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Genetic Approaches to Microbial Pathogenicity

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Preface

Important progress in the elucidation of the mechanisms influencing bacterial pathogenicity has recently been made through the introduction of modern genetic techniques. Molecular cloning allows the isolation of genes for phenotypes that epidemiological surveys have suggested play an important role in pathogenesis. The structural analysis of determinants for pathogenic traits can lead to the identification not only of the primary sequence but also of the possible secondary and tertiary structures for important virulence factors such as toxins and adhesins. From these data, the prediction of antigenic domains suitable for the development of new vaccines appears to be feasible. The regulation of virulence determinants by endogenous and exogenous factors can be more clearly understood through the functional analysis of the cloned virulence genes.

This volume surveys representative virulence properties of gram-positive and gram-negative bacteria to which the genetic approach has been successfully applied. The examples described here include important bacterial toxins (e.g., diphtheria toxin, cholera toxin, toxic shock syndrome toxin, hemolysins), adhesion structures from *E. coli* and *Neisseria gonorrhoeae*, and factors supporting iron uptake, serum resistance, and invasiveness in a variety of bacteria. Both the present state and the possible futural developments of these systems are described.

W. GOEBEL

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Genetic Analysis of Bacterial Virulence Determinants in *Bordetella pertussis* and the Pathogenic *Yersinia*

R.R. ISBERG and S. FALKOW

1 Introduction

With the advent of recombinant DNA technology, rapid advances have been made in understanding the molecular organization of bacterial pathogens (for review, see Macrina 1984). This has been especially true in the analysis of enterotoxin structure and function (So et al. 1976, 1978; MOSELEY and FALKOW 1980; PEARSON and MEKALANOS 1982) as well as in the evaluation of bacterial pili and hemolysins in gastrointestinal or extraintestinal infections (SVANBORG-EDEN and HANSSON 1978; WELCH et al. 1981; HARTLEIN et al. 1983). Similarly, such studies have clarified the role of iron uptake in the infectious process (CROSA 1984) and have been helpful in understanding better the molecular epidemiology of disease agents (HULL et al. 1984). We suppose that in the near future most virulence factors defined by classic studies in medical microbiology will be analyzed at the genetic level in some manner or other.

The cloning of a bacterial virulence factor does not, in itself, determine how the factor contributes to the infection of an animal host. For example, while we could clone and subsequently introduce mutations into the IgA protease gene of *Neisseria gonorrhoeae* (KOOMEY et al. 1982), the absence of a convenient and inexpensive model infection system hampered our ability to demonstrate clearly that this interesting protein was essential for gonoccal virulence. Similar problems have been encountered in analyzing potential determinants of chlamydial virulence. In this latter case, not only are animal infection studies difficult, but the absence of a method of DNA exchange makes it currently impossible to perform meaningful mutational analyses that are necessary and desirable if specific gene products are to be evaluated for their role in virulence.

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It seems to us that the analysis of microbial pathogenicity is most likely to proceed rapidly if one can construct mutants in the organism of interest and, in turn, if these mutants can be evaluated for virulence in either an animal host or in some kind of model infection system. This concept, though not at all original (for example, see BURROWS 1962), means that the true power of the recent advances in DNA technology lies as much in the construction and analysis of well-defined mutants as in the isolation and sequencing of genes. For instance, we believe one of the most useful avenues in our study of *Yersinia* virulence (PORTNOY and FALKOW 1981; PORTNOY et al. 1983; PORTNOY and MARTINEZ, this volume) was the ability to define the physical structure of avirulent mutants located on a plasmid in *Y. pestis*. Although such mutants had been originally isolated 20 years previously (HIGUCHI and SMITH 1961), their pleiotropic nature had remained a mystery. With the ability to isolate and map plasmid DNA, these mutations could be mapped physically, now rendering it a rather straightforward proposition to define the gene products that have been eliminated by these lesions.

Unquestionably, the increased application of genetic analysis to the study of bacterial pathogens will result in the discovery of new factors not previously known to play a role in virulence. This type of analysis permits us to go beyond the cloning of toxins and adherence factors into the realm of defining the roles various gene products play in allowing intracellular parasites to invade and survive within the tissues of their hosts. In order to define the factors that allow the intracellular pathway to take place, the investigator must rely on isolating mutants that eliminate or alter the phenotype of interest. In this sense, genetic analysis can achieve its full resolving power: it allows the investigator to discover new proteins whose mode of action may turn out to be quite novel. In our view, therefore, the goal of most analyses is to develop a method of screening or selecting for simple phenotypes that make possible the discovery of new gene products and the isolation of new classes of avirulent mutants.

In this article, we will describe how the development of genetic systems for the study of two organisms has made possible the description of new gene products involved in the infection process. Studies on these organisms relied on the ability to introduce mutations eliminating virulence in one of them (*Bordetella pertussis*) and the ability to select for a simple phenotype in the other (*Y. pseudotuberculosis*). We will conclude by suggesting some genetic techniques that have, in our judgment, been underused in the evaluation of virulence.

2 Insertion Mutagenesis To Define Virulence Factors

During the past decade the easiest factors to describe genetically have been those that could be cloned in *Escherichia coli* from another organism by selecting or screening for a particular phenotype. Unfortunately, this is not always possible particularly when dealing with fastidious pathogens; perhaps a simple phenotype does not exist or the desired gene does not express a product when cloned into *E. coli*. Under these circumstances, it is necessary to develop an alternative

method. One such approach is to isolate mutations in the pathogenic organism and analyze the lesions for their effects on virulence. The results will often suggest more fruitful approaches than cloning the desired genes (WEISS and FALKOW 1983).

We initiated our studies on the pathogenesis of *B. pertussis* infection by attempting to clone and characterize the genes encoding the pertussis toxin. Attempts to clone these genes using standard recombinant DNA technology failed (WEISS and FALKOW 1983), so it became apparent that more information had to be obtained concerning the genetic organization and expression of these genes. For this reason, mutants were isolated that lacked various virulence determinants, and the effects of these mutations on the pathogenesis of disease were evaluated. The availability of an animal model for *B. pertussis* infection made such a proposition feasible (PITTMAN et al. 1980).

Until recently, the isolation and mapping of mutations in an organism that had no established system of genetic exchange was a monumental task. Mutants could be isolated with hard work, but determining how many loci were affected by the lesions was often impractical. Fortunately, utilization of insertion mutagenesis now bypasses many of the classic problems of mutant isolation and mapping. The advantages of using transposable elements to isolate such mutants are manifold (KLECKNER et al. 1977). Insertion mutations usually result in totally eliminating the expression of the gene; their phenotype is rarely leaky. They are easy to map physically and genetically. The insertion of a drug-resistance transposon enables a mutation to be located either by changes in the restriction pattern of a DNA fragment or by genetically mapping the site of drug resistance. Finally, they are easy to isolate and are almost always the result of only a single insertional event, so having secondary mutations in the same strains is not a problem. For this reason, a protocol was devised for isolating insertions of the transposon Tn*5* into the chromosome of *B. pertussis* (WEISS et al. 1983).

Insertions of Tn*5* onto the chromosome were isolated by selecting for kanamycin resistance after introduction of a replication-defective plasmid that harbored the transposon (WEISS et al. 1983). Each kanamycin-resistant colony that grew was the result of a transposon-induced mutation, so relatively few colonies had to be screened in order to isolate lesions in the desired genes. Pools of insertions were then screened for their ability to produce pertussis toxin, hemolysin, an extracellular adenylate cyclase, a filamentous hemagglutinin (FHA), and dermonecrotic toxin. Mutants were isolated that reduced expression of hemolysin, pertussis toxin, and FHA individually, or reduced expression of the cyclase and hemolysin coordinately. Most importantly, several insertions were identified that eliminated expression of all of these factors simultaneously, implying that expression of these proteins is under the positive control of a single locus (WEISS and FALKOW 1983), as had been suspected from previous studies (KASUGA et al. 1953).

The mapping of mutations and the proof that they cause the phenotype being studied are the most important features of genetic analysis. There is no gross map of the *B. pertussis* chromosome, so mapping was performed by locating the site of insertion mutations on single restriction fragments, using Southern blot hybridization. It was found, in general, that each mutation that eliminated

the expression of a particular gene product mapped in the same restriction fragment, while mutations affecting different proteins were located on different DNA fragments. It appeared, therefore, that the affected genes were not closely linked physically. Proof that each mapped insertion was the cause of the negative phenotype was almost as straightforward. To accomplish this, one usually attempts to isolate and analyze revertants of the mutation. An alternative method had be pursued, however, since no selection existed that enabled spontaneous revertants to be isolated. To this end, it was possible to exploit a system of genetic exchange that involved mobilization of *B. pertussis* chromosomal genes by a conjugative plasmid integrated into the chromosome (WEISS et al. 1983). Crosses were performed between an avirulent Tn*5* insertion mutant and a fully virulent kanamycin-sensitive donor. Recombinants that regained virulence always simultaneously lost the Tn*5* insertion, showing that this insertion was the cause of the avirulent phenotype.

Perhaps the most useful insight from this work was the results on the regulation of virulence factors and the relative contribution of distinct virulence determinants to pathogenesis. In both cases, progress depended on the development of a convenient system for the isolation and characterization of mutants. Inactivation of a single locus, *vir,* resulted in simultaneous elimination of expression of a whole battery of virulence-associated factors, including all known toxins and adhesins encoded by *B. pertussis*. This suggested that *vir* encoded a positive regulator of these factors, and that cloning of these factors in *E. coli* might be facilitated by the presence of *vir* in trans. Subsequently, this has proven to be the case (WEISS et al. 1985). Secondly, the use of an appropriate animal model, lethality in the infant mouse following intranasal inoculation, provided important insights into the mechanism of pathogenesis. For instance, mutants that were defective only in the production of pertussis toxin were avirulent, establishing conclusively the importance of this protein (WEISS et al. 1984). Similarly, for the first time, the adenylate cyclase protein could be demonstrated to play an essential role in disease.

We believe that the main lessons learned from studies on *B. pertussis* are applicable to the analysis of virulence factors encoded by most bacterial pathogens. The inability to clone a virulence factor does not imply that studies in molecular genetics are impossible for that organism. One should not overlook the fact that the use of insertion elements as mutagens has become almost as powerful a genetic technique as the use of recombinant DNA technology. In the case of *B. pertussis,* insertional analysis permitted the isolation of both specific and pleiotropic mutants that could be mapped physically and characterized genetically in a detailed manner. Previously, such mutants were either not isolatable or not as easily understood. In addition, this analysis suggested a new approach for the cloning of genes that were previously found to be refractory to straightforward recombinant DNA methods. The other lesson that is reemphasized from these results is that the genetic analysis of pathogenic traits is best done in conjunction with a reasonable animal infection model. When such applicable animal systems do not exist, or existing systems are not exploited, the relevance of even the most elegant genetic and molecular analysis may be unclear.

3 Genetics of Invasiveness

Our studies on the pathogenesis of *B. pertussis* illustrate how genetic analysis can be utilized to gain insight into well-recognized virulence factors such as toxins and adhesins. Can such an approach be used to investigate less familiar factors? In this section, we will describe how we have used a genetic approach to study the physiology and pathogenesis of a more poorly understood process. Using a simple infection system and insertion mutagenesis, we have investigated the genetic basis for the ability of a pathogenic bacterium to invade epithelial cells.

Many pathogenic species of bacteria are able to invade the tissues of their hosts. For some of these species, such as *Shigella dysenteriae, Mycobacterium tuberculosis,* and *Legionella pneumophila* (FORMAL et al. 1983; WONG et al. 1980), invasion appears to be central in the evolution of their host-parasite relationship. Invasiveness appears to be important to these bacteria for several reasons, not the least of which is that it provides the bacterium with a mechanism to avoid host defenses. Hence, the bacterium is sequestered from antibody, antibiotics, and phagocytes. In addition, the invading microorganism has the ability to evade intracellular killing mechanisms while multiplying intracellularly in a "safe" niche. Furthermore, the ability to invade enables the bacterium to cross epithelial barriers, enter the lymphatic system, and subsequently become systemically distributed. Although most workers agree that invasiveness is an important factor in the pathogenesis of disease, the factors encoded by the bacterium that allow it to be internalized by the animal cell had not been identified to date (SANSONETTI et al. 1983). Our interest in this problem stems from our belief that invasive bacteria represent a class of pathogens that is basically different from specifically adherent toxin-producers, such as *B. pertussis,* in its pathway of infection.

The invasive phenotype can be easily studied in vitro by assaying for the endocytosis of the pathogen in cultured epitheloid cells (DEVENISH and SCHIEMANN 1981; SANSONETTI et al. 1983). The most convenient assay simply involves the addition of bacterial cells to a monolayer of animal cells for a few hours. The monolayer is then washed exhaustively, and fresh medium containing the antibiotic gentamicin is added. This antibiotic kills extracellular bacteria; bacteria survive if they have invaded the epithelial cell, since gentamicin does not efficiently cross the animal cell membrane. The degree of invasion can then be quantitated by gently lysing the animal cells in nonionic detergent, releasing the intracellularly located bacteria so they can be titered on a suitable bacteriological growth medium. We will show that this simple assay allows a rather detailed analysis of a factor that promotes invasion of a pathogen.

We have chosen *Yersinia pseudotuberculosis* as a model system for studying the invasive phenotype. This microorganism usually causes a zoonotic illness (MERCHANT and PARKER 1962), although it can cause an enteric disease in humans that leads to mesenteric lymphadenitis. A fatal disease that parallels this systemic illness can be induced in the mouse or guinea pig with this organism. We believe there are three advantages to studying *Y. pseudotuberculosis.* First it efficiently invades cultured epithelial cells (BOVALLIUS and NILSSON 1975).

Second, it is a member of the *Enterobacteriacae,* (as is *E. coli*) and is sensitive to coliphage P1, allowing DNA to be easily introduced into the bacterium via transduction (BOLIN and WOLF-WATZ 1984). Third, animal models exist that allow the pathogenicity of mutants to be evaluated (BOLIN et al. 1982).

It would be desirable to define all the determinants encoded by this bacterium that promote the invasion of animals cells. As a first step, we decided to take a simple approach and attempt to clone the genes responsible for this process into *E. coli* K12, an organism that is normally noninvasive. To this end, we introduced into *E. coli* a cosmid clone bank of the *Y. pseudotuberculosis* chromosomal DNA, and enriched for strains that were able to invade cultured HeLa cells. To enrich for the desired *E. coli* strain, a mixed culture containing representatives from the entire clone bank was pooled and allowed to infect a monolayer of HeLa cells. After allowing time for binding and invasion of the desired clone into the monolayer, the cells were washed exhaustively to remove noninvasive organisms. Bacteria that had bound or invaded the animal cells were then released from the monolayer by gentle detergent treatment.

Using the above protocol, about one-half of all the bacteria that survive the enrichment contain recombinant plasmids encoding *Y. pseudotuberculosis* gene products that convert *E. coli* K12 into a HeLa cell-invasive organism. Each of the clones surviving the enrichment invade cultured animal cells about as efficiently as *Y. pseudotuberculosis,* implying that the relevant factors encoded on the plasmids in these strains are expressed efficiently and localized properly in *E. coli.* Furthermore, the kinetics of invasion appear identical for *Y. pseudotuberculosis* and the *E. coli* clone.

In order to localize the region of DNA that encodes the factors conferring invasiveness, Tn*5* insertion mutations were isolated in the plasmid in a manner similar to that described above for *B. pertussis.* All of these mutations mapped in a 3.2-kilobase region of the cosmid clone. Deletion and more insertion mutations, this time with the nonpolar Tn*1000* (IIDA et al. 1984), were isolated in this region and used in functional complementation tests with previously isolated Tn*5* mutations. All mutations were found to fall into only one complementation group, indicating that only one protein may be necessary to confer the invasive phenotype.

We have begun physiological and electron microscopic studies of *E. coli* cells containing either the intact invasion gene or various insertion mutations in that gene (Fig. 1). Thin-section electron microscopy shows that the bacteria first bind HEp-2 cells at 0 °C without invading (Fig. 1 b). This is identical to what is seen with *Y. pseudotuberculosis.* The bacteria are soon surrounded by microvilli after being bound by the animal cell. When the temperature is raised to 37 °C, vacuoles containing large numbers of bacteria are observed within the HEp-2 cells, although some vacuoles containing only one bacterium are also present. There may be an occasional bacterium in vacuole-free cytoplasmic environments, but these are distinctly in the minority. Thin sectioning of an infection by an insertion mutant, in contrast, shows no bacteria present in the environment of the animal cell. When radiolabeled bacteria were used to study binding to the epithelial cells, we found that each insertion mutation that had lost the invasive phenotype was also unable to bind the animal cells

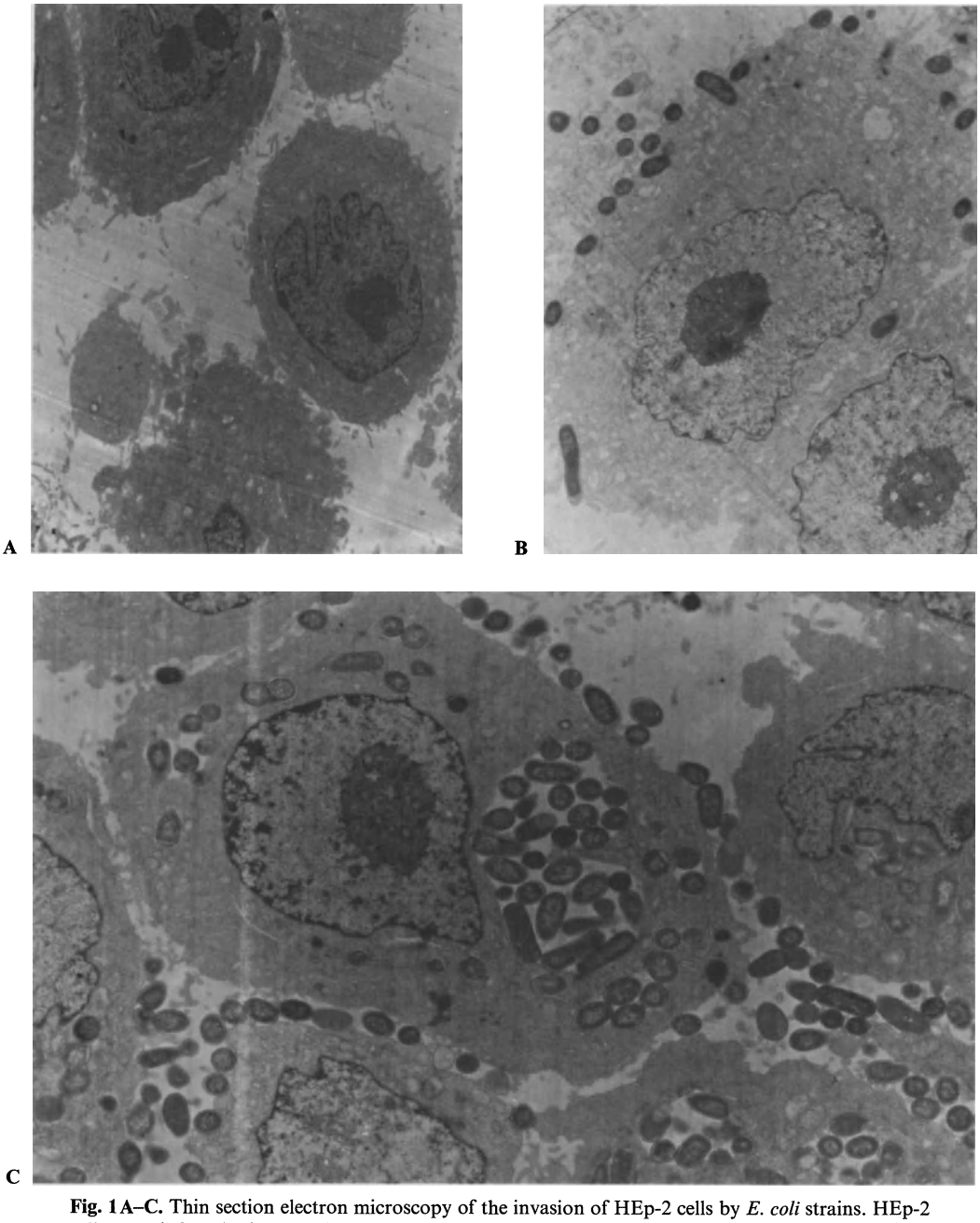

Fig. 1A–C. Thin section electron microscopy of the invasion of HEp-2 cells by *E. coli* strains. HEp-2 cells were infected with *E. coli* strains that harbored either a plasmid encoding the factor required for tissue invasion or an insertion mutation in this plasmid that eliminated invasion. Infection by an insertion mutant (**A**); infection at 0 °C by strain harboring intact invasiveness plasmid (**B**); infection at 37 °C by strain harboring intact invasiveness plasmid

at 0 °C. Both binding and invasion, therefore, are apparently mediated by the same *Y. pseudotuberculosis* gene product.

The physiological data show that the *E. coli* strain encoding the invasion determinant behaves almost identically to *Y. pseudotuberculosis* in its invasiveness properties. This is circumstantial evidence, but not proof, that this gene product is essential for invasiveness of *Y. pseudotuberculosis*. Genetic proof of its essentiality for invasion requires that specific mutations in this gene be crossed into *Y. pseudotuberculosis*. If this gene is required for *Y. pseudotuberculosis* invasion, then these mutants should be unable to invade. Clearly, being able to introduce DNA into *Y. pseudotuberculosis* with phage P1 should allow us to cross into this organism the mutants we have constructed in *E. coli*. In addition, such mutants will be useful in experimental animals infections, to study the role of invasion in the pathogenesis of *Yersinia* infection.

4 Future Prospects

The mode of action of the invasion determinant is unclear. It seems most likely that the protein modifies the bacterial cell surface in some manner, allowing binding and invasion. There are two possible ways it is able to accomplish this: (a) the protein is actually localized on the cell surface, allowing it to be bound via an interaction between this protein and the animal cell surface or (b) the protein modifies, perhaps enzymatically, some existing structure on the bacterial cell. One of the predictions of the latter model is that if this existing structure is required for invasion, it should be possible to identify it genetically by isolating mutations in it that result in a noninvasive phenotype. Such an approach should be possible by mutagenizing the *E. coli* chromosome, since it is clear that *E. coli* must encode this structure for invasion to occur. The advantage of this approach is that a great many genes that encode the proteins involved in the biogenesis of the cell surface have already been mapped in *E. coli*.

One of the aspects of the *Y. pseudotuberculosis* system which is most appealing is the possibility of constructing mutants in vitro or in *E. coli* and then reintroducing them into the *Yersinia* chromosome (BOLIN and WOLF-WATZ 1984). Currently, we have only isolated insertions or deletions in the *E. coli* clones – mutations that have gross effects on the structure of the protein being studied. In the future, it should be possible to isolate point mutations in this protein, using a variety of in vitro mutagenesis techniques (SHORTLE and BOTSTEIN 1983; SHORTLE et al. 1982). Such mutations can be crossed into the *Yersinia* chromosome by selecting for a linked marker and screening for the desired phenotype (Fig. 2). Mutants generated in vitro can be isolated in *E. coli*, screening for the desired phenotype on a plasmid that has a nearby drug resistance marker inserted in *Y. pseudotuberculosis* DNA. This construction may then be introduced into *Y. pseudotuberculosis* under conditions that are nonpermissive for replication of the plasmid. As can be seen in Fig. 2, selection for the appropriate drug resistance occasionally results in introducing the desired mutation onto the chromosome.

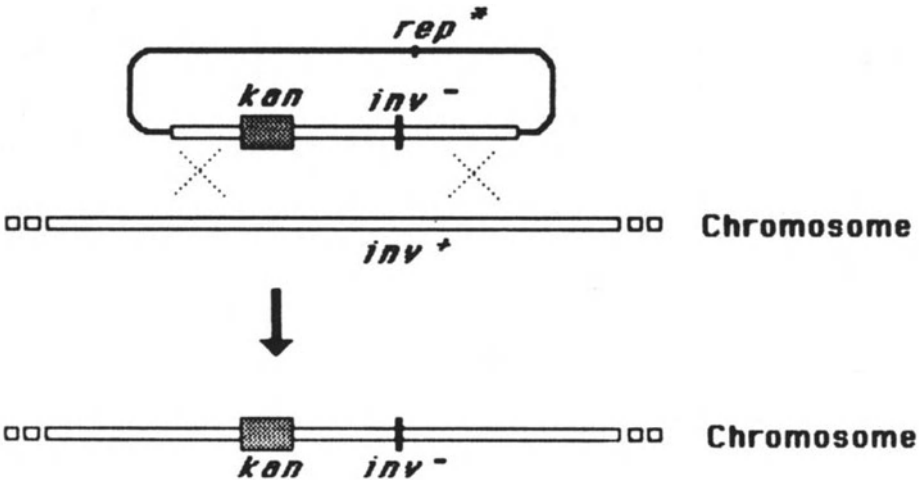

Fig. 2. Introduction of point mutations constructed in vitro onto the *Y. pseudotuberculosis* chromosome. A plasmid that is replication-deficient in *Y. pseudotuberculosis* (represented by *rep**) and contains a point mutation in the desired gene (*inv*) is introduced into this organism. Recombination between this plasmid and the chromosome is selected by a drug resistance marker (denoted *kan*) that is located nearby, but outside, the gene of interest. Among the *kan* recombinants that arise, some should now carry the *inv*$^-$ mutation

We believe that the isolation and analysis of point mutations, as in the manner just described, has been neglected in the investigation of pathogenic mechanisms. In addition to the possibility of isolating nonpolar mutations, such lesions allow insight into the functions of various regions of the proteins being studied. For instance, in the case of yersiniae invasiveness, we are especially interested in isolating point mutations that result in altering the binding properties of whatever structure is on the surface of the bacterium, so that the bacteria are able to bind animal cells, but are no longer able to invade. In this manner, we may be able to define which structures are important for binding and which structures are important for invasiveness. Certainly, a similar approach could be taken in the analysis of the mechanism of *Bordetella* toxin entry into the animal cell.

5 Conclusions

We believe that the studies on *B. pertussis* and *Y. pseudotuberculosis* show that a considerable amount of insight can be gained from a genetic analysis that is independent of the ability sequence genes directly. Although the sequencing of factors such as cholera toxin (MEKALANOS et al. 1983) and diphtheria toxin (GREENFIELD et al. 1983) has yielded great insights into the structures of these obviously important proteins, we believe that the study of bacterial pathogenesis in the near future will focus on defining more subtle elements in the infection

process. The discovery of these genes and their products will require the development of simple genetic systems for their analysis, such as those used to investigate the adenylate cyclase of *B. pertussis* and the invasion determinant of *Y. pseudotuberculosis*. In addition, the ability to isolate and analyze mutants will surely result in cases in which well-established paradigms will be shown to be incorrect. Recently, this was most graphically illustrated with the discovery that pilin is not the protein responsible for the adhesiveness exhibited by piliated strains of *E. coli* (LINDBERG et al. 1984). This result was only possible because a simple system existed that made possible the isolation of mutants and the analysis of their phenotypes. We suspect that many such surprises will occur in future genetic analyses.

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Gonococcal Pilus: Genetics and Structure

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1 Introduction

Antigenic variation is a means by which many infectious organisms evade the host immune response. This phenomenon has been observed in animal viruses (e.g., influenza virus), parasites (e.g., *Trypanosoma brucei*), and bacteria (e.g., *Borrelia recurrentis*). Recent work has shown that two surface proteins of *Neisseria gonorrhoeae* undergo antigenic variation as well. These two virulence factors are the pilus and the opacity (PII) proteins, both of which promote gonococcal binding to host (human) epithelial cells (Buchanan and Pierce 1976; Swanson 1973). In this chapter, we shall emphasize the work done on the pilus system. We present evidence that phase and antigenic variation are closely linked phenomena; that the regulation of pilus expression is reminiscent of yeast mating type interconversion and of immunoglobulin gene rearrangement; and that the gonococcal pilin belongs to a class or family of bacterial pilins with a common subunit structure.

The gonococcal pilus is a homopolymer of approximately 10000 subunits (pilins) of ~18000 daltons (Brinton et al. 1978). It is a major virulence factor: piliated, translucent (P^+, PII^-) cells are infectious whereas nonpiliated, translucent (P^-, PII^-) cells are not (Kellog et al. 1963). Among the many clinical isolates are found a range of pilus serotypes (Buchanan 1975; Robertson et al. 1977). Antigenically different pilins all share a common hydrophobic N-terminal sequence (Rothbard et al. 1984; Hagblom et al. 1985). Variation occurs in the C-terminal region. Antibodies raised against one pilus type will cross-react minimally with heterologous pili (Brinton et al. 1978; Virji and Heckels 1983), and pilus from one strain protects only against challenge by

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the homologous strain (J. SADOFF, personal communication). Thus the conserved region is immunorecessive. Antigenic variants of the ~28000 PII protein have also been observed (BLACK et al. 1984), and PII variants also share common regions (SWANSON and BARRERA 1983).

2 Phase Variation

The gonococcus undergoes phase variation in vitro. Cells of one colonial morphology will segregate others with a different morphology. This is easily observed by microscopy and is the result of the presence or absence of pilus and/or PII (KELLOGG et al. 1963; WALSTAD et al. 1977). Thus, one colonial variant can give rise independently to three other variants, the four variants being: P^+ PII^-, P^+ PII^-, P^- PII^+, and P^- PII^+. The frequencies of the pilus and PII switches vary from isolate to isolate and with culture conditions. In

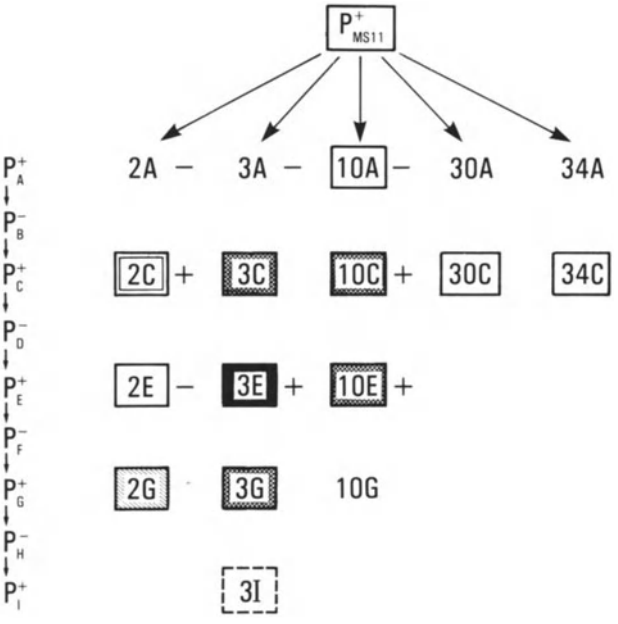


Fig. 1. *On the left side* is a schematic representation of the procedure used for obtaining lines of MS11 pilus variants. See text for a detailed description. The *right side* of this figure contains a summary of the pilus sequence information obtained from the P^+ variants from lines 2, 3, 10, 30 and 34. The MS11 P^+ progenitor is *at the top*. Each different pilus sequence is represented by the design of the box surrounding the strain number. Thus the progenitor P^+_{MS11}, 10A, 30C, 34C, and 2E all express the same pilin gene variant. 3C, 10C, 10E, and 3G express a second pilin gene variant. 2C, 3E, 2G, and 3I each express a unique gene variant. Those strains which produce a pilin antigenically different from that of the progenitor are indicated by a + *next to the boxes*. Those strains which produce pilin identical to that of the progenitor are marked with −. The absence of + or − indicates the immunological identity of the pilin from these variants has not been determined

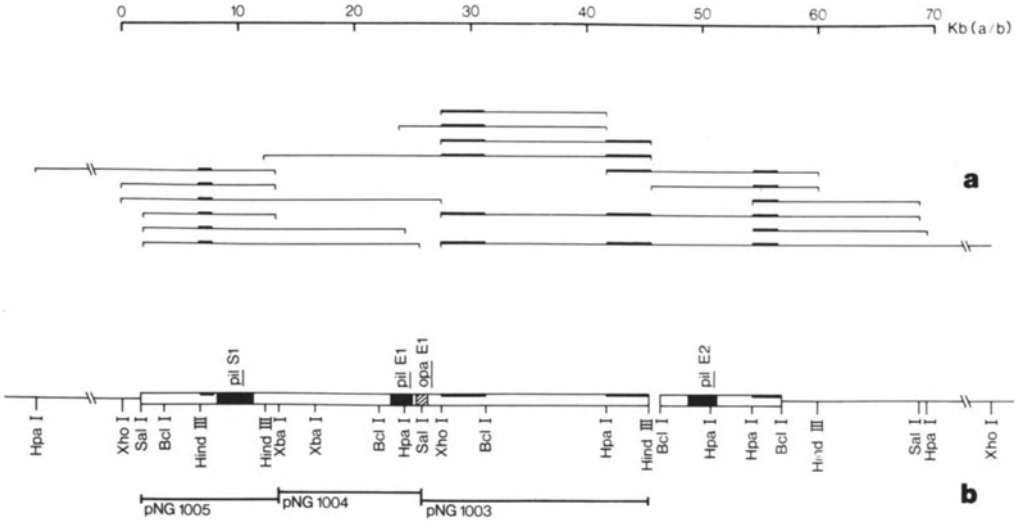


Fig. 2a, b. Chromosomal map of three pilus-related loci (*pil*S1, *pil*E1, and *pil*E2; *black boxes*) and one PII-related locus (*opa*E1; *hatched box*) is represented in **b**. *Thin lines* in **a** represent various clones from this 70-kb region used to map these loci. *Thickened lines* in corresponding positions in **a** and **b** represent unique flanking sequences used to orient the loci

MS11, the frequency of the P^+ to P^- switch is $\sim 10^{-3}$; the frequency of the P^- to P^+ switch is $\sim 10^{-4}-10^{-5}$. The PII^+ to PII^- and PII^- to PII^+ switch frequencies in MS11 are similar to the pilus switch frequencies.

We have made isogenic derivatives of MS11 which differ only in pilus expression (leftmost lane, Fig. 1). Thirty-four lines of MS11 have been established. Members of the A series have never undergone a phase switch and are therefore identical to the MS11 progenitor. Members of the B series are P^- variants derived from the A series; these have undergone the phase switch once. Members of the C series are P^+ variants derived from the B series; these have undergone the phase switch twice. Using this approach, we have generated lines of variants in which the members have undergone the phase switch six to eight times.

Cloning of the expressed pilin genes from P^+ cells and Southern blot analysis of the pilus variants using these genes and unique flanking sequences as probes (Fig. 2a) show that two regions of the gonococcal chromosome act as expression sites (MEYER et al. 1982, 1984). These two loci, *pil*E1 and *pil*E2, are 20 kb apart (Fig. 2b) and are contained in 4.0- and 4.1-kb ClaI fragments (Fig. 3). There are several other chromosomal regions which contain silent pilus sequences. One, *pil*S1, is ~ 15 kb away from *pil*E1. In addition, *opa*E1 apparently acts as an expression locus for the PII gene and is ~ 700 bp downstream of the pilin stop codon in *pil*E1 (STERN et al. 1984; Fig. 2b). This arrangement of pilin sequences is reminiscent of the organization of genes involved in mating type interconversion in *Saccharomyces cerevisiae*. In this latter system, the expression locus, MAT, receives a copy of the *a* or α gene from the silent locus, HMLa or HMRα, respectively (see HERSKOWITZ 1983).

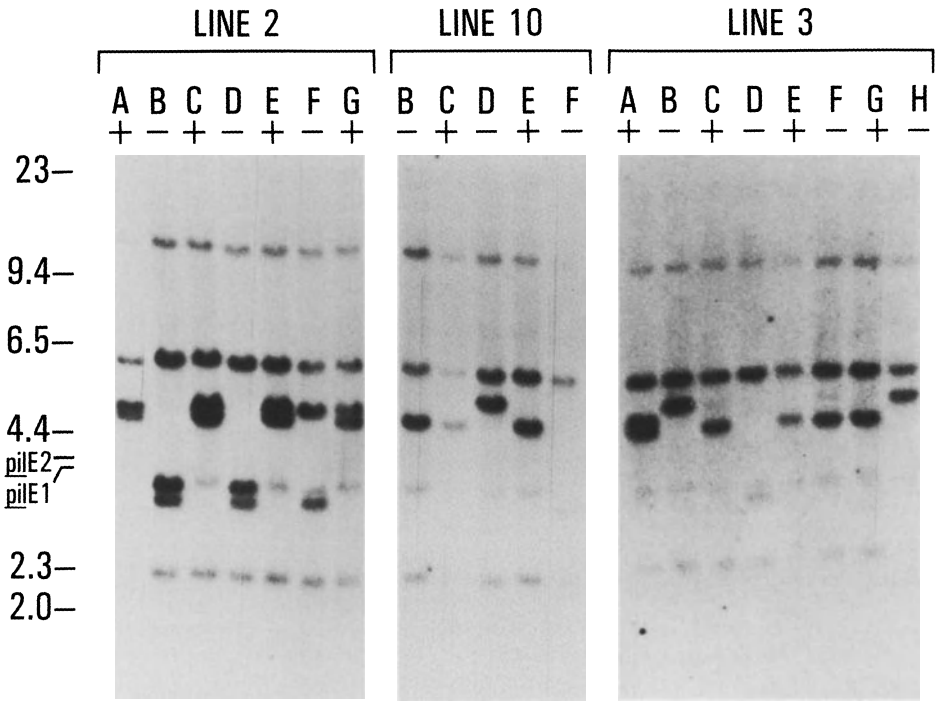


Fig. 3. Southern blot analysis of the two pilus expression loci, *pil*E1 and *pil*E2, in MS11 pilus variants from lines 2, 10, and 3. The pilus phenotype of these variants is indicated by + or − above each lane. Chromosomal DNA was digested with ClaI; the probe is pNG1100 (MEYER et al. 1982), a 2-kb insert containing the pilin gene as well as flanking sequences shared by *pil*E1 and *pil*E2

Southern analysis of members of lines 2, 10, and 3 derived from MS11 shows that the P^+ to P^- switch involves a rearrangement of either *pil*E1 (2F) or *pil*E2 (10B, 3F) or both 2B, 2D, 10D, 3D; Fig. 3). In the reverse P^- to P^+ switch is seen a restoration of one or the other or both loci. Thus, only one expression locus is needed for pilus expression (10C, 10E, 3C, 3E, 3G). *pil*E1 from several B series P^- variants have been cloned into *Escherichia coli*. Restriction mapping of these inserts shows that the chromosomal reorganization observed during the P^+ to P^- switch is due to a deletion of the pilin gene in the expression locus (Fig. 4). The deletions are of varying sizes and in many cases are the result of multiple recombination events which have occurred within directly repeated sequences found within *pil*E1 (SEGAL et al. 1985). At present we do not know whether the deleted pilin sequences are lost (as in the case of an outgoing MAT *a* or α gene) or whether it has now recombined with homologous silent pilin sequences.

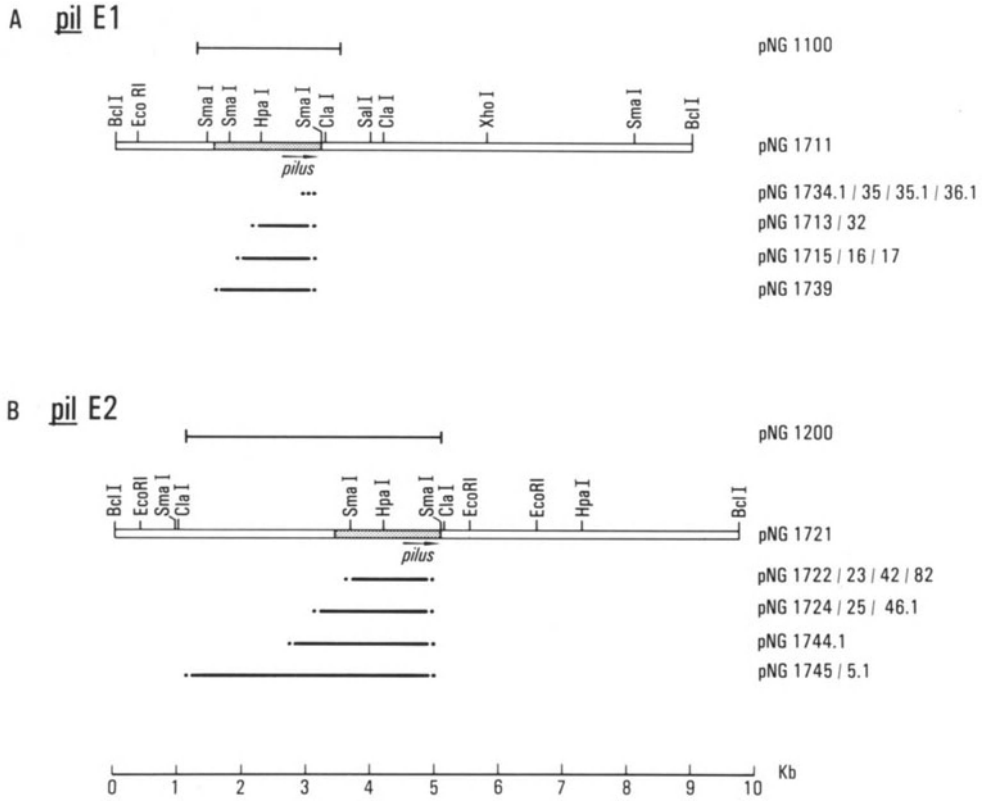


Fig. 4A, B. Restriction analysis of clones of *pil*E1 and *pil*E2 from several P^- MS11 variants. The ~10-kb BclI fragment containing either *pil*E1 or *pil*E2 is denoted by the *empty boxed region* in **A** and **B**. The *stippled area within each box* represents homology shared by *pil*E1 and *pil*E2. *pil*E1 and *pil*E2 were cloned from the P^- variants as BclI inserts, and recombinants were detected with DNA probes containing sequences unique to each expression locus. *Darkened lines flanked by dots* indicate the deletion found in each insert by restriction mapping. The *series of dots without a darkened line* indicates small regions of nonhomology found between the P^- and the original P^+ expression locus, as seen by S1 protection experiments. However, in these cases, no major deletions were observed. The names of the clones containing the various BclI inserts are listed *in the right-hand column*

Many P^- derivatives still contain one intact expression site (e.g., 2F, 10B, 3F). These cells are phenotypically P^- (that is, they do not have cell surface pilus). Some do not produce pilin while others produce very low levels of pilin ($<0.1\%$ of the amount produced by a P^+ cell; data not shown). Sequence analysis of these intact P^- loci shows that the pilin gene is still present and that the promoter sequences are identical to that of an expressing gene (SEGAL et al., to be published; HAGBLOM et al. 1985). Thus, pilus expression is regulated by deletionogenic events as well as by an as yet unidentified factor which is itself regulated by the phase switch. It is unclear why these P^- low-level pilin producers do not assemble pili. It is possible that the assembly function(s) is also switch regulated. An alternative explanation is that pilus assembly is

finely tuned to pilin concentration and a threshold must be reached before subunit assembly occurs. Furthermore the intact expression loci in these P^- cells have undergone sequence variation: pilin sequences found here in three out of three cases are different from each other and also from the sequence found in the P^+ MS11 progenitor. Our observations suggest that pilus phase and antigenic variation are closely, if not obligately, linked.

The phenomenon of pilus phase variation has so far been studied in vitro. Its significance, if it does occur in vivo, remains unclear. To our knowledge there have been no studies done to determine the state of piliation of gonococci during the intracellular or bacteremic stages of the disease. Aside from the initial attachment step, pili may not be necessary for virulence. As mentioned earlier, it is also possible that the P^- phase may be an intermediate step necessary for antigenic variation.

The details of PII phase variation are not known. A PII^+ to PII^- switch and vice versa is not accompanied by any large rearrangement of the *opa*E1 site or of the other cross-hybridizing bands on the chromosome (MEYER et al. 1984). A partial sequence analysis of the PII coding region in *opa*E1 shows that minor base changes and substitutions have occurred during the switch.

The pilus and PII phase switching events are probably unrelated, although the PII^+ to PII^- switches in some clinical isolates result in the production of pilins of different sizes (SALIT et al. 1980). P^+, PII^+ cells give rise to cells of the other three phenotypes at close to equal frequencies. Even though the *pil*E1 and *opa*E1 loci are quite close to each other, there is no sequence overlap and each structural gene has its own promoter. The region between these loci may be a hot spot for mutations generating PII phase variants which fortuitously affect the pilin gene as well. In this context we would like to point out that in some P^+ to P^- switches the *Cla*I fragment containing the *pil*E1 locus has now become larger in size (10D, 3H). This size increase could best be explained by a deletion of the ClaI site between *pil*E1 and *opa*E1 which now brings the two loci closer, and perhaps affecting the expression of PII.

3 Antigenic Variation

To examine pilus sequence variation in detail, we have used the technique of primer extension sequencing of pilin mRNA (MATTI et al. 1983). This technique involves using synthetic oligodeoxynucleotides complementary and specific to the pilin-coding sequence (known from Maxam/Gilbert sequencing of the cloned pilus gene from the P^+ MS11 progenitor; MEYER et al. 1984) to prime the synthesis of a cDNA copy from the pilin mRNA. The addition of dideoxynucleotides to the reaction yields cDNA sequences. Figure 1 summarizes the results of our sequence determinations on several lines of MS11 variants. MS11 can express at least seven variant pilin genes. Pilin information is not lost during the switch: 2A and 2E express the same pilin variant sequence while 2C expresses a different sequence. Thus, sequence variation, in which a new pilin gene is put into place in the expression locus, is best explained mechanistically

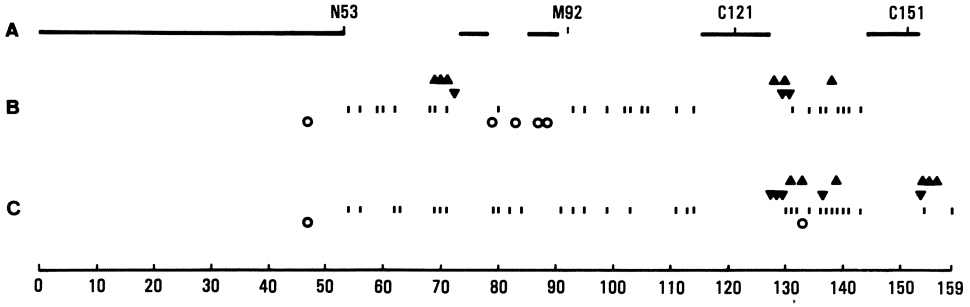


Fig. 5A–C. Positions of the original MS11 pilin sequence at which amino acid changes were found during antigenic variation. Bear in mind that this is a summary diagram. Each variant pilin may not have changes in all the positions indicated. The pilin produced by the MS11 progenitor, deduced from the DNA sequence (MEYER et al. 1984), is 159 residues in length and is denoted by the scale *at the bottom. The marks above the scale* represent residue changes seen at these positions as observed by primer extension sequencing of pilin-specific mRNAs. *Circles,* silent codon changes; *vertical slashes,* single amino acid changes; *triangles,* deletions of one or more residues; *inverted triangles,* insertions of one or more residues. The *marks* in **C** represent a summary of all positions at which changes were seen in the MS11 variants. Those in **B** represent changes observed in our epidemiological studies. *Thickened lines in* **A** mark those sections within the pilin sequence at which no changes were observed when we compared the sequences of 20 variants

by a gene conversion event. Those derivatives which express a variant gene produce pili immunologically different from that of the progenitor. We do not know at present how many epitopes each variant pilin sequence represents. However, work by others has shown that for the P9 strain of *N. gonorrhoeae* there are at least three epitopes contained in the variable region (VIRJI and HECKELS 1983).

A comparison of the different variant sequences at the DNA level shows that the pilin-coding sequence can be divided into three regions (Fig. 5). The conserved region occurs between residues 1 and 53. This is followed by a semi-variable (SV) region, in which are found only single amino acid changes which are the result of one to three base changes within each codon. The hypervariable (HV) region is located at the C-terminal end. Here are observed not only single amino acid changes, but also insertions of one to four amino acids and deletions. Within the variable region are two highly conserved areas (*cys*1 and *cys*2) centering around cysteines at positions 121 and 151. The conserved regions within the pilin sequence almost certainly play structural and/or functional roles (see next section). Furthermore "mixing and matching" of SV and HV gene segments occurs: members of line 3 have the same SV sequence but different HV sequences.

The lack of an animal model makes studying in vivo pilus antigenic variation difficult. We have approached this problem by sequencing pilin mRNA from clinical isolates from a gonorrhea epidemic in which all strains can be traced back to a single source (FLETCHER et al. 1983; FARUKI et al., to be published). Our results indicate that antigenic variation does occur with time during dissemination. Furthermore, isolates obtained from the same patient at different times

also produce antigenically variant pili. This variation is probably not the result of immunological pressures from the host since antibiotics were administered within a few days after each culturing was done. Rather, these variant strains were probably acquired by the patient through sexual contact with partners already carrying the variants. The pattern of intragenic diversity seen here closely matches that observed for our MS11 derivatives: the pilin consists of conserved, semivariable, and hypervariable regions. Thus, those events which have generated the pilin variants in vitro are an accurate reflection of in vivo events.

We cannot explain the curious tendency of MS11 to express two particular pilin genes (30C, 34C, 2E; 3C, 10C, 10E, 3G). This could be the result of gene organization. Gene conversion requires homologous pairing of sequences from the two loci undergoing conversion. If the two variant sequences are located close to the paired regions, they would be expected to be copied and converted at a higher frequency than those variant sequences located farther away. It is also possible (although not likely) that the expression of the variant genes is temporally programmed, as is the case for the VSG genes of *T. brucei* (BORST and CROSS 1982). In this instance, a certain set of pilin variant genes, for unknown reasons, preferentially would be expressed early and other genes late.

A good candidate for a sequence which functions in the pairing event during gene conversion is located in the 3′ untranscribed region of the pilus gene (between the rightmost SmaI/ClaI sites in Fig. 4). This sequence occurs in both *pil*E1 and *pil*E2, in both the P^+ and P^- state, as well as in several silent loci (E. SEGAL, T.F. MEYER, unpublished data). In some P^+ to P^- switches, the DNA sequence in this region has been altered partially (SEGAL et al. 1985). In addition the 3′ region of the pilin transcripts of a few P^+ variants also have altered sequences (HAGBLOM et al. 1985). These observations strongly suggest that this region is a site at which recombination occurs.

That SV and HV gene segments can be mixed and matched suggests that pilin sequences in the silent loci occur in a disjunct or discontinuous form. We have observed that in *Pil*S1 is a region which contains many directly repeated copies of variant sequences (MEYER et al. 1984). The conserved sequences are fewer in number and occur in a separate region. A constant region probe hybridized to only two chromosomal regions while a variable region probe (containing the *cys*1 and *cys*2 sequences) hybridized to many more (E. SEGAL, unpublished data). The generation of a functional pilin gene, then, would almost certainly entail a multiple recombination event. At present, we feel that the gonococcal chromosome already contains many variable region sequences. The matching of SV and HV segments would be another mechanism used by the organisms to generate more diversity. A probe specific to a variant sequence unique to 3G hybridized to one silent locus as well as to the expression locus. In this case at least, this particular variant sequence has not been generated de novo. However, it is quite possible that somatic mutations could occur in variant sequences with time to bring about more pilin diversity. The insertions and deletions are all seen at the SV/HV junction, suggesting that further diversity may be generated as a consequence of imprecise joining events.

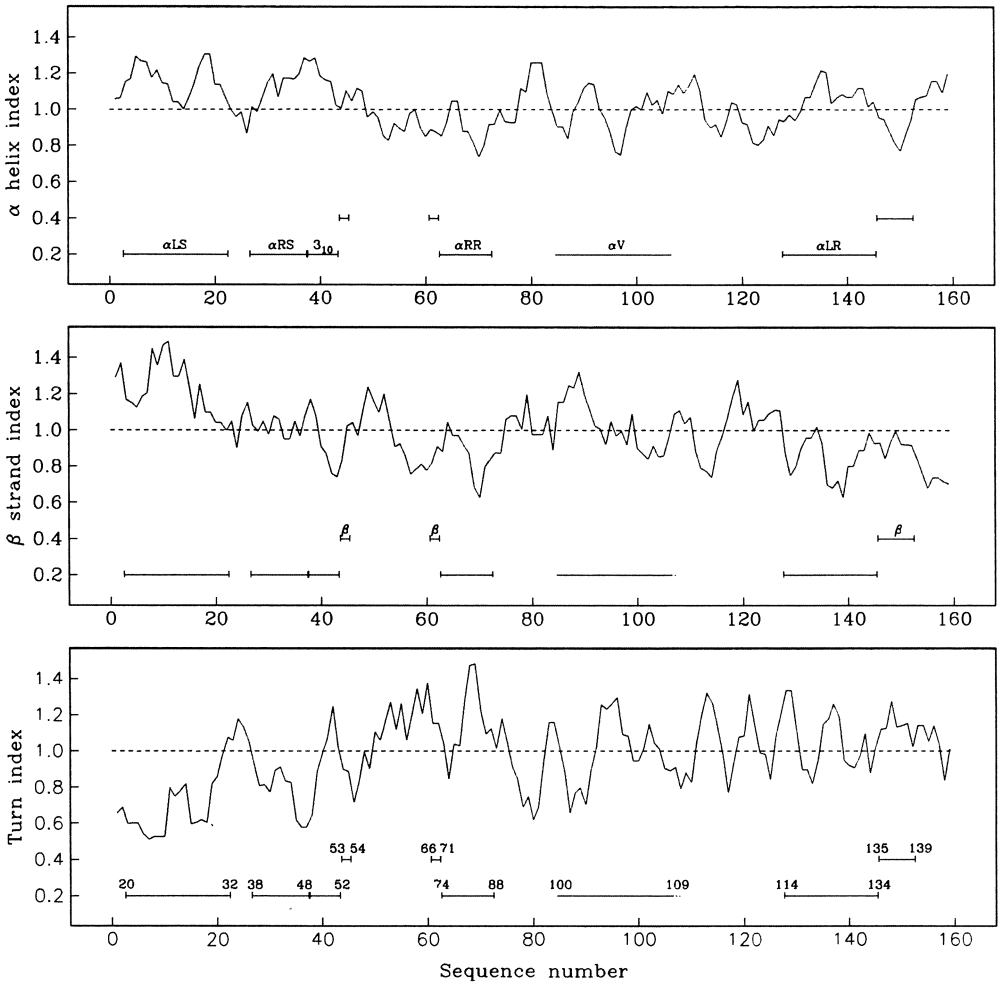


Fig. 6. The alpha helix, beta strand, and tight-turn conformational potential plotted versus sequence number for the MS11 pilin sequence. In each curve, values above the indicated cutoff of 1.0 define probable secondary structures. *The bars at the bottom of each plot* represent the secondary structural elements of the TMV coat protein positioned from the sequence alignment shown in Fig. 7. In the top plot TMV helices are labeled by the nomenclature used in describing the crystallographic structure (BLOOMER et al. 1978); beta strands are identified in the middle plot, and sequence ranges for both helices and beta strands are identified in the bottom plot

The similarity between the pilus and immunoglobulin system is striking. Immunoglobulins consist of conserved and variable domains. Within the latter are also found hypervariable segments. In hemotopoietic stem cells, the V and J coding segments for Ig light chains are widely separated (JOHO et al. 1983). During differentiation these segments are brought together, in most cases, by a deletion of intervening segments. In addition to somatic mutation, imprecise joining of Ig gene segments also contributes to diversity of antigen-binding

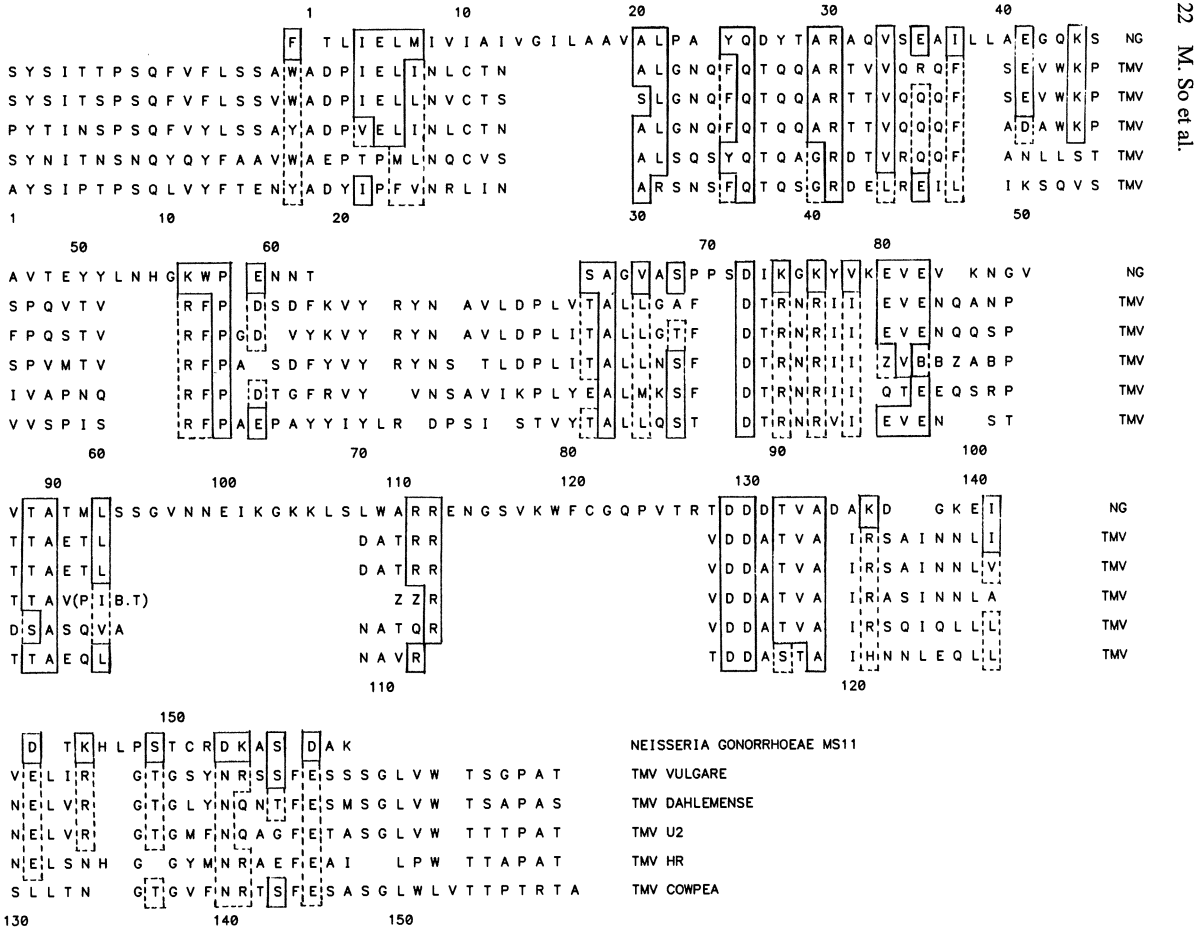


Fig. 7. The alignment of the amino acid sequence of *N. gonorrhoeae* MS11 pilin (MEYER et al. 1984) with sequences of five strains of TMV coat protein (BARKER et al. 1984). Sequence-conserved residues found in both TMV and pilin are *boxed:* identical residues are indicated by *solid boxes*, similar residues by *dashed boxes*. The sets within which residues were considered similar are: *(F, W, Y), (F, I, L, M, V), (A, G), (D, N), (Q, N), (E, Q), (B, D, E, Z), (H, K, R)*, and *(S, T)*. The sequences are shown in one-letter code: *A*, alanine; *C*, cysteine; *D*, aspartic acid; *E*, glutamic acid; *F*, phenylalanine; *G*, glycine; *H*, histidine; *I*, isoleucine; *K*, lysine; *L*, leucine; *M*, methionine; *N*, asparagine; *P*, proline; *Q*, glutamine; *R*, arginine; *S*, serine; *T*, threonine; *V*, valine; *W*, tryptophan; *Y*, tyrosine

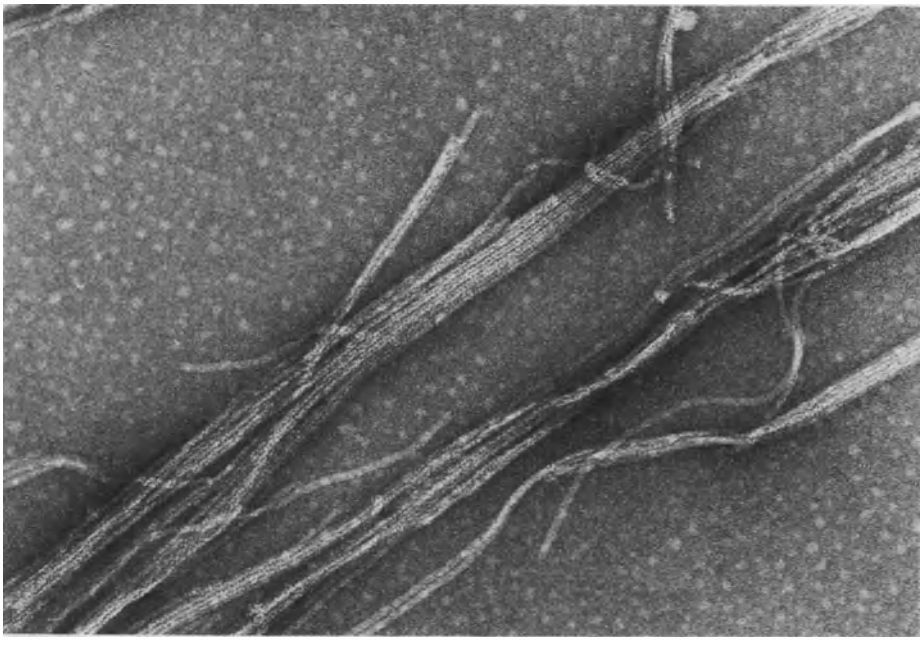


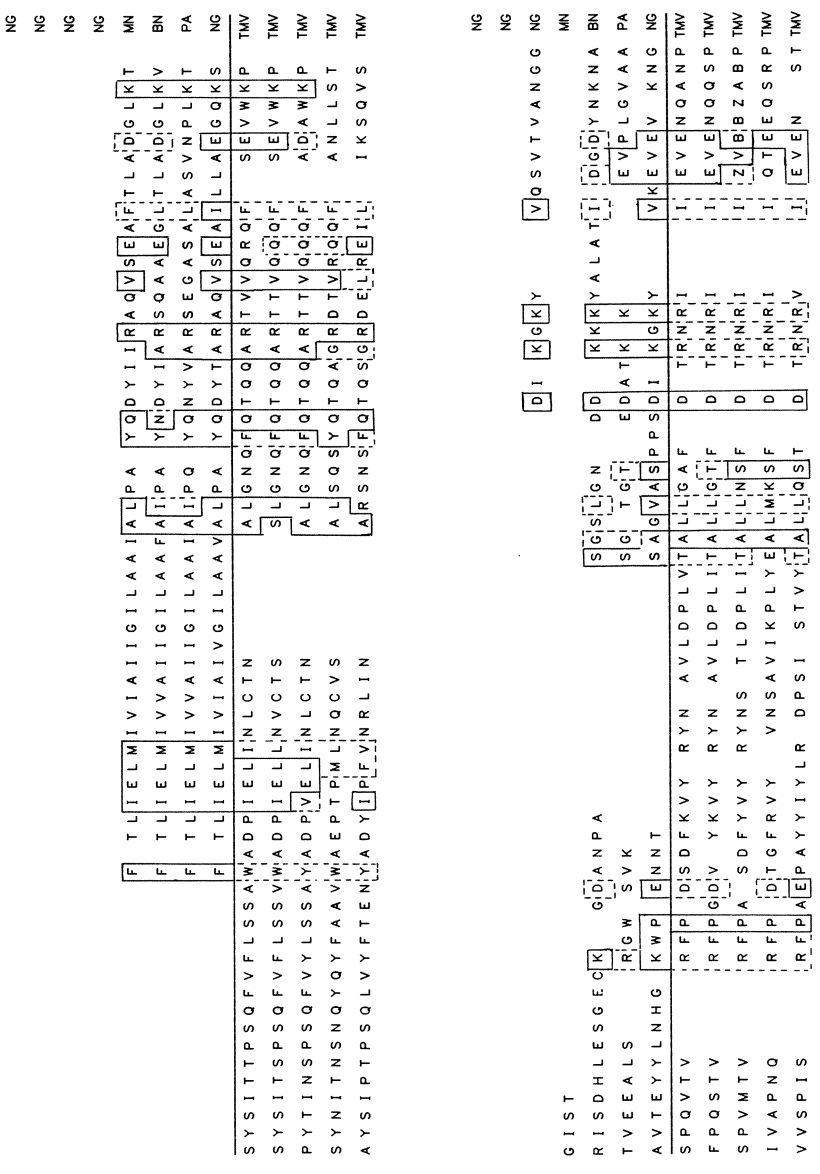
Fig. 8. Electron micrograph (×210000) of purified pilin subunits reassembled into filaments. In solution at pH 8–9, the 60-Å wide filaments occur both singly and in aggregated sheets or bundles; the narrow fibers are indistinguishable by electron microscopy from negatively stained native pili

sites. The vertebrates have evolved this elaborate system to deal with foreign substances which find their way into the body. It is curious to see that the gonococcus, with a genome size one-third that of *E. coli,* has also evolved an equally complicated system to avoid immune surveillance.

4 Pilus Structure

Pilus antigenic variation must occur within the constraints imposed by structural and functional requirements. Variants must produce pilin subunits capable of assembling into pili which can bind to host epithelial cells. Those with nonfunctional pili will be avirulent and at a selective disadvantage since gonorrhea is a disease with no intermediate hosts. This requirement is reflected in the conserved region located at the amino terminal end of the protein, as well as at the two cysteine regions. The conserved N-terminal region has been suggested to contain the epithelial cell receptor binding function (SCHOOLNIK et al. 1984). The HV region, on the other hand, is located in the carboxy terminal end of the protein and allows the bacterium to escape the host immune response.

To develop an understanding of the structural constraints imposed on the molecule, we have initiated a series of studies directed at determining the pilin three-dimensional structure. Based on the amino acid sequence deduced from



the DNA sequence (MEYER et al. 1984), we have predicted the secondary structure (Fig. 6) utilizing a modification of the Chou and Fasman empirical method (CHOU and FASMAN 1978; D. MCREE and E. GETZOFF, unpublished method). This initial secondary structure assignment suggested the categories of antiparallel alpha domains and parallel alpha/beta domains as possible structural motifs (RICHARDSON 1981), while arguing against motifs organized around antiparallel beta structure or known parallel alpha/beta topologies. Of the known alpha helical domains, the topology of tobacco mosaic virus (TMV) coat protein

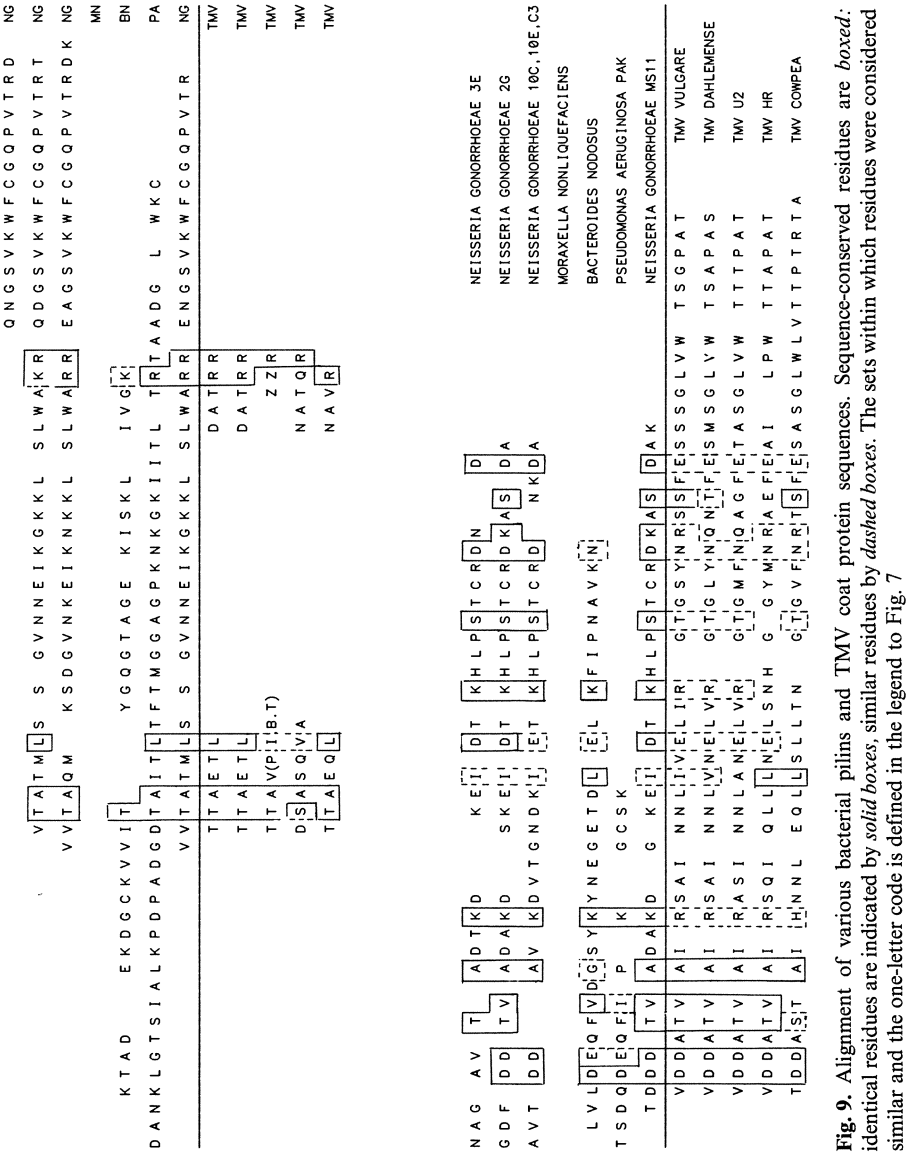


Fig. 9. Alignment of various bacterial pilins and TMV coat protein sequences. Sequence-conserved residues are *boxed*: identical residues are indicated by *solid boxes*, similar residues by *dashed boxes*. The sets within which residues were considered similar and the one-letter code is defined in the legend to Fig. 7

(BLOOMER et al. 1978), an antiparallel up and down helix bundle (WEBER and SALEMME 1980), was identified as the most compatible with the predicted pilin secondary structure (Fig. 6). Thus, the pilin molecule is predicted to be composed of a bundle of four antiparallel alpha helices aligned along the long axis of a roughly $25 \times 25 \times 70$-Å molecule. It was also noted that TMV coat protein and gonococcal pilin shared similarities in polypeptide chain length and in the formation of a long rod-like assembled structure consisting of many identical subunits.

Sequence similarities between gonococcal pilin and TMV coat protein were found when the pilin sequence was aligned simultaneously with five known variant TMV coat protein sequences obtained from the protein sequence database (BARKER et al. 1984). The concurrent alignment with multiple sequences enabled the regions conserved among different TMV strains to be identified and emphasized during the pilin sequence alignment. Amino acids conserved in all five TMV coat protein sequences were assumed to have structural and/or functional significance and were therefore considered to be most important in defining the concurrent alignment of the pilin sequence. The resulting alignment (Fig. 7) showed that sequence similarities between pilin and TMV coat protein include regions identified in TMV to be important for intrasubunit folding, for intersubunit contacts, and for the binding to nucleic acid. Analysis of the secondary structure prediction for the sequenced antigenic variants of MS11 indicates that the general structural parameters identified for the original MS11 serotype are maintained in the additional serotypes, thus supporting the hypothesis that antigenic variability occurs within those regions not important for pilus structural or functional integrity.

A comparison of biochemical and biophysical properties for TMV coat protein and gonococcal pilin revealed additional similarities between the two proteins. Both are acidic proteins with isoelectric points between 4.3 and 5.3 (GALLAGHER and LAUFFER 1983; ROBERTSON et al. 1977). The solubility properties of pilin (BRINTON et al. 1978) generally match the phase diagram of TMV coat protein subunit assembly (DURHAM et al. 1971). Both proteins are soluble at high pH and low ionic strength, but polymerize at lower pH and higher ionic strength. The TMV phase diagram was used as a guideline for possible conditions suitable for obtaining dissociated pilin subunits, reassembled pili, and protein crystals. Starting from dissociated subunits, we have reassembled filaments that are indistinguishable in electron micrographs from negatively stained native pili, as well as pilin sheets showing longitudinal filaments 60 Å apart with clearly distinguished and regular fine structure that is not present in the individual fibers (Fig. 8). Additionally conditions have been identified for growing blocky three-dimensional crystals with clean faces averaging 50 μm in each dimension. Crystal lattice spacings of about 200×320 Å in one projection and 30 Å in the perpendicular projection observed in negatively stained specimens of crushed crystals are compatible with the packing predicted from the subunit structure based on the proposed similarity with TMV coat protein. Further studies will concentrate on solving the pilin crystal structure, from which we can then build models for the association of the pilin into the pilus supramolecular structure.

An interesting finding from an extension of these studies on the gonococcal pilin is that pilins from widely disparate bacteria (*Pseudomonas aeruginosa* and *Bacteroides nodosus*) also show similarity in both sequence and predicted structural motif. Sequence-conserved regions shared between TMV coat protein variants and gonococcal pilin variants are also seen in the pilins from these two bacteria (Fig. 9). All three bacterial pilins have a homologous N-terminal amino acid sequence (identical at 20 out of 22 residues), and the N-terminal phenylalanine is methylated (MCKERN et al. 1983; SASTRY et al. 1983). Partial amino

acid sequence data from *Moraxella nonliqueficiens* and *Neisseria meningitidis* pillins show that these proteins also have the same two characteristics (FROHOLM and STETTEN 1977; HERMODSON et al. 1978). Thus detailed studies on gonococcal pilin structure and pilus assembly should have a large impact on our understanding of a class or family of bacterial pilins.

In summary, the genetic and structural studies were aimed at developing a clearer understanding of the pilus protein, a major virulence factor of the gonococcus. Elucidation of the mechanisms used by this organism to evade the host immune response should increase our understanding of bacterial pathogenesis in general and ultimately contribute to efforts aimed at disease prevention. A commonality of biological themes has emerged from our studies. Pilus gene organization is similar to that of yeast mating type system. The intragenic recombination events which lead to pilus antigenic variation are reminiscent of the mechanism used by vertebrates to generate immunoglobulin diversity. Finally we have observed a similarity in the structural motifs of several bacterial pilins.

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Role of a Plasmid in the Pathogenicity of *Yersinia* Species

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1 Introduction

1.1 Plasmids and Pathogenicity

Plasmids are self-replicating extrachromosomal elements found in bacteria of many genera (BRODA 1979). They range in size from approximately 2 to 500 kilobases (kb). Thus, the genetic coding capacity of these plasmids may vary from one or two polypeptides to several hundred polypeptide chains. They may, therefore, impart considerable genetic information to plasmid-bearing strains. In most instances plasmids are dispensable for the growth of bacteria in the laboratory environment. Plasmids, however, often carry genes whose expression

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provide bacteria with a selective advantage in certain adverse environments. During the past decade, it has become apparent that plasmids frequently encode determinants of bacterial pathogenicity (ELWELL and SHIPLEY 1980). The most thoroughly examined example of the latter type of plasmids is those encountered in the enterotoxigenic *Escherichia coli,* whose two major determinants of pathogenicity, colonization factors (which afford bacteria the ability to adhere to host cells) and toxin production, are plasmid-encoded traits (ELWELL and SHIPLEY 1980). In addition, all of the invasive pathogenic members of the Enterobacteriaceae have been shown to have a plasmid which is essential for pathogenicity. Invasive *E. coli* strains often harbor plasmids of the colicin V class. These plasmids code for either an iron-sequestering system and/or resistance to the bactericidal action of normal human serum (BINNS et al. 1979; WILLIAMS and WARNER 1980). *Shigella flexneri* strains carry a large plasmid whose expression appears necessary for inducing keratoconjunctivitis in guinea pigs and invasion of HeLa cell monolayers (SANSONETTI et al. 1982). *Shigella sonnei* often harbors a plasmid which mediates O antigen biosynthesis and which is also considered essential for virulence (SANSONETTI et al. 1981). Further, *Salmonella typhimurium* possesses a plasmid which encodes determinants involved in the invasion of HeLa cell monolayers and virulence (JONES et al. 1982). In all of these enteropathogens, genetic determinants associated with virulence are located on plasmids. It must be emphasized, however, that plasmid-mediated determinants must act in concert with essential chromosomally encoded elements for the full expression of virulence (SANSONETTI et al. 1983). This review is concerned with the role of the plasmid-mediated determinants of pathogenicity from bacterial strains classified within the genus *Yersinia,* with a major emphasis given to *Y. enterocolitica.*

By way of background we will present brief sections on the disease syndromes caused by the yersiniae. Literature citations to this material are minimal. The interested reader is referred to excellent reviews which have appeared dealing with more detailed aspects of the biology and pathogenesis of the yersiniae (BOTTONE 1977; BRUBAKER 1972; BRUBAKER 1983).

1.2 The Yersiniae

Three species, all of which are pathogenic for humans and animals, have been included in the genus *Yersinia. Y. enterocolitica* and *Y. pseudotuberculosis* are causative agents of gastrointestinal illness in humans, and *Y. pestis* is the etiological agent of plague (BRAUDE 1981). While the clinical manifestations of disease and the routes of infection vary among the yersiniae, there are common elements in the pathogenetic process of the three species. All have a very broad temperature range for growth, and all interact intimately with eukaryotic cells during their residence within a mammalian host. At the molecular level, all yersiniae possess a particular species of plasmid DNA which is required for the full expression of virulence. In addition, possession of this plasmid species enhances the survival potential of the invasive pathogens within the mammalian host. We present evidence supporting the suggestion that a plasmid found in all pathogenic *Yersinia* encodes virulence determinants common to all three species.

1.3 Disease Syndromes

1.3.1 Yersinia pestis

Yersinia pestis is endemic in rodents in many areas of the world. It is transmitted between rodents, and from rodents to humans, by a flea vector. Thus, the organisms are exposed sequentially to high and low temperatures of growth in their alternating hosts. The hallmark of bubonic plague is the appearance of a bubo, a massive inflammatory response in a lymph node draining the site of a bite by an infected flea. In bubonic plague, the infection often proceeds through the lymphatics to the bloodstream, by which the organisms are disseminated to distal organs, especially the liver, spleen, nervous system, and lungs. If untreated, the case fatality rate of bubonic plague may be as high as 75%. Infection of the lungs results in pneumonic plague, which is fatal in more than 90% of untreated cases and can be transmitted from person to person via respiratory droplets (POLLITZER 1954). Several animal models have been used to study the pathogenesis of *Y. pestis,* the subcutaneous infection of guinea pigs and mice being the most common. LD_{50} doses vary from host to host and strain to strain, but, in general, virulent strains have LD_{50}s for small animals of less than ten bacteria (POLLITZER 1954).

Several properties of *Y. pestis* have been implicated as virulence determinants, including envelope (fraction I) production, production of a plasmid-encoded pesticin (FERBER and BRUBAKER 1981), pigmentation, toxin production, and the elaboration of the V and W antigens. These properties, and their putative roles in virulence, have been reviewed by BRUBAKER (1979).

1.3.2 Yersinia enterocolitica

Yersinia enterocolitica infection presents a spectrum of clinical manifestations in humans ranging from a mild diarrhea to a typhoid-like septicemia. The most common clinical picture of infection is an acute gastrointestinal illness (BOTTONE 1977; LARSEN 1979). The disease predominantly affects young children, although all age groups are susceptible. Approximately 90% of the cases are self-limiting, although complications can occur in patients whose normal host-defense mechanisms have been compromised. We restrict our discussion to those serotypes of *Y. enterocolitica* which cause serious invasive disease such as serotype 0:8. Animal models used to investigate the pathogenesis of disease include both oral and intraperitoneal inoculation of mice, gerbils, or rabbits (CARTER 1975; PAI et al. 1980; WETZLER et al. 1968).

The generally accepted stages of host invasion by *Y. enterocolitica* presume the following sequence of events: ingestion of the bacteria with contaminated food or water by a susceptible host; transit of the bacteria to the ileum where they are assumed to attach to the columnar epithelial cells lining the ileum; penetration of the epithelial cells by a parasite-induced endocytic event, a process that is not at all understood; translocation of the yersiniae to the basal region of the eukaryotic cells; egestion of the bacteria into the lamina propria; evasion

of the constitutive host-defense mechanisms; and growth of the invading organisms in the terminal ileum with the concomitant and/or subsequent induction of a massive inflammatory response giving rise to a mesenteric lymphadenitis and terminal ileitis (BOTTONE 1977). Most *Y. enterocolitica* strains elaborate a heat-stable enterotoxin (PAI and MORS 1978). The toxin has been shown to activate guanyl cyclase in vitro much like the ST toxin of *E. coli* (RAO et al. 1979; ROBINS-BROWNE et al. 1979). The toxin may be responsible for the diarrhea seen during infection, although its role in this process is questionable since the toxin is known to be produced only at low temperatures of growth in vitro (PAI and MORS 1978; ROBINS-BROWNE et al. 1979), and it has not been detected in the diarrheal intestinal contents of infected animals (PAI et al. 1980). Further, toxigenicity is not related to pathogenicity in *Y. enterocolitica* (PAI et al. 1980; SCHIEMANN 1981). The genetic information for toxin production appears to reside on the chromosome.

1.3.3 Yersinia pseudotuberculosis

Yersinia pseudotuberculosis shares 90% DNA sequence homology with *Y. pestis,* but only about 50% homology with *Y. enterocolitica* (BERCOVIER et al. 1980; BRENNER et al. 1976). Nevertheless, the epidemiology of infection and the disease syndromes in humans caused by *Y. pseudotuberculosis* closely resemble those described for *Y. enterocolitica,* and will not be redescribed. Virulent strains of this organism are lethal to mice after oral inoculation (BOLIN and WOLF-WATZ 1984).

1.4 Similarities Among *Yersinia* Species

Although the disease syndromes and many determinants of pathogenicity may be different among the yersiniae, there are a number of similarities common to all three plasmid-bearing species. The most striking is the need to adapt to widely different temperatures of growth during the biology of infection. *Y. pestis* alternates between growth in a mammalian environment at 37 °C and growth in its flea vector at ambient temperatures. *Y. enterocolitica* and *Y. pseudotuberculosis* appear to alternate between the external environment, with its ambient temperature, and a mammalian host. Consistent with the ability to grow at these different temperatures, the organisms show a number of temperature-regulated characteristics. For example, *Y. enterocolitica* shows minimal nutritional demands for growth at low temperature, whereas at elevated temperatures complex nutritional demands are observed (BOTTONE 1977). Similarly, there are marked morphological and structural changes in response to growth temperature. Flagella are not formed at the high temperatures of growth; cells are elongated, tend to grow in short chains, and autoagglutinate. In contrast, those grown at 25 °C are highly motile, are coccobacillary, and remain as single cells. Clearly, the pleiotrophic effects of temperature on the physiology of yersiniae are pronounced, especially on the plasmid-bearing strains of *Y. enterocolitica*

and *Y. pseudotuberculosis*. The response to temperature may afford yersiniae the luxury of behaving as facultative parasites.

All three species of pathogenic yersiniae have a transient intracellular existence, *Y. pestis* in macrophages (CAVANAUGH and RANDALL 1959; JANSSEN and SURGALLA 1969) and *Y. enterocolitica* and *Y. pseudotuberculosis* in epithelial cells (BOVALLIUS and NILSSON 1975; LEE et al. 1977; DEVENISH and SCHIEMANN 1981) and, possibly, in macrophages also (UNE 1977b). Therefore, not only must these bacteria have the ability to adapt to widely varying temperatures, but they must also tolerate the intracellular and extracellular environments of their hosts. Finally, all virulent *Yersinia* species show a requirement for Ca^{2+} for growth at 37 °C (BRUBAKER 1983). This Ca^{2+} requirement has been associated with the presence of a particular species of plasmid DNA in yersiniae. Strains cured of the resident plasmid lose the Ca^{2+} requirement for growth at 37 °C. In *Y. pestis,* where the Ca^{2+} requirement is most pronounced, transfer of cultures growing at 25 °C in media deficient in Ca^{2+} to 37 °C results in the cessation of growth, i.e., no further increase in cell mass, after two to four divisions (ZAHORCHAK et al. 1979). The Ca^{2+} dependence is less pronounced in *Y. enterocolitica* and *pseudotuberculosis,* where a reduced growth rate is observed at 37 °C in the absence of Ca^{2+} compared with 25 °C (CARTER et al. 1980). The role of Ca^{2+} in the physiology of yersiniae is not understood, but it is known that the V and W antigens, considered as virulence factors, are not produced at 37 °C by yersiniae in media containing 2.5 m*M* Ca^{2+} (CARTER et al. 1980).

2 *Yersinia* Virulence Plasmids

2.1 Historical Perspective

During the 1950s it became apparent that avirulent mutants of *Y. pestis* arose spontaneously in some growth media after growth at 37 °C but not at 25 °C (OGG et al. 1958). Incubation at 37 °C in the absence of added Ca^{2+}, and in the presence of Mg^{2+}, resulted in the cessation of growth after a few generations (HIGUCHI et al. 1959). This was referred to as Ca^{2+} dependence. Incubation at 37 °C, especially in Ca^{2+}-deficient media, often selected for the growth of Ca^{2+}-independent mutants which, upon animal inoculation, were shown to be avirulent (BURROWS and BACON 1958). BURROWS and BACON (1956) described two antigens, the V and W antigens, which were expressed concomitantly with Ca^{2+} dependence at 37 °C, and were not expressed by the Ca^{2+}-independent mutants, nor were they expressed at 25 °C. Since VW^--, Ca^{2+}-independent mutants arose at a frequency of approximately 10^{-4} (HIGUCHI and SMITH 1961), SURGALLA (1960) and BURROWS (1962) suggested that the VW trait was associated with a plasmid. Many recent reports have confirmed and extended these early speculations that Ca^{2+} dependence and V and W antigen production are plasmid-encoded traits and that these traits are associated with virulence. Plasmid loss is invariably correlated with avirulence, Ca^{2+} independence, and lack

of V and W antigen production. However, the large coding capacity of the yersiniae virulence plasmids suggests that concurrent loss of these traits and virulence is not of itself sufficient evidence for assigning a major role in virulence to the V and W antigens, or to Ca^{2+} dependence. Plasmid curing results in the loss of many other genes besides those involved in Ca^{2+} dependence and V and W antigen expression. BRUBAKER and SURGALLA (1962) described streptomycin-resistant mutants of *Y. pestis* which were Ca^{2+} independent, V and W antigen positive, but avirulent. This led them to suggest that expression of Ca^{2+} dependence was essential for virulence. The actual role of V and W antigens in the virulence process remains unresolved, and an understanding of the physiological basis for Ca^{2+} dependence is not available at present.

2.2 Discovery of the Virulence Plasmid

Although there was evidence which suggested the existence of a plasmid in *Y. pestis,* early efforts to demonstrate its presence were unsuccessful (LITTLE and BRUBAKER 1972). In 1980, ZINK et al. showed that the ability of an 0:8 serotype strain of *Y. enterocolitica* to cause conjunctivitis in the guinea pig was associated with the presence of a 41×10^6 dalton (Mdal) plasmid (ZINK et al. 1980). They concluded that plasmid expression mediated tissue invasion. In the same year, GEMSKI et al. (1980a) showed that curing *Y. enterocolitica* strains of a resident 42-Mdal plasmid rendered them avirulent and Ca^{2+} independent. CARTER et al. (1980) demonstrated that the virulence of 0:8 strains of *Y. enterocolitica* for mice, and Ca^{2+} dependence, was related to their ability to produce V and W antigens, which were antigenically identical to the *Y. pestis* antigens. FERBER and BRUBAKER (1981) subsequently demonstrated that Ca^{2+} dependence was associated with a plasmid in *Y. pestis,* and GEMSKI et al. (1980b) reported similar results for *Y. pseudotuberculosis.* In 1981, PORTNOY et al. (1981) examined 100 isolates of *Y. enterocolitica* and found a good correlation between plasmid carriage and virulence determined by lethality for gerbils. They further showed that the pathogenic strains of *Y. enterocolitica* harbored a family of virulence plasmids related in DNA sequence homology by 55%–100%. In a subsequent study the virulence plasmids from *Y. pestis* EV76 and *Y. enterocolitica* were shown to share approximately 55% DNA sequence homology distributed over 80% of the plasmid genome (PORTNOY and FALKOW 1981). Thus, it seemed clear that a family of related plasmids existed in *Yersinia* which appeared essential for virulence, Ca^{2+} dependence, and V and W antigen production. Verification of the role of the plasmid-encoded functions in the pathogenesis of the disease awaited the transfer of the plasmid to a plasmid-free avirulent strain of yersiniae.

2.3 Transfer of the Virulence Plasmid to Plasmid-Cured Strains

The original reports concerning plasmid carriage and virulence within the yersiniae relied on epidemiological and plasmid-curing data. Conclusive evidence that a plasmid confers a particular trait requires a molecular version of Koch's

Table 1. Effect of *Tn5* insertions within pYV019 on *Y. pestis* and *Y. pseudotuberculosis*

	Y. pestis		*Y. pseudotuberculosis*		
	Cal[b]	Vir(LD_{50})[c]	Cal	Omp[d]	Vir[e]
Wild type[a]	+	1.6	+	+	+
Plasmid-cured	−	>107	−	−	−
Tn5 insertion # 1[f]	+	2.4	+	+[g]	−
Tn5 insertion # 2	+	NT[h]	+	+	+
Tn5 insertion # 3	−	NT	−	−	−
Tn5 insertion # 4	−	NT	−	−	−
Tn5 insertion # 5	−	>107	−	−	−
Tn5 insertion # 6	−	NT	−	−	−
Tn5 insertion # 7	−	>107	−	−	−
Tn5 insertion # 8	+/−[i]	2.9	−	+	+

[a] Wildtype: *Y. pestis* 195-P, *Y. pseudotuberculosis* YPIII (pIB1)
[b] Cal: Calcium dependence was determined by the ability of a strain to grow on magnesium oxalate as compared to the wildtype strain and plasmid-cured strain. (+indicates Ca^{2+} dependent)
[c] Vir(LD_{50}): LD_{50} doses were determined after subcutaneous injection of mice. These data were taken from PORTNOY et al. (1983)
[d] Omp: Presence of all the plasmid-encoded outer membrane proteins (see Fig. 2). (BOLIN et al. 1985)
[e] Vir: Lethality for mice after oral feeding. (BOLIN et al. 1985)
[f] *Tn5* insertion # refers to those insertions depicted in Fig. 1
[g] This strain was temperatur-sensative for growth at 37 °C
[h] NT: Not tested
[i] This strain showed an intermediate Ca^{2+} dependence response in *Y. pestis*

postulates (KOOMEY et al. 1982); a plasmid-cured strain must be isolated and shown to be avirulent in an appropriate animal model, and reinfection of that strain with the plasmid must result in the acquisition of full virulence. Unfortunately, there were no natural markers on any of the putative virulence plasmids which could be used to select for plasmid transfer. Two recent reports using different strategies and different *Yersinia* species have provided genetic evidence for the role of the plasmid in pathogenesis.

The bacteriophage P1 will infect and lysogenize *Y. pestis*. Based on this observation, PORTNOY et al. (1983) introduced the transposable element *Tn5*, which encodes resistance to kanamycin, into the avirulent, plasmid-bearing *Y. pestis* strain EV76 using P1::*Tn5*. They isolated a series of Ca^{2+}-independent *Tn5* insertion mutants most of which mapped in the *Y. pestis* resident plasmid pYV019. They also isolated a few *Tn5* insertions within the plasmid which did not affect Ca^{2+} dependence. The pYV019 plasmids harboring *Tn5* (pYV019::*Tn5*) were transformed into *E. coli* LE392 (Fig. 1), where the plasmid was stably maintained. The various derivatives of pYV019::*Tn5* were transduced by P1 into the potentially virulent strain of *Y. pestis* 195-P1 which lacked pYV019. Introduction of plasmid pYV019::*Tn5*, with *Tn5* insertions not affecting Ca^{2+} dependence, restored full virulence to strain 195-P1. *Tn5* insertions within the plasmid genes associated with Ca^{2+} dependence did not restore virulence to *Y. pestis* 195-P1 (Table 1). This analysis provided direct genetic evidence

demonstrating that expression of at least some plasmid-encoded genes are essential for virulence in *Y. pestis*. The data also supported earlier conjectures that Ca^{2+} dependence is a virulence determinant in this organism, although, as discussed below, expression of Ca^{2+} dependence per se is not essential for virulence.

HEESEMANN and LAUFS (1983) used a different approach to mobilize a *Y. enterocolitica* virulence plasmid (p0:8) into a plasmid-cured derivative. They cloned a small segment of p0:8 into the wide host range mobilizable cloning vehicle pRK290. Using a triparental mating system (DITTA et al. 1980), this recombinant plasmid was mobilized into a wild-type strain of *Y. enterocolitica,* where the cloned plasmid apparently recombined with the resident plasmid. The cointegrant plasmid was transformed into *E. coli* HB101, where the plasmid was characterized. The cointegrant plasmid was mobilized, using the triparental mating system, into an avirulent plasmid-free strain of *Y. enterocolitica,* and this strain was converted to full virulence. This analysis demonstrated that the *Y. enterocolitica* plasmid is essential for virulence in this organism.

Using a strategy analogous to that described for *Y. pestis,* BOLIN et al. (BOLIN et al., to be published) demonstrated that a plasmid from *Y. pseudotuberculosis,* pIB1, is also essential for expression of virulence.

2.4 Comparison of the Plasmids from *Yersinia enterocolitica, Yersinia pseudotuberculosis,* and *Yersinia pestis*

PORTNOY and FALKOW (1981) and PORTNOY et al. (1984) compared the virulence plasmids from the three species of *Yersinia* using restriction enzyme analysis (Fig. 1), solution hybridization, and nitrocellulose filter hybridization. While the plasmids from *Y. enterocolitica* and *Y. pestis* shared only about 55% DNA sequence homology, the plasmids from *Y. pseudotuberculosis* and *Y. pestis* were almost identical in this respect. A striking observation was that the region of the plasmids shown to encode determinants associated with Ca^{2+} dependence was highly conserved among the three plasmid species. The significance of this observation is unclear, but suggests a strong selective pressure to maintain those genes involved with the expression of Ca^{2+} dependence among these organisms. Although the Ca^{2+} dependence-encoding regions of the plasmids are virtually identical by DNA sequence homology, nevertheless, the physiological expression of Ca^{2+}-dependent growth is not identical in all three species of yersiniae. The Ca^{2+} requirement is more pronounced in *Y. pestis* than in the two other yersiniae (CARTER et al. 1980). This may be the result of differential chromosomal gene expression. Clarification of the situation may eventually occur when we understand the biochemical basis of Ca^{2+} dependence.

Yersinia enterocolitica mutates to Ca^{2+} independence at a frequency of about 1% (BERCHE and CARTER 1982) which, in each case examined, resulted from plasmid loss (PORTNOY and FALKOW 1981). The mutation rate of *Y. pestis* to Ca^{2+} independence occurs at a frequency of 10^{-4} (HIGUCHI and SMITH 1961), and about half of these mutants have lost their plasmid (PORTNOY and FALKOW 1981). This suggests that the plasmid from *Y. enterocolitica* is far

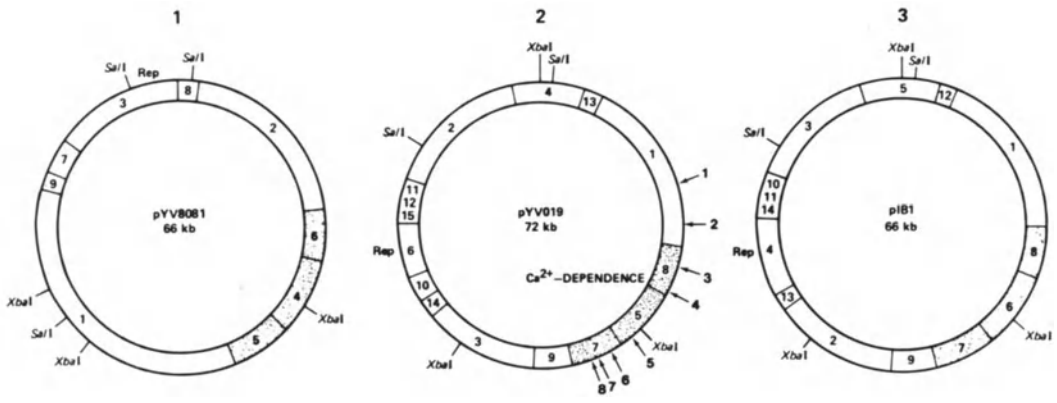

Fig. 1. *Bam*H1 restriction fragment maps for *Yersinia* virulence plasmids: *Y. enterocolitica* 8081 (pYV8081), *Y. pestis* EV76 (pYV019), and *Y. pseudotuberculosis* YPIII (pIB1). The *stippled areas* refer to three common *Bam*H1 fragments in the Ca^{2+} dependence locus. The *arrows* designate the sites of *Tn*5 insertions in plasmid pYV019 which are referred to in Table 1. (PORTNOY et al. 1984)

less stable than that of *Y. pestis*. After transformation into *E. coli* K12, the *Y. enterocolitica* plasmid pYV8081 was found to be temperature sensitive for maintenance, while the *Y. pestis* and *Y. pseudotuberculosis* plasmids were quite stable to passage at 37 °C. To examine the stability differences, the origins of replication of the *Y. enterocolitica* and *Y. pseudotuberculosis* plasmids, pYV8081 and pIB1, were cloned (PORTNOY et al. 1984). Transformants carrying the recombinant plasmids were shown to exhibit the same properties as the parental plasmids with regard to stability and maintenance at 37 °C. Further, the plasmid DNA restriction fragments containing the origins of replication of the plasmids shared little DNA sequence homology. We may conclude that, although the plasmids from the yersiniae are highly conserved in the region specifying Ca^{2+} dependence, they differ in that region of the plasmids necessary for essential plasmid maintenance functions. This implies that there may have been more than one ancestral *Yersinia* plasmid with different origins of replication and that the region encoding Ca^{2+} dependence was acquired from a common source. The conserved maintenance of the genes specifying Ca^{2+} dependence further suggests the view that these functions play a significant role in the biology of these organisms.

3 Plasmid-Encoded Outer Membrane Proteins

3.1 Expression of the Plasmid-Mediated Outer Membrane Proteins

Yersinia enterocolitica cells are morphologically different when cultured at 37 °C and 25 °C (BOTTONE 1977). This observation led PORTNOY et al. (1981) to examine the outer membrane protein profiles of *Y. enterocolitica* grown under different conditions. The altered cellular morphology was correlated with the appear-

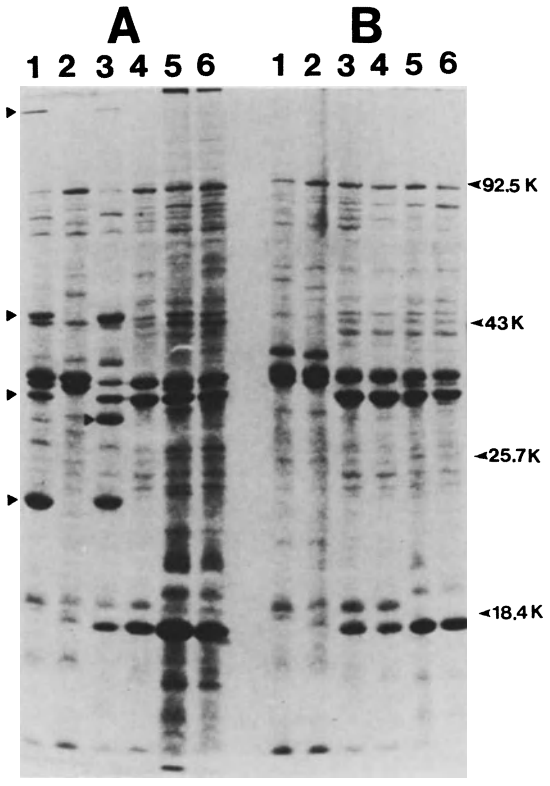

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel of *Yersinia* outer membrane proteins. *A*, *Yersinia* strains grown at 37 °C; *B*, strains grown at 28 °C. *Lane 1*, *Y. enterocolitica* 8081; *lane 2*, *Y. enterocolitica* 8081C (plasmid-cured); *lane 3*, *Y. pseudotuberculosis* YPIII (pIB1); *lane 4*, *Y. pseudotuberculosis* YPIII (plasmid-cured); *lane 5*, *Y. pestis* EV76 (pYV019); *lane 6*, *Y. pestis* EV76-6 (plasmid-cured). The *arrowheads* designate the major plasmid-mediated outer-membrane proteins. (PORTNOY et al. 1984)

ance of at least four major outer membrane proteins in cells grown at 37 °C (Fig. 2). These novel outer membrane proteins were shown to be plasmid mediated since they were absent from outer membrane preparations obtained from plasmid-cured derivatives grown at 37 °C. The expression of the novel proteins in the outer membrane was also shown to be temperature regulated since the proteins were not observed in outer membrane preparations from plasmid-bearing strains grown at 25 °C (PORTNOY et al. 1981; MARTINEZ 1983). In this paper, we use the word expression to mean that the proteins are located on the surface of the bacteria; that is, synthesis and transport of the proteins.

The outer membrane protein profiles of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* have been examined by a number of investigators (BOLIN et al. 1982; CHANG and DOYLE 1984; DARVEAU et al. 1980; PORTNOY et al. 1981; MARTINEZ 1983; STRALEY and BRUBAKER 1981). *Y. pseudotuberculosis* expresses a number of plasmid-mediated outer membrane polypeptides virtually identical in molecular mass to those produced by *Y. enterocolitica* (Fig. 2). Further, the plasmid-encoded outer membrane proteins from the two species cross-react antigenically, attesting to a close similarity, if not identity. *Y. pestis*, however, does not express any of these polypeptides in its outer membrane at either temperature of growth (DARVEAU et al. 1980; PORTNOY et al. 1984; STRALEY and BRUBAKER 1981).

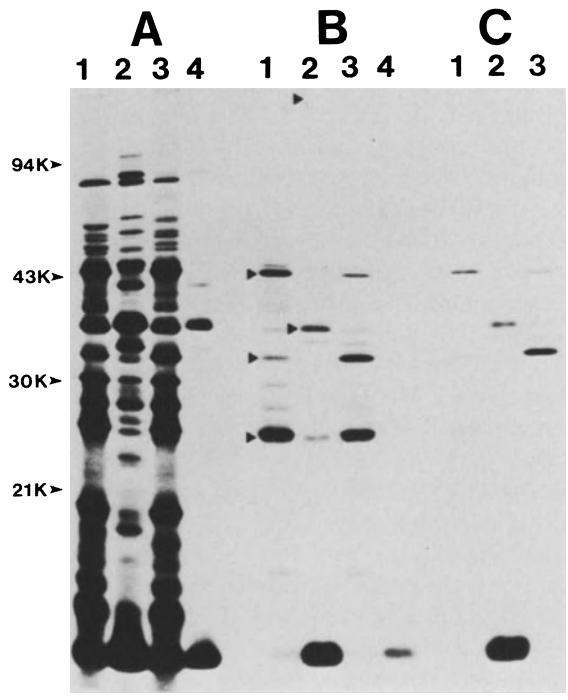

Fig. 3. Detection of plasmid-encoded outer membrane proteins in *E. coli* minicells. *A*, autoradiograph of SDS-acrylamide slab gel of ^{35}S-labeled outer membranes; *B*, immunoprecipitate of the same samples reacted with antiserum directed against the surface of *Y. enterocolitica* 9576 grown at 37 °C; *C*, identical to *B*, except that 2 μg purified 24000-dalton outer-membrane protein isolated from *Y. enterocolitica* was included in the reaction. The *arrowheads* designate the plasmid-encoded outer-membrane proteins shown in Fig. 2. *Lane 1*, minicells containing pYV019::*Tn5*; *lane 2*, minicells containing pYV8081::*Tn5*; *lane 3*, minicells containing pIB1::*Tn5*; *lane 4*, minicells alone. (PORTNOY et al. 1984)

Discrepancies have appeared in the literature regarding the plasmid-mediated outer membrane proteins of *Y. enterocolitica* and *Y. pseudotuberculosis*. BOLIN et al. (1982) have reported the appearance of a high molecular weight plasmid-mediated outer membrane protein, which they have termed protein 1. Other laboratories (STRALEY and BRUBAKER 1981; PORTNOY et al. 1981; MARTINEZ 1983; CHANG and DOYLE 1984) have observed several plasmid-mediated outer membrane proteins, including protein 1, in profiles from these species. The discrepancy may be based on the use of different growth media and growth conditions by different investigators. The precise environmental conditions for optimal expression of these proteins in the outer membrane of the yersiniae have not been determined. Nevertheless, it is clear that plasmid-bearing *Y. enterocolitica* and *Y. pseudotuberculosis* have the potential to alter dramatically their outer membrane protein profiles as a result of plasmid gene expression (see below). Relative to the chromosomally encoded major outer membrane proteins, the plasmid-mediated proteins are expressed in high concentration in the outer membrane.

Conclusive evidence that the plasmid-mediated outer membrane proteins were plasmid encoded was presented by PORTNOY et al. (1984). Plasmid DNA from each of the three species of *Yersinia* was genetically labeled by *Tn5* insertions and transformed into the minicell-producing strain of *E. coli* P678-54. The plasmid-encoded polypeptide products were detected by autoradiography (Fig. 3). Each plasmid encoded more than 20 polypeptides in the minicell system. Since the *Yersinia* virulence plasmids measure about 70 kb in mass, they contain

sufficient DNA to encode many more than these 20 polypeptides. The polypeptide patterns obtained from minicells harboring pYV019 and pIB1 appeared to be identical, while some differences were seen in comparison with the pattern obtained from pYV8081. This is consistent with the high degree of DNA sequence homology between the former two plasmids. Antiserum directed to the outer membrane proteins from *Y. enterocolitica* was used to immunoprecipitate the radio-labeled minicell protein products. It was clear from the data that the plasmid-encoded outer membrane proteins were synthesized in the minicell system, thus demonstrating that the genetic information encoding the novel outer membrane proteins was located on the virulence plasmids of all three species. The data in Fig. 3 also demonstrate that the outer membrane proteins from the three species are antigenically related. The fact that the *Y. pestis* plasmid has the DNA-coding capacity for the novel outer membrane proteins, yet fails to express the proteins on its surface in vitro, suggests that these are silent genes in *Y. pestis*. Alternatively, *Y. pestis* might have the capacity to express the proteins, but the appropriate growth conditions for in vitro expression have not yet been discovered. The fact that pYV019 has the genes coding for the outer membrane proteins allows the speculation that, even if not expressed in vitro, they may be expressed in vivo in a mammalian host, and, as noted below, there is immunological evidence for this.

The epidemiology of *Y. enterocolitica* infection suggests that the ingested organisms are progeny of low-temperature-grown cells. Hence, they would lack the plasmid-encoded outer membrane proteins since these are not expressed at temperatures below 28 °C–30 °C, at least in laboratory culture. Since antisera from patients convalescing from yersiniosis detected the novel outer membrane proteins in Western blot analyses (Fig. 4), the comparable proteins must have been expressed in the body of their host during the infection. Recently, WOLF-WATZ et al. (WOLF-WATZ et al., to be published) have shown that mice infected with *Y. pestis* EV76 generate antibody to some, but not all, of the *Y. enterocolitica* outer membrane polypeptides. It appears that *Y. pestis* also expresses at least some of the plasmid-encoded outer membrane proteins, but thus far only in vivo. It seems possible that the plasmid-encoded outer membrane proteins may play a role in the infectious process in all the yersiniae, but their failure to be expressed in vitro by *Y. pestis* has precluded their inclusion as putative pathogenetic determinants in this species. This observation is consistent with the arguments presented by SMITH (1968), that certain essential virulence determinants may be expressed by pathogenic microorganisms only in the in vivo environment and not in the in vitro environment.

The stringent control of plasmid-encoded outer membrane protein expression in the yersiniae may reflect their regulation by possibly both chemical and physical influences of an in vivo environment. Certainly, temperature would fulfill the criteria of a regulatory component for the expression of genes which may be essential for survival and growth in the in vivo situation. Nutritional differences between the in vitro and in vivo environment should not be overlooked as possible regulatory signals for expression of these genetic elements. The concentration of Ca^{2+} in the growth environment may act as such a regulatory signal for plasmid-gene expression.

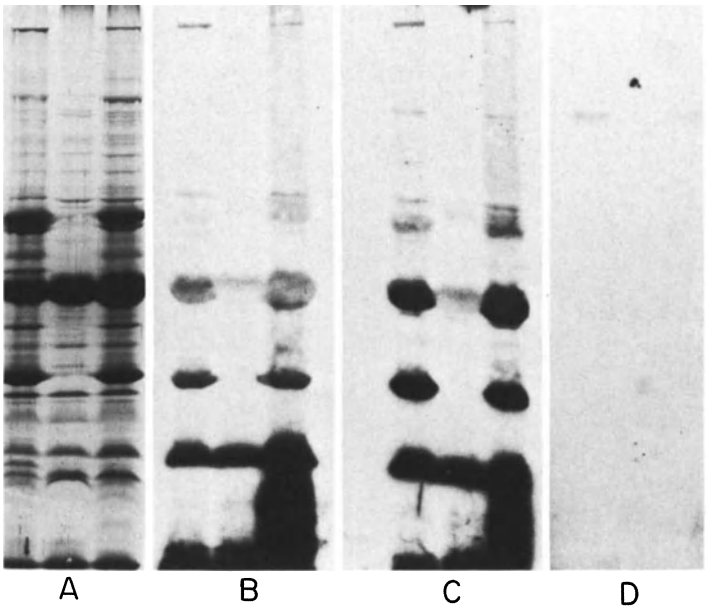

Fig. 4. Detection of *Y. enterocolitica* outer-membrane proteins with serum from a convalescent patient by the Western blot technique. Four sets of outer-membrane preparations from strains 8081 (pYV8081, grown at 37 °C), 8081 (pYV8081, grown at 25 °C), and 9576 (pYV9576, grown at 37 °C) were loaded on an SDS-acrylamide gel in that order, with an empty lane between each set, and electrophoresed. One set *A* was stained with Coomasie blue; the remaining gel was electroblotted to nitrocellulose paper, and the transfer of *B* was reacted with antiserum raised against strain 9576 grown at 37 °C; the transfer of *C* was reacted with antiserum obtained from a patient convalescing from yersiniosis; and that of *D* was reacted with a normal human serum pool. The nitrocellulose papers were subsequently reacted with ^{125}I-labeled staphylococcal A protein and exposed to X-ray film for autoradiography. (MARTINEZ 1983)

3.2 Surface Properties of Plasmid-Bearing *Yersinia*

MARTINEZ (1983) has demonstrated that all of the plasmid-encoded outer membrane proteins are susceptible to proteolytic digestion of intact bacteria. These, and other data, indicate that the proteins are externally exposed on the surface of the organisms. Further, the expression of the novel proteins on the outer membrane of *Y. enterocolitica* imparts a hydrophobic character to the bacteria. The molecular architecture of the plasmid-encoded outer membrane proteins on the cell surface is currently under examination.

The precise role of the plasmid-encoded outer membrane proteins in the biology of infection has not been defined. However, there is indirect evidence which suggests that these proteins may confer resistance to the bactericidal action of normal human serum (Fig. 5) (HEESEMANN et al. 1983; MARTINEZ 1983; PAI and DESTEPHANO 1982), a crucial property for invasive microorganisms. Plasmid-bearing cultures expressing the outer membrane proteins were quite resistant to the bactericidal action of serum, whereas their plasmid-cured derivatives, as well as cultures grown at 25 °C, were sensitive to killing by

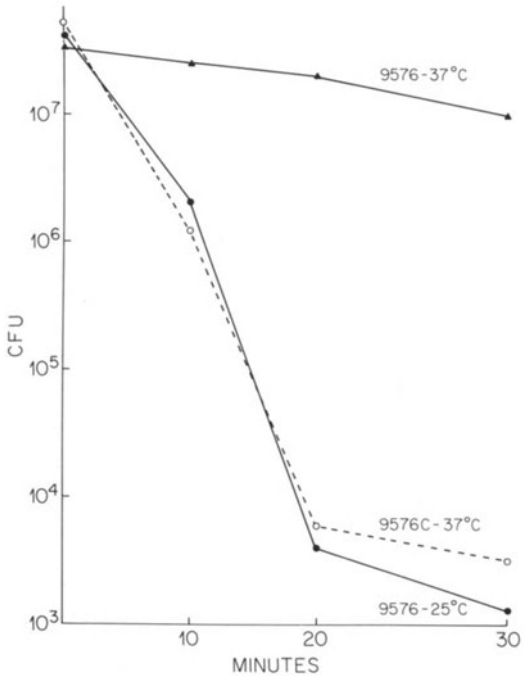

Fig. 5. Kinetics of killing of *Y. enterocolitica* 9576 (pYV9576) and 9576C (plasmid cured) by 10% normal human serum. Approximately 2×10^7 cells per ml of the strains grown at the indicated temperatures were treated with 10% normal human serum and sampled at intervals, diluted, and plated on BHI agar. (MARTINEZ 1983)

normal human serum. Proteolytic digestion of serum-resistant bacteria rendered the organisms sensitive to the killing action of serum (MARTINEZ 1983). Although the cited results strongly support the suggestion that these outer membrane proteins are responsible for resistance to the bactericidal action of serum, genetic evidence is required to complete the formal proof. PAI (personal communication) has shown that a serum-resistant plasmid-containing strain of *Y. enterocolitica* consumed 100-fold less complement than a serum-sensitive plasmid-free strain, suggesting that the proteins may block sites normally accessible to antibody and/or complement.

Yersinia pseudotuberculosis grown at 37 °C is resistant to the bactericidal action of serum, even among strains which lack the plasmid, while *Y. pestis* is constitutively serum-resistant (PERRY and BRUBAKER 1983). PERRY and BRUBAKER (1983) have argued that since the serum resistance of *Y. enterocolitica* is not affected by growth in Ca^{2+}-containing medium at 37 °C, and since the other two species are resistant regardless of plasmid content, the plasmid-encoded outer membrane proteins probably do not influence serum resistance. A genetic analysis of those elements involved in serum resistance will resolve this issue. It is possible that the outer membrane proteins may play additional, or alternate, roles in the infectious process in the other species of *Yersinia*.

Although there is ample evidence suggesting that expression of the plasmid is responsible for an altered cell surface, plasmid gene-products must act in concert with chromosomally encoded determinants. Under apparent chromosomal control is the chemistry of the lipopolysaccharide (LPS), another bacterial

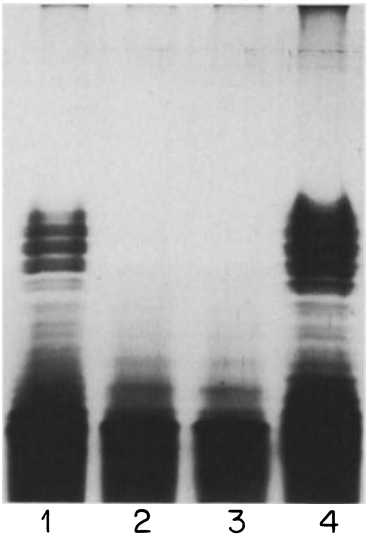

Fig. 6. SDS-acrylamide gel profile of LPS isolated from *Y. enterocolitica* strain 8081 and 8081C grown at 25 °C and 37 °C. LPS was isolated by a modification of the method of DARVEAU and HANCOCK (1983). *Lane 1,* LPS isolated from strain 8081C grown at 25 °C; *lane 2,* LPS isolated from 8081C grown at 37 °C; *lane 3,* LPS isolated from strain 8081 (pYV8081) grown at 37 °C; LPS isolated from 8081 (pYV8081) grown at 25 °C. LPS was detected by silver staining. (MARTINEZ, unpublished data)

cell-surface component which undoubtedly plays a pronounced role in the biology of the organisms. Irrespective of plasmid carriage, the nature of the LPS of *Y. enterocolitica* appears to be regulated by the temperature at which the cultures were grown (KAWAOKA et al. 1983; Fig. 6). Organisms grown at 25 °C express a "smooth" LPS with a full complement of O-side chains, whereas cultures grown at 37 °C appear "rough," i.e., deficient in O-side chains. It is tempting to correlate this observation with the temperature regulation of plasmid-encoded outer membrane protein expression. Recall that the novel outer membrane proteins are expressed on the cell surface only at high temperatures of growth (37 °C) and not at low temperatures. It is possible that the plasmid-encoded proteins may only be inserted into an outer membrane environment lacking O-side chains. Also possible is that the phospholipid composition of the envelope of these bacteria when grown at low temperature, which may be richer in unsaturated fatty acids than that of high-temperature-grown cells (MARR and INGRAHAM 1962), cannot accommodate the novel proteins, or their export mechanism. Regardless of the mechanism involved, the end result is that the plasmid-encoded outer membrane proteins are exposed to the external environment of the cell and are not "masked" by the presence of long O-side chains. Thus, the proteins are able to exert a major influence on cell-surface-associated phenomena, such as intercellular interactions and resistance to adverse environmental conditions.

DARVEAU et al. (1983) have shown that *Y. pestis* exhibits temperature and plasmid-associated changes in its LPS chemistry, although this organism lacks O-side chains at both temperatures of growth. It has yet to be determined whether the other yersiniae show plasmid-associated LPS modifications.

Preliminary data suggest that the plasmid-encoded outer membrane proteins are present in the cytoplasmic compartment of 25 C-grown *Y. enterocolitica* (R.J. MARTINEZ, unpublished observation), even though the cell envelope is

free of the polypeptides. If these preliminary data prove correct, then the virulence plasmid is not silent at low temperature; rather, the transport of the outer membrane proteins to the external membrane does not occur at low temperature. This is consistent with the observation that the envelope of the yersiniae changes in response to temperature.

3.3 Relationship Between Ca^{2+} Dependence, Outer Membrane Protein Expression, and Virulence

Because *Y. pestis* does not express the plasmid-encoded outer membrane proteins in vitro, it has not been possible to assess the relationship between *Tn*5 insertion mutations within pYV019 which abolish Ca^{2+} dependence, and outer membrane protein expression. To circumvent this obstacle, BOLIN et al. (BOLIN et al., to be published) have introduced pYV019::*Tn*5 derivatives into a cured strain of *Y. pseudotuberculosis,* and examined the phenotypes. The rationale for these experiments was that they would allow for a comparison of the phenotypic effects of mutations in genes involved in Ca^{2+} dependence, plasmid-encoded outer membrane protein production, and virulence for mice. The data (Table 1) show that the *Y. pestis* virulence plasmid, pYV019, is functionally interchangeable with the virulence plasmid from *Y. pseudotuberculosis* (*Tn*5 insertion # 2). This observation is consistent with earlier results which showed that these two plasmids were virtually identical at the molecular level. Also, most *Tn*5 insertions within pYV019 which abolished Ca^{2+} dependence also abolished the expression of the outer membrane proteins, and virulence (*Tn*5 insertions # 3–7). It would seem, therefore, that, as described for *Y. pestis,* there is a genetic linkage between Ca^{2+} dependence and virulence in *Y. pseudotuberculosis*. However, one *Tn*5 insertion mutation resulted in a Ca^{2+}-independent phenotype, but the strain retained the ability to express the outer membrane proteins and was virulent (*Tn*5 insertion # 8). This argues that Ca^{2+} dependence per se may not be a virulence determinant in *Y. pseudotuberculosis*.

BOLIN and WOLF-WATZ (1984) have isolated a *Tn*5 insertion mutation in the *Y. pseudotuberculosis* plasmid (pIB1) which was probably within the structural gene for the large outer membrane protein, protein 1. This strain retained full virulence for mice, implying that protein 1 was not an essential virulence determinant in this model. The strain retained the ability to express all of the other plasmid-encoded outer membrane proteins. Isolation of insertion mutations within the structural genes for each of the plasmid-encoded outer membrane proteins may elucidate their role, if any, in the pathogenetic process.

The fact that the *Yersinia* plasmid-encoded outer membrane proteins are only expressed on the bacterial surface at 37 °C, and then only in certain growth environments, suggests that they are regulated by both temperature and nutritional factors. Whether these regulatory influences act independently or in concert is not known. Along with the observation that the outer membrane proteins are expressed in vivo in all three species, this argues that the proteins are expressed in response to a specific environment within the mammalian host. The

temperature differential between the natural environment, or the body of a flea, and a mammalian host would certainly be a useful trigger to initiate synthesis of essential plasmid-encoded gene products.

BRUBAKER (1979) has speculated that Ca^{2+} dependence, a trait seen in all virulent *Yersinia* but expressed to different extents, may reflect the response of yersiniae to the mammalian intracellular environment which is low in free Ca^{2+}. However, once ingested by either professional phagocytes or by the columnar epithelial cells of the intestinal tract, *Yersinia* reside within a phagocytic vesicle, or phagolysosome, if lysosomal fusion occurs. Thus, the bacteria are not in contact with the cytoplasmic compartment of the eukaryotic cells, but rather are bathed in the extracellular fluid internalized during the phagocytic event (SILVERSTEIN et al. 1977), a fluid rich in Ca^{2+}. Since the membrane of the phagocytic vesicle is inverted relative to the cell membrane, i.e., the inner leaflet of the cytoplasmic membrane becomes the outer leaflet of the phagocytic vesicle (SILVERSTEIN et al. 1977), it is unlikely that the Ca^{2+} pump of the cytoplasmic membrane, from which the phagocytic vesicle membrane is derived, can function to free the luminal vesicle contents of Ca^{2+}. Thus, while it is appealing to invoke a mechanism by which *Yersinia* can discriminate between the extracellular and intracellular milieu, the physical basis for such a discrimination is not clear.

4 Speculations on the Role of Virulence Plasmid Expression in the Pathogenicity of Yersiniae

From epidemiological considerations, the portal of entry of *Y. enterocolitica* into a susceptible host is via contaminated food or water. Transit of the bacteria to the ileum in humans takes approximately 4 h, and it is at the ileum where the invading bacteria appear to initiate infection (UNE 1977a). The physiological state of the organisms, with respect to plasmid gene expression during this interval, is unknown, i.e., we do not know whether expression of plasmid-encoded functions has occurred during the transit time. This could have significance in many aspects of the pathogenetic process, such as attachment, parasite-induced phagocytosis, and excretion. We do not know whether replication and colonization by the yersiniae occur in the lumen of the intestines prior to induction of their internalization. This point is of considerable importance since growth in the lumen may presumably be accompanied by plasmid-encoded outer membrane protein expression. On the other hand, if the phagocytic event is induced by the original invaders, then it is possible that the outer membrane proteins are not yet expressed on the outer surface prior to internalization by the epithelial cells. This question may be approached experimentally by periodically examining the nature of the surface antigens on luminal yersiniae after oral challenge using anti-plasmid-encoded outer membrane protein sera as a molecular probe. This approach may also reveal the anatomical site within the mammalian host where the plasmid-encoded outer membrane proteins are

expressed on the bacterial cell surface. These are critical issues in the pathogenetic process if we are to understand the function of these novel proteins.

LACHICA and ZINK (1984) have recently suggested that the adherence of *Y. enterocolitica* to eukaryotic cells may be mediated by a plasmid-associated anionic fibrillar structure. They support this suggestion with the observation that plasmidless strains are cleared from the bowel of orally infected mice rapidly, whereas plasmid-bearing virulent strains were recovered from the stools of infected mice for the length of the experimental period (14 days).

This suggestion counters the observations that adherence to, and infection of, eukaryotic epithelial cells in culture is a temperature-regulated and chromosomally encoded function rather than plasmid encoded. In vitro, bacteria expressing the plasmid-encoded outer membrane proteins attached much less effectively to Hep 2 and Henle intestinal epithelial cells than their counterparts lacking these proteins (MARTINEZ 1983; unpublished results). It may be that intestinal proteases hydrolyze the proteins from the bacterial cell surface. This discrepancy between the in vitro and in vivo results requires further inquiry for clarification. It is possible that in vitro adherence measurements do not generate data that are relevant to the in vivo situation.

If the plasmid-encoded outer membrane proteins are not synthesized or externalized to the bacterial surface in the lumen of the intestines, then the bacteria may recognize the intravesicular environment, via Ca^{2+} concentration, the pH of the vesicle, or by way of yet another signal, and commence with the expression of the outer membrane proteins. Other plasmid gene-products, such as those shown to be involved with the expression of Ca^{2+} dependence, may be necessary for recognition of the intracellular environment.

Once internalized by an epithelial cell, the outer membrane proteins may exhibit a cytotoxic effect since this occurs in tissue culture (PORTNOY et al. 1981). If so, this could reflect the growth of the bacteria, or, alternatively, may be involved with their escape into the lamina propria. It is known that considerable intestinal epithelial cell damage occurs during yersiniosis and this may be a reflection of the cytotoxic effect.

For growth in the lamina propria, the yersiniae must tolerate the potentially bactericidal action of complement bathing the interstitial spaces. Plasmid-bearing *Y. enterocolitica* resist the killing action of complement, presumably in response to the expression of the outer membrane proteins. The biochemical mechanism of serum resistance is not known and is most probably multifactorial (TAYLOR 1983). The bacteria may resist opsonization by the complement cleavage product C3b, and may thus be more resistant to phagocytosis. This is, of course, completely speculative. A detailed examination of the mechanism of serum resistance in the yersiniae is needed to clarify the situation.

Another stage in the infectious process where the plasmid-encoded outer membrane proteins might play a role would be in the interaction with the phagocytic cells in the lamina propria. The outer membrane proteins could conceivably serve as antiphagocytic factors, thus eliminating the dangers of intracellular killing by the polymorphonuclear neutrophilic leukocytes (PMN) and macrophages. Preliminary data (TABRIZI and MARTINEZ, unpublished observations) suggest that this is not so. The adherence of *Y. enterocolitica* to mouse

peritoneal macrophages appears to be a temperature-regulated rather than a plasmid-encoded phenomenon. Bacteria grown at 25 °C adhere in greater numbers to these phagocytes than bacteria grown at 37 °C, regardless of plasmid carriage. Adherence to human blood leukocytes has also been examined in some preliminary experiments. The data indicate that bacteria grown at 37 °C regardless of plasmid content adhered much less effectively to both PMN and monocytes than bacteria grown at 25 °C. Further, high temperature-grown yersiniae attached more effectively to monocytes than to PMN, and the numbers of bacteria/eukaryotic cell were higher on monocytes than on PMN. However, low-temperature-grown yersiniae were seen adherent on 100% of both PMN and monocytes, and many more bacteria became associated with the phagocytes under these growth conditions. Opsonization rendered both high- and low-temperature-grown yersiniae adherent. The data imply that adherence to phagocytic cells may be a temperature-regulated chromosomal trait rather than a plasmid-encoded trait. A more detailed analysis of the interactions between *Y. enterocolitica* with phagocytic cells is in progress.

We may speculate that it is in the intravesicular environment of the phagocyte where the plasmid-encoded outer membrane proteins play a crucial role in the survival and growth of yersiniae. CAVANAUGH and RANDALL (1959) presented evidence suggesting that low-temperature-grown *Y. pestis* were readily ingested and killed by PMN, but resisted killing by monocytes, and reproduced therein. The progeny of *Y. pestis* which had grown within the monocytes were then resistant to ingestion by both PMN and monocytes, implying that the V and W antigens might have been synthesized in the intravesicular environment, and the expression of these antigens, or other plasmid-encoded gene products, rendered the bacteria resitant to phagocytosis. Other in vitro experiments with *Y. pestis,* however, have revealed that plasmid-bearing as well as plasmid-cured strains are capable of growth within macrophages (JANSSEN and SURGALLA 1969; STRALEY 1984). It would be premature to extrapolate these data to *Y. enterocolitica* considering that very significant differences in DNA homology exist between the two virulence plasmids and especially between the total cellular DNA of these bacteria. Chromosomally encoded genetic elements appear to be involved in the intracellular growth of *Y. pestis,* and this observation may extend to the other yersiniae.

It is apparent that excretion is a necessary prerequisite for dissemination of the bacteria. Whether the disseminated organisms are progeny of bacteria growing in the lumen of the bowel or are derived from those that have invaded the lamina propria is unknown. It is not known whether *Y. enterocolitica,* once they have entered the lamina propria, can return to the lumen of the bowel. If this does not occur, deposition into the lamina propria would be a dead end for the bacteria, an unlikely event. Relevant to this are the observations of RICCIARDI et al. (1978), who reported that mice infected intraperitoneally with *Y. enterocolitica* serotypes 0:3 and 0:9 excreted the organisms for several months in their stools. Although intraperitoneal injection is not a normal route of infection, these observations imply that bacteria growing in the tissue spaces can exit to the lumen of the intestines.

Yersiniosis is a self-limiting infection in most individuals, although fatalities

are not infrequent (BRAUDE 1981). Patients convalescing from the disease possess specific antibody directed to the plasmid-encoded outer membrane proteins (Fig. 4). This antibody may be protective and may serve to opsonize the bacteria, rendering them susceptible to ingestion and possibly destruction by phagocytes. Specific antisera to the plasmid-encoded outer membrane proteins may serve to overcome serum resistance. In addition, if the outer membrane proteins or other plasmid-encoded surface structures are involved in the colonization process, antibody may serve to block colonization and aid in the elimination of the bacteria.

It is apparent that studies dealing with the mechanisms of pathogenesis of yersiniae are in their infancy. The organisms can serve as very useful models for a detailed examination of several fascinating aspects of pathogenesis: parasite-induced phagocytosis, a common theme among many invasive microorganisms; survival and growth within phagocytes, the hallmark of the biology of facultative intracellular parasites; and resistance to the bactericidal action of normal serum. The latter property is an essential attribute of tissue invaders which must be exercised in the earliest stages of host invasion in order to express other virulence attributes they might possess. In addition, the pleiotrophic effects of temperature on the physiology of the organisms is another area worthy of study. The next few years of investigation in these areas should be exciting ones. The powerful experimental approaches made available by the progress of molecular genetics to the field of infectious diseases will greatly accelerate the pace at which these problems will reach resolution.

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Biochemical and Genetic Aspects of *Pseudomonas aeruginosa* Virulence

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1 Introduction

The *Pseudomonas* species includes at least 30 recognized strains, widely distributed in natural environments (e.g., soil and water) and as part of normal intestinal flora of mammals. Of these only a handful can cause infections in human hosts, and only *Pseudomonas aeruginosa* has been identified as a potentially life-threatening pathogen. This organism is usually associated with patients with an underlying disease who are subjected to extensive chemotherapy and hospitalization.

Pseudomonas aeruginosa possesses a number of features that make it easily identifiable in the clinical laboratory. The bacteria are aerobic rods with simple growth requirements. They grow well at elevated temperature and elaborate many identifiable exoproducts, such as fluorescent pigments, proteolytic enzymes, and hemolysins.

The fine structure of *P. aeruginosa* reveals a typical Gram-negative double-membrane cell envelope. Some strains have an extensive exopolysaccharide layer, revealed by special staining. Most cells have a single polar flagellum

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and are piliated. Additional characteristics include motility; acid production when grown on glucose, arabinose, or xylose; and inability to utilize the disaccharides lactose, sucrose, and maltose.

2 Pathogenesis

Up to 2 decades ago *P. aeruginosa* was found in about 10% of humans as a harmless component of normal flora. However, in recent years it has become the predominant pathogen of immunocompromised patients. *P. aeruginosa* is found frequently in patients suffering from cystic fibrosis and cancer. *P. aeruginosa* infections result when normal host defenses are not fully effective, as a result of trauma such as serious burns and wounds, drastic surgical procedures (e.g., tracheotomy, catheterization), and prolonged hospitalization for unrelated chronic conditions (BODEY et al. 1983).

The patients that are highly predisposed to *P. aeruginosa* infections thus can be divided into two groups, based on the nature of the host defense impairment. The first group involves conditions where the immune system is weakened as a result of the disease (cystic fibrosis, leukemia) or where there is deliberate immune suppression as a result of cancer therapy or organ transplantations. The most common type of infection is that of the respiratory tract. The bacteria isolated from such patients are always highly mucoid. However, under standard culture conditions, they revert to "rough" colonial morphology, similar to isolates from other sources. What selective advantage mucoid bacteria have in the lung is not clear.

A different type of infection involves destruction of the physical protective barriers, as in the case of burns, eye damage, or surgery. In many cases the typical "succession pattern" of invasion is observed. The initial flora of the wound, comprising predominantly gram-positive microorganisms, is progressively replaced by a population of gram-negative bacteria, frequently dominated by *P. aeruginosa*. Hospitalized patients usually are subject to antibiotic therapy, which seems to be an important factor in selection of *P. aeruginosa* as a predominant pathogen from a mixed population.

3 Virulence Factors of *Pseudomonas aeruginosa*

Many cellular components contribute to the survival and virulence of the bacteria in an animal host. We will limit our discussion of virulence factors to those cellular components that fit one of the following criteria: (a) mimic one of the pathological effects of *Pseudomonas* infection when administered to animals or cells in purified or partially purified form, (b) when injected into an animal elicit at least a partially protective immune response against the subsequent challenge with virulent strains of *P. aeruginosa,* or (c) mutations that abolish the synthesis of such protein markedly decrease the virulence of the microorgan-

ism. These criteria will narrow the potentially important virulence determinants to extracellular factors (exotoxins) and some of the components of the bacterial cell envelope.

Some of these factors are not yet well characterized biochemically, and genetic approaches to studying the virulence of *Pseudomonas* have just recently been undertaken.

3.1 Extracellular Components

3.1.1 Exotoxin A

Most (>90%) *P. aeruginosa* elaborate a "lethal toxin," exotoxin A, which is the most toxic component ($LD_{50}=1$ μg/kg in mice) of all cellular and extracellular fractions of *P. aeruginosa* (LIU 1973; LIU et al. 1973; BJORN et al. 1977). Significant antitoxin immunoglobulin titers can be found in patients recovering from *P. aeruginosa* infection (KLINGER et al. 1978; JAGGER et al. 1982; POLLACK et al. 1976), and survival of bacteremic patients can be correlated with the presence of a high antitoxic titer in their serum at the onset of infection (CROSS et al. 1980).

The molecular mode of action of exotoxin has been elucidated by IGLEWSKI and KABAT (1975). Exotoxin A was shown to inhibit mammalian protein synthesis by ADP-ribosylating elongation factor 2 (EF-2), like diphtheria toxin (see review by COLLIER 1975). Both of these toxins modify the same unusual amino acid, called diphthamide, in EF-2 (VAN NESS et al. 1980). The kinetic parameters for the ADP-ribosylation reaction are nearly identical for both of these toxins, indicating highly conserved structural features of their active site. However, comparison of nucleotide or amino acid sequences has shown no similarity between these two toxins (GRAY et al. 1984), even though a low level of immunological cross-reactivity has been detected (SADOFF et al. 1982).

Exotoxin A, like diphtheria toxin, is excreted as an enzymatically inactive proenzyme of 66000 daltons, which is highly toxic to animals as well as cytotoxic in cell culture. In order to elicit enzymatic activity, exotoxin A has to undergo one of two activation pathways. Reduction of two out of four disulfide bonds in the presence of urea renders the molecule fully enzymatically active (LEPPLA et al. 1978; LORY and COLLIER 1980). Alternatively, limited proteolytic digestion of thiol-activated toxin yields an enzymatically active fragment of 26000 daltons (LORY and COLLIER 1980). Both of these treatments abolish toxicity in animals and intact mammalian cells. Cleavage of the toxins to the smaller fragments can occur following prolonged incubation or storage, presumably as a result of the action of *Pseudomonas* proteases (CHUNG and COLLIER 1977; VASIL et al. 1977). In analogy with structure-function arrangement of diphtheria toxin, exotoxin A is thought to be composed of two domains: one is enzymatically active for ADP ribosylation and the other is responsible for delivering the toxin molecule to membrane receptors and possibly across the membrane of the mammalian cells.

However, it has not yet been possible to isolate the receptor-binding fragment of exotoxin A. The target cell receptor specified by a distinct domain of the

toxin is quite different from that of diphtheria toxin. Exotoxin A, in contrast to diphtheria toxin, is highly active against rodents and their cell lines. Furthermore, diphtheria toxin with a missense mutation in fragment B, which blocks the cytotoxic action of wild-type diphtheria toxin, fails to inhibit binding of exotoxin A (VASIL and IGLEWSKI 1978).

Exotoxin A is excreted into the growth medium without any detectable cell-associated pool. Using membrane perturbant, we were able to show that it is synthesized as a precursor with an additional 2500-dalton sequence (LORY et al. 1983). In the presence of ethanol that inhibits the processing, the active precursor accumulated in the outer membrane. In addition to the block in processing, the cysteines of the toxin molecule remain in sulfhydryl form, and so the ADP-ribosyltransferase activity of the precursor does not require additional treatment with thiols.

Exotoxin A, like most other exported proteins, is synthesized by membrane-associated polysomes. When peptide chains are completed in vitro and immunoprecipitated, the products are largely the mature 66000-dalton species, suggesting that a signal peptidase acts on the precursor very shortly after the attachment of the polysome to the inner membrane (LORY et al. 1983). Once the precursor is allowed to accumulate in the outer membrane, the resumption of toxin export following removal of the perturbant fails to process and to cause release of the precursor form, presumably because it is topologically removed from the site of cleavage. It therefore seems likely that the excretion of exotoxin A, and possibly some other proteins by gram-negative bacteria, involves specialized regions of the cell envelope. We have therefore proposed a mechanism of protein excretion in which a protein is laterally transferred from the inner surface of the inner membrane through the Bayer junction (BAYER 1979) to the exterior surface of the outer membrane from which it is released (LORY et al. 1983). These Bayer junctions have been implicated in the flow of phospholipids from inner into outer membrane, and assembly of lipopolysaccharide and membrane proteins into the outer membrane (JONES and OSBORN 1977; LANGLEY et al. 1982).

Synthesis and excretion of exotoxin A by *P. aeruginosa* is regulated by the composition of the media and growth conditions (LIU 1973). High levels of iron inhibit synthesis of exotoxin A, like that of diphtheria toxin in *Corynebacterium diphtheriae*. In *P. aeruginosa* the iron regulation of the toxin synthesis is pleiotropic: in addition to exotoxin A, synthesis of other exported proteins is also suppressed (BJORN et al. 1978, 1979). It seems likely that the effect of iron is at the level of transcription or translation and not in the export process, since the precursor of toxin A is not synthesized in medium containing a high concentration of iron.

A model can be postulated in which an iron-requiring repressor coordinately represses a number of genes coding for extracellular proteins. Several classes of mutants not responsive to iron in the medium were isolated by SOKOL et al. (1982). In addition to mutations affecting iron uptake, two distinct classes of regulatory mutations were isolated. One class was specific for exotoxin A, in that these mutants synthesized wild-type levels of exotoxin A in the presence of iron, but repressed synthesis of the proteases. In another class of mutants

elevated concentrations of iron repress synthesis of exotoxin A and one of the proteases, but not the synthesis of elastase. It is not yet possible to distinguish between an alteration in a diffusible regulatory protein or in a specific regulatory sequence (an operator) linked to the structural genes.

Additional regulatory mutants, altered in the synthesis of toxin A, have been isolated. Among the mutants in PA103, one class is pleiotropic: exotoxin A and proteases are reduced (OHMAN et al. 1980a, b). Another mutant class is more specific: synthesis of exotoxin A is reduced but the yields of all other extracellular proteins are not affected. This locus therefore may involve an element specific for exotoxin A. None of these mutations appeared to be altered in the genes involved in the iron-regulated synthesis of exotoxin A, since the presence of iron represses further the synthesis of the extracellular products by all mutants of this class.

A genetic analysis of mutants in another strain, PAO1, revealed two loci involved in toxin regulation (GRAY and VASIL 1981a). One such locus, *tox*1, near the 38-min region of the chromosome, specifies functions that regulate synthesis of proteases as well as exotoxin A. The second class of mutants (*tox*2) is specific for the toxin alone and is located near the 35-min region of the chromosome. Since direct comparison of chromosomal organization of PA103 and PAO is not possible, it is not clear whether these mutations are in the same genes. These studies suggest the presence of a complex genetic system that regulates the synthesis of a group of exported proteins.

A mutant isolated from strain PAO specifies an altered toxin polypeptide that is immunologically indistinguishable from native exotoxin (CRYZ et al. 1980). This polypeptide, CRM66, is nontoxic in cell culture and lacks ADP-ribosyltransferase activity in vitro. The locus (*tox*A1) therefore defines the structural gene for exotoxin A. This mutation is mapped at the 85-min region (HANNE et al. 1983) on the circular chromosome map of *P. aeruginosa* PAO1 (ROYLE et al. 1981).

The structural gene for exotoxin A has recently been cloned and the protein sequence deduced from the DNA sequence (GRAY et al. 1984). The N-terminus contains a typical cleavable signal sequence of 25 amino acid residues. Surprisingly, the enzymatically active domain of the toxin is on the carboxyl end of the molecule, in contrast to the N-terminal domain for the diphtheria toxin. Studies on the regulation of synthesis and on structure-function relationships of the toxin are expected to be greatly facilitated by the isolation of the gene.

3.1.2 Proteolytic Enzymes

Two major proteases have been isolated from culture fluids of *P. aeruginosa*. They have been classified as alkaline and neutral protease on the basis of their pH optima against various substrates (MORIHARA 1964). Both of these enzymes have been implicated as important virulence factors during colonization. They contribute to the breakdown of physical barriers of the host, as well as enhancing bacterial proliferation by supplying amino acids and peptides from tissue proteins. Furthermore, during later stages of infection *Pseudomonas* proteases have

been shown to interfere with immune defense mechanisms by degrading immunoglobulins (MILAZO and DELISLE 1984).

The neutral protease has a broad specificity, and, interestingly, it can degrade elastin, a major tissue component (MORIHARA 1964; MORIHARA et al. 1965; WRETLIND and WADSTRÖM 1977). This elastase has been studied in several laboratories in greater detail, but significant discrepancies in regard to some of its biochemical properties still exist. The molecular weights reported vary, depending on the methods used. Gel filtration on two different resins gave M_r values of 12000 or 21000, while sodium dodecyl sulfate (SDS)-gel electrophoresis of the same preparation gave a value of 33000 (KRIEGER and GRAY 1978). Analytical ultracentrifugation of elastase prepared from a different clinical isolate of *P. aeruginosa* yielded an M_r of 37000 (JENSEN et al. 1980), while elastase prepared by affinity chromatography migrated as a single 21000-dalton band on SDS-polyacrylamide gels (NISHINO and POWERS 1980). Isoelectric points of apparently the same elastase vary from 6.6 (WRETLIND and WADSTRÖM 1977) to 7.2 (JENSEN et al. 1980). These discrepancies are probably due to different methods used to purify the enzymes, but the autocatalytic activity of this protease may contribute to the observed differences. This elastase is a heat-stable zinc metalloprotein. It has a broad specificity for proteins and peptides, but peptide bonds adjacent to leucine and phenylalanine are preferentially cleaved. Thus, the elastase resembles thermolysin in its specificity. It is inhibited by ethylenediaminetetraacetate (EDTA), *o*-phenanthroline, and a number of synthetic substrate analogs, the most effective of which are peptides containing hydroxamic acids (NISHINO and POWERS 1980). A thermolysin inhibitor, phosphoramidon, and α_2-macroglobulin are both natural inhibitors of the elastase (JAGGER et al. 1980; MORIHARA et al. 1979).

Alkaline protease (pH optima between 7 and 9) has been purified and characterized by MORIHARA (1963). This enzyme has an apparent M_r of 48000 under nondenaturing conditions and a pI of 4.1. Like elastase, it is inhibited by metal-chelating compounds such as *o*-phenanthroline and EDTA, as well as heavy metals. The active site does not contain an active serine residue, since it is not affected by serine protease inhibitors. This protease hydrolyzes many native proteins but not any synthetic compounds tested; hence its specificity has not yet been determined.

Genetic characterization of protease production by *P. aeruginosa* implicated the activities of several genes in regulation of biosynthesis and excretion. Mutations affecting formation of the active elastase have been isolated by several groups. One such mutation results in the synthesis of enzymatically inactive elastase protein, thus defining the structural gene. This locus (*las*A1) has been mapped in the 75-min region of the *Pseudomonas* chromosome (HOWE et al. 1983).

Additional mutants have been isolated from ethyl methane sulfonate (EMS)-mutagenized cells of PAKS-1 (WRETLIND et al. 1977). Since these mutants are deficient in the synthesis or export of several extracellular proteins, the lesion appears to be in a membrane export machinery or in a central regulatory element.

Similar mutations have been isolated in strain PAO (WRETLIND and Pavlovs-

kis 1984). Loci specifying formation of extracellular proteins, including proteases, have been mapped at a different location than structural genes for exotoxin A or elastase. One class of such unlinked *xcp* mutations affects the release of several exoenzymes: the enzymes synthesized are comparable to the wild type but are cell-associated. Such mutations therefore are likely in genes specifying components of an export machinery. The second class of mutants fails to elaborate the exoproteins in liquid medium. Since no cell-associated pools of such proteins have been detected in this class of *xcp* mutants, they are probably involved in the regulation of biosynthesis of these proteins at the transcriptional or translational level.

A different class of mutants from strain PAO has been isolated by transposon mutagenesis (STAPLETON et al. 1984). In such mutants low levels of elastase activity were detected, but wild-type amounts of the antigen were detected immunologically. It was subsequently found that the insertion of the transposon into the *Pseudomonas* chromosome abolished production of another enzyme, alkaline protease, which is apparently needed to activate the inactive precursor of elastase to its active form.

It is clear that both major proteases play a role during infection: their respective contributions depend on the site of infection (WOODS et al. 1982a). The most pronounced effect was observed during experimental eye infection. Studies of KAWAHARAJO and HOMMA (1975) demonstrated clear correlation between the ability of variants to elaborate proteases and to cause severe corneal damage in mice. However, OHMAN et al. (1980a, c) showed that while exotoxin A was important in establishing infection, elastase was not required. On the other hand, most test strains produced alkaline protease, which may be responsible for the observed pathological effect. Recent isolation of alkaline protease-deficient mutants of nonelastolytic strain PA103 (thus making it completely nonproteolytic) provided the ideal isogenic test strains for examination of the role of proteases in model eye infection (HOWE and IGLEWSKI 1984). The loss of ability to elaborate alkaline protease resulted in concomitant loss of virulence of various mutants, as measured by decreased visible corneal damage as well as by decreased recovery of viable bacteria from the infected site. Survival of the protease-deficient mutant strain was enhanced by administration of low amounts of purified alkaline protease or by mixed infection with the protease-producing parental strain. When protease was added with the mutant corneal damage was identical to that produced by the wild type. Since the concentration of added protease alone was too low to elicit visible damage, this experiment demonstrated the importance of other cellular or extracellular factors in causing the disease.

The importance of elastase during an acute lung infection was demonstrated with an isogenic set of mutants of *P. aeruginosa* in a guinea pig pulmonary infection model (BLACKWOOD et al. 1983). A strain of PAO that elaborates a temperature-sensitive elastase was markedly less virulent than the parental strain. Since the two *Pseudomonas* proteases have different substrate specificities, their importance for the organism is clearly determined by the type of tissue proteins in the immediate environment, e.g., the lung is rich in elastin, which is a good substrate for the elastase.

3.1.3 Leukocidin

A toxic protein capable of destroying leukocytes from a variety of sources has been described by SCHARMANN (1976a, b; SCHARMANN et al. 1976). This protein causes drastic changes in the appearance of leukocytes (and of a number of different cultured cells): cells become enlarged and rounded up, and their nuclear segments fused. The high level of toxin activity of this protein toward polymorphonuclear leukocytes is significant since these cells are the primary host defense mechanism against *Pseudomonas* infection in most human tissues.

Leukocidin is a 27000-dalton polypeptide released from *P. aeruginosa* by autolysis. The cell-associated form, which is not toxic to leukocytes, can be released in an active form by added proteases, the most effective of which is *Pseudomonas* elastase. The leukocidin acts on the membrane (without causing lysis of the target cell) by altering its phospholipid composition and causing influx of Ca^{2+}. This in turn results in phosphorylation of a lysosomal membrane protein and leakage of lysosomal content into the cytosol (HIRAYAMA and KATO 1984).

The importance of leukocidin in pathogenesis has not been assessed. The cytopathic effect seen in vitro cannot be confirmed in vivo during infection, since *P. aeruginosa* produces a number of other toxic substances that may also be active against leukocytes. Clearly, it would be desirable to isolate mutants defective in leukocidin and assess their survival in a suitable animal model.

3.1.4 Hemolytic Substances

Pseudomonas aeruginosa excretes two hemolysins, differing in their mode of lysis. A small molecular weight glycolipid acts primarily by solubilizing lipids (and membranes) by a detergent-like mechanism, while a heat-labile hemolysin is a protein with phospholipase C activity. Since both hemolysins are excreted by *P. aeruginosa* under phosphate-limiting growth conditions, their biosynthesis is likely controlled by similar regulatory elements.

Of the many exocellular products elaborated by *P. aeruginosa,* phospholipase C has been identified as the principal agent responsible for fleece-rot dermatitis in sheep in Australia (J. CHIN, personal communication). The extracellular phospholipase C is a single-chain polypeptide of 78000 daltons with an isoelectric point at pH 5.5 (BERKA and VASIL 1982). The protein has been recently purified to homogeneity and characterized biochemically (BERKA and VASIL 1982). The enzyme lacks sugars or lipids, but its amino acid composition reveals the presence of two unusual residues, hydroxyproline and ornithine. Furthermore, unlike many excreted proteins, phospholipase C lacks cysteine and is not stabilized by intramolecular crosslinking. Phosphatidyl choline, lysophosphatidyl choline, and sphingomyelin are the best substrates for the phospholipase C. Moreover, the enzyme lyses red blood cells from a variety of sources, all of which have membranes rich in phosphatidyl choline.

A number of mutants altered in synthesis of phospholipase C have been isolated in *P. aeruginosa* PAO. One such mutant is constitutive for phospholipa-

se C synthesis: it fails to repress the synthesis of this enzyme in the presence of high levels of phosphate (BERKA et al. 1981). Furthermore, this mutation affects synthesis of other phosphate-regulated proteins, including alkaline phosphatase. This regulatory locus (*plc*B) is mapped in the 22- to 23-min region of the PAO chromosome. Interestingly, another mutation (*plc*A) that causes cessation of the synthesis of phospholipase C and alkaline phosphatase under phosphate starvation is mapped in the same region of the chromosome. Thus, this region may include several genes that regulate biosynthesis of proteins involved in phosphate metabolism.

The structural gene for *P. aeruginosa* phospholipase C has recently been cloned in *E. coli* (VASIL et al. 1982; COLEMAN et al. 1983; LORY and TAI 1983). In the new host, unlike *P. aeruginosa,* the formation of the active enzyme is not regulated by phosphate. The transcriptional initiation site in one such vector (pBR322) can be inactivated by insertion of a transposable element between the vector promoter and the cloned gene, or by deletion of the vector promoter. The amount of phospholipase C synthesized from its own promoter then drops to low levels, but the constitutive, phosphate-independent nature of expression still persists despite a fully functional *pho* operon in the *E. coli* host (DING et al. 1985).

The phospholipase C is normally excreted by *P. aeruginosa* into the growth medium without any cell-associated pools (STINSON and HAYDEN 1979). In contrast, the enzyme synthesized from the recombinant plasmid by *E. coli* is not exported but remains cell associated, and a major portion is localized in the outer membrane. This location probably reflects the failure of *E. coli* export machinery to transport a foreign protein or its failure to make a required enzyme. The signal peptidase may be one such export-specific protein. Preliminary data show that the cell-associated form in *E. coli* is about 2000 daltons larger than the extracellular enzyme excreted by *P. aeruginosa;* presumably the export signal peptide has been retained (S. LORY, P.C. TAI, unpublished observations).

Heat-stable glycolipid hemolysin is produced by about 80% of clinical isolates during the stationary phase of the growth cycle. While the chemical structure of this hemolytic substance has been known for some time (2-*o*-alpha-rhamnopyranosyl-alpha-L-rhamnopyranosyl-beta-hydroxydecanoyl-beta-hydroxydecanoate) very little information is available about its biosynthesis or mode of action (JARVIS and JOHNSON 1949). Since this glycolipid is capable of dissolving precipitated lysophosphatidyl choline (generated by phospholipase C from egg yolk lecithin like a detergent), the hemolysis probably results from intercalation in the erythrocyte membrane or disruption of the lipid bilayer (KURIOKA and LIU 1967).

A simple purification scheme recently developed yielded preparations of high specific activity (JOHNSON and BOESE-MARRAZZO 1980). Analysis of the preparation yielded two similar glycolipids, both of which were hemolytic. Clearly, additional structural work is needed to determine the relationship between these two hemolysins. Furthermore, the relative ease of preparation of the glycolipid-hemolysin should facilitate studies of its role in *Pseudomonas* pathogenesis.

3.1.5 Exoenzyme S

This protein is another ADP-ribosyltransferase produced by *P. aeruginosa,* elaborated by at least 38% of all clinical isolates (SOKOL et al. 1981). Unlike exotoxin A, its acceptor is not EF-2, but a number of yet unidentified proteins from eukaryotic cytoplasm (IGLEWSKI et al. 1978). Recently, the enzyme has been purified and characterized (NICAS and IGLEWSKI 1984). Two immunologically related forms (53- and 49-kD polypeptides) are found in the culture fluids. Only the smaller of the two has ADP-ribosyltransferase activity. It has not been possible to convert the larger form into the smaller, active form by either urea-thiol treatment or limited proteolysis which generates the active forms of other toxins. Thus the precise relationship of the two forms of exoenzymes is not clear.

What is clear, however, is that this enzyme is an important virulence determinant. Mutagenesis of *P. aeruginosa* P88 with a transposon resulted in isolation of a strain deficient in production of enzyme S. While the parental strain was highly virulent in the burned mouse infection model, reduced synthesis of enzyme S (and none of the other extracellular toxins) in this mutant resulted in at least a 2000-fold increase in LD_{50}. Because of the dramatic decrease in virulence as a result of a mutation in a single extracellular protein, it will be highly informative to elucidate the molecular action of this molecule in vivo; and purified preparations are available.

3.2 Cell Surface Components

The bacterial cell envelope contains components that interact with the environment, including the human host during infection. In gram-negative pathogens the outer membrane includes attachment factors, toxic lipopolysaccharide, and an exopolysaccharide slime layer that shields the bacterial cell further from the host immune defense mechanisms.

3.2.1 Pili

Pili are hair-like organelles 6–8 mm in diameter and of different lengths present on the surface of most *P. aeruginosa* strains. In addition to being involved in attachment to surfaces (mammalian during colonization and recipient during conjugal DNA transfer) they serve as receptors for bacteriophages and are organs of "twitching" motility (BRADLEY 1980). The presence of pili enhances the virulence of a microorganism by allowing it to adhere to solid surfaces despite fluid and air movement or ciliary action. In several instances, pili have been implicated in resistance of microorganisms to phagocytic killing. These two characteristics of pili allow us to consider them as potentially important virulence factors.

Pili of *P. aeruginosa* are chains of protein composed of a single subunit of M_r 16000–18000 (PARANCHYCH et al. 1979). They represent a strain-specific, antigenically heterogeneous group. Comparison of protein sequences from two

different pili provided clues to the antigenic diversity among the various pili. The amino terminal one-third of the pilin molecule from *P. aeruginosa* strains PAK and PAO are nearly identical in sequence, while the rest of the molecule shows very little homology (PARANCHYCH et al. 1979). Several strong antigenic determinants have been found, including those from the C-terminal region specific for each strain. [More remarkably, the N-terminal 20 amino acids are highly homologous to pili from two rather unrelated pathogens: *Neisseria gonorrhoeae* and *Moraxella nonliquefaciens* (PARANCHYCH et al. 1978).] In all cases, the conserved region is highly hydrophobic and the N-terminal residue is a modified amino acid, *N*-methylphenylalanine, presumably formed by post-translational modification (SASTRY et al. 1983).

Genetic characterization of gene(s) related to the expression of pili takes advantage of their function as receptors for RNA bacteriophages. Following adsorption of bacteriophages to the distal end of pili, the filament retracts and the injecting phage is brought to the bacterial surface. Thus it is possible to isolate *P. aeruginosa* mutants that are resistant to killing by pilus-specific phages, due either to lack of pili or to structurally altered, noncontractible pili. Mobilization of the chromosome of a piliated donor strain PAO by plasmid FP2 results in transfer of the pilus determinant into an isogenic nonpiliated recipient, and the pilus genes are mapped between 0 and 27 min on the PAO chromosome (BRADLEY 1979). The gene(s) specifying the nonretractible, multipiliated phenotype of strains PAO and PAK have not been identified.

The role of pili in attachment of nonmucoid strains to mammalian cells has been unambiguously established. An in vitro attachment system was used by WOODS et al. (1980) to demonstrate binding of pili, and of piliated *P. aeruginosa,* to buccal epithelial cells. Attachment of both was blocked by preincubation of epithelial cells with purified pili, or with antibody against pili from homologous strains. While homologous antibody was most effective in interfering with bacterial attachment, heterologous antibody also competed with attachment of most strains, indicating that certain epitopes specifying receptor recognition may be conserved among various pili serotypes.

Similarly, pilus-mediated attachment of nonmucoid *P. aeruginosa* to injured mouse tracheal cells has been demonstrated by competitive inhibition by purified pili (RAMPHAL et al. 1984). Both homologous and heterologous pili were effective, implying a common receptor for *Pseudomonous* pili on the mammalian cell surface. Binding of mucoid *P. aeruginosa,* while comparable to that of their nonmucoid counterparts, was not inhibited by pili. Thus, there is an additional adhesion factor (adhesin) present on the surface of mucoid strains.

The nature and number of pilin receptors on mammalian cells is not known. Buccal epithelial cells from cystic fibrosis patients that have been colonized by *P. aeruginosa* bind a significantly greater number of *P. aeruginosa* than cells from noncolonized patients. Trypsin treatment of cells from noncolonized control patients significantly increased the ability of these cells to bind *P. aeruginosa* (WOODS et al. 1982b). Since most clinical isolates of *P. aeruginosa* secrete proteolytic enzymes, it is conceivable that colonization of the respiratory tract of patients begins by proteolytic action to generate new receptors or to expose masked receptors for bacterial adhesion.

Furthermore, a receptor moiety in the lower respiratory tract is a *N*-acetylneuraminic acid-containing glycoconjugate, since this simple sugar can inhibit binding of *P. aeruginosa,* whether mucoid or nonmucoid, to injured tracheal cells (RAMPHAL and PYLE 1983). The attachment factor on the surface of the mucoid strains evidently recognizes the same receptor as pili.

The significance of pili in pathogenesis may be assessed once the virulence of various isogenic nonpiliated mutants is tested in vivo. It will be useful to isolate specific adhesion-defective mutants of several mucoid and nonmucoid isolates and to test their survival in appropriate animal models.

3.2.2 Lipopolysaccharide

In its purified form, lipopolysaccharide (LPS) of *P. aeruginosa* is considerably less toxic in mice than that of other gram-negative bacteria. LPS of *P. aeruginosa* is similar in structure to that of Enterobacteriaceae, containing three basic units: lipid A, core polysaccharide, and *O*-specific side chain (KROPINSKI et al. 1979). The composition of lipid A resembles *Salmonella* lipid A: glucosamine disaccharide backbones are linked to long-chain fatty acids. The core region polysaccharide is also fairly uniform among various *Pseudomonas* strains, consisting of 2-keto-3-deoxyoctanate, heptose, glucose, rhamnose, galactosamine, phosphate, and alanine. The *O*-side chains are, however, variable and therefore specify part of the serological heterogeneity of *P. aeruginosa* LPS. The *O*-side chain has a high content of amino sugars, including some rare ones, present in novel structural configurations in linkages differing from other gram-negative bacteria (TAHARA and WILKINSON 1983).

The importance of LPS in pathogenesis is shown by the observation that neutropenic mice can be protected from *P. aeruginosa* by treatment with type-specific anti-LPS antibody (CRYZ et al. 1983a, b). Similarly, a high molecular weight polysaccharide derived from the *O*-side chain of *P. aeruginosa* LPS was shown to be highly immunogenic in humans. Antibodies against the LPS may well play a key role in opsoic killing of *P. aeruginosa* at the onset of infection (PIER et al. 1978a, b).

3.2.3 Exopolysaccharide

The mucoid appearance of *P. aeruginosa* from chronic infections is due to production of a polysaccharide capsule: an alginate-like polymer, consisting of 1-4-linked D-mannuronic and L-guluronic acid. The relative amount of each sugar varies with the strain, as does the amount of polymer produced in laboratory cultures as well as in patients (LINKER and JONES 1966; EVANS and LINKER 1973).

The ability to elaborate an alginate exopolysaccharide under laboratory conditions is not a stable trait. Nonmucoid variants appear in pure *Pseudomonas* cultures at high frequency, and their mucoid mutants can be isolated, based on their increased resistance to certain antibiotics or bacteriophages (GOVAN

and FYFE 1978; MARTIN 1973). This stable genetic trait of *muc* mutants can be introduced into nonmucoid variants with the aid of sex factors, and the mutation can thus be mapped (FYFE and GOVAN 1980). In strain PAO a chromosomal locus in the 12-min region was shown to regulate biosynthesis of alginate, in an unknown way. Two additional loci involved in alginate biosynthesis have been characterized in strain FRD, isolated from a cystic fibrosis patient (OHMAN and CHAKRABARTY 1981). Mutations in *alg* loci, located on the bacterial chromosome, lead to a permanent loss of the mucoid character. These genes can be transferred by interrupted mating into mucoid recipients, and nonmucoid mutants can be isolated. The *alg* mutations all map in the same region of the chromosome, linked closely to the *his* and *met* markers. A different mutant of strain FRD, unable to synthesize the alginate exopolysaccharide at 41 °C, has recently been isolated (GOLDBERG and OHRMANN 1984). This locus (*alg* 50) is linked to a *trp* gene and is therefore different from the *alg* gene(s) in the *his* and *met* region described.

4 Concluding Remarks

Pseudomonas aeruginosa is an opportunistic pathogen that causes disease only when weakened conditions of the host (immune deficiences, cystic fibrosis, burns) allow invasion and colonization in various tissues. Virulence is therefore enhanced by many factors that allow survival of the organisms in an environment where nutrients are scarce or are present in polymeric forms that must be hydrolyzed. This organism thus excretes a large number of enzymes capable of degrading complex polypeptides, carbohydrates, and lipids, as well as toxins and factors that enhance the survival or colonization in other ways. Some not only break down the natural physical barriers of the host, thus aiding in the rapid spread of the organism, but also combat the natural defense mechanism by killing phagocytic cells or inactivating immunoglobulins. It is therefore not surprising that a new "virulence factor" of this organism is still being discovered.

The biosynthesis and localization (i.e., export to or outside the cell surface) of the virulence factors are clearly important for the pathogenesis of the organism. Elucidation of the regulatory mechanisms that govern the expression of these factors and genetic manipulations that would shed light on gene regulation and on structure-function relationships will advance our knowledge of the pathogenesis of this opportunistic organism. Since many regulatory mutants are now available and structural genes of many virulence factors have recently been cloned, the ever-advancing techniques of molecular genetics will play an essential role in our efforts to control *P. aeruginosa* infections.

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Genetic Determinants of Virulence in *Shigella* and Dysenteric Strains of *Escherichia coli*: Their Involvement in the Pathogenesis of Dysentery

D.J. KOPECKO, L.S. BARON, and J. BUYSSE

1 Introduction

Bacillary dysentery, caused mainly by *Shigella* and infrequently by certain dysenteric strains of *Escherichia coli,* is responsible for 10%–20% of acute diarrheal disease worldwide. Recognized as a clinical disease entity since the time of Hippocrates, dysentery is characterized by painful abdominal cramps and frequent defecation of blood and mucus. Primates are considered to be the only natural hosts susceptible to *Shigella* infection, a consideration that has seriously hindered the development of inexpensive animal models for study of the disease.

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Dysentery occurs predominantly in areas of low sanitation and poor nutrition in developing countries. However, shigellosis continues to be a localized problem in industrialized countries in closely housed populations (e.g., in prisons, nursery schools, mental institutions, or on Indian reservations). Bacillary dysentery is a serious intestinal malady and may be particularly severe in infants and elderly patients. Moreover, outbreaks of bacillary dysentery have determined the outcome of many important military campaigns and still cause high levels of morbidity among military and other personnel deployed in areas of low sanitation. A natural ecological niche for the causative bacteria is unknown. *Shigella* strains have been found to remain viable in water for periods of up to 6 months and, in rare cases, previously diseased individuals have been observed to excrete *Shigella* for periods of greater than 1 year (LEVINE et al. 1973b; DUPONT and PICKERING 1980). *Shigella* is unique among enteric pathogens in that as few as 10–100 virulent cells have been shown to cause dysentery in adults (reviewed by HORNICK 1978). Despite considerable research effort, there are no proven effective antidysentery vaccines available for widespread use.

Genetic analyses of *Shigella* have helped to provide insight into the gross pathobiology of shigellosis without yet defining the specific biochemical events involved. There have been several excellent general reviews of shigellosis (e.g., DUPONT and PICKERING 1980; HORNICK 1978) as well as brief reviews directed at genetic studies of *Shigella* virulence (GEMSKI and FORMAL 1975; PETROVSKAYA and LICHEVA 1982). However, recent advances in the genetic analysis of *Shigella* now allow for a better definition of the disease process. Thus, this review is aimed at summarizing our current genetic understanding of *Shigella* virulence; due to space limitations, only representative references/examples have been included. For a better appreciation of the genetic data, however, we feel it is appropriate first to define dysentery as compared with other common acute intestinal disease mechanisms and to discuss *Shigella* virulence assay systems briefly.

2 Dysentery: A Definition

2.1 Comparison with Other Common Intestinal Disease Mechanisms

Presently, there are at least four distinct mechanisms recognized by which bacteria cause acute gastrointestinal (GI) illness in humans. One mechanism, often termed "intoxication," occurs by bacterial secretion of an exotoxin that usually is preformed in food prior to ingestion by the host. This process is exemplified by staphylococcal food poisoning. These enterotoxins generally cause excessive fluid and electrolyte secretion from the bowel, do not cause histopathological changes in the intestine, and result in nausea, vomiting, and diarrhea. In contrast, the remaining three mechanisms require living and multiplying disease agents in the intestine.

In the second or "enterotoxigenic" mechanism, ingested bacteria adhere to and multiply on the surface of the small intestine, usually in the jejunum

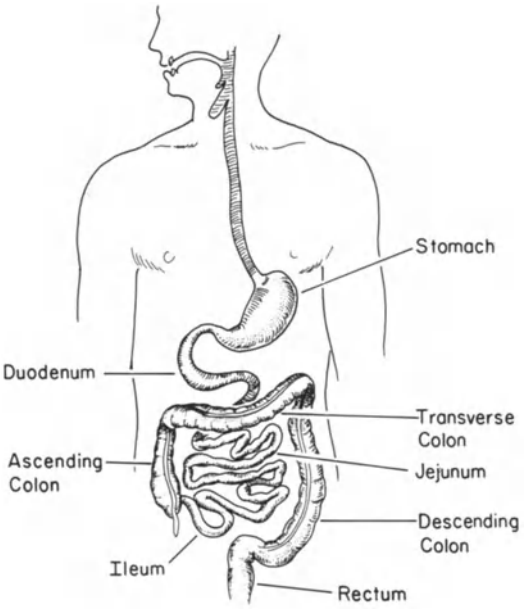

Fig. 1. The human gastrointestinal tract, with emphasis on portions of the small intestine (i.e., duodenum, jejunum, and ileum) and the large intestine/colon

and duodenum (Fig. 1), but cause no apparent mucosal damage. These organisms elaborate an enterotoxin(s) that stimulates excessive fluid/electrolyte efflux resulting in a profuse diarrhea. *Vibrio cholera* and the enterotoxigenic strains of *E. coli* (ETEC) serve as typical examples. This mechanism of GI tract disease specifically affects the proximal small bowel.

A third pattern of intestinal infection, presently being characterized, is typical of another group of *E. coli* strains termed enteropathogenic (EPEC). These bacterial agents do not make detectable levels of heat-stable or heat-labile enterotoxins like ETEC, but, nevertheless, cause diarrhea in newborns and young children. Once ingested, these organisms cause a local disruption of the intestinal microvilli, do not penetrate but attach to and colonize the underlying epithelial cell membrane throughout the intestine, and appear to produce a mucosal inflammation (reviewed by FORMAL et al. 1983). The precise cause of intestinal fluid loss is not known, but could involve the shigella-like enterotoxin recently found to be produced by these strains (WADE et al. 1979; O'BRIEN et al. 1982).

The fourth group of organisms, generally termed "invasive," actually penetrate the epithelial mucosa of the distal small intestine (i.e., ileum) or the large intestine (Fig. 1). These bacteria locally disrupt the microvilli, like the EPEC strains, and then invade the intestinal epithelial cells with ensuing intracellular multiplication. In some cases (e.g., *Salmonella typhi*) dissemination throughout the host may occur. The invasive mechanism(s) of disease, classically typified by *Shigella* and *Salmonella,* is now thought to be used by invasive strains of *E. coli, Yersinia, Vibrio parahemolyticus, Campylobacter,* as well as possibly *Aeromonas hydrophila* and *A. (Plesiomonas) shigelloides.* Beyond invasive ability, these bacteria may encode cholera-like enterotoxins, shigella-like enterotoxins, hemolysins or other cytolysins, or other potential virulence factors, and,

consequently, can evoke a variety of GI disorders including mild gastroenteritis, mesenteric adenitis, diarrhea, dysentery, and systemic infections.

Unlike other invasive bacterial diseases such as typhoid fever in which the invading bacteria are disseminated throughout the host, dysentery is a disease in which the bacteria are normally confined to the intestinal mucosa. Toxigenic organisms generally require an oral dose of $>10^5$ organisms to cause significant levels of disease within a population, but *Shigella* uniquely requires $<10^3$ cells to cause significant levels of shigellosis (reviewed by HORNICK 1978). Thus, these features broadly distinguish the pathogenic path of *Shigella* from that used by other intestinal disease agents.

2.2 Pathophysiology

How do *Shigella* cause disease? The overall process of bacillary dysentery has thus far been characterized in microscopic, but not yet in biochemical, detail. Intestinal analyses of infected monkeys or humans have shown that the colon is the primary site of bacterial invasion with some evidence for occasional involvement of the terminal ileum (LEVINE et al. 1973a; ROUT et al. 1975). The colonic mucosal surface is smooth in appearance, but contains numerous finger-like indentations, or crypts. Both the mucosal surface and the crypts are covered by an epithelial cell monolayer which overlays connective tissue containing blood and lymphatic vessels (i.e., the lamina propria). In addition, the lumen-exposed plasma membrane of each epithelial cell is normally extended into many cylindrical processes termed microvilli. Specialized goblet epithelial cells secrete a glycoprotein, mucin, which forms with water a lubricating, protective mucus coat over the epithelial mucosa.

Chromosomal mutants of *Shigella* strains that fail to penetrate epithelial cells, or that penetrate but cannot multiply intracellularly, have been isolated. Study of these mutants and virulent strains in animal models has revealed that in order to cause dysentery, *Shigella* must first invade epithelial cells of the colonic mucosa and then must be able to multiply intracellularly (LABREC et al. 1964; VOINO-YASENETSKY and KHAVKIN 1964; FORMAL et al. 1965a; OGAWA et al. 1967). Ultrastructural studies of infected intestine have revealed that the first visible alteration in the host colonic epithelium is a localized disruption of the microvilli, the outermost structure of the intestinal lining. The invading bacteria are then engulfed by an endocytic process involving invagination of the epithelial cell membrane (FORMAL et al. 1976, 1983). The intracellular bacteria are initially contained within the endocytic vacuole and later are found free in the cytoplasm, apparently following digestion of the vacuole. Subsequently, the microvilli are reestablished, and intracellular bacterial multiplication occurs. The intracellular bacteria disseminate laterally to adjacent epithelial cells and to the lamina propria. Shigellae rarely invade beyond the mucosa or enter the circulatory system. Intracellular multiplication of shigellae leads to epithelial cell necrosis, possibly due to the *Shigella* cytotoxin/enterotoxin. This process results in an acute inflammatory response involving an outpouring of polymorphonuclear leukocytes from the lamina propria. Apparently, these

neutrophils limit the infection to the superficial layers of the colon. However, lateral dissemination of the bacteria and consequent cell death leads to focal ulcerations of the epithelium. The resulting classical dysentery, observed within 24–48 h following ingestion of shigellae, is characterized by painful abdominal cramps, nausea, fever, tenesmus, and a bloody and mucus rectal discharge of small volume.

Patients with shigellosis may exhibit classical dysentery, only a watery diarrhea, or a combination of both. The small-volume rectal discharges normally associated with shigellosis are due to fluid malabsorption in the colon, the precise cause of which has not been defined, plus mucosal tissue debris. Experimental studies with monkeys indicate that the profuse diarrhea associated with some cases of dysentery may somehow be due to *Shigella*-induced abnormal jejunal export of fluid and electrolytes (ROUT et al. 1975). Gots and co-workers (1974) have shown that the intense inflammatory response caused by invasive pathogens like *Shigella* is associated with increased levels of prostaglandins which can stimulate fluid secretion. This mechanism may be primarily responsible for the large-volume diarrhea associated with some shigellosis cases.

Though a severe intestinal disease, bacillary dysentery is self-limiting and generally persists for 1–2 weeks. New colonic epithelial cells arise by division of undifferentiated epithelial cells at the bottom of the crypts and, with time, move up along the sides of the crypts. Old cells are cast off at the lumen surface, with complete replacement of the epithelial cell population every 4 to 6 days. This normal mucosal cell replacement system together with effective inflammatory and immune responses to control the spread of *Shigella* may be largely responsible for the self-limiting nature of shigellosis. These disease steps are summarized in Table 1.

2.3 Dysenteric *Escherichia coli* Strains

Evidence accumulated over the past 10 years indicates that some strains of *E. coli* (often called enteroinvasive) also cause bacillary dysentery. These *E. coli* strains (referred to herein as dysenteric *E. coli*) should be distinguished from the apparently different, intestinally acquired, invasive *E. coli* strains that cause animal septicemias. Interestingly, dysenteric *E. coli* strains exhibit many of the key diagnostic biochemical traits (SILVA et al. 1980), the *Shigella*-like enterotoxin (O'BRIEN et al. 1982), as well as the O-antigenic structures of some shigellae. It appears that these *E. coli* encode the same chromosomal and plasmid virulence traits as shigellae, with perhaps one exception. In contrast to *Shigella,* dysenteric *E. coli* strains may require a large infectious dose ($>10^8$ cells) to cause disease (FORMAL et al. 1971a).

2.4 Virulence Assay Systems

A number of whole animal, laboratory models as well as tissue culture and isolated intestinal loop assays are available with which one can assess some

or many of the virulence traits of *Shigella*. Monkeys are natural hosts for *Shigella* infection, present a disease apparently identical to that seen in humans, and serve as the model of choice to study *Shigella* pathogenesis and host immune defense mechanisms (ROUT et al. 1975). However, because of cost, animal availability, and other concerns, this model is unsuitable for preliminary screening of bacterial mutants. A very reliable, whole animal assay for *Shigella* virulence is the starved, opiated guinea pig model in which guinea pigs are infected orally, acquire characteristic dysenteric intestinal lesions, and finally succumb to the infection (FORMAL et al. 1958). Other whole animal systems (reviewed by VOINO-YASENETSKY and BAKACS 1977) have specific merits, but also are generally too difficult to use for assaying large numbers of genetic mutants. One of the most reliable models to assess *Shigella* virulence is the Sereny reaction (SERENY 1957) in which fully virulent *Shigella,* when inoculated into the conjunctival sac of a rabbit or guinea pig, provoke a keratoconjunctivitis and mimic an intestinal infection by invading and multiplying within the corneal epithelial cells. However, a dependable infectious dose is considered to be $>10^6$ cells/eye, making this assay unsuitable for detecting low-frequency mutational events within a large cell population. The ligated rabbit ileal loop model, which measures the same virulence traits as above plus fluid production, is also cumbersome, but has been employed to examine virulence properties of a few specific bacterial genetic hybrid strains (LA BREC et al. 1964; SANSONETTI et al. 1983). Recently, there has been renewed interest in assessing epithelial cell penetration ability in HeLa or Henle cell monolayers (SANSONETTI et al. 1983). Very useful for viewing epithelial cell invasion, the standard tissue culture invasion assay does not require a smooth lipopolysaccharide bacterial cell surface and does not measure other essential virulence properties (e.g., intracellular multiplication). However, Oaks and co-workers (1985) have developed a HeLa cell monolayer plaque assay which assesses the ability of invasive bacteria to invade, to multiply intracellularly, to disseminate to adjacent cells, and to result in the formation of dead host cell plaques. These latter two procedures offer considerable potential in the genetic analysis of these particular virulence properties. Finally, there are a series of cytotoxicity and immunological assays available for examining *Shigella*-like enterotoxin activity (KEUSCH et al. 1972a; O'BRIEN et al. 1982). Despite a great deal of effort and ingenuity on the parts of many investigators during much of the past 25 years, relatively few avirulent *Shigella* mutants have been isolated, due mainly to the limitations imposed by the available virulence assay systems. The recent renewed interest in and refinement of tissue culture assays and the development of new molecular genetic and immunological techniques have slightly eased the difficulties associated with detailed genetic analyses of the virulence properties of all invasive bacteria.

2.5 Diagnostic Biochemistry and Serology

The four *Shigella* species, *S. sonnei, S. flexneri, S. dysenteriae,* and *S. bodyii,* are divided into 1, 6, 10, and 15 serotypes, respectively, based on O-antigen stereochemistry. *Shigella* are typically classified as: nonmotile, lysine decarboxyl-

ase negative, lactose nonfermenting (except slow reaction by *S. sonnei*), and glucose utilizing without gas production (except for some strains of *S. flexneri* 6 and *S. boydii* 13). Members of this genus have not been shown to express common pili/fimbriae, but *Shigella* capsular (K) antigens have been reported.

3 Genetic Determinants of Virulence in Bacillary Dysentery

Though *Shigella* sp. have been studied since their discovery in 1896, the lack of suitable assays essentially prevented virulence analysis until about 1960. During the past 25 years genetic studies have revealed a series of chromosomal and plasmid-encoded properties that are involved in *Shigella* virulence. Despite the fact that *Shigella* virulence can be defined as multifactorial, little is yet known of the specific biochemical functions involved. More recently, dysenteric *E. coli* strains have been analyzed both in animal models and genetically, and they appear to mimic *Shigella* in their virulence characteristics.

Prior to discussing genetic studies of virulence, it is important to emphasize the fact that some mutations in bacteria can pleiotropically affect virulence without being a direct virulence property; e.g., mutations that result in the bacterial growth requirement of a factor that is not sufficiently available in the host. In addition to eliminating possible auxotrophic causes of avirulence, one should minimally define a suspected virulence gene mutation by studies in which the avirulent strain is restored to virulence by genetic transfer experiments, preferably by the insertion of a defined DNA fragment as occurs in transduction or via recombinant DNA procedures. Thus, some of the "virulence" traits already reported in shigellae may not be direct virulence properties. In addition, only relatively recently have plasmids been shown to be essential for *Shigella* virulence and some reported avirulent strains may simply be missing the necessary virulence plasmid.

3.1 Chromosomal Determinants of Virulence

Shigella are as highly related in nucleotide sequence to *E. coli* K12 as among the individual *Shigella* serotypes, with 80%–90% general homology and less than 3% base mismatch in these related sequences (BRENNER et al. 1973). *Shigella* encode most of the same genes as *E. coli* and the gene order and map position are thought to be, for the most part, identical to that of *E. coli* (for *E. coli* chromosomal map, see BACHMANN 1983). PETROVSKAYA and LICHEVA (1982) have reviewed many of the *Shigella* chromosomal genes that have been mapped.

Most of the studies on chromosomal virulence genes have been conducted in *S. flexneri* 2a, with a few studies conducted in *S. dysenteriae* 1 and *S. flexneri* 5. Though probable, it is not known whether analogous virulence traits exist in most other shigellae.

3.1.1 Colonial Morphology Variants: Noninvasive for Epithelial Cells

Virulent *Shigella* plated on nutrient agar medium form colonies that are smooth and translucent when examined under oblique illumination by microscopy. An apparent variety of stable, colony morphology variants of *Shigella* have been observed. Some variants appear unaltered in major lipopolysaccharide (LPS) antigen structure, but have lost virulence in various animal assay systems (COOPER et al. 1957; KEREKES 1962; LA BREC et al. 1964; FORMAL et al. 1965 b). For example, opaque colonial variants of *S. flexneri* 2a, occurring once per 10^4–10^5 cell divisions, were reported to have lost virulence (i.e., Sereny negative and HeLa cell noninvasive). Animal studies of one opaque mutant, termed 2457-0, versus the virulent parent led Formal and co-workers to conclude that epithelial cell invasion is an essential and early step in the pathogenesis of dysentery (LA BREC et al. 1964). The unusually high frequency of occurrence of these colonial variants, reminiscent of plasmid loss, prompted an analysis of their plasmid content. However, both the virulent parent and avirulent, opaque mutant strains were found to contain plasmids of approximately 140, 105, 2.6, and 2.0 megadaltons (Mdal) with no apparent differences in preliminary restriction endonuclease fragment patterns (KOPECKO et al. 1979). Similar opaque variants of *S. dysenteriae* 1 were shown to be avirulent (GEMSKI et al. 1972a). OGAWA et al. (1967) reported the isolation of an apparently different set of avirulent *S. flexneri* 2a colony morphology variants that are granular in appearance. Although colony morphology variants are stable mutants, some of these variants have been observed to mutate, at the same frequency as the forward mutation, to wild-type colony morphology in an apparent direct reversion (OGAWA et al. 1967).

In another report, three separate, flat, transparent colonial variants were isolated from *S. flexneri* 5 strain M90T. Attempts were made to restore virulence to these colonial variants by conjugally transferring in chromosomal DNA from *E. coli* K12 Hfr strain W1895 (FORMAL et al. 1965b). Several genetic hybrids of one *Shigella* variant which had received one or more of the *E. coli* genes for *ara*$^+$, *mal*$^+$, *xyl*$^+$, or *fuc*$^+$ were restored to virulence (i.e., positive in Sereny and starved guinea pig assays). These data define a very broad chromosomal region, probably between min 80 and 100, that encodes an undefined function(s) somehow involved in the expression of *Shigella* epithelial cell invasion ability and virulence (Fig. 2). Two of the above avirulent *S. flexneri* M90T colony variants could not be restored to virulence in similar conjugal matings, suggesting that different functions might be affected in these look-alike avirulent variants.

The precise genetic bases for the above colonial variants remain uncharacterized. However, it seems likely that colony morphology can be altered by many different chromosomal or plasmid mutations affecting, at a minimum, cell LPS or outer membrane structures. Some mutations may directly affect virulence functions while others may affect properties that indirectly inhibit virulence (e.g., mutation in outer membrane protein that induces a conformational membrane change affecting nearby virulence functions and overall colony morphology). This argument is strengthened by the fact that a number of pleiotropic

effects, seemingly unrelated to virulence (e.g., loss of glycerol kinase activity; KIM and CORWIN 1974; discussed below), have been associated with avirulent opaque colonial variants of *S. flexneri* (reviewed in GEMSKI and FORMAL 1975).

KIM and CORWIN (1974) found that the avirulent, opaque mutant *S. flexneri* strain 2457-0 (LA BREC et al. 1964) was deficient in glycerol kinase activity (i.e., phenotypically *glpK*$^-$). Spontaneous reversion of 2457-0 to glycerol fermentation ability occurred at a frequency of one in 10^4 cells and resulted in approximately 50% of the GlpK$^+$ strains regaining the ability to invade HeLa cells. Similarly, virulence was restored to about 50% of the GlpK$^+$ 2457-0 transductants receiving genes from the virulent *S. flexneri* 2a strain M42-43. None of nine separate 2457-0 recipients that had regained GlpK$^+$ activity following conjugational or transductional transfer from *E. coli* K12 was observed to regain virulence. Though the *E. coli* K12 and *Shigella* glycerol kinase enzymes appeared to be very similar biochemically, the *glpK* genes mapped in different locations in each host. Furthermore, when a virulent M42-43 strain was mutated to be glycerol nonfermenting, about half of the resulting GlpK$^-$ mutants were noninvasive for HeLa cells. These results are difficult to reconcile without further information. However, one interpretation of the data is that a single gene in *Shigella* specifies glycerol kinase synthesis and mutations in this gene do not affect virulence. A second adjacent gene(s) altered in strain 2457-0 somehow inhibits HeLa cell penetration ability and alters colony morphology. However, unusual mutation events, occurring at a frequency of 10^{-4}, can turn on or off only one or both of these adjacent genes. An alternative explanation would be that one gene product affecting several activities (e.g, virulence, GlpK activity) could undergo high-frequency mutations that affect one or more of these functions. Regardless of interpretation, these studies have identified a locus which is closely linked to *glpK* in *Shigella* (around min 65 on the chromosome; Fig. 2) and that is involved directly or indirectly with epithelial cell penetration.

3.1.2 O-Polysaccharide Antigen: Protection from Host Defenses

A smooth lipopolysaccharide cell surface has been directly associated with the virulence of many pathogenic enteric bacteria and is thought to protect the bacteria from normal host defense mechanisms. This is also true for shigellae. Rough mutants of shigellae have been recognized for many years to be avirulent in animal assays. OKAMURA and NAKAYA (1977) were the first to show that a rough mutant of *S. flexneri* 2a, though unable to provoke a positive Sereny reaction, could penetrate cultured HeLa cells. These data, now verified in other labs, suggest that a smooth LPS surface is needed to protect shigellae in the bowel lumen but that the LPS may not specifically be involved in cell invasion (OKAMURA et al. 1983). This hypothesis is supported by recent studies, discussed in detail below, which have shown that a rough *E. coli* K12 carrying a *Shigella* plasmid that encodes cell invasive ability can invade cultured HeLa cells (SANSONETTI et al. 1983).

The genes controlling *Shigella* LPS biosynthesis have been found to map, for the most part, in chromosomal regions analogous to the LPS genes of

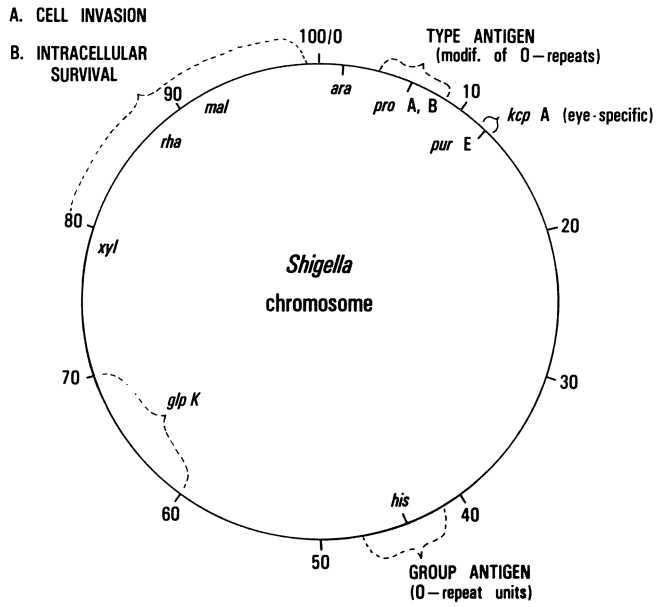

Fig. 2. Genetic map of the *Shigella* chromosome showing the locations of the determinants of virulence. This map has been divided into 100 equal divisions or min. Chromosome size, gene location, and gene symbols are based largely on the highly related, well-characterized *E. coli* genetic map (BACHMAN 1983). The glycerol kinase (*glpK*) gene of *Shigella* has been located between 60 and 70 min, which is very different than its location in *E. coli*. Most of the virulence genes identified above have been detected in *S. flexneri;* analogous loci in other shigellae are suspected. The symbols *his* and *pro* refer to genes for the biosynthesis of histidine and proline, and the symbols *ara, rha, xyl,* and *mal* refer to genes for the catabolism of arabinose, rhamnose, xylose, and maltose. The locus controlling keratoconjunctivitis provocation (*kcpA*) is cotransducible with the purine E (*purE*) gene

E. coli and *Salmonella*. Some *Shigella* LPS core oligosaccharide genes have been mapped at chromosomal min 80 (reviewed by PETROVSKAYA and LICHEVA 1982). Shigellae are classified into serotype on the chemical basis of O-polysaccharide antigens. Most shigellae are thought to encode the O-antigenic determinants at a single locus that is closely linked to the histidine biosynthetic genes at min 44 on the chromosomal map (Fig. 2). In contrast, *S. flexneri* contain an additional locus encoding the type-specific antigen genes, which are situated close to the proline determinants at min 6 on the map (Fig. 2). The type-specific antigen genes are responsible for enzymatic modification of an O-antigen chemical backbone, termed the group antigen in *S. flexneri,* which is encoded close to chromosomal min 44 on the map (FORMAL et al. 1970; SIMMONS 1971). At least some of the type-specific antigenic conversion systems are encoded by lysogenic viruses whose chromosomal attachment site is situated next to the *pro* genes (reviewed in FORMAL et al. 1970 and PETROVSKAYA and LICHEVA 1982). Naturally occurring (e.g., Y strains) or mutant *S. flexneri* containing a group antigen without type antigenic modification are virulent in animals (FORMAL

et al. 1971b). Thus, type-specific modification does not appear to be essential for virulence.

Using *E. coli* K12 Hfr donor strains GEMSKI and co-workers (1972) constructed numerous *S. flexneri* 2a genetic hybrid strains to express uniquely either *E. coli* 0–8 or 0–25 antigens. Only the 0–25 expressing hybrids were found to have retained virulence. Since the 0–25 antigen was more similar chemically to the *S. flexneri* 2a antigen than the 0–8 antigen, these workers logically suggested that the specific chemical composition and structure of the O-antigen may be important for virulence. This suggestion may prove correct, but in light or our present understanding of the multifactorial nature of *Shigella* virulence, the exact cause of the avirulence of the 0–8-expressing *Shigella* hybrids would appear to require further study.

Though most *Shigella* LPS genes are chromosomally located as described above, two exceptions have now been noted. All strains of *S. sonnei* carry a 120-Mdal plasmid that encodes the form I O-antigen genes of this species (KOPECKO et al. 1980; SANSONETTI et al. 1980; also discussed below in detail). Additionally, studies of *S. dysenteriae* 1 strains have recently revealed that a small 6-Mdal plasmid encodes one or more functions necessary for O-antigen expression (WATANABE and TIMMIS 1984).

3.1.3 Ability to Survive Intracellularly

In an attempt to locate *Shigella* chromosomal virulence genes, FALKOW et al. (1963) transferred *E. coli* K12 Hfr chromosomal regions into a virulent *S. flexneri* 2a recipient. Haploid and merodiploid genetic hybrid strains of reduced virulence and some that were avirulent were detected. One *Shigella* hybrid, X16, that had incorporated *E. coli ara*$^+$, *rha*$^+$, *xyl*$^+$, and *mal*$^+$ traits was studied in detail. This hybrid strain was determined to be capable of provoking a keratoconjunctivitis in the guinea pig eye and could invade cultured HeLa cells. However, strain X16 caused an abortive intestinal infection in the starved guinea pig; X16 cells could penetrate the intestinal epithelium and stimulate an inflammatory response, but could not persist intracellularly and the intestine returned to normal within 4 days after challenge (FORMAL et al. 1965a). These studies define a large region of the chromosome (from about min 80 to min 100; Fig. 2) that controls an uncharacterized function(s) involved in the ability of *Shigella* to multiply and persist within the epithelial mucosa. The function(s) affected in strain X16 is apparently different from the determinant that was found to affect epithelial cell penetration ability, as described in Sect. 3.1.1, and which is located in the same large chromosomal region.

Mutations, introduced by intraspecies transduction, that map at chromosomal min 72–73 in the *Shigella rpsL* (Str^R), *rpsQ* (*neaA,* neamine resistance), or *neaB* genes have been reported to result in the loss of *Shigella's* ability to survive intracellularly, but do not affect epithelial cell penetration ability (reviewed by PETROVSKAYA and LICHEVA 1982). The causative effects on virulence of these mutations are not understood.

3.1.4 KcpA Locus Controlling Keratoconjunctivitis Provocation

Using *E. coli* K12 Hfr strains to mate with and replace *Shigella* chromosomal virulence genes, FORMAL and co-workers (1971a) identified a region on the *S. flexneri* 2a chromosome, located between the *lac* and *gal* genes, which is necessary for keratoconjunctivitis provocation in the guinea pig. *E. coli* K12 does not appear to encode a similarly located complementing function. P1 transduction analysis revealed that the responsible locus, termed *kcpA,* was cotransducible with *purE* (Fig. 2). More recent studies have revealed that *kcpA*$^-$ *S. flexneri* can invade HeLa cells and are invasive in other assays (T.L. HALE and S.B. FORMAL, unpublished data). Presently, it is not clear if the *kcpA* locus specifically affects the Sereny reaction (i.e., may not be necessary for human intestinal infection), what biochemical factors are involved, and if a similar locus exists in other shigellae.

3.1.5 Enterotoxic and Cytotoxic Activities of Shiga Toxin

A definitive role for *Shigella* toxin in the pathogenesis of dysentery, though likely, has not been established. Discovered initially in *S. dysenteriae* 1, Shiga toxin appears to be produced at 100- to 1000-fold lower levels in other *Shigella* species and in dysenteric strains of *E. coli* (KEUSCH et al. 1977; O'BRIEN et al. 1977 and 1982). Shiga toxin is now known to display three different toxic activities; enterotoxicity in ligated rabbit ileal loop assays, cytotoxicity for various cultured eukaryotic cells, and neurotoxicity for mice (see KEUSCH et al. 1972b). The neurotoxic activity is not evident in infected monkeys (GEMSKI et al. 1972b) and apparently does not play a role in dysentery. However, the enterotoxic activity may be involved in the fluid secretion (diarrhea) often associated with dysentery and the cytotoxicity may be responsible for intestinal cell death (GEMSKI and FORMAL 1975; BROWN et al. 1980). Noninvasive but toxigenic mutants of *S. dysenteriae* 1 are unable to cause disease in monkeys and starved guinea pigs (GEMSKI et al. 1972b), suggesting that Shiga toxin cannot be taken up directly by intestinal epithelial cells and that epithelial cell invasion by *Shigella* may be necessary to internalize the Shiga toxin. Furthermore, other studies indicate that protein synthesis inhibition of mammalian cells may be the primary cytotoxic mechanism of Shiga toxin (THOMPSON et al. 1976; BROWN et al. 1980). For those interested, a current, brief review of the mechanisms of action of Shiga toxin is available (MIDDLEBROOK and DORLAND 1984).

The genes encoding the multi-subunit Shiga toxin or regulating its expression have not been identified. Shiga toxin-negative mutants of *S. dysenteriae* 1, though previously thought to have been isolated (GEMSKI et al. 1972b), have now been shown with more sensitive assays to produce low levels of toxin (O'BRIEN et al. 1977). Definition of a precise role for Shiga toxin in the pathogenesis of dysentery awaits the isolation of a Tox$^-$ *Shigella* strain.

3.1.6 Potential Virulence Factors

Recent studies of *S. sonnei* and *S. flexneri* have revealed that these shigellae contain chromosomally mediated Fe^{3+}-uptake systems (PERRY and SAN CLEMENTE 1979; PAYNE et al. 1983). The genes involved in these enterochelin and hydroxamate systems have not yet been mapped. Iron-sequestering systems are generally a virulence requirement for pathogenic invasive bacteria. Enteric bacteria require approximately 10^{-5} *M* Fe^{3+} in order to sustain growth, but the effective free [Fe^{3+}] in tissue is generally $<10^{-20}$ *M*. Therefore, it seems likely that these iron-sequestering systems will prove to be necessary for shigellae to multiply and persist intracellularly.

Limited studies with one *Shigella flexneri* 4b strain have led to the identification of an extracellular alpha-galactosidase that could be involved in degrading intestinal mucin glycoprotein sugars (PRIZONT 1982). Shigellae may carry mucinases which allow them to uncover epithelial cell membrane receptors that initiate endocytosis.

3.2 Plasmid-Borne Virulence Properties

The first observed drug resistance (R) plasmids, reported in the late 1950s, were detected in strains of *S. flexneri* in Japan. Although *Shigella* R plasmids have been studied intensively over the past 30 years, plasmids were not thought to play a role in the virulence of dysentery bacilli until 1980. Studies conducted over the past 5 years have demonstrated that the ability of all shigellae to invade epithelial cells is plasmid mediated. In addition, plasmid involvement in O-antigen synthesis of two *Shigella* serotypes has been noted.

3.2.1 Plasmid-Mediated O-Antigen Expression

Shigella sonnei strains, responsible for two-thirds of the shigellosis cases in many parts of the world, exist as a single serotype due to expression of the form I O-antigen consisting of disaccharide repeat units comprising two unusual amino sugars (KEENE et al. 1980). Smooth (i.e., form I antigen-expressing) *S. sonnei* have been observed for many years to undergo a spontaneous dissociation to rough (i.e. form II) cells on agar medium. KEENE and co-workers showed that rough, form II cells were devoid of O-antigen, but synthesized a complete core oligosaccharide. Two independent groups reported in 1980 that (1) the form I to form II colony type transition occurs in different strains at frequencies of from 0.5% to 50%, (2) the transition is irreversible (i.e, form II colonies never generate form I cells), and (3) form II cells are avirulent (i.e., Sereny negative) in contrast to the virulent form I parental cells (KOPECKO et al. 1980; SANSONETTI et al. 1980). The unusually high frequency and irreversibility of this transition suggested the involvement of a plasmid. Indeed, when form I *S. sonnei* strains

obtained from different geographical regions of the world were compared in plasmid analyses to form II derivatives of each strain, all virulent *S. sonnei* were observed to harbor an unstable 120-Mdal (180 kilobase pair, kb) plasmid necessary for form I antigen synthesis and virulence. Form II *S. sonnei* derivatives of these strains had invariably and spontaneously lost only this 120-Mdal plasmid species (KOPECKO et al. 1980; SANSONETTI et al. 1980). Similar results have now been obtained with more than 30 different *S. sonnei* strains (KOPECKO and FORMAL, unpublished data). Both form I and form II cell types often carried other large and small plasmid species not directly involved in virulence, some of which were subsequently shown to encode colicin E1 production and antibiotic resistance genes (SANSONETTI et al. 1981). These experiments indirectly demonstrated the association of the 120-Mdal plasmid (i.e., the form I plasmid) with virulence and the ability to synthesize form I antigen.

Neither wild-type nor transposon-tagged 120-Mdal form I plasmids were found to be conjugally self-transmissible to form II recipient *S. sonnei* cells. Also, no easily identifiable phenotypic properties with which one could monitor plasmid transfer were found to be associated with these plasmids. However, Kopecko and co-workers (1980) were able to mobilize conjugally a Tn*3*-tagged 120-Mdal form I plasmid from a virulent *S. sonnei* donor by in vivo recombination with a newly introduced F′ts*lac*::Tn*3* plasmid. Form II *S. sonnei* recipient cells that had received the Tn*3*-tagged 120-Mdal plasmid were restored to form I antigen-synthesizing ability, but not to virulence (see below). Similarly, this 120-Mdal plasmid was conjugally transferred to *S. flexneri* 2a, *Salmonella typhi, E. coli* K12, *S. typhimurium, Proteus mirabilis,* and *Serratia marcescens* (KOPECKO et al. 1980; BARON, KOPECKO, and FORMAL, unpublished data) and all transconjugants for this 120-Mdal plasmid were observed immunologically to express the form I O-antigen as well as antigens of the parental strain. These data directly demonstrated that all of the structural genes necessary for form I O-antigen synthesis are encoded on this 120-Mdal plasmid. More recently, the plasmid DNA region encoding a functional form I O-antigen has been cloned onto a cosmid vector. Insertion of these form I antigen-expressing recombinant cosmids into form II *S. sonnei* cells restored them to form I cell type but did not restore virulence (i.e., they remained Sereny negative and HeLa cell noninvasive; KOPECKO et al. 1983). These data indicate that the 120-Mdal form I plasmid encodes properties that are necessary for invasive ability and which are separate from O-antigen synthesis (see next section).

In preliminary studies of *S. dysenteriae* 1 strains, WATANABE and TIMMIS (1984) have identified a 6-Mdal plasmid that encodes one or more properties involved in O-antigen expression of this serotype. Further study of *S. dysenteriae* 1 O-antigen expression has revealed that some of the O-antigen genes are located adjacent to the *his* chromosomal locus and that the 6-Mdal plasmid encodes a function(s) involved in LPS core modification and, possibly, another function(s) involved in O-antigen expression (HALE et al. 1984). Thus, in contrast to the *S. sonnei* 120-Mdal plasmid that encodes all of the genes necessary for form I O-antigen synthesis and other genes necessary for epithelial cell penetration (see below), the 6-Mdal *S. dysenteriae* plasmid encodes one or more functions involved in *S. dysenteriae* serotype 1 O-antigen expression.

3.2.2 *Shigella sonnei*: Plasmid-Determined Invasiveness

Mobilization of the Tn*3*-tagged 120-Mdal *S. sonnei* form I plasmid via recombination with F′*lac*::Tn*3* restored a *S. sonnei* form II recipient's ability to synthesize the form I antigen, but did not restore virulence (KOPECKO et al. 1980; described above). This initial study also noted that *S. sonnei* strain 9774 was a smooth, form I^+ isolate but lacked virulence and contained only a 90-Mdal plasmid (probably a deletion mutant of the 120-Mdal plasmid). These facts strongly suggested that the typical 120-Mdal form I plasmid encodes factors that are essential for virulence and separate from the O-antigen. In further attempts to monitor plasmid transfer in efforts to restore virulence to form II *S. sonnei* cells, SANSONETTI and co-workers (1981) tested but could not correlate any of more than 165 different, easily identifiable, phenotypic properties (i.e., antibiotic resistance, colicin production or immunity, metabolic traits, or iron-sequestering ability) with the presence or absence of several separate form I plasmids. In phenotypically tagging the form I plasmid with transposons, these workers noted that the resulting *S. sonnei* strains carrying certain transposon-tagged form I plasmids were smooth and form I^+, but avirulent; these data indicated that the transposon had inserted into other plasmid-encoded virulence genes in these tagged plasmids. None of the transposon-tagged plasmids were found to be conjugally self-transmissible or transformable. In attempts to mobilize a transposon-tagged form I plasmid from virulent *S. sonnei* to form II *S. sonnei* cells with ten separate conjugative plasmids, each representing a different incompatibility group (inc gp), only the inc gp FI plasmid R386 was found to be successful. Of four *S. sonnei* transconjugants that had reacquired form I antigen expression, only two were virulent (i.e., Sereny test positive). Plasmid analyses revealed that the virulent transconjugants contained only the tagged 120-Mdal plasmid whereas the two avirulent isolates had coinherited the R386 plasmid along with the form I plasmid. Upon spontaneous loss of R386, the avirulent form I^+ transconjugants were observed to have regained virulence. It appeared that R386 had interfered with the expression of virulence, as had the Fts′*lac*::Tn*3* mobilizing plasmid (KOPECKO et al. 1980; discussed below). Despite experimental difficulties, these data directly demonstrated that the 120-Mdal plasmid encodes properties that are necessary for virulence and separate from form I O-antigen expression (SANSONETTI et al. 1981). More recent studies, discussed in Sect. 3.2.1, have shown that form II *S. sonnei* cells are noninvasive for HeLa cells. Furthermore, form II *S. sonnei* recipients of the form I antigen determinants cloned via recombinant DNA techniques express a smooth form I^+ cell surface, but are still avirulent and noninvasive for HeLa cells (KOPECKO et al. 1983). These latter data define more specifically that the form I plasmid encodes virulence genes controlling epithelial cell penetration.

Utilizing plasmids of different incompatibility groups, SANSONETTI et al. (1981) have determined that the 120-Mdal form I plasmids are most likely members of inc gp FI, although the observed incompatibility was weak. Also, the natural instability of form I plasmids appears to be plasmid determined; each form I plasmid was spontaneously eliminated at a characteristic frequency regardless of host background (SANSONETTI et al. 1981). Thus, the 120-Mdal

form I plasmids encode form I O-antigen synthesis as well as genes involved in epithelial cell invasion. These FI inc gp plasmids are highly unstable and are spontaneously lost at frequencies of from 0.5% to 50%. One wonders why a pathogen would carry essential virulence properties on an unstable plasmid and what selective pressures maintain the virulent state of *S. sonnei*.

3.2.3 Experimental Problem: FI inc gp Plasmids Inhibit the Sereny Reaction

As discussed above, both R386 and the F′ts*lac*::Tn*3* plasmids were observed to inhibit virulence of form I^+ *S. sonnei* when assayed by the Sereny reaction (KOPECKO et al. 1980; SANSONETTI et al. 1981). To examine this phenomenon in more detail, plasmids of many different incompatibility groups were transferred separately to virulent strains of *S. sonnei, S. flexneri, S. dysenteriae,* and dysenteric *E. coli*. All invasive strains responded identically; only FI incompatibility group plasmids inhibited virulence in the Sereny assay. In fact, F plasmid conjugal transfer genes cloned on a 45-kb *Bam*H1 fragment into the colicin E1 plasmid (i.e., pED830) also inhibited the Sereny reaction of virulent dysentery bacilli (KOPECKO et al. 1981). More recent studies have revealed that, although FI inc gp plasmids inhibit the Sereny reaction, dysentery bacilli carrying a FI inc gp plasmid are invasive for HeLa cells and virulent in some animal assays (SANSONETTI, KOPECKO, HALE, and FORMAL, unpublished data). Thus, some function(s) encoded within the F plasmid conjugal transfer gene region specifically inhibits the Sereny reaction. One speculative explanation is that an F plasmid-encoded outer membrane protein involved in conjugal transfer interferes with the attachment/invasion of bacilli into the guinea pig corneal epithelium. The Sereny reaction remains a good test for assessing the ability of *Shigella* to cause disease. However, these experiments demonstrate one difficulty associated with correlating *Shigella* virulence to a positive Sereny reaction.

3.2.4 Shigella flexneri: A Large Plasmid Specifies Epithelial Cell Penetration Ability

Plasmid analyses of wild-type virulent *S. flexneri* 2a strain 2457 and its avirulent, opaque colonial variants demonstrated the presence of large plasmids in cells of both colony types, but no involvement of plasmids in virulence was detected (KOPECKO et al. 1979). In light of the definition of *S. sonnei* virulence plasmids, SANSONETTI and co-workers (1982b) reexamined *S. flexneri* for virulence-associated plasmid traits. Virulent isolates of representative strains of the six *S. flexneri* serotypes (i.e., strains M25-8, M4243, J17B, M7639, M90T, and CCH060, respectively) were initially analyzed for plasmid content. All strains contained a large plasmid of approximately 140 Mdal in size; other large and small plasmids were present but not consistently in all strains. Upon restreaking on nutrient medium aged slant cultures of these virulent strains, about 10% of the resulting smooth, translucent colonies of strains M25-8 (serotype 1b), M4243 (serotype 2a), and M90T (serotype 5) were observed to be avirulent

(i.e, Sereny negative and HeLa cell noninvasive; note that avirulent isolates are not easily obtained by restreaking freshly grown virulent *Shigella*). However, these avirulent variants were found to express typical cell surface LPS antigens. Plasmid analyses revealed that all of these avirulent isolates had either spontaneously lost the 140-Mdal plasmid or undergone a specific 50-Mdal deletion in this large plasmid species. These data suggested that the large 140-Mdal plasmid is required for epithelial cell penetration ability. However, direct proof required reintroduction of this plasmid and virulence to an *S. flexneri* strain that had lost the corresponding plasmid. No phenotypic traits of selective value (e.g., antibiotic resistance, iron-sequestering ability, colicin production) for monitoring plasmid conjugal transfer were identified on this large 140-Mdal plasmid. Thus, the 140-Mdal plasmid of the serotype 5 strain M90T was tagged with Tn*5*; in several tagged plasmids the transposon insertion resulted in loss of virulence. One resulting virulent isolate of M90T carrying the Tn*5*-tagged 140-Mdal plasmid, pWR110, was used in attempts to transfer pWR110 to appropriate avirulent *S. flexneri;* no conjugal self-transfer of pWR110 was observed. However, any of three mobilizing conjugative plasmids (R16, R64*drd*11, and R386) were found to be capable of mobilizing pWR110 to avirulent *S. flexneri* strains of serotype 1b (M25-8) or serotype 2a (M4243) which had lost the corresponding 140-Mdal plasmid species. *S. flexneri* serotype 1b or 2a transconjugants that received the pWR110 plasmid were restored to virulence. *S. flexneri* transconjugants that had coinherited R386 and pWR110 were Sereny negative (see Sect. 3.2.3). However, segregation of R386 from these isolates occurred at a high frequency, suggesting that these 140-Mdal virulence plasmids are members of inc gp FI. These studies unequivocally showed that these 140-Mdal plasmids encode functions involved in *S. flexneri* epithelial cell penetration ability (SANSONETTI et al. 1982b). The ability of the serotype 5 plasmid, pWR110, to restore virulence to serotypes 1b and 2a strains demonstrates the functional homology among these plasmids. These 140-Mdal plasmids appear to be nonconjugative members of plasmid inc gp FI, do not encode any major LPS antigen, and are generally more stable than the *S. sonnei* virulence plasmids.

Recently, MAURELLI and co-workers (1984a) reported that cells of virulent *S. flexneri* 2a strain 2457-T form pigmented colonies on Congo red dye agar. Avirulent derivatives detected as nonpigmented colonies were found to have lost or deleted portions of the 140-Mdal virulence plasmid. This dye selection system may prove useful for studying other *S. flexneri* plasmids, but preliminary results show that some virulent serotypes of *Shigella* do not bind Congo red dye (BUYSSE, KOPECKO, and BARON, unpublished data).

In a separate study, MAURELLI et al. (1984b) found that *S. flexneri* 2a, *S. sonnei,* and *S. dysenteriae* 1 strains all require growth at 37 °C for virulence (i.e., Henle cell invasion). Cells grown at 30 °C were phenotypically avirulent until shifted to 37 °C and allowed to undergo protein synthesis. These results suggested that expression of one or more of the virulence genes is subject to growth temperature regulation. MAURELLI and CURTISS (1984c) have recently determined, using Mu*d*1 (Ap^R*lac*) fusions, that expression of at least one of the *S. flexneri* 2a 140-Mdal plasmid-mediated virulence proteins is growth temperature regulated.

3.2.5 *Plasmids of Other Shigellae and Dysenteric Escherichia coli*

SILVA and collaborators (1982) examined the plasmid content of a total of 58 virulent and spontaneously occurring avirulent (i.e., Sereny negative) strains representing all four species of *Shigella*. All 27 virulent strains contained a large plasmid (120- to 140-Mdal) species that was absent in all avirulent isolates. Taken together with data presented above, these findings suggest that all shigellae harbor a large plasmid that is required for virulence.

SANSONETTI et al. (1982a) reported that dysenteric *E. coli* strains of serotypes 0124 and 0143 contain large 140-Mdal plasmids. Spontaneous *E. coli* variants that had lost the large plasmid were isolated and invariably determined to be avirulent (i.e., Sereny negative and HeLa cell noninvasive), but contained a normal smooth LPS cell surface. Conjugal mobilization of the *S. flexneri* pWR110 virulence plasmid into these avirulent *E. coli* variants was found to restore their virulence (i.e. Sereny positive). Thus, it appears that all dysenteric *E. coli* and shigellae carry a large plasmid that is necessary for epithelial cell penetration. In recent studies, *E. coli* K12 strains carrying pWR110 were found to invade HeLa cells (SANSONETTI et al. 1983), indicating that most of the functions required for epithelial cell penetration are encoded by these large virulence plasmids.

3.2.6 *Virulence Plasmid Comparisons: DNA Sequences and Encoded Proteins*

The 140-Mdal virulence plasmids of several *S. flexneri* strains and a dysenteric *E. coli* strain were compared by agarose gel electrophoretic analysis of *Eco*R1- or *Bam*H1-generated restriction endonuclease fragments to similarly treated virulence plasmids of several *S. sonnei* strains. Though no two virulence plasmids had identical restriction patterns, the *S. sonnei* plasmids shared many restriction fragments and appeared not to have undergone much evolutionary divergence. In contrast, the *E. coli* and *S. flexneri* plasmids shared very few fragments in common with one another or with the *S. sonnei* plasmids. Though seemingly dissimilar by restriction endonuclease fragmentation patterns, DNA hybridization studies showed that all of these virulence plasmids share homologous sequences distributed throughout the plasmid molecule. The *S. flexneri* and *E. coli* virulence plasmids have apparently undergone significant microevolutionary mutations leading to variations in restriction sites. All of these virulence plasmids were found to share *Eco*R1 fragments of 11.5, 6.5, 5.5, and 2.6 Mdal, and these DNA regions may encode some of the epithelial cell penetration functions (HALE et al. 1983).

HALE and collaborators (1983) also made minicell mutants in various *Shigella* and dysenteric *E. coli* strains to examine plasmid-encoded protein synthesis. These studies established that (1) minicells from a virulent *Shigella* strain are invasive for HeLa cells, (2) plasmids in virulent *Shigella* or *E. coli* minicells synthesize a minimum of 10–15 outer membrane polypeptides ranging from 12 to 64 kilodaltons, and (3) seven outer membrane polypeptides appear to be associated with the virulent phenotype. Since epithelial cell infection involves

cell-to-cell interactions, it is likely that these outer membrane polypeptides play a direct role in bacterial invasion of the epithelium. Cloning of these virulence-associated regions via recombinant DNA procedures will allow for a better understanding of the invasion process.

3.3 Transfer of *Shigella* Virulence Genes to *Escherichia coli* K12

Recently, SANSONETTI and co-workers (1983) have used Hfr chromosomal gene transfer and plasmid mobilization techniques to transfer one or more of the *Shigella* virulence traits to a strain of *E. coli* K12. The resulting hybrid *E. coli* strains were assayed for HeLa cell invasiveness, Sereny reaction, and fluid production and mucosal pathology in the rabbit ileal loop model. *E. coli* K12 cells carrying only the *S. flexneri* plasmid pWR110 were observed to invade HeLa cells, but were negative in the Sereny reaction. A positive keratoconjunctivitis reaction and fluid production, as well as significant mucosal pathology in the rabbit ileal loop, were provoked only by hybrids that contained the *Shigella* kcp^+, his^+ (O-antigen genes) and arg^+-mtl^+ regions of the chromosome in addition to pWR110. Hybrids containing pWR110 plus some, but not all, of these chromosomal regions demonstrated an intermediate level of virulence in these assay systems. These observations reemphasize the fact that virulence in *Shigella* is multideterminant. Definition of the precise virulence-associated regions and functions of the *Shigella* chromosome using genetic engineering procedures will allow for a better understanding of how these determinants interact to provoke disease.

4 A Perspective Overview of Dysentery; Defined and Potential Bacterial Virulence Properties

Key events in the pathogenesis of bacillary dysentery, presented in Sect. 2.2, are summarized in Table 1. In this table, shigellosis has been divided arbitrarily into four disease steps and bacterial involvement is reviewed in comparative terms of disease-associated events, potential bacterial requirements, and defined bacterial virulence genes. Following host ingestion of a small bacterial inoculum, the bacterial cells likely possess cell surface and internal mechanisms to resist low pH, bile salts, antimetabolites produced by the normal gut flora, and host immune defenses. The only defined bacterial traits needed for survival in the bowel lumen are genes controlling LPS biosynthesis, specifically the O-antigen genes. In the next step of the disease, the cause of abnormal jejunal fluid transport is undefined. Also, the requirement for mucinases and the bacterial function(s) involved in microvillus disruption (possibly cytoskeletal collapse) have not been established. Bacterial requirements for attachment to epithelial cells remain undefined. Note, however, that IZHAR et al. (1982) found that adherence of shigellae to guinea pig colonic epithelial cells was Ca^{2+} dependent and apparently mediated by a mucosal adhesin that recognized bacterial LPS. Endocytic

Table 1. Bacterial involvement in the pathogenesis of shigellosis. The pathogenesis of shigellosis has been divided into four arbitrary steps. Known and suspected bacterial virulence traits and their potential involvement in each step of the disease process are shown

Disease steps	Probable associated events	Potential bacterial involvement	Established genetic loci	Usual disease symptoms
1. Ingestion	1. Low infectious dose; bacterial survival and multiplication in the gut lumen	1a. Resistance to low pH, bile salts, and gut flora/anti-metabolites	1a. None	None
		1b Restistance to host immune defenses	1b. O-Antigen genes	
2. Attachment/ invasion	2. Sometimes, water and electro-lyte secretion from the jejunum	2. Possible in-volvement of Shiga toxin	2. None	During first 48 h abdominal pain, fever nausea, sometimes watery diarrhea
(preferential for colon)	3. Bacterial passage through mucin	3. Possible glyco-protease or pro-tease production	3. None yet proven (see PRIZONT 1982)	
	4. Disruption of microvilli	4. Undefined	4. None	
	5. Bacterial attachment to epithelial cell (Ca^{2+} require-ment?)	5. Undefined bac-terial surface properties	5. Possibly plasmid gene(s); *kcpA*?	
	6. Internalization of bacteria by the epithelial cell (receptor-mediated endocytosis?). Bacteria contained in phagosome	6. Bacterial sur-face properties outer membrane protein receptor	6. Invasion plas-mid gene(s)	
3. Intracellular multiplication and dissem-ination	7. Disruption of phagosomal membrane	7. Undefined lytic enzymes	7. None	
	8. Intracellular bacterial multi-plication	8. Iron-seques-tering systems, other undefined systems	8. Iron acqui-sition – not yet proven virulence trait (see PAYNE et al. 1983); *xyl-rha* region; *kcpA*?	Same as above

Table 1 (continued)

Disease steps	Probable associated events	Potential bacterial involvement	Established genetic loci	Usual disease symptoms
	9. Invasion of adjacent epithelial cells and lamina propria	9. Undefined; possible lytic enzymes or normal invasion mechanisms	9. None	
4. Ulceration of colonic mucosa	10. Bacterial multiplication leading to inflammation and epithelial cell death	10. Same as (8) above plus Shiga toxin; lytic enzymes?	10. Shiga toxin gene(s) are not mapped	Severe abdominal cramps; tenesmus; frequent, small-volume rectal discharge of blood, mucus, and pus. Usually, self-limiting disease that persits 7–14 days
	11. Impaired colonic fluid absorption	11. Shiga toxin	11. None	
	12. Stimulation of intestinal motility	12. Undefined	12. Undefined (see FERNANDEZ et al. 1984)	
	13. Adjacent necrotic cells forming focal ulcerations. Discharge of blood, mucus, and pus	13. Probably none	13. None	
	14. Colonic mucosa epithelial cell replacement	14. Probably none	14. None	

uptake appears to involve several plasmid-encoded outer membrane proteins; chromosomal involvement is not well defined and may be indirect (e.g. *glpK* region, *mal-xyl* region) or specific for one assay system (i.e., *kcpA*). Bacterial release from the phagosome, as yet uncharacterized, may require bacterial lytic enzymes. Next, free intracellular bacteria must multiply and disseminate to adjacent cells and the lamina propria. Although iron acquisition is probably crucial to intracellular bacterial survival and iron-sequestering systems have been observed in shigellae, their essentiality for *Shigella* virulence has not been established. The *xyl-rha* region of the chromosome contains an undefined function(s) that is somehow needed for intracellular survival. The dissemination of shigellae, however, remains uncharacterized but may require bacterial lytic enzymes or may involve the plasmid-mediated invasion system. Epithelial cell death probably results from the cytotoxicity of Shiga toxin and fluid secretion may be

stimulated by the enterotoxic activity of Shiga toxin; the genes involved in Shiga toxin biosynthesis remain unmapped. Finally, undefined bacterial products have recently been reported to stimulate intestinal motility (FERNANDEZ et al. 1984) and may be involved in provoking abdominal cramps, tenesmus, or other disease symptoms. This brief overview was presented to convey our current understanding of the genetics of virulence and to point to undefined potential bacterial virulence properties.

Genetic dissection of *Shigella* virulence properties has, to date, contributed to the general micro- and macroscopic understanding of how *Shigella* cause disease. The biochemical events involved in the pathogenesis of dysentery remain largely uncharacterized. Currently available experimental techniques should aid in the detection of new virulence properties. In addition, each of the various defined *Shigella* virulence factors can now be cloned on a small DNA fragment so that biochemical analyses of the products and their involvement in disease pathogenesis can be initiated.

5 Application of Genetic Knowledge of Virulence to Diagnosis and Prevention of Dysentery

Genetic studies of *Shigella* virulence, in addition to augmenting our understanding of the pathogenesis of dysentery, provide unique insights that should aid in the development of new methods for disease diagnosis and prevention. For example, cloned virulence-associated DNA fragments should serve as specific DNA hybridization probes that can be used for rapid and improved detection of dysentery bacilli. DNA probes would be extremely beneficial in studying disease epidemiology and may prove useful in standard clinical microbiology labs.

Similarly, genetic characterization of shigellae major cell surface antigens has already aided in the development of antidysentery vaccines. Early vaccine efforts demonstrated that parenteral *Shigella* vaccines are not effective in preventing shigellosis, probably because the organisms are confined to the epithelial mucosa. Attenuated mutant *Shigella* oral vaccines, thought to stimulate local intestinal immunity, have provided serotype-specific immunity, but have not proven safe or effective enough for widespread use. Recently, however, FORMAL and collaborators (1981) have employed a proven safe and effective oral typhoid vaccine strain, *S. typhi* Ty21a, as a carrier of *Shigella* antigenic determinants. It appears that this novel approach of transferring major surface antigen genes, from the *Shigella* serotypes that are predominant in disease, to an attenuated carrier strain may provide a system for constructing oral vaccines against many different intestinally acquired diseases.

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Cholera Toxin: Genetic Analysis, Regulation, and Role in Pathogenesis

J.J. MEKALANOS

1 Introduction

One of the most well studied and earliest recognized mechanisms of bacterial pathogenesis is the production of potent exotoxins. Protein toxins are clearly involved in the pathogenesis of a wide variety of bacterial diseases affecting mankind. These include cholera, diphtheria, pertussis, anthrax, dysentery, tetanus, gas gangrene, and opportunistic infections caused by *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes,* and *Clostridium difficile.* Even *Escherichia coli* does on occasion possess the necessary genetic information for the production of several protein toxins which can enhance its pathogenicity. Yet, as of the date of this review, a detailed understanding of the molecular events involved in the genetic regulation of any one of these bacterial toxins has not been achieved. Moreover, the specific advantage or benefit imparted to the microbe by the production of these often lethal proteins has never been clearly elucidated.

Vibrio cholerae offers an amenable genetic system in which to study regulatory mechanisms controlling toxin production. This system also offers the opportunity to define the precise role that a toxin plays in pathogenesis, because the intestine apparently provides a selective environment for bacterial cells which have the capacity to produce cholera toxin. The purpose of this chapter is

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to review what is known about the genetics of toxinogenesis in *V. cholerae* and to speculate on what is the function of the toxin in the pathogenesis of cholera and other related diarrheal diseases.

2 Cholera Toxin Structure and Activity

The structure and mode of action of cholera toxin has been largely elucidated over the past 2 decades through the efforts of researchers too numerous to acknowledge adequately here. The reader is instead referred to several excellent recent reviews on these and related topics (Moss and Vaughan 1979; Holmgren and Lonnroth 1980; Field 1980; Gilman 1984). A brief summary of the most salient features of the structure and biological activity of cholera toxin follows.

Cholera toxin is a multimeric protein composed of two types of subunits. A (MW, 27215) and B (MW, 11677), present in the holotoxin in a ratio of one to five, respectively (Gill 1976). The A subunit is initially synthesized as a single polypeptide chain which is nicked by bacterial proteases to give a pair of disulfide-linked fragments, A1 and A2 (Gill and Rappaport 1977). This proteolytic processing is required for expression of the toxin's enzymatic activity (Mekalanos et al. 1979a), which resides exclusively in the A1 fragment (Gill and King 1975). Upon reduction, the A1 fragment can catalyze several NAD-dependent reactions including the hydrolysis of NAD and ADP ribosylation of proteins and low molecular weight compounds that contain a guanidinium group side chain (Moss and Vaughan 1977; Mekalanos et al. 1979b).

The B subunits each possess a high binding affinity for the toxin's cell-surface receptor ganglioside GM1 (van Heyningen et al. 1971; Cuatrecasas 1973). Binding of the holotoxin to target cells induces a translocation of the A1 fragment through the cell membrane, where it catalyzes ADP ribosylation of Gs, a guanylnucleotide-binding protein which regulates the activity of adenylate cyclase (Cassel and Pfeuffer 1978; Gill and Meren 1978). This covalent modification results in the activation of adenylate cyclase and accumulation of cAMP in the target cells. Elevated levels of cAMP are responsible for the various secondary effects that cholera toxin induces in different types of tissue. In the case of the intestinal mucosa, high levels of cAMP bring about (by a mechanism that is not understood) changes in ion transport in villus and crypt cells (Field et al. 1968). The net effect is an increase in chloride secretion and an inhibition in sodium absorption. This in turn results in a strong osmotic driving force for loss of water from these tissues and production of the watery diarrheal syndrome observed in Asiatic cholera (Field 1980).

Like cholera toxin, certain toxins produced by several different pathogenic organisms including *Corynebacterium diphtheriae* (Honjo et al. 1968), *Pseudomonas aeruginosa* (Iglewski and Kabat 1975), *Bordetella pertussis* (Katada and Vi 1982), and *Escherichia coli* (Gill and Richardson 1980) also produce their toxic effects by catalyzing the ADP ribosylation of specific eukaryotic, GTP-binding proteins. Furthermore, in each toxin molecule the catalytically active domain is associated either covalently or noncovalently with another

portion of the molecule (the B domain) which has strong binding properties for receptors on eukaryotic cells. This biochemical homology suggests that the ADP-ribosylating exotoxins may share a common evolutionary origin (COLLIER and MEKALANOS 1980). This is apparent in the case of cholera toxin and the heat-labile enterotoxin (LT) of *E. coli* which share identical modes of action and extensive structural, antigenic, and DNA sequence homology (see below). In contrast, diphtheria toxin and *Pseudomonas* exotoxin A also share identical modes of action, yet display little if any detectable amino acid sequence homology (GRAY et al. 1984). The existence of endogenous ADP-ribosyl transferases in eukaryotic cells which have substrate specificities similar to those of the bacterial toxins has also led to the speculation that the ADP-ribosylating exotoxins may be evolutionarily related to these eukaryotic enzymes (MOSS and VAUGHAN 1983; IGLEWSKI 1984). Understanding the role that these toxins play in pathogenesis and the mechanisms by which they are regulated may help to identify other common characteristics.

3 Factors Influencing Toxin Production in the Laboratory

Early work by several laboratories established the conditions for production of cholera toxin in the laboratory (CRAIG 1966; EVANS and RICHARDSON 1968; FINKELSTEIN and LOSPALLUTO 1970; RICHARDSON 1969; CALLAHAN et al. 1971; CALLAHAN and RICHARDSON 1973). Optimal conditions are low temperature (e.g., 25°–30 °C), rich media, and high aeration. Only a small amount of toxin is produced in glucose, sucrose, or glycerol minimal media, but the addition of certain amino acids (e.g., asparagine, arginine, and serine) stimulate production of toxin. Recently, SAGAR et al. (1979 and 1981) have proposed that phosphate and trace elements may regulate toxin production by affecting the uptake of these amino acids.

In general all these regulatory studies are difficult to evaluate because different strains of *V. cholerae* and methods of toxin assay were used by these various workers. For example, strains of *V. cholerae* can vary as much as three orders of magnitude in their level of toxin production in a given laboratory medium. There is also evidence that media composition, culture conditions, and strain backgroud can affect the extracellular secretion of the toxin (CALLAHAN et al. 1971; J. CRAIG, personal communication). Furthermore, the quantitation of toxin produced can be influenced by factors such as protease production (which can affect toxin processing and stability) or neuraminidase production (which can alter the GM1 receptor density and therefore the sensitivity to toxin of various assay systems).

However, the idea that cholera toxin production may be regulated by some nutritional factor is a good hypothesis and parallels observations made on the regulation of two other ADP-ribosylating exotoxins. Diphtheria toxin (MURPHY et al. 1978) and *Pseudomonas* exotoxin A (BJORN et al. 1978) are both regulated by the concentration of iron in the growth media. In the case of diphtheria toxin, this control appears to be at the transcriptional level (MURPHY et al.

1978). Analysis of the nutritional factors which control cholera toxin production will probably be best examined after the molecular components that mediate toxin regulation have been defined. At least two of these components, the *ctx* promoter and the *toxR* gene, have been identified and show structural variations among different strains of *V. cholerae* (see below).

4 Genetic Analysis of Toxinogenesis: Early Studies

Isolation of *V. cholerae* mutants altered in toxin production has provided another means of investigating cholera toxin regulation. These studies were largely prompted by the possibility of developing attenuated mutants of *V. cholerae* suitable for use as live, oral cholera vaccines. Most of this work was done with the highly toxinogenic 569B strain of *V. cholerae* isolated after passage in infant rabbits by DUTTA and HABBU (1955). While mutations in the toxin structural genes would have been most appropriate for vaccine development, all of these genetic studies yielded regulatory mutations.

HOWARD (1971) reported the use of the rabbit skin test (CRAIG 1966) to isolate nontoxinogenic mutants of strain 569B after mutagenesis with nitrosoguanidine (NTG). The mutants were avirulent in the rabbit intestinal loop model (i.e., unable to induce a secretory response) but apparently did not survive or multiply in the intestinal environment. These mutant strains were not characterized further.

FINKELSTEIN et al. (1974) employed an immunoprecipitation assay and NTG mutagenesis to isolate nontoxinogenic mutants of 569B. Loss of toxin production in these mutants was correlated with inability to induce a fluid secretion response in infant rabbits. These workers also noted that the ability of various mutants to grow in the intestinal environment seemed to correlate with the mutant's capacity to induce a residual secretory response in vivo (HOLMES et al. 1975). Some of these mutants were reported to be unstable on intestinal passage in animals; but one mutant, M13, was stably nontoxinogenic. This mutant was later shown to be hypotoxinogenic, producing 1000th the amount of toxin that the parental strain produces (HOLMES et al. 1978). M13 was tested in human volunteers as a possible vaccine strain but was found to be unstable, producing toxinogenic revertants during intestinal growth (WOODWARD et al. 1975).

MEKALANOS et al. (1978) reported the isolation of NTG-induced hypotoxinogenic and hypertoxinogenic mutants of strain 569B using a ganglioside binding-dependent immunoassay. Several of these hypotoxinogenic strains were noted to be unstable in the rabbit intestinal loop model; during passage they produced toxinogenic revertants which eventually displaced the mutant strain in vivo.

BASELSKI et al. (1978, 1979) in a exhaustive study isolated a variety of different avirulent mutants of *V. cholerae* including toxin-deficient mutants. Characterization of the toxin-deficient mutants in the infant mouse model suggested that these mutants showed enhanced killing and mechanical clearance in the intestinal environment compared with the toxinogenic parental strain CA401.

RUCH et al. (1978) and NICHOLS et al. (1979) also reported the isolation of hypotoxinogenic mutants of strain 569B after growth at 42 °C and NTG mutagenesis. One of these mutants (*toxTI-101N3*) was found to be unstable in the intestine of germ-free rats, giving rise to toxinogenic revertants which eventually displaced the mutant strain in vivo (J. NICHOLS, J. MURPHY, and J. MEKALANOS, unpublished observation).

Two conclusions became clear from these studies. First, unstable nontoxinogenic and hypotoxinogenic mutants of strain 569B could be reverted to toxinogenicity by intraintestinal passage in animals or humans. Second, stable mutations in the toxin structural genes of strain 569B were not readily obtainable. The explanation for the latter observation came with the recognition that strain 569B carried multiple copies of the toxin structural genes (MOSELEY and FALKOW 1980).

The molecular cloning of the heat-labile enterotoxin (LT) genes of *E. coli* by So et al. (1978) and their subsequent analysis by DALLAS and FALKOW (1979) clearly demonstrated the close structural similarity between LT and cholera toxin. Deletion mapping and DNA sequencing of the LT cistrons further identified the location of the coding sequences of the LT-A and LT-B subunits (DALLAS et al. 1979; DALLAS and FALKOW 1980; SPICER and NOBEL 1982). MOSELEY and FALKOW (1980) then showed that hybridization probes composed largely of the LT-coding sequence were homologous to *V. cholerae* DNA fragments in a Southern blot analysis. Because strain 569B displayed multiple fragments which hybridized to both the LT-A and LT-B, these investigators concluded that this strain of *V. cholerae* probably carried multiple copies of the cholera toxin structural genes. This prediction was proven correct through the cloning of the two cholera toxin operons carried by strain 569B (see below).

The probable existence of multiple toxin gene copies in 569B as well as other considerations prompted several investigators to attempt the isolation of toxin structural gene mutations in the El Tor biotype of *V. cholerae*. After two rounds of NTG mutagenesis, HONDA and FINKELSTEIN (1979) succeeded in isolating a mutant of the El Tor strain 3083 that produced only the B subunit of cholera toxin. This mutant was called Texas Star-SR and may represent the first *V. cholerae* strain isolated with a mutation in one of the cholera toxin structural genes. However, because the strain was subjected to heavy mutagenesis, other phenotypic alterations unrelated to toxin production that are seen in this mutant are probably the result of secondary mutations. The reduced capacity of Texas Star-SR to grow intraintestinally in infant rabbits (HONDA and FINKELSTEIN 1979) and adult rabbits (TOKUNAGA et al. 1984) cannot be reliably attributed to its toxin-related genetic lesion because the mutant grows more slowly than the parental strain in the laboratory.

MEKALANOS et al. (1982) employed the mutagenic properties of vibriophages VcA1 (JOHNSON et al. 1981) and VcA2cts1 to isolate nontoxinogenic mutants of the El Tor strain RV79. Southern blot analysis utilizing the LT-A and LT-B probes of MOSELEY and FALKOW (1980) indicated that these nontoxinogenic mutants lacked sequences homologous to the LT structural genes. Therefore, these *V. cholerae* strains were deleted for the cholera toxin structural genes and they represented the first definitive examples of such mutant strains. These

mutants were found to grow within ligated intestinal loops of rabbits but attained cell densities that were on average two- to sixfold lower than the parental strain. These observations once again did suggest that the toxin might play a beneficial role in the growth and/or survival of *V. cholerae* in vivo. Although these mutants were good candidates for testing as live oral cholera vaccine strains, recombinant DNA technology offered the possibility of constructing more precise mutations in the toxin genes.

5 Molecular Cloning of the *ctxAB* Operon and the Construction of Nontoxinogenic Mutants

Before 1981 it was prohibited by the Guidelines for Recombinant DNA Research to clone the genes encoding cholera toxin. Recognition of the structural and functional homology between the LT and cholera toxin was one of several reasons why the Recombinant DNA Advisory Committee voted to allow the cloning of the cholera toxin genes at the P1 level of physical containment. Within a few months of this vote one group reported the cloning of a *V. cholerae* DNA fragment which showed homology to the LT gene probes (KAPER and LEVINE 1981) while two other groups showed that other LT-homologous fragments cloned from a classical and an EI Tor strain encoded the production of biologically active cholera toxin in *E. coli* (PEARSON and MEKALANOS 1982; GENNARO et al. 1982).

PEARSON and MEKALANOS noted four observations about the expression of cholera toxin in *E. coli*. First, the amount of toxin made by *E. coli* was about 100-fold less than that produced by the donor *V. cholerae* strain (569B). Second, the specific activity of the toxin, in terms of S49 tissue culture toxicity versus toxin antigen, was over 200-fold less than the toxin purified from *V. cholerae*. This result was shown to be due to the lack of proteolytic processing (nicking) of the A subunit synthesized in *E. coli*. Third, a frameshift mutation introduced early in the A subunit gene caused a reduction in the amount of B subunit produced, suggesting that the A and B subunit genes were arranged in a single transcriptional unit (the *ctxAB* operon; see below). Finally, the cholera toxin produced by *E. coli* was found to be cell associated rather than extracellularly secreted as it is when produced by most *V. cholerae* strains. A similar result was reported by GENNARO et al. (1982), who concluded that cholera toxin made in *E. coli* was located in the periplasm of these cells. Evidence suggests that the extracellular secretion of cholera toxin is a property of *V. cholerae* that can be altered in some mutants (HOLMES et al. 1975; NEILL et al. 1983).

The molecular cloning of the cholera toxin operon allowed investigators to delete specifically the genes encoding the toxin or one of its subunits without using any form of chemical mutagenesis on the organism. MEKALANOS et al. (1983) did this by first constructing in vitro deletions in the cloned toxin structural genes, *ctxA* and *ctxB*, and then incorporating these defined mutations into *V. cholerae* by an in vivo marker exchange procedure. Initially, a DNA fragment

encoding kanamycin resistance was inserted between two restriction endonuclease sites known to lie within the *ctxA* and *ctxB* genes. The marker exchange procedure of RUVKUN and AUSUBEL (1981) was then used to recombine this construction into the chromosome of *V. cholerae* in place of the resident copies of the *ctxA* and *ctxB* genes. A second marker exchange step with a plasmid containing an internal deletion in *ctxA* (generated with nuclease Bal31) then replaced the kanamycin-resistant construction to give the strain if *V. cholerae* deleted only for *ctxA*. The parental strain used in this procedure was Ogawa 395, a virulent classical strain; the resultant A-B− and A-B+ derivatives were called 0395-NT and 0395-N1, respectively. Similar mutants of Ogawa 395 have also been constructed by KASPER et al. (1984a). The construction of mutants such as 0395-NT and 0395-N1 would have been essentially impossible by conventional chemical mutagenesis because Ogawa 395 contains two copies of the *ctxAB* operon (see below). The characterization of these mutant strains in laboratory animals is discussed in the last section of the review.

Introduction of *ctxAB* deletion mutations into El Tor strains of *V. cholerae* has also been accomplished by methods similar to those described above (SPORECKE et al. 1984; KAPER et al. 1984b). It is important to note that these methods of mutant isolation are limited in their specificity by the precision used in the in vitro construction step. For example, KAPER et al. (1984b) concluded that JBK70, a *ctxAB* deletion mutant they had constructed, retained all other antigens important to immunity. Close examination of their construction and knowledge of the nucleotide sequence adjacent to the cholera toxin operon (MEKALANOS et al. 1983) shows that the *AccI* sites used in the generation of their *ctxAB* deletion mutation lie outside the toxin structural genes and therefore may affect the expression of gene products other than the toxin. Indeed, one of these *AccI* sites lies within a large open reading frame located upstream of the toxin operon (J. MEKALANOS, unpublished results).

However, in general, the cloning of the toxin structural genes has allowed the construction of precise mutations in the *ctxAB* operon which will eventually find utility in the study of cholera pathogenesis and in the development of more effective cholera vaccines. The use of genetically engineered *ctx* deletion mutant strains as living cholera vaccines seems certain in light of the encouraging results seen with chemically induced mutants such as Texas Star-SR when tested in human volunteers (LEVINE et al. 1984)

6 Genetic Mapping of *ctx* Locus in *Vibrio cholerae*

Early work by VASIL et al. (1975) established that a locus called *tox* mapped in the *his* region of the *V. cholerae* chromosome. This locus turned out not to be the toxin structural genes but rather a regulatory gene controlling toxin expression (BAINE et al. 1978) (see below). The availability of *ctx* deletion mutations and methods for the construction of site-specific mutations in the *ctx* operon has made possible the unambiguous genetic mapping of *ctx* in *V. cholerae*.

SPORECKE et al. (1984) used a combination of VcA1-facilitated recombination and three-factor conjugative crosses to map the *ctx* locus of the El Tor strain RV79. Two types of mutant *ctx* alleles were used: a total deletion of the *ctx* region and an in vitro-constructed *ctxA* deletion marked with an insertion encoding kanamycin resistance. The data obtained placed the *ctx* locus between the *his* and *nal* markers on the *V. cholerae* map. Heterologous crosses between RV79 and the classical strain 569B established that one of the two *ctx* copies of 569B also maps at this position. However, these heterologous crosses were subject to linkage artifacts probably due to chromosomal inversions between the two strains.

Conflicting genetic mapping data were reported by SAUNDERS et al. (1982). These investigators used an antigenic variation between the toxins of El Tor strains RJ1 (same RV79) and 3083 to map a locus called *vct* between *met* and *trp* on the *V. cholerae* chromosome. The possibility exists that *vct* and *ctx* are the same loci, because the overall gene order in the region is *met trp nal ctx his tox* (SPORECKE et al. 1984). Evidence that *vct* recombinants are altered in toxin gene structure supports this possibility (SAUNDERS et al. 1983). However, SAUNDERS et al. (1982) used heterologous strains in their crosses and scored their recombinants by a method that was subject to interference by toxin regulatory mutations. Thus, the map order of SPORECKE et al. (1984) is more likely to be correct.

7 Nucleotide Sequence of the *ctxAB* Operon

The complete nucleotide sequence of a *ctxAB* operon derived from the El Tor strain 2125 has been reported (MEKALANOS et al. 1983). Partial sequences for *ctx* operon copies cloned from the El Tor strains 1621 (GENNARO and GREENAWAY 1983), 62746 (LOCKMAN and KAPER 1983), RV79, and E7946 (MEKALANOS et al. 1983) have been determined as well as partial sequences for both of the *ctx* operon copies present in the classical strain 569B (MEKALANOS et al. 1983). The complete amino acid sequence of the cholera toxin B subunit (purified from strain 569B) has been reported (LAI 1977; KUROSKY et al. 1977) and varies from nucleotide sequence of the 2125 B subunit at four residues. Strain 569B carries two copies of the *ctxAB* operon but the available nucleotide sequence data suggest that these are identical in sequence (MEKALANOS et al. 1983). However, the minor differences in the amino acid sequences of the 569B toxin subunits and the deduced sequences of the El Tor toxin subunits are probably real. The variation of the toxin gene sequences between the two biotypes may simply be due to genetic drift or alternatively may represent antigenic variation due to immunological selective pressure.

Comparison of the nucleotide sequence of *eltAB* (encoding *E. coli* LT; DALLAS et al. 1979) with that of *ctxAB* confirms the close evolutionary relationship between structural genes of LT and cholera toxin (MEKALANOS et al. 1983). Overall homology is about 76% at the nucleotide sequence level and 77% at the amino acid sequence level. In regard to regions upstream of the structural

genes, there is the conspicuous absence of any significant homology between these toxin operons (MEKALANOS et al. 1983). This simply reflects the fact that the *ctx* promoter must interact with regulatory elements unique to *V. cholerae* while the LT promoter may be broadly active in different gram-negative genera. Indeed, the *elt* promoter is very active in *V. cholerae* while the *ctx* promoter is only weakly active in *E. coli* (NEILL et al. 1983; MEKALANOS and PEARSON 1982).

The nucleotide sequence of the *ctxAB* operon has also provided information regarding translational control in *ctx* expression and the mechanism of toxin secretion. The *ctxA* and *ctxB* genes each encode separate hydrophobic signal sequences presumably involved in the secretion of the toxin subunit polypeptide chains through the bacterial cell membrane (GENNARO and GREENWAY 1983; LOCKMAN and KAPER 1983; MEKALANOS et al. 1983). The two subunit genes also possess their own ribosome-binding sites, each consisting of a Shine-Dalgarno (SD) sequence and ATG initiation codon. The SD sequence of *ctxB* appears to be larger than that of *ctxA* (TAAGGA versus GGAG) and this may be involved in determining the 1:7 ratio of *ctxA* to *ctxB* expression (MEKALANOS et al. 1983). The properties of a genetic fusion of *ctxA* to *ctxB* support this prediction since nine times less B subunit is synthesized when its translational signals are derived from *ctxA* (MEKALANOS et al. 1983). A one-base-pair overlap in the *ctxA* termination codon TGA and the *ctxB* initiation codon ATG may also be involved in the translational coupling of *ctxB* expression to *ctxA*, although this is probably not the case because *ctxB* expression can still be very high in the absence of *ctxA* translational initiation signals (J. MEKALANOS, unpublished results).

8 Duplication and Amplification of the *ctx* Genetic Element

The first indication that *V. cholerae* strains might carry multiple copies of the cholera toxin structural genes came from the observations of MOSELEY and FALKOW (1980), who noted that multiple bands appeared in a Southern blot analysis of *V. cholerae* strains of classical biotype when their DNA was hybridized to either the LT-A or LT-B gene probes. In contrast, the El Tor strains examined showed only a single band hybridizing to the LT probes. They concluded that the two toxin subunit genes were close together but that the copy number of the toxin genes and adjacent sequence environment varies among strains of *V. cholerae*. The molecular cloning of multiple copies of the *ctxAB* operon from both classical and El Tor strains proved that *V. cholerae* strains of both biotypes could carry more than one copy of the toxin operon (PEARSON and MEKALANOS 1982; MEKALANOS et al. 1983; MEKALANOS 1983).

Structural analysis of cloned copies of the *ctxAB* operon and their adjacent sequences revealed that about 5–6 kilobase pairs of DNA upstream of all toxin operon copies thus far examined are similar in structure (MEKALANOS et al. 1983; MEKALANOS 1983). This is remarkable in light of the high degree of variation in *ctx* DNA structure that has been observed in Southern blot analyses

of *V. cholerae* chromosomal DNA (MOSELEY and FALKOW 1980; KAPER et al. 1981; PEARSON and MEKALANOS 1982; MEKALANOS 1983). These and other observations described below lead MEKALANOS (1983) to propose that these upstream conserved sequences are part of a larger genetic element responsible for *ctx* duplication and rearrangement events.

Detailed Southern blot analysis has indicated that all *V. cholerae* strains of the classical biotype carry two copies of the *ctxAB* operon which are apparently widely separated on the *V. cholerae* chromosome (MEKALANOS 1983). These two *ctx* operon copies are located at the same two chromosomal sites in the 14 different classical strains that were examined. Both of these *ctx* copies have been cloned from strain 569B (MEKALANOS et al. 1983). Comparison of the restriction maps of the two cloned inserts shows that, in contrast to the upstream DNA, the DNA downstream of *ctxB* is different for the two *ctx* copies. This result suggests that in 569B one end of the genetic element encoding *ctxAB* is located within a few hundred base pairs of the end of *ctxB*.

El Tor strains of *V. cholerae* can also carry multiple copies of the *ctx* genetic element (MEKALANOS 1983). About 70% of the El Tor strains that were examined carried only a single *ctx* copy while the remaining had two or more *ctx* copies arranged on large tandem repeats which were either 7 or 9.7 kilobase pairs in length.

The difference in size between these two types of tandem repeats was found to be due to a variation in the copy number of a smaller tandemly repeated sequence. This 2.7-kb sequence was called RS1 (repetitive sequence-1) RS1 is always found one or two times at the novel joints of the large *ctx* tandem duplications found in El Tor strains. Copies of RS1 can also be found approximately 3.2 kb upstream of *ctx* and also immediately downstream of the B cistron. However, there is considerable variation in the copy number of RS1 in both locations with the downstream site found to be unoccupied in *ctx* copies derived from all classical strains as well as a few El Tor strains (MEKALANOS 1983).

Figure 1 shows a schematic representation of the structures that have been observed in the *ctx* regions of several *V. cholerae* strains. The RS1 repeat is shown as arrows in this diagram. Because RS1 contains an asymmetrically located *BglII* site, it is possible to infer what the RS1 structure is around several additional cloned *ctxAB* copies for which only restriction map data have been reported (GENNARO et al. 1982; KAPER et al. 1984a). These comparisons show that the *ctx* genetic element is variable in size and structure depending upon the copy number and location of the RS1 repeats. If the RS1 repeats are actually insertion sequences, then it is also apparent that transposon-like elements carrying *ctx* probably exist in a variety of *V. cholerae* strains (MEKALANOS 1983).

If the *ctx* genetic element is capable of transposition, then this may provide a genetic mechanism for the introduction of the *ctx* genes into other strains of *V. cholerae* that initially lacked the toxin structural genes. KAPER et al. (1981) reported that naturally occurring strains of *V. cholerae* isolated from the environment frequently lack DNA sequences homologous to the LT gene probes and therefore are *ctxAB* negative. Other data support the conclusion that some of these nontoxinogenic strains are clonally related to toxinogenic strains iso-

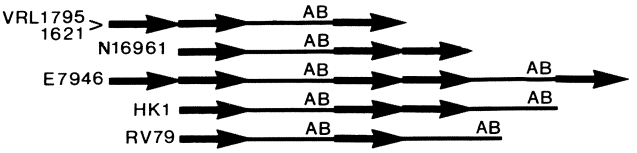

Fig. 1. RS1 structure adjacent to *ctxAB* copies of selected El Tor strains of *V. cholerae*. Copies of RS1 are represented by *arrows*. Structures were determined for strains E7946, RV79, and VRL1796 by cloning and Southern blot analysis (MEKALANOS 1983). Structures for strains 1621 and N16961 were inferred from published restriction maps of cloned *V. cholerae* DNA fragments. (GENNARO et al. 1982; KAPER et al. 1984a)

lated from a similar geographical location (GOLDBERG and MURPHY 1983). Recently, MILLER and MEKALANOS (1984) showed that these nontoxinogenic strains of *V. cholerae* also lack sequences homologous to DNA probes known to define regions of the *ctx* genetic element that are upstream from the toxin operon. Thus, these nontoxinogenic strains probably lack the entire *ctx* genetic element including RS1 sequences. These data support the possibility that the *ctx* genetic element is involved in the conversion of these environmental nontoxinogenic strains of *V. cholerae* to the toxinogenic state.

Recently, additional support for this hypothesis has been obtained. We have observed that conjugative transfer of the *ctx* genetic element from toxinogenic strains to these nontoxinogenic strains is possible and occurs by a mechanism which may involve illegitimate recombination (GOLDBERG and MEKALANOS 1985a). Namely, the site of insertion of the *ctx* genetic element in the chromosome of the nontoxinogenic strain is the same as that of the toxinogenic donor strain however, the *ctx* genetic element is tandemly duplicated at this new chromosomal insertion site even though the donor strain in this experiment contains only a single copy of trhe *ctx* genetic element. The structure of the *ctx* tandem duplication seen in these genetic recombinants appears to be the same as that seen in several toxinogenic clinical isolates. These data suggest that tandem duplications of the *ctx* genetic element may be intermediates in the transposition of this element by a mechanism that may involve some type of illegitimate or site-specific recombinational event.

The existence of *ctx* tandem duplications in El Tor strains of *V. cholerae* suggests that gene amplification might play a role in the regulation of cholera toxin expression. Accordingly, MEKALANOS (1983) tested the possibility that amplification of the toxin region occurred in hypertoxinogenic variants of *V. cholerae* selected during intestinal passage in rabbits. Significant amplification was observed, with some variants acquiring as many as six tandem copies of the *ctx* region. This increase in *ctx* copy number is probably responsible for the two- to fourfold increase in toxin production seen in the in vivo-selected variants. The nature of the selective pressure causing this enrichment of amplified, hypertoxinogenic variants in vivo is unknown but is probably related to the in vivo selection process that was involved in the reversion of hypotoxinogenic mutants observed in earlier animal studies (HOLMES et al. 1975; MEKALANOS et al. 1978) (see below).

Copy number of the *ctx* region can be shown experimentally to affect the level of toxin production by *V. cholerae*. SPORECKE et al. (1984) isolated an insertion mutation of vibriophage VcA1 into one of two *ctxAB* copies present in the El Tor strain RV79. The mutant strain, DC24, was found to produce about one-half the amount of toxin that the parental strain produced (I. SPORECKE, D. CASTRO, and J. MEKALANOS, unpublished observation). Introduction of multicopy plasmids carrying *ctxB* (fused to the *ctx* promoter) into strains of *V. cholerae* deleted for their chromosomal toxin genes results in levels of B subunit production which are 20–100 times higher than the same strain carrying as single copy of *ctxB* (MEKALANOS et al. 1983; J. MEKALANOS, unpublished results). These results support the conclusion that the copy number of the *ctxAB* operon has an effect on toxin production and therefore are consistent with the idea that gene amplification plays a role in the regulation of toxin expression in *V. cholerae*.

Gene amplification has been observed in other prokaryotic systems (ROWND et al. 1975; YAGI and CLEWELL 1976; ANDERSON and ROTH 1977; EDLUND et al. 1979). In these other systems the amplification events occur by unequal crossing over between directly repeated sequences and are consequently *recA* dependent. The structures of the *ctx* tandem duplications suggest that unequal crossing over between directly repeated copies of RS1 may be responsible for this phenomenon in *V. cholerae* (MEKALANOS 1983). We have recently shown that amplification of the *ctx* genetic element is also dependent on the activity of the *V. cholerae recA* gene (GOLDBERG and MEKALANOS 1985b).

To do this we first developed a system for studying *ctx* amplification events in the laboratory without the use of an animal model. This was done by "tagging" the *ctx* genes with a selectable marker (i.e., kanamycin resistance), which was then used to select for amplification events by virtue of their phenotype of high-level resistance to kanamycin. Amplification to as high as 20 copies of the *ctx* genetic element can easily be obtained by this procedure. We then constructed a *recA* mutant of *V. cholerae* essentially by the procedure of BETTER and HELINSKI (1983), which involves the cloning and subsequent inactivation of a foreign *recA* gene in *E. coli*. After introduction of the mutant *recA* gene into *V. cholerae,* we found that amplification of the *ctx* genetic element could no longer be detected (GOLDBERG and MEKALANOS 1985b). Therefore, the *recA* gene of *V. cholerae* also plays a role in toxin expression via its essential involvement in the amplification of the *ctx* genetic element.

9 Positive Control in *ctxAB* Transcription

While translational control and variation in copy number of the *ctxAB* are important in the control of toxin expression in *V. cholerae,* there are clearly other regulatory mechanisms involved. Until recently, the only mutations that were known to affect cholera toxin production were in regulatory genes. This was in part due to the use of strain 569B in most of these genetic studies.

Because 569B carries two copies of the *ctxAB* operon, the only mutations that reduced the level of toxin production by this strain were necessarily in genes that encode products required for the expression of both toxin operon copies.

Mutations in a locus called *tox* cause a 1000-fold decrease in toxin production in strain 569B (BAINE et al. 1978). The prototype of this type of mutation is the *tox-2* allele carried by mutant M13 (FINKELSTEIN et al. 1974; HOLMES et al. 1975). This mutant was tested in human volunteers as a possible live oral vaccine and was found not to induce a diarrheal syndrome. This result argues that the *tox* gene product plays an important role in the regulation of cholera toxin in vivo (i.e., in the intestinal environment).

MILLER and MEKALANOS (1984) showed in strain 569B and several *tox* mutant strains by Northern blot analysis that *tox* mutations drastically decrease the level of *ctx* mRNA. This suggests that *tox* mutations act at the transcription level and that the *tox* locus might encode a trans-acting regulatory element required for the transcriptional activation of both copies of the *ctx* operon in strain 569B.

Both of these *ctx* copies have been cloned and were found to express about 100-fold less cholera toxin in *E. coli* than in *V. cholerae* 569B (PEARSON and MEKALANOS 1982; MEKALANOS et al. 1983). One explanation for this result is that *E. coli* lacks the regulatory gene corresponding to the *tox* locus. Accordingly, MILLER and MEKALANOS (1984) were able to clone the *tox* locus by screening for increased *ctx* transcriptional activity in *E. coli*. They did this by constructing a genetic fusion of the *ctx* promoter region to the gene for β-galactosidase (*lacZ*) and then moved this genetic construction onto the *E. coli* chromosome. Thus, the activity of the *ctx* promoter could be followed conveniently with this strain by using lactose indicator media or chromogenic substrates of β-galactosidase. Introduction of a plasmid-based, *V. cholerae* 569B genomic library to this *E. coli* indicator strain allowed these investigators to screen for recombinant plasmids which encoded factors that activate the transcription of the *ctx* promoter/*lacZ* fusion (Fig. 2). Using this procedure they were able to clone a gene called *toxR* which can increase the expression of *ctx* over 100-fold in *E. coli* (MILLER and MEKALANOS 1984). The cloned *toxR* gene was shown to complement the defect present in several independently isolated hypotoxinogenic mutants of strain 569B, increasing the toxin production by these strains at least 300- to 1000-fold. Thus, *tox* mutants such as M13 (FINKELSTEIN et al. 1974) almost certainly have mutations in the *toxR* gene.

Southern blot analysis with a probe derived from the cloned *toxR* gene demonstrated that all other *V. cholerae* strains tested carry DNA sequences homologous to the *toxR* gene (MILLER and MEKALANOS 1984). These included both classical and El Tor biotypes as well as environmental nontoxinogenic strains of *V. cholerae*. The latter group is of particular interest because these strains of *V. cholerae* lack the toxin structural genes (KAPER et al. 1981) and sequences associated with the *ctx* genetic element (MILLER and MEKALANOS 1984).

Additional genetic analyses indicate that these *toxR* homologous sequences are indeed active. We have observed that in contrast to all other *V. cholerae* strains examined strain 569B carries a small 1.2-kb pair deletion downstream

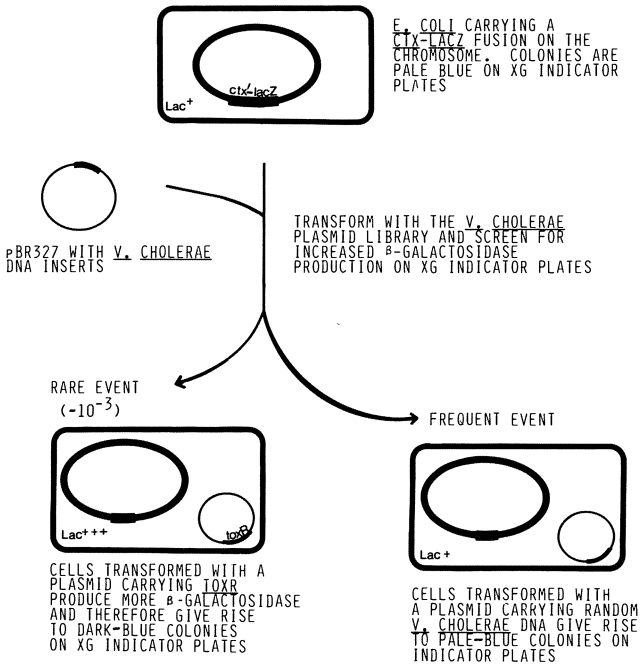

Fig. 2. Method used to clone the *V. cholerae*-positive regulatory gene *toxR*. *ctx'-lacZ*, the *ctx* promoter fused to the *β*-galactosidase gene; *XG*, 5-bromo-4-chloro-3-indolyl-D-galactoside

of the *toxR* gene (MILLER and MEKALANOS, to be published). This deletion provides a structural polymorphism with which to follow the *toxR* region in genetic crosses between 569B *tox* mutants and other strains of *V. cholerae*. It was found that the El Tor strain RV79 can donate to these *tox* mutants in conjugative crosses a gene that both reverses their mutant phenotype and shows the same genetic linkage properties as the *tox* locus of 569B (MILLER and MEKALANOS, to be published). Southern blot analysis showed that these genetic recombinants had received the *toxR* homologous sequences of RV79, thus proving that these sequences are active. The 569B recombinants that had received the RV79 *toxR* sequences produced about tenfold less toxin than 569B, suggesting that the deletion mutation near the *toxR* locus of 569B might also have a positive effect on toxin expression. Whether this deletion alters the structure of the *toxR* gene product or another gene involved in the regulation of *toxR* or *ctxAB* expression is unknown.

Differences in the *toxR* locus do not account for all the variation in toxin production seen with different strains of *V. cholerae*. There is evidence that these strain differences are due in part to a sequence variation in the *ctx* promoter region. MEKALANOS et al. (1983) reported that the only difference found in the 5′ sequence upstream of five *ctxAB* operon copies cloned from four different *V. cholerae* strains was a variation in the copy number of a 7-bp sequence,

T-T-T-T-G-A-T. This sequence was found tandemly repeated three to eight times 77 bp upstream of the start of *ctxA*. Both *ctx* promoter copies of 569B had eight of these small tandem repeats, while the two *ctx* promoters from RV79 each had only three copies of the small repeat. MILLER and MEKALANOS (1984) obtained evidence that the repeats were important in *ctx* expression by assaying the toxin production of strain SM201 (SPORECKE et al. 1984). This strain has the two *ctxAB* copies of RV79 replaced by a single copy of the *ctxB* gene and *ctx* promoter of strain 569B. Because SM201 produced about tenfold more toxin than RV79, it was concluded that the *ctx* promoter of 569B was 20 times more active than the *ctx* promoter of RV79. This suggests that the copy number of tandem repeats may have an effect on the activity of the toxin promoter.

Additional support for this conclusion has come from a deletion analysis of the *ctx* promoter region that has shown that the two most important structural features of this promoter's ability to be activated by the *toxR* gene are the −10 or Pribnow box and the repetitive region (R. TAYLOR, V. MILLER, and J. MEKALANOS, unpublished results). Given the position of the repetitive region relative to the start of *ctx* transcription, these results suggest that the *toxR* gene product activates the *ctx* promoter by binding to the repetitive region.

DNA sequencing, maxicell analysis, and site-specific mutagenesis have revealed the nature of the *toxR* gene product (V. MILLER, R. TAYLOR, and J. MEKALANOS, unpublished results). The *toxR* gene contains an open reading frame large enough to encode a 34-kilodalton protein. Introduction of deletion mutations in this open reading frame eliminates the ability of the *toxR* gene to activate *ctx* expression. A protein of approximately 34 kilodaltons is produced by maxicells carrying the wild-type *toxR* plasmid but not by maxicells carrying plasmids which have mutations in the open reading frame. These data leave little doubt that the *toxR* gene product is the 34-kilodalton protein.

Activation of the *ctx* promoter probably occurs by binding of the *toxR*-encoded protein to one or more of the 7-bp repetitive sequences. This binding may be cooperative, thus explaining the influence of repeat copy number on activity of the toxin promoter. The molecular mechanism by which binding of the *toxR* protein to the repetitive region activates *ctx* gene expression is unknown but may involve either protein-protein interactions with RNA polymerase or a conformational change in the *ctx* promoter DNA induced by the binding of the *toxR* protein to the repetitive sequence.

In addition to positive control mediated by *toxR*, there is also evidence of other regulatory loci influencing cholera toxin expression. Hypertoxinogenic mutants of strain 569B have mutations in a locus called *htx* (MEKALANOS et al. 1979c; MEKALANOS and MURPHY 1980). These mutations cause a three- to sevenfold increase in toxin production by strain 569B and map between the *str* and *rif* loci of *V. cholerae*. Mutations in a locus called *ltx* cause an opposite effect – a 100-fold decrease in toxin production. These mutations may represent different allelic states of the same toxin regulatory locus, because *htx* and *ltx* mutations map in the same location and can both be isolated at an increased frequency after NTG-induced, *rif* comutagenesis (MEKALANOS and MURPHY

1980). The phenotypes of these mutations suggest that the *htx*/*ltx* locus encodes a negative control element involved in *ctx* expression. If so, then *htx* alleles might represent null mutations in this repressor-like element while *ltx* mutations might alter the response of the repressor toward inducers of toxin synthesis (MEKALANOS and MURPHY 1980).

Strains carrying *htx* mutations grow more slowly than the parental strain and are consequently unstable. Faster-growing clones are either revertants of *htx* or carry suppressor mutations unlinked to *htx* (MEKALANOS and MURPHY 1980). Recently, we have shown that these second-site mutations are probably in the *toxR* gene (MILLER and MEKALANOS, to be published). Given that *htx* mutations do not affect the hypotoxinogenic phenotype of the *tox* mutant strain M13 (MEKALANOS and MURPHY 1980), these results indicate that *toxR* mutations are dominant over *htx* mutations. Therefore, if the *htx* gene product is a repressor-like factor it may affect either *ctx* or *toxR* expression.

10 Speculation on the Role of Cholera Toxin in Pathogenesis

The pathogenesis of cholera is remarkably complex, involving the correct interaction of both bacterial and host factors for a productive infection to result. Orally ingested *V. cholerae* must survive the acid environment of the stomach and reach the upper bowel in sufficient numbers to colonize. Peristalsis and the flow of mucus secretions act to wash out these bacterial cells before they have a chance to multiply in the intestinal lumen or reach their ultimate attachment sites along the brush border of the intestinal epithelium. *V. cholerae* resists these mechanical clearance mechanisms by using motility (GUENTZEL and BERRY 1975) and chemotaxis (FRETER et al. 1981) to direct its swimming through the mucus gel and deep within the intervillous spaces and crypts. Production of protease, mucinase, neuraminidase, and other degradative enzymes probably aids in this penetration process or at least provides substrates for growth during this stage. Eventually the cells reach the epithelial cell surface, where adherence occurs via an attachment mechanism that probably involves surface pili and lectins. Multiplication continues at an accelerated rate (FRETER et al. 1981), but the nature of the substrates being used and other nutritional properties of this environment (i.e., the availability of oxygen, phosphate, and trace elements) are unkown. At some point during this process the production of cholera toxin commences. The secretory diarrhea induced by the toxin further changes the physical environment around the vibrios, perhaps aiding in their eventual release from the tissue surfaces and excretion from the host.

However, do we really understand the role that cholera toxin plays in the pathogenesis of cholera and related diarrheal diseases? Is its job simply to cause diarrhea and in this way disseminate the organism to its next victim (FINKELSTEIN 1973) or could the toxin be providing another function? The in vivo characterization of toxin-deficient mutants of *V. cholerae* suggests that this may well be the case.

Hypotoxinogenic mutants do not survive and multiply in the intestine as well as wild-type strains (HOWARD 1971; HOLMES et al. 1975; BASELSKI et al. 1979). Furthermore, revertants of hypotoxinogenic mutants can be selected by intestinal passage in rabbits (HOLMES et al. 1975; MEKALANOS et al. 1977). One can conclude that these hypotoxinogenic mutants probably represent *toxR* mutants because the parental strains used in these studies (569B and CA401) were classical in biotype and therefore have two copies of the *ctxAB* operon (MEKALANOS 1983). It is possible that *toxR* may regulate the production of other *V. cholerae* virulence factors besides the toxin inasmuch as *toxR* is a regulatory gene present even in nontoxinogenic strains of *V. cholerae* (MILLER and MEKALANOS 1984). Thus, the in vivo defect observed in these early hypotoxinogenic mutants may reflect the cumulative effect of losing more than just cholera toxin production.

The Texas Star-SR mutant of HONDA and FINKELSTEIN (1979) and the *ctx* deletion mutants described by MEKALANOS et al. (1982) also showed a detectable reduction in their ability to multiply in vivo (TOKUNAGA et al. 1984). However, both Texas Star-SR and the phage-induced *ctx* deletion mutants have secondary mutations that may be responsible for this result.

The characterization in vivo of site-specific *ctx* mutants constructed by in vitro recombinant DNA methods has provided the most convincing evidence that the toxin is beneficial to growth in the intestinal environment. Recently it has been shown that strains 0395-NT and 0395-N1 (A-B− and A-B+ mutants, respectively, of *V. cholerae* Ogawa 395; MEKALANOS et al. 1983) colonize rabbit intestines about 10- to 100-fold less efficiently than their parental strain (N. PIERCE, personal communication). In these studies the open gut rabbit model was used (GRAY et al. 1983), which allows clearance of nonadherent organisms. Similar studies performed in ligated intestinal loops of rabbits (in which clearance should not occur) have also shown that the 0395-NT and 0395-N1 strains multipled on average three- to fivefold less than the wild-type strain (B. BOON, E. SIMOEN, M. DEWILDE and J. MEKALANOS, unpublished results). The fact that both mutants show similar reduced cell yield during growth in vivo suggests that the activity of the holotoxin and not just its B subunit is required to produce the increased intestinal colonization.

How does the production of cholera toxin enhance intestinal colonization by *V. cholerae?* At least two possible mechanisms could be proposed: (1) relief of nutritional deprivation and (2) inhibition of bactericidal activity produced by epithelial cells.

In the first case, it is hypothesized that *V. cholerae* can grow fastest when it is adhered to the brush border of intestinal epithelial cells because of the availability of oxygen leaking from the vascular bed underlying this tissue (FRETER et al. 1981). However, it is further postulated that a critical nutrient is missing or limiting in this environment and that the activity of the toxin is required to make this nutrient available. The nutrient could be a trace element like iron or magnesium which is released from epithelial cells along with other ions as a result of the action of the toxin. Thus, microcolonies derived from single toxinogenic cells would arise faster than those of the nutrient-limited nontoxinogenic cells. This would ultimately lead to the increased intestinal colo-

nization observed for toxinogenic versus nontoxinogenic strains. An attractive aspect of this model is that the concentration of the critical nutrient might be linked to the regulation of toxin synthesis.

In the second proposed mechanism, it is hypothesized that bactericidal activity is produced by the epithelial cells which is inhibited by the action of the toxin. There is evidence that bactericidal activity exists on the mucosal surface. KNOP and ROWLEY (1975) described such activity in ligated intestinal loops of mice and found that it did not involve normal flora and required an intact blood supply. BASELSKI et al. (1978 and 1979) also noted that in the intestines of infant mice there existed killing activity to which toxin-deficient mutants (probably *toxR* mutants) appeared more susceptible. Indeed, FRETER and O-BRIEN (1981) have hypothesized that such activity can actually select for nonchemotactic mutants of *V. cholerae* in infant mice, simply because the chemotactic parental strain is subject to enhanced killing after reaching the mucosal surface. The nature of this killing activity is not known but one can imagine that it involves either phagocytosis (OWEN et al. 1983) or a cell-surface-mediated cytotoxic event. Antibody-mediated bactericidal activity should not be involved because the animals being used above are presumably immunologically naive in regard to *V. cholerae*.

Regardless of the nature of the mucosa-associated killing activity, there is a good chance that cholera toxin could impair it given the detrimental effects that cAMP has on the function of neutrophils and macrophages (BOURNE et al. 1973; COX and KARNOVSKI 1973). It is also of interest to note that two other toxins produced by *Bordetella pertussis* and *Bacillus anthracis* are now known to be invasive bacterial adenylate cyclases which can effectively bind to and enter phagocytic cells and inhibit their function via the synthesis of cAMP (CONFER and EASTON 1982; LEPPLA 1982). Thus, a similar event induced by cholera toxin would not be unprecedented in toxin biology. A cholera toxin-mediated inhibition of mucosal killing activity would allow toxinogenic *V. cholerae* to occupy and colonize the surface of the intestinal epithelial cells more efficiently than nontoxinogenic strains. Moreover, if the postulated mucosa-associated killing activity is broadly effective against a variety of enteric pathogens, then the toxin-mediated inhibition proposed here could be the predominant reason for the occurrence of cholera toxin-like enterotoxins in other bacterial genera such as *Escherichia, Salmonella, Campylobacter,* and *Aeromonas*.

In conclusion, it is clear that the genetic analysis of toxinogenesis in *V. cholerae* has provided a number of surprises. The observed duplication and amplification of the *ctx* genetic element, RS1, and the 7-bp repeated sequence in the *ctx* promoter region have helped establish that repetitive DNA is no longer a topic confined to eukaryotic molecular biology. The observation that the *toxR* regulatory gene exists in nontoxinogenic as well as toxinogenic strains of *V. cholerae* has provided another system for studying the evolution of regulatory systems controlling the expression of accessory genetic elements. Finally, the characterization of *ctx* site-specific mutants in vivo has provided evidence that cholera toxin plays a more complex role in pathogenesis than previously envisioned.

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Molecular Biology of Fimbriae of Enterotoxigenic *Escherichia coli*

F.R. Mooi and F.K. de Graaf

1 Introduction

Particular strains of *Escherichia coli* that produce enterotoxins are an important cause of diarrheal disease in man and domestic animals. The ability of these enterotoxigenic *E. coli* (ETEC) strains to adhere to the intestinal epithelium is required as an initial step in establishing diarrheal disease. The bacterial cell surface structures that are responsible for adherence have been called adhesins or colonization factors (see also Gaastra and De Graaf 1982, for a recent review). A special class of adhesins is formed by the proteinaceous filamentous surface appendages called fimbriae (Duguid et al. 1955) or pili (Brinton 1959). These fimbrial adhesins are host specific and include K88 (Ørskov et al. 1964) and 987P (Nagy et al. 1976) on porcine strains; K99 (Ørskov et al. 1975) and F41 (De Graaf and Roorda 1982; Morris et al. 1982) on porcine, ovine and bovine strains; and CFA/I and CFA/II (Evans et al. 1975; Evans and Evans 1978) associated with strains of human origin. Some characteristics of these fimbrial adhesins are shown in Table 1. It should be noted that this list is not complete and that the number of discovered fimbrial adhesins is rapidly increasing.

Apart from their filamentous appearance the fimbriae of ETEC have a number of other characteristics in common which have facilitated their detection and analysis. In addition to their natural receptors located on the intestinal epithelial cells, these fimbriae also bind to receptors on erythrocytes of various animal species (Table 1), indicating that similar receptor structures may be present on both cell types. Agglutination of erythrocytes is often used to identify

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Table 1. Some characteristics of fimbriae of enterotoxigenic *E. coli* strains[a]

Fimbriae	Origin	Morphology	Diameter (nm)	Mol.wt. subunit	pI	Location genes	Erythrocytes agglutinated
K88	Porcine	Flexible	2.1	27540	4.2	Plasmid	Guinea pig, chicken
987P	Porcine	Rigid	7	20000	3.7	Chromosome	–[b]
K99	Bovine, ovine, porcine	Flexible	4.8	18200	9.7	Plasmid	Horse, sheep
F41	Bovine, ovine porcine	Flexible	3.2	29500	4.6	Chromosome?	Human, guinea pig, horse, sheep
CFA/I	Human	Rigid	7	15058	4.8	Plasmid	Human, bovine, chicken
CFA/II[c]	Human					Plasmid	
CS1		Rigid	7	16800	ND		Bovine, chicken, human[d]
CS2		Rigid	7	15300	ND		Bovine[d], chicken, White leghorn hens
CS3		Flexible	2	14500[e] 15500	ND		Bovine[d]

ND, not determined
[a] See text for references
[b] 987P fails to agglutinate horse, guinea pig, rabbit, sheep, pig, or bovine erythrocytes
[c] CFA/II is composed of three components, see text
[d] Different degrees of hemagglutination are observed with erythrocytes from different individuals
[e] Purified CS3 appears on SDS-PAGE as two distinct subunits

and classify adhesins. Furthermore, the production of fimbriae is regulated by the growth temperature, and at temperatures of 18°–25 °C very few fimbriae are produced. This phenomenon is often used to obtain specific sera against fimbriae by absorption of sera raised against whole cells grown at 37 °C with cells grown at 20 °C. The effect of temperature on the production of fimbriae has probably evolved to prevent expression of fimbrial genes outside the host, where the temperature will generally be much lower than 37 °C.

This review will be confined to the well-characterized fimbriae of ETEC, with emphasis on their molecular aspects.

2 Genetics

The genes involved in production of fimbriae of ETEC strains generally reside on plasmids (Table 1), and this has facilitated their analysis.

The CFA/I fimbria is encoded by a group of closely related plasmids [size approximately 89 kilobase pairs (kbp)] that also carry the genes for heat-stable enterotoxin, ST (WILLSHAW et al. 1982). The genes coding for CFA/I have been mapped by transposon mutagenesis, and it appeared that two regions on the plasmid, separated by 37 kbp, are required for CFA/I production (SMITH et al. 1982). Both regions were isolated by molecular cloning (WILLSHAW et al. 1983), and it was shown that the ST genes are closely linked to one of these regions (designated region 1). Region 1 is contained within a DNA fragment of 6 kbp. The second region (region 2) is much smaller and was contained within a 2-kbp fragment. Cell-free extracts of bacteria harboring only region 1 contained protein that reacted with antibodies directed against CFA/I fimbriae, suggesting that the CFA/I fimbrial subunit gene is located within this region. The separate location of region 2 might indicate that it is not only involved in production of CFA/I, but also of other adhesins encoded by CFA/I plasmids. That more than one fimbrial adhesin may be encoded by a particular plasmid is not without precedent (see below).

CFA/II was originally described as a fimbrial colonization factor produced by ETEC of serogroups 06 and 08, and mediating mannose-resistant hemagglutination of bovine erythrocytes at 4 °C (EVANS and EVANS 1978). Later it was determined that the entity referred to as CFA/II by EVANS and EVANS is composed of three different components (SMYTH 1982; CRAVIATO et al. 1982), designated coli-surface-associated antigens CS1, CS2, and CS3 by SMYTH (1982). The three CFA/II components can be distinguished serologically, and on the basis of the molecular weights of their subunits, their hemagglutination patterns, and their morphology (Table 1). It seems probable that the fimbriae described by EVANS and EVANS (1978) were CS1, while the antiserum they produced was directed against CS3 (SMYTH 1984). The genes responsible for production of CS1, CS2, and CS3 reside within the same plasmid (size approximately 89 kbp), which generally also codes for heat-labile (LT) and heat-stable (ST) enterotoxin (PENARANDA et al. 1980; MULLANY et al. 1983; SMITH et al. 1983). Although CFA/II plasmids code for all three components, a strain containing a CFA/II plasmid may produce CS1 and CS3, CS2 and CS3, or CS2 or CS3 only. The decisive factor determining whether CS1 or CS2 is produced is the genetic character of the host, which is recognized by its serotype and biotype. Practically only ETECs of serotype 06:H16 have been shown to produce CS1 or CS2. Furthermore, CS1 is produced only by 06:H16 strains of biotype A (rhamnose negative), while CS2 is produced only by 06:H16 strains of biotype B, C, and F (rhamnose positive) (CRAVIATO et al. 1982; SMYTH 1982). CS3 is produced independent of these biotypes and serotypes (CRAVIATO et al. 1982). When CFA/II plasmids are transferred to *E. coli* K12, only CS3 is produced (MULLANY et al. 1983). It is not clear what mechanism underlies the selective phenotypic expression of the CFA/II components.

K99 fimbriae are encoded by conjugative plasmids with sizes of approximately 75 kbp (SMITH et al. 1972; ØRSKOV et al. 1975). The genes coding for K99 fimbria production have been isolated by molecular cloning (VAN EMBDEN et al. 1980), and the cloned DNA contained in the recombinant plasmid pFK99 has been shown to contain eight structural K99 genes, at least seven of which have

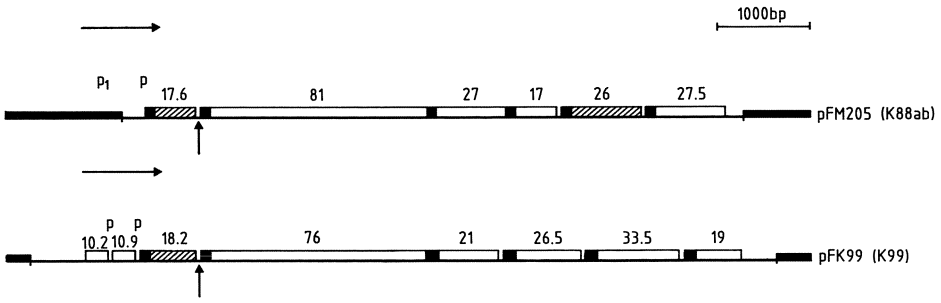

Fig. 1. Genetic maps of cloned K88ab and K99 DNA. The *thick black lines* and *thin lines* represent pBR322 and cloned DNA, respectively. The locations of the various structural genes are indicated by *boxes*. The *black ends of the boxes* indicate the parts of genes coding for the signal peptide. The *numbers above the boxes* refer to the molecular weights ($\times 10^3$) of the corresponding polypeptides. Fimbrial subunit genes are *shaded*. The *horizontal arrows* indicate the direction of transcription. The *vertical arrows* indicate regions with dyad symmetry: *P*, promoter sequence; *P1*, pBR322 promoter P1 (STUEBER and BUJARD 1981); *bp*, basepair

been implicated in the biosynthesis of the K99 fimbria (Fig. 1) (DE GRAAF et al. 1984 and ROOSENDAAL, unpublished). Originally, an additional structural gene, coding for a 21 500-dalton polypeptide (p21.5), was assigned to the region located between the genes for p76 and p21 (Fig. 1) (DE GRAAF et al. 1984). However, DNA sequence data indicate that the gene for p76 is directly followed by the gene for p21 (ROOSENDAAL, unpublished). p21.5 is produced in very low amounts in minicells, and probably translated from within the C-terminal part of the gene for p76. DNA sequence data have revealed two possible promoters which are located proximal to the genes for p10.9 and the K99 fimbrial subunit (p18.2) (ROOSENDAAL et al. 1984, ROOSENDAAL, unpublished). Especially the promoter proximal to the gene for p10.9 complies well with the consensus sequence of strong *E. coli* promoters, and the K99 genes are probably mainly transcribed from this promoter in pFK99. Thus it seems likely that the K99 genes are transcribed from their natural promoter(s) in the constructed recombinant plasmid. This is also suggested by the observation that expression of the K99 genes is independent of the orientation of the cloned DNA within the cloning vehicle used (VAN EMBDEN et al. 1980).

K99 fimbria production in strains containing wild-type K99 plasmids is repressed at temperatures below 30 °C and by L-alanine (DE GRAAF et al. 1980a and 1980b). The effect of temperature and L-alanine on K99 fimbria production is still observed in strains containing the recombinant plasmid pFK99 (VAN EMBDEN et al. 1980), indicating that the genes involved in these regulatory phenomena are still contained within this plasmid.

The K99 operon contains a small intracistronic region, showing dyad symmetry, between the fimbrial subunit gene and the gene for p76 (ROOSENDAAL et al. 1984). The stem and loop structure that can be formed by the K99 transcript of this region (Fig. 2) might function as a rho-dependent terminator, which introduces a natural polarity in the K99 operon. The polypeptides encoded

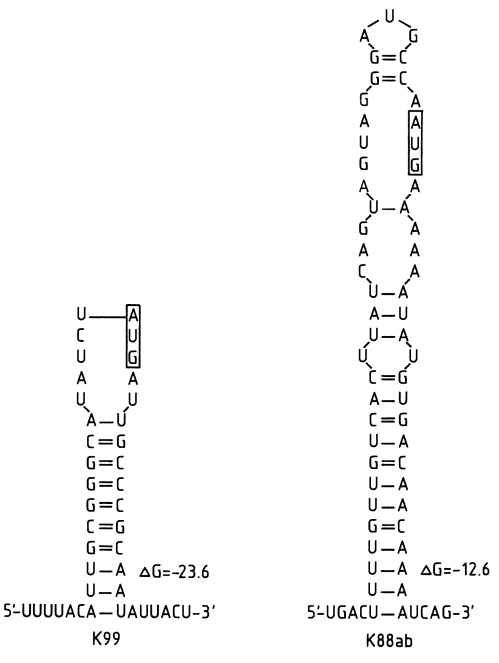

Fig. 2. Possible secondary structures formed by K99 and K88ab mRNA. The DNA regions coding for these structures are indicated by *vertical arrows* in Fig. 1. The putative initiation codons for the genes for p81 and p76 have been blocked. ΔG was calculated according to TINOCO et al. (1973)

by the DNA located downstream from the putative terminator are probably required in lower amounts than the K99 fimbrial subunit, the gene of which is located upstream from the terminator. Another possible function for this region is suggested by the finding that the putative initiation codon for the gene for p76 is located within the stem and loop structure (Fig. 2). Therefore, this region might also be involved in temperature-dependent regulation, because a low temperature will stabilize the stem formed by the transcript and thus prevent initiation of translation of the gene for p76.

K88 fimbriae are encoded by plasmids which frequently also code for the ability to utilize raffinose (Raf) (ØRSKOV and ØRSKOV 1966; SHIPLEY et al. 1978). The K88 and Raf genes are not closely linked, but separated by 30 kbp (MOOI et al. 1979). The association of K88 and Raf genes might reflect the abundance of raffinose or related sugars in the porcine intestine. It is also possible that this association endows porcine ETEC with a selective advantage, because the Raf genes code for enzymes involved in degradation of sugars that compete with the intestinal receptor for the fimbrial binding site.

At least three different K88 variants, K88ab, K88ac, and K88ad, have been described (ØRSKOV et al. 1964; GUINEE and JANSEN 1979), which can be distinguished serologically. The K88ab and K88ac genetic determinants have been cloned (MOOI et al. 1979; SHIPLEY et al. 1981) and studied extensively. Probably the only significant differences between these two determinants reside in the fimbrial subunit genes. Six structural genes have been located on the cloned

K88ab DNA contained within the recombinant plasmid pFM205, at least five of which are located within a single transcriptional unit (i.e., the genes for p17.6, p81, p27, p17, and p26) (Fig. 1) (MOOI et al. 1981, 1982a, 1982b). Similar results have been obtained with the K88ac determinant (KEHOE et al. 1981, 1983). Two different fimbrial subunit genes have been located within the K88ab operon (Fig. 1) (MOOI 1982, MOOI et al. 1984). The large subunit (p26) constitutes the major component of the K88ab fimbriae. The existence of the small fimbrial subunit (p17.6) was inferred from DNA sequence data and the analysis of K88ab mutants. It is produced in too low amounts to be detected and might be a minor component of the K88ab fimbria (see Sect. 4.2).

Although the K88ab recombinant plasmid pFM205 (Fig. 1) contains all the structural genes necessary for production of functional fimbriae, this plasmid does not contain all the K88ab genes. Expression of the K88ab genes in pFM205 is mainly dependent on the pBR322 promoter P1 (Fig. 1) (STUEBER and BUJARD 1981), indicating that the natural K88ab promoter is not contained within this plasmid (MOOI 1982). When P1 is deleted a 10- to 20-fold drop in K88ab production is observed. A weak promoter proximal to the gene for p17.6 might be responsible for the low level of K88ab production still observed in the absence of P1 (MOOI et al. 1984). It is interesting that although the K88ab genes are mainly transcribed from P1 in pFM205, strains containing this plasmid still show temperature-dependent production of K88ab fimbriae (MOOI et al. 1979). This suggests that temperature does not (only) affect initiation of transcription of K88ab genes. Preliminary experiments indicate that the temperature might also affect translation of K88ab mRNAs (OUDEGA and MOOI, unpublished).

The K88ab operon contains a region with dyad symmetry located at the same relative position as in the K99 operon (Fig. 1). However, the stem and loop structure that can be formed by the K88ab transcript of this region is less stable than that of the K99 transcript (Fig. 2). In the K88ab operon, the most highly expressed gene (the gene for the large fimbrial subunit) is located downstream from the region of dyad symmetry. Thus, since there is no evidence for the presence of a promoter between the region of dyad symmetry and the gene for the large fimbrial subunit (MOOI 1982), it is unlikely that the stem and loop structure functions as a terminator. However, since the initiation codon of the gene for p81 is located within the stem and loop structure (Fig. 2), it might be involved in temperature-dependent regulation, as was suggested for the K99 operon.

The K99 and K88ab operons appear to be organized very similarly (Fig. 1). In both operons a putative promoter sequence, probably coding for a weak promoter, is followed by a fimbrial subunit gene and, next, a gene coding for a large polypeptide. Furthermore, both operons contain a small intracistronic region showing dyad symmetry between the fimbrial subunit gene and the gene for the large polypeptide. Most of these common features of the K99 and K88ab operons are also found in operons coding for the Pap and F_{7_2} fimbriae of uropathogenic *E. coli* strains (NORGREN et al. 1984; VAN DIE et al. 1984). Also, fimbrial subunits encoded by these operons show homology (see Sect. 3), indicating that they are evolutionarily related. These observations suggest that these fimbrial operons have evolved from common ancestral genes.

3 Structure and Function of Fimbriae

Many *E. coli* strains have been shown to produce more than one type of fimbria, and often two or more different fimbriae can be detected on the same cell (MORRIS et al. 1980; LEVINE et al. 1984). Generally these fimbriae are serologically unrelated and have different receptor specificities. The occurrence of more than one fimbrial species within a single strain has often resulted in contradictory data with respect to subunit molecular weight, amino acid composition, and hemagglutination pattern of a particular fimbria.

At least two different morphological types of fimbriae can be distinguished (Table 1). One morphological type, represented by type I (BRINTON 1965), 987P (ISAACSON and RICHTER 1981), CFA/I (EVANS et al. 1979), CS1, and CS2 fimbriae (MULLANY et al. 1983; SMYTH 1984), consists of rigid fimbriae with a diameter of approximately 7 nm. Generally an axial hole is visible in these fimbriae under the electron microscope. The second morphological type is represented by K88 (STIRM et al. 1967), K99 (DE GRAAF et al. 1980c), F41 (DE GRAAF and ROORDA 1982), and CS3 fimbriae (LEVINE et al. 1984; SMITH et al. 1984), and consists of very thin (diameter 2–5 nm) and flexible fimbriae. The term fibrillae or fibrillar fimbriae has been used for these structures to distinguish them from the more rigid fimbriae (LEVINE et al. 1984). Fimbriae and fibrillae may occur within the same strain and may even be present simultaneously on a single cell (LEVINE et al. 1984). Whether these differences in morphology reflect an adaptation to different functions remains to be determined.

The fimbriae of ETEC form a group of functionally and structurally closely related proteins, and might have evolved from common ancestral genes. Therefore, it is of interest to compare the primary structures of different fimbrial subunits. From this comparison one may infer evolutionary relationships. Furthermore, it allows one to discern between conserved and variable sequences, to correlate these with similarities and differences in function and ultrastructure, and, ultimately, to link certain sequences to particular function. Also, the identification of conserved sequences may facilitate the development of synthetic vaccines, directed against a number of fimbriae.

In the context of structure-function relationships it should be noted that there is evidence that some fimbriae of uropathogenic *E. coli* strains attach to the P-receptor by means of an adhesin that is distinct from the fimbrial subunit that constitutes the major component of the fimbria (NORGREN et al. 1984). Thus it is possible that some fimbrial subunits only have a structural function, i.e., to expose the adhesin molecule some distance from the cell surface, probably to facilitate interaction with the host receptor.

The primary structure of a number of fimbrial subunits has been partially or completely resolved. Comparison of these sequences reveals that fimbrial subunits show homology at their N-and C-termini (Fig. 3). The conservation of amino acid residues in limited areas of fimbrial subunit polypeptide chains would be explained if these areas define domains involved in functions shared by all these subunits. Such functions could involve transport across the outer membrane, anchorage, and subunit-subunit binding. Conservation of particular amino acid residues is most pronounced at the C-terminus, suggesting that

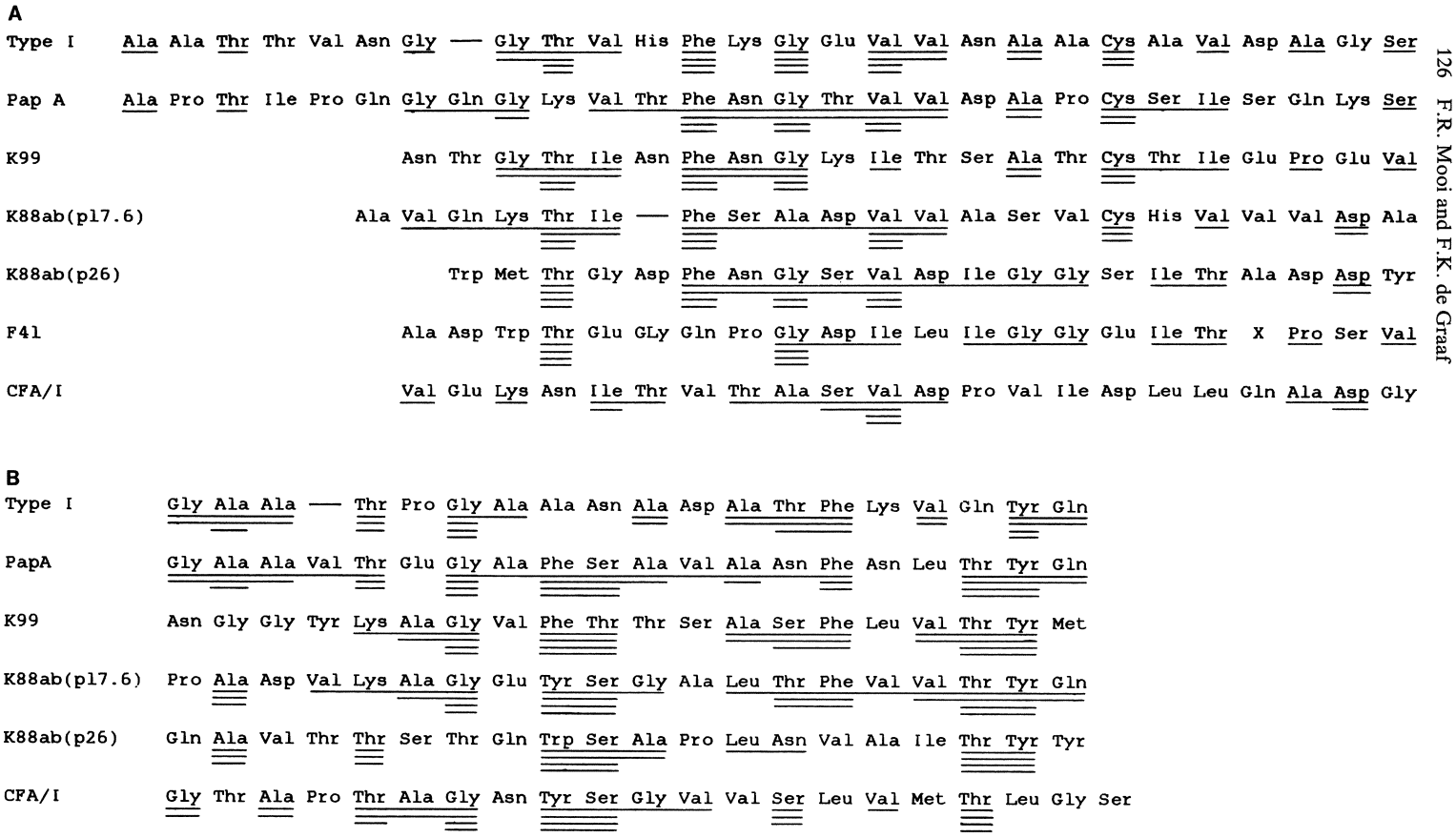

Fig. 3. Comparison of amino-(*A*) and carboxy- (*B*) terminal amino acid sequences of fimbrial subunits. Identical or functionally identical amino acid residues are *underlined*. The amino acids ser, thr and phe, trp, tyr were assumed to be functionally identical. The number of times a residue is *underlined* indicates how often it occurs at that particular position. The amino acid sequences were obtained from the following references: K88ab (p26) (GAASTRA et al. 1981); CFA/I (KLEMM 1981); F41 (DE GRAAF and ROORDA 1982); K99 (ROOSENDAAL et al. 1984); PapA (BAGA et al. 1984); K88ab (p17.6) (MOOI et al. 1984); type I (KLEMM 1984)

its function has been more conserved than that of the N-terminus. For several reasons it seems possible that C-termini of fimbrial subunits are involved in subunit-subunit binding. With the exception of the C-terminus of type I fimbriae, the C-termini are hydrophobic and therefore likely to be buried inside the fimbrial structure. Furthermore, at three positions aromatic amino acid residues, which have been implicated in the maintenance of quarternary structure of fimbriae, appear to be conserved. WATTS et al. (1983) have shown that two tyrosine residues, and at least one tryptophane residue, become exposed when *Pseudomonas* fimbriae are dissociated into dimers, suggesting that these amino acid residues are located at the dimer-dimer interface. In addition, MCMICHAEL and OU (1979) have shown that disruption of type I fimbriae of *E. coli* K12 by acid treatment results in unmasking of tyrosine residues, also implicating an aromatic amino acid in subunit-subunit binding. In this context it is interesting to note that the type I fimbrial subunit derived from *E. coli* K12 contains only two tyrosine residues, both located at the C-terminus of the molecule (KLEMM 1984).

The primary structure of only a few fimbrial subunits has been determined completely. The large K88 fimbrial subunit was the first fimbrial subunit of which the amino acid sequence was resolved. Compared with most *E. coli* fimbrial subunits, it has a large molecular weight (Table 1). Nevertheless, the large K88 fimbrial subunit shows homology at its N- and C-terminus with other fimbrial subunits, suggesting an evolutionary relationship. Possibly, the large K88 fimbrial subunit has evolved from a putative ancestral fimbrial subunit gene, by gene fusion or gene duplication. At least three serological variants of the K88 fimbria have been described, which have been designated K88ab, K88ac, and K88ad (ØRSKOV et al. 1964; GUINEE and JANSEN 1979). Apparently, the K88 fimbriae contain regions which are conserved and form the a determinants, and regions which are variable and form the b, c, and d determinants. Chemical analysis of the K88 variants only revealed differences in amino acid compositions (MOOI and DE GRAAF 1979), suggesting that the serological differences are solely due to differences in primary structure. The primary structure of the large K88ab fimbrial subunit was determined by DNA (GAASTRA et al. 1981) and protein sequencing (KLEMM 1981). Later the primary structure of the large K88ad fimbrial subunit was resolved (GAASTRA et al. 1983). Comparison of the two primary structures (Fig. 4) reveals highly conserved and highly variable regions. The first 37 and last 28 amino acid residues are completely identical between the two variants. A large stretch of amino acid residues showing complete identity is also found between residues 104 and 132. Two antigenic determinants have been predicted within these conserved sequences (Fig. 4) (KLEMM and MIKKELSEN 1982), and they might represent the a determinants. Antigenic determinants have also been predicted within variable regions (Fig. 4), and these regions might contribute to the b and d antigenic determinants. We assume that the conserved sequences of the large K88 fimbrial subunits are involved in assembly and maintenance of the fimbrial structure and receptor recognition. With respect to receptor recognition it should be noted, however, that the possibility cannot be excluded that the receptor-binding site is located on the small K88 fimbrial subunit. The variable regions of the large subunits

```
          1                   20                  40                  60
          .         .         .         .         .         .         .
K88ab     WMTGDFNGSVDIGGSITADDYRQKWEWKVGTGLNGFGNVLNDLTNGGTKLTITVTGNKPI
K88ad                                             S         E         S

         61                  80                 100                 120
          .         .         .         .         .         .         .
K88ab     LLGRTKEAFATPVSGGVDGIPQIAFTDYEGASVKLRNTDGETNKGLAYFVLPMKNAEGTK
K88ad          R         V         H           E   P   IE

        121                 140                 160                 180
          .         .         .         .         .         .         .
K88ab     VGSVKVNASYAGVFGKGGVTSADGELFSLFADGLRAIFYGGLTTTVSGAALTSGSAAAAR
K88ad                AL R          M    E SH       P N QNS  PG

        181                 200                 220                 240
          .         .         .         .         .         .         .
K88ab     TELFGSLSRNDILGQIQRVNANITSLVDVAGSYREDMEYTDGTVVSAAYALGIANGQTIE
K88ad             K                  N P  FN N A    S   V        R

        241                 260
          .         .         .
K88ab     ATFNQAVTTSTQWSAPLNVAITYY
K88ad
```

Fig. 4. Comparison of the primary structures of the large K88ab and K88ad fimbrial subunits. The amino acid sequences are given in the single-letter code. For the K88ad subunit only amino acid residues that differ from K88ab are indicated. The *horizontal bars* indicate predicted antigenic determinants. See text for references

might have evolved by genetic drift, or to evade the immune response of the host.

Apart from the large K88 fimbrial subunits, the primary structure of the CFA/I (KLEMM 1982), PapA (BAGA et al. 1984), K99 (ROOSENDAAL et al. 1984), type I (KLEMM 1984), and small K88ab fimbrial subunit (MOOI et al. 1984) has been resolved. With the exception of CFA/I, these fimbrial subunits are similar in a number of aspects (Fig. 5). They show homology at their N- and C-termini, are composed of approximately the same number of amino acid residues, and contain two cysteine residues located at approximately the same positions. The two cysteines are probably involved in the formation of an intra-chain disulfide linkage (MCMICHAEL and OU 1979; ISAACSON et al. 1981). Although homology is most evident at the N- and C-termini of these polypeptides, identical amino acid residues are also evident in the central parts of these molecules (Fig. 5). Homology is most evident between the type I and PapA fimbrial subunits, sug-

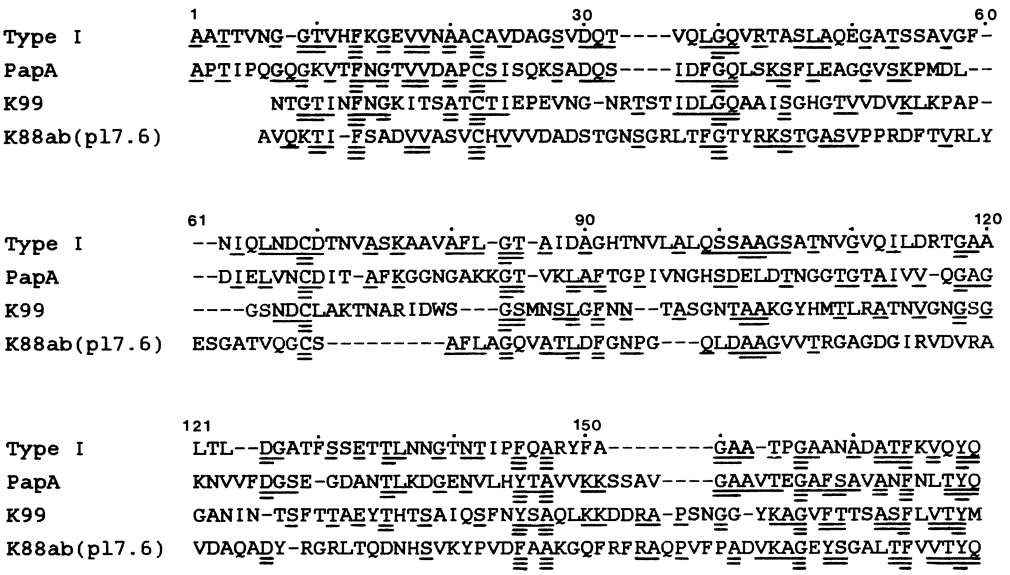

Fig. 5. Comparison of the primary structure of the type I, papA, K99, and small K88ab fimbrial subunit. The amino acid sequences are given in the single-letter code. *Underlining* of identical or functionally identical amino acid residues follows the convention described in Fig. 3.S, T and F, W, and Y were assumed to be functionally identical. *Dashes* indicate gaps introduced to increase the number of matches. See text for references

gesting that they are evolutionarily more closely related than the other fimbrial subunits. Except at its C-terminus, the CFA/I subunit shows very little homology with the above-mentioned fimbrial subunits. Furthermore, this polypeptide is approximately 15 amino acid residues shorter and contains no cysteine residues. This might indicate that CFA/I has evolved from the putative ancestral gene by one or more deletion events, possibly involving its N-terminus.

In summary it may be stated that within a large group of fimbrial subunits the conservation of amino acid residues is most apparent at the C-termini and to a lesser extent at the N-termini. These regions are probably involved in functions common to this group of proteins such as transport across the outer membrane, anchorage, and maintenance of the fimbrial structure. The more variable central parts have probably been adapted to the recognition of different receptors, or may simply have diverged as a response to immunological pressure, because less stringent structural or functional constraints are imposed on them.

4 Biogenesis of Fimbriae

4.1 Introduction

Fimbriae are supramolecular structures composed of low molecular weight subunits, and this section will deal with the problem of how these low molecular

weight subunits are transported across the inner and outer membrane and assembled into a filamentous structure at the cell surface. Generally five to eight polypeptides have been implicated in the phenotypic expression of *E. coli* fimbriae (Mooi et al. 1982; Norgren et al. 1984; Van Die et al. 1984; De Graaf et al. 1984), and this makes them simpler subjects to study biogenesis than, for example, F-pili and flagellae, which require a much larger amount of gene products for their biogenesis (Manning and Achtman 1979; Iino 1977; Silverman and Simon 1977).

Most information available on biogenesis of fimbriae has been gained with the K88ab fimbria, and we concentrate here on this fimbria. However, it seems likely that other fimbriae are produced in a similar way, because fimbrial genetic determinants have probably evolved by divergent evolution from common ancestral genes, and this is probably reflected in similar routes for their biogenesis.

4.2 Functions of K88ab Polypeptides

Insight into the functions of the K88ab polypeptides was obtained by the analyses of derivatives of pFM205 (Fig. 1), which contain small deletions in the various K88ab genes (Mooi et al. 1982, 1983). It appeared that at least five of the six K88ab polypeptides encoded by pFM205 are involved in the biogenesis of the K88ab fimbria (Table 2). It is not clear whether the sixth polypeptide (p27.5) is also involved in this process. Although a deletion in the gene for p27.5 results in a reduction of K88ab fimbriae production, it does not affect their function since p27.5 mutants still bind to porcine intestinal epithelial cells and agglutinate erythrocytes (Table 2). The low amount of K88ab fimbriae produced by the mutant lacking p27.5 cannot be enhanced by complementation in trans, suggesting that the effect of the mutation is not (only) due to the absence of p27.5, but (also) to the deletion of a cis-acting locus. One observation suggests that the K88ab fimbrial subunit and p27.5 are somehow connected; a deletion in the gene for p27 affects the stability of both polypeptides (Table 2). More mutants lacking p27.5 will have to be analyzed to clarify the role of this polypeptide.

All six polypeptides encoded by the cloned K88ab DNA are synthesized as precursors containing a signal peptide (Mooi et al. 1981, 1982, 1984), indicating that they are located in the periplasmic space or outer membrane. This has been confirmed by determining the subcellular localization of these polypeptides (Table 2) (Van Doorn et al. 1982). It appeared that p27, p17, and p27.5 are located in the periplasmic space, whereas p81 is located in the outer membrane. Of course p26, the major component of the K88ab fimbria, is normally located on the outside of the cell, attached to the outer membrane. The location of p81 in the outer membrane suggests that it is involved in this attachment. The subcellular location of p17.6 could not be determined, because it is produced in too low amounts to be detected. The fact that p17.6 is a fimbrial subunit or evolutionarily related to one suggests that it is located on the cell surface.

Since p26 is synthesized as a precursor containing a signal peptide and accumulates transiently in the periplasmic space (see below), it is probably trans-

Table 2. Properties of K88ab polypeptides and the effects of mutations in their genes

Polypeptide	Subcellular location	Effect of a mutation in the gene for this polypeptide on:		Putative function polypeptide
		Hemagglutination (HA)	Biogenesis K88ab fimbria	
p17.6	Extracellular?	No HA	1. No fimbra produced 2. Intracellular accumulation of p26	Minor component of the K88ab fimbria
p81	Outer membrane	No HA	1. No fimbria produced 2. Accumulation of assembly precursors (p26-p27-p17) in periplasmic space	Transport of p26 (and p17.6?) across the outer membrane and anchorage of fimbria
p27	Periplasmic space	No HA	1. No fimbria produced 2. Degradation of p26 and p27.5	Transport p26 across outer membrane
p17	Periplasmic space	No HA	1. Transport of p26 across outer membrane, and assembly of fimbriae reduced 2. Accumulation of assembly precursors (p26-p27) in periplasmic space	Modification of p26
p26	Extracellular	No HA	No fimbriae produced	Major component of the K88ab fimbria
p27.5	Periplasmic space	Reduced HA	Decrease in p26 synthesis, and fimbria production	?

ported across the inner membrane via the same route as *E. coli* periplasmic proteins (SILHAVY et al. 1983). In the periplasmic space, p26 associates with p27 before it is transported across the outer membrane. Normally, the p26–p27 complexes are too transient to allow analysis. However, in mutants lacking p17 or p81 these assembly precursors accumulate in the periplasmic space (Table 2), from where they can be isolated and analyzed. Electrophoretic analysis suggests that the assembly precursors are composed of p26–p27 dimers that have a low affinity for each other and continuously associate and dissociate to form oligomers of different size. This process might facilitate subsequent assembly of p26. In mutants lacking p27, p26 is synthesized and subsequently rapidly degraded (Table 2). Apparently association with p27 affects the conformation of the large fimbrial subunit, which becomes more resistant to proteolysis. The effect of p27 on the conformation of p26 suggests several possible

functions for p27, which might not be mutually exclusive. First, as is shown clearly with phage tail proteins (KING 1980) and flagellin subunits (IINO 1977), structural proteins are often synthesized in a form that does not spontaneously assemble to prevent the formation of a structure at the wrong site, and the function of p27 could be to induce or stabilize such a form of p26 to prevent it from premature polymerization in the periplasmic space. Indeed, fimbrial subunits present in the periplasmic space have a different, more thermolabile, conformation than fimbrial subunits assembled into fimbriae (MOOI, unpublished), confirming that they occur in different conformations in the course of fimbrial assembly. Second, association with p27 might be required to fold the p26 polypeptide chain into a particular conformation necessary for transport across the outer membrane. For example, p26 might be transported as an extended or relatively unstructured polypeptide chain through channels formed by p81 molecules in the outer membrane (see below). Finally, association of p26 with p27 might be required to deliver the energy necessary for transport or assembly of p26. For example, if p27 catalyzes the folding of the p26 polypeptide chain into a strained and energetically unfavorable conformation, some of the energy built into the protein during polymerization of amino acids might be retained. The conformational energy thus built into the p26–p27 complexes might be used to drive transport or assembly of p26.

Considering its instability in the absence of p27, it seems likely that p26 associates with p27 within a very short span of time after its synthesis and transport across the inner membrane is completed. Possibly, association takes place as the fimbrial subunit polypeptide chain emerges from the inner membrane. p27 is synthesized in large amounts, and a fast association between p26 and p27 is probably facilitated by a high concentration of p27 in the periplasmic space. Furthermore, p26 has a low isoelectric point (pI = 4.2), whereas p27 has a high isoelectric point (pI $\leq$ 9.3) (MOOI et al. 1983); therefore these proteins are expected to have opposite net charges at physiological pH, also facilitating their association in the periplasmic space.

Mutants lacking p17 are still able to transport p26 to the cell surface and assemble them into fimbriae, albeit very inefficiently (Table 2). However, these mutants do not agglutinate erythrocytes or bind to intestinal epithelial cells. This might be attributed to the small amounts of fimbriae produced by these cells. Alternatively, the fimbriae produced might not be functional. If the latter is true, this suggests that p17 (which is located in the periplasmic space) is involved in modification of p26 during its passage through the periplasmic space, since it is difficult to see how else p17 can affect the functioning of extracellular fimbrial subunits. If the fimbrial subunit is indeed modified by p17, this modification must be slight since fimbrial subunits derived from mutants lacking p17 have the same apparent molecular weight as fimbrial subunits derived from wild-type strains.

In mutants lacking p17 most p26 molecules are found in the periplasmic space associated with p27. Apparently, p17 is necessary for efficient transport of p26 across the outer membrane. It is possible that only modified p26 molecules are transported efficiently across the outer membrane. Another possibility is suggested by the observation that in mutants lacking p81, p17 is found asso-

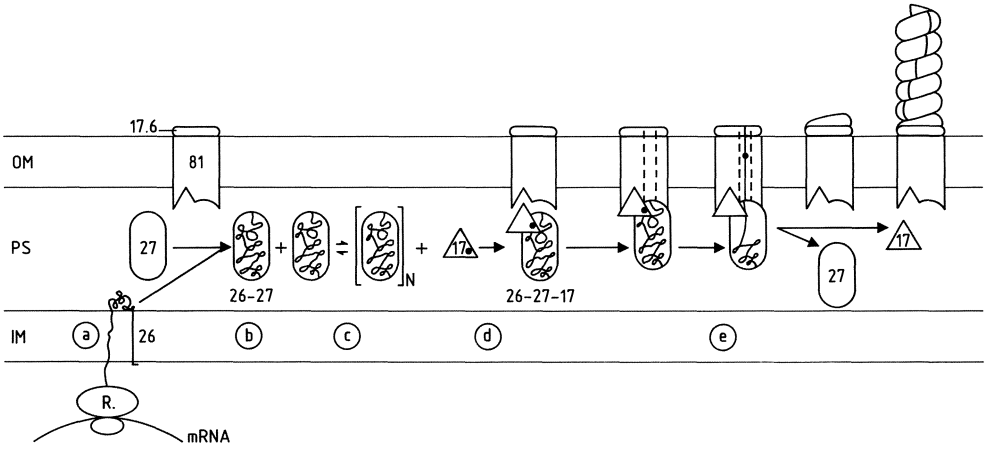

Fig. 6. Model for the biogenesis of the K88ab fimbria. *a* p26 (the large fimbrial subunit) is transported across the inner membrane following the normal secretory pathway of periplasmic proteins. *b* In the periplasmic space p26 associates with p27. This association induces or stabilizes a conformation of p26 that is required for transport across the outer membrane. *c* The p26–p27 dimers have a low affinity for each other and form oligomers of different size. This process might facilitate the subsequent assembly of p26. *d* p17 binds to the oligomers and enhances their affinity for p81 (for simplicity only p26–p27 dimers are depicted). *e* Binding of the p26-p27-p17 complexes to p81 induces conformational changes in these proteins which result in modification of p26 by p17, transport of p26 across the outer membrane through channels formed by p81, and dissociation of p27 and p17. p27 and p17 are reused for further cycles of assembly. At the cell surface p26 obtains a conformation with high affinity for other p26 molecules, allowing it to assemble into the fimbrial structure. The small fimbrial subunit (p17.6) has been assumed to be part of the basal structure of the fimbria. The *numbers* refer to the molecular weights ($\times 10^3$) of the polypeptides. The *thick black line* indicates the signal peptide of p26. The *large black spot* indicates the hypothetical group used to modify p26. *IM*, inner membrane; *PS*, periplasmic space; *OM*, outer membrane; *R*, ribosome

ciated with the p26–p27 complexes in low amounts (Table 2); association of p17 with the p26–p27 complexes might enhance transport of p26 across the outer membrane by enhancing the affinity of these complexes for export sites. Both possibilities prevent unmodified p26 molecules from being assembled into fimbriae in significant amounts.

The location of p81 in the outer membrane, and the accumulation of p26 in the periplasmic space of mutants lacking p81, suggests that p81 is involved in transport of fimbrial subunits across the outer membrane. It is possible that p81 is a trans-membrane protein that forms channels specific for fimbrial subunits. Association of complexes composed of p26, p27, and p17 with p81 in the periplasmic space might induce conformational changes in these proteins, resulting in modification of p26, opening of the channel, transport of p26 across the outer membrane, and release of p17 and p27. p17 and p27 are probably reused for further cycles of assembly. At the cell surface p26 probably folds into a conformation with a high affinity for other fimbrial subunits, enabling it to assemble into the fimbrial structure. At the base of the fimbria, some p26 molecules presumably remain associated with p81, anchoring the fimbria to the cell.

It is difficult to obtain insight into the function of p17.6, because it is produced in too low amounts to be detected. Since the primary structure of p17.6 suggests it is a fimbrial subunit, it is probably located at the cell surface. Furthermore, mutants lacking p17.6 do not produce K88ab fimbriae and accumulate p26 inside the cell (Mooi et al. 1984). These observations suggest that p17.6 is a minor component of the K88ab fimbria. If p17.6 and p26 are indeed part of the same structure, they must not only bind to themselves, but also to each other. This could be accomplished if both polypeptides contain similar binding sites. It is tempting to speculate that the C-termini of p17.6 and p26 constitute these binding sites, since they show homology (Fig. 3).

A model for the biogenesis of the K88ab fimbria is shown in Fig. 6.

4.3 Functions of K99 Polypeptides

With the exception of p10.9, all K99 polypeptides encoded by pFK99 (Fig. 1) have been implicated in the biogenesis of the K99 fimbria (De Graaf et al. 1984; Roosendaal and De Graaf, unpublished). It is not clear whether p10.9 is also involved, because mutants lacking this polypeptide have not yet been isolated. p10.2 and p10.9 are probably produced in very low amounts, because these polypeptides are not detected in minicells. The existence of these polypeptides was inferred from DNA sequence data (Roosendaal, unpublished). The predicted amino acid sequence of p10.2 shows that it is a highly charged polypeptide, which does not contain an N-terminal signal peptide. Thus, p10.2 is probably located in the cytoplasmic space. It might be involved in regulation at the level of transcription or translation, since nonpolar insertions in its gene result in a decrease in K99 fimbria production.

Several observations suggest that p76 and p21 have similar functions as the K88ab polypeptides p81 and p27, respectively (Table 2). First the genes for p76 and p21 have the same relative position as the genes for p81 and p27, respectively (Fig. 1). Second, the two K99 polypeptides show homology with the two corresponding K88ab polypeptides (Mooi and Roosendaal, unpublished). Finally, a small nonpolar deletion in the gene for p21 results in degradation of the K99 fimbrial subunit (De Graaf et al. 1984). Thus this mutant resembles a K88ab mutant lacking p27 (Table 2).

p19 might have a similar function as the K88ab polypeptide p17 (Table 2). The two polypeptides have similar molecular weights and are both produced in low amounts. Furthermore, p19 can be coprecipitated with the K99 fimbrial subunit from cell-free extracts using antibodies directed against the K99 fimbrial subunit (De Graaf et al. 1984). Since p19 is not found associated with purified K99 fimbriae, this observation suggests that p19 associates with the K99 fimbrial subunit prior to its assembly. A similar observation has been made for p17 and the large K88ab fimbrial subunit. Finally, a K99 mutant lacking p19 resembles a K88ab mutant lacking p17 in that it shows a reduced amount of fimbrial subunits on the cell surface and does not adhere to epithelial cells (De Graaf et al. 1984). The fact that these mutants do not adhere could be due to low amounts of fimbriae on the cell surface or to the absence of functional receptor-

binding sites. If the latter possibility is true, this suggests that p19 is directly or indirectly involved in receptor binding.

No clues about the functions of p26.5 and p33.5 have yet been obtained. Double mutants lacking p33.5 and p19 have the same phenotype as mutants lacking p19 only (see above). p26.5 probably associates with the fimbrial subunit prior to its assembly, because it can be coprecipitated with the fimbrial subunits from cell-free extracts, but is not found in purified K99 fimbria preparations.

In mutants lacking one or more of the polypeptides p76, p21, or p26.5, fimbrial subunits are synthesized and subsequently degraded. Thus as is observed for the large K88ab fimbrial subunit, the K99 fimbrial subunit requires other polypeptides to fold into a stable (proteolysis-resistant) conformation.

4.4 Some Remaining Questions

Several interesting questions concerning the biogenesis of fimbriae remain to be addressed. For example, it is not clear how the fimbria grows. Growth may occur by the addition of fimbrial subunits at the base or at the tip of the fimbria. Since the fimbria does not contain a hollow core large enough for transport of fimbrial subunits, growth at the tip requires that fimbrial subunits travel by one-dimensional diffusion along the outside of the fimbria to the tip where they must be assembled. Another more likely possibility is that the fimbria grows by insertion of fimbrial subunits at its base.

It is also not clear what determines the length of fimbriae. One possibility is that the relative concentration of different proteins is important. For example, it seems plausible that when the amount of anchorage proteins per cell increases relative to the amount of fimbrial subunits, the fimbriae produced will tend to be shorter. Since the K88ab and K99 genes are located within a single transcriptional unit, protein ratios are probably determined by polar effects (see Sect. 2) and by the efficiencies of initiation of translation.

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Genetics of *Escherichia coli* Hemolysin

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1 Introduction

Hemolysis, i.e., the lysis of erythrocytes, is a rather widespread capacity of bacteria and other microorganisms but the molecular basis of this character varies from organism to organism. Extracellular enzymes have been shown to be responsible for hemolysis by some bacteria, e.g., the phospholipases of *Pseudomonas aeruginosa* (VASIL et al. 1982) and *Chlostridium perfringens* (also called α-toxin; SMITH 1979) and also the sphingiomyelinase C produced by *Staphylococcus aureus* (β-toxin; FREER and ARBUTHNOTT 1983). "Nonenzymatic" proteins which disrupt the membrane of erythrocytes have been identified as the causative agents of hemolysis by, among others, *Staphylococcus aureus* (α-toxin; FREER and ARBUTHNOTT 1983), *Vibrio cholerae* (GOLDBERG and MURPHY 1984), *Aeromonas hydrophila* (CHAKRABORTY et al. 1984), and the enterobacteria *Escherichia coli* (SMITH AND HALLS 1967; JORGENSEN et al. 1980; GOEBEL et al. 1984), *Proteus mirabilis* (PEERBOOMS et al. 1982), and *Proteus morganii* (EMÖDY et al. 1983). Among other enterobacteria hemolytic strains of *Serratia marcescens* (LE MINOR and LE COUEFFIC 1975) and *Shigella sonnei* have been described and the possibility exists that at least *Shigella* strains bear plasmids originating from *E. coli* (STENZEL 1971 b). Forming another subclass of the "nonenzymatic" proteins are the sulfhydryl (SH)-activated hemolysins such as streptolysin O

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of *Streptococcus pyogenes,* pneumolysin of *Streptococcus pneumoniae,* the θ-toxin of *Clostridium perfringens,* as well as cereolysin and listeriolysin of *Bacillus cereus* and species of *Listeria* (for review see ALOUF 1980 and ALOUF and GEOFFROY 1984). These toxins are assumed to recognize cholesterol as a receptor in the erythrocyte membrane and are serologically related and oxygen-labile.

Epidemiological data, clinical observations, and results of animal tests with purified hemolysins have made it clear for some time that several of the bacterial hemolysins play a role in pathogenicity (for reviews see STEPHEN and PIETROWSKI 1981; ALOUF 1980; FREER and ARBUTHNOTT 1983). Whereas hemolysis in these cases may be regarded as a diagnostic marker in routine analyses, the in vivo role of hemolysin in the infection process is presumed rather to be physical dissolution of a variety of cells. In 1974 BERNHEIMER introduced the term "cytolysin" to define such bacterial products with a membrane-damaging capacity and for many of the hemolysins a cytolytic action on eukaryotic cells, especially leukocytes, has been well documented (SHEWEN and WILKIE 1982; MCGEE et al. 1983; Wannamaker 1983; Rogolsky 1979; Cavalieri and SNYDER 1982a, c).

At the turn of the century KAYSER (1903) and SCHMIDT (1909) described hemolysis by strains of *Escherichia coli.* The hemolysins of numerous bacterial species have subsequently been studied in the fields of classical and diagnostic bacteriology, toxicology, and more recently microbial pathogenicity in which since the 1960s they have become the subject for genetic analysis, particularly in *E. coli.* The location of the *E. coli* hemolysin (*hly*) genes on transmissible plasmids was postulated (analogous to the enterotoxin genes of pathogenic intestinal strains) and subsequently confirmed (SMITH and HALLS 1967; SMITH and LINGGOOD 1971) and the later discovery of chromosomal *hly* determinants has led to their location by Hfr mapping (RATINER 1972; HULL et al. 1982). After the establishment of recombinant DNA techniques, molecular analysis of hemolysis became more attractive to several investigators and molecular cloning was first used successfully in *E. coli* for the isolation of the plasmid-borne *hly* determinant (NOEGEL et al. 1979, 1981; NUMEROW et al. 1981; GOEBEL and HEDGPETH 1982) and its chromosomally located counterpart (WELCH et al. 1981; BERGER et al. 1982).

Cloning of these various genetic determinants has been the essential first step in their analysis by molecular techniques including transposon-mediated mutagenesis and sequencing, in addition to their use as hybridization probes in homology studies and as sources for specific gene product analysis (DE LA CRUZ et al. 1980a; MÜLLER et al. 1983; KNAPP et al. 1984; for review see MACRINA 1984). The isolation of hemolysin genes and their introduction into appropriate nonhemolytic hosts has also proved to be the most unambiguous way of clarifying their involvement in pathogenicity (see WELCH et al. 1981; HACKER et al. 1983a).

In this review we will summarize the biological and biochemical properties of the *E. coli* hemolysin and will focus our attention on the genetic studies performed on the responsible *hly* determinants, emphasizing the application of gene cloning.

2 *Escherichia coli* Hemolysin

2.1 Incidence

Hemolytic *Escherichia coli* strains can be isolated from the normal animal flora, from cases of neonatal diarrhea in piglets, as well as from the normal stool flora and both intestinal and extraintestinal infections of man. A relatively high incidence of hemolytic stains has been noted among *E. coli* isolated from the feces of healthy animals (53%–76% of isolates; see SMITH 1963). Similarly, enteropathogenic and exterotoxigenic *E. coli* isolates from pigs, which are often associated with rotavirus, frequently exhibit hemolysis and from the 2nd to the 8th day of diarrheal infection nearly 100% of the strains are hemolytic (LECCE et al. 1982). These strains characteristically carry the hemolysin determinant on large transmissible plasmids (SMITH and HALLS 1967; GOEBEL and SCHREMPF 1971; DE LA CRUZ et al. 1979) and they may also produce heat labile (LT) and/or heat-stable (ST) enterotoxin along with specific K88-adhesins (SMITH 1969). While these epidemiological data suggest a contribution of hemolysin to the pathogenesis of diarrhea in piglets all attempts to verify this directly have until now been unsuccessful (SMITH and LINGGOOD 1971; LECCE et al. 1982).

In man, the percentage of hemolytic strains found in the intestines of healthy individuals and patients with diarrheal diseases is unusually low (from 5% to 20%) but is very high (35% to 60%) among those strains causing urinary tract infections [first demonstrated by DUDGEON et al. (1921)]. While the colonization factor antigens I and II (CFAI and CFAII) and the ST and LT enterotoxins have not been detected among fecal hemolytic strains (SO et al. 1975; EVANS et al. 1978), Hly^+ isolates from urine generally demonstrate mannose-resistant hemagglutination and carry specific O- and K-antigens and often the ColV plasmid, all these being regarded as virulence factors for these strains (HUGHES et al. 1983; VÄISÄNEN-RHEN et al. 1984). The contribution of hemolysin to virulence of *E. coli* causing extraintestinal infections is now well established (WELCH et al. 1981; HACKER et al. 1983a) and, in contrast to Hly^+ *E. coli* strains isolated from animals, those obtained from man carry their *hly* determinants in nearly all cases on the chromosome (MÜLLER et al. 1983; HACKER et al. 1983b). The number of Hly plasmids isolated from human *E. coli* fecal strains is small (DE LA CRUZ et al. 1979; GRÜNIG and LEBEK 1985) and isolation of plasmids from uropathogenic *E. coli* has been reported in only a few cases (HÖHNE 1973; TSCHÄPE and RISCHE 1974; see Table 1).

2.2 Genetic Determination

The extrachromosomal nature of the genes encoding production of hemolysin in *E. coli* was first reported in 1967 by WILLIAMS SMITH and HALLS, who demonstrated transfer of 10 Hly^+ plasmids from *E. coli* strains of animal origin to *E. coli* K-12 recipients. Physical evidence for the plasmid location of the hemoly-

Table 1. Hly plasmids isolated from *E. coli* wild-type strains

Designation of Hly plasmids	Size	Transfer property	*inc* group	Source	Authors
pHly152[a]	62.2 kb	*tra*$^+$	I_2	Fecal, animal	SMITH and HALLS (1967) GOEBEL et al. (1974)
pHly167[a]	NT	*tra*$^+$	NT	Fecal, animal	SMITH and HALLS (1967) MACKMAN and HOLLAND (1984)
P212	190 kb	*tra*$^+$	F_{VI}	Fecal, animal	MONTI-BRAGADIN et al. (1975) DE LA CRUZ et al. (1979)
pIP241[b]	61 kb	*tra*$^+$	I_2	Fecal, animal	LE MINOR and LE COUEFFIC (1975)
pHly185	76 kb	*tra*$^-$	NT	Fecal, animal	MINSHEW et al. (1978) STARK and SHUSTER (1983)
pHly1405/71[c]	NT	*tra*$^+$	NT	UTI, human	HÖHNE (1973)
pHly33	150 kb	*tra*$^+$	F_{VI}	UTI, human	TSCHÄPE and RISCHE (1974)
pHly3451	155 kb	*tra*$^+$	F_{VI}	UTI, human	TSCHÄPE (personal communication)
pHly7880	NT	*tra*$^+$	I_2	UTI, human	TSCHÄPE (personal communication)
pSU316[d]	75 kb	*tra*$^+$	$F_{III, IV}$	Fecal, human	DE LA CRUZ et al. (1979, 1980b)
pSU105[d]	123 kb	*tra*$^+$	F_{VI}	Fecal, human	DE LA CRUZ et al. (1979, 1980b)
pCW*2*	68 kb	*tra*$^-$	NT	Fecal, animal	EMÖDY et al. (1980) WAALWIJK et al. (1982)
pGL680[e]	70 kb	*tra*$^+$	F_{IV}	Fecal, human	GRÜNIG and LEBEK (1985)
pGL681[e]	67 kb	*tra*$^+$	F_{VI}	Fecal, human	GRÜNIG and LEBEK (1985)

[a] SMITH and HALLS (1967) described ten Hly plasmids of fecal origin

[b] LE MINOR and COUEFFIC (1975) described six Hly plasmids of human and animal origin; three belong to *inc*F_{IV}; three belong to *inc*I_2

[c] HÖHNE (1973) described three Hly plasmids derived from human UTI strains

[d] DE LA CRUZ et al. (1979 and 1980b) studied five Hly plasmids, originally isolated from SMITH (1963)

[e] GRÜNIG and LEBEK (1984) described another three Hly plasmids of human fecal origin (all *inc*F_{VI}) two 67 kb and one 105 kb in size

sin genes in a natural isolate followed when GOEBEL and SCHREMPF (1971) isolated and characterized the CCC DNA of a wild-type hemolytic strain.

The Hly plasmids, which have been isolated from various sources (see Table 1), belong to different incompatibility groups although the incI_2 and incF_{VI} groups are predominant. Their molecular masses range from 60 kb to over 160 kb and even those Hly plasmids which are otherwise structurally rather unrelated share extensive homology in their hemolysin determinants. Specific *hly* probes derived from the incI_2 plasmid pHly152 (GOEBEL et al. 1974) hybrid-

ized with corresponding DNA restriction fragments of four Hly plasmids belonging to the inc groups I_2, $F_{III,IV}$, and F_{VI} and varying in their molecular mass and transferability. These data suggest that the hemolysin determinant is a discrete genetic entity that can undergo recombination without extended sequence homology (DE LA CRUZ et al. 1980a, b; see Sect. 2.4). Carriage by Hly plasmids of additional known extrachromosomal genes such as those coding for antibiotic or heavy metal resistance or enterotoxin production has never been observed (HÖHNE 1973; STENZEL 1971a; GOEBEL, unpublished results).

Hly plasmids have been transferred by conjugation into several gram-negative bacteria including *Salmonella typhimurium, Proteus mirabilis, Proteus morganii, Shigella sonnei,* and *Serratia marcescens* (SMITH and HALLS 1967; LE MINOR and LE COUEFFIC 1975; EMÖDY et al. 1983; HACKER et al. 1985a). By cloning a 14-kb *Sal*I-*Hind*III fragment of the Hly plasmid pHly152 (GOEBEL and HEDGPETH 1982; for details see Sect. 2.4.) into the broad host range plasmid pKT231 it has been possible to introduce the *hly* determinant into an *Aeromonas hydrophila* strain (GOEBEL and CHAKRABORTY, unpublished results). Transfer into *Shigella* and *Salmonella* strains often selects for rough forms, which are presumably better recipients for these plasmids (STENZEL 1971b; HACKER et al. 1985a), and from experiments with cloned hemolysin determinants it is obvious that the *E. coli* hemolysin does not increase the virulence of *S. typhimurium* (HACKER et al. 1985a).

While some data indicating chromosomal inheritance of *hly* determinants in strains from human origin have existed for some time [i.e., the absence of *hly* conjugative transfer and no loss of the Hly phenotype following treatment with curing agents (HÖHNE 1973; MINSHEW et al. 1978)], clear-cut evidence for the existence of chromosomally borne *hly* determinants has only recently become available:

1. The isolation of plasmid-free hemolytic *E. coli* strains (HUGHES et al. 1982; MÜLLER et al. 1983)
2. Hybridization of chromosomal DNAs from nine plasmid-free urinary and fecal isolates belonging to the 0-serogroups 04, 06, 018, and 075 with *hly*-specific probes (MÜLLER et al. 1983; see also WELCH et al. 1983)
3. Absence of hybridization between plasmids of *E. coli* urinary isolates and *hly*-specific probes (DE LA CRUZ et al. 1983)
4. Cloning of *hly* determinants from total DNA of plasmid-free *E. coli* isolates of serogroup 04, 06, 018, and 075 (BERGER et al. 1982)
5. Mapping of the *hly* determinant near the *ilv* locus on the chromosome of a 04 strain (HULL et al. 1982) and at another location on the chromosome of a 018 isolate (SCHMIDT, unpublished results)

Because the chromosomally and plasmid-borne hemolysin determinants share a high degree of sequence homology (MÜLLER et al. 1983; DE LA CRUZ et al. 1983), one cannot exclude the possibility that Hly plasmids integrate entirely or in part into the chromosome of *E. coli* strains (see STENZEL 1971a; MÜLLER, unpublished results).

2.3 Cloning of *hly* Determinants

The hemolytic *E. coli* strain PM152 harbors three transmissible plasmids, 97.6 kb, 62.2 kb, and 48.5 kb, and most of the *Hin*dIII and *Eco*RI fragments of the 62.2 kb plasmid coding for the hemolytic activity (ROYER-POKORA and GOEBEL 1976) were cloned using pACYC184 and RSF2124 as vectors. One of the resulting recombinant plasmids, pANN202, which comprises the pACYC184 vector and the *Hin*dIIIE fragment (2.2 kb), was cleaved with *Bam*HI and *Sal*I, and a large *Bam*HI/*Sal*I fragment of the plasmid pHly152 was introduced into this DNA molecule. The resulting high-copy plasmid, pANN202-312, expresses amounts of external and internal hemolysin in *E. coli* K-12 similar to that of the original pHly152 (NOEGEL et al. 1979, 1981; GOEBEL and HEDGPETH 1982) and a deleted derivative, pANN202-312 *Δ Hin*dIII, carries the entire *hly* determinant on a 9.6-kb insert, the resulting plasmid DNA being thus 13.4 kb (see Fig. 1, Table 2). Very recently a *hly*$^+$ 10,5 kb *Sal*I fragment of the plasmid pGL681, originally isolated from a human fecal *E. coli* strain (see Table 1), was ligated into pACYC184 and both *E. coli* K-12 and 06 transformants harboring this plasmid are clearly hemolytic (HACKER and GRÜNIG, unpublished results).

Similar results have been described by two other groups of investigators who have cloned the *hly* determinants of the plasmids pHly185 (STARK and SHUSTER 1983) and pIP241 (NUMEROW et al. 1979, 1981). From the first plasmid, originally isolated from animal feces (see SMITH 1963), a 13.3-kb *Eco*RI insert was ligated into pBR322; from the latter plasmid (LE MINOR and COUEFFIC 1975), a 16.2-kb *Sal*I fragment was cloned, also into pBR322.

Using the cosmid cloning technique (COLLINS and HOHN 1978), WELCH et al. (1981, 1983) and BERGER et al. (1982) were able to isolate hemolytic clones from gene banks which were constructed by inserting partial *Sau3*A fragments of chromosomal DNA into the cosmid vectors pJC74 and pHC79. BERGER et al. isolated cosmid clones from four different strains belonging to the important uropathogenic serogroups 04, 06, 018, and 075. The subcloning of the *hly* determinant was achieved as described for the pHly152 *hly* gene, i.e., *Bam*HI/*Sal*I fragments of the recombinant cosmids were ligated into pANN202, comprising pACYC184 and the left (*hly*C) protion of the pHly152 determinant including the regulatory region (see Fig. 1, Table 2). The *hly*$^+$ subclones of the chromosomal *hly* determinants and the plasmid-borne *hly* genes thus differed only in the structural gene for hemolysin (*hly*A) and the genes encoding the transport system (*hly*B_a, *hly*B_b, see Sect. 2.7).

WELCH and co-workers (1981, 1983) isolated a subclone from recombinant cosmid DNA derived from the uropathogenic strain J96 (04:K6) by ligating a *hly*$^+$ 11.7-kb *Sal*I fragment into pACYC184. Interestingly, they found that among numerous Hly$^+$ *E. coli* K-12 cosmid clones isolated from this strain and the 06 strain C1212 a high percentage also encoded fimbriae production (Fim) and mannose-resistant hemagglutination (Mrh). This confirmed the close linkage of these two virulence determinants (LOW et al. 1984) already suggested by HACKER et al. (1983b) following isolation of Hly$^-$, Fim$^-$, Mrh$^-$ mutants of a 06:K15:H31 strain (see Sect. 2.5).

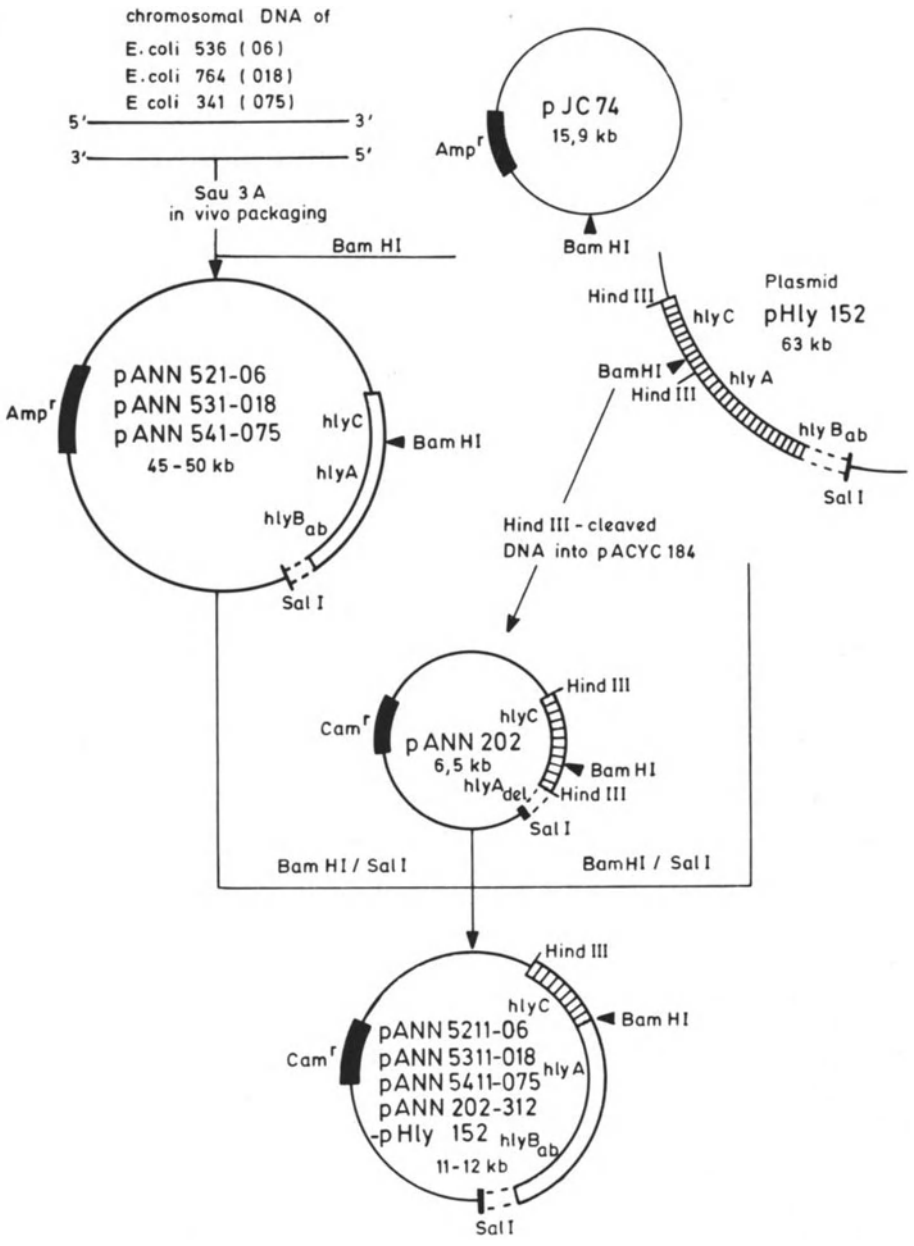

Fig. 1. Construction of the recombinant *hly*$^+$ plasmids. The *open boxes* indicate chromosomally encoded *hly* cistrons, the *hatched boxes* plasmid-encoded *hly* cistrons, and the *broken lines* a 2-kb DNA segment between the distal ends of *hly*B_b and the *Sal*I sites which encode no gene products (for details see Sect. 2.3)

Table 2. Cloned *hly* determinants from different *E. coli* strains

Designation of recombinant *hly* plasmid	Origin of the cloned *hly* determinant	Source	Cloned restriction fragment	Authors
pANN202-312 ΔH	Plasmid pHly152	Fecal, animal	9.6-kb *Hind*III	NOEGEL et al. (1979, 1981) GOEBEL and HEDGEPETH (1982)
pNR20	Plasmid pIP241	Fecal, animal	16.2-kb *Sal*I	NUMEROW et al. (1979, 1981)
pJS204	Plasmid pHly185	Fecal, animal	13.2-kb *Eco*RI	SHUSTER and STARK (1983)
pANN681	Plasmid pGL681	Fecal, human	10.5-kb *Sal*I	HACKER and GRÜNIG (unpublished)
pSF4000	Chromosome of J96 (04:K6)	UTI, human	11.7-kb *Sal*I	WELCH et al. (1981, 1983)
pANN5311	Chromosome of 764 (018:K5:H$^-$)	Fecal, human	9.0-kb *Bam*HI-*Sal*I[a]	BERGER et al. (1982)
pANN5411	Chromosome of 341 (075:K95)	UTI, human	9.0-kb *Bam*HI-*Sal*I[a]	BERGER et al. (1982)
pANN511 (cosmid)	Chromosome of 367 (04:K5:H5)	UTI, human	30-kb *Sau*3A	BERGER et al. (1982)
pANN5211[b]	Chromosome of 536 (06:K15:H31)	UTI, human	9.0-kb *Bam*HI-*Sal*I[a]	BERGER et al. (1982)
pANN532 (cosmid)[b]	Chromosome of 536 (06:K15:H31)	UTI, human	30-kb *Sau*3A	KNAPP et al. (1984)
pDAL2 5-23 (cosmids)[c]	Chromosome of C1212 (06)	UTI, human	30-kb *Sau*3A	LOW et al. (1984)
pLG570	Chromosome of LE2001	UTI, human	30-kb *Sau*3A	MACKMANN and HOLLAND (1984)

[a] The recombinant plasmids consist of the *hly*C part of pHly152 and of chromosomal *hly*A, *hly*B_a, and *hly*B_b DNA (see Fig. 1 and BERGER et al. 1982)

[b] The strain 536 bears two *hly* determinants on the chromosome (see KNAPP et al. 1984)

[c] The cosmid clones also code for mannose-resistant hemagglutination (*mrh*, see LOW et al. 1984)

2.4 Structure of *hly* Determinants

A physical map of the *hly* determinant cloned from the plasmid pHly152 is given in Fig. 2. This and restriction maps of the cloned chromosomal *hly* determinants pANN511 (UTI, serotype 04), pANN5211 (06), pANN5311 (018), pANN5411 (075; see BERGER et al. 1982), SF4000 (04; see WELCH et al. 1983), and the plasmid determinants pSJ204 (pHly185; see STARK and SHUSTER 1983) and pCW2 (WAALWIJK et al. 1984) show defined variations, particularly in the *hly* structural gene *hly*A, but the general composition of the various determinants is very similar.

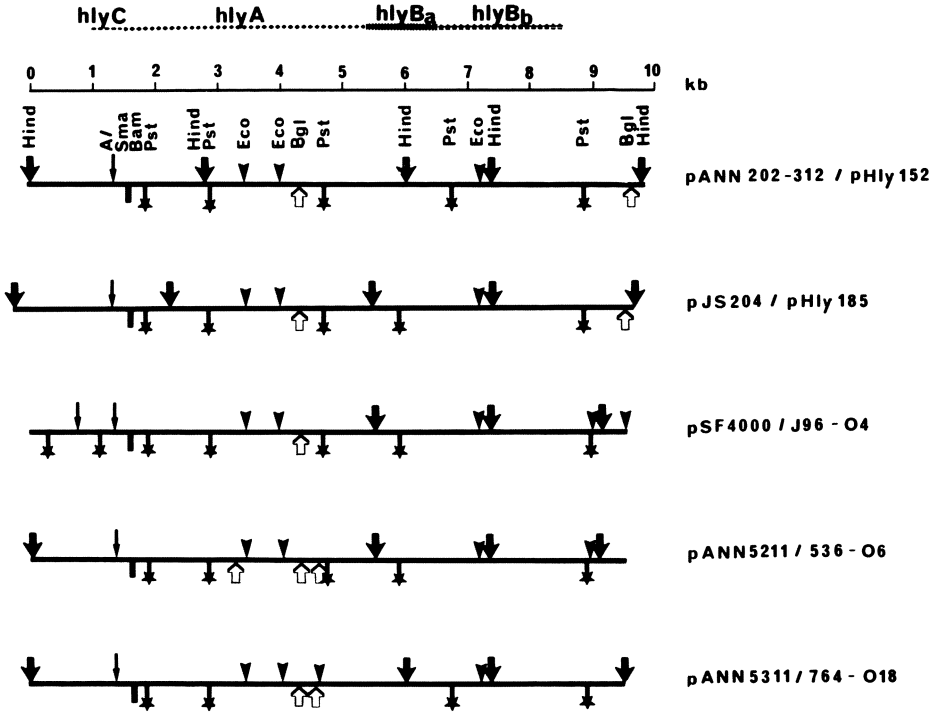

Fig. 2. Restriction map localization of the hemolysin determinant of plasmids pHly152 (pANN202-312) and pHly185 (pJS204) and of the chromosomal *hly* determinants from strains J96 (pSF4000), 536 (pANN5211), and 764 (pANN5311). The data are published by GOEBEL et al. (1984), STARK and SHUSTER (1983), WELCH and FALKOW (1984), and BERGER et al. (1982). Further data on the structure of different *hly* determinants are given by DE LA CRUZ et al. (1980a), NUMEROW et al. (1981), MÜLLER et al. (1983), MACKMAN and Holland (1984a, b), and WAALWIJK et al. (1984)

The region essential for hemolysin synthesis and secretion has been analyzed by transposition of Tn5 and Tn3 into different Hly plasmids or recombinant DNAs and selecting for mutants unable to form hemolytic zones on blood agar plates. For pANN202-312, the recombinant DNA derived from the Hly plasmid pHly152, a region of about 7 kb starting a few hundred base pairs to the left of the single *Sma* site (at coordinate 1.3 kb) and ending shortly before the *Pst*I site (at coordinate 8.8 kb), was found to be indispensable for the full hemolytic phenotype (see Fig. 2) and Hly^- mutants isolated could be divided into two groups:

1. Those which still produced internal hemolysin (i.e., hemolysin could be detected in the periplasmic space) but were unstable to secrete it (Tn3 insertions between coordinates 5.5 kb and ca. 8.6 kb)
2. Those which were unstable to synthesize hemolysin (Tn3 insertions between coordinates 1.0 and 5.5 kb).

By complementing both types of mutants with cloned fragments of the *hly* region, four complementation groups have been identified (NOEGEL et al. 1979,

1981; WAGNER et al. 1983). Two cistrons (*hly*C and *hly*A) are essential for the synthesis of active hemolysin and two other cistrons are required for its secretion (*hly*B_a and *hly*B_b). Similar functional definitions of the *hly* determinant have also been described for other plasmid-borne genes (STARK and SHUSTER 1982, 1983; NUMEROW et al. 1979, 1981) and the chromosomal *hly* determinants of 06 and 018 UTI strains (KNAPP et al. 1984; WAGNER, unpublished results).

As can be seen in Fig. 2 there is no *Sal*I cleavage site in the *hly* determinants of the strains investigated and this character was used to test whether hemolytic UTI *E. coli* strains carry multiple *hly* determinants on their chromosome. *Sal*I-cleaved chromosomal DNA of several strains was hybridized to nick-translated α-P^{32}-labeled DNA of cloned *hly* cistrons and the resulting data made it clear that two of the six strains investigated carry two *hly* determinants on the chromosome (KNAPP et al. 1984). Because Hly^- mutants of such strains can be isolated readily one may speculate that the *hly* determinants are located adjacent to each other (HACKER et al. 1983b). The two *hly* determinants of 06 strain 536 have been analyzed further and they differ slightly in their restriction patterns, thus making it unlikely that the *hly* genes are tandem duplications analogous to the *Vibrio cholerae* toxin genes (MEKALANOS 1983).

2.5 Regions Flanking *hly* Genes

In addition to the coding region of the four genes (*hly*C, A, B_a, and B_b), some of the plasmid *hly* determinants investigated so far seem to carry homologous flanking regions. On four out of nine Hly plasmids examined, a common region at the left *hly* boundary extending upstream beyond the N-terminal end of the *hly*C gene has been identified and part of this region from pHly152 was sequenced (KNAPP et al. 1984; GOEBEL et al. 1984). Comparison of this sequence with known IS elements of *E. coli* revealed identity with IS2. This and the restriction maps of the region in the four plasmids analyzed suggests strongly that a class of plasmid-borne *hly* determinants is generally flanked upstream by IS2.

The four plasmids also show a common sequence at the right end of the *hly* determinants, distal to *hly*B_b, and this sequence (in pHly152) has been localized between the *Pst*I site at 8.8 kb and the *Bgl*II site at 9.6 kb (Fig. 2). Sequencing and restriction analyses have revealed no identity with any of the known IS elements, and no clear-cut short repeats at the ends of this flanking sequence have yet been identified. This suggests that the sequence may be either a partially deleted IS element or not even a typical IS element. ZABALA et al. (1982, 1984) and DIAZ-AROCA et al. (1984) have described a new IS element, IS91, which is present distal to *hly*B_b on five Hly plasmids. It can not as yet be excluded that the 850-bp sequence located between *Pst*I and *Bgl*II found on the four plasmids mentioned above is a part of the IS91 element.

These two flanking sequences, IS2 and the 850-bp sequence, show no homology to each other (KNAPP et al. 1984) and appear to be a general feature of a class of plasmid-borne *hly* determinants, being found on Hly plasmids carried by *E. coli* strains isolated from both animal feces and human urinary tract

infections. The other five out of nine Hly plasmids (pGL680-pGL685; see above and Table 1) which are not directly flanked by the two IS elements seem to carry additional regulatory sequences adjacent to their *hly* determinants (GRÜNIG and LEBEK 1985; see Sect. 2.8). None of the chromosomal *hly* determinants examined thus far are flanked by IS2 at the *hly*C proximal end although several IS2 elements have been identified in other regions of the chromosome in some of these strains (KNAPP et al. 1984). The *hly*B_b-distal sequence of the *hly* determinant has been found close to both ends of the *hly* determinants in several hemolytic 06 strains and, although they do not lie directly next to *hly*C or *hly*B_b, evidence suggests that this sequence is involved in the frequent loss of *hly* determinants observed in 06 and 018 UTI strains (HACKER et al. 1983b; KNAPP et al. 1984). Such *hly* loss in the 06 *E. coli* strain 536 also affects the *mrh* determinant, and these data have been extended by LOW et al. (1984), who demonstrated the presence of a 1-kb sequence between the *hly* and the *mrh* determinant on the chromosome of several wild-type strains. It is interesting to speculate that these flanking sequences may be essential:

1. For the transfer of the *hly* determinant between hemolytic plasmids and the chromosome.
2. For "virulence gene pick-up", allowing the creation of a "virulence cluster" on the chromosome. This possibility is supported by the observation that spontaneous mutants can be isolated which have lost functional Hly, Mrh, and Msh (mannose-sensitive hemagglutination) and also show greatly increased sensitivity to human complement, another factor assumed to influence virulence (HUGHES, HACKER and GOEBEL, unpublished results).

2.6 Expression and Regulation

It has previously been shown for the Hly plasmid pHly152 that Tn5 insertions in *hly*C are polar, removing both *hly*A and *hly*B_a expression but not that of *hly*B_b (WAGNER et al. 1983), and similar results have also been reported using Tn3 (NOEGEL et al. 1979, 1981). These findings suggest that the *hly* determinant is transcribed from two promoters, one (*hly*P_L) located upstream of *hly*C and the other (*hly*P_R) near *hly*B_b. Similar observations have been reported for the plasmid pHly185 (STARK and SHUSTER 1983) and the cloned chromosomal *hly* determinants of 04 (WELCH et al. 1983) and 018 (WAGNER, unpublished results) isolates.

To extend information on promoter activity and regulation of transcription, protein fusion (see CASADABAN and COHEN 1979) and sequence studies have recently been performed (JUAREZ et al. 1984a, b; GOEBEL et al. 1984). Mud-1 *lac* insertions into pHly152 allowed determination of the strength of transcription along the *hly* determinant by measuring the *hly*-directed β-galactosidase activity. As shown in Fig. 3 the "operon" *hly*C, A, B of a pHly152 appeared to be transcribed rather weakly and this was confirmed by cloning the *Hin*dIII *Bam*HI fragment (see Fig. 2) into a promoter-probe vector such that the *hly*P_L regulatory region preceded the galactokinase gene (JUAREZ et al. 1984a, b).

The transcriptional activity observed, though stronger than that of $p\lambda_{482}$, a synthetic promoter determining very low transcription, was less than half that directed by the *gal* promoter. Activity of β-galactosidase in Mud-1*lac* fusions was considerably lower when Mud-1 was integrated in *hly*B_a than when it was inserted in *hly*C, suggesting a considerable decline in *hly* transcriptional activity from *hly*C through to *hly*B_a and thus strengthening the view that three of the *hly* genes are transcribed from *hly*P_L. Additional support for polycistronic transcription is provided by the observation that no transcriptional activity follows insertion of *hly*A and *hly*B_a-specific fragments into promoter-probe vectors (HUGHES, unpublished observations). Differences in Hly activity directed by *hly* determinants have been observed (WELCH and FALKOW 1984; PELLETT et al. 1984) and this has been correlated with the regulatory region preceding *hly*C. A 750-bp *Ava*I fragment containing the *hly*P_L promoter of a chromosomal *hly* determinant in place of the promoter region of the pHly152 *hly* determinant resulted in an increase in extracellular hemolysin production. This quantitative difference in hemolysin activity may be one reason why *hly* determinants are associated with distinct levels of virulence (WELCH and FALKOW 1984; HACKER et al. 1983a; see Sect. 2.8).

Current work, sequencing, and measuring transcriptional activity of various plasmid and chromosomal *hly*P_L regions will elucidate further the importance of transcriptional regulation in influencing Hly-determined virulence. Sequence data are already available for the plasmid pHly152. As can be seen in Fig. 3, almost perfect −10 (Pribnow box) and −35 consensus sequences have been found in the *hly*P_L region. There are, however, only 15 bp between them, and this could explain the weak transcriptional activity of this promoter. Recent data seem to indicate, however, that the actual start of transcription is further upstream.

A 11-bp interspace region between the C-terminal end of *hly*C and the N-terminal end of *hly*A has been identified and this probably functions as a ribosome-binding site ["Shine-Dalgarno" (SD) sequence]. The 69-bp interspace region between *hly*A and the putative start of *hly*B_a also contains an SD sequence beginning 10 bp in front of the putative start codon of *hly*B_a.

As mentioned above, the *hly*B_b cistron is transcribed from a second promoter *hly*P_R. The direction of transcription as determined by characterization of Mu-sensitive derivatives of Hly$^-$ Mud-1 insertions in *hly*B_b seems to be opposite to that of the *hly*C, A, B_a "operon." The strength of this promoter, based on the level of β-galactosidase activity of Mud-1 insertion mutants in *hly*B_b, is considerably higher than that of *hly*P_L (GOEBEL et al. 1984; JUAREZ et al. 1984b). A putative promoter sequence located upstream of the *Pst*I site at coordinate 8.8 (Fig. 2) is shown in Fig. 3 as well as a possible start codon for HlyB_b (Michel, unpublished results).

The additional isolation of HlyC-LacZ and HlyA-LacZ proteins has offered a suitable way to study more exactly observations previously made on hemolysin production:

1. Gene dosage had been assumed to have little or no influence on the level of internal and external hemolysin pools, i.e., the same hemolysin activity

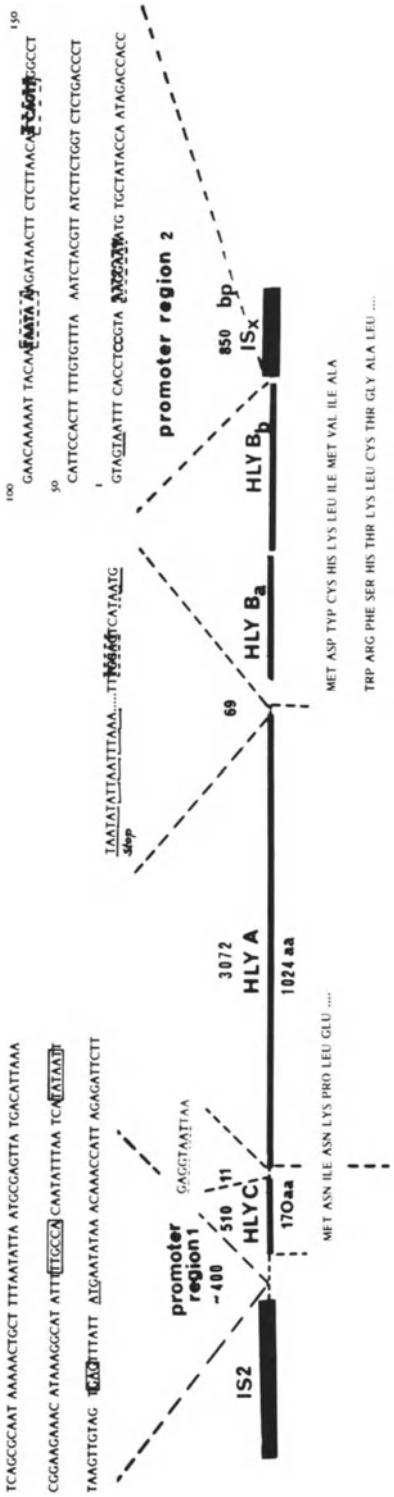

Fig. 3. Functional map of the *hly* determinant of pHly152 showing the interspace regions between *hly*C and *hly*A, and *hly*A and *hly*B$_a$, respectively, and the promoter regions 1 and 2. Characteristic sequences are specifically marked. N-terminal amino acid sequences deduced from the DNA sequences are shown for HlyC, HlyA, and HlyB (for details see Sect. 2.6 and GOEBEL et al. 1984)

had been observed irrespective of whether the hemolysin determinant was located on a low-copy plasmid (pHly152) or on a high-copy vector (pACYC184). In contrast, there is a decrease in β-galactosidase production by *hly*C-*lac*Z or *hly*A-*lac*Z constructions after cloning into pSC101 (low-copy plasmid) compared with activity in pACYC184. This strict gene-dosage effect of β-galactosidase is observed whether the other *hly* genes (*hly*A, B_a, and B_b for *hly*C-*lac*Z and *hly*B_a and B_b for *hly*C, *hly*A-*lac*Z) were present or not, ruling out possible negative regulatory functions of these *hly* gene products.

2. Hemolytic activity reaches a peak in late logarithmic phase then declines rapidly, prompting suggestions that hemolysin is synthesized and/or secreted most actively during this period. Beta-galactosidase activity by cells carrying *hly-lac*Z determinants increases directly with cell mass, reaching a plateau in early stationary phase. The kinetics of Hly activity therefore reflect the instability of the Hly protein.
3. Previous observations of Hly^+ strains growing in glucose and glycerol in addition to the use of cya^-, cap^- mutants have indicated that production of Hly is not influenced by cAMP levels (HUGHES, unpublished observations). This was confirmed by the *hly-lac*Z fusions, as cells carrying them produced equivalent amounts of β-galactosidase in the presence and absence of both cAMP and cGMP (JUAREZ et al. 1984a).
4. Significant differences observed in Hly activity when Hly^+ strains are grown aerobically or anaerobically are seen also when β-galactosidase activity is measured in *hly-lac*Z fusion-bearing strains. A threefold reduction in activity of *hly*C-*lac*Z strains was observed under conditions of reduced oxygen tension, *hly*B_b-*lac*Z fusions showing no such difference. This indicates that synthesis, but not secretion, of Hly is controlled at the transcriptional level by oxygen tension.

2.7 Gene Products and Their Function

As described above, the *hly* determinant comprises four different genes (*hly*C, hlyA, *hly*B_a, and *hly*B_b) and in order to identify their primary gene products, plasmids were constructed carrying the separate *hly* genes, isolated from the plasmid pHly152, under the control of various promoters (GOEBEL and HEDGPETH 1982; HÄRTLEIN et al. 1983; GOEBEL et al. 1984). These plasmids and pANN202-312ΔH (the entire *hly* determinant, i.e., between the *Hin*dIII sites at 0.0 kb and 9.6 kb cloned into pACYC184) were transformed into minicells and maxicells of *E. coli* and the plasmid-specific gene products radioactively labeled. Transcribed from *hly*P_L the *hly*C product could be readily identified as a 18-kilodalton protein. The primary product of *hly*A was identified as a protein of 106 kilodaltons after putting this gene under the control of the λP_R promoter (GOEBEL and HEDGPETH 1982). Expression of *hly*B_a and *hly*B_b was difficult to detect in mini- and maxicells of *E. coli*. Two proteins of about 50 kilodaltons and >60 kilodaltons were identified which may be the gene products of *hly*B_a and *hly*B_b, respectively (HÄRTLEIN et al., 1983).

While recombinant DNA techniques in conjunction with the use of mini- and maxicells have recently allowed the identification of the gene products of the *hly* determinant, much effort has previously been invested in the biochemical characterization of the active protein endproduct and its lytic action. Hemolytic activity (both intracellular and extracellular) reaches a maximum in the late logarithmic phase after which it typically decreases rapidly (LOVELL and REED 1960; SMITH 1963; SPRINGER and GOEBEL 1980). It is stimulated by growth in complex media and by the presence of hemoglobin (RENNIE and ARBUTHNOTT 1971; JORGENSEN et al. 1976). It remains open whether these conditions enhance synthesis or secretion but it is evident that active hemolysin is extremely unstable and appears to be stabilized by the presence of molecules such as hemoglobin, with which it appears to associate (HÄRTLEIN, unpublished results). The instability and apparent association of mature hemolysin explains varying molecular weights cited in the literature including 58 kilodaltons (NOEGEL et al. 1979, 1981), 120 kilodaltons (WILLIAMS 1979), and 600 kilodaltons (RENNIE and ARBUTHNOTT 1974). More recently MACKMAN and HOLLAND (1984a, b) have identified in the supernatant of hemolytic *E. coli* strains proteins of 107 kilodaltons, i.e., corresponding to the primary *hly*A gene product.

In *E. coli* K-12 carrying the cloned *hly* determinant of pHly152, a 58-kilodalton protein seems to be associated with hemolytic activity in the supernatant. Active hemolysin outside the cytoplasm is only found when the two *hly* genes, *hly*A and *hly*C, are present in the cell and it has been proposed that in the presence of the 18-kilodalton HlyC protein the 106-kilodalton (HlyA) protein is processed and the major proteolytic product of 58 kilodaltons is hemolytically active and transported across the cytoplasmic membrane (HÄRTLEIN et al. 1983; WAGNER et al. 1983; GOEBEL et al. 1984). MACKMAN and HOLLAND (1984a, b) have found the 107-kilodalton protein in the supernatant of several strains, some of which harbor hemolytic plasmids whereas others bear the *hly* determinant on the chromosome. They claim that the secreted 107-kilodalton protein carries the hemolytic activity (MACKMAN and HOLLAND 1984b). Thus, it is difficult to decide at the moment whether the proteolytic degradation of the 106-kilodalton protein is an artifact of the system used (mini- and maxicells) or whether proteolysis of HlyA is occurring during the transport of HlyA.

The function of HlyC is not yet understood but it appears to convert the 107-kilodalton protein (or a proteolytic product of it) to active hemolysin since strains harbouring the cloned *hly*A gene or Hly^- mutants with Tn3 within *hly*C do not yield active hemolysin in logarithmic growth phase (WAGNER, unpublished results; EMÖDY et al., 1982 and unpublished results).

Hemolysin is secreted through the outer membrane of the *E. coli* cell only when *hly*C and *hly*A are complemented by $hlyB_a$ and $hlyB_b$. The two gene products of $hlyB_a$ and $hlyB_b$ were found predominantly in the outer membrane fraction (HÄRTLEIN et al. 1983; WAGNER et al. 1983). Mutants defective in $hlyB_a$ form no hemolysis zones on blood agar and accumulate amounts of hemolysin in the periplasm, whereas $hlyB_b$ mutants form small hemolysis zones around growing colonies. These and other data have led to a model which suggests that $HlyB_a$ protein fixes hemolysin to the outer membrane from where it is released by the $HlyB_b$ protein (see GOEBEL et al. 1984; WAGNER et al. 1983).

The cell-bound β-hemolysin of *E. coli* described by SMITH (1963) and subsequently by others could be the hemolysin produced by $HlyB_b$ mutants of α-hemolytic strains.

The observation (TSCHÄPE and RISCHE 1974; WAGNER, unpublished results) that the same hemolysin plasmid can determine different levels of hemolysin in different host strains infers a possible influence of chromosomal genes on the production and/or secretion of hemolysin. Recent description of chromosomal mutations that specifically inhibit (JUAREZ and GOEBEL 1984) or increase (JUAREZ, personal communication) secretion of hemolysin without affecting transport of periplasmic proteins or secretion of colicins E1, E2, and E3 suggests that, in addition to the *hly*-specific gene products $HlyB_a$ and $HlyB_b$, other specific cellular proteins may be involved in the hemolysin transport.

2.8 Role in Pathogenicity

From epidemiological studies it is evident that there is a high incidence of hemolysin production among *E. coli* strains, causing urinary tract infection (UTI, see Sect. 2.1 and HUGHES et al. 1982); and it is commonly associated with other factors assumed to contribute to virulence, i.e., mannose-resistant hemagglutination (GREEN and THOMAS 1981; VÄISÄNEN-RHEN et al. 1984) and specific O- and K-antigens (EVANS et al. 1981; VAN DEN BOSCH et al. 1982b). Such strains are also found in the fecal flora, where they constitute a "pool" of potential urinary pathogens (HACKER et al. 1983c).

The view that hemolysin contributes to *E. coli* nephropathogenicity has been supported by the use of different in vivo models (FRIED and WONG 1970; FRIED et al. 1971; MINSHEW et al. 1978; KETYI et al. 1978; VAN DEN BOSCH et al. 1982a; WAALWIJK et al. 1982). Nevertheless data from such studies have been obtained with clinical isolates or genetic variants of strains which have been mutagenized or have lost or received large plasmids or parts of the chromosome (EMÖDY et al. 1980; LINGGOOD and INGRAM 1982; HULL et al. 1982). They are, therefore, indicative rather than conclusive as the genetic background of the organisms examined was undefined and the specific contribution of hemolysin itself cannot readily be deduced.

The use of recombinant DNA techniques has resolved this problem. WELCH et al. (1981) (see Table 3) cloned a chromosomal *hly* determinant as 11.7-kb DNA fragment (see Sect. 2.3) and introduced the recombinant DNA into a nonhemolytic 022 fecal strain. The results show clearly that the *hly* determinant increases virulence in a rat peritonitis model and similar data have been obtained following introduction of four different cloned *hly* determinants into a Hly^- mutant of the 06 urinary isolate (HACKER et al. 1983a). The plasmid pHly152 *hly* determinant increased the toxicity of strains for mice by a marginal degree whereas the cloned chromosomal *hly* determinants from two *E. coli* strains of serogroup 018 and 075 resulted in markedly greater toxicity without changing the serum resistance of the strains (HACKER et al. 1983a; HUGHES, HACKER and GOEBEL, unpublished results). Recent studies with strains carrying different cloned *hly* determinants in various animal models (see Table 3; HACKER et al.

Table 3. Contribution of cloned *E. coli hly* determinants to virulence in different animal models

Host strain	Recombinant *hly* plasmid	Origin of the *hly* determinant	Animal model	Degree of virulence effect	Authors
J198(022)[a]	pANN202-312	Plasmid pHly152	Rat peritonitis	No	WELCH et al. (1981)
536-21(06)[b]	pANN202-312	Plasmid pHly152	Rat pyelo-nephritis	Low	MARRE et al. (to be published)
536-21(06)[b]	pANN202-312	Plasmid pHly152	Mouse toxicity	Low	HACKER et al. (1983a)
536-21(06)[b]	pANN202-312	Plasmid pHly152	Mouse lung edema	No	EMÖDY (unpublished)
536-21(06)[b]	pANN202-312	Plasmid pHly152	Mouse nephro-pathogenicity	No	EMÖDY (unpublished)
536-21(06)[b]	pANN202-312	Plasmid pHly152	Embryo chicken	Low	HACKER and JUNGWIRTH (unpublished)
J198(022)	pJS204	Plasmid pHly184	Rat peritonitis	Inter-mediate	WELCH and FALKOW (1984)
536-21(06)	pANN681	Plasmid pGL681	Mouse toxicity	High	HACKER (unpublished)
536-21(06)	pANN681	Plasmid pGL681	Mouse sepsis	High	HOF and HACKER (unpublished)
J198(022)	pSF4000	Chromosome J96(04)	Rat peritonitis	High	WELCH et al. (1981)
J198(022)	pANN5211	Chromosome 536(06)	Rat peritonitis	Low	WELCH and FALKOW (1984)
536-21(06)	pANN5211	Chromosome 536(06)	Rat pyelo-nephritis	Inter-mediate	MARRE et al. (to be published)
536-21(06)	pANN5211	Chromosome 536(06)	Mouse toxicity	Inter-mediate	HACKER et al. (1983a)
536-21(06)	pANN5211	Chromosome 536(06)	Mouse nephro-pathogenicity	High	EMÖDY (unpublished)
536-21(06)	pANN5211	Chromosome 536(06)	Embryo chicken	Low	HACKER and JUNGWIRTH (unpublished)
536-21(06)	pANN5311	Chromosome 764(018)	Rat pyelo-nephritis	High	MARRE et al. (to be published)
536-21(06)	pANN5311	Chromosome 764(018)	Mouse toxicity	High	HACKER et al. (1983a)
536-21(06)	pANN5311	Chromosome 764(018)	Mouse lung edema	Low	EMÖDY (unpublished)
536-21(06)	pANN5311	Chromosome 764(018)	Mouse nephro-pathogenicity	High	EMÖDY (unpublished)
536-21(06)	pANN5311	Chromosome 764(018)	Mouse sepsis	High	HOF and HACKER (unpublished)

[a] Hly^- fecal strain
[b] Hly^- mutants of uropathogenic *E. coli* strain (see HACKER et al. 1983b)

Table 3 (continued)

Host strain	Recombinant *hly* plasmid	Origin of the *hly* determinant	Animal model	Degree of virulence effect	Authors
536-21(06)	pANN5311	Chromosome 764(018)	Embryo chicken	High	HACKER and JUNGWIRTH (unpublished)
536-21(06)	pANN5411	Chromosome 341(075)	Mouse toxicity	High	HACKER et al. (1983a)
536-21(06)	pANN5411	Chromosome 341(075)	Mouse lung edema	Low	EMÖDY (unpublished)
536-21(06)	pANN5411	Chromosome 341(075)	Mouse nephro-pathogenicity	High	EMÖDY (unpublished)

to be published) clearly show the contribution of hemolysin to virulence and data from an experimental rat pyelonephritis model in these studies strongly indicate an involvement of hemolysin in renal infections (MARRE et al. to be published).

While the contribution of hemolysin to virulence of *E. coli* strains appears to be established, the mechanism by which it is exerted remains unclear. One might assume that hemolysin would increase the pathogenicity of urinary strains by damaging kidney cells or destroying leukocytes and results are indeed available which demonstrate a cytotoxic effect of hemolysin on eukaryotic cells (CHATURVEDI et al. 1969; DEPAUW et al. 1971; FRY et al. 1975; CAVALIERI and SNYDER 1982b, c; RATINER et al. 1976; PEERBOOMS et al. 1984). In addition destruction of monocytes and leukocytes by Hly$^+$ *E. coli* has been reported (GADEBERG et al. 1983; GADEBERG and ØRSKOV 1984; CAVALIERI and SNYDER 1982a) but as with many animal tests only wild-type isolates and not isogenic strains with and without cloned *hly* determinants have been employed for comparison. Preliminary studies using the latter in genetically manipulated variants indicate an influence of hemolysin on leukotriene generation and histamine release from human granulocytes and rat mast cells (SCHEFFER and KÖNIG, unpublished results).

In addition to the presumptive cytotoxic potency of hemolysin, a bacteriolytic effect has been reported (JORGENSEN et al. 1983). Nevertheless this seems to be a strain-specific phenomenon without common relevance as it has not been observed among other Hly$^+$ strains (GOEBEL, unpublished results).

Hemolysin may also act as a virulence factor by enabling hemolytic strains to obtain iron (LINGGOOD and INGRAM 1982; WAALWIJK et al. 1983) and this effect has been confirmed by the observation that strains bearing some Hly plasmids show a reduced secretion of hemolysin in the presence of $FeCl_3$ and an increase when grown in the presence of iron chelators (GRÜNING and LEBEK 1985). A 10.5 kb *Sal*I *hly*$^+$ fragment cloned into pACYC184 still confers this "Fe^{3+} effect" and as these plasmids (e.g., pGL680, pGL681, see Table 1) have flanking regions different from those of the common plasmid type (see Sect. 2.5),

sequences upstream of the *hly*C gene are assumed to be responsible for this effect.

Cloned hemolysin determinants exert different levels of virulence to strains tested in various in vivo systems (see HACKER et al. 1983a). As described above (Sect. 2.3) these clones were constructed in such a way that the DNA upstream of *hly*C remained constant (always deriving from pHly152), thus excluding the influence of different regulatory regions (see Fig. 1). It was proposed that small differences in *hly*A, the structural gene for hemolysin, are responsible for the differences in virulence and this has been supported by subsequent work showing sequence differences at the C-terminal and of *hly*A (WAGNER, JUAREZ, GOEBEL, unpublished results). Recent experiments in which hybrid determinants of chromosomal (serogroup 018) and plasmid (pHly152) DNA were constructed seem to indicate that a major cause of these differences directed by hemolysin is not only the sequence composition of *hly*A but also its combination with *hly*C (WAGNER, GOEBEL and HACKER, unpublished results). In addition to the specific Hly transport system, differences in the promoter sequences of *hly* determinants (WELS, KNAPP, GOEBEL, unpublished results) and also the host background (WAGNER, KNAPP, HACKER, unpublished results) influence the level of virulence evoked by hemolysin.

3 Conclusion

Recombinant DNA techniques, particularly the use of gene cloning and hybridization probes, have provided new insights into the determination of bacterial virulence, allowing initiation of extensive studies into the genetics and biochemistry of the responsible virulence factors. This has been especially true of toxins, including the hemolysins produced by a wide variety of species (HACKER and HUGHES 1985).

The first bacterial virulence determinant to be cloned was that encoding *E. coli* ST toxin (SO et al. 1976) and initial focus on this organism and plasmid-borne genes continued with the isolation of the extrachromosomal *E. coli* hemolysin determinants (NOEGEL et al. 1979, 1981). This and the subsequent cloning of chromosomal *hly* genes (WELCH et al. 1981; BERGER et al. 1982) has provided the basis for precise analysis of *E. coli* hemolysin production. Nevertheless a number of questions remain unanswered. How are the two *hly* transcriptional units regulated? How do the four characterized gene products interact, i.e., what is the precise function of the *hly*C product and what is the nature of the specific transport process? What is the mode of action of hemolysin on erythrocytes and eukaryotic cells in general? The different levels of virulence exerted by the various *hly* clones is still to be clarified and the biological activity of hemolysins may also be studied now by the use of hybrid genes, site-specific mutagenesis, and sequence analysis in conjunction with biological assays.

The parallel cloning of genes encoding hemolysin production (*hly*), mannose-resistant hemagglutination (*mr*h), mannose-sensitive hemagglutination (*ms*h), and serum resistance (see BINNS et al. 1979; SVANBORG-EDÉN 1984) will allow

further elucidation of their interaction in determining multifactorial virulence and this should prove to be particularly pertinent in view of their close genetic association.

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Note Added in Proof

After finishing this manuscript another review on *E. coli* hemolysin has been published: CAVALIERI, SJ, BOHACH GA, SNYDER IS (1984) *Escherichia coli* α-hemolysin: Characteristics and probable role in pathogenicity. Microbiol Rev 48:326–343

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Genes Determining Adhesin Formation in Uropathogenic *Escherichia coli*

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1 Introduction

The ability to adhere to the site of infection is considered to be a prerequisite for most bacterial infections. For a number of gram-negative species there is evidence that filamentous surface structures on the bacteria, called pili or fimbriae, are the adhesins that bind to epithelial cells of the host. The adhesins interact with complementary receptors which are carbohydrate-containing polymers on the target cells. The importance of adhesion in pathogenesis of infectious disease has stimulated intensive research aimed at elucidating the molecular basis of such interactions. It is hoped that an increased knowledge about bacterial adhesion will facilitate more rational developments of therapeutic or preventive measures against a given pathogen. Analysis of genetic determinants has provided a great deal of insight into the biogenesis of adhesins. By molecular cloning techniques, in vitro mutagenesis, DNA sequencing, and other procedures, individual gene products are identified and their functions assessed. Studies of regulatory features help our understanding of what molecular mechanisms determine expression of the virulence function.

We will here focus on recent studies on uropathogenic *E. coli* strains with special regard to the molecular genetics of the adhesins.

2 Binding Specificity and Structure of *Escherichia coli* Pili Adhesins

Bacterial adherence classically has been visualized by the hemagglutination assay. The binding of a bacterium to two or more erythrocytes causes visible

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aggregation of the red blood cells. That carbohydrate-containing components could be involved was suggested by the fact that sugars could inhibit the agglutination of erythrocytes. Mannose-specific adhesins, defined by the inhibition of hemagglutination when D-mannose is present, were shown to be widely distributed among enteric gram-negative bacteria (DUGUID 1968). The mannose-*sensitive* hemagglutination (MSHA) was found to be correlated to the presence of fimbriae on the bacteria (DUGUID and GILLIES 1957). Purified preparations of such fimbriae, also referred to as type 1 pili (BRINTON 1965), agglutinate guinea-pig erythrocytes and bind to monkey kidney cells (SALIT and GOTSCHLICH 1977a; SALIT and GOTSCHLICH 1977b). Type 1 piliated *E. coli* also bind to Tamm-Horsfall uromucoid, which suggests that the biological role of the adhesin could be to mediate attachment to mucus in the intestinal tract (ØRSKOV et al. 1980). The data from studies on the MSHA adhesins clearly suggest that type 1 pili bind to mannose-containing receptors. The pili were shown to represent polymers of a single polypeptide subunit, pilin (BRINTON 1965), and it is therefore likely that the pilin protein mediates the binding specificity.

Bacterial adhesins causing mannose-*resistant* hemagglutination (MRHA) have also been correlated to the presence of pili structures on the bacterial surface. Enterotoxigenic *E. coli* (ETEC) isolated from piglets often possesses the K88 fimbrial adhesive antigen (ØRSKOV et al. 1961). Similarly, ETEC isolated from calves may express MRHA and pilus-like surface antigens such as K99 (ØRSKOV et al. 1975), and different colonization factor antigen (CFA) adhesins are found in human diarrheal ETEC strains (EVANS et al. 1975; EVANS and EVANS 1978). The exact structures of the receptors to which the bacteria bind remain to be elucidated for most of the adhesins associated with ETEC isolates. The present evidence clearly implicates carbohydrates in the form of glycolipids and glycoproteins (KEARNS and GIBBONS 1979; FARIS et al. 1980).

The most well characterized case at present in terms of binding specificity and receptor structure is that of *E. coli* isolates associated with urinary tract infection (UTI) and pyelonephritis. Such strains may express MRHA and have been shown to adhere to human uroepithelial cells in vitro (SVANBORG-EDÉN et al. 1976; SVANBORG-EDÉN et al. 1977; KÄLLENIUS and WINBERG 1978; KÄLLENIUS and MÖLLBY 1979; KÄLLENIUS et al. 1980a). Studies with pyelonephritic *E. coli* reveal that P blood group antigens belonging to the globoseries of glycolipids are receptors for the bacteria (KÄLLENIUS et al. 1980b; LEFFLER and SVANBORG-EDÉN 1980; VÄISÄNEN et al. 1981). Those glycolipids contain a digalactoside moiety, α-D-Gal*p*-(1→4)β-D-Gal*p*, and the presence of a synthetically made form of the sugar inhibits the binding of P-specific *E. coli* to uroepithelial cells (SVENSON et al. 1983). The majority of uropathogenic *E. coli* strains show this digalactoside-binding specificity and pili on the bacterial surfaces presumably mediate the adhesion (KORHONEN et al. 1982; VÄISÄNEN-RHEN et al. 1984). Purified pili from such bacteria also bind to synthetic digalactoside-receptors on latex beads and are referred to as P-fimbriae, Pap (*p*ili *a*ssociated with *p*yelonephritis) pili, or Gal-Gal-binding pili (KORHONEN et al. 1982; NORMARK et al. 1983; O'HANLEY et al. 1983). The MRHA adhesins of uropathogenic *E. coli* that do not show the digalactoside-binding specificity were provisionally named X-adhesins until their receptors are characterized (VÄISÄNEN et al. 1981).

Table 1. Properties of some pili adhesins cloned from uropathogenic *E. coli*

E. coli isolate	Pilus serotype	Pilin M_r (daltons)	Hemagglutination of erythrocytes	Carbohydrate specificity	Reference
J96	F13[a]	19500	MRHA-human monkey	Gal-Gal	NORMARK et al. (1983)
IA2	–[b]	17000	MRHA-human	Globoside[c]	CLEGG and PIERCE (1983)
C1212	F7	22000	MRHA-human monkey	Gal-Gal	LOW et al. (1984)
AD110	$F7_2$	17000	MRHA-human monkey	Globoside[c]	VAN DIE et al. (1983)
KS71	"KS71A"	22000	MRHA-human	Globoside	RHEN et al. (1983a)
KS71	"KS71B"	19000	MRHA-human	Globoside	RHEN et al. (1983a)
536	–[b]	16500[d]	MRHA-bovine	X-type	BERGER et al. (1982)
J96	F1A (type 1A)	17000	MSHA[e]	D-Mannoside	O'HANLEY et al. (1983)
KS71	F1C (type 1C)	17300	–[f]	–	RHEN et al. (1983a)

[a] F. ØRSKOV and I. ØRSKOV, personal communication
[b] Not defined
[c] LUND et al. (1985)
[d] J. HACKER, personal communication
[e] Agglutination of human, guinea pig, horse, and sheep erythrocytes; binds to Tamm-Horsfall uromucoid
[f] No HA detected

Purified pili from *E. coli* strains expressing pili adhesins seem to consist mainly of single pilin polypeptide subunits. The molecular weights of the subunits typically range between about 15000 and 25000 daltons and pilins often contain two cysteine residues, presumably forming a disulfide loop in the N-terminal half (O'HANLEY et al. 1983). Studies of type 1 pili structure showed that the pilin subunits form a right-handed helix with $3^1/_8$ subunits per turn and a diameter of 7 nm (BRINTON 1965). Presumably, the arrangement of subunits is similar in a number of *E. coli* pili representing different binding specificities. The predicted secondary structures of Pap, K99, and type 1 pilin subunits, as deduced from primary sequence data, show a great deal of similarity (BÅGA ET AL. 1984; ROSENDAAL et al. 1984; KLEMM 1984). Some pili adhesins from uropathogenic *E. coli* which are currently under study by the molecular genetic approach are listed in Table 1.

Although it has been assumed that the binding specificity resides within the structure of the pilin, in no system is there direct evidence that the pilin itself binds to a receptor. The biochemical studies have not excluded the possibility that some very minor component in a pilus preparation might be the actual adhesin. In this context it should also be noted that nonfimbrial adhesins also exist in *E. coli* (DUGUID et al. 1979; WILLIAMS et al. 1984).

3 Genetics

Recombinant DNA-aided analyses show that pili adhesins have common features in that they are produced by multicistronic gene clusters. Commonly, such determinants consist of a major subunit structural gene and three to seven auxiliary genes. The latter genes presumably provide functions involved in translocation and assembly of the subunits during biogenesis. The K88 and K99 adhesins, which are plasmid encoded, are among those that have been most extensively studied (for reviews see: GAASTRA and DE GRAAF 1982; MOOI and DE GRAAF, this volume).

The genes involved in production of type 1 pili and the determinants encoding MRHA and pili adhesins in uropathogenic *E. coli* have been found to reside on the bacterial chromosome (BRINTON et al. 1961; SWANEY et al. 1977; HULL et al. 1981). Recently, a number of MRHA determinants have been cloned in *E. coli* K-12 after construction of genomic libraries from *E. coli* associated with pyelonephritis or upper urinary tract infections (HULL et al. 1981; BERGER et al. 1982; CLEGG 1982; RHEN et al. 1983a; VAN DIE et al. 1983; LOW et al. 1984). Characterization of a clone from the pyelonephritic *E. coli* strain J96 showed that a digalactoside-specific pilus adhesin is produced from a multi-

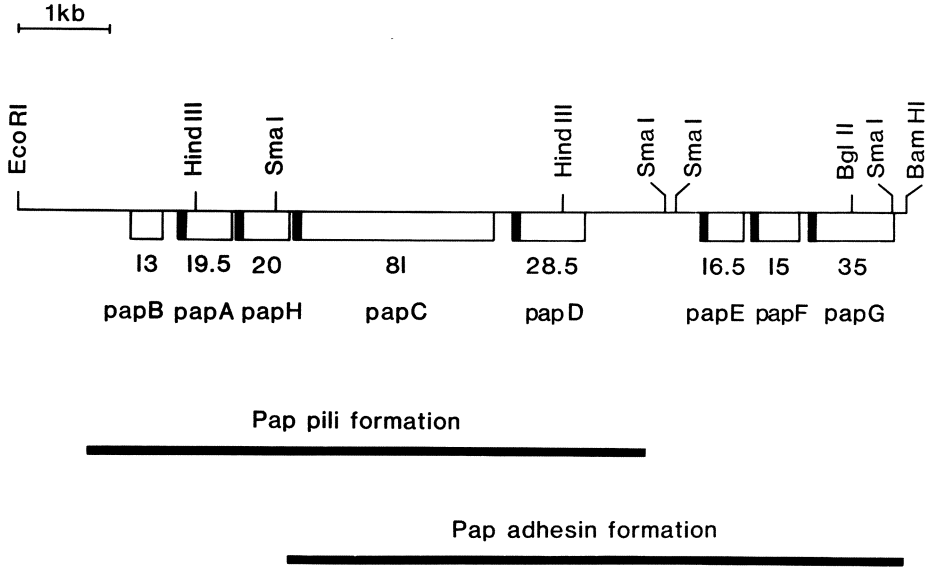

Fig. 1. Gene organization of the Pap pilus-adhesin determinant carried by plasmid pPAP5. The physical map represents DNA originating from the pyelonephritogenic *E. coli* strain J96. The cloning vector (pBR322) DNA is not shown. The *boxes* indicate positions of individual *pap* genes (*A–H*), and the molecular weights (in kilodaltons) of the corresponding gene products are also given. The *thick vertical bars at one end of the boxes* indicate the parts of genes coding for signal peptides. In the case of the *papH* gene the presence of a signal peptide has been hypothesized from DNA sequence data although as yet a precursor has not been found. The *horizontal bars* below the gene map indicate the minimal segments required for Pap pili formation and for Pap adhesin formation as defined by studies of mutant derivatives

cistronic cluster of genes denoted *pap* genes (NORMARK et al. 1983; NORGREN et al. 1984; Fig. 1). At least eight *pap* genes have been identified within a segment of about 8.5 kilobase pairs (kb) of DNA required for Pap pilus formation and digalactoside-binding. The *papA* gene codes for the Pap pilin subunit protein (NORMARK et al. 1983; BÅGA et al. 1984). A structural comparison of the *pap* DNA in plasmid pPAP5 with determinants that have been characterized from other uropathogenic *E. coli* (Table 1) indicates that digalactoside-specific clones may be rather similar. The original clone from strain IA2 was shown to express pili and MRHA, and subsequent studies of a subclone (pDC5) showed that at least four cistrons were concerned with adhesin production (CLEGG 1982; CLEGG and PIERCE 1983). Similarly, the clone pPIL110-35 from strain AD110 was shown to carry at least five genes involved in MRHA expression and pilus biosynthesis (VAN DIE et al. 1984). Alignment of the physical maps and DNA hybridization experiments with the three clones show that they are largely homologous in the region covering the *papC–D* genes of pPAP5 (Fig. 2; LUND et al., 1985). The pilin subunit gene in pPIL110-35 is located at a position similar to that of the *papA* gene in pPAP5 and hybridizations reveal that there is homology although the patterns of restriction endonuclease sites differ. The pDC5 clone does not carry DNA corresponding to the *papB-A* region.

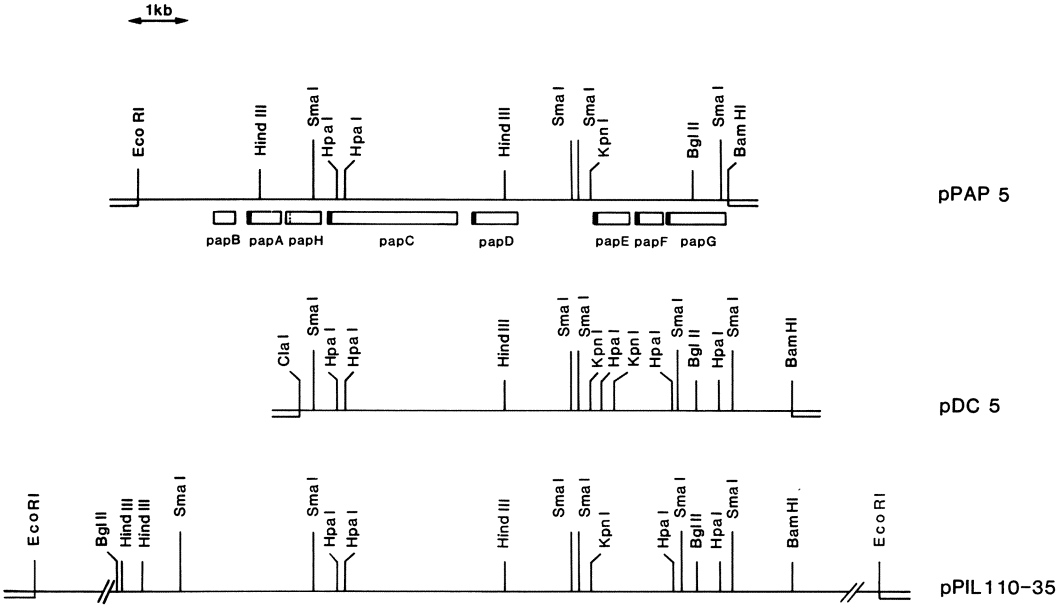

Fig. 2. Structural comparison of cloned pilus-adhesin determinants of three uropathogenic *E. coli*. The cloned chromosomal DNA (*single lines*) in pPAP5, pDC5, and pPIL110-35 originated from *E. coli* isolates J96, IA2, and AD110, respectively (see Table 1; HULL et al. 1981; CLEGG 1982; VAN DIE et al. 1983). After restriction endonuclease analysis and hybridization studies the maps have been aligned to indicate regions of similarity (LUND et al., 1985; see the text for further explanation). Only portions of the vector plasmids are shown at the junctions with the cloned DNA

Pap DNA probes do not hybridize to chromosomal DNA of *E. coli* K-12 laboratory strains and only in rare cases do probes show homology to other isolates lacking MRHA properties (HULL et al. 1984). The uropathogenic *E. coli* which express MRHA therefore seem to have acquired a segment of additional DNA when compared with the normal fecal *E. coli*.

There may also be multiple MRHA gene clusters in a given uropathogenic strain. Two cosmid clones mediating MRHA and one mediating MSHA were recently obtained in cloning experiments with the pyelonephritogenic *E. coli* strain KS71 (RHEN et al. 1983a; Table 1). The two MRHA clones show P-blood group specificity and are thought to produce different, although serologically cross-reacting, fimbriae (RHEN et al. 1983a). Similarly, two different clones mediating Gal-Gal specific binding were isolated from the pyelonephritogenic strain C1212 (LOW et al. 1984).

Production of alpha-hemolysin (Hly) is another virulence-associated property for which the genetic determinants are found mainly in virulent strains. In some uropathogenic *E. coli* the Hly and MRHA determinants have been shown to be closely linked (HACKER et al. 1983; LOW et al. 1984). The presence of repeated sequences flanking those determinants led to the suggestion that acquisition might have involved transposition events (HACKER et al. 1983; LOW et al. 1984).

3.1 Genetic Separation of Adhesin and Pilus Formation

The bacterial component which directly interacts with the host receptor glycoconjugate has been a focus of attention in the molecular genetic analysis of Gal-Gal binding pili of *E. coli* J96. The identification and characterization of individual *pap* genes by transposon insertion mutagenesis gave results indicating that Pap pili formation alone is not sufficient for expression of adhesion (NORGREN et al. 1984). Insertions of transposon Tn*5* in the *papE–G* region (see Fig. 1, Table 2) abolished binding although pili were formed on the bacterial surface. Furthermore, one of the transposon insertions in the Pap pilin subunit structural gene, *papA*, did not inactivate MRHA expression (NORMARK et al. 1983; NORGREN et al. 1984). Two other mutagenesis approaches were therefore devised to test directly whether the PapA protein, and thereby the Pap pilus, is required for digalactoside-specific binding. Firstly, in vitro frameshift mutagenesis of the *papA* gene by insertion of a DNA linker did not alter the MRHA expression or specificity (Table 2; LINDBERG et al. 1984). Secondly, derivatives lacking the entire *papA* gene due to deletion were shown to be proficient in mediating digalactoside-specific MRHA and in providing *E. coli* K-12 with the capacity to attach to urinary bladder cells (Table 3; UHLIN et al. 1985). The *papA* mutant clones do not mediate formation of Pap pili as analyzed by electron microscopy. It could thereby be concluded that digalactoside-specific binding does not require the presence of Pap pili.

Analysis of DNA linker insertion mutants in the *papF* and *papG* cistrons confirmed the results from Tn*5* mutagenesis showing that digalactoside-specific agglutination can be abolished although Pap pili are formed (LINDBERG et al.

Table 2. Mutational analysis of the *pap* gene cluster of *E. coli* J96

Method	Mutation	MRHA[a]	Pili[b]	Reference
Transposon insertion	*papB*::Tn*5*	−	+	NORGREN et al. (1984)
	papA::Tn*5*[c]	+	−	
	papC::Tn*5*	−	−	
	papD::Tn*5*	−	−	
	papF::Tn*5*	−	+	
	papG::Tn*5*	−	+	
Linker insertion	*papA1*	+	−	LINDBERG et al. (1984)
	papE1	+	+	
	papF1	−	+	
	papG1	−	+	
	papA1, *papE1*	−	−	

[a] MRHA was shown to be Gal-Gal specific by assaying both P_1 and p-erythrocytes. The digalactoside specificity was also confirmed by agglutination tests with latex beads carrying synthetic digalactoside receptor

[b] Presence of pili on bacteria was monitored by electron microscopy

[c] One of two *papA*::Tn*5* mutants shows MRHA-positive phenotype in some *E. coli* K-12 backgrounds (NORMARK et al. 1983)

Table 3. Phenotypes of *pap* deletion derivatives[a]

pap genes present	Adhesion[b]	Pili[c]	Relative amount of Pap antigen
B A H C D E F G	+	+	1
B A H C D	−	+	0.9
H C D E F G	+	−	0.3
E F G	−	−	0.1
None	−	−	<0.02

[a] Different parts of the *pap* determinant (see Fig. 1) were subcloned on an expression vector (UHLIN et al. 1985)

[b] Adhesion was monitored as agglutination of erythrocytes and latex beads (Gal-Gal specific; see Table 2) and as binding to urinary bladder tissue culture cells

[c] Electron microscopic examination

[d] Presence of Pap antigen in cell extracts was determined by a competitive ELISA using Pap pili and antiserum raised against purified Pap pili (NORMARK et al. 1983; NORGREN et al. 1984)

1984). Furthermore, no digalactoside-specific hemagglutination was observed when purified Pap pili from a *papF*, or a *papG* mutant, were assayed. The evidence therefore shows that Pap pilus formation and Pap adhesin formation can be separated genetically (Fig. 1).

Results indicating that formation of adhesins and pili can be separated have also been obtained from studies of a clone from a UTI strain expressing X-type binding specificity (BERGER et al. 1982). Derivatives which could mediate MRHA, but no longer pili, were found among subclones of the cosmid carrying

DNA from strain 536 (J. HACKER, personal communication). From studies of the clone from strain IA2 which was discussed above (i.e., pDC5), it appears that, also in that case, synthesis of the major pilus subunit is abolished although the subclone still expresses MRHA (LUND et al., 1985).

3.2 Genes Mediating Digalactoside Specificity

The products of the *papF* and *papG* genes seem both to be required for expression of the digalactoside-binding. As mentioned above inactivation of either of them results in loss of adhesion although Pap pili are formed (Table 2). With respect to piliation there is some difference between *papF* and *papG* mutants. Whereas a *papG* mutant appears to mediate normal amounts of pili there is significantly less pili produced by a *papF* mutant in comparison with the wild-type Pap clone (LINDBERG et al. 1984). At present neither of the two genes can be excluded as a candidate for being the Pap adhesin structural gene.

The fact that pili purified from bacteria carrying a wild-type Pap clone do show digalactoside-binding argues for that it should be possible to detect the adhesin component in such pili preparations. Analysis by polyacrylamide gel electrophoresis failed to provide evidence for presence of polypeptides other than the *papA* gene protein (O'HANLEY et al. 1983). However, serological data clearly indicate that some minor Pap protein(s) in fact is associated with the PapA protein polymers (UHLIN et al. 1985; LINDBERG et al., unpublished data). Antiserum raised against purified Pap pili reacts in an ELISA with bacteria which carry the *papC–D* genes and express MRHA but lack the *papA* gene (Table 3). Furthermore, external labeling of a Pap pilus preparation with radioactive iodine (^{125}I) and subsequent gel electrophoretic analysis has revealed that the *papE* gene product is present as a minor component (LINDBERG et al., unpublished data). The PapE protein cannot be the actual adhesin since a frameshift mutation, *papE1*, does not alter the binding properties of the bacteria (Table 2). However, pili purified from the *papE1* mutant did not on their own agglutinate erythrocytes and a *papA1*, *papE1* double mutant does not express digalactoside-specific binding (LINDBERG et al. 1984). Presumably, the 16500-dalton PapE protein interacts at some stage which determines how the adhesin is presented on the bacterial surface.

Nucleotide sequence analyses indicate that the proteins encoded by the *papE* and *papF* genes are structurally similar to the PapA protein and other pilins (LINDBERG et al., unpublished data). The predicted secondary structure, hydrophilicity profile, and location of two cysteine residues show resemblance to what is found for most pilins. The findings support the idea that these proteins could constitute minor components of a pilin polymer although they do not seem to form pili by themselves. In this context it should also be mentioned that the Pap DNA carries a fourth gene which codes for a pilin-like protein. Nucleotide sequencing showed that an open reading frame for a 20000-dalton protein with the mentioned attributes of a pilin is located immediately downstream of *papA* (Fig. 1; BÅGA et al., unpublished data). The gene, denoted *papH*, is very poorly expressed in *E. coli* K-12 and from studies with insertion and

deletion mutants it appears not to be essential for Pap pili formation or adhesin expression. Perhaps it may be regarded as a pilin "pseudogene."

The amino acid sequence of the 35000-dalton PapG protein, as deduced from the DNA sequence, does not suggest any secondary structure similarities with the pilin proteins (LUND et al., unpublished data). However, similar to the pilin-like proteins, the PapG polypeptide is also made in a precursor form, suggesting that it is translocated to the periplasm or outer membrane (NORGREN et al. 1984).

Genes required for MRHA expression have been identified also within regions of the pDC5 and pPIL110-35 clones corresponding to the *papE–G* part of pPAP5 (CLEGG and PIERCE 1983; VAN DIE et al. 1984). Probes of pPAP5 from the *papG* gene do not hybridize to the other two clones although they seem to encode PapG protein analogs of similar molecular weight (LUND et al., 1985). Loss of hemagglutination capacity, due to a deletion abolishing the PapG analog in pDC5, can be complemented *in trans* by a plasmid carrying only the *papE,F,G* region of pPAP5. A plausible explanation for the results would be that the PapE, PapF, and PapG proteins of a given gene cluster interact during adhesin biogenesis. Because of differences between the protein analogs a PapG protein from one gene cluster might not interact with the PapE and PapF proteins from another gene cluster. A comparison of primary sequences of the *E*, *F*, and *G* genes and proteins from different digalactoside-binding clones should provide further insight into how expression of the adhesin is mediated.

3.3 Genes Mediating Auxiliary Functions in Pilus Adhesin Biogenesis

The characterization of different pilus-adhesin gene clusters from *E. coli* shows that they all contain a cistron for a protein with molecular weight of about 80000 daltons (KEHOE et al. 1981; MOOI et al. 1981; CLEGG and PIERCE 1983; DE GRAAF et al. 1984; NORGREN et al. 1984; ORNDORFF and FALKOW 1984; VAN DIE et al. 1984). In the Pap gene cluster the *papC* gene encodes an 81000-dalton protein (Fig. 1). Inactivation of the *papC* gene by mutation abolishes both Pap pili formation and expression of Pap adhesin (NORGREN et al. 1984; Table 1). Cell extracts of bacteria carrying a *papC* mutant clone do contain appreciable amounts of PapA protein as detected by ELISA (NORGREN et al. 1984). A defect in the 81000-dalton protein therefore seems to block the process of surface localization and assembly of Pap pilin. Similar results have been obtained from studies of the K88 gene cluster, which also encodes an 81000-dalton polypeptide (MOOI et al. 1982).

In the Pap gene cluster, inactivation of the *papD* gene, which encodes a 28500-dalton polypeptide, gives phenotypic consequences indistinguishable from those of *papC* mutations. Both adhesion and piliation are abolished (NORGREN et al. 1984). However, a difference between the *papD* and *papC* mutants is seen when the amount of pilin accumulated within the cells is determined. In *papD* mutant cells there is much less PapA protein, and the amounts of PapE and PapF proteins seem very much reduced as monitored by minicell

analysis (NORGREN et al. 1984; NORGREN et al., unpublished data). Apparently, the PapD protein is required for surface localization of pilus-adhesin subunits. Presence of the PapD protein seems to stabilize the subunits and presumably protects them from degradation. One hypothesis would be that the PapD protein may form a complex with subunits (e.g., PapA protein) in the periplasmic space. Such complexes could interact with PapC protein which would catalyze translocation and polymerization at the outer membrane. Similarly, biogenesis of the adhesin would involve complex formation between PapD protein and either of the PapE or PapF proteins. The fact that PapE protein can be detected in preparations of pili suggests that it might be incorporated as a minor constituent in PapA polymers. Also PapF protein could be an integral part of pili although there is at present no direct evidence at hand.

To understand how different pilus-adhesin subunits interact functionally it will be important to determine their topological locations on the cell surface and in pili. Perhaps the PapF and PapG proteins must interact with complexes containing PapE and/or PapA in order to constitute active adhesin. This would explain the lack of binding activity in pili from *papE* mutants and the adhesin-negative phenotype of *papA*, *papE* double mutant cells (Table 2). The process of surface localization and assembly might also involve some, yet undetected, modification(s) of the subunits. Such a modifying function would be an alternative role for the PapG protein in the complex.

It is at present premature to propose any detailed molecular model for pilus-adhesin biogenesis. However, it is evident that the process must involve intricate protein-protein interactions. An intriguing finding in the studies of the Pap system is that the PapC and PapD proteins presumably act on different sets of subunits. Biogenesis of Pap pili and biogenesis of Pap adhesin can occur independently but in both cases PapC and PapD are required. Comparative structural studies of different pilin-like proteins and systematic alterations in such proteins (e.g., by in vitro mutagenesis) should help elucidate what requirements there are in terms of protein-protein recognition.

3.4 Regulatory Functions

It may be anticipated that gene expression in a cluster mediating pilus-adhesin biogenesis is regulated such that production of catalytic and structural components is coordinated. Minicell analysis of polypeptides produced by cloned determinants shows, as may be expected, that the major pilus subunit protein is more abundant than the other gene products (NORGREN et al. 1984; VAN DIE et al. 1984). The initial characterization of transcriptional organization in the Pap clone from strain J96 suggests that there may be at least four functional promoters. Operon fusion studies, using the *E. coli lacZ* gene, show that subclones containing: the *Eco*RI-*Hin*dIII fragment carrying *papB* and part of *papA*; the *Hin*dIII fragment carrying *papC* and part of *papD*; or the *Sma*I-*Bgl*II fragment carrying *papE*, *papF*, and part of *papG* all promote transcription (NORGREN et al. 1984; GÖRANSSON et al., unpublished data). Northern-blot hybridization has revealed that there are two different transcripts produced from the *papB*-

papA region (BÅGA et al., unpublished data). A probe from the *papA*-coding sequence hybridized to a 0.8-kb- and to a 1.3-kb-long transcript whereas a probe from within *papB* only hybridized to the longer of the two. The 2-kb *Eco*RI-*Hin*dIII fragment carrying those genes therefore appears to contain two different promoters. Immediately after the *papA*-coding sequence, i.e., between *papA* and *papH* (Fig. 1), there is a sequence which presumably terminates transcription. The position of the terminator and the lengths of the transcripts indicate that one promoter is located between *papB* and *papA* and one upstream of *papB*. Furthermore, the 0.8-kb-long transcript is more abundant than any of the other *pap* transcripts from pPAP5.

The *papB* gene has been suggested to play some regulatory role since transposon Tn*5* insertions cause reduced expression of Pap pili (NORGREN et al. 1984). Additional evidence for such a role has been obtained in studies of how glucose affects Pap expression. The presence of glucose in the growth medium reduces expression of both pili and adhesin, and experiments with *cya* and *crp* mutants of *E. coli* indicate that Pap is subject to catabolite repression (GÖRANSSON et al., unpublished data). Production of β-galactosidase from a *papA-lacZ* transcriptional fusion plasmid was 10- to 20-fold lower in *crp* or *cya* mutant strains as compared with a wild-type host. When cyclic AMP (cAMP) was added to the growth medium the β-galactosidase activity was the same in *cya* and *cya*$^+$ strains. DNA sequence analysis of the *papB-papA* region strongly suggests that there is a CAP-cAMP binding site near the presumed *papB* promoter (BÅGA et al., unpublished data). One possibility would be that the *papB* gene encodes an activator which influences *papA* gene expression (and perhaps expression also of other *pap* genes) and that catabolite repression of Pap operates at the level of *papB* expression. However, it remains to be tested if *papB* affects expression of other genes *in trans*. Furthermore, the observation that one of the two transcripts from the *papB-papA* region appears to cover both genes must also be taken into consideration.

The question of glucose effects and catabolite repression has also been investigated in the case of type 1 pili biogenesis. In *Salmonella typhimurium* type 1 pili production was reported to depend on cAMP in accordance with what one would expect for a system subject to catabolite repression (SAIER et al. 1978). However, in *E. coli* similar studies led to the conclusion that type 1 piliation is not subject to regular catabolite repression, but that absence of glucose favors outgrowth of piliated bacteria (EISENSTEIN and DODD 1982). With the molecular cloning of type 1 pili genetic determinants, it should now be possible to study this in more molecular detail.

A regulatory gene has recently been defined in the type 1A pili gene cluster from *E. coli* strain J96. Mutational inactivation of a cistron encoding a 23000-dalton protein located near the type 1A pilin gene resulted in hyperpiliation of the bacteria (ORNDORFF and FALKOW 1984; and personal communication). The presumptive regulatory gene showed positive *trans*-complementation and it appears as if the 23000-dalton protein acts as an inhibitor of type 1 piliation.

The fact that clinical isolates often seem to possess more than one pilus-adhesin gene cluster raises the question whether or not their regulatory functions interact. Studies of bacterial populations of an *E. coli* strain known to carry

different Gal-Gal-specific and type 1 pili adhesins indicated that a minority of the individual cells express more than one antigen at a given time (NOWICKI et al. 1984). Subpopulations, presumably expressing only type 1 pili or only Gal-Gal pili adhesins, were obtained by treatments with antisera raised against the specific type of pili antigens (RHEN et al. 1983b). After further growth such subpopulations were soon found to express all pili serotypes of the original population, and the results were taken as evidence that expression of the pili is subject to a phase variation mechanism (RHEN et al. 1983b). Studies on type 1 piliation of *E. coli* K-12 have shown that expression is switched on and off in a mode consistent with a proposed phase variation (EISENSTEIN 1981). However, the molecular mechanism behind the variation is not yet clear.

3.5 Thermoregulation of Pilus Adhesin Expression

In addition to the effects by growth medium composition (e.g., catabolite repression), a general feature of pili adhesins is that production very much depends on the growth temperature (GAASTRA and DE GRAAF 1982). The binding properties and piliation of the bacteria seem optimally expressed at 37° C whereas both are absent at 20°–25° C. The influence of growth temperature on expression of virulence properties seems to be a common characteristic of bacterial pathogens of mammals. Production of K1 capsular antigen in *E. coli* (BORTOLUSSI et al. 1983) and expression of invasive virulence by pathogenic *Shigella* (MAURELLI et al. 1984) and *Yersiniae* (BRUBAKER 1979) have been shown to depend on growth temperature. However, little is known about the molecular mechanisms determining expression of such virulence properties.

Many of the cloned pilus-adhesin determinants show the temperature dependence also in the *E. coli* K-12 hosts used. In the case of the Pap clone from *E. coli* J96, quantitative analyses of pili antigen, by a competitive ELISA, showed that there is at least a 20-fold reduction at 22° C as compared with 37° C (Table 4). Since this indicated that the temperature effect operates at the level of pilus subunit synthesis, rather than pilus assembly, the expression of *papA-lacZ* fusion hybrids was studied at different temperatures (GÖRANSSON and UHLIN 1984). Both translational and trancriptional fusions showed reduced expression of β-galactosidase at temperatures below 37° C. The fact that the extent of reduced transcription, as monitored by the *papA-lacZ* fusion, corresponded to that of Pap antigen production led to the conclusion that thermoregulation of *pap* gene transcription is the reason for the observed temperature dependence (Table 4; GÖRANSSON and UHLIN 1984). The experiments with *papA-lacZ* fusions also demonstrate that some component involved in thermoregulation must be contained within the 2-kb *Eco*RI-*Hin*dIII fragment carrying *papB* and part of *papA* (Fig. 1). At present it is unclear whether or not the *papB* gene product is directly involved in thermoregulation. Since expression of adhesion is also temperature dependent, the present evidence indicates that cistrons in separate transcriptional units might be subject to this kind of regulation.

It is clear that we need to know more about when, and how, the virulence properties are expressed. In particular one may ask how expression is regulated

Table 4. Thermoregulation of *pap* gene expression[a]

Assay	Relative amount at	
	37° C	22° C
ELISA[b] (Pap antigen)	1	0.06
β-Gal activity[c] (*papA-lacZ* hybrid)	1	0.04

[a] The expression from the cloned Pap determinant of *E. coli* strain J96 was analyzed at different growth temperatures (GÖRANSSON and UHLIN 1984)

[b] *Escherichia coli* K-12 strain HB101 carrying the Pap plasmid pRHU845 was subject to a competitive enzyme-linked immunosorbent assay (ELISA) for quantitation of Pap antigen

[c] The *papA-lacZ* transcriptional fusion plasmid pHMG1 in the *E. coli* K-12 strain MC1061 was assayed for relative expression of β-galactosidase activity

under the conditions present at the site of infection. Comparisons of bacterial surface composition show that there are distinct differences between cells that are grown under laboratory in vitro conditions and cells that grow under in vivo conditions (FINN et al. 1982). The cloned determinants have made it possible to monitor expression from each individual gene and to define further the role of a given virulence function in the pathogenicity of the bacteria.

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Genetic Basis of Iron Assimilation in Pathogenic *Escherichia coli*

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1 Introduction

Progress in sanitation and medical practice have combined to eliminate certain classical bacteremic infections in the United States and throughout Europe. In these societies the common gram-negative bacilli now isolated in the clinic are those which are prominently associated with man, such as *Escherichia coli*, or are, like *Pseudomonas aeruginosa*, those which are equipped with special devices to take advantage of the compromised host (YOUNG et al. 1982).

It has been apparent for some time that pathogenicity involves a multifaceted interaction between host and microbe. The acquisition of virulence by *E. coli* would seem to afford a tractable avenue of approach for an understanding of this phenomenon at the molecular level. This is because, in the first instance, the bacterium is a normal, generally innocuous, endosymbiont of man. Secondly, more is known about the genetic endowment of *E. coli* than for any other form of life. Any experiments directed at conversion of *E. coli* K12 strains to virulence by use of genetic material from clinical isolates of this bacterium would doubtless reveal that a number of plasmid and chromosomal determinants are required for this transformation. However, we can now assert with

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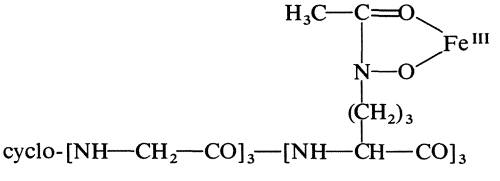
confidence that the acquisition of an effective iron-gathering mechanism is one of the significant factors specifying virulence.

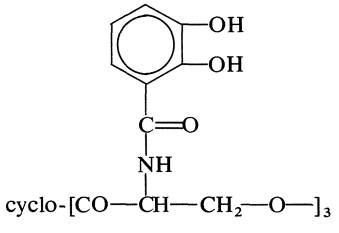
This laboratory set out several decades ago to investigate the mechanisms(s) whereby microorganisms acquire iron which, with the possible exception of certain lactic acid bacteria (ARCHIBALD 1983), is for all species a universal growth requirement. Initially we focused on very aerobic bacteria, such as *Bacillus subtilis* and *Bacillus megaterium*, and on certain members of the lower eukaryotes, such as *Ustilago*, in the belief that these species would exhibit a more robust iron uptake pathway as a consequence of their need to synthesize relatively higher levels of iron enzymes in an oxidizing environment. The smut fungus *Ustilago sphaerogena*, for example, forms cytochrome *c* to the extent of several percent of its dry weight. Indeed, *U. sphaerogena* and other fungi yielded a line of ferric hydroxamate cyclohexapeptides, the ferrichromes, the deferriform of which was greatly overproduced on culture of the organisms under low iron stress. With the detection of ferrichrome (1) in *Aspergillis niger* and a related hydroxamate



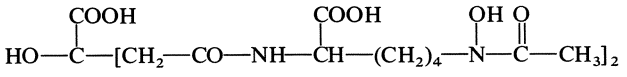
(1) Ferrichrome

in *B. megaterium* the common occurrence of this type of iron carrier seemed assured (NEILANDS 1957). Other bacilli, such as *B. subtilis*, however, produced a different ligand in response to low iron stress, subsequently shown to be a catechol. Growth of *E. coli* and a *Pseudomonas* sp. in the unpurified medium gave no indication of the presence of a ferric iron ligand and it was speculated that the reason might be found in the less vigorous growth and hence lower iron requirement of these bacterial species (GARIBALDI and NEILANDS 1956).

However, with the refinement of minimal media for cultivation of enteric bacteria the year 1970 saw the isolation of a tricatechol from *Salmonella typhimurium* (POLLACK and NEILANDS 1970) and *E. coli* (O'BRIEN and GIBSON 1970) independently named, respectively, enterobactin and enterochelin (2). At about the same time GIBSON and MAGRATH (1969) isolated aerobactin (3) from *Aerobacter aerogenes* 62-I as the first and as yet the only fully characterized hydroxamate compound from enteric bacteria.



(2) Enterobactin



(3) Aerobactin

Research on the mechanism and regulation of microbial iron assimilation has lately centered on *E. coli* and related enteric species since these forms are the most amenable to genetic manipulation. This chapter will review our present understanding of the genetic basis for iron assimilation in invasive strains of *E. coli*, with particular emphasis on the system specified by pColV. The subject will be prefaced by some background material on the biogeochemistry of iron and the role of this element as a virulence factor.

2 Iron in Evolution

The "discovery" of O_2-evolving photosynthesis by cyanobacteria some 3×10^9 years ago did not immediately result in an oxidizing environment owing to the presence on the surface of the planet of an abundant supply of reduced ferrous mineral. Ultimately, however, the exposed iron was converted to ferric oxyhydroxide polymers and precipitated from solution as demanded by the solubility product of ca. 10^{-38} M for $Fe(OH)_3$. The aerobic and facultative anaerobic microflora responded (NEILANDS 1972) to this challenge by the development of a line of virtually ferric-ion-specific ligands now known as siderophores (Greek, "iron bearers"), of which ferrichrome (1) and enterobactin (2) are prototypical examples of the hydroxamate and catechol series, respectively. Indeed, the K_{sol} of 10^{-38} M for $Fe(OH)_3$ limits the maximum concentration of soluble ferric iron at pH 7 to 10^{-17} M, and hence all metabolically active ferric iron must be chelated or in some manner protected from hydrolysis.

3 Iron in Infection

So far as an iron supply in an aerobic environment is concerned, the problems faced by microorganisms invading living tissues or plant roots "infecting" soil are similar in nature and may require elaboration of siderophores and/or reducing agents. The availability of iron in the host will depend on the particular site in the host. For example, iron may be readily available in hematoma. In serum, on the other hand, the presence of iron-binding proteins (transferrin) reduces the availability of iron and siderophores may be essential for bacteria to survive.

Most studies of the effect of iron in infection have involved the injection of iron into the host. These studies are complicated because it is assumed that the increased availability of iron directly affects the growth of the bacteria. However, the iron may also alter host defenses. The subject has been reviewed

by WEINBERG (1978, 1984), BULLEN (1983), and FINKELSTEIN et al. (1983). In most cases it is thought that the invading microbe must synthesize siderophore(s) but in a few instances, notably *Legionella pneumophilia* (REEVES et al. 1983) and *Neisseria meningitidis* (SIMONSON et al. 1982), it has not been possible to detect elaboration of such carrier molecules, at least as the species are cultivated in vitro. Despite considerable speculation that siderophores play a significant role in virulence there is as yet limited direct experimental evidence that siderophores are significant virulence factors in infections with *E. coli*. In the case of *E. coli* one study has shown that the enterobactin system was fully derepressed in an infection of the peritoneum in experimental animals (GRIFFITHS 1983). Enterobactin production, however, did not correlate with virulence of *E. coli* in the development of renal infection or proportion of deaths in a murine model of urinary tract infection (MONTGOMERIE et al. 1979). MILES and KHIMJI (1975) were also unable to show a relationship between enterobactin production and virulence of *E. coli*.

It is possible that aerobactin may be more significant than enterobactin in the virulence of *E. coli*, at least in the development of bacteremia. Strains of *E. coli* carrying plasmids for biosynthesis and transport of the siderophore aerobactin were found to be more virulent than strains carrying plasmids defective in the aerobactin system in a murine model of bacteremia (WILLIAMS and WARNER 1980). STUART et al. (1982) observed a high incidence of hydroxamate-positive strains in isolates from the blood of humans and poultry. In other studies the presence of aerobactin was associated with bacteremia in patients and aerobactin-positive strains survived better in heat-inactivated serum (MONTGOMERIE et al. 1984). In a mouse model of urinary tract infection aerobactin-positive *E. coli* were associated with a higher proportion of early deaths which were probably the result of bacteremia (MONTGOMERIE et al. 1984).

4 Iron in *Escherichia coli*

Escherichia coli appears to contain the normal complement of iron enzymes expected of a facultative anaerobic microorganism. The conditions of growth can be expected to modify drastically the iron demand of the cell. Propagation on citrate or on one of the other trichloroacetic acid (TCA) cycle intermediates will invoke a higher demand for iron since these substrates can only be oxidized through the aerobic, cytochrome-containing pathways. Thus a *hem*A mutant, blocked in porphyrin synthesis, fails to proliferate aerobically but its growth is unimpaired on glucose as carbon source. No such bypass is known to exist around the ribotide reductase enzyme needed for DNA synthesis and in *E. coli* this enzyme contains iron (LAMMERS and FOLLMANN 1983).

Recent results show that each cell of an *E. coli* K12 strain cultured to early stationary phase in minimal medium (M9), nutrient broth, and Luria broth contains 3.3×10^{-19}, 11×10^{-19}, and 260×10^{-19} gram atoms of iron, respectively, as measured by atomic absorption spectrophotometry (NAKAMURA, unpublished data). This demonstrates the capacity of the organism to store iron under favorable growth conditions.

5 High- and Low-Affinity Iron Assimilation in *Escherichia coli* K12

Escherichia coli K12 is equipped with multiple systems for the acquisition of iron, which may be approximately described as either "low" or "high" affinity in nature (NEILANDS 1984). The low-affinity system is relatively insensitive and is not thought to involve specific transport agents or receptors. Very little is as yet understood about the genetics and mechanism of operation of this system. In contrast, the various high-affinity systems have been studied in some detail. All vestiges of these latter systems can be eliminated by mutation. The resulting auxotrophes can then be seen to grow normally on complex media or even on minimal media containing glucose; growth on minimal media with TCA cycle intermediates is, however, severely restricted. The genetic functions for high-affinity systems indigenous to the K12 strain of *E. coli* which are known to date are listed in Table 1.

The citrate system comprises at least two genes, *fecA* and *fecB*. These represent, respectively, the outer membrane receptor (M_r, 80.5K) and the permease for ferric citrate (HUSSEIN et al. 1981). The system is induced by growth on ferric citrate and hence its relevance to virulence is open to question. It is absent from *S. typhimurium*, which, however, can use citrate as a carbon source. The converse is true in *E. coli* where, apparently, not enough citrate can be transported as the iron complex to sustain growth.

Table 1. Genetic functions involved in high-affinity iron assimilation in *Escherichia coli* K12[a]

Gene	Chromosomal map locus (min)	Product/function
tonA (*fhuA*)	3	Outer membrane receptor for ferrichromes, albomycin, T1, T5, ϕ80, UC-1, colicin M
fhuCDB	3	Utilization of hydroxamate-type siderophores
fecA	7	Outer membrane receptor for ferric citrate
fecB	7	Ferric citrate permease
fepA	13	Outer membrane receptor for ferric enterobactin and colicin B
fepB	13	Ferric enterobactin permease
fes	13	Esterase-reductase step in ferric enterobactin utilization
entABCDEFG	13	Enterobactin biosynthesis
fhuE	16	Outer membrane receptor for coprogen and ferric rhodotorulate
fiu	18	Induced at low iron, function unknown
tonB	27	Utilization of all iron chelates and vitamin B12
cir	43	Induced at low iron; colicin Ia receptor
exbB	64	Hyperexcretion of enterobactin
perA	74	Affects synthesis of several outer membrane proteins, including the ferric enterobactin receptor
iucABC	?; pColV	Aerobactin biosynthesis
iutA	?; pColV	Ferric aerobactin transport
iutB	?; pColV	Outer membrane receptor for ferric aerobactin
fur	near *lac*?; 15	Regulation of high-affinity iron assimilation

[a] References cited in the text

Table 1 lists the substantial number of genes making up the enterobactin system. The latter has been cloned in bacteriophage Mu (LAIRD et al. 1980; LAIRD and YOUNG 1980) and shown to be organized into several transcriptional units extending across some 26 kb DNA. More recently, operon fusions have been constructed with the vector Mu *d*(Ap^r *lac*) (FLEMING et al. 1983). Thus far the only gene product examined in any detail is the 81K outer membrane receptor for ferric enterobactin (FISS et al. 1982). It is a water-insoluble protein with phenylalanine as a N-terminal amino acid residue. The protein appears to be particularly susceptible to cleavage by a second outer membrane component, protein *a*. The significance of this event is not apparent at the moment. Polarity effects arising from Mu *d* insertions into *entA*, *entC*, and *entE* suggest that these genes are part of the operon *entA(CGB)E* (FLEMING et al. 1983). There is as yet no sequence data for the enterobactin cluster, which is located between *purE* and *lip*, and much work remains to be done to disclose its transcriptional organization and control.

Escherichia coli K12 strains contain a cluster of genes involved in utilization of hydroxamate type siderophores, such as ferrichrome. This siderophore is formed by all *Penicillia* and by many other fungi although not, so far as is known at present, by any bacterial species. The first of these genes discovered was the one required for ferrichrome transport through the outer membrane, a gene known for ca. 4 decades as *tonA* inasmuch as it imparts resistance to several phages, including T1 (WAYNE and NEILANDS 1975). Its biochemical function as the ferrichrome receptor became apparent as a result of work in *S. typhimurium* showing resistance to albomycin, a ferrichrome analog, to map close to *panC* (LUCKEY et al. 1972). The analogous locus in *E. coli* confers sensitivity/resistance to a range of lethal agents. In the *fhuB* mutation the 78K outer membrane receptor for ferrichrome remains intact and functional for most lethal agents, such as T1, T5, ϕ80, and colicin M, while ability to use ferric hydroxamates and sensitivity to albomycin are lost (KADNER et al. 1980). The *fhuB* gene has been localized to a 1.4-kb *PstI* fragment of DNA cloned in pCPN12; the product has been tentatively identified in a maxicell strain, a *lon* mutant, as a 20K polypeptide (PRODY and NEILANDS 1984). The cellular location of the *fhuB* protein, whether outer or inner membrane, periplasmic or cytoplasmic, as well as the function of this unit of the hydroxamate transport system, remains to be determined. The *fhuB* gene is situated several kilobases clockwise from *tonA* on the *E. coli* linkage map. Two of the intervening genes have been designated *fhuC* and *fhuD* but no function has been assigned to them (FECKER and BRAUN 1983). At minute 16 an additional gene, *fhuE*, is believed to be required for utilization of the siderophores coprogen and rhodotorulic acid (HANTKE 1983).

A second classic genetic lesion of *E. coli*, *tonB*, affords resistance to T1 and imparts failure to use all iron chelates and vitamin B12. The function of this component is still a matter of debate but the gene has been sequenced and the promoter-terminator sequences shown to resemble those commonly found in *E. coli* (POSTLE and GOOD 1983). The protein is unusually proline rich and its general hydrophilic character indicates that its major bulk is not located within a membrane. The predicted size, some 26K, is 10K less than

that measured on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The *exbB* mutation (GUTERMAN and DANN 1973), for some unknown reason, results in excessive excretion of enterobactin.

Work with *Salmonella* sp. disclosed the existence of a mutation, designated *fur*, which results in constitutive expression of all components of the siderophore apparatus (ERNST et al. 1978). Similar mutants have been reported in *E. coli* but the precise nature of the genetic lesion has yet to be defined (BRAUN and BURKHARDT 1982; HANTKE 1982). The constitutive phenotype may represent, at the gene level, the loss of a repressor, which binds iron as corepressor, or acquisition of some internal defect, which results in failure properly to metabolize iron.

Finally, in *E. coli* K12 strains there are two additional outer membrane proteins which are induced by low iron stress. The product of gene *fiu* has an M_r of 83K and is absent in *E. coli* B strains (NEILANDS 1979); no function has been assigned to this polypeptide. The colicin Ia receptor, M_r 74K, is also induced at low iron. These two proteins still "weep" for a siderophore. It appears that the *fiu*, *fepA*, and *cir* (colicin Ia) genes are regulated by iron in some coordinate fashion while *tonA* is under a different type of iron regulation (KLEBBA et al. 1982). Administration of ferrichrome or ferric enterobactin to a low iron culture followed by a pulse label indicates repression of biosynthesis of the three proteins proceeds along a common kinetic path. The same kinetics apply to induction of synthesis of the three proteins in a high iron culture suddenly stressed for iron, but in this case the response is delayed as the cell consumes its internal iron stores. The *perA* mutation affects synthesis of several outer membrane proteins, including the ferric enterobactin receptor (81K), while enterobactin production remains normal (LUNDRIGAN and EARHART 1981).

6 Iron Assimilation in Pathogenic *Escherichia coli*

6.1 Background

Some 6 decades ago GRATIA (1925) observed that filtrates of a culture of a virulent strain of *E. coli* contained a factor, "V," which killed sensitive strains of the same bacterium (HUTTON and GOEBEL 1961). It was also recognized that, although resembling bacteriophage, the new principle did not replicate on serial passage. Subsequently FREDERICQ (1950) described the specificity and distribution of these factors and laid the foundation for our present understanding of the class of "killer proteins" or "protein antibiotics" designated generically in *E. coli* as colicins. It is significant and pertinent to the present discussion that component V is the original colicin.

In a search for transmissible pathogenic characters in strains of *E. coli* causing invasive infections of man and animals, SMITH (1974) discovered a plasmid-controlled toxin and a plasmid-controlled lethal agent, the latter apparently identical to colicin V. The lethal character was associated not with a toxin but with enhanced ability to survive in body fluids. The ColV plasmids of

six wild strains of *E. coli* upon transfer to K12 strains increased the lethality of the latter and it was concluded that colicin V itself might be the agent responsible. It was noted that strains of *E. coli* causing bacteremia in man and animals commonly carry the ColV plasmid. In a subsequent study SMITH and HUGGINS (1976) confirmed and extended the correlation between the ColV plasmid and virulence.

6.2 A Novel Iron Assimilation System Encoded by Colicin V Plasmids

In view of the critical role of iron for proliferation of bacteria in tissues and body fluids, WILLIAMS (1979) considered the possibility that *E. coli* strains carrying the ColV plasmid were enhanced as regards their capacity to acquire iron. He reported that pColV conferred strong selective advantage upon bacteremic strains unless iron was administered in the course of experimental infections. In minimal medium, transferrin inhibited growth of cured strains but had no effect on pColV$^+$ strains. Radioiron was rapidly accumulated by the plasmid-containing strains, resulting in repression of the outer membrane proteins commonly associated with iron status. The pColV system was shown to depend on *tonB* but to be in other respects distinct from the systems already known in *E. coli.*

STUART et al. (1980), working with other ColV plasmids, reported results similar to those found by WILLIAMS and concluded that plasmid-determined hydroxamate constitutes a cell-bound system for iron transport. It soon became clear that the virulence component of pColV could be assigned to the hydroxamate-type siderophore iron uptake system rather than to colicin V (WILLIAMS and WARNER 1980; QUACKENBUSH and FALKOW 1979). The latter bacteriocin, however, may play some role in selection among strains of *E. coli* in the gut (SMITH and HUGGINS 1976).

6.3 Aerobactin as the Siderophore of pColV-K30

We have already noted that aerobactin was detected as a product of the enteric bacterium *A. aerogenes* 62-I, where it was shown to be expressed side-by-side with enterobactin (GIBSON and MAGRATH 1969). Although PERRY and SAN CLEMENTE (1979) detected a hydroxamate-type siderophore as a product of low iron-grown *Shigella sonnei* the substance was stated not to be aerobactin. In the following year, however, PAYNE (1980) characterized aerobactin from *Shigella flexneri.*

With this background information in hand, WARNER et al. (1981) isolated the hydroxamate siderophore of pColV K-30 bearing strains of *E. coli* and identified it by field desorption mass spectroscopy as aerobactin. The siderophore was shown to be excreted into the medium under conditions of low iron stress. Use of a mutant blocked in aerobactin synthesis indicated that the small fraction of the siderophore bound to cellular material is probably associated with a surface receptor.

6.4 The Ferric Aerobactin-Cloacin Receptor

The molecular weight of ferric aerobactin, 616, is sufficient to prevent free diffusion through the small, water-filled pores of the outer membrane of enteric bacteria. This suggests that, as is the case for ferrichrome and ferric enterobactin (NEILANDS 1979), a ferric aerobactin receptor should exist. Identification of this receptor came from a Dutch group working with a bacteriocin, cloacin, from *Enterobacter cloacae*. Thus van TIEL-MENKVELD et al. (1981, 1982) observed that *E. coli* strains harboring pColV-K30 became susceptible to cloacin. The M_r of the ferric aerobactin-cloacin receptor, 74K, is very similar to that of the colicin Ia receptor and it is hence most easily detected in outer membrane preparations of iron-stressed *cir* mutants (BINDEREIF et al. 1982; GREWAL et al. 1982).

6.5 Cloning and Genetic Organization of the Aerobactin Gene Cluster

Cloning of the aerobactin system from the large pColV-K30 was facilitated by the use of cloacin sensitivity screening, the proper recombinant plasmid rendering the cell susceptible to the bacteriocin (BINDEREIF and NEILANDS 1983). Digestion of pColV-K30 to completion with *Hin*dIII gave at least 11 different fragments ranging in size up to 30 kb. The mixture of fragments were ligated into the *Hin*dIII site of the 2.0-kb expression vector pPlac (BINDEREIF 1984) carrying the origin of replication and the ampicillin resistance gene of pBR322. Ampicillin-resistant transformants of *E. coli* K12 294 were replicated to Luria broth plates containing ampicillin and cloacin. By this combination of positive and negative screening a cloacin-sensitive transformant was isolated and shown to harbor a plasmid, designated pABN1, which contained a 16.3-kb *Hin*dIII segment of pColV-K30. A subclone plasmid, pABN5, 8.7 kb, was constructed by deleting an *Eco*RI fragment of pABN1.

Bioassays for aerobactin with *E. coli* LG1522, which contains a mutant (*iuc*) plasmid defective in synthesis of aerobactin (WILLIAMS and WARNER 1980), and chemical assays with ferric perchlorate were applied to pABN1 and pABN5. These tests showed the larger plasmid to contain all of the determinants for biosynthesis and transport of aerobactin while the smaller plasmid could perform only the biosynthetic steps. Moreover, culture of cells harboring pABN1 at differing levels of iron disclosed that the regulatory sequences had been captured on pABN1. Evidently, the *Eco*RI site in pABN1 occurs within the gene for the 74K outer membrane receptor.

KRONE et al. (1983a) cloned a 6.5-kb *Bam*HI fragment of pColV-K30 into pBR322 and obtained pSF8, a plasmid specifying a 50K and a 74K polypeptide in minicells, the latter component representing the ferric aerobactin-cloacin receptor. In a subsequent paper, KRONE et al. (1983b) reported construction of plasmids specifying the 74K and 50K polypeptides and the 50K polypeptide alone and showed by complementation tests that both proteins were required for uptake of ferric aerobactin. Evidence was obtained to indicate that the 50K protein participates in ferric aerobactin transport and that the protein resides in the cytoplasmic membrane.

A systematic study of pABN1 and a series of deletion plasmids using [^{35}S]-methionine in a minicell system indicated the formation of a minimum of five polypeptides with sizes of 63K, 33K, 32K, 53K, and 74K, arranged in that order. The results also suggested the presence of a promoter/regulatory site preceding the 63K protein (BINDEREIF et al. 1983; BINDEREIF and NEILANDS 1985b).

In vitro runoff transcripts of pABN5 and a series of deletion plasmids constructed therefrom confined the promoter region to a 0.7-kb *Hin*dIII-*Sal*I fragment preceding the first structural gene in the aerobactin complex (BINDEREIF and NEILANDS 1985b). Use of S1-nuclease mapping in vitro located a major and a minor transcriptional start site within this fragment; only the major site was found in vivo. The presence of the two promoter regions was confirmed by DNA sequence analysis. The major and minor initiation start sites were shown to be located at 30 and at about 80 bp upstream from the first structural gene (63K). This region proved to be rich in potential secondary structures. Expression of the aerobactin operon is transcriptionally regulated by the iron status of the cell, based on quantitative S1-nuclease mapping assays and on β-galactosidase activity measurements in a strain carrying a *iucA′ – 'lacZ* fusion construction. Iron-regulatory sequences are contained in a 152-bp *Sau*3A fragment.

In a more detailed investigation of the aerobactin gene cluster, BINDEREIF (1984) reported the five polypeptides of the complex to have M_r values of 63, 33, 32, 53, and 74K and to be arrayed in this order in an operon. The first three polypeptides were shown to be involved in synthesis of aerobactin and were designated *iucABC*. The remaining two genes, *iutAB*, were shown to specify the 53K and 74K proteins required for transport of ferric aerobactin. A small deletion in the gene for the 32K polypeptide resulted in the accumulation of two precursors of aerobactin, an anionic and a neutral hydroxamate. Both of these were isolated and characterized. The neutral compound was shown to be N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine while the anionic compound was demonstrated to be N^{α}-citryl-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine, viz., aerobactin minus one side chain. These results lend further support to the proposed biosynthetic route for aerobactin, namely, oxidation of lysine at the N^{ε} atom, acetylation of the hydroxylamino-*N*, and condensation of the resulting neutral amino acid with the distal carboxyl groups of citrate to yield aerobactin. The genes probably required for these transactions are believed to be *iucA*, *iucB*, and *iucC*, respectively (BINDEREIF 1984). The enzyme oxidizing the di-basic amino acids at the ω-*N* atom has so far eluded isolation and characterization. A specific inhibitor of this enzyme might be a highly useful chemotherapeutic agent.

6.6 Is the Aerobactin Gene Cluster a Transposable Element?

We have already noted the presence of aerobactin in *A. aerogenes* 62-I and *S. flexneri* as well as in pColV-bearing and clinical isolates of *E. coli*. A survey of several *Salmonella* strains revealed the fairly common presence of aerobactin, sometimes alone but generally in company with enterobactin. Table 2 lists all

Table 2. Hydroxamate-type siderophores formed by enteric bacteria[a]

Species	Siderophore	Reference
Aerobacter aerogenes 62-I	Aerobactin	GIBSON and MAGRATH (1969)
Shigella sonnei	"Not aerobactin"	PERRY and SAN CLEMENTE (1979)
Shigella flexneri	Aerobactin	PAYNE (1980)
Escherichia coli ColV K-30	Aerobactin	WARNER et al. (1981)
Escherichia coli ColV : RJ1003, RJ1000, RJ1001, RJ1002	"Hydroxamate"	STUART et al. (1980)
Escherichia coli, environmental isolates	"Hydroxamate"	STUART et al. (1982)
Escherichia coli, clinical isolates	Aerobactin	BINDEREIF and NEILANDS (1985a)
Enterobacter cloacae	Aerobactin	VAN TIEL-MENKVELD et al. (1982)
Salmonella arizona: SA1, SLS, SL5301, SL5302	Aerobactin	MCDOUGALL and NEILANDS (1984)
Salmonella austin	Aerobactin	MCDOUGALL and NEILANDS (1984)
Salmonella memphis	Aerobactin	MCDOUGALL and NEILANDS (1984)

[a] With the exception of *S. austin* and *S. memphis*, most, or perhaps all, of these species also form enterobactin

of the enteric bacteria presently known to synthesize hydroxamate siderophores. Among all of these species, only the pColV-bearing *E. coli* and the original source of aerobactin, *A. aerogenes* 62-I, have been confirmed to carry the genes for the siderophore on a plasmid. The rest, including a series of clinical isolates of *E. coli*, some of which produce no colicin, presumably carry the aerobactin genes on the chromosome (BINDEREIF and NEILANDS, 1985a).

The finding that aerobactin is produced in various enterics and is sometimes plasmid-coded and sometimes chromosomal prompted us to consider that it might be on some type of mobile genetic element. The presence of a repeated sequence downstream from the aerobactin gene complex was tested by using pABN1- and pABN5-insert DNA as differential hybridization probes. The insert of pABN5 terminates with the 74K gene while pABN1 contains an additional ca. 8 kb DNA downstream from the 74K gene. If there should be a repeated sequence in the downstream region then the pABN1 probe would hybridize to more ColV-K30 fragments than the pABN5 probe. The result showed that the 16.3-kb *Hin*dIII fragment of pABN1 hybridized to four *Hin*dIII fragments of pColV-K30 (MCDOUGALL and NEILANDS 1984). As the probe similarly hybridized to digests of *E. coli* chromosomal DNA, insertion sequences were suspected. These were located by Southern hybridization analysis directly adjacent to the 74K gene on the right side of the aerobactin gene complex, and on the left side some 7 kb upstream from the transcriptional start site. The repeated sequences were subcloned, mapped, and identified as IS1, or a close relative thereof. Whether one or both of the IS1 sequences flanking the aerobactin operon are actually functional in promoting transposition and the significance of the upstream (intervening) DNA (which is conserved in several plasmid-coded systems) remain unknown at this time.

Our present understanding of the organization and structure of the aerobactin gene cluster of pColV is shown in Fig. 1.

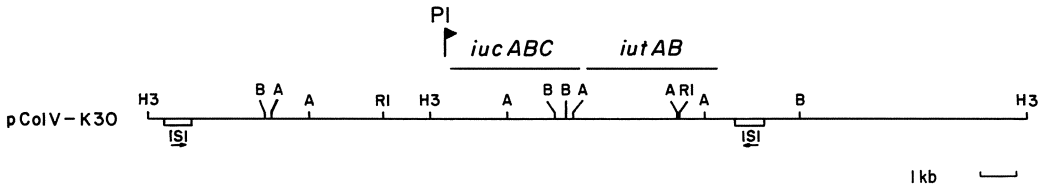


Fig. 1. Organization of the aerobactin biosynthesis and transport operon of the ColV-K30 plasmid. The genetic organization of the pColV-K30 aerobactin operon is shown above the map of the pColV-K30 aerobactin region. Restriction sites to the right of the downstream IS1 sequence have not been mapped completely. The position and orientation of the two IS1 copies flanking the pColV-K30 aerobactin genes are represented by *open boxes* under the pColV-K30 map. The location of the restriction sites is abbreviated as follows: *H3*, *Hin*dIII; *B*, *Bam*HI; *A*, *Ava*I; *RI*, *Eco*RI. The position and direction of transcription from the major promoter are indicated by P1 and P, respectively

The data in Table 2 indicate that *Shigella* must be included among the aerobactin-forming enteric genera which are potentially pathogenic in the human. Transfer of a Tn5-labeled derivative of a 140-Mdal plasmid specifying virulence in *S. flexneri* to *E. coli* K12 enabled determinants for invasion of epithelial cells to be identified in vitro, while full expression of virulence in animal models was shown to require loci linked to the *his*, *arg-mtl*, and *purE* regions of the chromosome (SANSONETTI et al. 1983). We do not know if aerobactin, which is formed by *S. flexneri* (PAYNE 1980), should be added to this list.

7 Rationale for Presence of Aerobactin in Pathogenic *Escherichia coli*

Reference to the published stability constants for transferrin and aerobactin with ferric iron suggests that efficient transfer of the metal ion from iron transferrin to aerobactin is a thermodynamic impossibility. Equilibration of enterobactin and aerobactin with iron transferrin proved that both siderophores can abstract the iron although the catechol compound does so at a superior rate in buffer (KONOPKA et al. 1982). Similar results were found for transfer of the transferrin-bound iron to cells of *E. coli* BN3040Nal^r (pColV-K30 *iuc*) was mediated by the two siderophores. However, when the reaction was run in serum, aerobactin proved more efficacious than enterobactin. The latter, but not aerobactin, was demonstrated to form a stable 1:1 complex with serum albumin (KONOPKA and NEILANDS 1984). In contrast, the nonaromatic aerobactin could not be shown by equilibrium dialysis to possess any degree of binding affinity for albumin.

8 Summary and Conclusions

Dating from the seminal observation of SCHADE and CAROLINE (1944, 1946) that iron could reverse the bacteriostatic action of egg white and serum, substan-

tial progress has been registered in our comprehension of the various mechanisms whereby microorganisms acquire the element from a reluctant host. Reference to Table 1 indicates a bewildering variety of genetic functions to be required in the different high-affinity iron uptake systems in *E. coli*. Some of the biosynthetic steps for specific siderophores, such as enterobactin and aerobactin, have been partially clarified and the outer membrane receptors have been identified. The function of certain genes, such as *ton*B, remains an enigma. Given the essential nature of iron on one hand and on the other the positively lethal role which the element plays in catalyzing the Haber-Weiss-Fenton reaction (HALLIWELL and GUTTERIDGE 1984), it is apparent that regulation of assimilation is the key to efficient cellular performance of the metal. This aspect of iron assimilation can be expected to be actively investigated in the next decade.

Clearly, iron is only one of the various virulence factors in strains of *E. coli* empowered to cause disseminating infections. Furthermore, no single iron absorption pathway can be expected to occur in all virulent strains of the organism. The following mechanisms can be envisaged for uptake of iron from the host:

Excretion of a Siderophore. This mechanism may appear to be more common than it is in reality owing to the ease of detection of the low-iron-induced, soluble chelating agents. Among the siderophores known at this time to be available to *E. coli*, the nonaromatic character of aerobactin would seem to offer certain advantages to the pathogen and may account for the relatively high incidence of aerobactin producers by blood isolates. The very high concentration of albumin in serum ensures that in this environment enterobactin would be tightly bound to protein. Nonetheless, capacity to synthesize enterobactin is certainly going to be an advantage over no siderophore. This may explain the several reports for *S. typhimurium* in which enterobactin was found to be a virulence factor (YANCEY et al. 1979).

Degradation of Iron-Binding Proteins. Surface proteases have been detected in *E. coli*. For instance, protein *a* cleaves the ferric enterobactin receptor (FISS et al. 1982) and protease IV (REGNIER 1981) exhibits endoprotease activity against casein. However, neither of these enzymes has been shown to make iron available from mammalian iron-binding or heme proteins.

Reduction. The very substantial solubility of ferrous ion at neutral pH renders this mechanism attractive, provided that the iron is promptly taken into the cell. This pathway, which may be quite important in plants, has yet to be explored in bacteria.

Absorption of Polymer Iron. Based on work with *S. typhimurium* (POLLACK et al. 1970), a K12 strain of *E. coli* stripped of its enterobactin production would be expected to show a doubling time of <1 h in glucose-minimal medium where the iron concentration is $\sim 1.0\ \mu M$. In this situation the accumulation of organic acids, which would be anticipated as the norm in growth at relatively low iron, might induce pathways such as the one for ferric citrate. Alternatively,

enhanced uptake of polymeric iron through some as yet unrecognized adaptation of envelope structure is possible.

Iron Porphyrin. The "hemoglobin adjuvant effect" for *E. coli* bacteria has been traced to the heme moiety of this conjugated protein (DAVIS and YULL 1964). There is, as yet, no detailed genetic analysis of heme iron utilization in *E. coli*. The chelate is large enough to require an outer membrane receptor but may be sufficiently hydrophobic to pass the envelope without need for such specialized surface proteins.

In strains harboring the ColV-K30 plasmid, it is agreed that production of the bacteriocin is not necessary for virulence. In the case of pColV, I-K94, which specifies virulence and serum resistance (BINNS et al. 1979), no hydroxamate can be detected (STUART et al. 1980). NILIUS and SAVAGE (1984) found that three out of eight ColV plasmids conjugated into plasmidless *E. coli* K12 conferred resistance to the bacteriocidal effect of serum. The factor(s) could not be assigned to detectable products excreted with the growth medium. As if to underline the somewhat tenuous correlation between bacteriocin and siderophore synthesis, some of the aerobactin-positive clinical isolates of *E. coli* examined in this laboratory form no colicin (BINDEREIF 1984).

Note Added in Proof

Two recent studies have appeared on the aerobactin gene cluster of ColV plasmids. CARBONETTI and WILLIAMS [Infect Immun (1984) 46:7–12], using pABN1 and derivatives, conclude from Tn1000 insertional inactivation that four polypeptides are required for biosynthesis of the siderophore. Our results in general agree with these findings, apart from some discrepancy in the molecular weights of the gene products. GROSS et al. [Mol Gen Genet (1984) 196:74–80], working with pColV-K311, re-named *iuc* as *aer* and assigned the gene order as B→C→A. Although we specify the oxygenase, acetylase and synthetase by the identical letters, namely, A, B and C, respectively, our data with pColV-K30 suggest that the first gene in the series is at least in part involved in the oxygenation of lysine. Regarding the letter designation of the two *iut* genes we concur that it is desireable to assign A to the gene for the 74K outer membrane ferric aerobactin receptor since this follows the custom already established for genes encoding receptors of other siderophores.

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Surface Components of *Escherichia coli* That Mediate Resistance to the Bactericidal Activities of Serum and Phagocytes

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1 Introduction

Microorganisms that enter the animal body, either actively or passively, encounter a battery of nonspecific and specific chemical and cellular defenses whose role is to inactivate and eliminate foreign materials. Successful pathogens have the ability to avoid, resist, or inactivate these defenses for at least the period necessary for initiation of the disease process.

Two well-defined, interacting humoral components that constitute the major first-line host defenses against invading bacteria are the complement system and the phagocytes (e.g., see MIMS 1982; TAYLOR 1983). The complement system is a protein cascade whose activation leads to deposition of complement component C3b, a major serum opsonin, on the surface of activating structures which in turn renders such structures highly susceptible to phagocytosis. Moreover, in the case of particles or cells containing accessible lipid bilayers, insertion of a lytic complex, or membrane attack unit, composed of components C5b, C6, C7, C8, and C9 into such bilayers results in destruction of their integrity and thus their biological functions. As a result, most gram-negative bacteria, having outer membranes as integral components of their cell envelopes, are readily killed by complement in serum. Complement may be activated either by the classical pathway, usually as a result of the formation of antigen-antibody

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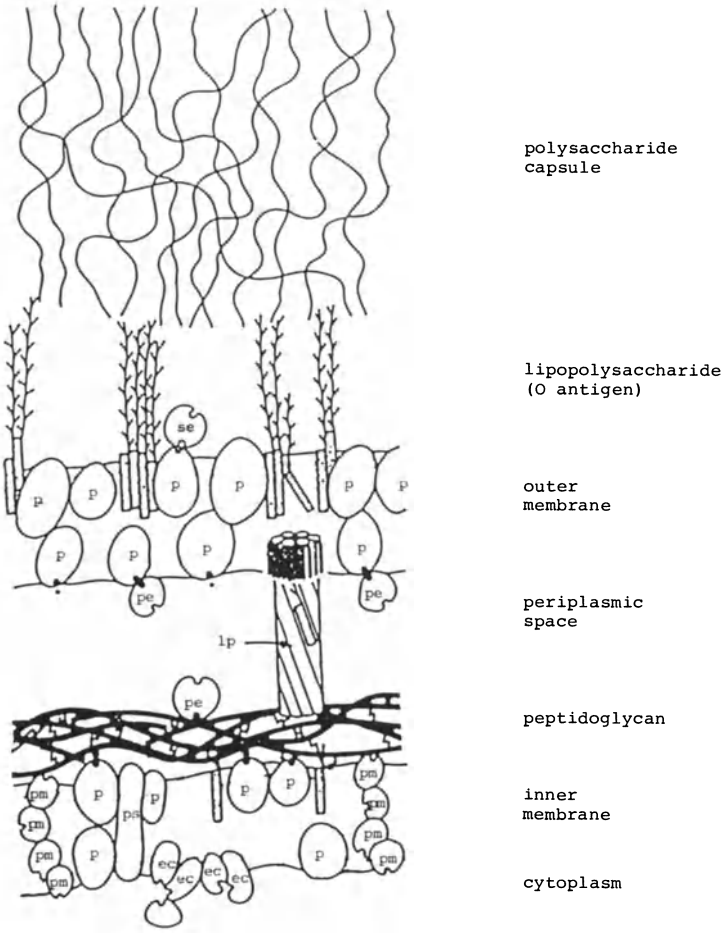

Fig. 1. Schematic diagram of the *E. coli* cell envelope. In this diagram, adapted from COSTERTON (1977), the lipid bilayer structure of the inner and outer membranes has for clarity been omitted. *p*, membrane protein; *lp*, lipoprotein; *pe*, periplasmic enzyme; *se*, cell surface enzyme; *ps*, permease; *pm*, inner membrane protein involved in synthesis and translocation of surface macromolecules; *ec*, membrane-bound cytoplasmic enzyme

complexes between the activating structure and preexisting circulating IgM or IgG antibodies, or by the alternative pathway, when the structure is such that it effects C3b-mediated amplification of the alternative pathway (escape from regulation). The alternative pathway is particularly important in neonates and infants that have limited repertoires of circulating antibodies.

Professional phagocytes, i.e., macrophages and polymorphonuclear leukocytes, are present in enormous quantities in the animal body and on its mucosal surfaces, and either circulate in the blood or are localized on or within tissues. They are the principal agents for the clearing from the animal body of dead cells, cellular debris, and foreign particles. Phagocytes have specific receptors for Fcγ, C3b, and C3b degradation products. Following receptor-ligand interac-

tion, they destroy target particles by engulfing them in vacuoles which subsequently fuse with lysosomes that contain a battery of hydrolytic enzymes, various bacteriostatic and bactericidal compounds, and free radical-generating enzymes that kill and digest cells and organic materials.

Although invasive microorganisms have developed various strategies to defend themselves against complement and phagocytes (e.g., see BUCHANAN and PEARCE 1979; MÄKELÄ et al. 1980; MIMS 1982; PENN 1983; FALKONE et al. 1984), we shall restrict our discussion to those which are exhibited by pathogenic *E. coli* strains and which have been subjected to genetic analysis.

Thus far, three types of component of the *E. coli* cell surface have been demonstrated to inhibit bacterial killing by serum and phagocytosis, namely acidic polysaccharide capsules, O-antigen lipopolysaccharides, and outer membrane proteins (see Fig. 1 for schematic diagram of the structure of the cell envelope of gram-negative bacteria). In this review, we shall discuss recent advances in the genetic analysis of certain of these surface components.

2 Acidic Polysaccharide (K) Capsules

Essentially all strains of bacteria capable of producing meningitis in humans (e.g., meningococcus, *Haemophilus influenzae*, *E. coli*, group B streptococci, and *Streptococcus pneumoniae*) possess a polysaccharide capsule as the outermost structure of the cell surface (e.g., see MÄKELÄ et al. 1980; ROBBINS et al. 1980; JENNINGS 1983; Fig. 1). The majority of invasive bacteria producing other types of infections are also encapsulated. The severity of disease caused by capsulated bacteria has been correlated with the amount of capsular material produced during infection (MACLEOD and KRAUSS 1950; MCCRACKEN et al. 1974). Noncapsulated relatives of pathogens do not generally produce disease, and noncapsulated variants of disease-producing strains exhibit greatly reduced pathogenicity in animal models (WOLFBERG and DEWITT 1969; AGÜERO and CABELLO 1983; Table 1). Thus, the polysaccharide capsules of invasive pathogens are important virulence factors. However, of the many types of bacterial capsules thus far identified, only few are associated with virulent bacteria and thereby play a role in bacterial pathogenesis: of the six types of polysaccharide capsule found in *Haemophilus influenzae* (PITTMAN 1931), only type b is associated with the majority of strains isolated from serious infections (TURK and MAY 1967), and of the greater than 100 capsule types of *E. coli* (ØRSKOV et al. 1977), only a few (e.g., K1, K5, K12) are associated with pathogenic strains (e.g., KAIJSER et al. 1977). The fact that capsular polysaccharides of similar or identical chemical structure are often found in unrelated pathogens (ØRSKOV et al. 1977) is consistent with the view that only a few capsular types are important in bacterial virulence.

Capsules of pathogenic bacteria usually inhibit phagocytosis (MIMS 1982). The exact mechanism(s) whereby they do so is not conclusively established but capsules probably function by preventing opsonization of bacterial cells and thereby reducing the efficiency of phagocytosis (ROBBINS et al. 1980). The

Table 1. Influence of the K1 capsule and the ColV plasmid on virulence of *E. coli* and on its susceptibility to phagocytosis and killing by serum[a]

Property[b]	LD_{50}[c]	% Phagocytosis[d]	% Survival in serum[e]
$K1^+$ $ColV^+$	10^5	26	170
$K1^+$ $ColV^-$	10^6	36	120
$K1^-$ $ColV^+$	$>10^9$	90	30
$K1^-$ $ColV^-$	$>10^9$	90	5

[a] Taken from AGÜERO and CABELLO (1983) and DELUCA and CABELLO (unpublished data)

[b] The $K1^+$ phenotype and carriage of the ColV plasmid of *E. coli* FC001 (018ab,ac:K1:H7) and its $K1^-$ and $ColV^-$ derivatives obtained as K1-specific phage-resistant mutants and colicin V nonproducing derivatives obtained by SDS curing, respectively

[c] The 50% lethal dose obtained by intraperitoneal injection of Swiss-Webster adult mice with bacteria plus hog gastric mucin

[d] Percentage of mouse peritoneal macrophages having phagocytosed the indicated bacteria

[e] Percentage of bacteria surviving incubation for 90 min in presence of 10% human serum. Values greater than 100% indicate that bacterial multiplication occurred during the incubation period

K1 capsule is thought to mask cell surface structures that directly activate complement, and thereby to prevent cell opsonization by C3b. Moreover, the K1 polysaccharide, which is composed of polymers of α-(2–8) residues of *N*-acetyl neuraminic acid (NeuNAc) (MCGUIRE and BINKLEY 1964), is very poorly immunogenic, perhaps because it is not recognized as foreign due to the fact that α-(2–8)-linked NeuNAc residues are found in gangliosides and in most mammalian serum and cell membrane glycoproteins (SCHAUER 1982). At any rate, even though circulating antibodies to a number of *E. coli* antigens are found in high titers in human blood, significant levels of anti-K1 antibodies that could opsonize invading *E. coli* K1 bacteria have not been detected (CROSS et al. 1983).

Capsular polysaccharides may be O-acetylated. Form variants of the K1 capsule have been identified in which the C_7 and C_9 atoms of neuraminic acid are randomly acetylated (ØRSKOV et al. 1979). The nonacetylated form is the most common type found in clinical isolates of *E. coli* K1, and is less immunogenic than the acetylated polysaccharide. However, O-acetylation at positions C_7 or C_8 of the group C meningococcus capsular polysaccharide, which is also a polymer of NeuNAc, is associated with a reduction in capsule immunogenicity. Again, it is the more weakly antigenic form, i.e., in this case the O-acetylated polysaccharide, which predominates in organisms isolated from cases of meningitis (GLODE et al. 1979).

The weak immunogenicity of the K1/meningococcus B capsules [until recently, only one high-titer antibody had been raised in an experimental animal (ØRSKOV et al. 1979)] has hindered immunological analysis of capsule structure

and function. Recently, however, the raising of two IgM-type, anti-K1 monoclonal antibodies was reported (CROSS et al. 1983; SÖDESTRÖM et al. 1983). Moreover, the raising of a high-titer IgG-type anti-K1/meningo B antibody from B-cell hyperreactive NZB mice has been described (FROSCH et al. 1985). The availability of these antibodies will considerably facilitate functional studies on the important K1 and meningococcus B capsules.

2.1 K1 Capsule

Over 80% of cases of *E. coli* neonatal meningitis, and a high proportion of *E. coli* bacteremias and urinary tract infections, are caused by strains that produce the K1 capsule (MCCRACKEN et al. 1974; ROBBINS et al. 1972; SARFF et al. 1975; KAIJSER et al. 1977). K1 polysaccharide is chemically and immunologically similar to the capsule of meningococcus B (LUI et al. 1971; BHATTACHARJEE et al. 1975), the principal cause of sporadic meningococcal meningitis in adults.

In a recent study (AGÜERO and CABELLO 1983), a comparison of a $K1^+$ *E. coli* strain (018ab,ac:K1:H7) isolated from a case of neonatal meningitis and a $K1^-$ derivative of this strain (obtained as a K1-specific phage-resistant mutant) showed that the K1 capsule significantly reduced the susceptibility of bacteria to phagocytosis by peritoneal macrophages and to killing by serum, and greatly increased their pathogenicity for a mouse infection model (Table 1). This and other investigations (e.g., WOLFBERG and DEWITT 1969; GEMSKI et al. 1980; TIMMIS et al. 1981; CROSS et al. 1984) have clearly demonstrated the ability of the K1 capsule to protect bacteria from killing by serum and phagocytes and its central role in the pathogenesis of some $K1^+$ *E. coli* strains.

The K1 capsule is the most extensively studied of the capsules (ØRSKOV et al. 1977; ROHR and TROY 1980; SCHAUER 1982), although our knowledge of the genetics and biochemistry of its synthesis and assembly as a functional organelle is still quite incomplete. The process of capsule production can be divided into three stages: (a) the synthesis and activation of NeuNAc, (b) polymerization of the activated sugar units, and (c) translocation of the polymer to the cell surface.

Although the biochemistry of NeuNAc synthesis has not yet been elucidated, in theory only two enzymes are required, namely an epimerase for the transformation of *N*-acetylglucosamine to *N*-acetylmannosamine, and an aldolase for the condensation of N-acetylmannosamine and phosphoenolpyruvate to yield NeuNAc. Activities corresponding to both of these types of enzyme have been found in K1 antigen-producing cells of *E. coli* (COMB and ROSEMAN 1960; GHOSH and ROSEMAN 1965). NeuNAc is subsequently activated by linkage to cytidine monophosphate by neuraminate cytidyl transferase (COMB et al. 1959).

The polymerization of NeuNAc is carried out by sialyl transferase, a poorly defined enzyme complex located in the inner membrane of K1-producing bacteria, which transfers NeuNAc residues from activated NeuNAc to an endogenous acceptor (KUNDIG et al. 1971; TROY and MCCLOSKEY 1979). The lipid undecaprenol acts as a carrier of NeuNAc in this process (TROY et al. 1975; TROY and MCCLOSKEY 1979; ROHR and TROY 1980). The endogenous acceptor, com-

posed of the growing chain of NeuNAc residues, is thought to receive new residues at its nonreducing terminus. The reducing terminus may be blocked, perhaps by a polypeptide which could function to secure the growing polysaccharide chain to the cytoplasmic membrane (ROHR and TROY 1980).

The process of translocation of the complete polysaccharide from its site of synthesis to the cell surface has not yet been characterized. It has been suggested that this process occurs at sites of contact between the bacterial cytoplasmic and outer membranes (BAYER 1979). It is noteworthy that the majority of encapsulated strains of *E. coli* produce a specific outer membrane protein, termed K protein, that is not found in nonencapsulated strains (PAAKKANEN et al. 1979). This protein was recently shown to function as a porin (SUTCLIFFE et al. 1983; WHITFIELD et al. 1983) and could conceivably function in translocating the polysaccharide to the outer surface of the bacterial cell.

Analysis of isolated capsular material has revealed that polysaccharide on the cell surface is attached to a phospholipid (GOTTSCHLICH et al. 1981; SCHMIDT and JANN 1982). It was postulated that this phospholipid may anchor the polysaccharide in the outer membrane, although the apparent lability of the lipid-polysaccharide linkage would seem to argue against a function of this type. Another possible function of the phospholipid would be in translocation of the polysaccharide to the cell surface.

Results from genetic mapping studies with a variety of encapsulated strains of *E. coli* have suggested that genes encoding the production of acidic polysaccharides in most 0 serotypes map near the *serA* locus of the chromosome (ØRSKOV and NYMAN 1974; ØRSKOV et al. 1976). Recombinants isolated from genetic crosses involving donors and recipients elaborating different capsular antigens expressed only the antigen of the donor parent; genes for the polysaccharides analyzed in this study were therefore concluded to be allelic (ØRSKOV et al. 1977). The genes for certain other K polysaccharides in a few 0 serotypes (08, 09, 020) were found to map near the *his* locus, i.e., near O-antigen biosynthesis genes (ØRSKOV et al. 1977).

2.1.1 Cloning and Analysis of K1 Capsule Genes

In order to analyze in greater detail the genetics of K1 capsule production, cosmid gene banks from $K1^+$ strains of *E. coli* were recently generated, and clones in the gene banks that contained hybrid plasmids carrying the K1 biosynthesis genes were identified by their production of precipitin haloes in agar plates containing anti-meningococcus B antiserum (SILVER et al. 1981; TIMMIS et al. 1981). Two K1 gene-containing hybrid plasmids, pSR23 and pKT172, that have similar restriction endonuclease cleavage maps, and their deletion mutant derivatives, have been studied in some detail (ECHARTI et al. 1983; SILVER et al. 1984; BOULNOIS, JANN, and TIMMIS, to be published).

Plasmid pKT274, a deletion derivative of pKT172, contains the cosmid-cloning vector pHC79, the transposon Tn*5*, and about 20 kb of chromosomal DNA from *E. coli* Bi7509/41 ($07:K1:H^-$), and directs the synthesis of K1 capsular material in the K-12 laboratory strain of *E. coli*. Deletion and transpo-

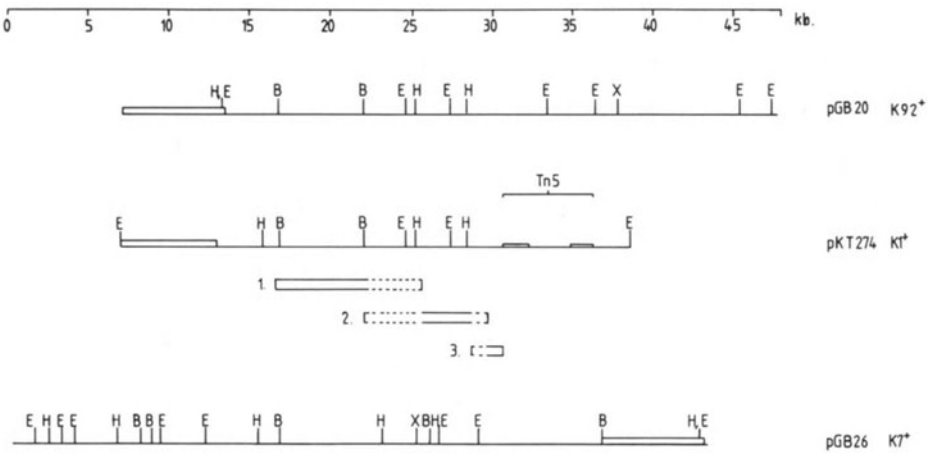

Fig. 2. Genetic and physical map of plasmid pKT274 ($K1^+$) and restriction enzyme maps of plasmids pGB20 ($K92^+$) and pGB26 ($K7^+$). *H,E,B,X,* cleavage sites for the *Hin*dIII, *Eco*RI, *Bam*HI, and *Xho*I endonucleases. *Single-line regions* represent cloned chromosomal DNA, whereas the *double-line regions* at the end of the maps represent vector DNA. The *short double-line segments* that terminate the Tn*5* element of pKT274 indicate the inverted repeats of this element. Regions *1*, *2*, and *3* are the functional regions of the K1 gene cluster described in the text

son mutagenesis analysis of pKT274 has demonstrated that a DNA segment about 15 kb in length specifies production of the K1 capsule. The K1 production genes are organized in three distinct regions (Fig. 2; ECHARTI et al. 1983; BOULNOIS, JANN, and TIMMIS, to be published; BOULNOIS, unpublished).

Mutations in region 1 prevent K1 capsule formation, as judged by the inability of bacteria harboring such mutant plasmids to develop a precipitation zone when grown on agar-containing antimeningococcus B antiserum. However, immunodiffusion of cell lysates obtained by several cycles of freezing and thawing of mutant bacteria demonstrated the presence of K1 antigen (ECHARTI et al. 1983; FROSCH et al. 1985). Osmotic shock experiments revealed that the K1 polysaccharide in these mutants is located in the periplasmic space. Immunoelectrophoretic analyses indicate that the K1 polysaccharide made by region 1 mutants is probably identical to that made by the strain from which the genes were originally cloned (BOULNOIS, JANN, and TIMMIS, to be published). Thus, functions encoded by region 1 seem to be involved in the process of translocation of the mature polysaccharide from the periplasmic space to the cell surface.

Mutations in region 2 completely prevent the formation of any polysaccharide that reacts with anti-meningococcus B antiserum and are assumed to affect the genes of enzymes required for the synthesis, activation, and polymerization of NeuNAc (ECHARTI et al. 1983; BOULNOIS, unpublished). Consistent with this conclusion is the finding that bacteria carrying mutant derivatives of pSR23 containing Tn*5* insertions in region 2 produce a K1 capsule when supplied with exogenous NeuNAc (SILVER et al. 1984).

Mutations in region 3 prevent both halo formation by bacteria carrying mutant hybrid plasmids and the release of antimeningococcus B antiserum-reacting material by osmotic shock. However, the antiserum does precipitate

intracellular material that is released by cell lysis. Immunoelectrophoretic analysis of this material revealed that it has a very low electrophoretic mobility compared with that of mature polysaccharide (FROSCH et al. 1985; BOULNOIS, JANN, and TIMMIS, to be published). Its chemical structure is presently unclear but it is tempting to speculate that it may consist of polysaccharide linked either to undecaprenol or the endogenous acceptor. The intracellular location of this material and its electrophoretic behavior indicate that the functions inactivated by mutations in region 3 may involve transfer of the NeuNAc polymer from either the lipid carrier or the acceptor to the phospholipid.

A similar study of the pSR23 plasmid has been carried out and, with one exception, findings were obtained that are consistent with those described above for pKT274. The main difference was a proposal of the existence of additional genes for capsule production in a DNA region, designated region C, that had not been identified in the study of the pKT172/pKT274 plasmids (SILVER et al. 1984). The evidence in support of this proposal was that a mutant plasmid containing a Tn*5* insertion some 3 kb from region C failed to express not only genes known to encode capsule production functions but also genes located in region C. The Tn*5* insertion of this mutant plasmid was concluded to have inactivated a control region involved in the expression of capsule biosynthesis genes. However, an examination of the restriction endonuclease cleavage maps of pSR23, pKT274, and pKT172 reveals that region C is present on pKT172 but absent from pKT274. All available data indicate that the capsule specified by pKT274 is indistinguishable from that produced by bacteria containing pKT172 and from that produced by the $K1^+$ strain from which the genes were originally cloned. It seems possible therefore that the pSR23 mutant plasmid on which the existence of region C is based is defective in more than one function, and that region C is not involved in K1 capsule production.

Analysis of the polypeptides encoded by the pSR23 plasmid has identified some of the proteins that are probably involved in capsule production (SILVER et al. 1984). Thus far, the analysis is not sufficiently advanced to permit correlation of specific polypeptides with individual enzyme activities. However, an operon composed of two genes that encode polypeptides of molecular weights of 45000 and 50000 which are involved in NeuNAc biosynthesis has been defined on the basis that a defect in their production could be rectified by the provision of exogenous NeuNAc. These polypeptides would be encoded by region 2 of pKT274 (Fig. 2). A minimum of five polypeptides encoded by region 1 and involved in the translocation process were also identified in this study (SILVER et al. 1984).

A comparison of the polypeptides present in outer membranes prepared from the $K1^+$ donor strain and *E. coli* K-12 derivatives carrying the pKT172 and pKT274 plasmids revealed the presence of the K protein (see above) in the former strain but its absence in the *E. coli* K-12 derivatives (G.J. BOULNOIS, unpublished). Therefore, the K protein is not essential for K1 capsule production, at least in the *E. coli* K-12 background. Further studies are clearly needed to establish whether or not K protein plays a role in capsule production in encapsulated clinical isolates of *E. coli* and, if so, which other outer membrane protein is able to substitute for it in *E. coli* K-12.

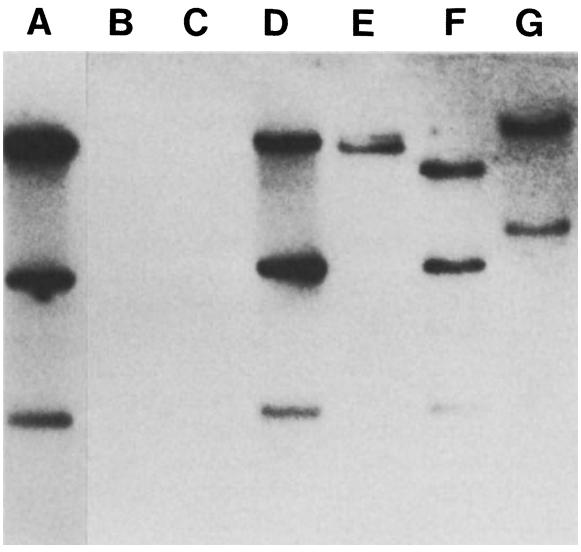

Fig. 3. Southern blot analysis of sequences homologous to cloned K1 genes in different encapsulated bacteria. Plasmid pKT274 DNA was used as probe in a standard Southern blotting experiment (1975). Lane *A* contains *Eco*RI-digested DNA of pKT172, the original K1 gene-containing cosmid clone; lanes *B–G* contain *Eco*RI-digested chromosomal DNAs of strains *Neisseria meningitidis* B (*B*), *N. meningitidis* C (*C*), *E. coli* K1 (*D*), *E. coli* K7 (*E*), *E. coli* K92 (*F*), and *E. coli* K100 (*G*) (ECHARTI et al. 1983)

2.1.2 Polynucleotide Sequence Relatedness of K1 Genes and Those of Other Capsules

The chemical and immunological similarity of the capsules of *E. coli* K1 and group B meningococcus suggested the possibility of related genetic determinants for capsule production in these unrelated bacteria. However, Southern Blot analysis (SOUTHERN 1975) of total DNA from group A, B, and C meningococci, using as probe a cloned DNA segment carrying the K1 capsule determinants (Fig. 3), failed to reveal any polynucleotide sequence homology, even under experimental conditions that permitted 25% mismatch in hybrid molecules (ECHARTI et al. 1983). Thus, either entirely different proteins are used to accomplish similar functions in *E. coli* K1 and meningococcus B, or similar proteins accomplish similar functions and, despite there being little divergence of the protein sequences, there exists extensive divergence of the sequences of their determinants, principally in the third and second codon positions. Cloning of the meningococcus B capsule determinants and comparison of the polynucleotide sequences of these genes with equivalent genes in *E. coli* K1 will answer this question.

Although in this study no relatedness was detected between the determinants of similar capsules in different organisms, it is to be anticipated that some of the determinants of the more than 100 distinct capsules produced by different strains of *E. coli* will be similar, particularly those encoding functions which may be common to the production of all capsules, e.g., involving the undecaprenol carrier molecule, attachment of the terminal phospholipid, and the translocation process. This was tested by Southern Blot analysis of total DNA obtained from *E. coli* producing the following capsules: K7, a polymer of *N*-acetylmannosaminuronic acid and D-glucose residues (ICHIHARA et al. 1974),

which is antigenically related to the *Streptococcus pneumoniae* type III capsular polysaccharide; K92, a polymer of alternating α-(2–8)- and α-(2–9)-linked NeuNAc residues which is antigenically related to the meningococcus C capsular polysaccharide; K100, a phosphodiester-linked copolymer of ribose and ribitol, which is antigenically related to the *H. influenzae* b capsular polysaccharide; and K5, a polymer of *N*-acetylglucosamine and glucuronic acid (VANN et al. 1981).

When a DNA fragment containing all of the K1 capsule genes was used as a probe, significant homology was found in the DNA of encapsulated strains of *E. coli*, but not in the noncapsulated laboratory strain *E. coli* K-12 (a feature that has proven invaluable for the cloning and analysis of capsule determinants; see below). In the case of *E. coli* K5 and K92, some of the restriction endonuclease-generated DNA fragments that bound the probe were identical in size to those generated from the probe itself, indicating considerable conservation of polynucleotide sequence within the determinants of these different capsules (Fig. 3; ECHARTI et al. 1983; BOULNOIS, unpublished). In the case of the determinants of the K92 capsule, this was not surprising because it is chemically similar to the K1 polysaccharide. However, the K5 polysaccharide is structurally distinct (VANN et al. 1981). The use as probes of smaller DNA fragments derived from the different functional regions of the K1 gene complex revealed that at least part of the homology between the K1 determinants and equivalent determinants of strains producing other capsules resides in the region that encodes translocation functions. Thus, chemically different polysaccharides of *E. coli* would appear to be translocated to the cell surface by a common mechanism involving the products of some common genes (see below).

The restriction endonuclease-generated DNA fragments from *E. coli* K7 and K100 which bound the K1 gene probe have sizes that are different from those of the probe, and thus their sequences appear to have diverged more extensively from that of the K1 determinants than have those of the K5 and K92 determinants.

2.2 Cloning and Analysis of Genes of Other *Escherichia coli* Capsules

In order to define which steps in capsule production in *E. coli* are common and which are capsule specific, it will be necessary to characterize extensively the genetics and biochemistry of several capsules. In particular, it will be crucial to determine which defects in the formation of one capsule can be complemented by equivalent functions of others (see below). To this end, cosmid gene banks of DNA from *E. coli* K5, K7, K12, and K92 strains have been produced in *E. coli* K-12 and screened by colony hybridization (GRUNSTEIN and HOGNESS 1975) for homology to a DNA segment carrying part of region 1 of the K1 capsule determinant region (translocation functions). Positive clones in the banks were subsequently shown by means of anticapsular antiserum or capsule-specific phages to produce capsular material of the expected type. The prescreening of the banks for capsule translocation determinants by colony hybridization not only economizes utilization of valuable antisera, but also permits very care-

ful tests to be carried out on a limited number of clones that may not produce large quantities of capsular material and that may not readily be detected by less-sensitive tests which must be used for the screening of entire gene banks. However, the prescreening will only identify capsule-producing clones under conditions where the K-12 host strain synthesizes the appropriate sugar components of the capsule, or where sugar biosynthesis genes are closely linked to the polysaccharide translocation genes, as is the case with the K1 antigen.

Figure 2 shows restriction endonuclease cleavage maps of plasmids pKT274, pGB20, which specifies production of the K92 capsule, and pGB26, which specifies production of the K7 capsule. Alignment of the pKT274 and pGB20 maps was made on the basis of restriction endonuclease-generated fragments that are common to both plasmids. In the case of pKT274 and pGB26, alignment relied on hybridization of probes from specific regions of pKT274 to various restriction enzyme digests of pGB26. From these maps it is immediately obvious that while the genes for the K1 and K92 capsules are closely related, those for the K7 capsule appear to be different.

Detailed analysis of regions of homology between the K1 and K7 capsule gene clusters indicates that these clusters have a similar organization. DNA probes derived from regions 1 and 3 of the K1 gene region hybridize to segments of pGB26. Moreover, some DNA fragments from this plasmid are able to complement certain region 1 mutations that prevent K1 polysaccharide translocation to the cell surface (R. MOUNTFORD, N. HIGH and G.J. BOULNOIS, unpublished data). Thus, at least some steps in the translocation process are common to capsules having different structures. In contrast, DNA probes from region 2 of the K1 gene cluster failed to hybridize to the K7 gene region. This is not surprising, given the fact that region 2 is thought to encode enzymes for sugar biosynthesis and polymerization, enzymes that presumably are unique to strains that produce K1 and other capsules consisting of NeuNAc polymers. Thus, it is likely that functions encoded by region 2 of the K1 gene cluster, and its equivalent in other capsule biosynthesis gene clusters, are unique and constitute the primary determinants of capsule type.

3 Outer Membrane Proteins

The discovery that outer membrane proteins of *E. coli* could mediate resistance to host defenses arose from an investigation of possible plasmid involvement in such resistance. Plasmids, promiscuous extrachromosomal genetic elements that may be transmitted horizontally among populations of bacteria at high frequencies under appropriate selection conditions, have been known for a long time to encode important pathogenesis factors, such as enterotoxins, adhesion antigens, and high-affinity iron uptake systems (for reviews, see CABELLO and TIMMIS 1979; ELWELL and SHIPLEY 1980). More recently, plasmids of enteroinvasive strains of *E. coli* and *Shigella* have been shown to encode functions for the invasion of human epithelial cells (*E. coli* and *Shigella*: HARRIS et al. 1982; SANSONETTI et al. 1982) and the biosynthesis of lipopolysaccharide (*Shigella*;

KOPECKO et al. 1980; WATANABE and TIMMIS 1984; WATANABE et al. 1984). Plasmids also encode resistance to a wide range of antibiotics (e.g., see LEVY et al. 1981).

Examination of a selection of plasmids led to the identification of several belonging to the F incompatibility group of conjugative plasmids, such as ColV (FI), R100 (FII), and R6-5 (FII), that increased the ability of *E. coli* K-12 to survive in dilute solutions of serum (WILLIAMS SMITH 1974; REYNARD and BECK 1976; FIETTA et al. 1977; TAYLOR and HUGHES 1978; BINNS et al. 1979; TIMMIS et al. 1979; MOLL et al. 1980). Subsequent studies revealed that these endowed resistance to high concentrations of serum upon some but not all serum-sensitive wild strains of *E. coli* (TAYLOR and HUGHES 1978). The fact that in no case did the plasmids permit *E. coli* K-12 to survive in high concentrations of serum emphasized that plasmid-specified resistance involves additional bacterial components, in particular O-antigen lipopolysaccharide (TAYLOR and HUGHES 1978; MOLL et al. 1980; TIMMIS et al. 1981). Genetic studies on serum resistance specified by these plasmids has thus far identified two determinants of resistance, namely *traT* carried by R100, R6-5 and ColV, and *iss* carried by ColV.

3.1 *traT*p

The cloning of R6-5 plasmid DNA fragments containing the serum resistance gene of the plasmid, and the generation of transposon mutant derivatives of hybrid plasmids containing such fragments, made possible localization of the serum resistance gene to a region previously known to contain the *traT* gene (MOLL et al. 1979, 1980). The *traT* gene, together with the adjacent *traS* gene, specifies the phenomenon of surface exclusion (ACHTMAN et al. 1977), the reduced ability of a strain carrying a conjugative plasmid to act as a recipient when mated with a donor strain carrying a related plasmid. Comparison of the polypeptides specified by hybrid plasmids carrying an intact serum resistance gene with those specified by mutant plasmids no longer mediating serum resistance confirmed that the serum resistance factor specified by plasmid R6-5 was the product of the *traT* gene, *traT*p, a major outer membrane protein having an apparent molecular weight of 25000 daltons. This protein is present in 5000–10000 copies per cell in wild-type bacteria carrying IncFII plasmids (BITTER-SUERMANN et al. 1984) and in 20000 copies per cell in *E. coli* K-12 bacteria carrying pKT107, a *traT* gene-carrying pACYC184 hybrid plasmid (MOLL et al. 1980). Studies with a related *traT*p encoded by plasmid F have shown that it is a lipoprotein that probably exists in the outer membrane as oligomers (MINKLEY 1984; PERUMAL and MINKLEY 1984). Iodination of tyrosine residues of cell surface proteins of pKT107-carrying bacteria revealed that *traT*p is highly exposed on the bacterial surface (TIMMIS et al. 1981; MANNING et al. 1982). Bacteria carrying one particular type of mutant plasmid overproduced an altered *traT*p species having a reduced accessibility to iodination and, hence being less exposed on the cell surface, were defective in the serum resistance and surface exclusion properties (MANNING et al. 1982). These results are consis-

tent with both of the known biological properties of *traT*p, which involve interactions of the bacterial cell surface with external factors.

Interestingly, despite the fact that *traT*p is exposed on the cell surface, attempts to obtain an anti-*traT*p monoclonal antibody from mice immunized with $traT^+$ whole bacteria, or outer membranes prepared from such bacteria, were unsuccessful. Three anti-*traT*p antibodies that reacted strongly with solubilized bacteria were obtained after immunization with purified *traT*p, although none of them reacted strongly with whole bacteria (BITTER-SUERMANN et al. 1984). Thus, *traT*p epitopes located on the cell surface would appear to be poorly immunogenic, a feature reminiscent of the K1 capsule.

The mechanism by which *traT*p mediates resistance to complement has not yet been elucidated, although the possibility that it acts either by degradation or nonproductive binding of one or more complement components seems to have been ruled out: addition of $traT^+$ serum-resistant bacteria to a solution of serum does not significantly reduce its subsequent capacity to kill isogenic $traT^-$ serum-sensitive cells (TIMMIS et al. 1981).

Studies on the binding of complement components to isogenic $traT^+$ and $traT^-$ cells have failed to detect any significant differences in the amounts of individual components bound to $traT^+$ and $traT^-$ derivatives of *E. coli* K-12 suspended in dilute solutions of serum (BINNS et al. 1982) or in the amounts of C3b bound to $traT^+$ and $traT^-$ derivatives of a wild strain of *E. coli* suspended in higher concentrations of serum (up to 50%; AGÜERO et al. 1984). Thus, the serum resistance property of $traT^+$ cells would seem not to result from a *traT*p-mediated reduction in the binding of complement, particularly in view of the fact that anti-*traT*p antibody does not render $traT^+$ serum-resistant bacteria sensitive to serum killing (AGÜERO and CABELLO, unpublished data). A current best guess therefore is that *traT*p mediates resistance to serum by interfering with the correct assembly or functioning of the complement membrane attack complex. It has been estimated that the *E. coli* cell surface can bind in excess of 10000 membrane attack complexes. Bacteria carrying R6-5 contain at most 5000–10000 copies of *traT*p monomers in their outer membranes, but a much smaller number of functional units of the protein, due to oligomer formation. These two considerations suggest that only a fraction of the complement-binding sites on the surface of *E. coli* are important for complement killing and that *traT*p is localized and exerts its protective activity at these sites. In this regard, the recent observation that the presence of *traT* in bacteria suspended in serum results in an altered distribution of C3b bound to the cell surface (AGÜERO et al. 1984) many be significant.

In addition to mediating bacterial resistance to serum killing in certain strains of *E. coli*, *traT*p reduces their susceptibility to phagocytosis by peritoneal macrophages (AGÜERO et al. 1984). Whereas the approximately 5000 copies of *traT*p per cell in R6-5-carrying bacteria provide maximal resistance to serum killing, they provide less protection against phagocytosis than do the 20000 copies per cell in pKT107-carrying bacteria. That is, resistance to phagocytosis is a quantitative property that reflects the copy number of the $traT^+$ plasmid [this gene dosage effect appears to be a consequence of constitutive expression of the R6-5 *traT* gene from its own promoter (C.D. O'CONNOR and K.N. Timmis,

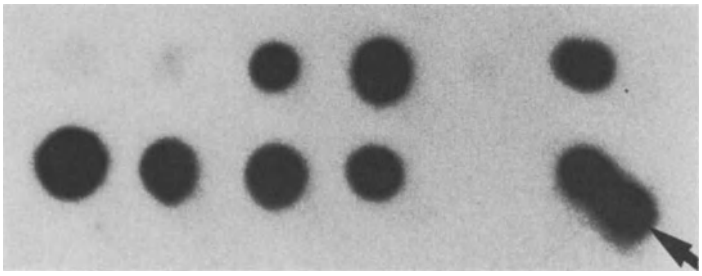

Fig. 4. Colony hybridization of clinical isolates of *E. coli* with a *traT* gene probe. Part of a nitrocellulose filter is shown that contains lysed colonies of clinical isolates of *E. coli* and that has been probed with a 700-bp *Bst*EII fragment of R6-5 containing the *traT* gene (GRUNSTEIN and HOGNESS 1975; MOLL et al. 1979; MONTENEGRO et al. 1985). The *arrow* indicates the positive control colony *E. coli* K-12 (pKT107)

to be published)]. As indicated above, the distribution of C3b molecules bound to the surface of *traT*$^+$ bacteria that show reduced susceptibility to phagocytosis is not uniform (AGÜERO et al. 1984). Moreover, despite the finding that anti-*traT*p antibody does not reduce *traT*p-mediated resistance to serum it does abolish *traT*p-mediated resistance to phagocytosis, although Fab fragments prepared from anti-*traT*p IgG molecules do not. It is likely, therefore, that *traT*p exerts its antiphagocytic effect by reducing opsonization of *E. coli* cells by C3b.

Finally, *traT*p was found to increase to a limited but significant extent the pathogenicity of an *E. coli* strain for a mouse infection model (L. DELUCA and F.C. CABELLO, unpublished data).

It should be emphasized that although most or all IncF group plasmids specify closely related *traT* proteins, such proteins do not have identical primary sequences and need not necessarily have identical functional properties. The recent cloning and analysis of a *traT* gene of a ColV plasmid revealed that although its product mediated serum resistance, it mediated a level of resistance to phagocytosis considerably lower than that of *traT*p of R6–5 (PARADA et al. to be published).

A crucial aspect of any potential pathogenesis factor is its prevalence among pathogenic and nonpathogenic microbes. Two procedures for detecting the presence of *traT* or *traT*p, namely colony hybridization (GRUNSTEIN and HOGNESS 1975), with a 700-bp *Bst*EII fragment containing part of the *traT* gene as probe (Fig. 4), and immunoblotting (TOWBIN et al. 1979), with an anti-*traT*p monoclonal antibody (Fig. 5), have been used in such studies. The colony hybridization procedure has revealed the presence of *traT* gene sequences in isolates of *E. coli*, *Shigella*, *Salmonella*, and *Klebsiella*, but not in *Enterobacter*, *Citrobacter*, *Yersinia*, *Proteus*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Aeromonas*, or *Plesiomonas* (MONTENEGRO et al. 1985). Within *E. coli*, the *traT* gene was found to be plasmid-borne in all cases examined and only present on plasmids belonging to the F incompatibility group. The monoclonal antibody detected *traT*p in essentially all *E. coli* and *Salmonella typhimurium* strains that had been shown by colony hybridization to be *traT*$^+$. Interestingly, antibody-reacting proteins having molecular weights greater than that of *traT*p were detected in 31%

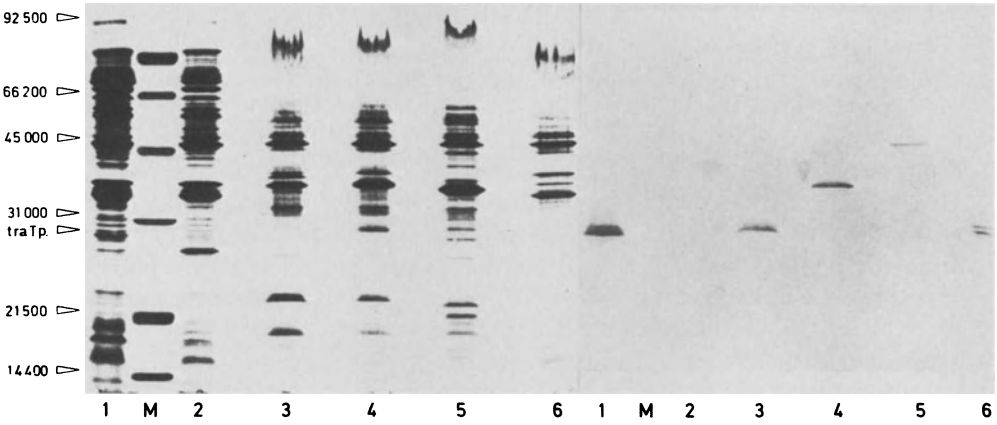

Fig. 5. Immunoblot of total cellular proteins of clinical isolates with an anti-*traT*p monoclonal antibody. Bacteria from overnight liquid or agar plate cultures were solubilized in sodium dodecyl sulfate (SDS)-containing buffer and subjected to electrophoresis on an SDS-polyacrylamide gradient slab gel. Proteins were then transferred to a nitrocellulose filter, blotted with the monoclonal antibody, and bound antibody subsequently revealed by an anti-mouse peroxidase conjugate (TOWBIN et al. 1979; JURS et al. 1984). *Left side*, stained gel; *right side*, immunoblot. Lanes *1–6* contain solubilized bacteria of the following strains; *1*, *E. coli* K-12 (pKT146; a plasmid from which *traT*p is expressed at high levels; see MANNING et al. 1982); *2*, *E. coli* K-12 *traT*$^-$; *3*, *Salmonella typhimurium*; *4*, *S. derby*; *5*, *Enterobacter cloacae*; *6*, *E. coli traT*$^+$. Lane *M* contains molecular weight markers. Note that whereas the reacting species of *S. typhimurium* and the *E. coli* clinical isolate have the same molecular weights as that of *traT*p specified by R6-5 (pKT107), those of *S. derby* and *E. cloacae* have higher molecular weights. Note also the double band in the case of the *E. coli* clinical isolate; this is frequently observed when bacteria are grown on blood plates as they were in this particular case (JURS et al. 1984)

of isolates of salmonellae of serogroups B, C, and D, and in 91% of isolates of *Enterobacter cloacae* (Fig. 5; JURS et al. 1984). The extent of structural and functional similarity to *traT*p of these *traT*p-like proteins in non-*E. coli* isolates remains to be determined.

*traT*p was found in about 60% of *E. coli* isolates that caused bacteremia, in about 60% of those that caused enteric infections, in about 50% of those which produced urinary tract infections, and in about 40% of isolates from feces of healthy persons. Thus, although *traT* is found in a high proportion of isolates assumed to be nonpathogenic, it is present in a significantly higher proportion of pathogenic strains, in particular invasive strains causing bacteremia. On the other hand, a high degree of correlation of the *traT*$^+$ character and serum resistance was not found: although 60% of *E. coli* isolates from cases of sepsis were serum resistant, only 34% were concomitantly *traT*$^+$ and serum resistant (MONTENEGRO et al. 1985). Thus, if *traT*p does play a role in the pathogenesis of some invasive pathogens, another cell surface component must fulfill an analogous function in others. Moreover, if the role of *traTp* is to mediate resistance to complement, it is obviously unable to manifest this property in some isolates, namely those serum-sensitive strains that are *traT* positive.

3.2 *iss*p

ColV plasmids are carried by a high proportion of clinical isolates of *E. coli* and have been shown to increase the virulence of test strains of this organism in experimental infection models (WILLIAMS SMITH 1974; MINSHEW et al. 1978; AGÜERO and CABELLO 1983; Table 1).

Genetic studies in conjunction with a chicken infection model demonstrated that a large cloned *Bam*HI fragment of the ColV, I-K94 plasmid, not derived from the plasmid transfer region (and hence presumably not carrying *traT*), conferred upon host bacteria increased virulence and resistance to serum (BINNS et al. 1979). It was assumed that a single gene, designated *iss* for *i*ncreased *s*urvival in *s*erum, was responsible for the two phenotypes.

In another study (PARADA et al., to be published), a ColV plasmid present in an *E. coli* K1 strain isolated from a case of neonatal meningitis (AGÜERO and CABELLO 1983), was analyzed by Southern blotting, using as probes a DNA fragment containing the *traT* gene of R6-5 and a DNA fragment containing the *iss* gene of ColV, I-K94, and was shown to contain *traT* and *iss* determinants which were located on different DNA fragments. These DNA fragments were cloned separately and each shown to specify an increase in resistance to serum of a wild-type *E. coli* strain. Analysis of the proteins expressed from a hybrid plasmid containing the cloned *iss* gene and derivative plasmids containing transposon insertions that inactivated serum resistance identified the *iss* gene product as an outer membrane protein having a molecular weight similar to that of *traT*p. The *iss* protein is present in fewer copies per cell than is the case for *traT*p. Interestingly, although the ColV plasmid-encoded *traT* and *iss* proteins mediate serum resistance, they do not decrease the susceptibility of host cells to phagocytosis by macrophages. Experiments to determine whether or not the cloned *iss* gene is the determinant of increased virulence specified by ColV plasmids of isolates from cases of sepsis remain to be carried out.

4 Concluding Remarks

In this review we have summarized recent studies on the molecular genetics of *E. coli* cell surface structures that cause selected strains of bacteria to exhibit one or more of several pathogenicity-associated properties, namely resistance to complement killing, resistance to phagocytosis, and the increase in bacterial virulence for infection models. The genetics of O-antigens of *E. coli* have received little attention in recent years and therefore are not dealt with in this review, although of course some types are known to mediate resistance to complement and to increase bacterial virulence (MOLL et al. 1979; PLUSCHKE et al. 1983; GOLDMAN et al. 1984). Moreover, it is almost certain that new factors will be discovered that contribute to the resistance of pathogenic *E. coli* strains to host defenses (HACKER et al., to be published).

After identification of microbial factors exhibiting virulence-associated properties, it is necessary to evaluate their actual contribution to bacterial pathogeni-

city, i.e., to determine their relative importance for pathogenicity, to ascertain their prevalence among related pathogens, and to evaluate their mode of action. The former two objectives have previously been approached by epidemiological studies. Nevertheless it is clear that such studies can never establish causal relationships. Current genetic approaches are able to establish causal relationships because they enable isogenic pairs of strains to be constructed that differ only in the factor under investigation. However, the comparison of the pathogenicity of such pairs of isogenic strains is problematic because few animal infection models exactly parallel natural human infections. For this reason models must be chosen with care and data obtained interpreted conservatively.

Moreover, even if a factor is clearly shown to be causally related to the pathogenic potential of a particular strain, and even if this factor is found in a high proportion of strains causing similar infections, only tentative conclusions relating to the general role and importance of the factor can be drawn. This is particularly true for gram-negative bacteria, which have complex cell envelopes, and in which a certain degree of structural and functional overlap between different surface components is known to exist. Another aspect of the interplay of cell surface structures is the fact that certain pathogenicity factors function only in concert with other cell surface components. For example, full expression of *traT*p-mediated serum resistance requires an intact O-antigen (MOLL et al. 1979), although not all O-antigens can fulfill this need (TAYLOR and HUGHES 1978). Thus, the presence of an O-antigen and *traT*p does not necessarily signify that a strain is serum resistant or, if it is, that the resistance is *traT*p mediated. Obviously, a clear understanding of the role and importance of cell surface pathogenicity factors requires detailed studies of their activities in several different test strains.

We have described here genetic studies on two types of cell surface structure, namely acidic polysaccharide capsules and outer membrane proteins. It should be emphasized that, although there are many different types of capsules and outer membrane proteins in *E. coli*, only a few actually exhibit pathogenicity-related properties. The evidence that the K1 capsule is a major virulence factor of some invasive strains of *E. coli* would seem to be irrefutable. It mediates resistance to complement killing, resistance to phagocytosis, and increases the virulence of capsule-less strains for infection models, probably by acting as an anti-opsonin. The *traT* protein encoded by plasmid R6-5 also exhibits these three properties. The mechanism whereby it mediates resistance to complement seems to be through interference with the functioning of the membrane attack complex of complement. The increase in virulence mediated by *traT*p is considerably less dramatic than that observed with the K1 capsule. Any role *traT*p may have in virulence remains to be established by the generation of specific *traT*$^-$ deletion mutants of *traT*$^+$ O$^+$ serum-resistant, pathogenic isolates, and by the subsequent comparison of their virulence levels with those of their isogenic parent strains. The *iss* protein mediates resistance to serum, probably by a mechanism similar to that of *traT*p, but not resistance to phagocytosis.

Although not thus far definitively established, recurring features of the *E. coli* factors discussed here are poor immunogenicity of the exposed epitopes, and a change in the binding of C3b by bacterial cell surfaces. These properties

contrast with those of O-antigens which can have similar roles and which nevertheless are extremely antigenic. However, in the absence of specific antibody the O-antigen is antiphagocytic and, even in the presence of antibody, mediates resistance to complement killing. It appears to achieve the latter due to the fact that the extremely long O side chains [a recently described immunoblotting procedure revealed lipopolysaccharide (LPS) molecules isolated from *Shigella sonnei* containing greatly in excess of 30 side chain monomer units (STURM et al. 1984)] cause nonproductive binding of complement components at sites far removed from their membrane targets. Thus, through apparently different mechanisms, O-antigens would appear to contribute to aspects of virulence similar to those effected by polysaccharide capsules and outer membrane proteins.

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Molecular Cloning of Genes Encoding Gram-Positive Virulence Factors

M.S. GILMORE

1 Introduction

Among both scientific and commercial reasons for cloning genes related to bacterial virulence, the most compelling is the contribution that analysis of cloned virulence determinants can make to advancing an understanding of infectious disease. Establishment of an infectious disease is the result of an imbalance in the steady-state "host-parasite" relationship. As the host defense mechanisms are many and interrelated, the ability of a bacterium to invade and cause disease is the result of the complex interaction of many bacterial traits. To understand bacterial pathogenicity, then, it is essential to isolate and characterize the components of this multifactorial process, identify regulatory features, and assess their respective contribution to the infectious disease. Recombinant DNA technology provides the necessary tool for isolating the genes encoding virulence factors.

A review has recently been published which surveys many bacterial antigens and virulence determinants that have been cloned (MACRINA 1984). In this article I have attempted to review the rapidly expanding literature on cloning medically relevant gram-positive virulence factors (excluding those originating in the oral streptococci, which are covered in more detail elsewhere in this volume, see CURTISS p. 235ff). The purpose of this review is twofold: (1) To provide the scientist contemplating molecular cloning of genes encoding gram-positive virulence factors with a basic body of information which details the advantages

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of cloning systems currently in use or development and (2) to describe the results of recent applications of recombinant DNA technology toward similar ends.

2 Cloning in Gram-Positive Organisms

The first experiments on transforming avirulent variants of a species to the virulent state by the introduction of virulence-factor-encoding DNA sequences were the landmark studies on the gram-positive organism *Streptococcus pneumonia* by Avery and co-workers (AVERY et al. 1944; MCCARTY and AVERY 1946). Despite this, much of the emphasis in the development of recombinant DNA technology has been placed on methods using strains of *Escherichia coli* as the host. Several comprehensive manuals describing state-of-the-art techniques for manipulating genes and their introduction into *E. coli* are available (DAVIS et al. 1980; MANIATIS et al. 1982; RODRIGUEZ and TAIT 1983). As will be discussed in subsequent sections, however, the gram-positive virulence factors which have thus far been cloned are uniformly extracellular products. The presence of the outer membrane unique to gram-negative organisms presents a formidable barrier to externalization of cloned gram-positive extracellular gene products. As a result, detection of the cloned phenotype among transformants of this heterologous host is often made more difficult. For this reason it would be advantageous to clone extracellular gene products of gram-positive origin in a gram-positive host. Additionally, it would be desirable to limit the dissemination of genes encoding virulence factors to organisms known to undergo genetic exchange with the natural host.

The first systems developed which were applicable to molecular cloning in a gram-positive organism utilized transformable strains of *Bacillus subtilis* as the host (EHRLICH 1978; GRYCZAN and DUBNAU 1978; KEGGINS et al. 1978). However, because of the inefficiency of competent *B. subtilis* transformation by recombinant DNA molecules – only multimeric forms which enter the bacterium as single DNA strands can regenerate a circular duplex molecule (CANOSI et al. 1978; MOTTES et al. 1979) – these cloning systems were not amenable to "shotgun" cloning chromosomal DNA fragments. Two technical improvements alleviated this inefficiency somewhat. CHANG and COHEN (CHANG and COHEN 1979) described a technique for transforming protoplasts of *B. subtilis*, thereby circumventing the process of single-stranded DNA uptake employed by naturally competent cells. GRYCZAN and co-workers (GRYCZAN et al. 1980) developed a helper plasmid system which uses a resident plasmid-sharing partial homology to the cloning vector to permit recombinational rescue of the entering single-stranded vector-chromosomal DNA hybrid.

Because of the medical importance of the genera, molecular cloning in naturally competent strains of *Streptococcus sanguis* and *S. pneumoniae* has been intensively investigated (BEHNKE and FERRETTI 1980a; MACRINA et al. 1980; BEHNKE et al. 1981; STASSI et al. 1981). Although several well-characterized vectors have been described for use in streptococcal cloning (BEHNKE and FERRETTI 1980b; BURDETT 1980; MACRINA et al. 1980; BEHNKE and GILMORE 1981;

MALKE et al. 1981), their application has primarily been limited to cloning selectable chromosomal genes which can complement auxotrophic mutations (STASSI et al. 1981) and antibiotic resistance determinants (BEHNKE and FERRETTI 1980b; MACRINA et al. 1980; BEHNKE et al. 1981). Again the limitation of these systems is implicit in the inefficiency of the two-hit kinetic, single-stranded DNA uptake mechanism employed by competent streptococci (BEHNKE 1981; MACRINA et al. 1981; SAUNDERS and GUILD 1981). As for *B. subtilis*, resident plasmid marker rescue cloning systems have increased the efficiency of cloning in streptococci (BEHNKE et al. 1982; MACRINA et al. 1982a; MALKE and HOLM 1982).

Additional gram-positive species which naturally achieve a competent state for DNA uptake include strains of *S. mutans* (PERRY and KURAMITSU 1981) and *Staphylococcus aureus* (PAKULA and WALCZAK 1958) [the latter as the result of a phage-associated competence factor (THOMPSON and PATTEE 1981)]. Transformation of group N streptococci (MCKAY and BALDWIN 1982), group D streptococci (M SMITH, B EHRENFELD, and D CLEWELL, personal communication), staphylococci (KELLER et al. 1983), and various bacilli (MARTIN et al. 1981; IMANAKA et al. 1982; TAKAHASHI et al. 1983) has also been reported as has the development of appropriate cloning vectors (LÖFDAHL et al. 1978; WILSON and BALDWIN 1978; WILSON et al. 1981). Despite these advances, the limitations of gram-positive cloning systems inherent in their respective DNA uptake mechanisms and the necessity for cell wall regeneration by protoplasts have prevented their general application for direct cloning of nonselectable chromosomal genes.

The ability to clone large chromosomal DNA fragments in *E. coli* using cosmids (COLLINS and HOHN 1978; COLLINS and BRÜNING 1978) and lambda substitution vectors (BLATTNER et al. 1979; LOENEN and BRAMMAR 1980) makes these the methods of choice for establishing primary banks of cloned chromosomal genes from gram-positive bacteria. Such large-capacity vectors make possible construction of gene banks in fewer than 500 clones with a high statistical probability that any given gene has been cloned intact (COLLINS and BRÜNING 1978). However, to study expression of the cloned determinant in its native background, or a background approximating that of the natural host, the ability to subclone gram-positive virulence determinants in a gram-positive organism is critical. Shuttle vectors, which have the ability to replicate in *E. coli* and in various gram-positive species (EHRLICH 1978; KREFT et al. 1978; MACRINA et al. 1982b; ML DAO and JJ FERRETTI, personal communication), greatly facilitate transfer of the cloned determinant from *E. coli* to an appropriate transformable gram-positive organism. Using shuttle vectors, fragments of the large chromosomal insert in the primary clone can be subcloned to identify the DNA region necessary for expression of the desired virulence trait (FAIRWEATHER et al. 1983; KREFT et al. 1983). The smaller shuttle vector/virulence factor gene chimera can then efficiently be introduced into a transformable gram-positive species. Broad host range transmissible plasmids may then be of value for mobilizing the shuttle vector chimera into nontransformable gram-positive species (CLEWELL 1981).

The recent development of a technique for cloning specific DNA sequences using a conjugative antibiotic resistance transposon from *Streptococcus faecalis* promises a quantum advance in cloning gram-positive virulence factors (GAW-

RON-BURKE and CLEWELL 1984). The tetracycline-resistance transposon Tn916 (Tc) can be introduced into the gram-positive pathogen by conjugation, or transformation with transposon-carrying derivatives of broad host range plasmids. Using such procedures, the transposon has been introduced into a variety of gram-positive organisms (GAWRON-BURKE and CLEWELL 1984; CLEWELL et al. 1985). Antibiotic-resistant strains of the pathogen generated by random insertion of the transposon into the chromosome are selected and screened for variants made deficient in the virulence trait of interest. Chromosomal DNA is then ligated into an appropriate vector, introduced into *E. coli* via transformation, and selection is made for the transposon-encoded antibiotic resistance (the transposon-encoded resistance is expressed in *E. coli*). In the absence of continued selection for Tc resistance, Tn916 has been observed to excise (apparently precisely) in *E. coli*, thus regenerating the particular gene of interest (GAWRON-BURKE and CLEWELL 1984). A similar strategy has been independently developed using the *S. pneumoniae* transposon Tn1545 (CARLIER and COURVALIN 1982; P COURVALIN, personal communication).

These new methods introduce considerable flexibility into the process of cloning gram-positive virulence factors. As suggested by the authors, if the restriction endonuclease used for cloning the chromosomal-DNA-flanked transposon did not cleave within the virulence-factor-encoding-region, upon precise excision of the transposon this factor may be expressed in *E. coli* (GAWRON-BURKE and CLEWELL 1984). If the regenerated virulence-factor-encoding sequence is not expressed at detectable levels in this heterologous host, it can be subcloned into a shuttle vector and introduced into a gram-positive organism where the phenotype may be more readily detected (GAWRON-BURKE and CLEWELL 1984; CLEWELL et al. 1985). Use of the above-described transposon methods for cloning gram-positive virulence factors then has three obvious advantages over previously existing approaches. To obtain the desired clone it is no longer necessary to isolate it from gene banks or libraries of the entire chromosome of the pathogen in question. Secondly, cloning of gram-positive virulence-factor-encoding DNA is no longer dependent upon its expression in *E. coli*. Finally, during the process of marking the desired chromosomal gene by insertion of the transposon, insertional inactivation of the virulence factor will allow determination of the complexity of the respective cistron.

3 Cloned Gram-Positive Virulence Factors

That many of the following cloned determinants are contributory to and not merely correlated with bacterial pathogenicity must be stated with some caution. The most convincing demonstration that any given bacterial gene is actually involved in the disease process is made by constructing isogenic variants of the pathogen specifically deficient only in the putative virulence trait under study (SPARLING 1979). A comparison of the pathogenicity of the variant with the wild-type strain in an appropriate model system can then be made, allowing proof and quantitation of the putative factor's contribution to virulence. How-

Table 1. Cloned gram-positive virulence determinants

Virulence factor	Natural host species	Principle cloning vector	Primary localization in *E. coli*	Detection	Ref.
Cereolysin X	*Bacillus cereus*	Cosmid	OM	Hemolysis	KREFT et al. (1983)
Streptolysin O	*Streptococcus pyogenes*	Lambda	CA	Hemolysis	KEHOE and TIMMIS (1984)
α-Hemolysin	*Staphylococcus aureus*	Lambda	CA	Hemolysis	KEHOE et al. (1983)
Staphylokinase	*Staphylococcus aureus*	Plasmid	P	Proteolysis	SAKO et al. (1983)
Streptokinase	*Streptococcus equisimilis*	Lambda	CY	Proteolysis	MALKE and FERRETTI (1984)
Protein A	*Staphylococcus aureus*	Plasmid	P	ELISA	LÖFDAHL et al. (1983)
M Protein	*Streptococcus pyogenes*	Cosmid	P	Immunoblot	SCOTT and FISCHETTI (1983)
Diphtheria toxin	*Corynebacterium diphtheriae*	Plasmid	P	Colony hybridization	LEONG et al. (1983)
Erythrogenic toxin	*Streptococcus pyogenes*	Plasmid	CA	Immunological	JOHNSON and SCHLIEVERT (1984)
Pyrogenic exotoxin C	*Staphylococcus aureus*	Plasmid	P	Radioimmunoassay	KREISWIRTH et al. (1983)

OM, outer membrane; CA, cell associated; P, periplasmic; CY, cytoplasmic

ever, a prerequisite for constructing such deficient variants is the cloning and isolation of putative virulence-factor-encoding DNA so that it singly and specifically can be modified and used to displace the wild-type gene. Thus far, none of the cloned virulence factors of gram-positive origin has undergone such a rigorous test. Because of the large body of evidence implicating the involvement in bacterial pathogenicity of those factors described in this review, they will be referred to as virulence factors nonetheless. A summary of the gram-positive virulence determinants which have been cloned, the vector systems used, the localization of the gene products synthesized by *E. coli*, and the methods used to detect the desired transformant is given in Table 1.

3.1 Hemolysins

Hemolysins have been convincingly demonstrated to contribute to the virulence of *E. coli* strains causing extraintestinal infections (WELCH et al. 1981; HACKER et al. 1983; WELCH and FALKOW 1984). Much evidence also exists implicating the involvement of hemolysins in the virulence of many gram-positive pathogens (ALOUF 1977; SMYTH and DUNCAN 1978; ROGOLSKY 1979; ALOUF 1980; WAN-

NAMAKER 1983). Since transformants of *E. coli* expressing cloned hemolysin determinants can readily be identified on blood agar plates, and because of their medical importance, several laboratories have successfully undertaken the cloning and characterization of hemolysin determinants of gram-positive origin.

Several pathogenic, gram-positive genera produce hemolysins (or more accurately cytolysins since they lyse not only erythrocytes but a variety of eukaryotic cells) of the thiol-activated class (for a review of these cytolysins, see BERNHEIMER 1974; BERNHEIMER 1976; SMYTH and DUNCAN 1978). Among these are *S. pyogenes* (streptolysin O), *Listeria monocytogenes* (listeriolysin), *Clostridium tetani* (tetanolysin), *B. alveus* (alveolysin), and *B. cereus* (cereolysin). All of the SH-activated cytolysins share common biochemical properties; in addition to being activated by sulfhydryl-reducing agents, all are inactivated by cholesterol, the probable cell-surface-binding molecule (JOHNSON et al. 1980). The SH-activated cytolysins also share immunological cross-reactivity (COLWELL and BERNHEIMER 1977). Because of these similarities, the genes encoding the SH-activated hemolysins (cytolysins) likely share common evolutionary ancestry.

KREFT et al. (1983) reported the cloning of cereolysin, the SH-activated cytolysin produced by *B. cereus*. It was observed that cereolysin was poorly expressed in *E. coli* and that hemolytic zones around colonies harboring the cosmid-cloned determinant appeared on blood agar plates only after prolonged incubation. In contrast, expression of the cloned determinant in *B. subtilis* was found to parallel that of the wild-type *B. cereus* strain when cultivated in a chemically defined medium (KREFT et al. 1983). To further identify the cloned determinant as that encoding cereolysin, it was shown that the hemolytic activities of cell-free supernatants from cultures of *B. cereus* and the *B. subtilis* clones were similarly enhanced by dithiothreitol and inhibited by cholesterol.

Further research on the expression of the cloned cereolysin determinant in *E. coli* revealed that about 70% of the cytolytic activity was bound to the outer surface of the outer membrane, and this remained consistent throughout the growth phase (GILMORE and GOEBEL, to be published). The cytolytic activity attributable to the presence of this cloned gene in *E. coli* is therefore difficult to observe on blood agar as the protein apparently does not readily dissociate from the membrane and diffuse through the medium. There are also no measurable cytoplasmic or periplasmic pools of cereolysin, making detection of the cytolytic activity in soluble cell fractions impossible. Although the cloned cereolysin gene product is synthesized in appreciable amounts in *E. coli*, its localization renders it difficult to detect.

The sequence of the cloned cereolysin gene has also been determined (GILMORE et al., to be published). Surprisingly, no open reading frame capable of encoding the 55.5-KD cereolysin protein described by COLWELL et al. (1976) was found. Two shorter, tandem open reading frames, separated by multiple-stop codons in each frame, were identified. Deletion analysis and site-specific mutagenesis have shown that both inferred polypeptides are necessary for hemolytic activity. Putative signal peptides can be identified from the inferred sequence of both proteins. Unique bands corresponding to the expected molecular weights of 31.1K and 28.5K for the precursor and mature forms of the first

protein, and 36.6K and 34K for the precursor and mature forms of the second protein, respectively, have been identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of labeled minicell proteins derived from *E. coli* strains harboring the cloned cereolysin determinant (W. GOEBEL and S. KATHARIOU, personal communication). Although the predicted molecular weights of the processed forms combined (62.5K) approximate the average molecular weight for cereolysin (55.5K) determined by several methods (COLWELL et al. 1976) – suggesting the possibility of covalent linkage of the two subunits – the inferred amino acid composition differs significantly from that published for cereolysin (COLWELL et al.). The relationship of the two cytolysins derived from different strains of *B. cereus* remains to be determined.

Alternatively, the cloned and sequenced cereolysin (henceforth referred to as cereolysin X to distinguish it from the cereolysin more nearly confirming to the physical parameters characteristic of the SH-activated cytolysins) may be more closely related to other previously described two-component hemolysins. THOMPSON et al. (1984) have recently published a report on the chromatographic purification of an enterotoxin produced by a *B. cereus* strain isolated from a food-poisoning outbreak. Toxic activity required two proteins, one estimated to be 43.0 kilodaltons and the other 38.0 kilodaltons. Importantly, enterotoxic activity could not be resolved from hemolytic activity (THOMPSON et al. 1984). A two-component hemolysin (possessing in addition bacteriocin activity) produced by *S. fecalis* subsp. *zymogenes* has also been described (GRANATO and JACKSON 1969) although little is known about the physical or biochemical nature of the proteins. The relationship between the cloned cereolysin X and the *S. fecalis* subsp. *zymogenes* produced hemolysin (no bacteriocin activity has yet been detected for the former) or the *B. cereus* enterotoxin is at present only speculative. DNA fragments related to the production of the *S. fecalis* subsp. *zymogenes* hemolysin have recently been cloned (LEBLANC et al. 1983) and partially sequenced (D. BEHNKE, personal communication), and sequence comparison with that for the *B. cereus* cloned cytolysin should illuminate the relationship between these genes.

KEHOE and TIMMIS (1984) have recently reported on the cloning of streptolysin O from *S. pyogenes* strain Richards. Hemolytic zones around recombinant phage plaques were detected at a low frequency. Subcloning of the DNA region determined to encode streptolysin O on a high-copy number plasmid in *E. coli* appeared to be deleterious to the host. Minicells derived from *E. coli* harboring the plasmid-borne gene were found to produce proteins of 69K and 61K molecular weight which interacted with anti-streptolysin O (KEHOE and TIMMIS 1984). The size of these proteins corresponded well with reported determinations of the molecular weight for streptolysin O. Because the size of the cloned determinant was too small to code for two separate proteins of these molecular weights, and since they reacted specifically with anti-streptolysin O, it was suggested that they may represent the precursor and processed form of the cytolysin. KEHOE and TIMMIS (1984) as well as KREFT et al. (1983) were unable to detect any DNA homology between the cloned cytolysin determinants and the chromosomal DNA from SH-activated cytolysin-producing strains of other genera under stringent conditions. Research is in progress directed toward cloning the

listeriolysin gene (J. KREFT and W. GOEBEL, personal communication) and the alveolysin determinant (J. ALOUF, personal communication). Sequence comparison at the nucleotide and inferred amino acid levels should reveal a considerable amount of information on the evolution, active site structure, and conserved antigenic determinants of these highly toxic cytolysins.

KEHOE et al. (1983) have reported the cloning of α-hemolysin from *Staphylococcus aureus* strain Wood 46. Hemolytic zones in an erythrocyte agar overlay above recombinant phage plaques were observed at the expected frequency of about 1 in 1000. Hemolysis was specifically inhibited by incorporating anti-α-hemolysin serum in the overlay. The α-hemolysin determinant was subcloned into pACYC184, and *E. coli* transformants were found to express hemolytic activity only after prolonged incubation. Liquid culture supernatants from these transformants were devoid of hemolytic activity. A transient pool of periplasmic α-hemolysin was found in cultures of fresh transformants; however, after subcloning, hemolytic activity could only be found when the transformants were lysed. *E. coli* minicells harboring the α-hemolysin determinant were found to produce unique proteins of 34 kilodaltons and 33 kilodaltons. The reported size of about 1620 bp for the α-hemolysin determinant appears too small to encode two distinct proteins of this size, assuming a typical amino acid composition. The two proteins may represent precursor and processed forms of the *S. aureus* α-hemolysin (KEHOE et al. 1983).

The α-hemolysin determinant has been subcloned into a shuttle vector and used to transform competent *B. subtilis* and protoplasts of *S. aureus* (FAIRWEATHER et al. 1983). *B. subtilis* transformants were found to express and release α-hemolysin into the supernatant. Proteins of 33 kilodaltons and 34 kilodaltons were specifically immunoprecipitated from these culture supernatants, the latter protein being the major species. As with α-hemolysin expression in *B. subtilis*, expression in transformed nonhemolytic *S. aureus* mutants approximated that of the nonmutagenized parent strain *S. aureus* 8325-4. It is suggested that the cloned *S. aureus* Wood 46 α-hemolysin determinant might therefore be recognized by factors which regulate the α-hemolysin native to strain 8325-4 (FAIRWEATHER et al. 1983). Introduction of the plasmid-borne Wood 46 determinant into wild-type 8325-4 already synthesizing native α-hemolysin resulted in a tenfold increase in the measured hemolytic activity (FAIRWEATHER et al. 1983). This finding discounts the possibility that the Woods 46 determinant and the 8325-4 are regulated by a factor acting in trans with similar binding affinities for the two determinants.

3.2 Plasminogen Activators

The therapeutic application of bacterial plasminogen activators is well known. Bacterial plasminogen activators may contribute to pathogenicity by activating plasminogen, resulting in proteolysis of the protective fibrin network of a clot at a wound site (MALKE and FERRETTI 1984). The *S. aureus* phage S-C encoded staphylokinase has been cloned in *E. coli* by SAKO et al. (1983). Clones expressing staphylokinase were detected by the production of cleared halos in agar made

with heat-treated serum. The fibrinolytic activity was only observed when plasminogen was included in the agar, positively identifying the cloned determinant as responsible for synthesis of the plasminogen activator. A 15.3-kilodalton protein capable of mediating plasminogen activation was detected in *E. coli* culture supernatants. The protein corresponded in size to the plasminogen activator produced by phage-lysogenized *S. aureus* (SAKO et al. 1983). Further evidence that the cloned determinant was staphylokinase was obtained from Ouchterlony tests. The staphylokinase produced by the heterologous *E. coli* host was found predominantly (60%) in the periplasmic space and in the culture supernatant (20%). These results suggest that passage through the outer membrane of *E. coli* is limiting for staphylokinase phenotypic expression (SAKO et al. 1983). The nucleotide sequence of the staphylokinase-encoding *sak* gene has recently been reported (SAKO and TSUCHIDA 1983).

The gene-encoding streptokinase has recently been cloned from the genome of *S. equisimilis* (MALKE and FERRETTI 1984). Detection of streptokinase synthesized by recombinant phage-infected *E. coli* was made by overlaying phage plaques with agar-containing plasminogen and casein. Zones of clearing were observed above streptokinase-containing plaques as the result of caseinolytic activity of active plasmin. Among other lines of evidence, identity was demonstrated between concentrated supernatants of the streptokinase-producing *E. coli* clone and the *S. equisimilis* natural producer, using monospecific IgG in double diffusion tests. In contrast to staphylokinase, streptokinase was found predominantly in the cytoplasm (52%) among soluble fractions of *E. coli* harboring a recombinant plasmid (MALKE and FERRETTI 1984). Additionally, the periplasmic pool (30%) was found to be transient and devoid of activity if obtained from cultures in the stationary phase. The authors suggest that the cytoplasmic membrane limits externalization in this case (MALKE and FERRETTI 1984). Determination of the nucleotide sequence of the encoded determinant has revealed an inferred signal peptide not unlike those previously described in the literature (H MALKE, JJ FERRETTI, personal communication). The reason for the barrier at the cytoplasmic membrane is at present unclear.

3.3 Immune-System-Impeding Factors

Staphylococcal protein A, a cell surface protein, binds the Fc region of immunoglobulins. In addition to and as a consequence of this Fc reactivity, several biological activities of staphylococcal protein A have been described which implicate its involvement in staphylococcal virulence (GROV 1977). The Fc-binding activity of protein A has also been used to considerable advantage in immunological laboratory methods.

Staphylococcal protein A has recently been cloned (LÖFDAHL et al. 1983).The production of protein A by transformants was detected using an enzyme-linked immunosorbent assay. A subcloned 2.15-kb DNA fragment was found to encode protein A completely, and the nucleotide sequence of the 5′ end of the gene was determined. The inferred amino acid sequence was found in good agreement with that reported in the literature, and a signal peptide could be deduced

(LÖFDAHL et al. 1983). In *E. coli* transformants, a periplasmic pool was found to account for all cell-associated soluble protein A detected (LÖFDAHL et al. 1983).

Streptococcal M protein mediates an antiphagocytic activity, affording some protection for *S. pyogenes* from the host's immune system (LANCEFIELD 1962). This antiphagocytic activity is neutralized by antibody to the type of M protein expressed. Neutralization is type specific, and about 70 distinct M protein types have been characterized (SCOTT and FISCHETTI 1983). The chromosomal sequence encoding M protein from *S. pyogenes* D471 (type M6) has been cloned (SCOTT and FISCHETTI 1983). Immunological identity was demonstrated between purified M6 protein produced by *S. pyogenes* D471 and chromatographically purified *E. coli*-synthesized M protein. The size of the M protein synthesized by the heterologous host was found to correlate well with literature reports on the size of M protein released into the medium by streptococcal L forms (SCOTT and FISCHETTI 1983). *E. coli*-synthesized M protein was found to be predominantly localized in the periplasm with only trace levels detected elsewhere (FISCHETTI et al. 1984). M protein types 5 and 19 (POIRIER et al. 1984), 24 (BURDETT and BEACHY 1984), and 12 (SPANIER et al. 1984) have also recently been cloned. Comparison of the nucleotide sequences encoding the various M types should contribute significantly to an understanding of the genetic basis for the unusual variability of this virulence trait.

3.4 Phage-Associated Toxins

The involvement of bacteriophage β in toxinogenic conversion of *Corynebacterium diptheriae* is well established (FREEMAN 1951; FREEMAN and MOORE 1952; for reviews see PAPPENHEIMER 1977; and MURPHY and BACHA 1979). The diptheria toxin structural gene is encoded by β-phage sequences and regulated by bacterial host factors and iron (MURPHY and BACHA 1979). Two groups have recently reported cloning the amino-terminal end of the toxin which includes the domain possessing enzymatic protein synthesis inhibiting activity from corynebacteriophage β (LEONG et al. 1983; TWETEN and COLLIER 1983). Immunologically identified proteins synthesized by *E. coli* transformants were observed to catalyze ADP ribosylation of eukaryotic elongation factor 2, the protein-synthesis-inhibiting activity. In both instances, the majority of the cloned enzyme activity was localized in the periplasmic space (68%–89%), with no activity detectable in the culture supernatants (LEONG et al. 1983; TWETEN and COLLIER 1983). Peptides synthesized from the cloned determinants, however, were found to be very unstable in *E. coli* (TWETEN and COLLIER 1983). This instability may have been attributable to the incomplete structure of the toxin peptides produced (TWETEN and COLLIER 1983). The gene encoding the immunologically identical inactive *tox* 228 protein (CRM228) has been cloned and sequenced (KACZOREK et al. 1983). The *tox* 228 gene was found to be expressed only at low levels. The authors suggest that this may be attributable to the short spacing between the -10 and -35 promoter sequences, or the use of rare codons in the structural gene (KACZOREK et al. 1983).

Streptococcal erythrogenic toxins have been shown to elicit a variety of host reactions including erythema, enhanced susceptibility to endotoxin shock, and pyrogenic effects (for recent reviews, see ALOUF 1980; WANNAMAKER 1983). The effects of erythrogenic toxin on the host immune system include a biphasic suppression followed by enhancement of antibody response to heterologous erythrocytes, and a mitogenic effect on T-lymphocytes (ALOUF 1980). Production of the three immunologically distinct erythrogenic toxin types by strains of *S. pyogenes* has been shown to be associated with the presence of phage (ALOUF 1980; WANNAMAKER 1983).

Recently, two laboratories have cloned the type A erythrogenic toxin [or streptococcal pyrogenic exotoxin A, *spe*A (JOHNSON and SCHLIEVERT 1984)] from the streptococcal phage T12 chromosome. In addition to immunological identity between the erythrogenic toxin produced by transformed *E. coli* and that purified from streptococcal culture supernatants, JOHNSON and SCHLIEVERT demonstrated that type A exotoxin produced by transformants was also biologically active, eliciting a pyrogenic response and enhanced endotoxin sensitivity in rabbits (JOHNSON and SCHLIEVERT 1984). Suppression of murine plaque-forming spleen cell response and nonspecific lymphocyte mitogenicity activities were also attributed to the heterologously produced erythrogenic toxin (JOHNSON and SCHLIEVERT 1984).

Using a similar approach, WEEKS and FERRETTI (1984) also cloned the *spe*A gene from streptococcal bacteriophage T12. Further evidence that *spe*A-related sequences were absent from the *S. pyogenes* host chromosome was obtained by hybridization experiments using cloned T12 sequences to probe chromosomal DNA. The T12 *spe*A gene was further subcloned into a shuttle vector and used to transform *S. sanguis* strain Challis. Type A streptococcal exotoxin secreted from streptococcal transformants was found to be immunologically identical to exotoxin from *E. coli* transformants and to exotoxin from *S. pyogenes* toxin-producing strains.

Staphylococcal pyrogenic exotoxin type C (SPE-C) [apparently identical to enterotoxin F (BONVENTRE et al. 1983)], produced by 92% of *S. aureus* isolates derived from acute toxic shock syndrome cases (ALTEMEIER et al. 1982; BERGDOLL et al. 1982), shares many of the biological activities ascribed to streptococcal erythrogenic toxin (for a detailed series of manuscripts on this topic, see "The Toxic Shock Syndrome," *Annals of Internal Medicine* 96(2) 1982). Among these include pyrogenicity, ability to enhance endotoxin sensitivity (SCHLIEVERT 1982), mitogenic activity toward T-lymphocytes, and ability to suppress IgM synthesis (SCHLIEVERT et al. 1981). Staphylococcal isolates from the earliest cases of toxic shock syndrome were found to belong to a single phage-group (TODD et al. 1978). Surveys broader in scope found 60% of toxic shock syndrome (TSS) isolates to be of staphylococcal phage-group I (ALTMEIER et al. 1982) and 92% of such isolates to be lysogenic for some bacteriophage compared with 18% for non-TSS-associated strains (SCHUTZER et al. 1983). These results suggested the possible role of phage conversion in SPE-C production in toxic shock syndrome (SCHUTZER et al. 1983).

The staphylococcal pyrogenic exotoxin type C (or toxic shock syndrome exotoxin) encoding gene has recently been cloned (KREISWIRTH et al. 1983).

Most of the cell-associated SPE produced by *E. coli* was found localized in the periplasm. Additionally, these researchers demonstrated that none of the phage isolated from TSS strains and used to lysogenize SPE-C nonproducers was capable of conversion to SPE-C production, suggesting phage association to be circumstantial in this syndrome (KREISWIRTH et al. 1983).

4 Conclusion

Significant advances have been made in both the development of technology for molecular cloning of gram-positive virulence-factor-encoding genes and its application. In many cases, cloning gram-positive genes in *E. coli* is advantageous not only because of the efficiency of cloning in this organism, but also because of the often-observed localization of the heterologously synthesized virulence factor in the periplasmic space. This allows a tremendous preliminary concentration of the gene product – the *E. coli* clones need only to be harvested and the periplasmic proteins can then be specifically released in a small volume by osmotic shock (FISCHETTI et al. 1984). On the other hand, lack of efficient secretion of the cloned gene product through the outer membrane can hinder detection of the desired phenotype among the transformants (KREFT et al. 1983). In such cases, and where regulation of the cloned gene is to be studied in the natural host or a related organism, it is necessary to introduce the cloned determinant into a gram-positive host. Strategies and vectors facilitating the transfer of cloned gram-positive determinants back into a gram-positive strain have been developed and extensively used.

The many results cited above clearly attest to the feasibility and value of undertaking the molecular cloning of genes encoding gram-positive virulence factors. Further study on the regulation of virulence factor gene expression, on the biological activities of the encoded gene products, and on the contribution of cloned determinants to bacterial virulence is needed to gain a comprehensive understanding of the pathogenicity of gram-positive bacteria.

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The Diphtheria Toxin Structural Gene

J.R. MURPHY

I Diphtheria Toxin and Its Structural Gene

While there was considerable indirect evidence that toxinogenesis in *Corynebacterium diphtheriae* was related to lysogeny (FREEMAN 1951; FREEMAN AND MORSE 1952; GROMAN 1953a, b, 1955; GROMAN and EATON 1955; HOLMES and BARKSDALE 1969), it was not until the report of UCHIDA et al. (1971) that it was clear that corynebacteriophage β carried the structural gene for diphtheria toxin. UCHIDA and coworkers (1973a, b) described the isolation and characterization of a family of β-phage mutants which directed the synthesis of nontoxic serologically cross-reacting proteins that were related to diphtheria toxin. These defective toxin-related molecules were instrumental in defining the major structural/functional domains of diphtheria toxin, as well as positioning the N-terminal amino acid of toxin to fragment A. In general, the diphtheria *tox* mutants that were isolated fell into two major classes: (i) those that carried a missense mutation affecting the adenosine diphosphate (ADP) ribosyl-transferase activity of fragment A; and (ii) those that carried a nonsense mutation in the fragment B encoding portion of the structural gene which resulted in the expression of a truncated toxin-related molecule that is devoid of the eukaryotic cell receptor binding domain (UCHIDA et al. 1973a, b; LAIRD and GROMAN 1976b; HOLMES 1976).

Diphtheria toxin has been recently shown to be synthesized in precursor form on membrane-bound polysomes in *C. diphtheriae*, and cotranslationally secreted into the culture medium as a single polypeptide chain (SMITH et al. 1980). The intact toxin molecule (58348 daltons) has two disulfide bridges, between Cys_{211}–Cys_{226} and Cys_{486}–Cys_{496}. The fourteen amino acid polypeptide subtended by the first disulfide bridge contains three arginine residues, and is clearly exposed since this region of the toxin molecule is exquisitely sensitive to proteolytic "nicking" with trypsin. Following mild trypsin digestion and reduction of the disulfide bond, diphtheria toxin can be separated into an N-terminal 21167 dalton fragment A, and a 37199 dalton fragment B under

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denaturing conditions. These two polypeptide fragments contain at least three functional domains that are essential for the cytotoxic action of diphtheria toxin. Fragment A is enzymatically active and catalyzes the nicotinamide adenosine diphosphate (NAD^+) dependent ADP-ribosylation of eukaryotic elongation factor 2 (EF-2). It has recently been shown that a single molecule of fragment A introduced into the cytosol is sufficient to cause cell death (YAMAIZUMI et al. 1978). Fragment B of diphtheria toxin contains the eukaryotic cell receptor binding domain, as well as two lipid associating domains that appear to be essential in the membrane translocation of the A fragment into the eukaryotic cell cytosol (UCHIDA et al. 1973a; BOQUET et al. 1976; FALMAGNE et al. 1980; BACHA et al. 1983). The lipid associating regions of fragment B are contained within the amino terminal (ca. 23000 daltons). The first region is amphipatic and hydrophilic and is located in the amino terminal 77 amino acid residues of fragment B. The region resembles the surface lipid associating domain found in human apolipoprotein A1 (FALMAGNE et al. 1980; LAMBOTTE et al. 1980). The second lipid associating domain is positioned ca. 40000–45000 daltons from the N-terminus of the toxin molecule, and is hydrophobic (BOQUET et al. 1976). This region resembles the transverse lipid associating domain observed in many intrinsic membrane proteins.

While a great deal is known about the protein chemistry of diphtheria toxin molecule, until recently very little was known of the genetic organization of the diphtheria toxin structural gene, *tox*. Early studies had positioned the *tox* character on the β phage genetic map (HOLMES and BARKSDALE 1969); however, the position of *tox* on the restriction endonuclease digestion map has only recently been determined. COSTA et al. (1981) and BUCK and GROMAN (1981a, b) localized the diphtheria toxin structural gene on a ca. 3900 base pair (bp) *Bam*H1 restriction endonuclease fragment of the corynebacteriophage β genome. Figure 1 shows the *Bam*H1, *Eco*R1, *Hin*dIII, and *Kp*n1 restriction endonuclease digestion maps of the clear-plaque forming mutant of corynebacteriophage β, β_c.

LAIRD and GROMAN (1976a) had previously demonstrated that corynebacteriophage β, like coliphage lambda, integrates into the *C. diphtheriae* chromosome following circularization of the linear vegetative genome through its cohesive ends, *cos*, and insertion through the phage attachment site, *att*P. In addition, LAIRD and GROMAN (1976a) have demonstrated that the prophage genetic map of corynebacteriophage β is a circular permutation of the vegetative map, and that the *tox* character was positioned adjacent to *att*P. Since it was shown that the transcription of the *tox* operon proceeded toward the *att*P site (LAIRD and GROMAN 1976b), the localization of *att*P on the physical map allowed for the determination of the orientation of *tox* on the *Bam*H1 fragment of corynephage β. These experiments took advantage of the fact that integration of the β-phage genome into the *C. diphtheriae* chromosome resulted in the splitting of the *att*P containing restriction endonuclease fragment of the vegetative genome into two novel fragments in the prophage genome. MICHEL et al. (1982) and BUCK and GROMAN (1981a, b) have used Southern blot analysis of restriction endonuclease digests of corynebacterial chromosomal DNA to determine the orientation of the *tox* gene.

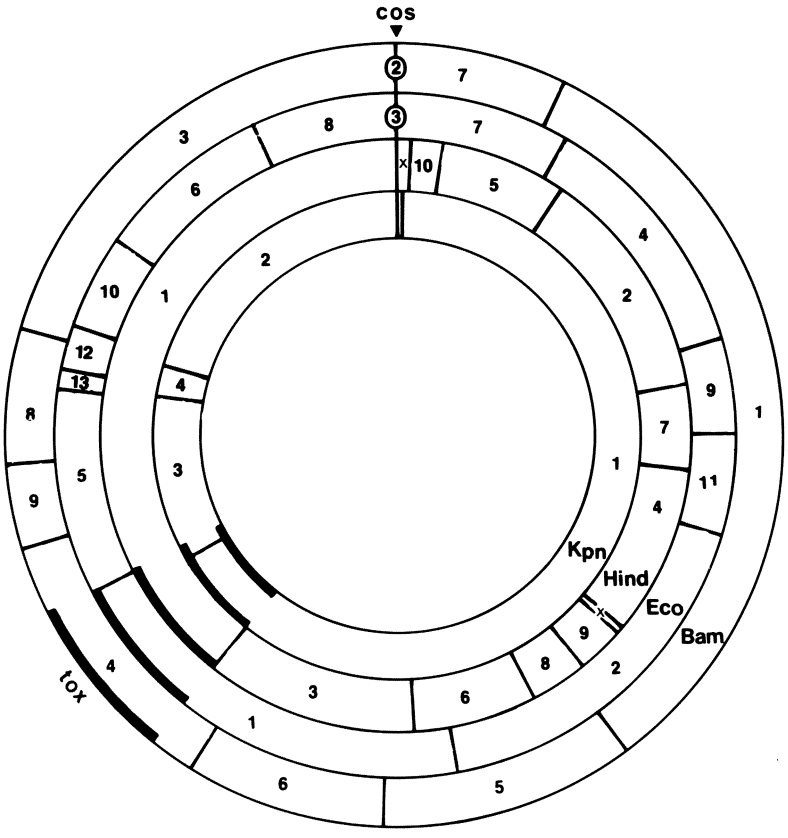

Fig. 1. Restriction endonuclease digestion map of corynebacteriophage β_c. *cos*, cohesive ends of the linear vegetative genome; *tox*, diphtheria toxin structural gene. Fragments which contain an internal cos site are *circled*. *Kpn*, *Kpn*1; *Hin*d, *Hin*dIII; *Eco*, *Eco*R1; *Bam*, *Bam*H1

The positioning of *tox* on the *Bam*H1–4 fragment of the β-phage and the generation of the detailed restriction map of this fragment aided the subsequent cloning of portions of the diphtheria toxin structural gene into both the pBR322, and pUC8 vectors in *Escherichia coli* K-12 (LEONG et al. 1983a, b; KACZOREK et al. 1983; TWETEN and COLLIER 1983). Figure 2 shows the relative position of the *Sau*3A1 endonuclease sites within the 3900 bp *Bam*H1 fragment of corynebacteriophage B. As can be seen, *Sau*3A1 divides the diphtheria toxin structural gene into two major segments. The first segment, which contains a *Hin*dIII site, has been shown to encode the *tox* regulatory region, the diphtheria toxin signal sequence, and all of fragment A (KACZOREK et al. 1983; RATTI et al. 1983; GREENFIELD et al. 1983). In fact, the *Sau*3A1 site between fragments A and B of toxin is located immediately after the codon for the third arginine (Arg_{218}) in the exposed disulfide loop. The second *Sau*3A1 fragment encodes all of fragment B except for the C-terminal 17 amino acids. The molecular cloning of both of these fragments, as well as the molecular cloning of the

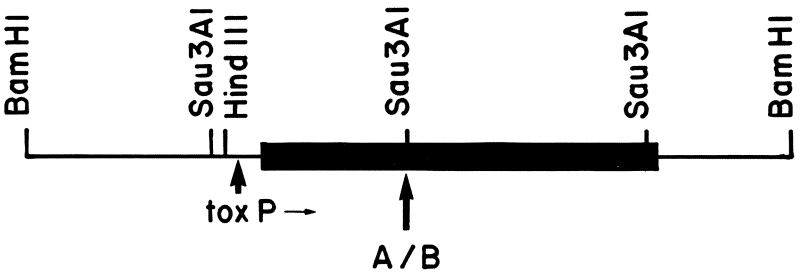

Fig. 2. Diagram of the *Bam*H1–4 fragment of corynebacteriophage β_c showing the *Sau*3A1 sites dividing the diphtheria *tox* gene into two major segments. The relative position of the *tox* promoter, the direction of transcription, and the function between fragments A and B are indicated by arrows

*Bam*H1 fragment encoding the *tox*-228 allele, has allowed for the complete nucleotide sequence of the diphtheria toxin structural gene to be determined (KACZOREK et al. 1983; RATTI et al. 1983; GREENFIELD et al. 1983; GIANNINI et al. 1984). Figure 3 summarizes the nucleotide base sequence data of the diphtheria *tox* alleles that have been obtained from the analysis of two different wild-type alleles and three mutant alleles. It is remarkable to note that in the case of the wild-type *tox* alleles, the nucleotide sequence of both the β-phage and ω-phage genes are identical. The complete DNA sequence of three mutant *tox* alleles (*tox*-45, *tox*-197, and *tox*-228) have also been determined (KACZOREK et al. 1983; GIANNINI et al. 1984).

The molecular cloning of the *Sau*3A1–2 fragment A encoding segment in *E. coli* K-12 results in the expression of diphtheria toxin fragment A-related proteins in transformants. Since it was shown that diphtheria toxin was synthesized in precursor form on membrane-bound polysomes in *C. diphtheriae*, it was of interest to determine the localization of the fragment A-related proteins that were expressed in *E. coli*. LEONG et al. (1983a, 1983b) and TWETEN and COLLIER (1983) have reported that diphtheria toxin fragment A-related polypeptides that are expressed in *E. coli* are exported to the periplasmic compartment. Interestingly, the nucleotide sequence analysis of the *tox* structural gene demonstrated that the *tox* signal sequence was analogous to the signal sequences that were previously described for many secreted proteins in gram-negative bacteria (MICHAELIS and BECKWITH 1982). The *tox* signal sequence was found to have a positively charged amino terminus with arginine and lysine residues at positions 3 and 4, respectively. This region is followed by a stretch of 20 amino acids ending at a histidine at position 24. The sequence Ala_{25}–Gly_{26} is found at the junction of the signal peptide and the mature form of the toxin, and its cleavage results in the release of the mature form of toxin into the culture medium of *C. diphtheriae*, or periplasmic compartment of *E. coli*. The one feature of the diphtheria toxin signal sequence that differs from gram-negative signal sequences is that of the length of the hydrophobic core region. In the case of many secreted proteins in *E. coli*, the hydrophobic core region of the signal peptide is 16–18 amino acids, whereas it is 21 amino acids in diphtheria toxin.

Immediately upstream from the GTG *tox* initiation codon, DNA sequences characteristic of those required for a ribosome binding site and promoter can be found. As shown in Fig. 4, an AAGG ribosome binding sequence is found at −10 to −13, and sequences, related to the consensus "−10" and "−35" sequences of *E. coli* promoters can also be found. LEONG et al. (1983), KACZOREK et al. (1983), and TWETEN and COLLIER (1983) have reported that the cloning of diphtheria toxin gene segments encoding fragment A, or CRM228, in either orientation in the pBR322 and pUC8 vectors resulted in comparable levels of *tox* expression. This observation suggested that the expression of diphtheria toxin-related proteins in *E. coli* was being directed by a *tox* specific promoter. LEONG et al. (1983b) reported the subcloning of the *tox* promoter in the promoter probe vector, pk0–1. In this instance a 232 bp segment of the *Sau*3A1–2 insert was found to direct the expression of galactokinase. This DNA fragment was found to have promoter activity that was intermediate between the *lac* promoter and the *gal* promoter in *E. coli* when inserted in pk0–1 vector in the appropriate orientation. As can be seen in Table 1, a DNA fragment as small as 67 bp that contains the diphtheria *tox* "−10" and "−35" sequences is just as active in directing the expression of galactokinase as the larger 232 bp fragment. Both KACZOREK et al. (1983) and RATTI et al. (1983) identified two sequences that were similar to the highly conserved TATAAT at the "−10" position for *E. coli* promoters: TATAAT and TAGGAT at positions −54 and −48 upstream from the *tox* initiation signal. In addition, a TTGA sequence found at position −74 of *tox* is similar to the conserved TTGACA sequence found at the "−35" position in *E. coli* promoters. It is of interest to note that the spacing between the proposed "−10" and "−35" sequences of the putative *tox* promoter is 12 bp for the TATAAT sequence, and 18 bp for the TAGGAT sequences. In the case of the *E. coli* promoters that have been characterized to date the spacing between the "−10" and "−35" sequences has been 15 to 21 bp, with 17 bp being the optimal spacing (HAWLEY and MCCLURE 1982).

In order to gain a further understanding of the diphtheria *tox* promoter, LEONG and MURPHY (1985) have mapped the 5′-end of the *tox* transcript by S1 nuclease analysis. The results of this study have indicated that the site of the 5′-end of the transcript is at positions −40 and −41 from the initiation codon. As can be seen in Fig. 5, the diphtheria *tox* mRNA extracted from the lysogenic, toxigenic *C. diphtheriae* strains C7(*β*) and PW8, as well as *E. coli* recombinant strains that carry the *Sau*3A1 insert all protect the single stranded DNA probe to positions −40 and −41 from the start of the structural gene. These studies suggest that RNA polymerase in both the gram-positive *C. diphtheriae* and the gram-negative *E. coli* recognize comparable promoter sequences. These observations have been independently confirmed by KACZOREK et al. (1985) by both S1 nuclease and primer extension analysis.

It has been known for many years that the expression of diphtheria toxin by toxinogenic strains of *C. diphtheriae* was dependent upon the physiologic state of the diphtheria bacillus. In particular, toxin was only expressed during the decline phase of the cell growth cycle when iron became the growth rate limiting substrate. While the precise mechanism of regulation of the diphtheria

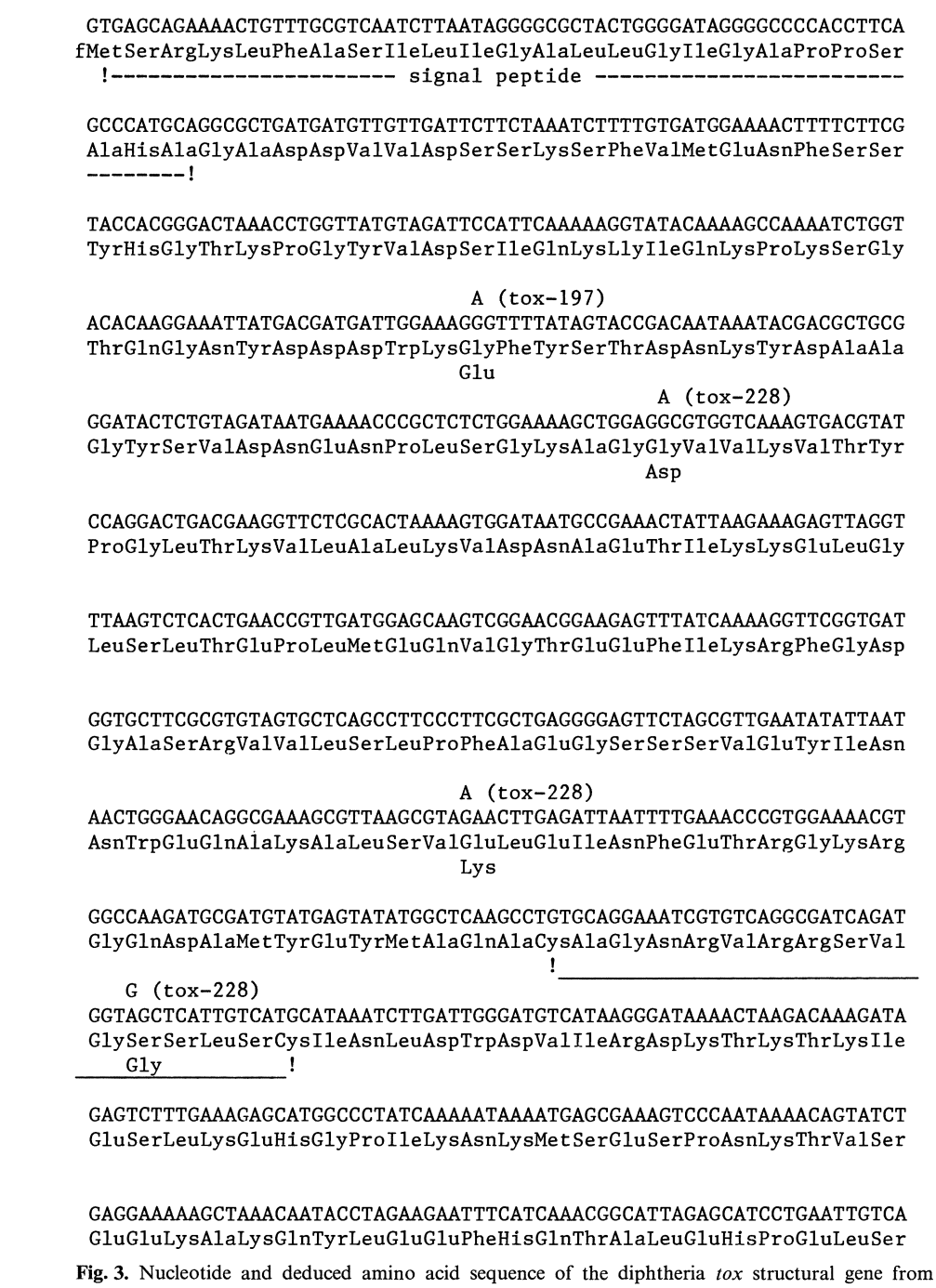

Fig. 3. Nucleotide and deduced amino acid sequence of the diphtheria *tox* structural gene from the initiation codon (GTG) to the translational stop signal (TGA). The point mutations that have been sequenced for the *tox*-45, *tox*-197, and *tox*-228 alleles are shown. Data is compiled from KACZOREK et al. (1983), RATTI et al. (1983), GREENFIELD et al. (1983), and GIANNINI et al. (1984)

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GAACTTAAAACCGTTACTGGGACCAATCCTGTATTCGCTGGGGCTAACTATGCGGCGTGGGCAGTA
GluLeuLysThrValThrGlyThrAsnProValPheAlaGlyAlaAsnTyrAlaAlaTrpAlaVal

AACGTTGCGCAAGTTATCGATAGCGAAACAGCTGATAATTTGGAAAAGACAACTGCTGCTCTTTCG
AsnValAlaGlnValIleAspSerGluThrAlaAspAsnLeuGluLysThrThrAlaAlaLeuSer

ATACTTCCTGGTATCGGTAGCGTAATGGGCATTGCAGACGGTGCCGTTCACCACAATACAGAAGAG
IleLeuProGlyIleGlySerValMetGlyIleAlaAspGlyAlaValHisHisAsnThrGluGlu

ATAGTGGCACAATCAATAGCTTTATCGTCTTTAATGGTTGCTCAAGCTATTCCATTGGTAGGAGAG
IleValAlaGlnSerIleAlaLeuSerSerLeuMetValAlaGlnAlaIleProLeuValGlyGlu

CTAGTTGATATTGGTTTCGCTGCATATAATTTTGTAGAGAGTATTATCAATTTATTTCAAGTAGTT
LeuValAspIleGlyPheAlaAlaTyrAsnPheValGluSerIleIleAsnLeuPheGlnValVal

                                                    T (tox-45)
CATAATTCGTATAATCGTCCCGCGTATTCTCCGGGGCATAAAACGCAACCATTTCTTCATGACGGG
HisAsnSerTyrAsnArgProAlaTyrSerProGlyHisLysThrGlnProPheLeuHisAspGly
                                                    STOP

TATGCTGTCAGTTGGAACACTGTTGAAGATTCGATAATCCGAACTGGTTTTCAAGGGGAGAGTGGG
TyrAlaValSerTrpAsnThrValGluAspSerIleIleArgThrGlyPheGlnGlyGluSerGly

                                                    A (tox-228)
CACGACATAAAAATTACTGCTGAAAATACCCCGCTTCCAATCGCGGGTGTCCTACTACCGACTATT
HisAspIleLysIleThrAlaGluAsnThrProGluProIleAlaGlyValLeuLeuProThrIle
                                                    Ser

CCTGGAAAGCTGGACGTTAATAAGTCCAAGACTCATATTTCCGTAAATGGTCGGAAAATAAGGATG
ProGlyLysLeuAspValAsnLysSerLysThrHisIleSerValAsnGlyArgLysIleArgMet

CGTTGCAGAGCTATAGACGGTGATGTAACTTTTTGTCGCCCTAAATCTCCTGTTTATGTTGGTAAT
ArgCysArgAlaIleAspGlyAspValThrPheCysArgProLysSerProValTyrValGlyAsn
    !_____________________________!

GGTGTGCATGCGAATCTTCACGTGGCATTTCACAGAAGCAGCTCGGAGAAAATTCATTCTAATGAA
GlyValHisAlaAsnLeuHisValAlaPheHisArgSerSerSerGluLysIleHisSerAsnGlu

ATTTCGTCGGATTCCATAGGCGTTCTTGGGTACCAGAAAACAGTAGATCACACCAAGGTTAATTCT
IleSerSerAspSerIleGlyValLeuGlyTyrGlnLysThrValAspHisThrLysValAsnSer

AAGCTATCGCTATTTTTTGAAATCAAAAGCTGA
LysLeuSerLeuPhePheGluIleLysSerSTOP
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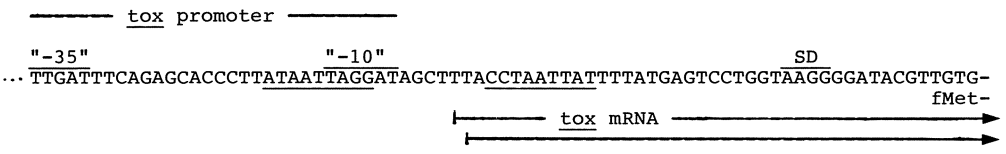

Fig. 4. Nucleotide base sequence of the region immediately upstream of the diphtheria toxin structural gene. Those sequences that are related to the conserved "−35" and "−10" promoter sequences in *E. coli* are indicated. The *underlined* sequences indicate inverted repeat sequences. The Shine-Dalgarno sequences (*SD*), and the two start points (−41 and −40) for the diphtheria *tox* mRNA are indicated. The GTG sequence and fMet indicate the point of initiation of translation of pro-diphtheria toxin

Table 1. Number of base pairs of cloned corynephage *β Bam*H1–4 DNA from the *tox* promoter region and galactokinase activity of *Escherichia coli* N100 strains carrying the promoter probe vector pk0–1 or derivative recombinant plasmids.

E. coli strain	bp *β*-phage *Bam*H1–4 DNA	Galactokinase activity[a]
N100	–	0
N100(pk0–1)	–	0.81
N100(pDT299)[b]	232	26
N100(pDT251)[c]	232	209
N100(pHin)	174	200
N100(pAlu)	67	225
N100(pLac)	–	252
N100(pGal)	–	154

[a] Galactokinase activity was assayed in extracts of *E. coli* N100 derivatives. Galactosidase activity is expressed as units of activity per ml of culture at $A_{590nm} = 1.0$

[b] pDT299 carries the promoter region insert in the pk0–1 vector in the opposite orientation with respect to the *tox* structural gene

[c] pDT251 carries the promoter region insert in the pk0–1 vector in the same orientation as the *tox* structural gene

tox operon by iron is not known, there are several lines of investigation that support the hypothesis that the regulation of *tox* expression is directed at the level of transcription and is mediated by a corynebacterial-specific factor. Murphy et al. (1976), Leong and Murphy (1985) and Kaczorek et al. (1985) have shown that *tox* mRNA can only be detected in *C. diphtheriae* strains that are iron starved and are producing diphtheria toxin. In addition, the regulation of the corynephage *tox* gene appears to be mediated by a corynebacterial-determined factor. Kanei et al. (1977) have reported the isolation of corynebacterial mutants that are constitutive for diphtheria toxin. The target for this factor is likely to be the diphtheria *tox* operator locus that has been postulated by Murphy et al. (1976) and Welkos and Holmes (1979), who have reported the isolation of corynebacteriophage *β* mutants that direct the constitutive expression of toxin. In both instances the expression of *tox* was found to be *cis-*

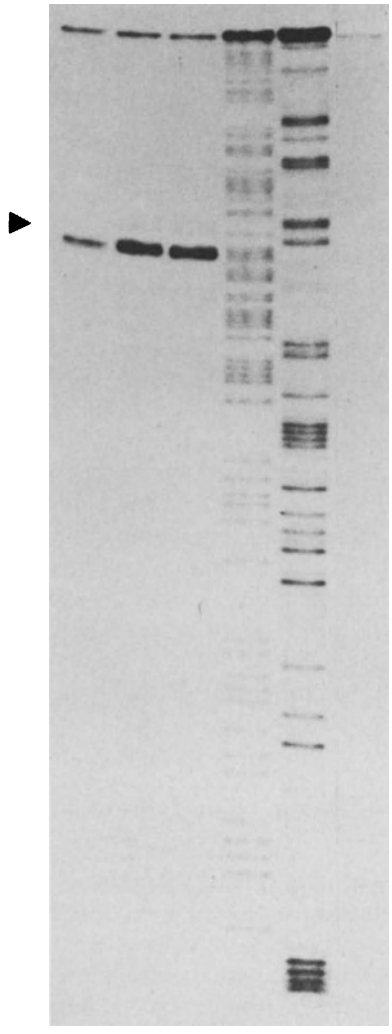

Fig. 5. S1 nuclease analysis of diphtheria *tox* mRNA extracted from strains of *E. coli* and *C. diphtheriae*. *Lane A*, Digestion products for RNA extracted from *E. coli* (pDT201); *lane B*, digestion products for RNA extracted from *C. diphtheriae* C7 (β^{tox+}) grown under iron limiting conditions; *lane C*, digestion products for RNA extracted from *C. diphtheriae* PW8 (ω^{tox+}) grown under iron limiting conditions; *lane D*, G + A sequencing reactions of the probe; *lane E*, C + T sequencing reactions of the probe; *lane F*, digestions products for RNA extracted from *C. diphtheriae* C7(β^{tox+}) grown under conditions of iron excess; *lane G*, digestion products for yeast tRNA. *Arrow* indicates that portion of the *tox* gene probe that is protected from S1 digestion following S1 nuclease treatment

dominant, and WELKOS and HOLMES (1979) mapped the mutation to the N-proximal end of the *tox* operon. These observations have supported the hypothesis that the diphtheria *tox* operon is regulated by a corynebacterial determined apo-repressor, which in the presence of iron forms a complex that has affinity for a *tox* operator locus and acts as a negative controlling element (MURPHY and BACHA 1976). Under conditions when iron becomes the growth rate limiting substrate, the intracellular iron would become sequestered by the most avid iron binding proteins in the corynebacterial cytoplasm and the dissociation of the repressor-iron complex would result in the derepression of the *tox* operon.

Based solely upon the mutants that have been isolated to date, one cannot rigorously rule out the possibility that a positive controlling element is involved in the regulation of *tox* expression. There are, however, several lines of indirect evidence that suggest that this is not the case. Perhaps the best evidence that supports the hypothesis that a negative controlling factor is involved in the regulation of the *tox* operon comes from the recent observation that the molecular cloning of the diphtheria *tox* promoter in the promoter probe vector pk0–1 in *E. coli* results in the constitutive synthesis of relatively high levels of galactokinase. Since a DNA insert as small as 67 bp that carries the "−35" and "−10" sequences of the *tox* promoter has been shown to be active (Table 1), one would have to argue that *E. coli* produces a diphtheria *tox* positive controlling element which allows for high level expression were this mode of regulation to be operative.

While the understanding of the regulation of the *tox* operon is far from complete, the recent molecular cloning of the regulatory region in promoter probe vectors in *E. coli* should allow for the application of molecular genetic methodology to the study of this problem. Since it has been shown that the diphtheria *tox* promoter is functional in *E. coli* and will direct the expression of galactokinase, one would anticipate that the molecular cloning of corynebacterial-determined regulatory factors into such transformants may result in the regulated expression of the *gal*K gene. This is an especially attractive possibility since, as yet, conjugation, transformation, and transduction have not been reported for *C. diphtheriae*. The inability to manipulate the *tox* gene and its regulatory region, other than by the isolation of mutants, has been a serious handicap in the study of the molecular genetics of this system.

2 Diphtheria Toxin-Related Polypeptide Hormone Gene Fusions

Over the past several years there has been a growing interest in the development of hybrid protein toxins that are directed to specific surface receptors on target cells. In concept, this line of investigation stems from Ehrlich's hypothesis that "magic bullets" could be developed for the treatment of specific diseases. Once it was recognized that many microbial toxins could be divided into structural domains which had a specific function in the intoxication process, it became attractive to assemble hybrid toxin molecules that were composed of the enzymatically active portion of diphtheria or ricin toxin coupled to a variety of ligands. This early work was also stimulated by the observation that fully active diphtheria toxin could be reconstituted by combining the A fragment from the nontoxic CRM45 with the B fragment from the nontoxic CRM197 (Uchida et al. 1973a, b), as well as by the observation that a biologically active hybrid toxin molecule (ricin A/abrin B) could be formed from the receptor binding domain of one toxin molecule and the enzymatically active portion of another toxin molecule (Olsnes et al. 1974).

Since Yamaizumi et al. (1978) demonstrated that the introduction of a single molecule of diphtheria toxin fragment A into the cytoplasm of a eukaryotic

cell was lethal, many investigators have attempted the construction of hybrid toxins that would take advantage of this extreme toxicity and "target" it to specific subsets of cells by substituting the binding domain. Early attempts to combine the enzymatically active fragments of toxins with polypeptide hormones as cell receptor specific ligands were disappointing. CHANG et al. (1977) were successful in synthesizing a hybrid toxin composed of fragment A of diphtheria toxin and human placental lactogen. Even though the hybrid molecule retained that ADPR-transferase activity and lactogen-binding properties, it was not cytotoxic for intact cells (CHANG et al. 1977; OELTMANN and HEATH 1979a, b).

In marked contrast, however, hybrid toxin molecules that retained biological activity could be assembled from the A-chain of the plant toxin ricin and human chorionic gonadotropin. Furthermore, it was of particular interest that this hybrid toxin demonstrated some degree of receptor-specific cytotoxicity in that it was biologically active against rat R2C cells which carry the chorionic gonadotropin receptor, and was not toxic toward mouse L-cells which do not carry the receptor. The major problem that was encountered in much of the early work on hybrid toxins was the relatively high concentrations of the hybrids that were required to intoxicate intact target cells. In many instances concentrations of 100 μg hybrid toxin per milliliter were required to inhibit protein synthesis in the target cell population. At this concentration it is difficult to separate the effect of nonspecific intoxication due to fluid phase pinocytosis from specific intoxication due to receptor mediated endocytosis of the hybrid toxin molecules.

GILLILAND et al. (1978) were more successful in the assembly of highly active hybrid toxin molecules. These investigators have coupled the A-fragment of diphtheria toxin to the lectin concanavalin A, and found that the toxic moiety had to be coupled through a disulfide linkage in order to be cytotoxic. Furthermore, this hybrid toxin was active in the microgram per milliliter range. In addition, this group also reported the construction of hybrid toxins composed of epidermal growth factor (EGF), the A-chain of ricin, and the A fragment of diphtheria toxin. Interestingly, the ricin A-chain/EGF hybrid was specifically cytotoxic at concentrations similar to those required for the biological activity of EGF alone.

Many investigators have observed that hybrid toxins composed of diphtheria toxin fragment A coupled to a variety of ligands were nontoxic for intact eukaryotic cells, whereas the analogous hybrids that were assembled with ricin A-chain were highly toxic. Since the hydrophobic domains of fragment B might have been essential in the membrane translocation of the A fragment, BACHA et al. (1983) separately coupled thyrotropin-releasing hormone (TRH) to two nontoxic diphtheria toxin related polypeptides: CRM26 and CRM45. Both proteins are ADP-ribosyl transferase positive, but only CRM45 contains the lipid associating domains of fragment B. BACHA et al. (1983) have clearly shown that the CRM45-TRH conjugate was cytotoxic at a concentration of 3×10^{-9} *M* for rat pituitary GH3 cells which have TRH surface receptors. This concentration is similar to that required for TRH alone to effect the release of 50% thyrotropin-stimulating hormone (TSH) from this cell line. In contrast, 3T3 cells which are devoid of TRH receptors are not affected by the CRM45-TRH

hybrid. Interestingly, the CRM26-TRH conjugate was not cytotoxic for either the GH3 or 3T3 cell line in vitro. TRH receptor binding experiments demonstrated that both the CRM45-TRH and CRM26-TRH conjugates bound to the cell surface receptor with comparable affinities. Since only the CRM45/TRH conjugate was cytotoxic, it is apparent that the B fragment retained in CRM45 plays an essential role in the membrane translocation of fragment A into the cell cytosol.

There were several observations that were essential for the application of recombinant DNA technology to the development of hybrid toxin genes: (i) the ability to employ small polypeptide hormones to direct conjugate toxin molecules to specific cell surface receptors, (ii) the successful cloning and expression of portions of the diphtheria toxin structural gene in *E. coli* K-12, and (iii) the recent development of both the chemistry and instrumentation for the synthesis of relatively large defined oligodeoxyribonucleotides on solid-phase supports.

My colleagues (MIYANOHARA, BISHAI, and BOYD) and I (unpublished) have recently turned our attention to the genetic construction and expression of a family of diphtheria toxin-related-α-melanocyte stimulating hormone (α-MSH) hybrid genes. The selection of the small polypeptide hormone α-melanocyte stimulating hormone (α-MSH) as the cell receptor ligand in the construction of the diphtheria toxin-related peptide hormone gene fusions was made for the following reasons: the α-MSH receptor binding domain is positioned on the C-terminal end of this polypeptide, thereby allowing for the fusion between the C-terminal portion of the diphtheria toxin-related protein and the N-terminal region of α-MSH, and the oligonucleotides that are required to encode α-MSH are small enough to be synthesized using methodology that is currently available.

The strategy that we have adopted for the genetic assembly of hybrid diphtheria toxin-related-α-MSH genes involved the separate cloning of both the *Sau*3A1–2 and *Sau*3A1-1 DNA segments that encode fragments A and B of diphtheria toxin in *E. coli* K-12. Following modification of the fragment B encoding *Sau*3A1-1 insert at appropriate restriction endonuclease sites with oligonucleotide linkers, a synthetic gene for α-MSH was then cloned in different positions within fragment B. Figure 6 shows the flow diagram that we have used in the assembly of a diphtheria toxin-related-α-MSH gene fusion at the *Cla*1 site within the *Sau*3A1-1 insert.

It should be noted that the modification of the DNA encoding fragments A and B of the toxin gene were conducted separately due to the specificiations of the Recombinant DNA Guidelines in the United States. The current guidelines allow for the molecular cloning of nontoxic portions of the diphtheria toxin gene at the P1 level of containment. In this way modifications of the fragment B encoding insert could be made, and the gene fusion between the truncated fragment B and α-MSH could be efficiently made and sequenced before recloning the fragment A encoding *Sau*3A1-2 insert in the correct orientation completed the assembly of the hybrid toxin gene(s). Since the potency of each of the hybrid toxins that are encoded by the gene fusions is unknown, as is the pathogenic potential of the recombinant *E. coli* that carry the hybrid

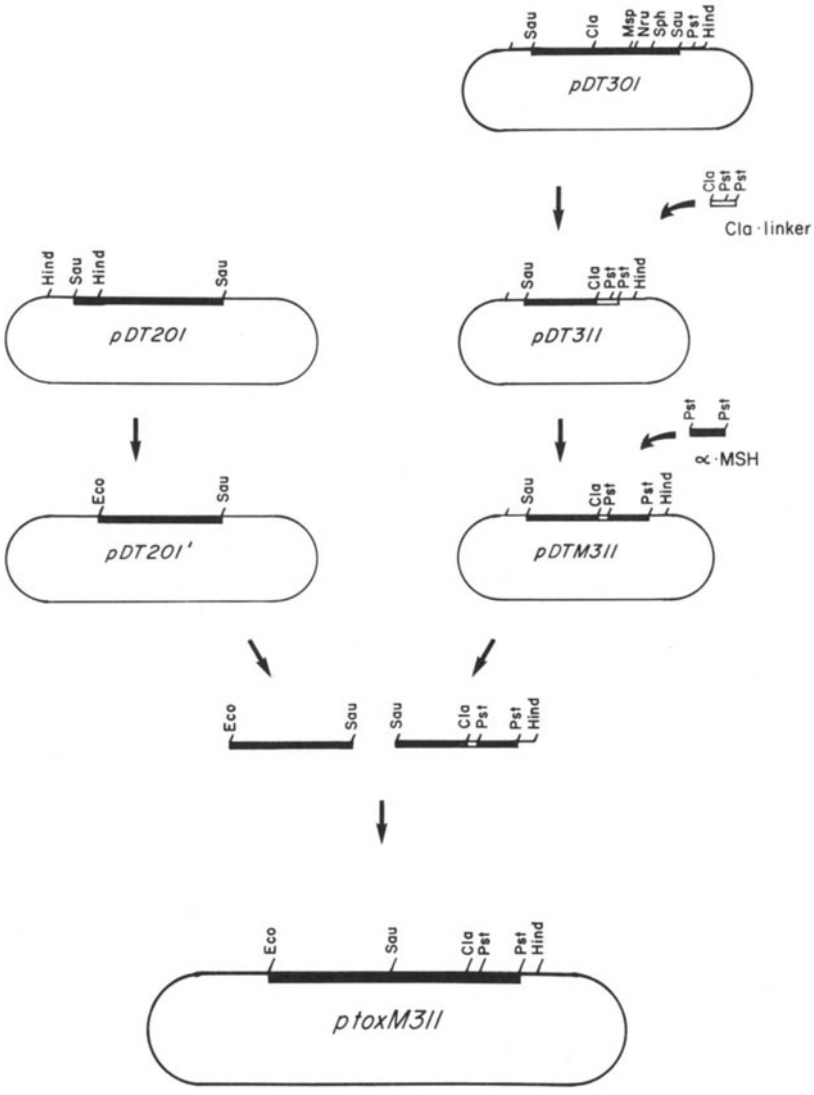

Fig. 6. Flow diagram for the construction of the diphtheria toxin-related-α-melanocyte stimulating hormone hybrid toxin gene. Modifications of pDT201 and the cloning of the *Cla*1-linker and *Pst*1-α-melanocyte stimulating hormone encoding oligonucleotide in pDT301 and pDT311 were performed under P1 conditions. The final transformation, isolation, and characterization of ptoxM311 was performed under P4 conditions

toxin gene, the final genetic assembly of the diphtheria toxin-related peptide hormone gene fusions must be made under the P4 level of containment.

As can be seen in Fig. 7, the *Sau*3A1-1 insert was modified at the *Cla*1 site by the introduction of an oligonucleotide linker that encodes a unique *Pst*1 site, a Cys codon, and a TAG stop signal which are in correct translational reading frame with respect to fragment B. The molecular cloning of the

```
 ValAlaGlnValIleAspAlaAlaAlaCysSTOP
.ggtgcgcaagttatCGATGCTGCAGCATGTTAGTAGCTGCA-3'
.ccacgcgttcaatagcTACGACGTCGTACAATCATCG-5'
                  Pst1          1/2 Pst1
```

Fig. 7. Nucleotide sequence of the oligonucleotide linker required to modify the diphtheria *tox* structural gene at the *Cla*1 site with the introduction of a unique *Pst*1 restriction site, a C-terminal cysteine codon, and a translational stop codon. Diphtheria *tox* gene sequences immediately upstream from the *Cla*1 site are shown in lower case. The oligonucleotide linker sequence is shown in upper case. It should be noted that the linker was constructed with $^1/_2$ *Cla*1 and $^1/_2$ *Pst*1 sites at each end in order to use a vectorial cloning strategy with *Cla*1 + *Pst*1 digested pDT301

```
          SerTrySerMetGluHisPheArgTrpGlyLysProValSTOP
     5'-GCAAGTTATAGTATGGAGCACTTCAGGTGGGGAAAGCCAGTATAGCTGCA-3'
 3'-ACGTCGTTCAATATCATACCTCGTGAAGTCCACCCCTTTCGGTCATATCG-5'
1/2 Pst1                                          1/2 Pst1
```

Fig. 8. Synthetic β-melanocyte stimulating hormone encoding gene. The gene was assembled following hybridization of two 50 bp oligonucleotides through a 46 bp homologous core region. The 4 bp single stranded ends are one-half *Pst*1 restriction endonuclease digestion sites that allow for cloning in *Pst*1 digested pDT311 (MIYANOHARA, BISHAI, BOYD, and MURPHY, in preparation)

*Sau*3A1-2 upstream, and in the appropriate orientation from the modified *Sau*3A1-1 insert, will give rise to a diphtheria toxin-related gene whose protein product would be anticipated to be nontoxic and have a molecular weight of 29267. Most importantly, this modified CRM would have a C-terminal cysteine residue which could possibily be used in the formation of hybrid toxin molecules through disulfide linkage at this defined site.

We have used the unique *Pst*1 site in the *Cla*1-linker for the molecular cloning of a synthetic gene for α-MSH. As can be seen in Fig. 8, the gene for α-MSH was synthesized on a 50 bp oligonucleotide. An additional 3 bp were included before the start of the encoding region in order to insure appropriate translational reading frame at the fusion joint in the linker, and to give a $^1/_2$ *Pst*1 restriction site at each end. The second 50 bp oligonucleotide was synthesized such that a 46 bp core region was directly homologous to the first strand, leaving a 4 bp single stranded *Pst*1 recognition site on each end. Following purification of each strand and hybridization to form a DNA duplex, the synthetic α-MSH gene was readily cloned into *Pst*1 digested plasmid vectors. Preliminary results have suggested that the molecular cloning of this synthetic gene for α-MSH into an expression vector results in the expression of α-MSH related peptides in recombinant strains of *E. coli*.

As can be seen in Fig. 6, the formation of the toxin-related peptide hormone gene fusions involved the ligation of DNA fragments that encode fragment A of diphtheria toxin and the modified fragment B-α-MSH fusion protein, and then a second ligation into a plasmid vector. In order to maximize the probability of our being successful in this genetic construction, we adopted a vectorial cloning strategy. This was accomplished by changing the *Hin*dIII site upstream of the *tox* promoter in the *Sau*3A1-2 insert (pDT201) to an *Eco*R1 site. This change made it possible to purify an *Eco*R1-*Sau*3A1 fragment from pDT201′ that encodes all of fragment A of diphtheria toxin. This fragment could then be ligated to the *Sau*3A1–*Hin*dIII fragment from pDTM311 which

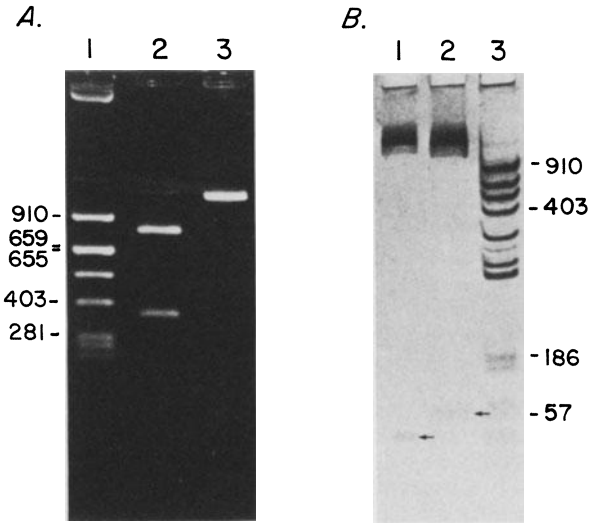

Fig. 9A, B. Restriction endonuclease digestion profiles of the *Eco*R1–*Hin*dIII fragment of ptoxM311. **A** 2% agarose gel stained with ethidium bromide. *Lane 1 Alu*1 digest of pBR322; *lane 2 Sau*3A1 digest of the purified *Eco*R1–*Hin*dIII fragment; *lane 3* purified *Eco*R1–*Hin*dIII fragment. **B** 8% polyacrylamide gel stained with ethidium bromide. Photograph is printed in reverse contrast. *Lane 1 Pst*1 digest of the *Eco*R1–*Hin*dIII fragment; *lane 2 Cla*1 digest of the *Eco*R1–*Hin*dIII fragment; *lane 3 Alu*1 digest of pBR322. Size markers are given in base pairs

encodes the fragment B-related-αMSH fusion protein. Following ligation, and digestion with *Eco*R1 and *Hin*dIII, the toxin hormone gene fusion could then be inserted into *Eco*R1 + *Hin*dIII digested vector.

The plasmid pDT201 was digested with *Hin*dIII, and after filling in the single-stranded ends with T4 DNA polymerase, *Eco*R1 linkers were ligated into position. Since the *Hin*dIII site is upstream from the diphtheria *tox* promoter, this modification of the *Sau*3A1-2 insert does not affect the expression of fragment A in recombinant *E. coli* strains. The unique *Pst*1 that was introduced into the fragment B encoding region of pDT311 was used as the site for the cloning of the synthetic α-MSH gene. Following DNA sequence analysis to insure both the correct orientation and the maintenance of appropriate translational reading frame through the two fusion junctions (fragment B sequences through the *Cla*1 site and linker, as well as sequences through the *Pst*1 site into the α-MSH encoding region), the *Eco*R1-*Sau*3A1 segment of pDT201′ and the *Sau*3A1–*Hin*dIII segment of pDTM311 were ligated together and cloned under P4 containment in the pEMBL8 vector in *E. coli* K-12. Plasmid DNA has been isolated from appropriate recombinant strains and has been analyzed by restriction endonuclease digestion. Figure 9A, B shows that the *Eco*R1–*Hin*dIII fragment from ptoxM311 has the expected *Sau*3A1, *Pst*1, and *Cla*1 digestion patterns. In addition, preliminary data clearly show the expression, and export to the periplasmic compartment, of an M_r 34000 ADP-ribosyl transferase activity in recombinant *E. coli* strains that carry ptoxM311.

We are currently completing the analogous genetic construction of additional diphtheria toxin-related-α-MSH gene fusions at the *Msp*1 and *Sph*1 restriction sites in the *Sau*3A1-1 insert. The comparative study of the hybrid toxins assembled through disulfide linkage or through gene fusion at these three different sites within fragment B of diphtheria toxin should provide additional insight into those structural-functional domains of fragment B that are required to

facilitate the membrane translocation of fragment A into the eukaryotic cell cytosol, as well as provide additional information on the nature of toxin-cell interactions which lead to intoxication and cell death.

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Genetic Analysis of *Streptococcus mutans* Virulence

R. CURTISS III

1 Introduction

CLARKE (1924) first isolated and described a streptococcus from a human carious lesion and inferred that it was a potential causative agent of dental caries. He named the organism *Streptococcus mutans*. This observation went unnoticed for some 35 years until ORLAND (1959) demonstrated that enterococci could cause dental caries in germ-free rats and FITZGERALD and colleagues (FITZGERALD and KEYES 1960; FITZGERALD et al. 1960) found that maximum cariogenicity in germ-free animals was observed following infection with streptococci of the type originally described by CLARKE. It was subsequently demonstrated that *S. mutans* is the principal etiological agent of dental caries in humans (see NEWBRUN 1978). *S. mutans* only colonizes humans following tooth eruption during the 1st year of life, with most humans being colonized during early childhood (STILES et al. 1976).

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Serological characterization (BRATTHALL 1970; PERCH et al. 1974), DNA isolation and characterization (COYKENDALL 1970), and biotyping (SHKLAIR and KEENE 1976) have led to the subdivision of the *S. mutans* group into four distinct genospecies. Biotypes I and V organisms have the type-specific antigens representing serotype *c*. *e* and *f*, 36%–38% guanine plus cytosine (G+C) in their DNA, and are designated as *S. mutans* (COYKENDALL 1977). Biotype II represents the serotype *b* group, has 41%–43% G+C in its DNA, and has been named *S. rattus* (COYKENDALL 1977). Biotype III strains express the serotype *a* antigen, have 42%–44% G+C in their DNA, and have been named *S. cricetus* (COYKENDALL 1977). Biotype IV strains possess serotype *d* and *g* antigens, have 44%–46% G+C in their DNA, and have been named *S. sobrinus* (COYKENDALL 1977).

The genetic distinctions between *S. mutans* and *S. sobrinus* have also been confirmed using hybridization with fragments of DNA cloned in *E. coli*. Thus, an *S. mutans* gene for a glucosyltransferase cloned from *S. mutans* PS14 serotype *c* (ROBESON et al. 1983) is only able to hybridize to DNA sequences from serotype *c*, *e* and *f* strains and not with DNA from any of the other serotypes (M. PUCCI and F. MACRINA 1985). Conversely, a DNA sequence cloned from *S. sobrinus* 6715 (serotype *g*) which specifies surface protein antigen A (SpaA) (HOLT et al. 1982) will hybridize with DNA from serotype *d* and *g* strains, but not with DNA from strains of any of the other serotypes (R. ALICO, personal communication).

Dental caries induced by the *S. mutans* group (includes *S. mutans*, *S. rattus*, *S. cricetus*, and *S. sobrinus*) have worldwide distribution (NEWBRUN 1978). It is the most frequent bacterial infectious disease, and is probably the most costly, at least in industrialized nations (NEWBRUN 1978). *S. mutans* is the most prevalent cause of dental caries in humans in Europe and North America, with *S. sobrinus* being the next most common etiological agent (NEWBRUN 1978).

2 Stages for Display of *Streptococcus mutans* Pathogenicity

HAMADA and SLADE (1980a) have thoroughly reviewed the literature pertaining to the characterization of *S. mutans* and its involvement in dental caries. The unique abilities of *S. mutans* to colonize the tooth surface and induce tooth decay have enhanced interest by scientists in elucidating the process of plaque formation which leads to the onset of dental caries. Although the process and factors necessary for *S. mutans*-induced caries are not completely understood, the results obtained in various laboratories have suggested a tentative sequence of events. A brief synopsis of these steps or stages will be given to provide a context for presenting the genetic studies undertaken which will eventually permit enumeration of the number of genes and gene products, the nature of their interactions, and the means for their control in the overall process of dental caries formation.

The first step involves a sucrose-independent reaction by which *S. mutans* cells attach to glycoproteins or proteins that are contained in the pellicle coating

of the tooth surface as extensively reviewed by GIBBONS and VAN HOUTE (1980). These glycoproteins and proteins that form the pellicle are deposited on the tooth surface from saliva (CLARK et al. 1978; STAAT et al. 1980). This sucrose-independent attachment is undoubtedly a complex reaction in part because of the diversity of different glycoproteins or proteins in the pellicle and because of non-specific factors such as trapping in retentive areas of teeth (GIBBONS and SPINELL 1970; HAY et al. 1971; KASHKET and DONALDSON 1972; ERICSON et al. 1976; MIRTH et al. 1981). In any event, there is evidence that *S. mutans* cell surface proteins may be necessary for this initial sucrose-independent adherence (LILJEMARK and SCHAUER 1975; ERICSON and MAGNUSSON 1976b; CLARK and GIBBONS 1977; CLARK et al. 1978; STAAT et al. 1980; ORSTAVIK and ORSTAVIK 1982; DOUGLAS and RUSSELL 1982; WESTERGREN and OLSSON 1983; MCBRIDE et al. 1984; OGIER et al. 1984; DOUGLAS and RUSSELL 1984; M. RUSSELL, personal communication). Other data would implicate negatively charged teichoic acids on the *S. mutans* cell surface along with bridges involving Ca^{2+} ions reacting with the negatively charged glycoproteins on the pellicle (RÖLLA et al. 1978).

The second step is sucrose dependent and involves adherence of bacteria in a much firmer fashion to the tooth surface and also the aggregation (or agglutination) reactions between adjacent *S. mutans* cells in different chains of streptococci. Both adherence and aggregation in the sucrose-dependent phase involve the synthesis of water-soluble and water-insoluble glucans under the control of cell-associated glucosyltransferase enzymes (GIBBONS and NYGAARD 1968; MUKASA and SLADE 1973, 1974a; CHLUDZINSKI et al. 1976; CIARDI et al. 1976; AKSNES 1978; FIGURES and EDWARDS 1979; HAMADA and SLADE 1980b; NEWMAN et al. 1980; KOGA et al. 1982; WALKER et al. 1984). Several groups have made significant contributions to our understanding of these sucrose-dependent stages. SLADE and colleagues (MUKASA and SLADE 1973, 1974a; HAMADA and SLADE 1980b) have provided evidence to indicate there are specific binding sites for glucans on the surface of cells of *S. mutans* such that one can get intercell or interchain aggregation facilitated by the glucans or by dextran. In addition, glucosyltransferases obviously bind to glucans and can constitute an additional means for facilitating aggregation (GERMAINE and SCHACHTELE 1976) or adherence (MUKASA and SLADE 1974b; HAMADA and SLADE 1980b). It is likely that different glucosyltransferases are responsible for synthesizing various linkages in glucans and play different roles in adherence and aggregation. Dextranase may also play some role in adherence and aggregation as originally suggested by GUGGENHEIM and BURCKHARDT (1974). Evidence consistent with such a role includes:

1. Exogenously added dextranase inhibits water-insoluble glucan synthesis (SCHACHTELE et al. 1975) and inhibits sucrose-dependent adherence (WALKER 1972; HAMADA et al. 1975) and aggregation (GRAVES and VERRAN 1984).
2. Dextranases can modify glucans (STAAT and SCHACHTELE 1974; CHLUDZINSKI et al. 1976; GERMAINE et al. 1977; ELLIS and MILLER 1977; HARE et al. 1978) possibly yielding acceptor molecules for glucosyltransferases (FREEDMAN et al. 1978).

The third stage of caries formation is the result of a composite of metabolic activities appearing in the plaque resulting from adherence and aggregation. *S. mutans* is a homolactic fermenter and can degrade a diversity of mono-, di-, and trisaccharides to yield lactic acid. The production of lactic acid with a concomitant drop in pH eventually causes demineralization of tooth enamel, thus beginning dental caries development (CHARLTON et al. 1971a, b). Most of the free sugars are transported into the *S. mutans* cytoplasm via a phosphotransferase system that leads to the phosphoenolpyruvate-dependent phosphorylation of glucose (SCHACHTELE and MAYO 1973), lactose (CALMES 1978; HAMILTON and LEBTAG 1979), and sucrose (ST. MARTIN and WITTENBERGER 1979; SLEE and TANZER 1979).

The dental plaque arising from adherence and aggregation of *S. mutans* provides a suitable niche for multiplication of other microorganisms that are not able – or not as able as *S. mutans* – to colonize on the tooth surface by themselves, but which, nevertheless, produce acid by sugar metabolism and therefore contribute to the rate of tooth decay (NEWBRUN 1978). Several metabolic activities of *S. mutans* may contribute to this phenomenon. For example, in addition to the metabolism of free sugars, *S. mutans* liberates fructose during polymerization of the glucosyl residues of sucrose by glucosyltransferases and glucose during the polymerization of fructosyl residues of sucrose by fructosyltransferases (CARLSSON 1970). Furthermore, *S. mutans* synthesizes intracellular glycogen-like polysaccharides (GIBBONS and SOCRANSKY 1962) that can serve as a source of metabolizable carbohydrate, as can the extracellular polysaccharides when free sugars are in minimal supply.

3 Approaches to the Genetic Analysis of *Streptococcus mutans* Virulence

Much of what we know about the ability of *S. mutans* to cause dental caries has been learned from biochemical characterization of the proteins and cell surface components that seem to contribute to each stage of the cariogenic pathway. More recently, genetic approaches have been undertaken, most extensively with regard to the isolation and characterization of mutants defective in adherence, aggregation, and acid production. Association of a genotypic alteration, as responsible for a phenotype characteristic of each mutant, has not been possible due to the paucity of classical means of genetic analysis. Early descriptions of bacterial viruses capable of phage conversion or transduction of *S. mutans* (GREER et al. 1971) have not been confirmed. PERRY and KURAMITSU (1981) have only recently described a reproducible method for transformation of several restricted strains of *S. mutans* and *S. cricetus*. Conjugational transfer of plasmid DNA to *S. mutans* has been described (LEBLANC et al. 1978) but a usable conjugational system for genetic analysis of *S. mutans* has not yet been developed (CLEWELL 1981). Because of this lack of a suitable gene transfer system in *S. mutans*, recombinant DNA techniques have been applied for the molecular genetic analysis of *S. mutans* virulence and metabolic activities (CURTISS et al. 1982a, b), as an alternative way to determine the

number of genes contributing to a given phenotype and also to study their mechanism of regulation. These techniques, coupled with a diversity of biochemical and immunological procedures, and, with the design of plasmid shuttle vectors capable of replicating both in *E. coli* and transformable *S. mutans* strains (MACRINA et al. 1982a, b, 1983; DAO and FERRETTI 1985; MURCHISON et al. 1985a), will make possible induction of specific mutational defects and their rigorous genetic analysis by complementation, recombination, and cloning. These shuttle vectors will also facilitate construction of gene and operon fusions (SILHAVY et al. 1984) to evaluate mechanisms of regulation for *S. mutans* genes contributing to virulence.

4 Mutant Isolation and Characterization

FREEDMAN et al. (1981) have prepared a compendium describing all the mutants isolated and characterized in the *S. mutans* group up until the date of their review. Many of the mutants isolated and described have been well characterized phenotypically in terms of adherence, aggregation, acid production, and even for cariogenicity in various animals; nevertheless, specific biochemical lesions have not often been established. In the absence of being able to subject the mutants to genetic analysis one is also faced with the possibility that the phenotype is due to two or more genetic alterations. This has been potentiated on the one hand by the frequent use of potent mutagens such as *N*-methyl-N^1-nitro-*N*-nitrosoguanidine that frequently cause multiple mutations (GUEROLA et al. 1971) and on the other by isolating mutants after repetitive subculturing or enrichment procedures. Thus, FREEDMAN et al. (1981) cautioned investigators to isolate and characterize mutants obtained from separate mutagenesis or enrichmant regimens so as to avoid studying sibs. They also indicated the importance of fully characterizing mutants for testable properties thought to be important for virulence and seeking revertants which should regain the full wild-type phenotype if the original mutant was due to a single genetic lesion.

Mutagenesis with mutagens such as ethyl methane sulfonate or nitrous acid under conditions giving cell survival of 10% or higher enhances the frequency of single genetic defects but does not preclude the occurrence of double mutation events (MURCHISON et al. 1981). In using mutagens with chain-forming streptococci it is important to investigate the chain length before and after mutagen treatment. This is particularly important with mutagens such as nitrous acid, which leads to formation of cells as singlets and doublets (MURCHISON et al. 1981).

Many of the first mutants described and analyzed were obtained as colony morphology variants on mitis salivarius agar. *S. mutans* colonies are typically rough, irregular, and hard on this medium, but variants with smooth, soft, colony morphologies could readily be found and shown to be defective in adherence to smooth surfaces and in forming plaque (DESTOPPELAAR et al. 1971; FREEDMAN and TANZER 1974; MICHALEK et al. 1975; KURAMITSU 1976; BULKACZ and HILL 1977; HAMADA et al. 1978). Some of these mutants such as

those isolated by KURAMITSU (1976) and by MICHALEK et al. (1975) were shown to be defective in glucosyltransferase activity, specifically in synthesizing water-insoluble glucans. Unfortunately, these biochemical characterizations were conducted prior to the realization that there are at least two and probably more glucosyltransferases in *S. mutans* and *S. sobrinus* strains (FUKUSHIMA et al. 1981; CIARDI 1983; MCCABE et al. 1983; ROBESON et al. 1983). It is also possible to obtain colony morphology variants on media such as brain heart infusion agar (MURCHISON et al. 1982). Some of these mutants have alterations in cell wall composition, making them readily lysable. Others are altered in cell morphology or in chain length.

A variety of mutant enrichment protocols have been devised to obtain mutants defective in a given function thought to be important in virulence. Thus, it has been possible to enrich for mutants unable to adhere to smooth surfaces in the presence of sucrose (MURCHISON et al. 1981). Likewise, many enrichment strategies have been devised to enrich for mutants that fail to aggregate after addition of sucrose or dextran to nongrowing cells suspended in a buffer (CURTISS et al. 1985). Based on the observation of STAAT et al. (1980) that an agglutinin from the pit of the avocado *Persia americana* will prevent sucrose-independent adherence of *S. mutans* cells to saliva-coated hydroxyapatite, a method was devised to enrich selectively for mutants that are less able to be agglutinated by this agglutinin (MURCHISON et al. 1985b).

In evaluating mutants obtained, one can examine them for defectiveness in adhering in a sucrose-independent phase to saliva-coated hydroxyapatite (CLARK and GIBBONS 1977), in adhering to smooth glass or plastic surfaces in the presence of sucrose, and in being aggregated by sucrose or dextran when nongrowing cells are suspended in buffer (GIBBONS and FITZGERALD 1969). Many mutants with similar phenotypes are nonetheless likely to have a different biochemical basis and the biochemical defect is most likely to be on the cell surface. Thus, it was possible to show complementation, both in vitro and in the gnotobiotic rat (HIRASAWA et al. 1980), between one adherence-defective mutant deficient in a glucosyltransferase but proficient in aggregation, and a mutant deficient in aggregation but proficient in synthesizing glucosyltransferase. More recently, mutants of similar phenotype have been examined and shown to be capable of complementation for adherence ability in vitro and for cariogenicity during mixed infection in germ-free rats (LARRIMORE et al. 1983).

The existence of mutants that are both adherence defective and dextran-induced aggregation defective, as well as mutants that are defective in one of these traits but appear wild-type with respect to the other, indicates there must be some gene products unique to each activity as well as some that might be shared (MURCHISON et al. 1981). FREEDMAN and GUGGENHEIM (1983) and HAMADA et al. (1983) have isolated and described mutants proficient in adherence but defective in dextran-induced aggregation. The observations by HAMADA et al. (1983) and FREEDMAN et al. (1983) that defectiveness in dextran-induced aggregation has little effect on cariogenicity in specific pathogen-free rats suggests that aggregation, at least in this rat model of cariogenicity, is not terribly important. FREEDMAN and GUGGENHEIM (1983) infer from most of their results

that neither dextranase nor glucosyltransferase serves as a receptor for dextran-induced aggregation but show, nevertheless, that antibodies against dextranase and against glucosyltransferase inhibit dextran-induced aggregation. This paradox in addition to the disagreement with MUKASA and SLADE, 1974b suggests that one must be careful in ascribing a mutant phenotype that is revealed by a defect in enzyme activity or a specific quantifiable reaction to a total loss of the protein. It is quite reasonable to assume that proteins, especially on the surface of the bacterial cell, might display several functions and that a mutation leading to alteration of one of them might leave other activities displayed by the protein completely normal. On the other hand, antibodies may have steric effects which can also lead to misinterpretations.

RUSSELL et al. (1983) have isolated a mutant devoid of a 74000 molecular weight glucan-binding protein which also displays a fructosyltransferase activity. There are two other non-glucan-binding fructosyltransferases. The mutant was defective at sucrose-induced adherence but not at either sucrose-induced or dextran-induced aggregation (RUSSELL et al. 1983). Thus, there must be distinct differences between dextran-binding proteins which recognize the $\alpha 1 \rightarrow 6$ glucosyl linkage as opposed to glucan-binding proteins which might recognize $\alpha 1 \rightarrow 3$ linkages or other structures in water-insoluble glucans.

Some of the more straightforward work making use of mutant isolation and characterization concerns the isolation of *S. mutans* mutants altered in carbohydrate utilization. TANZER et al. (1976) isolated from two serotype *c S. mutans* strains mutants that were defective in the synthesis of intracellular polysaccharides. They showed that these mutants were of lower cariogenicity when used to infect specific pathogen-free rats. More recently, BIRKHED and TANZER (1979) have shown that one of the mutants designated 805 was deficient in the ADP-glucose pyrophosphorylase but had more or less comparable levels of the ADP-glucose-glycogen glucosyltransferase and glycogen phosphorylase activities to those of the wild-type parent. The serotype *d* and *g S. sobrinus* strains have very little intracellular polysaccharide synthesis ability. Thus, this process, which is very important for the biotype I *S. mutans* strains (serotype *c*), is of less importance to the virulence of the biotype IV *S. sobrinus* strains (serotypes *d* and *g*) (TANZER et al. 1976). Of interest is the fact that the mutant 805, while giving lower caries scores in rats, nevertheless competes with other microflora and colonizes quite well (TANZER et al. 1982).

HILLMAN (1978) has isolated mutants devoid of lactate dehydrogenase in a serotype *b S. rattus* strain and has shown that these mutants colonize in mice and rats quite well, and compete with other oral flora, but are of reduced cariogenicity (JOHNSON and HILLMAN 1980a, b).

Several groups have isolated mutants defective in fermenting various carbohydrates and specifically those altered with regard to some of the phosphotransferase activities. Nevertheless, these mutants have not been evaluated for cariogenicity and thus one does not know what influence they might have on acid production in vivo.

In evaluating virulence of mutants in various animal models one must recognize that the model might not reflect what goes on in humans, particularly if the mutant is in an *S. mutans* group serotype other than *c*. Also, unfortunately,

the same well-characterized mutants have not been compared for virulence in the various animal systems currently being used to evaluate virulence. Future progress to provide a complete characterization of the number and functions of proteins involved in adherence and aggregation will require conjoint use of gene-cloning techniques, complementation of *S. mutans* mutants obtained in transformable *S. mutans* strains, induction of specific mutations by insertional inactivation, site-specific mutagenesis of cloned genes, and selection of mutants for loss or alteration of surface proteins using polyclonal and monoclonal antibodies against cloned gene products (CURTISS et al. 1985).

5 Classical Means of Gene Transfer

5.1 Conjugation

LEBLANC et al. (1978) demonstrated that the conjugative *S. faecalis* plasmid pAMβI was transmissible by conjugation during filter matings with *S. sobrinus* strain 6715-10. Although others could repeat this observation, the pAMβI plasmid is unstable in *S. sobrinus* and no one has been able to develop a conjugative system that will allow mobilization of chromosomal DNAs and recombinational analysis between mutant strains. More recently, HARTLEY et al. (1984) have described a disseminated tetracycline-resistance gene from a tetracycline-resistant *S. mutans* strain (TOBIAN and MACRINA 1982; TOBIAN et al. 1984). They used this probe to hybridize against DNA from a random collection of tetracycline-sensitive and tetracycline-resistant streptococci. Of interest was the finding that an *S. sanguis* isolate with the same DNA sequence was able to transfer tetracycline resistance during filter matings to other oral and enteric streptococci in the absence of any plasmid DNA. It is, thus, possible that this newly described tetracycline-resistant element represents a conjugative transposon analogous to the Tn916-, Tn918-, and Tn919-encoding tetracycline resistance which act as conjugative transposons in the enterococci (GAWRON-BURKE and CLEWELL 1984).

Nonconjugative plasmids were first described in a *S. mutans* serotype *e* strain by DUNNY et al. (1973). Plasmids having the same apparent 3.6-Mdal size were shown to be widely distributed in biotype I *S. mutans* strains (MACRINA et al. 1977; MACRINA and SCOTT 1978). Such small plasmids are now found with a frequency of 5%–10% among clinical isolates of serotype *c* and *e S. mutans* strains (KATAMAYA et al. 1978; CAUFIELD et al. 1982). CAUFIELD and colleagues have used this cryptic plasmid as a tag in epidemiological studies in humans to indicate transmission of *S. mutans* strains from parent to offspring in clinical isolates (CAUFIELD et al. 1982). HANSEN et al. (1981) have cloned this plasmid in *E. coli* and shown it to produce a cytoplasmic 20000 molecular weight protein as revealed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Although there have been reports from time to time that these small plasmids somehow contribute to adherence or to the production of bacteriocins, there

is no evidence to substantiate such claims (see HAMADA and SLADE 1980a), since strains cured of their cryptic plasmid have the same properties as their parents. The reason for the prevalence of this widespread plasmid is not apparent. However, CAUFIELD et al. (1985) have found a correlation between bacteriocin production and possession of the plasmid. CAUFIELD et al. (1985) have also shown that there are slight variations in these small cryptic plasmids in that there are two types in one incompatibility group (MUCRHISON et al. 1985a) that have slightly different sites recognized by several restriction enzymes and that are even correlated with expression of a different bacteriocin activity. The plasmid originally described by DUNNY et al. (1973) is in a separate incompatibility group (MURCHISON et al. 1985a). Loss or substitution of a cryptic plasmid by introducing a shuttle plasmid (MURCHISON et al. (1985a) does not change the type of bacteriocin activity expressed. Thus, these plasmids effect but cannot encode bacteriocin activities.

5.2 Transformation

PERRY and KURAMITSU (1981) demonstrated transformation of several serotype *c S. mutans* and serotype *a S. cricetus* strains with chromosomal DNA conferring drug resistance. This observation was quickly confirmed in a number of laboratories. It is now evident that some 25%–30% of the serotype *c*, *e*, and *f S. mutans* strains are transformable with either chromosomal or plasmid DNA (WESTERGREN and EMILSON 1983; MURCHISON et al. 1985a). We have observed that strains possessing cryptic plasmids are more often transformable with both plasmid and chromosomal drug-resistant markers than are recipients lacking a resident cryptic plasmid (MURCHISON et al. 1985a). With saturating concentrations of plasmid and chromosomal DNA, no discernible differences in transformant frequencies could be observed between plasmid-containing and plasmid-free transformable recipients (MURCHISON et al. 1985a). KURAMITSU and TRAPA (1984) demonstrated genetic transfer for both chromosomal and plasmid markers between *S. mutans*, *S. sanguis*, and *S. milleri* during mixed cultivation which they ascribed to transformation. It is, thus, possible that the prevalent cryptic plasmid of *S. mutans* is disseminated by transformation in nature and this would explain why plasmid-containing strains are more frequently transformable than are recipient strains lacking the disseminated cryptic plasmid. A test of this hypothesis would require using cloned fragments of chromosomal DNA for the purpose of examining by Southern blot analysis for restriction fragment length polymorphisms among the chromosomes of strains possessing the same cryptic plasmid.

PERRY et al. (1983) have demonstrated using *S. mutans* strain GS5 transformation of smooth colonizing nonadherent mutants to a rough colony type capable of adherence and, also, ability to synthesize bacteriocin into mutants unable to synthesize bacteriocin. PERRY and KURAMITSU have recently observed linkage between the gene for a glucosyltransferase cloned by ROBESON et al. (1983) and another metabolic attribute.

The mechanism of transformation in *S. mutans* has yet to be defined. It is clear that DNA exposed to *S. mutans* cells is almost completely taken up by 15-min incubation as shown by it becoming insensitive to deoxyribonuclease (MURCHISON et al. 1985a). This is similar to the observed uptake of plasmids by *S. pneumoniae* (LACKS 1977). Thus, presumably, like in *S. sanguis* and *S. pneumoniae*, double-stranded DNA is converted to single-stranded DNA during the process of DNA penetration across the *S. mutans* cell surface with insertion of a single donor strand in place of a single recipient strand. In keeping with this, MACRINA et al. (1981) have demonstrated that transformation of *S. sanguis* with plasmid DNA in monomeric form follows two-hit kinetics which would be expected if the covalently closed circular DNA were linearized and one of the two strands degraded during DNA uptake.

6 Host-Vector Systems to Facilitate Gene Cloning

A great diversity of host-vector systems are available for the cloning of foreign genes into various strains of *E. coli* (MANIATIS et al. 1982). In the belief that *S. mutans* genes might not be well expressed in *E. coli*, BEHNKE and FERRETTI (1980), BEHNKE et al. (1981), and MACRINA et al. (1980, 1982a) have constructed a number of plasmids with suitable drug resistance markers and restriction enzyme cleavage sites that are capable of being transformed into, and replicated in, the transformable Challis strain of *S. sanguis*. With the discovery that *S. sanguis* presumably linearizes plasmid DNA with destruction of one of the single strands during DNA uptake, it became necessary to devise additional strategies for the successful introduction into *S. mutans* and *S. sanguis* of genes cloned from another streptococcus species on those vectors. TOBIAN and MACRINA (1982) developed such a means using a resident helper plasmid lacking the drug resistance marker on the cloning vector but, otherwise, homologous to it.

An alternate method to get around the transformability difficulty of streptococci is to construct shuttle vectors capable of replication in both streptococci and *E. coli*. In this case, identification of recombinants cloned in *E. coli* can be carried out with return of a desired cloned gene to a transformable streptococcal strain. This works well since most competent recipient cells take up two or more recombinant plasmids and can generate one complete recombinant plasmid by recombination. Such shuttle vectors have been constructed by MACRINA et al. (1982b, 1983), by DAO and FERRETTI (1985), and by G.A. CARDINEAU (CARDINEAU et al. 1983; MURCHISON et al. 1985a). All these shuttle vectors are 9–11 kb in size and possess erythromycin-resistance determinants selectable in streptococci and tetracycline-resistance – or tetracycline- and chloramphenicol-resistance – markers selectable in *E. coli*. Shuttles have been constructed using replicons from pACYC184, as well as from a derivative of pBR322. Several different replicons functioning in streptococci have also been used and are most likely compatible.

7 Analysis of *Streptococcus mutans* Gene Products Using Recombinant DNA and Molecular Genetic Procedures

7.1 Analysis of Plasmid Functions

Genes conferring resistance to erythromycin (BEHNKE and FERRETTI 1980), gentamicin/kanamycin (BEHNKE et al. 1981), and tetracycline (TOBIAN and MACRINA 1982) have been cloned from several streptococcal species onto streptococcal plasmids and introduced into *S. sanguis* strain Challis. As expected, these drug-resistance genes are also expressible in *E. coli*, whereas *E. coli* drug-resistance genes are not expressed in any of the transformable streptococci.

HANSEN et al. (1981) cloned the cryptic plasmid pVA318 into *E. coli* using the pBR322 cloning vector. The recombinant plasmid was unable to replicate in a *polA*(Ts) mutant *E. coli* when the growth temperature was shifted to 42° C indicating that either the cryptic plasmid replicon did not function in *E. coli* or was dependent on the *polA* gene product. Using the ability to detect plasmid-specified proteins in minicells (ROOZEN et al. 1971), HANSEN et al. (1981) were able to demonstrate the synthesis of a new 20000 molecular weight protein which remained in the cytoplasm of *E. coli*. This protein had no bacteriocin-like activity when tested against a wide variety of streptococcal strains.

7.2 Analysis of Biosynthetic and Catabolic Functions

Shotgun cloning of chromosomal DNA from the serotype *c S. mutans* strain PS14 and the serotype *g S. sobrinus* strain 6715 into *E. coli* led to the discovery that many *E. coli* mutants lacking enzymes of amino acid, purine, and pyrimidine biosynthesis, and of carbohydrate utilization could grow by using enzymes encoded by streptococcal DNA sequences. Attempts to make an exhaustive screen for function of *S. mutans* enzymes in *E. coli* were not made, but the initial screens indicated that some 40% of the *E. coli* mutants tested were complementable by *S. mutans* or *S. sobrinus* sequences (CURTISS et al. 1982a). An *E. coli* with a deletion of the *lamB-malK* genes was able to grow on maltose using an *S. mutans* gene encoding a protein which substituted for the maltose transport protein encoded by the *malK* gene. The recombinant remained phage lambda resistant as expected (CURTISS et al. 1982a).

SMORAWINSKA et al. (1983) studied the expression of *S. mutans* genes for galactose utilization introduced into *E. coli* strains with a deletion of the *gal* operon. Whereas in *E. coli* galactose is metabolized solely via the LELOIR pathway, in *S. mutans* it is either via the LELOIR pathway or the tagatose pathway (HAMILTON and LEBTAG 1979). The enzymes of the latter pathway are used for lactose and galactose metabolism in *S. lactis*, as well as in certain other streptococci. *E. coli* strains with the *S. mutans gal* genes grew very slowly, in part, because the first enzyme of the tagatose pathway is an isomerase that converts galactose 6-phosphate to tagatose 6-phosphate. This reaction is dependent upon conversion of galactose to galactose 6-phosphate which only occurs inefficiently in *E. coli*, making use of the phosphotransferase system (HANSEN

et al. 1985). In addition, the products of the *S. mutans gal* genes appear to be subject to proteolytic degradation since mutants able to grow more rapidly on galactose can be isolated and can be shown to contain the *lon* mutation inhibiting proteolytic breakdown while conferring UV sensitivity and ability to produce colanic acid (HANSEN et al. 1985). The *S. mutans* DNA encodes not only for the isomerase but, also, for a tagatose 6-phosphate kinase that yields tagatose-1,6-disphosphate and an aldolase which splits the tagatose 1,6-diphosphate into triosephosphates (SMORAWINSKA et al. 1983; HANSEN et al. 1985). In *S. mutans* the gene activities are constitutively expressed but are induced to higher levels when the cells are grown on galactose. The *E. coli* recombinant clone gives constitutive expression with minimal effects on enzyme activities when grown on galactose or in the presence of other modulators of *E. coli gal* operon expression. HANSEN et al. (1985) have used Tn5 mutagenesis and subcloning to demonstrate that in *E. coli* there are two or three separate transcriptional units encoding the proteins of the tagatose pathway. Introduction of the *S. mutans gal* genes into *S. typhimurium* strains with *ptsH* or *ptsI* mutations block growth on galactose. It appears that the *ptsM* gene encoding the enzyme II for mannose but not the *ptsG* gene specifying the enzyme II for glucose is needed for growth on galactose (HANSEN et al. 1985) and must be responsible for generating and galactose 6-phosphate.

More recently, R. RUSSELL and colleagues (personal communication) and KURAMITSU and colleagues (personal communication) have cloned a gene from the serotype *c S. mutans* strains Ingbritt and GS-5, respectively, that encodes a 59000 molecular weight sucrase gene KURAMITSU's group has shown that this gene specifies a sucrose-6-phosphate hydrolase activity. This activity is distinctly different from the 55000 molecular weight glucosyltransferase specified by the *gtfA* gene cloned and characterized by ROBESON et al. (1983).

A gene encoding biosynthetic activity has been cloned and well studied (JAGUSZTYN-KRYNICKA et al. 1982). This is the *S. mutans* gene for aspartic acid semialdehyde dehydrogenase. The *S. mutans asd* gene functions very well in *E. coli* mutants having deletions of the *asd* gene and is constitutively expressed (JAGUSZTYN-KRYNICKA et al. 1982). When the proteins synthesized by the *E. coli* strain containing the *S. mutans asd* gene are examined by SDS-polyacrylamide gel electrophoresis, one finds that the *S. mutans asd* gene product represents some 5%–9% of the total *E. coli* protein synthesized (CURTISS et al. 1982a). Furthermore, in minicells the *asd* promoter outcompetes the *bla* promoter on pBR322 for RNA polymerase since the *asd* but not the *β*-lactamase protein is synthesized (JAGUSZTYN-KRYNICKA et al. 1982). The *S. mutans asd* gene has been fully sequenced (CARDINEAU and CURTISS 1985a, b). The gene has a very unusual promoter with five Pribnow boxes and a very strong Shine-Dalgarno sequence that leads to both very efficient transcription and translation. In addition, the gene possesses an upstream promoter that has attenuator-like properties in that it encodes for a 45 amino acid polypeptide (CARDINEAU and CURTISS 1985b). Unlike attenuators in biosynthetic operons in *E. coli*, the presence of this sequence increases rather than decreases transcription of the *asd* structural gene. In addition, the 45 amino acid polypeptide contains few of the end product amino acids – methionine, threonine, lysine, and isoleucine – that result from

the biosynthetic pathway which includes the activity of aspartate semialdehyde dehydrogenase. Studies are in progress using shuttle vectors and gene fusions to elucidate the mechanism by which this gene is regulated in *S. mutans* as well as in *E. coli*.

Based on the foregoing results, it is evident that one can select in *E. coli* for a diversity of *S. mutans* genes encoding enzymes for biosynthetic activities and for ability to hydrolyze various sugars. Since *S. mutans* gene products involved in sugar transport will function in *E. coli*, it should be possible to clone the genes specifying the components of the phosphotransferase system. It will also be worthwhile to clone and analyze the regulation of genes specifying the enzymes and proteins leading to lactic acid secretion. One can use these cloned genes to deliver insertionally inactivated genes to render the resultant *S. mutans* mutant acariogenic, but otherwise of wild-type phenotype.

7.3 Surface Protein Antigens

Shotgun cloning of size-fractionated *S. mutans* and *S. sobrinus* DNA using cosmid-cloning vectors and suitable strains of *E. coli* K-12 has led to the identification by immunological screening of numerous recombinant clones expressing cell surface proteins without detectable enzyme activity (HOLT et al. 1982; CURTISS et al. 1982b, 1983a, 1985). The *s*urface *p*rotein *a*ntigens of gram-positive microorganisms have been designated by the symbol Spa to distinguish them from the *o*uter *m*embrane *p*roteins specified by gram-negative microorganisms, which are designated by the symbol Omp. The use of the *spa* symbol for genes specifying Spa proteins seems reasonable given the increasing importance being ascribed to proteins on the surface of gram-positive microorganisms. The genetic designators for virulence traits will, of course, require adherence to the standard rules of genetic nomenclature originally proposed by DEMEREC et al. (1966).

HOLT et al. (1982) cloned a gene for the SpaA protein from *S. sobrinus* strain 6715 (serotype *g*). This protein, when synthesized by *S. sobrinus*, has an apparent molecular weight of 210000 but an apparent molecular weight of some 170000 when synthesized by the recombinant *E. coli*. The difference in molecular weight is due to a modification of the protein by *S. mutans* which is not made by *E. coli*. The entire coding sequence of the *spaA* gene is present in *E. coli* as revealed by expression of the gene product by various cosmid recombinants and by suitable subclonings. The nature of this modification is currently under study. The *spaA* gene is well expressed in *E. coli* and most of the protein synthesized is translocated across the cytoplasmic membrane into the periplasmic space (HOLT et al. 1982). Antisera raised against the SpaA protein synthesized by recombinant *E. coli* has been used to evaluate inhibition of various in vitro measures of virulence. Specifically, the antisera inhibits sucrose-induced aggregation but not dextran-induced aggregation or sucrose-dependent adherence to smooth surfaces (CURTISS et al. 1983b). The antibodies have also been used to isolate *S. sobrinus* mutants lacking the SpaA protein. These mutants are also defective in sucrose-induced aggregation and not in any of the other attributes. The vast majority of $SpaA^-$ mutants are, however,

dextranase deficient. All evidence currently available supports the notion that dextranase is a result of post-translational proteolytic cleavage of SpaA protein (CURTISS et al. 1985). Indeed, antiserum against the SpaA protein made by recombinant *E. coli* or native SpaA protein will immunoprecipitate not only the SpaA protein but also dextranase and a glucan-binding protein (J.F. BARRETT, personal communication). It now appears that the SpaA protein is also serving as a glucan-binding protein (J.F. BARRETT, personal communication). Of great interest is the observation that SpaA$^-$ mutants that are devoid of dextranase are totally avirulent in the germ-free rat model for cariogenicity.

The SpaA protein of *S. sobrinus* 6715 is equivalent to the antigen B (R. RUSSELL 1979) which is also equivalent to the antigen I/II described by RUSSELL et al. (1980) and isolated from the Guy's strain of *S. mutans*. Of interest is the fact that antibodies against antigens I/II or B will react with the SpaA protein of *S. sobrinus* and antisera against the SpaA protein purified from recombinant *E. coli* expressing this *spaA* gene will react with a high molecular weight protein from all members of the *S. mutans* group except for the serotype *b S. rattus* strains (HOLT et al. 1982). It is evident that this protein has retained by conservation of amino acid sequences some homology even though the DNA encoding the *S. sobrinus spaA* gene will not hybridize with DNA from *S. mutans*, *S. rattus*, or *S. cricetus* strains (R. ALICO, personal communication; J. HANLIN, personal communication). More recently, M. RUSSELL (personal communication) has shown that antisera against the antigen I/II will inhibit sucrose-independent adherence of *S. mutans* to saliva-coated hydroxyapatite. This has also been confirmed by DOUGLAS and RUSSELL (1985), who have used antisera against the antigen B to demonstrate a 50% inhibition of sucrose-independent adherence to saliva-coated hydroxyapatite. It, thus, appears that the *spaA* gene of *S. mutans* is involved in sucrose-independent adherence to saliva-coated hydroxyapatite, serves as a glucan-binding protein, is involved in sucrose-dependent aggregation, and is probably translationally modified to dextranase. The dextranase released into the medium could degrade dextrans produced by other oral microorganisms and thus, supply bacteria in plaque with another metabolizable source of carbohydrate.

Although an analysis of *S. mutans* cell surface proteins on SDS-polyacrylamide gels reveals great diversity, there is at present little progress in characterizing recombinant clones expressing each of the proteins specified. This is likely to change with more researchers using recombinant DNA techniques to analyze *S. mutans* virulence. Such work will be needed to determine which, if any, cell surface proteins induce heart cross-reactive antibodies. This information, in turn, will permit selection of proteins to use as components to safe vaccines against *S. mutans*-induced dental caries.

7.4 Glucosyltransferases

One strategy for cloning genes that hydrolyze sucrose was based on the fact that the trisaccharide raffinose could be transported into the *E. coli* cell via

the *lacY* permease, where it induces the product of the *melA* gene, which is an α-galactosidase that generates galactose and sucrose (CURTISS et al. 1983b; ROBESON et al. 1983). Thus, by using an *E. coli* unable to use galactose, into which DNA from the serotype *c S. mutans* strain PS14 was shotgun cloned, ROBESON et al. (1983) were able to recover recombinant clones that specified a 55000 molecular weight protein which was subsequently shown to be a glucosyltransferase, designated GtfA. This glucosyltransferase is more heat stable than other sucrose-hydrolyzing enzymes found in *S. mutans*. It is tenaciously associated with the *S. mutans* cell surface, not being released even by salt washing of the *S. mutans* cells. We now believe that the protein is a transmembrane protein since some – but not all – *E. coli* strains harboring this cloned DNA are able to grow on sucrose which can get into the periplasmic space; nevertheless, no more than 20% of the glucosyltransferase activity is released from recombinant *E. coli* following cold osmotic shock (ROBESON et al. 1983). The *gtfA* gene product synthesizes a water soluble glucan of some 2000 molecular weight that has predominantly α1→3 glucosyl linkages. The same *gtfA* gene has been cloned by R. RUSSELL and colleagues (personal communication), KURAMITSU (personal communication) and by PUCCI and MACRINA (1985). In the latter case, the *gtfA* gene which is from the serotype *e S. mutans* LM7 strain has been placed on a shuttle plasmid vector and introduced into strain Challis of *S. sanguis*. The recombinant now synthesizes elevated levels of alcohol-insoluble and water-insoluble glucan. PUCCI and MACRINA (1985) have also proven that in *S. sanguis* it is the glucosyl moiety of sucrose which is solely incorporated into the water-insoluble glucans. It is possible that the molecular weight of the glucan made by the purified GtfA enzyme synthesized by the recombinant *E. coli* is limited by the absence of some other critical protein or component. It should be noted that the activity of the *gtfA* gene product synthesized in *E. coli* is not stimulated by the addition of any dextran or glucan primer (ROBESON et al. 1983). Since enzyme activity of other glucosyl-transferases is enhanced by the addition of dextrans (CIARDI 1983), the GtfA protein might constitute a primase.

MORRISSEY et al. (1985) have devised an additional means to screen for cloned genes that possess sucrose-hydrolyzing activities. They have employed the λL47.1 vector (LEONEN and BRAMMAR 1980) for shotgun cloning of *S. mutans* DNA with subsequent plating of infected bacteria on medium containing sucrose. In this case, enzyme released upon cell lysis liberates free glucose and/or fructose for growth of the indicator bacteria. Clones containing genes for glucosyltransferases are readily detectable because, in addition to bacterial growth, on observes synthesis of glucans (R. RUSSELL, personal communication).

Using this approach, the genes for the glucosyltransferases that synthesize both water-soluble and water-insoluble glucans by serotype *c* and *h S. mutans* strains have been cloned and identified by R. RUSSELL and colleagues (personal communication). KURAMITSU and colleagues have also cloned the gene for the glucosyltransferase synthesizing water-soluble glucans (personal communication). It would, thus, appear that there are at least three glucosyltransferase enzymes specified by the genetic information in serotype *c S. mutans* strains.

7.5 Glucan-Binding Proteins

As stated above, we have recently shown that the SpaA protein of *S. sobrinus* is a glucan-binding protein (see CURTISS et al. 1985). RUSSELL et al. (1983) purified a glucan-binding protein of 74000 molecular weight that also possessed fructosyltransferase activity. RUSSELL et al. (1985) have cloned this glucan-binding protein, which has an unprocessed molecular weight of 76500 when synthesized in *E. coli*. The cloned gene product binds to dextran or glucan but does not possess fructosyltransferase activity. Antibodies against the glucan-binding protein made by *E. coli* will immunoprecipitate fructosyltransferase activity from *S. mutans* extracts. The failure of *E. coli* to process the 76500 molecular weight protein to the 74000 molecular weight form may be the basis for the paradox.

8 Use of Cloned Genes and Cloned Gene Products To Evaluate Importance of Gene Products to Virulence

Although not accomplished by anyone working on *S. mutans* genetics at present, it should be evident that one can take an *S. mutans* gene cloned in *E. coli*, insertionally inactivate the gene with some transposon expressible in *S. mutans*, and return the gene to *S. mutans* by transformation to determine the phenotype associated with a specific genetic lesion (CURTISS et al. 1985). In addition, one can make use of recent *lacZ* fusion technology (SILHAVY et al. 1984) to determine how the gene might be regulated in *S. mutans* under various in vitro and in vivo conditions.

A corollary approach would make use of various transposons such as Tn916 (GAWRON-BURKE and CLEWELL 1982, 1984) and Tn917 (YOUNGMAN et al. 1983). GAWRON-BURKE and CLEWELL (1984) have devised a strategy to introduce the tetracycline-resistance transposon Tn916 into chromosomal genes in streptococci and then use the expression of tetracycline resistance to clone the mutated gene in *E. coli*. In *E. coli* the Tn916 is precisely excised from the cloned DNA to result in the restoration of the wild-type nucleotide sequence of the gene. One can, thus, use this strategy not only to clone a specific gene that has been altered by insertional inactivation with the transposon but to obtain the wild-type sequence. YOUNGMAN et al. (1983) placed the Tn917 transposon encoding erythromycin resistance into a temperature-sensitive plasmid replicon and then selected for transpositions into the *Bacillus subtilis* chromosome. They then used selection for erythromycin resistance to clone the insertionally inactivated gene. Quite possibly, given the ability of streptococcal plasmids to replicate in *B. subtilis* (CLEWELL 1981), the vector constructed by YOUNGMAN et al. (1983) might be usable in transformable *S. mutans* strains.

Antibodies raised against the product of a cloned gene synthesized by recombinant *E. coli* can also be used to isolate specific mutants in instances where one is not dealing with a transformable streptococcal strain. This is particularly important in *S. sobrinus*, where no one has been able to detect transformation for chromosomal or plasmid genetic markers. We, thus, used antibodies against

the SpaA protein produced by *E. coli* to isolate *S. sobrinus* mutants that were almost totally devoid of the SpaA protein as evaluated immunologically using quantitative analytic methods.

Antibodies against a cloned gene product can also be used to evaluate various *S. mutans* mutants that may be devoid of enzyme activity or some specific quantifiable trait, but which can, nevertheless, be demonstrated to possess still the structural protein in an altered form. These antibodies can thus be used to detect cross-reactive protein and to define better the nature of the mutational defects in various *S. mutans* mutants.

Polyclonal antibodies against various cloned gene products are likely to cause steric hindrance in various in vitro assays for virulence. This has already been noted by a number of investigators. The use of monoclonal antibodies (raised against cloned gene products), however, might clarify the information obtained from these studies.

9 Prevention of *Streptococcus mutans*-Induced Dental Caries

JOHNSON and HILLMAN (1980a, b) demonstrated that a lactate dehydrogenase-deficient mutant obtained from *S. rattus* will compete very well against wild-type *S. mutans* in colonizing tooth surfaces while giving a very much reduced caries score. Similarly, TANZER et al. (1982) demonstrated that serotype *c S. mutans* mutant 805, which is unable to synthesize intracellular polysaccharide (i.e., glycogen), is also able to outcompete its parent when used to infect specific pathogen-free rats. In both cases, the notion is to colonize individuals with a less cariogenic organism to preclude colonization by more cariogenic wild-type *S. mutans* strains. The problem, of course, is that such use of these mutants will still cause a low level of caries.

An alternate strategy is to make use of the synthesis of a broad-spectrum bacteriocin. HILLMAN et al. (1984) have isolated a strain of *S. mutans* that produces a 1000 molecular weight bacteriocin that is capable of inhibiting 123 of 124 strains of *S. mutans*, *S. cricetus*, and *S. sobrinus* tested. IKEDA et al. (1982) have also isolated and described a broad-spectrum bacteriocin produced by the serotype *c S. mutans* strain C3603. This bacteriocin is a basic protein of molecular weight 4800 and is active not only against various serotypes of *S. mutans* but against other oral streptococci, as well. More recently, it has been shown that the bacteriocin C3603 inhibits the synthesis of proteins, DNA and RNA, in *S. mutans* cells (TAKADA et al. 1984). Although in vivo production of an antimicrobial agent is likely to lead to selection of bacteriocin-resistant mutants that are fully virulent, one can conceive of genetically engineering a noncariogenic oral streptococcus to produce both the bacteriocins described by HILLMAN et al. (1984) and by IKEDA et al. (1982).

The alternate and probably more acceptable approach to contend with *S. mutans*-induced dental caries is to develop a vaccine. Unfortunately, immunization of rabbits against whole *S. mutans* cells leads to the induction of heart cross-reactive antibodies (VAN DERIJN et al. 1976). There is an extensive literature on the subject, much of which is contradictory. Nevertheless, one must

be concerned since any anticaries vaccine which would be administered to children must be totally safe and no adverse side effects can be tolerated. Because of the potential for surface proteins purified from *S. mutans* cultures to contain contaminants that might produce heart cross-reactive antibody, there is some value of using the gene products produced by recombinant organisms.

COHEN et al. (1983) have thoroughly reviewed various means for immunization against dental caries although they have not considered the use of vaccines devised by recombinant DNA techniques. RUSSELL et al. (1982) demonstrated protective immunity against *S. mutans*-induced dental caries in monkeys (*Macaca fascicularis*) immunized with the 29000 molecular weight protein antigen A. HUGHES et al. (1983) are proceeding to develop a vaccine derived from this *S. mutans*-produced protein. SMITH et al. (1983) are making use of purified glucosyltransferase antigens obtained from both serotype *c S. mutans* and serotype *g S. sobrinus* strains for a vaccine against *S. mutans*-induced dental caries.

Since the secretory immune system is of paramount importance in protecting against colonization on a surface bathed by secretions, since stimulation of the production of secretory IgA by ingestion of whole *S. mutans* has been shown to protect rats against *S. mutans*-induced dental caries (MICHALEK et al. 1976, 1978), and since oral ingestion of *S. mutans* by humans induced production of secretory IgA against *S. mutans* in saliva, tears, and colostrum (MESTECKY et al. 1978), we devised a unique strategy to stimulate the secretory immune system to produce a response against *S. mutans* surface proteins (CURTISS et al. 1983b). In this we have made use of the observation by CARTER and COLLINS (1974) that *S. typhimurium* is able to attach to and invade through the gut-associated lymphoid tissues (GALT) or Peyer's patches in mice. It is known from the work of CEBRA et al. (1976), BIENENSTOCK et al. (1978), and WEISZ-CARRINGTON et al. (1979) that a specific secretory immune response can be obtained by stimulation of the gut-associated lymphoid tissue. We thus reasoned that some of the same genetic methods used to attenuate *E. coli* K-12 in our construction of χ1776 for safe recombinant DNA research (CURTISS et al. 1976) could be employed to attenuate salmonella without impairing their ability to attach to and invade the GALT and persist long enough to stimulate an immune response. Similar considerations lead HOISETH and STOCKER (1981) to develop *aroA* deletion mutants of *S. typhimurium* that were avirulent but could confer protective immunity to mice against subsequent challenge with virulent *S. typhimurium* cells. We have, thus, endowed avirulent derivatives of *S. typhimurium* with the ability to produce the SpaA and GtfA proteins and, in collaboration with my Birmingham colleagues, are investigating the ability of these recombinant strains to induce secretory IgA against the streptococcal proteins in mice and to confer protective immunity against *S. mutans*-induced dental caries in gnotobiotic rats.

10 Conclusion

The genetic analysis of virulence of the *S. mutans* group of etiological agents for dental caries is now progressing rapidly. The genetic, biochemical, and immu-

nological methods to accomplish this are now available and others are being developed. Information on the numbers and functions of cell surface gene products and their means of interaction and regulation in response to each other and the eukaryotic host should soon be forthcoming. In the process, basic information should be learned about the genetic control over the interaction between a pathogen and its eukaryotic host. This basic information will have direct application in presenting us with a diversity of means to intervene in further diminishing the incidence of *S. mutans*-induced dental caries.

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