:H7 producing the heat-stable enterotoxin (ST) and the colonization factor CFA/I were isolated from children with diarrhea in São Paulo.

Genetic studies and analysis of plasmids by agarose gel electrophoresis and/or electron microscopy revealed that: 1) In 3 strains of serotype 0128ac:H12 (strains TR438/1, TR14/1 and TR99/1) and in 1 strain of serotype 0128ac:H7 (TR780) the expression of CFA/I and ST is coded for by genes from a single plasmid; 2) The CFA/I-ST coding plasmids of these strains are not self-transmissible and need for transfer a conjugative plasmid (plasmid B) which was isolated from strain TR438/1; 3) The CFA/I-ST coding plasmid of strain TR780 differs from plasmids of the other strains, being incompatible with the conjugative plasmid B; 4) The CFA/I-ST plasmid isolated from strain TR438/1 is 97 kilobases long and plasmid B has a length of 64 kilobases as determined by electron microscopy; 5) The molecular weight of the CFA/I-ST plasmids of strains TR14/1 and TR99/1 is about 49×10^6 daltons while that of the CFA/I-ST plasmid of strain TR780 is 59×10^6 daltons, as determined by agarose gel electrophoresis.

ON THE TRANSFER SYSTEM DETERMINED BY PLASMIDS BELONGING TO INCOMPATIBILITY GROUP S

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All plasmids belonging to an incompatibility group appear to determine the sinthesis of a particular transfer system.

Plasmids isolated from Serratia marcescens that were originally classified as incompatibility group S (1) determine a transfer system which is optimum when host strains are grown at 22°C (2). This transfer system typified by plasmid R477, appears to be different from plasmids of all other incompatibility groups so far studied.

Plasmids isolated from other genera of bacteria ie. S.typhimurium, K.pneumoniae, S.flexneri, S.anatum, C.freundii, E.cloacae and S.typhi and classified into incompatibility group H2 also appear to produce a transfer system that is most efficient at low temperature.

Incompatibility relationship between plasmids belonging to inc. groups S or H2 have been studied in order to demostrate our hypothesis that they share a unique transfer system (3). Inc S plasmids are indeed incompatible with plasmids Mip235, R1022 and pSD114 (inc H2).

We have studied further the transfer system of inc S/H2 plasmids. Our results support those of Rodrîguez Lemoine et al (2) that the growth temperature of the donor affect significantly transfer of all those plasmids grouped previously into inc. group S. The incubation temperature of recipient cells has little effect on transfer efficiency. Temperature of mating appears to has not effect on transfer efficiency of any of the S/H2 inc. plasmids studied. We have used different conditions that Taylor et al (4) but we found no appreciable differences using temperatures of 24°C or 37°C during 1 h or 10 min of mating in liquid medium.

We have found that temperature of growth of cells (24°C-37°C) carryng plasmids pSD114, Mip235 and R1022 but not N-1 appears to have little or not effect on their transfer.

The growth temperature of the donor cells appears to effect one to the early stages related to the union formation of pairs or aggregate of donor and recipient cells. When donor cells grown at 37°are mixed with recipient cells and forced to bring together (i.e by conjugation on solid surfaces or using filters) the frequency of transfer is increased to close the efficiency of donor grown at 24°C.

Presence of common fimbriae in donor cells but not in recipient cells appears to play an important role in transfer of S/H2 plasmids.

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A SIMPLE PROCEDURE FOR THE DETECTION OF "CRYPTIC" CONJUGATIVE PLASMIDS OF THE INCOMPATIBILITY GROUP N

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Conjugative plasmids of the incompatibility group N are widely distributed and frequently isolated from natural populations of bacteria - often, but not exclusively, from Enterobacteriaceae. Sometimes, such plasmids, although conjugative, have been found to be otherwise cryptic. Their isolation supports the concept that in natural environments their exist pools of such plasmids that specify only functions related to bacterial conjugation and that they acquire other genes by recombination events such as those mediated by transposons. The detection of such "cryptic" conjugative plasmids is usually by indirect methods which involve their ability to mobilize genes from non-conjugative plasmids. Although bacteriophages specific for this group of plasmids exist, phage sensitivity tests frequently require first the transfer of the plasmid to a suitable standard host. We describe a relatively simpler procedure for detecting such plasmids. This procedure should facilitate and encourage the determination of the proportion of such "cryptic" conjugative plasmids in collections of N group plasmids from particular environments and provide information that could be useful in epidemiological or evolutionary studies.

The test is based on the observation that a variety of species of gram negative bacteria harbouring conjugative N group plasmids whether "cryptic" or "non-cryptic" kill *Klebsiella pneumoniae* strain M5al. Plasmids of the groups P and W but not other groups also mediate killing of the *K. pneumoniae* strain but to a degree that is less than with the N group plasmids. The mechanism of killing is being investigated.

Procedure - Grow the test culture and strain M5al to late exponential phase in Penassay Broth at 37 C with aeration. Spread 0.1 ml of M5al (10^7-10^8 cells) on Penassay Agar. After the surface has dried, place a drop of the test culture on the M5al-seeded surface. Incubate the plate for 24 hours at 37°C and observe for the inhibition of M5al at the area inoculated with the test culture. The procedure lends itself well to the use of multipoint inoculating devices enabling the simultaneous screening of large numbers of cultures. (We are grateful to M. Arroyo and H. Tschape for some of the test strains.)

INDICATOR PLATES FOR CHLORAMPHENICOL RESISTANCE IN ENTEROBACTERIACEAE MEDIATED BY CHLORAMPHENICOL ACETYLTRANSFERASE

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Rosanilin dyes such as crystal violet and basic fuchsin can be used as indicator dyes in agar plates to distinguish betwee chloramphenicol sensitive colonies and chloramphenicol resistant colonies containing the inactivating enzyme chloramphenicol acetyltransferase (CAT). On certain media containing rosanilins, enterobacterial colonies containing CAT are more darkly colored than colonies not containing this enzyme. This permits the direct detection of chloramphenicol sensitive cells in a population of chloramphenicol resistant cells by plating on agar medium containing rosanilin dyes but lacking chloramphenicol. This method should be valuable in cloning experiments using insertional inactivation of the CAT resistance gene. Enterobacteriaceae harboring unstable plasmids conferring chloramphenicol resistance form sectored colonies on rosanilin dye indicator plates. The color difference between chloramphenicol sensitive and resistance colonies is less dramatic than the color difference between Lac$^+$ and Lac$^-$ colonies on MacConkey lactose medium, but is sufficient for unambiguous distinction.

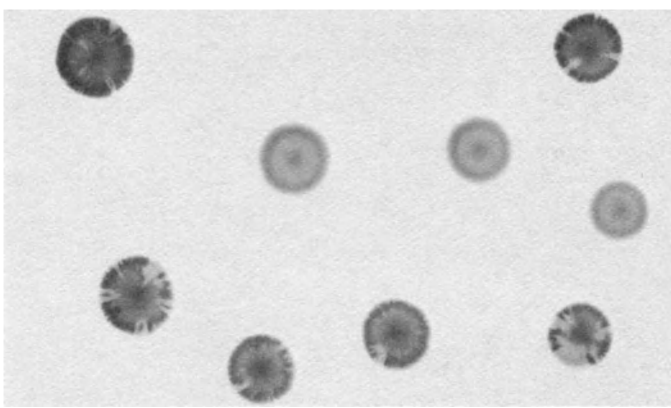

Figure Legend: Sectored colonies of *Salmonella typhimurium* harboring the R plasmid NR1 on broth plates containing 2 μg/ml crystal violet. The spontaneous loss of the r-determinants component of NR1 in *S. typhimurium* during colony formation results in the lighter colored sectors within individual colonies which contain chloramphenicol-sensitive cells. Non-sectored, light-colored colonies are colonies which do not contain the r-determinants component.

PLASMID-CODED DEGRADATION OF SALICYLIC AND ISOVANILLIC ACIDS IN THE SOIL BACTERIUM K17

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The soil bacterium K17[1] is able to degrade the lignan alpha-conidendrin via the intermediate isovanillic acid (4-methoxy,3-hydroxy benzoic acid) and grows on a number of aromatic carboxylic acids and lower aliphatic alcohols[2,3]. In K17, as in a great number of bacteria isolated by us from lignin-containing wastes[3], a number of phenotypes were found to be unstable and were irreversibly lost at high frequency with growth on nutrient media[4]. The frequency of loss was increased by introduction of the IncP plasmids RP4 and R68.44 into the strain.

K17 strain degrades salicylic acid via gentisic acid[2] after 5-hydroxylation of the aromatic ring. Isovanillic acid is degraded via protocatechuic acid. K17 easily lost the ability for growth on salicylic and isovanillic acids, but such "cured" mutants still grow on gentisic and protocatechuic acids.

The sal and the isovan phenotypes could be reintroduced into the cured mutants by conjugation with the parent strain, and also to heterologous recipients such as *Pseudomonas putida* PAW85, and with a lower frequency to *Agrobacterium tumefaciens* LBA202, provided that a conjugative P-plasmid was first introduced into the donor strain. The sal and the isovan phenotypes hitch-hiked into the recipient also when selection was made for the transfer of the R-plasmid only.

A large plasmid (over 100Md) was transferred from the K17 donor into the *Agrobacterium* recipient along with the sal^+ phenotype.

We conclude that the degradation of salicylic acid and isovanillic acid is in K17 coded for by a plasmid.

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MANIPULATION OF THE GENES CODING FOR THE HEAT-LABILE ENTEROTOXIN OF *ESCHERICHIA COLI*

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Plasmid P307, isolated from a porcine strain of *E. coli* (1) carries genes (*elt*A and *elt*B) (2) which specify the *synthesis* of a heat-labile *enterotoxin*. These genes have been cloned into pBR313 to produce plasmid EWD299 (3).

Plasmid pUB1841 was generated *in vitro* from EWD299 by deleting the small EcoRI fragment *carrying* most of the *elt*B gene. An intact and functional *elt*A gene is left (3). Plasmids pUB1844 and pUB1845 were constructed by cloning the small HindIII fragment of EWD299, which carries *elt*B, into pACYC184 (4) in the two possible orientations. When whole cell lysates (WCL) (or cell free supernatants CFS) of UB5201 (pUB1841) were mixed with WCL (or CFS) of UB5201 (pUB1844 or pUB1845) no toxin activity was detected using the Y-1 tissue culture cell assay. In contrast, when pUB1841 and pUB1844 were both carried by UB5201, toxin activity comparable to that found in UB5201 (EWD 299) was observed. When pUB1845 replaced pUB1844 toxin activity was decreased to about 1%. The host strain is a *rec*A strain of *E. coli* and tests confirmed that no recombination between pUB1841 and pUB1844 or pUB1845 had occurred to any detectable extent. The results indicate that either *elt*A and *elt*B have separate promoters or, when cloned into pACYC184 *elt*B is under the control of an external promoter.

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GENETIC AND PHYSICAL CHARACTERISTICS OF ENTEROTOXIN PLASMIDS FROM HUMAN STRAINS OF ESCHERICHIA COLI

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Enterotoxigenic strains of *E. coli* isolated from several sources were shown by genetic and physical analysis to possess plasmid DNA encoding the heat-labile enterotoxin genes. A study was conducted to investigate the relationship among six enterotoxin plasmids transferred into an *E. coli* K12 in the basis of incompatibility, repression and restriction analysis. The study have shown that all of them belongs to the incompatibility group L, and represses the *tra* genes of F-like plasmids. Analysis of plasmid DNA fragments on 1% agarose gel, revealed common genetic sequences. In two of them an identical cutting pattern was observed, indicating that they are a unique plasmid in different strains of clinical isolates. (This research was supported by grant 2222/15 81/77 - CNPq)

BINDING AND UPTAKE OF PLASMID DNA DURING TRANSFORMATION OF $CaCl_2$-TREATED *ESCHERICHIA COLI*

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Little is known of the mechanisms involved in uptake of plasmid DNA during transformation of $CaCl_2$-treated *E.coli*. Cells harvested in early exponential phase produce about 200 times more transformants at saturating DNA concentrations than cells in lag or stationary phases[1]. The efficiency of transformation (transformants/µg DNA at non-saturating concentrations), the amount of DNA required to saturate the transformation capacity of a fixed number of cells, and the total number of transformants at saturation increased to a maximum in early exponential phase (A_{660} = 0.1 - 0.2) and then declined progressively. Transformation was most efficient at a time when the modal volume of cells in the culture was greatest and when the size distribution was skewed towards cells of large volume. However, there cannot be a simple relationship between cell volume and transformability since during growth in batch culture the former varies over a two fold range whereas the latter varies about 200 fold.

About 10% of ^{3}H-NTP16 DNA in a transformation mixture remained tightly bound to the outside of cells in the presence of Ca^{2+}. $<$10% of such tightly bound DNA subsequently became DNase-resistant after a 42°C heat-pulse. When the system was just saturating, 1-2 molecule equivalents of NTP16 DNA (Mol. wt. 5.7×10^6)/viable cell became DNase-resistant, but $<$1% of viable cells became transformed. This suggests that a large proportion of DNase-resistant DNA may be located in the periplasm after the heat pulse. Separation of membrane fractions (in the absence of DNase) after transformation showed that $>$90% of bound DNA remained attached to the outer membrane. If cells were treated with DNase after the heat-pulse then most of the small amount of labelled DNA remaining was associated with inner membranes. Plasmid DNA bound equally well *in vitro* to isolated inner or outer membranes. The efficiencies of divalent cations in promoting binding to membranes or whole cells ($Ca^{2+} \gg Ba^{2+} > Sr^{2+} > Mg^{2+}$) paralleled their ability to induce transformability. DNA binding was greatly reduced if outer membranes were treated with trypsin. Proteolytic enzymes also reduced transformation frequencies in intact $CaCl_2$-treated cells, suggesting that protein components of the cell envelope are required for binding and/or transport of DNA during transformation.

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INTERGENERIC MOBILIZATION OF NONCONJUGATIVE R PLASMIDS BY 24.5 MEGADALTON CONJUGATIVE PLASMID OF *NEISSERIA GONORRHOEAE*

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Some strains of *N.gonorrhoeae* carry conjugative plasmids of 24.5 Mdal[1] that are capable of mobilizing gonococcal β-lactamase plasmids[3]. We investigated the ability of one such plasmid, pLE2451[2], to mobilize nonconjugative plasmids in intergeneric tri-parental matings. Strains of *N.gonorrhoeae* carrying pLE2451 could mobilize plasmids residing in an intermediate donor $hsdR_k^- M_k^+$ strain of *E.coli* to an $hsdR_k^- M_k^+$ recipient. However, pLE2451 itself could not be detected physically in either strain of *E.coli*. This indicated that pLE2451 was unstable in *E.coli* but could survive sufficiently long to express mobilization functions. 3.2 Mdal and 4.4 Mdal gonococcal β-lactamase plasmids, plasmids originally isolated from enteric bacteria (ColE1 and NTP5), from *H.parainfluenzae* (RSF0885) and from *H.ducreyi* (pJB1) were mobilized between strains of *E.coli* by pLE2451 at frequencies of 10^{-3} to 10^{-4} per initial donor. In contrast, the enteric plasmids NTP1 and NTP16, unlike other plasmids also encoding TEM β-lactamase, were not mobilized, presumably because appropriate mobility functions were not provided. However, transfer events involving transient survival of conjugative plasmids might play a general role in the dissemination of nonconjugative plasmids between bacterial species.

The molecular relatedness between β-lactamase plasmids found in *N.gonorrhoeae* and *Haemophilus* species has prompted speculation that the gonococcus may have acquired such plasmids from *Haemophilus*[1]. None of the β-lactamase plasmids tested in our experiments could be mobilized from *E.coli* by pLE2451 if the final recipients were strains of *N.gonorrhoeae* or *Haemophilus influenzae*. This suggests that a substantial restriction barrier operates against passage of plasmids from *E.coli* to these organisms. Furthermore intergeneric mobilization experiments indicated that transfer of β-lactamase plasmids mediated by pLE2451 occurs much more readily out of the gonococcus to *Haemophilus* than in the reverse direction. In addition, the conjugative plasmids pUB701, pFR16017 and pRI234, isolated originally in *H.influenzae*, were incapable of mobilizing either gonococcal or *Haemophilus* β-lactamase plasmids, even in intraspecies crosses. Thus conjugative transfer from *Haemophilus* to *Neisseria* species as an origin of gonococcal β-lactamase plasmids would seem to be an infrequent event *in vivo*.

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INTRASTRAND BASE PAIRING IN SINGLE-STRANDED DNA FROM pCR1 AND ITS POSSIBLE RELATIONSHIP TO ROLLING CIRCLE TRANSFER REPLICATION

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Numerous studies have indicated the existence of important short range intrastrand base pairing in DNA and RNA. The presence of such pairing can be inferred by inspection of the base sequence or by computer analysis. Long range interactions have not been as extensively studied and interacting regions at present cannot be readily predicted even by computer analysis. Our approach to this problem has been by electron microscopy to locate the interacting regions relative to an origin such as a transposon insertion site or unique ends created by a restriction enzyme. Standard formamide-cytochrome c spreading conditions modified by the addition of ammonium acetate are used to visualize reproducible stem and loop structures resulting from long range base pairing. The nucleotides responsible are then identified from the base sequence of the DNA.

Rolling circle DNA replication begins when a single strand nick is introduced at the origin of DNA synthesis, creating a 5' and a 3' end. The DNA strand displaced during synthesis may remain single-stranded. Transfer DNA replication probably proceeds by a rolling circle mechanism (Rupp, W.D. and Ihler, G. (1968) Cold Spring Harbor Symp. Quant. Biol 33, 647-650). Previous results with ϕX174 DNA (Edlind, T. and Ihler, G., (1980) J. Mol. Biol., 142, 131-144) demonstrated that base paired sequences bring the 5' and 3' ends of linear ϕX174 DNA, cleaved at the origin of viral strand replication, close together, which may facilitate their rejoining by ϕX174 gene A protein following DNA replication by the rolling circle mechanism. We have examined a small (13.7 kb) transferable plasmid, pCR1 carrying the transposon Tn903 for similar pairing. Unique ends were introduced with EcoRI. A 1.5 kb stem and loop often containing an internal hairpin was observed near the left end of the molecule and nucleotides likely to be responsible for this pairing have been located in the base sequence. Both the origins of vegetative and transfer DNA replication are located in this loop. Formation of the stem by long range base pairing would draw the 5' and 3' ends, created by cleavage at the origin of replication, closer together. Several molecules were observed in which the loop appeared completed paired, suggesting the existence of further, weaker base pairing which could hold the 5' and 3' ends much closer together and facilitate recircularization of the single-stranded DNA after transfer.

We have also found in pCR1 that Tn903 was inserted into a region already containing inverted repeat sequences and suggest that transposition may be facilitated by the presence of inverted repeats or potential long range base pairing.

This research was supported in part by NIH research grants GM24432 and GM27727.

CELL-TO-CELL TRANSFER OF R-PLASMIDS FROM *STREPTOCOCCUS FAECALIS* TO *STAPHYLOCOCCUS AUREUS*

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Erythromycin and clindamycin have proved useful as alternative therapies for *S. aureus* infections, especially in the penicillin-allergic patient as well as in some infections due to methicillin-resistant strains. Between 1978 and 1980, clinical isolates obtained at the University of Michigan Medical Center have exhibited an increase in resistance to these compounds with 5% of *S. aureus* strains showing resistance in 1978, while 40% of isolates are resistant in 1980. Resistance to these agents is frequently plasmid-mediated and thought to develop in *S. aureus* via transduction. However, a recent report by vanEmbden and coworkers [J. Bacteriol. 142:407 (1980)] suggested to us that transfer of macrolide-lincosamide-streptogramin (MLS) resistance plasmids from streptococci to *S. aureus* could contribute to the evolution of resistance in *S. aureus*. Mating experiments on filter membranes (overnight) using *S. aureus* 879 R-4 as a recipient and *S. faecalis* strain JH2-2 containing various known MLS R-plasmids as donors, we detected transfer as shown below:

Plasmid	Original Source of Plasmid	Transfer Frequency per Recipient
pAMβ1	*S. faecalis*	1×10^{-5}
pAM15346	*S. pyogenes*	5×10^{-5}
pAC1	*S. pyogenes*	7×10^{-6}
pIP501	*S. agalactiae*	1×10^{-6}

We also demonstrated transfer of pAMβ1 to three different clinical isolates of *S. aureus* at frequencies from 1×10^{-7} to 5×10^{-8}. When 25 clinical isolates of *S. faecalis* were examined, three could be shown to transfer MLS resistance to 879 R-4 at frequencies similar to pAMβ1. Matings were performed with *S. faecalis* carrying pAMα1 (a small non-conjugative tetracycline-resistance plasmid) in addition to pAMβ1 and mobilization of tetracycline resistance could be demonstrated at frequencies of 1×10^{-8}. These studies suggest that intergeneric R-plasmid transfer is a potential factor in the evolution of resistant strains of *S. aureus*.

PLASMID-CODED LOW-MOLECULAR WEIGHT RNA SPECIES

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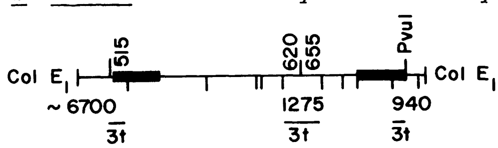
I. The evolution of antibiotic resistance transposons has selected two separate functions: antibiotic resistance and the ability to transpose between replicons. In some cases, it is possible to argue that antibiotic resistance can be "picked up" by a mobile DNA element to the mutual benefit of both. Such a model would explain, for example, the ability of the Tn5 inverted repeats (IS50 sequences) to transpose without the companionship of the neomycin phosphotransferase genes (see Berg, et al., this volume). On the other hand, the transposition and resistance functions may be functionally linked either *de novo* or by subsequent evolution of the Tn element (Reznikoff, et al., this volume). We (F. Schmidt, R. Jorgensen, M. De Wilde, and J. Davies) have recently identified a low molecular weight RNA species which is encoded by the inverted repeat of Tn10. By itself, this is not surprising; but this RNA (which when isolated contains two molecular species) is induced by tetracycline. Figure 1 shows the results of Southern hybridization of this RNA mixture to Tn10. Note particularly that the RNA hybridized to the outside 400 bp of Tn10 DNA. One can speculate that this RNA is in some way involved in transposition. Although we have not demonstrated such a connection, it is interesting that transposition of erythromycin resistance in *S. aureus* is induced by subinhibitory concentrations of drug (1).



II. We have also investigated in preliminary fashion, the tRNA-like RNA coded by the *tra* region of R100 (NR1 or R222). We showed earlier (2) that this RNA had a 3' C-C-A sequence and other characteristics (although not modified bases) of a tRNA. Furthermore, this RNA cross-hybridized to the cloned f6 fragment of the F plasmid which is contained in pRS5 (2). More recently, we have prepared and characterized an RNA similar to that from R100 which is coded by cloned F plasmid. The fingerprint analysis of this RNA shows that it is not identical to that of R100, but it is similar in size and, presumably, in sequence since R100 RNA hybridizes to the DNA from which it is derived. Supported in part by funds from the University of Missouri Medical School Research Council and NIH grant GM26756.

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CHARACTERIZATION OF MUTANTS OF A PLASMID ColE1 DERIVATIVE WHICH AFFECT PLASMID COPY NUMBER

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pDMS6642 (Fig. 1), which exhibits an increased copy number, was mutagenized, and plasmids conferring increased resistance to ampicillin were selected. One mutant plasmid, pLS103, showed an increase in β-lactamase proportional to the plasmid copy number (Table 1b). Two plasmids, pLS54 and pLS57, exhibited an enhanced β-lactamase production that was 3 to 8 times greater than that expected for the increased copy number found (see Table 1b). Ligation of the promoter of the β-lactamase gene of pDMS6642 or the mutants to the promoterless tetracycline resistance gene carried by plasmid pGA46 (2) indicated that pLS54 and pLS57 promoters exhibit a 3-fold greater transcriptional activity as measured by conferred drug resistance (Table 1d) than does the promoter carried by pDMS6642. In an *in vitro* linked translation system pLS54 and pLS57 DNA was found to be about 2.5 - 3-fold more effective as templates for the synthesis of β-lactamase than pDMS6642. The insertion of either plasmid pGA46 or a nucleotide sequence containing a chloramphenicol resistance gene derived from the transposon Tn9 (Table 1c,d) into the Pst site of pDMS 6642, pLS54 and pLS57 reduced the plasmid copy number to between 20-30 per chromosome while failing to reduce the copy number of pLS103 significantly (Table 1c and e). The results suggest that the copy number of pDMS6642, pLS54 and pLS57 are all higher than ColE1 (which is about 15 - 20/chromosome) because of a transcriptional-read-through from the β-lactamase promoter into the RNA-primer transcript (see Fig. 1) that normally governs replication initiation of ColE1. While the potential for copy number control exists in pDMS6642, a strong promoter upstream from the normal RNA primer of replication can apparently override the expression of that control as can mutants affecting the structure of RNA of ColE1 (1). Copy number therefore appears to be controllable at the transcriptional level in ColE1.

Table 1. Properties of plasmids.

	a) Copy #	b) β-lac/10^8 plasmids	c) Copy #	d) Tc^R µg/ml	e) X-Cm^R copy#
pDMS6642	57	0.05	22	5	23
pLS54	109	0.43	36	15	32
pLS57	84	0.15	24	15	21
pLS103	140	0.08	-	-	127

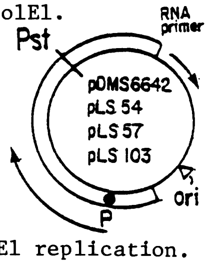

Fig. 1. pDMS6642 and its mutants. Origin of ColE1 replication. region of β-lactamase of *Tn*3. P. location of the β-lactamase promoter and its direction of transcription. Pst = site sensitive to endonuclease Pst1 into which pGA46 and a fragment of *Tn*9 was cloned.

INCOMPATIBILITY AMONG GROUP Y PLASMIDS

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Incompatibility expressed by the group Y plasmid prophages P1 and P7 was investigated by analysis of the behavior on non-selective medium of heteroplasmid cells immediately after introduction of the second plasmid by infection. Since a marker effect biased the segregation results when both plasmids were selected on solid medium, we followed plasmid segregation on nonselective medium. P1 and P7 derivatives fall into two sub-classes based on the rapidity of expression of incompatibility: homologous plasmids (P1-P1 or P7-P7) express incompatibility more rapidly than heterologous (P1-P7) plasmids. The determinant of this difference is genetically separable from the immunity determinant of the prophage.

After homologous superinfection, no colonies with both plasmids are recovered. We call this rapid expression of incompatibility. It apparently results because neither plasmid can replicate in cells containing two plasmids, possibly because of a plasmid specific inhibitor that controls copy number. At cell division, the two unreplicated plasmids segregate into different daughters. This interpretation is supported by kinetic data.

Following heterologous superinfection there is no increase in the number of cells with the marker of the incoming plasmid; the number of heteroplasmid cells remains constant. We call this slow expression of incompatibility and suggest that the resident plasmid has a very much greater probability of replication than the newly introduced one. The replicated plasmid is then partitioned between the daughter cells while the one that does not replicate is inherited essentially unilinearly. This suggest a physical relation between plasmid replication and partition, possibly by way of a membrane site.

CLONING OF FOREIGN GENES IN B.subtilis, NATURE OF BLOCKS TO THE EXPRESSION OF E.coli hisG GENE

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Several genes derived from E.coli have been cloned into B.subtilis, but for only two of them (thymidylate synthetase[1] and tetracyline resistance[2]) phenotypic expression of the traits has been demonstrated.

We have studied the ability of the E.coli hisG gene to complement a corresponding mutation in B.subtilis. The relevant gene, together with its E.coli vector pBR313, was inserted into a S.aureus/B.subtilis plasmid pCS194, a natural recombinant between two smaller S.aureus plasmids, pC194 and pS194. The resulting interspecific plasmid, pPV28, was found to be stable in E.coli, but highly unstable in B.subtilis, where the most frequent rearrangements involved the loss of the entire E.coli DNA sequence, plus the surrounding pS194 moiety, following a nearly precise excision process[3].

It has been possible to clone the E.coli hisG gene in B.subtilis via the interspecific E.coli-B.subtilis vector pHV14. The resulting plasmid, pPV48, could be faithfully replicated in B.subtilis but failed to complement the corresponding B.subtilis mutation in spite of the presence of a functional hisG gene.

Southern hybridization of mRNA produced in vivo by B.subtilis CU403 minicells, harboring pPV48, with restriction segments of this plasmid, as well as E.M. comparison of R-loops obtained through in vitro transcription of pPV48 with E.coli and B.subtilis RNA polymerases, suggest that B.subtilis RNA polymerase transcribes the E.coli hisG gene. Lack of his^+ phenotype could be due either to a wrong initiation of transcription or to a post-transcriptional event occurring on a functional mRNA.

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THE DETECTION OF TRANSPOSABLE RESISTANCE ELEMENT

Tn5 IN A PORCINE DIARRHEAL ISOLATE

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A transposable kanamycin resistance element was isolated from an enterotoxigenic (ST) E. coli strain isolated as the cause of diarrhea in pigs (strain 1710). The transposon was detected as insertions in phage λ b515 b519 (λkan's) that were detected as kan^R transducing phage in an induced lysated from a kan^R exconjugant of 1710 and a lysogenic E. coli recipient. These insertions were shown to be indistinguishable from Tn5 by:

1) HindIII digestion patterns of λkan DNA's.
2) Hybridization patterns of PstI and HincII cleaved λkan and λ::Tn5 DNA's probed with ^{32}P-ColE1::Tn5.
3) HaeIII digestion patterns of pVH51 transposition derivatives.
4) In vivo resistance pattern and in vitro substrate range of aminoglycoside-phosphotransferase activity.

Plasmid DNA prepared from a kan^R exconjugant of 1710 (R1710 DNA) was compared to R-plasmid JR67 DNA. JR67 was the original source of Tn5 and was found in Klebsiella strains causing urinary tract infections in humans. From restriction analysis and Southern hybridizations using as probes JR67, ColE1::Tn5, and restriction fragments of Tn5's inverted repeats or resistance gene, it was shown that:

1) The EcoR1 digestion patterns of the two plasmid preparations have no similarity.
2) R1710 DNA has more homology to JR67 than is attributable to Tn5. This may be explained in part by the fact that both plasmids code for resistance to streptomycin and to sulfisoxazole.
3) R1710 DNA contains an extra copy of sequences homologous to the inverted repeats of Tn5 (IS50), but not homologous to the resistance gene.

It was also shown by incompatibility testing that R1710 is not of the same incompatibility group as JR67 (Iα).

WIDESPREAD OCCURRENCE OF AN Ap^rST^+ *E. COLI* PLASMID OF HUMAN ORIGIN

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From 144 children admitted to the Hospital Infantil de Mexico with acute watery diarrhea during 1976-1977, five isolates of *E. coli* were tested for heat-stable(ST) and heat-labile(LT) toxin and for resistance to 14 antibiotics. Antibiotic resistance was determined by the agar dilution method.[12] ST^+ production was determined by the suckling mice assay [16] and LT toxin was determined by the adrenal cell assay.[14] Positive strains for either toxin were subsequently assayed in the rabbit ilium loop model.[15] The 31 LT^+ producing isolates came from 18 patients; 93.5% were resistant to between four and nine antibiotics. The 19 ST^+ producing strains came from 13 patients; 89.5% were resistant to as many as five antibiotics and 5% were resistant to seven antibiotics.

The linkage of antibiotic resistance and ST^+ enterotoxin activity was studied in six high level ST^+ isolates from five patients by conjugation to *E. coli* K-12 J54(Nal^r). The resistance markers used were ampicillin(Ap) and tetracycline(Tc)(three cases) and Ap, Tc, and streptomycin(Sm)(three cases). Transconjugants were selected for one antibiotic and then tested for resistance to the unselected antibiotics and for ST^+ production. In all cases Ap^r and ST^+ were tightly linked. In some instances we also found linkage of ST^+ with Tc^r and Sm^r.

Plasmid DNA was isolated from various ST^+ transconjugants representing the different patterns of antibiotic resistance; the partially purified DNA was analyzed on agarose gels. The only transconjugants that showed a single plasmid band(approximately 80 md)[17] were Ap^rST^+. All others exhibited at least two extrachromosomal bands, one of which was 80 md. Four Ap^rST^+ transconjugants, each derived from a separate clinical strain(different patients) and shown to have only one plasmid band, were further studied by EcoR1 restriction endonuclease analysis. Purified restricted plasmid DNAs from each were coelectrophoresed in a 1% agarose slab gel. The restriction pattern was identical in all cases: 11 fragments giving a total molecular weight of 81.5 md.[17] The four patients from whom these transconjugants were derived live in different section of Mexico City and had been hospitalized at different times of the year.

References

12,16,14,15,17: listed in Kupersztoch-Portnoy Y. M. this volume.

TRANSFER OF CHROMOSOMALLY INTEGRATED PLASMIDS IN *Haemophilus influenzae*

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Chromosomally integrated conjugative R plasmids transfer with an efficiency of 0.001 to 0.01 in standard isogenic genetic transformation crosses of *Haemophilus influenzae* Rd (relative to a high-efficiency point mutation). There is no transfer at all to *rec*⁻ or $CaCl_2$-treated recipients while free plasmids in such donor DNA lysates transfer infrequently (0.000001) to all these recipients. We have constructed strains with long nonhomologous plasmid-derived DNA inserts (from 6 to 14 megadaltons) at a given site in the chromosome. These inserts are transferred with efficiencies which vary inversely with size (0.3 to 0.03). Transforming out these inserts is independent of size; the efficiency is close to one. Replacing one insert by another is about as efficient as adding alone. A closely linked point mutation in the recipient reduces all transfer phenomena by 3 to 100 times while the same mutation in the donor DNA gave a much smaller effect. Thus the transfer of integrated plasmids from hospital isolates to strain Rd is infrequent because of large plasmid size and because of imperfect homology in the plasmid-flanking DNA regions. Spreading of integrated plasmids between heterologous populations should thus be limited.

pLEB1 DNA added to 2 different insert strains was integrated within the insert (Campbell model?). This was accompanied by loss of plasmid-controled ampicillin resistance. The integrated plasmids could be transferred by transformation. In one strain the plasmid was not excised spontaneously. In the other strain excision appeared to be *recA*-independent.

TRANSFER OF N PLASMIDS TO PSEUDOMONAS AERUGINOSA

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Our previous research, and the work of other investigators, has determined that the N plasmids transferred at very low frequencies to *Pseudomonas aeruginosa*, and that the retransfer of these plasmids from *P. aeruginosa* to either *E. coli* or *P. aeruginosa* was not detected. We have obtained *P. aeruginosa* mutants which show an increase in their recipient ability for N plasmids (Ren mutants). The N plasmids do not transfer at the same frequency to the Ren mutants; for example, the transfer of N3 is hardly affected by the mutations but there is a significant increase (10,000fold in some cases) in the transfer frequency of R46. Plasmid pCF290 is able to transfer from two Ren mutants to *E. coli*, but the retransfer of the other N plasmids is not detected. Differences among the N plasmids are also noted in their stability patterns: pCF290 is stable in each mutant but N3, R45 and R46 are lost at various frequencies depending on the host strain.

The mutations seem to be specific for N plasmid transfer since the transfer frequencies of several plasmids from other incompatibility groups (FII, Iα, C, W, P) are not affected. The antibiotic resistances mediated by the N plasmids are generally expressed by the Ren mutants, but the sensitivities to phages IKe and PRD1 could not be detected. In contrast, sensitivity to PRD1 was observed when the Ren mutants harboured RP1. The Ren mutations might possibly involve a membrane component as evidenced by a pyocin sensitivity test. One mutant is resistant to a particular pyocin, while two of the other mutants are more sensitive than the parental strain. Whether or not there is a direct correlation between N plasmid transfer and sensitivity to this pyocin remains to be determined.

COINTEGRATE FORMATION BETWEEN PLASMIDS CARRYING VIRULENCE FACTOR AND ANTIBIOTIC RESISTANCE GENES IN E. COLI

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Enterotoxigenic Escherichia coli (ETEC) strains which cause diarrhea in young pigs often possess the proteinaceous fimbrial surface antigen, K88, which enables the bacterium to adhere to the mucosal epithelium of the anterior small intestine of the pig. The genetic determinants for production of K88 fimbriae and utilization of the trisaccharide raffinose (Raf) are located on a 52 megadalton nonconjugative plasmid. The K88/Raf plasmid can be mobilized by a variety of conjugative plasmids in the ETEC strains Selection for Raf transfer frequently results in the isolation of cointegrate plasmids containing both the K88/Raf and mobilizing plasmid genomes. We have examined some parameters of cointegrate formation between a K88/Raf plasmid, pPS900, and pPS030, a conjugative R factor carrying the determinants for resistance to tetracycline and streptomycin. The K88/Raf plasmid was mobilized with equal efficiency from $RecA^+$ or $RecA^-$ donors. The percentage of transconjugants containing cointegrate plasmids varied markedly in repeated matings using the same donor and recipient strains. This variability is probably due, at least in part, to the instability of the cointegrates. Storage of strains containing cointegrates usually results in disassociation, but stable cointegrates can be isolated at a low frequency. Nine stable cointegrates have been analyzed by comparison of restriction endonuclease fragment patterns and filter blot hybridization against the cloned K88 determinant. These studies showed that cointegration involves a specific region in each plasmid. Each stable cointegrate had suffered a deletion of sequences from the K88/Raf plasmid. The deletions appear to have a single point of origin and most terminate at one of two sites resulting in loss of all or part of an 8.2 megadalton HindIII fragment containing the K88 determinant. We are currently examining the nature of the sequences involved in cointegration.

STABILITY OF PLASMID R1-19 IN HYPER-RECOMBINANT *Escherichia coli* K-12 STRAINS

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The plasmid R1-19 is a composite molecule cointegrated by two components: the RTF and the r-det. The r-det, which carries the drug resistance genes, is flanked by two insertion sequences IS1. In *E. coli*, R1-19 is a relatively stable composite molecule. In *S. typhimurium*, the RTF and the r-det, dissociate at a high frequency generating multi-sensitive cells which retain the RTF component. The dissociation involves a recombination (*recA*-dependent) between the two IS1.

In the present work, the stability of R1-19 in *recA*$^+$, hyper-rec (*polA1* and *dam*-3), and *recA*$^-$ *E. coli* strains was analized by subcultivating the cells without selective pressure and by checking for antibiotic resistance markers. In the *recA*$^+$ strains the plasmid was quite stable, segregating multi-sensitive cells at low frequencies. The plasmid, however, showed great instability in the hyper-rec strains and was completely stable in the *recA*$^-$ strain. In addition to the high percent of multi-sensitive cells, a low percentage of segregants Km^s, Km^s Ap^s, and Cm^s Sm^s -Sp^s, was found in the hyper-rec strains. Approximately 90 to 95% of the multi-sensitive cells retained the RTF component.

The instability found for R1-19 in *E. coli* hyper-rec strains is similar to that described for *S. typhimurium.* Therefore, the different behavior observed for R1-19 in *E. coli* and *S. typhimurium* *recA*$^+$ strains, is probably due to a lower recombination level in *E. coli* as compared to that of *S. typhimurium*.

STUDY OF pPK237, A BROAD-HOST RANGE MULTIRESTITANT PLASMID ORIGINATING FROM PSEUDOMONAS AERUGINOSA RESISTANT TO GENATAMICIN

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Plasmid PK237, originating from a Gm-Cb resistant Pseudomonas aeruginosa isolate, is a wide-host range, multiresistant and very stable plasmid, which carries genes for resistance to mercury and eleven antibiotics.

Three variants of it were obtained: PK237-2a, PK237-10 and PK237-16. Molecular weight determinations revealed that the original pPK237 is one molecule of 67 Md and that the variants are deletion mutants of it.

From crude preparation of E.coli K12+pPK237 two Gm-modifying enzymes were detected, which were characterized after purification as AAC(3)I and AAD(2"). On the other hand radioassay results from crude preparations of cultures carrying the variants distinguished them in: a) high or low level activity and b) with both or only one of the two enzymes detected. This variation explained the differences in levels of Gm resistance mediated by these plasmids.

Beta-lactamase detection and characterization showed that a TEM-I b-lactamase is mediated by pPK237 and pPK237-2a, while the variants pPK237-10 and pPK237-16 mediate a PSE-2 b-lactamase.

From the above findings and previous results a tentative mapping is proposed, which suggests the possible evolutionary relationships between the original plasmid and its variants.

HIGH EXPRESSION OF GENES IN *E. COLI* BY CLONING ON AMPLIFIABLE PLASMID VECTORS

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To increase expression of cloned genes in *E. coli* we used small derivatives of a "runaway"-replication mutant of plasmid R1[1]. The plasmids show temperature-dependent loss of control of copy number resulting in amplification of plasmid DNA in the cells. Therefore, gene products coded for by the plasmid may be overproduced due to the increased number of plasmid copies to levels as high as 1000-1500 per cell. New plasmid vectors constructed *in vitro* are shown in Table 1. A derivative of plasmid pBEU1 carrying the *E. coli recA* gene enabled us to overproduce the *recA* protein without stimulating its proteolytic activity and to obtain and purify proteins from *recA* mutants that cannot be derepressed by nalidixic acid or UV light treatment.

Table 1. Plasmid Cloning Vectors.

Plasmid	Kilobases	Single Restriction Sites	Antibiotic Resistance[c]
pBEU1[a]	17.4	*Bam*HI, *Hind*III, *Hpa*I, *Sst*I	Ap
pBEU27	10.8	*Bam*HI	Sp
pBEU28	9.2	*Eco*RI	Km
pBEU43	7.7	*Eco*RI, *Hpa*I	Ap
pBEU50[b]	9.9	*Bam*HI, *Eco*RI, *Hind*III, *Hpa*I	Ap, Tc

[a] pBEU1 carries the entire transposon Tn*3*.
[b] Sites for *Bam*HI and *Hind*III in region coding for Tc resistance.
[c] Ap, Sp, Km, and Tc denote resistance to ampicillin, spectinomycin, kanamycin, and tetracycline, respectively.

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PLASMID (pKM101)-MEDIATED MUTAGENESIS AND REPAIR

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The 35.4 kb N-incompatibility plasmid pKM101 makes cells more resistant to killing by agents such as UV and more sensitive to mutagenesis by these agents. These effects are $recA^{+}lexA^{+}$-dependent (1). *E. coli umuC* mutants seem to be specifically deficient in "error-prone repair" and these deficiencies are suppressed by pKM101 (2). These results are consistent with pKM101 coding for a unique component of "error-prone repair" and probably explain why pKM101 plays such a key role in the Ames test (3). By a combination of insertion mutagenesis, deletion mapping and cloning we have identified a region of at least 1900 bp but less than 2400 bp which is required for these effects (4). Interestingly the region of pKM101 responsible for mutagenesis/repair is surrounded by inverted repeats.

In addition we have used insertion and deletion mutants to localize several genetic regions on the plasmid genome. In clockwise order on the pKM101 map are: i) the *bla* gene - coding for a β-lactamase, ii) the "Slo" region - responsible for retarding cell growth on minimal medium, iii) the *tra* genes - enabling pKM101 to transfer conjugally, iv) sensitivity to IKe phage v) a single and double strand endonuclease vi) *fip* - fertility inhibition of P group plasmids (functions iv, v, and vi map within the *tra* region), vii) the *muc* gene(s) - responsible for enhancing UV and chemically-induced mutagenesis in the cell, and viii) the "Rep" region - essential for plasmid replication. In addition we have shown that pKM101 arose from its parental plasmid, R46, by the deletion of a 14 kb region of DNA.

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THE CONSTRUCTION OF NOVEL CLONING VEHICLES FOR USE WITHIN *STAPHYLOCOCCUS AUREUS* AND *BACILLUS SUBTILIS*

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Cm resistance plasmids pCW7 and pC221 of *Staphylococcus aureus* have been characterized by the construction of detailed restriction maps and by the identification of restriction sites on both plasmids which map within either the structural gene encoding CAT or its controlling elements. The number and order of recognition sites for endonucleases *Alu*I, *Hinf*I, *Mbo*I and *Taq*I on pCW7 and pC221 were determined. Circularization of the largest *Mbo*I fragment (1.8 kb) from pC221 formed a stable replicon (pCW41) which encoded an inducible CAT. To identify sites mapping within the Cm resistance determinant Cm Tc recombinant plasmids were constructed *in vitro* from pCW41 or pCW7 and Tc resistance plasmid pCW3 (Table 1). Then site-specific mutations were introduced by filling in the complementary ends of selected restriction sites present on DNA from pCW7 or pCW41 with *E. coli* polymerase I followed by recircularization of the recombinant plasmid by blunt-end ligation. Pol I treatment of the *Bst*EII site on pCW41 DNA and the *Bst*EII or *Bgl*II site present on pCW7 DNA resulted in the loss of both the recognition site and Cm resistance.

Cm Tc recombinant plasmids pCW48 and pCW59 should prove to be useful as molecular cloning vehicles in *S. aureus* and *Bacillus subtilis*. The *Bst*EII site on pCW48 and the *Bgl*II and *Bst*EII sites on pCW59 can be used for the insertional inactivation of Cm resistance. The versatility of plasmid pCW59 for cloning is increased by the ability of the *Bgl*II site (A↓GATCT) to accept restriction fragments produced by digestion with endonucleases *Mbo*I (↓GATC), *BamH*I (G↓GATCC), *Bcl*I (T↓GATCA) and *Xho*II (Pu↓GATCPy).

Table 1 - Description of Plasmids

Plasmid Number	Size	Phenotype[a]	Description
pC221	4.4 kb	Cm	Natural isolate.
pCW3	4.5 kb	Tc	" "
pCW7	4.2 kb	Cm	" "
pCW41	1.8 kb	Cm	*Mbo*I fragment A from pC221.
pCW48	6.1 kb	CmTc	*Hpa*II restricted pCW41 inserted into *Hpa*II site of pCW3.
pCW59	5.3 kb	CmTc	*Hind*III fragment A from pCW3 inserted into the *Hind*III site of pCW7. Spontaneous deletion of 1.2 kb of pCW7 DNA.

[a]Phenotype = phenotype of strain carrying the plasmid.

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EXPRESSION OF EUKARYOTIC GLYCOPROTEINS IN BACTERIA

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Sequential expression of variant surface glycoproteins (VSGs) enables the parasitic protozoan Trypanosoma brucei to evade the immune response of its mammalian hosts (Cross 1978). Studies of several isolated VSGs have indicated extensive amino acid diversity and the absence of a hydrophobic segment which might serve to anchor the carboxy-terminus to the membrane. Nucleotide sequence data suggests that the primary translation product of one VSG gene contains a hydrophobic tail at the carboxy-terminus which is not found on the isolated mature glycoprotein (J.C. Boothroyd et al (1980) Nature, 288:624). We are using clones of the VSG gene as a model system for studying the expression of eukaryotic glycoproteins in bacteria.

Complementary DNA (cDNA) molecules corresponding to the VSGs of several variants have been synthesised and cloned in to the Pst I site of pBR322 using G·C tailing (Hoeijmakers et al 1980). Immunological screening of eight cDNA clones for VSG 117 indicates that four clones produce VSG polypeptides at a low level ($\sim 1 - 5 \times 10^2$ molecules/cell). The bacterial synthesis of VSG polypeptide is probably directed from the β-lactamase promoter though not as a fusion product with the β-lactamase protein. The four expressing clones contain only part of the structural gene for the VSG so synthesis of this polypeptide in E.coli may be initiated at an internal methionine.

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PHEROMONE-INDUCED AGGREGATION SUBSTANCE IN STREPTOCOCCUS FAECALUS

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When exposed to specific sex pheromones excreted by recipient cells, certain plasmid-containing donor strains of S. faecalis modify their surfaces to become adherent, enabling them to aggregate with recipients (see Clewell, this volume). Pheromone-induced donors will also self-aggregate in the absence of recipient cells; this response is inhibited by chloramphenicol or rifampin. Aggregated cells are readily dissociated upon exposure to EDTA; the aggregation of induced cells has been found to require divalent cations as well as phosphate. Exposure of EDTA-dissociated cells to trypsin, SDS (0.05%), or heat prevents reaggregation when the cells are subsequently placed in an optimum environment for aggregation.

An antiserum was prepared against a gluteraldehyde-fixed preparation of an induced strain (39-5) carrying the conjugative plasmid pPD1. Absorption with uninduced cells resulted in an antiserum which, using a fluorescent antibody technique, was reactive only with induced cells. The inducible antigen has been designated aggregation substance (AS) and can be extracted (with 1% Triton X-100) and monitored by immunoelectrophoresis techniques. The absorbed antiserum was also used in combination with electron microscopic analyses using a horse-radish peroxidase immunological stain. The latter revealed an amorphous material present on the surface of induced, but not uninduced, cells.

In addition to pPD1, pheromone-induced aggregation responses are conferred by pAD1, pOB1, pAMγ1, pAMγ2 and pAMγ3. (At least three different compatibility groups are represented here.) By microscopic fluorescent antibody detection, the antiserum prepared against induced cells carrying pPD1 was found, in all cases, to readily cross-react with induced (but not uninduced) strains separately harboring these plasmids. It is not certain yet whether AS is directly determined by these plasmids or by a chromosome-borne determinant under plasmid-control.

A DNA-DIRECTED CELL-FREE SYNTHESIS SYSTEM CAPABLE OF USING LINEAR DNA FRAGMENTS AS TEMPLATE

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A new cell-free system (reconstructed cell-free system) has been recently developed which overcomes two major defects of the S-30 system. Since the extract is prepared from a recB mutant, defective in linear DNA specific nuclease, the introduced DNA is very stable. Contaminated chromosomal DNA which usually creates background synthesis has been eliminated by modifying the method of preparation of cell extract. This cell-free synthesis system has the following unique features; 1) it can use linear DNA or DNA fragments as templates, 2) it has a high efficiency of synthesis both at transcriptional and at translational level, 3) it has good fidelity of gene expression and, 4) it has very little background synthesis.

This RC system can be applied to identify the gene product to study the genetic structure and to analyze the regulatory mechanism.

SURVEY OF A CONFERENCE: TURISTA OR NOT TURISTA?

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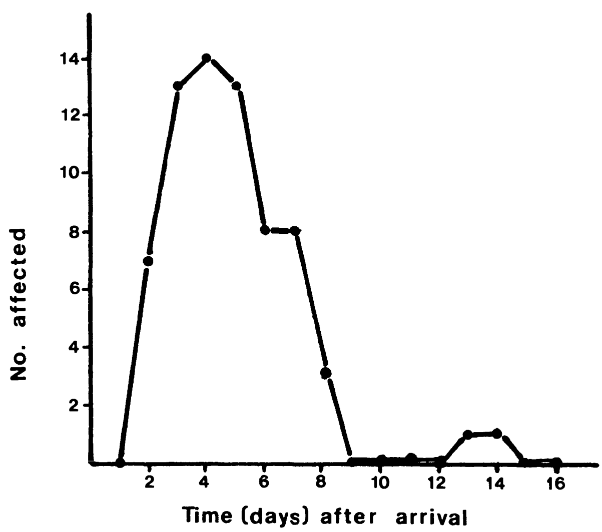
One of the topics under discussion at the conference was the relationship between plasmids and diarrhea. Since "turista" often afflicts travellers to foreign countries, a survey of the attack rate of gastrointestinal problems in participants was made. Among the more than 200 participants, about 190 were visitors to the Dominican Republic. 114 responded to the questionnaire.

The first reported illness occurred on the second day of the conference. By the end of the five-day meeting, 47 were affected. The post-conference survey showed that 67/114 had suffered some mild or more severe symptoms of "turista." The Figure below records the daily incidence of newly-affected individuals (mild + severe) over the conference and post-conference period. In both mild and severe cases, the incidence peaked at 4-5 days and then dropped off. 41.2% of the those who responded remained unaffected. 18 conferees had mild symptoms (cramps, loose stools) which lasted 1-3 days. The remaining 49, however, developed moderate to severe symptoms which lasted 2-14 days. In two individuals, a multiply-resistant *Shigella sonnei* was isolated.



Incidence of gastrointestinal symptoms among conference participants

Of the parameters examined, e.g. food and water consumption, previous travel history, the following conclusions could be made: a)no South or Central American visitor reported any illness; b) the unaffected and mildly-affected groups were highly-represented by persons who had previously travelled to countries in South and Central America or Asia (Table); c) the affected (mild + severe) were more apt to have eaten cold salads at the hotels or other restaurants. There was no correlation between where the salad was eaten and the attack rate.

Group	# of responders	% eating salads	% travelled+
Unaffected	34*	47.1	85.3
Mild	18	77.8	72.2
Mod-Severe	49	77.5	44.9

*excludes 13 responders who did not complete the questionnaire
+those with previous travel to Central or South America or Asia

44.4% of the unaffected and 6-12% of the affected had travelled before and ate no salads. Obviously there were lessons learned by some through travel that were not learned by others. In fact, those who had travelled and did not eat salads represented 61% of the unaffected and only 15-17% of the affected groups. Moreover, 63% of the affected group had been sick on prior travels, but only 40% of those in the unaffected group. Only three individuals,who remained unaffected,were taking prophylactic medication: two were taking trimethoprim-sulfonamides; one was taking Keflex.

It appears from this mini-survey that prior travellers who avoided uncooked foods, e.g. cold salads, would be less susceptible to diarrheal disease. Besides food habits, another possibility for the correlation between prior travel and less disease would be if the travellers retained an immunity from previous trips. This possibility my explain why certain salad-eating travellers were less affected than others: a larger proportion of the mild group than the severe group had travelled before.

At the least, the results would suggest that plasmid investigators should travel more and eliminate cold salads from their diets. In what percentage of cases plasmids were involved is still a question.

STATEMENT REGARDING WORLDWIDE ANTIBIOTIC MISUSE

Antibiotics have been developed to treat diseases caused by micro-organisms in humans, animals, and cultivated plants. However, these antimicrobial agents are losing their effectiveness because of the spread and persistence of drug-resistant organisms. Moreover, unless steps are taken to curtail the present situation we may find a time when such agents are no longer useful to combat disease.

We are faced with a worldwide public health problem. It is due in large part to the indiscriminate use of antibiotics, especially in the following practices: a) dispensing antibiotics without prescription; b) using clinically-useful antibiotics as growth promoters in animal feeds and on agricultural crops; c) prescribing antibiotics for ailments for which they are ineffective; d) misleading consumers by advertising antibiotics as "wonder drugs," especially in areas where dispensing is not regulated; e) using different labeling and advertising to sell the same product in different parts of the world.

Let no one suppose that widespread use of antibiotics is in any way a substitute for good sanitation and personal hygiene. Efforts in improving these mainstays of infectious disease prevention and control must be encouraged and strengthened. At the same time, it is imperative to increase awareness of the dangerous consequences of antibiotic misuse at all levels of usage: consumers, prescribers, dispensers, manufacturers, and government regulatory agencies. Only then can we begin to institute measures to curb the unnecessary use and flagrant misuse of these drugs.

We, the undersigned, have drafted this statement to instigate action towards halting this ever-increasing worldwide problem. We would like this communication to serve as the impetus for organizing national and international committees from which directives for prudent antibiotic use can be established. As a first step, we urge that a uniform practice in the prescription and distribution of antibiotics be implemented and enforced in those areas where adequate medical expertise is already available. Furthermore, we urge that proper standards of advertising and dispensing of these drugs be agreed upon and adhered to in all nations of the world.

The above statement evolved from presentations and discussions during the conference on Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids, held in Santo Domingo, Dominican Republic, January, 1981. All those who signed did so as individuals and not as representatives of their institutions.

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GLOSSARY

Reference should be made to the following publications; for information on plasmids to R. P. Novick, R. C. Clowes, S. N. Cohen, R. Curtiss, N. Datta, and S. Falkow (1976) Uniform nomenclature for bacterial plasmids: A proposal. Bacteriol. Rev. 40, 168-189, and for transposons and insertion sequences to A. Campbell D. Berg, D. Botstein, E. Lederberg, R. Novick, P. Starlinger, and W. Szybalski (1977) Nomenclature of transposable elements in bacteria. In "DNA insertion elements, plasmids and episomes". Eds. Bukhari, A. I., J. A. Shapiro and S. L. Adhya, Cold Spring Harbor Laboratory, New York.

The following items are particularly important.

Cointegrate - a genetic element composed of two or more complete replicons in covalent linear continuity where the component replicons are known to be capable of physically independent replication. Cointegrates may be formed by recombination, transposition, *in vitro* construction or other mechanisms (see Clowes, 1972).

Complementary DNA (cDNA) - a single or double-stranded sequence of DNA in which the sequence on one strand is complementary to that of a messenger RNA. Usually derived by *in vivo* or *in vitro* synthesis using a DNA polymerase (reverse transcriptase) from a retrovirus such as avian myeloblastosis virus (see Rougeon *et al*, 1975).

Conjugation (bacterial) - the process of genetic exchange between bacteria dependent upon cellular contact, in which genetic material is transferred from one organism (the donor) to another (the recipient).

Copy-number mutant - a plasmid in which the copy number (the number of molecules of a specific plasmid per genome equivalent or per host cell) has been changed by mutation.

Cosmid - a plasmid containing the sequence of and around the cohesive (cos) terminus of λ phage. The remaining sequences are usually those required for plasmid replication and for antibiotic resistance (see Collins & Brunning, 1978).

Direct repeat - two identical base sequences in a double-stranded DNA molecule.

Enterotoxin - a toxin synthesized by an enteric microorganism, usually *Escherichia coli*.

Hfr - the state of harboring a conjugative plasmid that is integrated into the chromosome and consequently is able to promote oriented chromosomal transfer to suitable recipients.

Insertion sequence (IS) element - a DNA segment, generally shorter than 2kb, which contains no known genes unrelated to insertion function, and which can insert into several sites in a genome. Symbols IS1, IS2, IS3, IS4, IS5, etc.

Inverse repeat (inverted repeat) - two DNA sequences (up to several hundred nucleotides in length) in a double-stranded DNA, one sequence of bases being repeated with the same polarity in the complementary strand.

e.g. 5' —— ACAAACT-------------------AGTTTGT—— 3'
3' —— TGTTTGA-------------------TCAAACA—— 5'

The two sequences are separated by other bases. If they are continuous or separated by only one base, they are termed a palindromic sequence.

K-antigen - a surface antigen of a bacterial cell that permits the cell to adhere to the cells of the intestinal mucosa of the alimentary tract.

Leader region - a sequence of DNA from which a messenger RNA is transcribed, which may be either terminated near the 3' end of this region, or may continue through the sequence of the adjacent structural gene(s). A mechanism of regulating certain enzymes.

Marker rescue - a recombination experiment in which the presence of certain genetic regions determining phenotypic properties can be detected by recombination of these regions into a replicon defective for these properties.

Maxicells - cells heavily irradiated by ultraviolet light to extensively damage chromosomal DNA which is consequently unable to produce active messenger RNA and protein. If plasmids are present in these cells, there is a lower probability that the plasmid DNA (due to its smaller size) will be damaged, and can in consequence be used to detect plasmid-specific messenger RNAs and proteins (see Sancar *et al*, 1979).

Minicells - the product of cell division of a mutant bacterial strain. At each cell division, one normal cell and one cell (minicell) without chromosomal DNA result. Many plasmid DNAs are transferred into minicells, which when separated from normal cells by centrifugation, can be used to determine plasmid-specific messenger RNAs and proteins.

Mobilization - the process by which a conjugative plasmid brings about the transfer of DNA to which it is not stably and covalently linked.(see N. Willetts, 1980).

Nick translation - a method to prepare highly radioactive DNA, resulting from treatment with DNA polymerase I, in which the 3'→ 5' exonuclease activity removes base sequences adjacent to a single-stranded 'nick' and in which the 5'→3' polymerizing activity replaces them with radioactive deoxyribonucleotides.

Northern blot - see Southern blot

Plasmid - a replicon that is stably inherited (i.e. readily maintained without specific selection, in an extrachromosomal state. Naturally occurring plasmids in prokaryotes are generally dispensable.

colicin - 'Col' plasmid, any plasmid that carries genetic information for the production of a colicin.

conjugative - a plasmid that can bring about the transfer of DNA by conjugation.

F - the prototype "fertility factor" responsible for conjugation in the *E. coli* K12 strain of Cavalli-Sforza *et al* (1953) and by Hayes (1953) in their early studies of bacterial mating.

F' - an F derivative incorporating a segment of the bacterial chromosome.

non-conjugative - a plasmid that cannot bring about the transfer of DNA by conjugation.

resistance (R) - a plasmid that carries genetic information for resistance to antibiotics and/or other antibacterial drugs.

Pribnow box - a DNA sequence of approximately seven bases situated approximately five bases in the 5' direction from the first base of a promoter, involved in the initiation of transcription (see Promoter).

Promoter - the site at which RNA transcription is initiated on the DNA template (see Rosenberg & Court, 1979).

R-loop - a segment of double-stranded DNA, in which one of the DNA strands has been displaced by homologous RNA, and thereby produces a structure visible by electron microscopy (see Thomas *et al*, 1976).

Replication origin - the DNA site (or sites) on a replicon from which the replication of DNA is initiated.

Southern blot - a method of DNA:RNA hybridization, by which a DNA preparation is cleaved into fragments with one or more restriction enzymes, the fragments are separated on an agarose gel, then denatured and transferred by capillarity to a sheet of cellulose nitrate paper, which is saturated with a radioactive labelled messenger RNA preparation, thereby identifying by autoradiography those DNA fragment(s) which have homology with the mRNA. The method has been modified for DNA:DNA and RNA:DNA hybridization, which are sometimes loosely referred to 'Northern' blots (see E. M. Southern, 1975).

Transfer (tra) genes - those genes carried on a conjugative plasmid that are responsible for the donor phenotype.

Translocation - see Transposition

Transconjugant - a bacterial cell that has received genetic material from another bacterium by conjugation.

Transposition element or sequence (or transposon) - a well-defined genetic element usually of constant size which contains genes unrelated to insertion function, and that transposes intact from one genetic locus to another. Transposition (Tn) elements are generally larger than 2kb, and a number (e.g. Tn1, Tn2, Tn3, etc.) is allocated to each independent isolate from nature, even if it is apparently identical to some previous isolate.

Transcription - the synthesis of a single-stranded RNA molecule by an RNA polymerase enzyme, from a double-stranded DNA molecule, the sequence of bases on the RNA molecule being complementary to those on one of the strands of the DNA molecule being transcribed.

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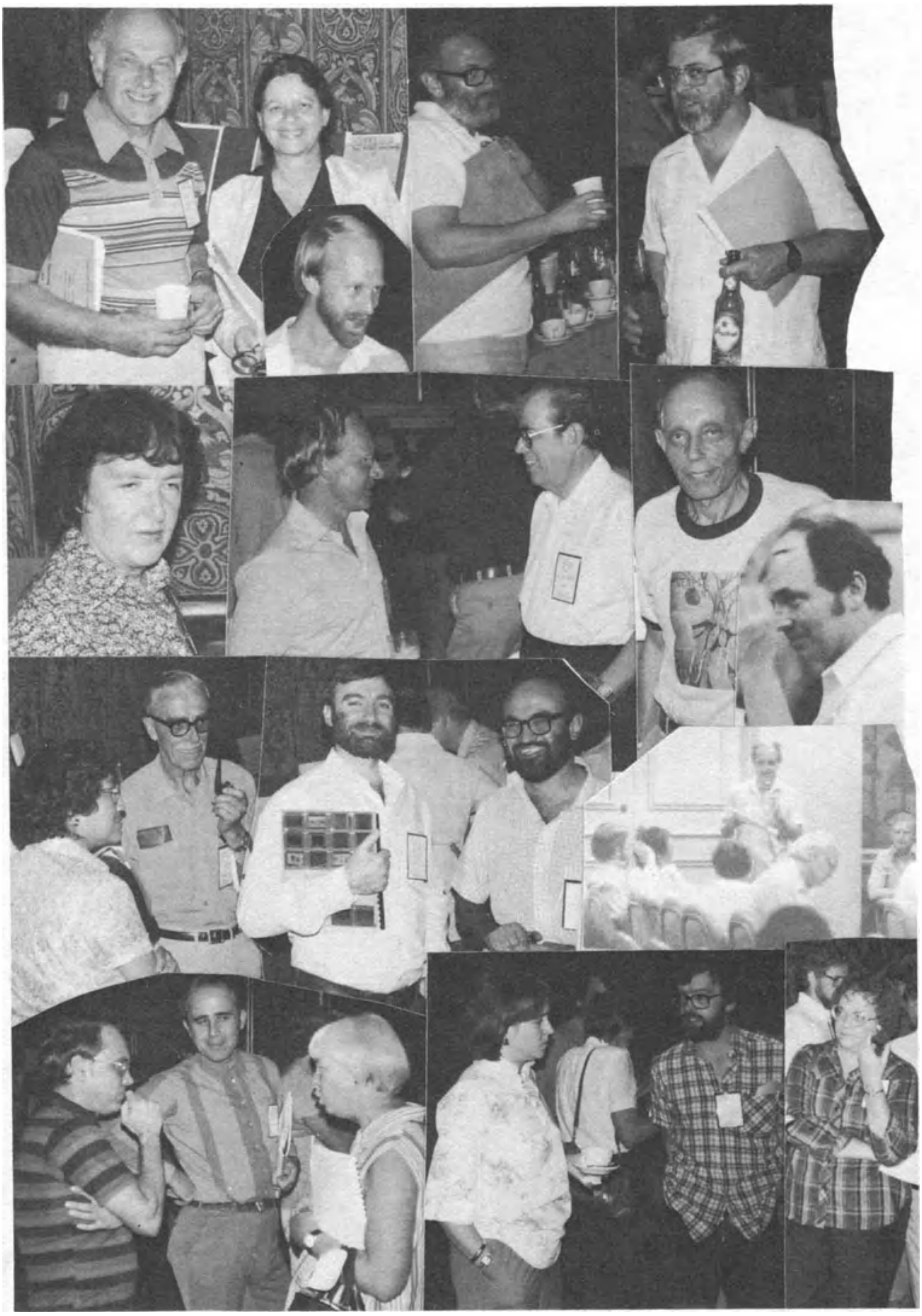

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