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Bacterial Capsules

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Prof. Dr. KLAUS JANN
Dr. BARBARA JANN

Max-Planck-Institut für Immunbiologie
Stübeweg 51, 7800 Freiburg, FRG

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Preface

Many bacteria, such as certain *Neisseria* and *Haemophilus* or *Escherichia coli*, are able to withstand the bactericidal activity of complement and phagocytes. This bacterial self protection is brought about by encapsulation. Bacterial capsules thus enable the pathogenic bacteria to survive in the host by counteraction or evasion of the nonspecific host defense in the early preimmune phase of an infection. It is only in the late immune phase of the infection, when specific anticapsular antibodies are formed and enforce the host's defense system, that this protective action is overcome. Encapsulated bacteria are then killed and eliminated. Interestingly, some capsules can not or only inefficiently be handled by the immune system. The ensuing lack of antibody formation results in a prolonged susceptibility of the host to the pathogenic bacteria exhibiting such capsules.

It was found that bacterial capsules consist of acidic polysaccharides. From this it followed that the role of the capsules in the interaction of encapsulated bacteria with the host may be due to the chemistry of the capsular polysaccharides. This led to intensive studies of capsular polysaccharides in many laboratories. Our increasing knowledge of the structural features of capsular polysaccharides prompted not only immunochemical studies analyzing the interactions of these polysaccharide antigens and characterizing the epitopes, but also investigations into their biosynthesis. These studies were complemented and supported by genetic analyses. Today many interdisciplinary investigations of capsular polysaccharides are in progress. One of these is concerned with the elaboration of vaccines on a polysaccharide basis which induce anticapsular antibodies. The biological significance and potency of antibodies induced in this manner is the topic of world-wide efforts today. One of the more recent contributions to the study of capsules and their polysaccharides is electron microscopic analysis. Electron microscopy is very helpful in the study of capsule expression and in the analysis of the interaction of capsules with complement and antibodies. Thus, the biomedical and biochemical research in this field is developing rapidly.

The expansion of the interdisciplinary activities, together with the accumulation of many new facts which highlight the importance of bacterial capsules in infections and which describe their structure, expression, and biological functions, have made it desirable to have a book containing pertinent new facts along with our knowledge of long standing. In this venture it was important not only to accumulate data, but also to emphasize the general principles behind them. Thus, the authors were encouraged to express their interpretations and thoughts, which, even though they may be biased, stimulate further activity in the areas presented. The main emphasis is placed on gram-negative bacteria, notably *E. coli*, *H. influenzae*, and *N. meningitidis*.

Freiburg, October 1989

Klaus Jann
Barbara Jann

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BOULNOIS, G. J.
CROSS, A. S.
JANN, B.
JANN, K.
JENNINGS, H. J.
KROLL, J. S.
MOXON, E. R.
ØRSKOV, F.
ØRSKOV, I.
ROBERTS, I. S.

Genetics of Capsular Polysaccharide Production in Bacteria

G. J. BOULNOIS and I. S. ROBERTS

1 Introduction

A feature of many bacteria of diverse genera is the production of extracellular acidic polysaccharides. These polysaccharides may be organised into distinct structures termed capsules, or may be excreted as an extracellular slime. However, this distinction is arbitrary and in practice may be of no functional significance.

Encapsulated bacteria are frequently associated with serious diseases in man and animals. This is particularly so for those organisms which are invasive, and encounter the host's immune system in normally sterile tissue. In these locations the capsule is thought to allow the invading organisms to evade the immune system by a number of mechanisms, which include reduction of complement- and antibody-mediated opsonophagocytosis as well as (for certain bacteria) resistance to the bactericidal activity of complement (ROBBINS et al. 1980). Because of their importance in the virulence of many bacteria and their usefulness as vaccines (LEE 1987) for the prevention of bacterial infections, capsules have been the subject of intensive investigation.

This review will focus on the synthesis, translocation and surface assembly of capsular polysaccharides in bacteria. In particular we will discuss recent advances in the genetic analysis of capsule biogenesis and, where possible, the genetic data will be interpreted in the light of information available on the structure and biosynthesis of the polysaccharide. We have not attempted to cover all bacterial capsules; rather we have focussed on those which are direct interest to us: by and large these represent the most studied of the bacterial capsules.

Department of Microbiology, Medical Sciences Building, University of Leicester, Leicester, UK

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2 Structure and Biosynthesis of Capsules: General Principles

In this chapter we will only consider biosynthesis of polysaccharides in general terms. For detailed discussions the interested reader is referred to several recent reviews (SUTHERLAND 1985; TONN and GANDER 1979; TROY 1979; JANN and JANN, this volume).

Production of a capsule begins with the synthesis of the sugar components of the polysaccharides and their activation by conversion to nucleotide derivatives. Sugar biosynthesis and activation are generally considered to be cytoplasmic-based activities. In contrast, the subsequent polymerisation is catalysed by an inner membrane-bound transferase complex. These transferases are poorly defined, with an unknown number of components and a catalytic mechanism which remains obscure. As for lipopolysaccharide (LPS) (SUTHERLAND 1985; JANN and JANN 1984) and enterobacterial common antigen (ECA) (BARR and RICK 1987), polymerisation is generally believed to involve a lipid carrier (undecaprenol or polyprenol phosphate) on which monosaccharides or oligosaccharides are assembled. Whether or not this lipid functions as a carrier for all *Escherichia coli* capsular polysaccharides is not clear. Recent studies (JANN, personal communication) suggest that a lipid intermediate does not exist in polymerisation of the *E.* coli K5 capsular polysaccharide. In the case of the K1 polysaccharide, which is composed of oligomeric *N*-acetylneuraminic acid (NeuNAc), monomeric and/or polymeric NeuNAc is transferred from the lipid carrier to an endogenous acceptor. The nature of the endogenous acceptor and the subcellular site at which this step in capsule synthesis occurs are unclear. For the *E. coli* K1 polysaccharide, the endogenous acceptor contains polyNeuNAc which may be linked to protein (TROY 1979). Since many capsular polysaccharides are found associated with phosphatidic acid, the endogeneous acceptor might also contain this phospholipid. This phospholipid has been suggested to function as an anchor for extracellular polysaccharide in the outer membrane (SCHMIDT and JANN 1982; GOTSCHLICH et al. 1981). However, a role for the phspholipid in capsule biogenesis cannot be excluded. The mechanism of addition of phosphatidic acid and the subcellular location at which this occurs is not clear. Similar substitutions have been observed on the capsules of *Neisseria meningitidis* (GOTSCHLICH et al. 1981) and *Haemophilus influenzae* type b (KUO et al. 1985).

Very recently (JANN, personal communication), a KDO residue has been found on the reducing terminus of the K5 capsular polysaccharide of *E. coli*. This residue links the specific polymer to the phosphatidic acid anchor and may be the initiation residue for polysaccharide biosynthesis.

The final stage of capsule production, the translocation of polysaccharide to the cell surface and its organisation into a capsule, is very poorly defined. The molecular genetic analysis of capsule production is now beginning to shed some light on this complex process and is revealing previously unknown aspects of the production of this most important surface structure.

3 Genetics of *Escherichia coli* Capsules

Escherichia coli has been shown to produce more than 70 capsular polysaccharides (ØRSKOV et al. 1977). Individual isolates can only produce one of these polymers, expression of which is stable, and switching of capsular type has not been documented.

The capsules of *E. coli* have been grouped into two classes on the basis of physical, chemical and biochemical criteria (JANN and JANN 1982, 1985). The type II capsules resemble those of *H. influenzae* (see Sect. 4) and *N. meningitidis* (see Sect. 5). These capsules characteristically have molecular weights of less than 50000 and frequently contain 2-keto-3-deoxy-D-mannooctulonic acid (KDO) and/or NeuNAc. In contrast, the type I capsules have molecular weights in excess of 100000 and resemble those produced by *Klebsiella*. In this review we will discuss only the type II capsules, which are exemplified by the K1, K5, K7, K12, K92 and K100 capsules. These have the structures shown in Table 1. Two forms of the K1 antigen have been described (ØRSKOV et al. 1979) which differ in O-acetylation.

Genetic analysis, involving Hfr crosses (ØRSKOV and ØRSKOV 1962), indicated that more than one locus was required for K antigen biosynthesis. One locus was thought to be involved in determination of antigen specificity and another in antigen production. Further crosses demonstrated that one locus termed *kpsA* mapped at about 62 min near *ser A* (ØRSKOV and NYMAN 1974). Furthermore, the determinants for K10 and K50 were allelic (ØRSKOV and NYMAN 1974). Subsequent analysis revealed that the determinants for K1 and K54 were also located at *kpsA* (ØRSKOV et al.

Further insight into the genetics of K antigen production awaited the cloning of the genes involved.

Two groups adopted a cosmid cloning strategy to isolate the genes required to allow laboratory strains of *E. coli* K-12 to produce the K1 antigen (SILVER et al. 1981;

Table 1. Repeating units of some group II *E. coli* capsules

K1 Ac$^+$	$\xrightarrow{8}$ NeuNAc $\underset{\alpha}{\overset{2}{\rightarrow}}$ (OAc at 7/9)	MCGUIRE and BINKLEY (1964)
K1 Ac$^-$	$\xrightarrow{8}$ NeuNAc $\underset{\alpha}{\overset{2}{\rightarrow}}$	ORSKOV et al. (1979)
K5	$\xrightarrow{4}$ GlcUA $\xrightarrow[\beta]{1,4}$ GlcNAc $\underset{\alpha}{\overset{1}{\rightarrow}}$	VANN et al. (1981)
K7	$\xrightarrow{3}$ ManNAcA $\xrightarrow[\beta]{1,4}$ Clc $\underset{\beta}{\overset{1}{\rightarrow}}$	TSUI et al. (1982)
K12	$\xrightarrow{3}$ Rha $\xrightarrow[\alpha]{1,2}$ Rha $\xrightarrow[\alpha]{1,5}$ KDO $\underset{\beta}{\overset{2}{\rightarrow}}$	SCHMIDT and JANN (1983)
K92	$\xrightarrow{8}$ NeuNAc $\xrightarrow[\alpha]{2,9}$ NeuNAC $\underset{\alpha}{\overset{2}{\rightarrow}}$	EGAN et al. (1977)
K100	$\xrightarrow{3}$ β-D-Ribf $\xrightarrow{1,2}$ Ribitol-5 $\rightarrow$ PO_4	TSUI et al. (1988)

Abbreviations: NeuNAc, *N*-acetylneuraminic acid; GlcUA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; ManNAcA, *N*-acetyl mannosamineuronic acid; Glc, glucose; Rha, rhamnose; KDO, 2-keto-3-deoxy-D-mannooctulonic acid; Ribf, ribose; OAc, O-acetylation

ECHARTI et al. 1983). Laboratory strains of *E. coli* K12 are not encapsulated, and in fact lack all of the capsule genes discussed below (ECHARTI et al. 1983). In both cases K1 antigen production was detected initially by searching for halo precipitin formation on antiserum agar plates containing equine meningococcal group B antiserum. This detection system was chosen because the K1 capsule and the capsule of group B meningococci had previously been shown to be structurally and antigenically identical (LIU et al. 1971 b; BHATTACHARJEE et al. 1975; LIFELY et al. 1981).

In the case of pSR23, the K1 cosmid isolated by SILVER et al. (1981), the plasmid was unstable in the absence of selection for vector-encoded drug markers. The polysaccharide produced by recombinants was compared using several techniques and was shown to be indistinguishable from that produced by the wild-type *E. coli* K1 from which the genes were isolated. *E. coli* harboring pSR23 were as sensitive to K1-specific bacteriophage (GROSS et al. 1977) as the wild-type *E. coli* K1 strain (SILVER et al. 1981).

ECHARTI et al. (1983) reported the isolation of a number of cosmid clones which conferred upon *E. coli* K-12 the ability to produce the K1 antigen. Although each recombinant gave rise to precipitation haloes on antiserum agar, their sensitivity to five K1-specific phages was variable. For example, LE392(pKT169) was only sensitive to one (K1 phage B) phage whilst LE392(pKT172) was sensitive to all five K1-specific phages. The reasons for this variability in phage sensitivity are unclear. Interestingly, in all cases the K1 phage titres on recombinant strains were higher (often by an order of magnitude) than the same phage grown on the *E. coli* K1 isolate from which the genes were cloned. Whether this reflects restriction of the phage on the wild-type *E. coli* and/or subtle differences in production of antigen by recombinant relative to wild strains remains unclear.

Genes for the production of K1 antigen have been subjected to a detailed analysis. The work done by our group will be considered in detail and attention will be drawn to points where this is in agreement or conflict with that of others.

Since LE392(pKT172) (see above) was the only clone isolated which produced K1 polysaccharide in a form which reacted with both equine anti-meningococcus group B serum and a K1 monoclonal antibody (FROSCH et al. 1985) and also conferred sensitivity to all five K1-specific bacteriophages, this plasmid was chosen for further study (ECHARTI et al. 1983). To locate and delineate the genes for K1 production a series of deletion derivatives of pKT172 was constructed (ECHARTI et al. 1983). The smallest plasmid still capable of directing production of the K1 antigen was pKT274 (see Fig. 1), and this plasmid was used in further studies.

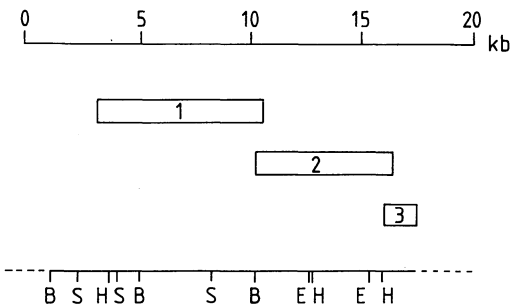

Fig. 1. The K1 capsule gene cluster. The (partial) restriction map of the K1 capsule genes showing cleavage sites for *Bam*H1 (*B*), *Sal*I (*S*), *Hind*III (*H*) and *Eco*R1 (*E*). The *boxes* refer to the various functional regions of the K1 genes as described in the text. The scale is shown in kilobase (kb) pairs

As for pKT172, bacteria harboring pKT274 produced the K1 antigen as judged by sensitivity to K1-specific bacteriophage (BOULNOIS et al. 1987) and reaction with equine meningococcal group B serum (ECHARTI et al. 1983) and a K1-specific monoclonal antibody (FROSCH et al. 1985). Electron microscopic analysis of polysaccharide produced by bacteria carrying pKT274 revealed that only 5%–10% of bacteria produced a capsule (ALLEN et al. 1987). Similar patterns of K1 capsule expression were observed when the wild-type bacteria were examined in the same way. These studies involved examination of ruthenium red-stained bacteria and must be interpreted with care since in preliminary studies (KRONCKE et al., to be published) using gold-labelled K1 monoclonal antibodies in conjunction with electron microscopy, all LE392(pKT-274) cells had K1 antigen on their surface. The extent of encapsulation exhibited by bacteria carrying either pKT172 (the progenitor of pKT274) or pSR23 (SILVER et al. 1981) has not been reported.

The properties of deletion and insertion mutations in pKT274 have been used to delineate K1 capsule genes (Fig. 1). Mutations that affected K1 antigen production fell into three phenotypic classes which were reflected in three physical groupings of these mutations. These groupings defined regions 1, 2 and 3 of the K1 capsule genes (Fig. 1). The same basic conclusions have been reported for pSR23 with the exception that a fourth region was identified (Silver et al. 1984b). It is worth notling that during the construction of pKT274 from pKT172, this fourth region identified by SILVER et al. (1984b) was lost, yet pKT274 retained the ability to direct production of the K1 antigen. These apparently conflicting observations await clarification.

All transposon insertions which fell in region 2 abolished production of K1 antigen (Fig. 1). No immunologically cross-reactive material was detected either in cell culture supernatants or intracellularly (ECHARTI et al. 1983; BOULNOIS et al. 1987). Several observations have led to the suggestion that this region encodes functions for

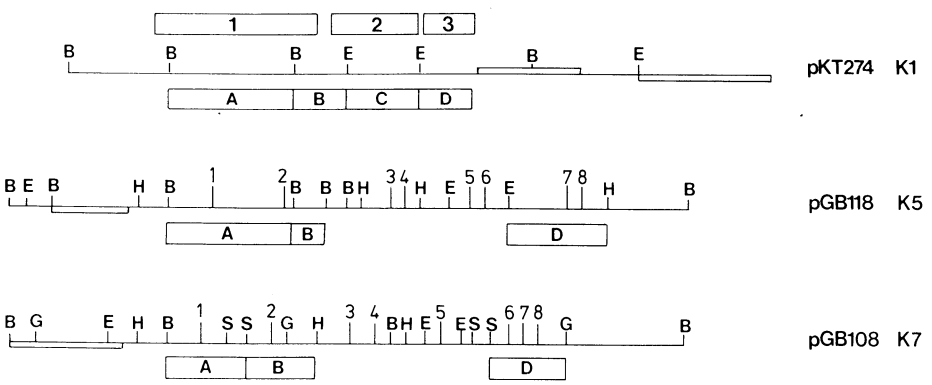

Fig. 2. The K1, K5 and K7 capsule gene clusters. The restriction maps are shown of plasmids which direct production of the K1 capsule (pKT274), K5 capsule (pGB118) and K7 capsule (pGB108). Restriction site designations are as in Fig. 1 and include sites for *BglII*(*G*). Boxes labelled *1–3* above pKT274 denote the functional blocks of genes within the K1 capsule gene cluster (see Fig. 1 and text). Boxes marked *A–D* under pKT274 denote gene probes used to search for homology in the K5 and K7 genes. Boxes *A*, *B* and *D* under pGB118 and pGB108 delineate minimal fragments in these plasmids which were homologous to the K1 gene-derived probes. *Numbered vertical lines* denote the site of insertion of Tn1000

the synthesis, activation and polymerisation of NeuNAc (SILVER et al. 1984b; BOULNOIS et al. 1987; and see below). In contrast to insertions in region 2, insertions in regions 1 and 3 result in a failure of *E. coli* to produce surface polysaccharide although intracellular K1 antigen has been detected (FROSCH et al. 1985; BOULNOIS et al. 1987). Insertions with a similar phenotype have been isolated also in pSR23 (SILVER et al. 1984a).

Several lines of evidence indicate that the biogenesis of different capsules have common steps. The determinants for different antigen types map to the same locus (see above). When the *E. coli* K1 genes were used as DNA probes, homology between the K1 genes and DNA from *E. coli* K7, K92 and K100 was observed (ECHARTI et al. 1983). The patterns of hybridisation observed suggested that region 1 determinants may be present in *E. coli*, producing different polymers (TIMMIS et al. 1985). In addition, the K1 genes carried on pSR23 could complement an undefined mutation in K100 capsule biosynthesis (SILVER et al. 1984b).

To explore the relationships between the different capsule biosynthetic pathways, genes for production of the K5, K7, K12 and K92 antigens have been cloned and characterised (ROBERTS et al. 1986).

Bacteria harbouring the cloned K5, K7 and K12 genes were sensitive to bacteriophage specific for the capsule in question. Whilst the phage formed plaques with almost equal efficiency on the recombinant and wild-type strains, the plaque morphology in each case was different: plaques were, in general, smaller and more turbid on recombinant strains. In the case of recombinants expressing the K5 antigen, polysaccharide (e.g., bacteria carrying pGB110) was apparent on all recombinant strains (KRONCKE et al., unpublished). Interestingly, the capsule produced by bacteria harboring pGB118, a deletion derivative of pGB110, is some ten times thicker than the wild-type and is present on only about 20% of cells (KRONCKE et al., unpublished data). The reasons for this are unclear. It may indicate that a regulatory region, located some distance from the capsule determinants, is required for full encapsulation. Such a region was tentatively identified by SILVER et al. (1984b); however, as discussed above, the nature of this putative regulatory region is unclear.

The cloned K1, K5, K7, K12 and K92 shared common restriction sites, and this was most apparent for DNA adjacent to or within region 1 of the K1 genes (ROBERTS et al. 1986). With the exception of the K92 genes, this similarity disappeared in regions that might be expected to correspond to the regions 2 and 3 of the K1 genes. In the case of K92, the restriction maps of the K1 and K92 encoding plasmids were very similar and extensive DNA sequence homology between the K1 and K92 genes was also apparent (ECHARTI et al. 1983). This is not surprising since the K1 and K92 polysaccharides are very similar in structure (Table 1).

In order to further define regions common to the various capsule genes, a series of Southern blotting experiments was performed in which probes representative of the various regions of the K1 genes (Fig. 1) were used to search for homologous regions in the other capsule genes. These data are summarised in Fig. 2. Probes originating from either within region 1 of the K1 genes (probe A) or at the junction between regions 1 and 2 (probe B) hybridised to fragments in the K5 and K7 genes and homology was detected in regions which were aligned on the basis of common restriction enzyme cleavage sites (Fig. 2). A similar finding was made when a probe carrying region 3 determinants (probe D) of the K1 genes was used. However, in this case

the region in the K5 and K7 capsule genes which was homologous to the probe did not always align on the physical maps of the K1 genes (Fig. 2). Sequences in the various capsule genes that were homologous to K1 regions 1 and 3 were not contiguous, and the intervening DNA was of variable size. The size of this segment was broadly in keeping with the complexity of the capsular polysaccharide in question. A DNA probe (probe C, Fig. 2) from within region 2 of the K1 genes did not hybridise to any fragment of DNA from the K5 and K7 genes (I. S. ROBERTS et al. 1988).

On the basis of these results it was concluded (ROBERTS et al. 1988) that the K1, K5, K7, K12 and K92 genes have a common organisation. Transposon 1000 mutagenesis of the K5 and K7 genes showed this to be the case. Insertions in regions of the K5 and K7 genes which were homologous to K1 genes gave similar phenotypes to inserts in the corresponding regions of the K1 genes. Insertions in the DNA between these regions which had homology with the K1 genes were completely devoid of antigen. Thus genes for different capsules have a similar organisation; i.e. a central segment of DNA variable in size (region 2) and unique for a given antigen is flanked by determinants (regions 1 and 3) that encode products which act in capsule biogenesis *post*-polymerisation and which may be responsible for export of mature polysaccharide to the cell surface and its assembly into a functional capsule.

Since regions 1 and 3 of the different capsule clusters shared extensive sequence homology, and mutations in each region gave rise to similar phenotypes, it seemed likely that the genes concerned code similar, if not identical functions. This was confirmed by demonstrating that mutations in region 1 of one capsule gene cluster were complemented by subclones carrying the equivalent region from a different K antigen gene cluster. In addition, subcloned fragments from within region 1 of capsule genes encoded a similar set of polypeptides (ROBERTS et al. 1986). Similar observations were made for region 3. Thus the products of regions 1 or 3 of different gene clusters carry but the same reactions in the biogenesis of chemically different polysaccharides.

The findings described above raise the question of what functions are coded by each region.

In the case of the K1 genes, certain insertion mutants which fell in region 2 and failed to produce K1, the defect was circumvented if NeuNAc was supplied in the growth medium. These insertions are therefore assumed to define genes for NeuNAc biosynthesis. Since some of these mutants did not produce K1 antigen in response to addition of exogenous N-acetylmannosamine (ManNAc), the defect was postulated to prevent the condensation of ManNAc and phosphoenolpyruvate to form NeuNAc. A 45-kd polypeptide was simultaneously lost in these insertions and it thus seems likely that this polypeptide is NeuNAc synthetase (SILVER et al. 1984a).

Region 2 of the K1 gene cluster also encodes the enzyme CMP-NeuNAc synthetase. A 3.3-kb region 2 fragment of pSR23, cloned in the vector pBR322, conferred upon *E. coli* K-12 the ability to synthesise CMP-NeuNAc. A 50-kd polypeptide was associated with enzyme activity (AARONSON et al. 1984). Insertions in the equivalent region of pKT274 fail to complement a chromosomal lesion which affects CMP-NeuNAc synthetase. In addition, subclones carrying this region complemented a CMP-NeuNAc synthetase mutation (GANGULI et al., unpublished observation).

Whether region 2 also encodes sialyl transferase, the poorly defined membrane-bound enzyme complex which catalyses polymerisation of NeuNAc (TROY 1979), remains to be clearly demonstrated. All insertions in pSR23 tested (SILVER et al.

1984b) retained sialyl transferase activity, although with the exception of the insertions which define the NeuNAc synthetase gene, this activity was only 25% of that found in the wild type. When the *Bam*H1-*Eco*R1 fragment (coordinates 10.5–13 kb, Fig. 1) was subcloned, the resulting plasmid, pSX51, when introduced into *E. coli* K-12, was capable of transferring sialic acid to exogenous colominic acid acceptor. This indicates that this fragment might encode sialyl transferase activity although the number of genes present and the size of their corresponding products are not known (VIMR et al., manuscript in preparation). The comprehensive collection of insertion mutants in pKT274 has yet to be analysed for sialyl transferase activity.

In the case of region 2 it is clear from studies on the K1 genes that enzymes for the biosynthesis of NeuNAc and its activation are specified. It is perhaps not surprising that this is so since NeuNAc is not found (as far as we know) as a component of other polymers common to *E. coli* of all serotypes. This is not the case for polymers like the K5 and K7 antigens, since each contain sugars which are key components of other polysaccharides. For example, the K12 polysaccharide contains KDO, which is also found in LPS (JANN and JANN 1985), and the K7 antigen contains *N*-acetyl mannosamine uronic acid (ManNAcA), which is also found in ECA (MAYER and SCHMIDT 1979). A priori, one might expect that the genes for KDO and ManNAcA biosynthesis are encoded by loci distinct from the *kps* locus because K antigen determinants are allelic whereas KDO and ManNAcA production is ubiquitous. It is possible that in *E. coli* K12 and K7 the determinants for KDO and ManNAcA synthesis are duplicated with one copy residing in region 2 at the *kps* locus. Recent studies have demonstrated that the cloned K7 genes can complement certain lesions in the *rff-rfe* locus (MEIER, personal communication). This locus contains genes for the biosynthesis of ECA (MAYER and SCHMIDT 1979; MEIER and MAYER 1985) and it remains a possibility that the observed complementation reflects some common step in the polymerisation of K7 polysaccharide and ECA. The map positions of the genes for KDO synthesis (39 min) and ManNAcA synthesis (84 min) have been determined in *Salmonella typhimurium* (RICK and OSBORN 1977; LEW et al. 1978) but not in *E. coli*. The functions encoded by region 2 of capsule genes other than K1 have not been studied extensively but we assume that thy encode activities for biosynthesis of polymer.

On the basis of the above findings it seems reasonable to suppose that within *E. coli* there are many different region 2-like determinants. In preliminary studies (ROBERTS et al. 1988) probes derived from region 2 of the K1, K5 and K12 genes only hybridise to DNA from *E. coli* shown previously, using standard serological techniques, to produce the capsule in question. This observation has a number of important implications. Firstly, *E. coli* producing one capsule type do not have "silent" region 2 genes located at chromosomal loci distinct from the capsule gene cluster near *serA*. Secondly, it supports the contention that within the *E. coli* population there are many such region 2 gene clusters. We have suggested that these region 2 determinants comprise a cassette of biosynthetic determinants. The relationship between different cassettes remains obscure, as do the genetic mechanisms which have ensured that the region 2 cassettes are always located between region 1 and 3. The DNA sequence of junction regions between various cassettes and regions 1 and 3 ought to be illuminating. Such studies are currently in progress.

The role of the gene products encoded by region 3 in the biogenesis of the K1 capsule remains unclear. Immuno-electrophoretic analysis of antigen isolated from insertions in this region revealed that it had a low electrophoretic mobility compared with the native polymer (BOULNOIS et al. 1987). It seems reasonable to suppose that these insertions result in the appearance of a previously unrecognised intermediate in capsule biosynthesis. Since these insertions have immunoreactive material intracellularly, the region 3 products have been postulated to function after polymerisation of NeuNAc. This might include transfer of polymer from the undecaprenol carrier used for oligomerisation (TROY and MCCLUSKEY 1979). Alternatively, these functions might mediate transfer of polymer to the phospholipid anchor molecule. Whatever the functions encoded by region 3, the same set of polypeptides function in the biosynthesis of all polymers so far analysed (I. S. ROBERTS et al. 1988; see below). On this basis we suggest that region 3 does not encode sugar transferase enzymes. Further light will be shed on the role of region 3 encoded functions in biosynthesis of K1 when sufficient of the intracellular polymer present in region 3 mutants has been purified and chemically characterised. It is interesting to note that when thin sections of bacteria harbouring plasmids carrying insertions in the region 3 determinants of the K5 genes were probed with gold-labelled, K5-specific monoclonal antibodies and examined in the electron microscope, the intracellular, immunoreactive material was found in the cytoplasm (KRONCKE et al., unpublished and see JANN and JANN, this volume). In addition, immuno-electrophoretic analysis of this material indicated that it is devoid of lipid (KRONCKE et al., unpublished; JANN and JANN, this volume). This suggests that the region 3 functions are involved in the translocation of polymer across the inner membrane to other acceptors in either the inner or outer membrane, or the periplasmic space. Implicit in this suggestion is the notion that K5 polysaccharide chain elongation occurs on the cytoplasmic, rather than periplasmic, face of the inner membrane (see JANN and JANN, this volume).

The intracellular material in region 3 mutants of the K1 genes has a low electrophoretic mobility relative to native polysaccharide, a situation which contrasts to that found in the equivalent mutations in the K5 genes. To what extent this reflects the finding that the K5 antigen, unlike the K1 antigen, appears not to be synthesised on a lipid carrier is unclear.

Insertions or deletions that fell within region 1 of the K1 genes resulted in the intracellular appearance of polysaccharide which to date has proved to be indistinguishable from mature, cell surface polysaccharide. The intracellular material reacts with K1 polyclonal and monoclonal antibodies, and has a mobility indistinguishable from native K1 polysaccharide. Rocket immuno-electrophoretic analysis of this material indicated that bacteria with a disrupted region 1 synthesised about 20% of the K1 antigen made by pKT274 (BOULNOIS et al. 1987). Since this material forms micelles, we have suggested that it is full-length polymer linked to the phospholipid anchor molecule although confirmatory chemical evidence is lacking (BOULNOIS et al. 1987; TIMMIS et al. 1985). A derivative of pSR23, termed pSR27, carried a deletion of a substantial proportion of region 1 (SILVER et al. 1984b). This deletion had normal levels of sialyl transferase activity and was unstable even in the presence of selection. We (ECHARTI et al. 1983; TIMMIS et al. 1985; BOULNOIS et al. 1987; I. S. ROBERTS et al. 1988; SILVER et al. 1984b) concluded that this region of the K1 genes was involved in translocation of polysaccharide to the cell surface.

The K1 polysaccharide present within bacteria with a disrupted region 1 was released from cells by procedures which release the contents of the periplasmic space (Boulnois et al. 1987). The defect therefore seemed to block translocation of polymer from the periplasmic space. Five polypeptides have been shown to be encoded by region 1 of the K1 genes (Silver et al. 1984b) and a similar set of polypeptides was found in the K5 and K7 polysaccharide translocation functions (Roberts et al. 1986). At least one of the proteins of 60 kd is located in the periplasmic space (Silver et al. 1987), an observation consistent with the notion that region 1 encodes functions for the translocation polysaccharide from the periplasm to the cell surface.

Several lines of evidence point to the conclusion that the functions encoded by region 1, which were shown in the case of K1 to be involved in export, carry out the same role for all K antigens. Firstly, there is considerable sequence homology between region 1 determinants from different capsule gene clusters, they all encode a similar set of polypeptides, and mutations in region 1 of one K antigen gene cluster can be

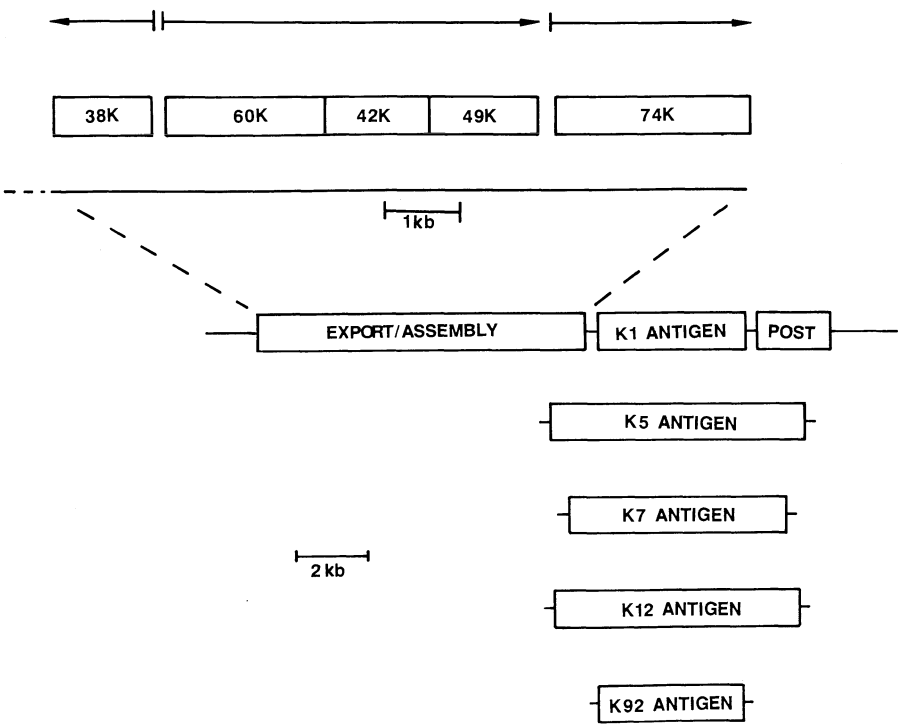

Fig. 3. Schematic representation of capsule gene organisation in *E. coli*. The K1 capsule gene organisation is shown in the *centre* of the figure. The box labelled *export/assembly* refers to those genes involved in the translocation of mature polysaccharide to the cell surface and its organisation into a capsule. The expanded region shows the organisation and direction of transcription of genes believed to act in this process. Boxes labelled *K1–K92 antigen* are "cassettes" of determinants required for the biosynthesis of each antigen. The box labelled "*post*" refers to region 3 (see Figs. 1 and 2) and encodes functions which are believed to act in capsule production after synthesis of polysaccharide. The precise boundaries of the various gene blocks have yet to be determined

complemented if the equivalent region from a second K antigen gene cluster is provided *in trans*.

Our work has focussed on the K7 region 1 determinants since they are the most convenient to study. The extent of these genes was mapped using a collection of Tn1000 insertions in a plasmid which carries the K7 export functions and asking whether or not these can complement large deletions in region 1 of the K1 gene cluster. These experiments defined a 9-kb region which was required for polysaccharide export (Fig. 3). Analysis of patterns of polypeptides produced by these insertion mutants in *E. coli* minicells allowed localisation of the coding sequences for each polypeptide required for K antigen transport (Fig. 3).

Comparison of membrane protein profiles of bacteria carrying the cloned K7 region 1 genes compared with those of the same bacteria lacking this plasmid has revealed that certain of these export proteins can be visualised. The most notable difference was the presence of a 46-kd protein in bacteria carrying the cloned genes (KRONCKE et al., unpublished). This may correspond to the 48-kd product seen in minicells (Fig. 3). The 46-kd K7 protein has been partially purified from membranes and antisera produced. This should be a valuable tool in the analysis of polysaccharide export. In addition, weakly expressed proteins with apparent molecular weights of about 75000 and 55000 were also visualised. These may correspond to the 72-kd and 58-kd products seen in minicells (Fig. 3). SILVER et al. (1984b) have reported that region 1 of the K1 genes (carried on a plasmid pSR43) encodes proteins of 77, 60 and 40 kd. The 60-kd periplasmic protein coded by the K1 region 1 genes is also probably coded by the translocation genes for the K2, K5, K7, K12, K13 and K92 polysaccharides, since a DNA probe isolated from within its structural gene (*kpsD*) hybridised to DNA from *E. coli* of these serotypes. A polypeptide of similar molecular weight has been seen in minicells carrying the cloned K5, K7 and K12 translocation function (ROBERTS et al. 1986). However, *kpsD* specific probes failed to hybridise to either K3 or K100 *E. coli*. This is interesting since a gene probe from within the K1 translocation function hybridised to DNA from *E. coli* K100 (ROBERTS et al. 1986). It will be interesting to determine the requirements for export of *E. coli* K100 polysaccharide.

In addition to functions for the transfer of phospholipid-linked polysaccharide from the periplasmic space and across the outer membrane, region 1 may also encode functions for assembly of the capsule. Indeed, preliminary evidence obtained using plasmid pGB14 (ECHARTI et al. 1983), a deletion derivative which removes only a small part of the left-hand end of the K1 region 1 genes, indicated that bacteria carrying this plasmid have small tufts of polysaccharide on their surface (ROBERTS et al., manuscript in preparation). Bacteria harbouring pGB14 were not sensitive to K1 bacteriophage. Thus the deletion in pGB14 might remove (or block expression of) functions required for assembly into capsules and/or for correct orientation of polysaccharide on the surface. Interestingly, this phenotype is minimicked in wild-type bacteria or recombinants expressing the K5 or K12 capsule. When these bacteria are grown at 18 °C to prevent capsule production and shifted to the capsule permissive temperature of 37 °C, newly synthesized polysaccharide is present initially as tufts on the bacterial surface (KRONCKE et al., unpublished). Thus the deletion in pGB14 may remove a gene for products required for assembly of surface expressed polysaccharide into the capsule. An examination of the K7 translocation function indicates that the deletion in pGB14 would remove only the gene for the 38-kd protein (Fig. 3).

It is clear that region 1 encodes functions involved in polysaccharide export. However, other proteins encoded at loci distinct from region 1 probably also play a role in this process. For example, polysaccharide export has been postulated to occur at junctions between the inner and outer membranes (BAYER 1979). Translocation has also been shown to require a functional porin in the outer membrane (FOULDS and AARONSON 1984). This can be provided by the *ompK*, *ompC* or *phoE* gene products or protein 2 of *E. coli* K-12 and, in clinical isolates, probably by the K protein. This protein, with an apparent molecular weight of 40000, which also functions as a porin (SUTCLIFFE et al. 1983; WHITFIELD et al. 1983), is found in encapsulated *E. coli* isolates (independent of serotype) but rarely in non-encapsulated strains (ACHTMAN et al. 1983; PAAKKANEN et al. 1979; VAN ALPHEN et al. 1983). We were unable to demonstrate (TIMMIS et al. 1985) that the K1 genes carried by pKT274 encoded this product, at least at sufficiently high levels to be readily observed in envelope extracts. However, the K7 transport genes encode proteins of 38 kd and 42 kd which may correspond to K protein.

The precise role each of these proteins plays in polysaccharide export is an important area for further studies.

4 Genetics of *Haemophilus influenza* Capsules

Haemophilus influenzae elaborates six serologically and chemically distinct capsular polysaccharides designated types a through f (ROBBINS et al. 1984). Of these only type b capsular polysaccharide is associated with invasive diseases in humans (ROBBINS et al. 1980). The structure of type b capsule (see MOXON and KROLL, this volume) is very similar to that of the K100 capsule of *E. coli* (Table 1).

Early studies, exploiting the natural transformability of *H. influenzae*, demonstrated that a non-capsulated organism originally selected from a typed organism could be transformed to any other capsular types depending on the serotype of the organism from which the transforming DNA had been isolated (ALEXANDER et al. 1954). Subsequent studies (CATLIN et al. 1972) indicated that the locus for encapsulation (termed *cap b*) was located in the *H. influenzae* chromosome near *bio* and that the *cap b* functions might be located over a segment of DNA as large as 50 kb.

Some of the genes involved in production of the type b capsule were cloned in lambda vectors (MOXON et al. 1984). Clones carrying *cap b* functions were identified following transformation of a non-encapsulated derivative of *H. influenzae* type b with lambda recombinant DNA and searching for encapsulated derivatives. One clone containing 11.4 kb of DNA was characterised and shown to contain some but not all of the genes required for type b capsule expression. Subsequent attempts to isolate *E. coli* clones, carrying *H. influenzae* DNA cloned in cosmid vectors, which expressed immunoreactive type b capsular polysaccharide, were unsuccessful (HOISETH et al. 1986). The insert DNA from the phage clones was subsequently used as a hybridization probe to isolate larger tracts of *cap b* DNA from cosmid libraries (HOISETH et al. 1986).

On analysis of a number of the isolated cosmid clones it became apparent that an 18-kb tandem duplication existed in the cloned DNA. This arrangement was only

stable in recombination deficient *E. coli* (HOISETH et al. 1986). Southern blot analysis demonstrated that this duplication was found in the vast majority of *H. influenzae* type b.

Spontaneous loss of encapsulation in *H. influenzae* type b occurs at a reasonably high frequency (0.1 %–0.3 %) and this was associated with loss of *cap b*-associated DNA segments. The biological significance of the irreversible loss of encapsulation remains obscure. To test the notion that this high frequency loss of encapsulation occurred by a *rec*-dependent recombinational event which resulted in the loss of one 18-kb duplication, the *rec*-1 mutation was introduced into a type b strain. In such a strain the appearance of capsule-less derivatives was not observed, indicating that loss by a recombinational event, probably involving the tandem duplication, was crucial to the instability of capsule expression (HOISETH et al. 1986). HOISETH et al. (1986) suggested two possible explanations for why, if one copy of the 18-kb duplication was still present in capsule-less derivatives, they failed to produce capsules. Firstly, the duplicated sequences were functionally different, and this difference was not apparent at the level of gross physical restriction maps. Alternatively, the 1.3-kb of DNA separating the tandem duplications which would be lost in the deletion contains essential information for capsule expression. That the latter is most likely was suggested by the observation that when either one or the other duplicated segments was mutagenesised, capsule expression was unaffected (ELY et al. 1986).

A gene, termed *bexA*, encoding a 24.7-kd protein has been mapped to this central region (KROLL et al. 1988). A frame shift mutation engineered into this gene resulted in bacteria which failed to express a capsule, but immunoreactive material was present intracellularly. This phenotype resembled that which results from the recombinational loss of the *cap b* duplication. This observation, together with the sequence similarities between *bexA* and various ATPases, has been used (KROLL et al. 1988) to support the argument that *bexA* encodes an energiser of oligosaccharide and/or polysaccharide export. It will be interesting to explore the relationship between *bexA* and the region 3 determinants of the *E. coli* capsule genes which may encode functions involved in the energy-dependent translocation of *E. coli* capsular polysaccharides across the inner membrane (see above).

Whilst the majority of *H. influenzae* type b isolates have a tandem duplication at the *cap b* locus, three isolates which are encapsulated have been analysed in which only one of the duplicated regions is present. In each case the 1.3-kb DNA segment that separates the duplications found in most strains was also present (KROLL and MOXON 1988). This finding supports the contention that this sequence is essential for expression of capsule functions. In the strains of *H. influenzae* carrying only one copy of the duplication, half the amount of polysaccharide was produced compared to the situation where both copies were present. Capsule expression by bacteria with a single 18-kb region was completely stable, supporting the notion that the duplication is in part responsible for the genetic instability observed. Even though these single copy variants produce half the normal amount of polysaccharide, they are virulent as judged by production of bacteraemia and meningitis in infant rats (KROLL and MOXON 1988).

A detailed genetic analysis of the organisation of capsule functions at the *cap* locus of *H. influenzae* is currently in progress (MOXON, personal communication). The data available to date support the notion that capsule functions are organised essentially

as for *E. coli* (see above). A region within *cap b* of 4.6 kb has homology only with DNA from type b organisms but not the other serotypes of *H. influenzae* or other *Haemophilus* species, non-typeable *H. influenzae* or other bacterial species which show serological cross-reaction with the type b capsule. In this sense the 4.6-kb DNA segment behaves like region 2 of the *E. coli* capsule genes (FROSCH et al. 1987; M. ROBERTS et al. 1988; see above). Two loci have been defined in the 4.6-kb segment. One is required for capsule synthesis and the other for maintenance of colonial morphology reminiscent of the wild-type organism. It seems reasonable to suppose that the former are involved in the biosynthesis of polysaccharide; the role of the latter remains obscure. Immediately adjacent to this region is a segment of DNA which hybridises to type b and type a strains. Mutations in this region abolish synthesis of the type b capsule and these can be rectified following transformation with DNA obtained only from type a or type b. *H. influenzae*. Since type a and type b capsules are the only *H. influenzae* capsules containing ribitol (see KROLL and MOXON, this volume), it has been suggested that this region encodes ribitol biosynthesis or modification.

As was the case for *E. coli* (ECHARTI et al. 1983; I. S. ROBERTS et al. 1986, 1988; ROBERTS et al. 1988; and see above), a segment from the *cap* locus of *H. influenzae* type b demonstrated broad hybridisation profiles with all *H. influenzae* serotypes (MOXON et al. 1984; HOISETH 1985; MOXON, personal communication). This region lies to the left of, and adjacent to, the region thought to be involved in ribitol synthesis or activation, and mutations in the region result in the intracellular appearance of immunoreactive type b polysaccharides. To the right of the b capsule specific region is another region with is *cap* locus specific and which hybridises to DNA of all serotypes. The nature of these genes and their role in capsule biogenesis remain unclear since it has not proved possible to isolate mutations in this region. These segments of DNA might be equivalent to region 1 or 3 of the *E. coli* capsule gene cluster.

5 Genetics of Meningococcal Capsules

At least eight serogroups have been described in *N. meningitidis* (GOTSCHLICH 1984) and four of these contain NeuNAc. The structure of the group B polysaccharide is identical to *E. coli* K1 and the group C polysaccharide (α2–9 linked NeuNAc) is similar to the K92 antigen of *E. coli* (Table 1). The biosynthesis of the group B polysaccharide of *N. meningitidis* (MASON and HOLBEIN 1985) has many steps in common with the synthesis of the K1 antigen of *E. coli* (TROY 1979), although some differences have been noted. For example, *N. meningitidis* group B, unlike *E. coli* K1, possesses a CMP-NeuNAc hydrolase (MASSON and HOLBEIN 1983).

Very little is known of the genetics of capsule production in *N. meningitidis*. The cloned K1 genes share little or no DNA sequence homology with DNA from *N. meningitidis* groups B and C (ECHARTI et al. 1983). Because of this a comparison between the *E. coli* K1 genes and the equivalent genes in group B meningococci will be particularly informative. To this end we have recently cloned some of the group B capsule determinants (REID et al., unpublished). This was achieved by introducing fragments

of meningococcal DNA cloned in plasmid vectors into *E. coli* K1 strains with a specific mutation which renders CMP-NeuNAc synthetase inactive. Clones in which production of K1 was restored were then detected by an immunoblotting procedure. One plasmid (pGB147) isolated in this way was shown to comprise about 5 kb of cloned meningococcal DNA which could complement several insertions in region 2 (see Fig. 1) of the K1 genes, indicating that multiple capsule determinants reside on pGB147. Fragments from within this cloned segment had an interesting hybridisation profile. Some hybridised only to meningococci which elaborate capsules containing NeuNAc whilst others hybridised to DNA from all meningococcal serogroups. The former presumably correspond to region 2-like determinants in the meningococcus whilst the latter may be equivalent to either region 1 or region 3 of the *E. coli* capsule genes. The cloned DNA hybridised extensively to carrier isolates of meningococci which were previously shown to be non-groupable by standard serological means (Ison et al., unpublished). Thus the majority of non-groupable meningococci contain capsule determinants. Some non-groupable isolates failed to hybridise to all probes, indicating that they lack capsule genes and are therefore presumably incapable of causing invasive disease. Of particular interest was the observation that the probe which hybridised to all serogroups also hybridised to some isolates of *Neisseria lactamica*. The significance of this remains unclear.

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Structure and Biosynthesis of the Capsular Antigens of *Escherichia coli*

B. JANN and K. JANN

1 Introduction

A characteristic feature of gram-negative bacteria, to which *Escherichia coli* belong, is the presence of an outer membrane on the external side of the murein sacculus. This layer consists of proteins, lipoproteins, phospholipids, and lipopolysaccharides (LPSs). LPSs of smooth wild-type bacteria, consisting of lipid A, core, and O-specific polysaccharide, are O antigens and those of rough mutants consisting only of lipid A and core are R antigens (JANN and WESTPHAL 1975; JANN and JANN 1984).

Serologic studies on *E. coli* (KAUFFMANN and VAHLNE 1945; ØRSKOV et al. 1977) had indicated that many strains exhibit surface antigens which are distinct from the O antigens. These additional antigens were assumed to be present in an extracellular envelope or capsule covering the cell wall O antigens. They were therefore termed K antigens ("*Kapselantigene*"). Electron microscopy has revealed that many coli bac-

Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, FRG

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teria are indeed surrounded by a capsule, which, however, can be demonstrated only after certain stabilizing procedures (BAYER and THUROW 1977; BAYER et al. 1985; BAYER, this volume).

It is generally accepted that capsules protect pathogenic bacteria against the unspecific host defense which is exerted in the preimmune phase of infection by serum complement and phagocytes. This can be imitated in vitro by the phagocytosis of uncapsulated *E. coli* and their resistance to phagocytosis in the encapsulated form. Anticapsular antibody, which is formed in the immune phase of infection, can neutralize the shielding effect of the capsule.

In this chapter we discuss the molecular characteristics of the *E. coli* capsular (K) antigens and present information on their structure, biosynthesis, and cellular expression.

2 Characterization of *Escherichia coli* Capsular (K) Antigens

Capsular antigens of *E. coli* are acidic polysaccharides with different chemical compositions. Several features have been used for a general classification of the capsular polysaccharides, such as chemical composition, molecular weight, mode of expression, and genetic determination:

1. *Molecular Weight:* Certain capsular polysaccharides, notably those originally characterized as A antigens (KAUFFMANN and VAHLNE 1945), have a very high molecular weight, their aqueous solutions being very viscous. These polysaccharides are heterogeneous with respect to apparent molecular weight, as demonstrated by selective extraction procedures and analysis of their hydrodynamic properties (HUNGERER et al. 1967; ØRSKOV et al. 1977). Other capsular K polysaccharides have lower molecular weights; in most cases their aqueous solutions are less viscous.
2. *Nature of the Acidic Component:* Most of the K polysaccharides belonging to the group of A antigens (KAUFFMANN 1954) contain glucuronic acid, although some contain galacturonic acid instead. Some are substituted ketosidically with pyruvate to give a cyclic 1-carboxyethylidene substitution with an additional negative charge. These polysaccharide consist of repeating units with a size of tri- to hexasaccharide.

Many other K polysaccharides do not contain hexuronic acids as the charged component but have other acidic constituents such as *N*-acetylneuraminic acid (NeuNAc), 2-keto-3-deoxymanno-octonic acid (KDO), *N*-acetylmannosaminuronic acid (ManNAcA), or phosphate. Their repeating units are mono-, di- or trisaccharides, which results in higher charge densities.

3. *Mode of Expression:* In a systematic study, ØRSKOV et al. (1984) reported that the capsular (K) polysaccharides of some *E. coli* strains are present at all growth temperatures and that those of other strains are present on bacteria grown at 37 °C but absent from bacteria grown at 20 °C or below.

Many capsular K polysaccharides occur only in O groups 8, 9, and 20, i.e., only together with the O8-, O9-, or O20-specific LPSs, whereas others are not restricted to distinct O groups and can occur together with different O antigens (LPSs).

4. *Lipid Components:* The capsular K polysaccharides were originally thought to be free polysaccharides. It was, however, found in several instances (GOTSCHLICH

et al. 1981; SCHMIDT and JANN 1982) that they are subsituted at the reducing end with a hydrophobic moiety. Today we assume that is the case with all K polysaccharides. The nature of the hydrophobic moiety may serve as a classification parameter.

5. *Genetic Determination of the K Polysaccharides:* All capsular K polysaccharides are determined by chromosomal genes, but not all from the same chromosomal site. Currently two distinct genetic systems are known which direct the biosynthesis of different K antigens. In one of these, two chromosomal locations are operative, one close to the *his* gene cluster and the other close to the *trp* gene (SCHMIDT et al. 1977). The other genetic system comprises a single chromosomal site KpsA, close to *serA* (ØRSKOV and NYMAN 1974; ØRSKOV et al. 1977). Genes characteristic of capsular polysaccharides have been cloned and gene probes can be used for the analysis of the relevant capsule genes (M. ROBERTS et al. 1988; BOULNOIS and ROBERTS, this volume).

6. *Biochemical Classification:* It was found very recently (FINKE et al., 1989) that in certain encapsulated *E. coli* strains the activity of the cytoplasmic enzyme CMP-KDO synthetase is significantly elevated as compared with uncapsulated and rough *E. coli*, whereas this is not the case in other encapsulated strains (see Sect. 6.2). This increased enzyme activity is also found in encapsulated strains the capsular polysaccharides of which do not contain KDO in their repeating unit.

Intergeneric Relationships: Structural and immunochemical analyses showed that several capsular polysaccharides of *E. coli* are very similar to those of other genera. In some cases there is even complete structural identity. The most striking intergeneric relationships are to *Haemophilus influenzae*, *Neisseria mengitidis*, and *Klebsiella*. The finding is also borne out by serologic cross-reactivities of the respective encapsulated bacteria (ROBBINS et al. 1974; ØRSKOV and ØRSKOV this volume). Interestingly, the O8-, O9-, and O20-specific LPSs with which the *Klebsiella*-like capsular poly-

Table 1. Grouping of capsular polysaccharide antigens of *E. coli*

Property	Capsular polysaccharide group	
	I	II
Molecular weight	> 100 kd	< 50 kd
Acidic component	Glucuronic acid Galacturonic acid Pyruvate	Glucuronic acid NeuNAc KDO ManNAcA Phosphate
Expressed below 20 °C	Yes	No
Coexpression with	08, 09, 020	Many O antigens
Lipid at the reducing end	Core-lipid A[a]	Phosphatidic acid
Removal of lipid at pH 5–6/100 °C	No	Yes
Chromosomal determination at (close to)	*rfb(his)*, *rfc(trp)*	*kpsA(serA)*
CMP-KDO synthetase activity activity elevated	No	Yes
Intergeneric relationship with	*Klebsiella*	*H. influenzae*, *N. meningitidis*

[a] This substitution has been verified only with a few polysaccharides (JANN, unpublished; WHITFIELD et al., personal communication)

saccharides are coexpressed are structurally identical with the *Klebsiella* O3, O5, and O4 LPSs, respectively (BJÖRNDAL et al. 1972; CURVALL et al. 1973; JANSSON et al. 1985; PAROLIS et al. 1986; PREHM et al. 1976; RESKE and JANN 1972; VASILIEU and ZAKHAROVA 1976).

Analysis of a great number of *E. coli* capsular K polysaccharides and organization of the data according to the above-described parameters made it possible to divide the *E. coli* capsular polysaccharides (K antigens) into two groups (JANN and JANN 1987) as shown in Table 1. It should, however, be pointed out that, much like any other classification, the grouping shown in Table 1 is not without exceptions.

3 Structures of Capsular K Polysaccharides

3.1 Group I Polysaccharides

Group I capsular polysaccharides can be subdivided according to the absence or presence of amino sugars. Their repeating units are listed in Tables 2 and 3, according to this subdivision. Those group I capsular polysaccharides which were originally termed A antigens generally do not contain amino sugars (Table 2). The same was observed with all capsular polysaccharide antigens of *Klebsiella* (HEIDELBERGER and NIMMICH 1976). The structures of the *E. coli* K28 and K55 antigens were found to be identical with the *Klebsiella* K54 and K5 antigens, respectively (ALTMAN and DUTTON 1985; ANDERSON and PAROLIS 1988).

3.2 Group II Polysaccharides

In contrast to group I capsular polysaccharides, which are a relatively homogeneous group, the capsular polysaccharides of group II differ widely in composition and general structural features. On the basis of their acidic components, there are several subgroups.

3.2.1 Polysaccharides Containing 2-Keto-3-deoxymanno-octonic Acid

2-Keto-3-deoxymanno-octonic acid (KDO) was previously thought to be a characteristic LPS constituent, providing the linkage between lipid A and the carbohydrate moiety of LPSs (JANN and WESTPHAL 1975). We now know that KDO is also a major constituent of a number of capsular polysaccharides. Whereas LPSs contain only about 2%–5% of KDO, this sugar contributes about 40%–60% of the capsular polysaccharide mass. For a review on KDO see UNGER (1981).

The repeating units of KDO-containing group II polysaccharides are shown in Table 4. It is striking that in 9 out of 13 polysaccharides KDO is present together with ribose, mostly as a disaccharide repeating unit. Although the structural differences between the KDO-ribose polysaccharides are small, they suffice to establish capsular

Table 2. Repeating units of amino sugar free (*Klebsiella*-like) group I capsular polysaccharide antigens of *E. coli*

K antigen	Repeating unit	Reference
K27	6)-Glc-(1,3)-β-GlcA-(1,3)-Fuc-(1- 3 1 Gal	JANN et al. (1968)
K28	3)-α-Glc-(1,4)-β-GlcA-(1,4)-α-Fuc-(1- 4 1 β-Gal	ALTMAN and DUTTON (1985)
K29	2)-Man-(1,3)-Glc-(1,3)-β-GlcA-(1,3)-β-Gal-(1- 4 1 Pyr^{4}_{6}-β-Glc-(1,2)-α-Man	CHOY et al. (1975)
K30	2)-Man-(1,3)-Gal-(1- 3 1 β-GlcA-(1,3)-Gal	CHAKRABORTY et al. (1980)
K31	2)-α-Glc-(1,3)-β-Gal-(1,3)-β-GlcA-(1,2)-β-Rha-(1,2)-β-Rha-(1, 4 1 α-Rha	DUTTON et al. (1988)
K34	2)-β-GlcA-(1,4)-β-Gal-(1,3)-β-Gal-(1. 3 1 α-Glc-(1,4)-β-Gal	DUTTON and KUMA-MINTAH (1987)
K37	3)-β-Glc-(1,3)-α-Gal-(1- 4 1 Pyr^{4}_{6}-α-Gal	ANDERSON et al. (1987)
K42	3)-Gal-(1,3)-GalA-(1,2)-Fuc-(1- 2-OAc	JANN et al. (1965)
K55	4)-β-GlcA-(1,4)-β-Glc-(1,3)-β-Man-(1 4 6 Pyr	ANDERSON and PAROLIS (1988)

specificities. O-acetylation, which in most cases is not statistical, contributes largely to antigenic specificity. Thus, the K13, K20, and K23 polysaccharide antigens have the same glycoside structure (VANN and JANN 1979; VANN et al. 1983). O-deacetylation converts the K13 and the K20 antigen into the K23 antigen.

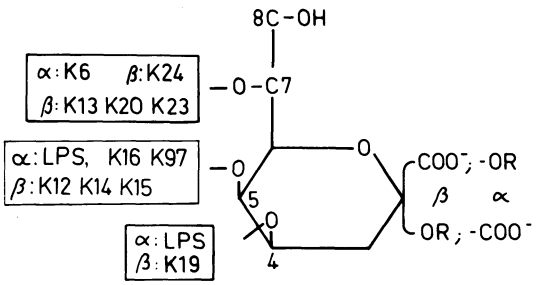
The structure of the K14 polysaccharide, which contains *N*-acetylgalactosamine in addition to KDO, is related to that of the capsular polysaccharide of *N. meningitidis* 29-e, which also consists of these two constituents (BHATTACHARJEE et al. 1978). The K24 antigen has the unusual combination of glycerol phosphate and KDO and therefore also belongs to the group of phosphate-containing group II polysaccharides.

KDO occurs in many linkages, both in pyranosidic and furanosidic ring forms, mostly as β anomer and as well as α anomer. The different substitution patterns of KDO in capsular polysaccharides and in LPSs are indicated in Fig. 1.

A KDO-related 4-deoxy-2-hexulosonic acid was found in the K3 antigen of *E. coli*

Table 3. Repeating units of amino sugar containing group I capsular polysaccharide antigens of *E. coli*

K antigen	Repeating unit	Reference
K8	3)-α-GlcNAc-(1,3)-β-GlcA-(1,3)-β-GalNAc-(1,2)-β-Gal-(1, 4-OAc	PAROLIS and PAROLIS (1988)
K9	3)-β-Gal-(1,3-β-GalNAc-(1,4)-α-Gal-(1,4)-α-NeuNAc-(2, 5-OAc	DUTTON et al. (1987)
K40	4)-β-GlcA-(1,4)-α-GlcNAc-(1,6)-α-GlcNAc-(1- | CO.NH (serine)	DENGLER et al. (1986)
K87	4)-β-Glc-(1,3)-L-FucNAc-(1,3)-GlcNAc-(1,6)-Gal-(1- 4 1 β-Glc	TARCSAY et al. (1971)



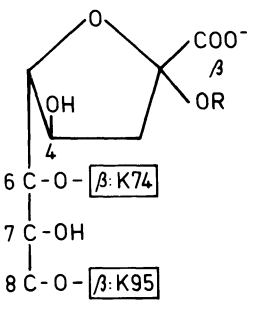


Fig. 1. Survey of KDO linkages found in capsular polysaccharides and in LPSs

Table 4. Repeating units of KOO containing capsular group II polysaccharide antigens of *E. coli*

K antigen	Repeating unit	Reference
K19	3)-β-Rib-(1,4)-β-KDO-(2, 8-OAc	JANN et al. (1988)
K97	2)-β-Rib-(1,5)-β-KDO-(2,	JANN et al. (unpublished)
K14	6)-β-GalNAc-(1,5)-β-KDO-(2, 8-OAc	JANN et al. (1983)
K15	4)-β-GlcNAc-(1,5)-β-KDO-(2,	VANN (personal communication)
K13	3)-β-Rib-(1,7)-β-KDO-(2, 4-OAc	VANN and JANN (1979)
K20	3)-β-Rib-(1,7)-β-KDO-(2, 5-OAc	VANN et al. (1983)
K23	3)-β-Rib-(1,7)-β-KDO-(2,	VANN et al. (1983)
K95	3)-β-Rib-(1,8)-β-KDOf-(2, 7/8-OAc	DENGLER et al. (1985)
K12	3)-α-Rha-(1,2)-α-Rha-(1,5)-β-KDO-(2, 7/8-OAc	SCHMIDT and JANN (1983)
K6	2)-β-Rib-(1,2)-β-Rib-(1,7)-α-KDO-(2,	JENNINGS et al. (1982)
K74	3)-β-Rib-(1,2)-β-Rib-(1,6)-β-KDOf-(2, 2-OAc	AHRENS et al. (1988)
K16	2)-β-Rib-(1,3)-β-Rib-(1,5)-β-KDO-(2, 3-OAc	LENTER et al. (1989, in press)
K24	1)-Gro-(3-P-7-)-β-KDO-(2,	LENTER and JANN (in preparation)

as a substituent of a polysaccharide backbone consisting of 2)-α-L-rhamnosyl-(1,3)-α-L-rhamnosyl-(1,3)-α-L-rhamnosyl repeating units (DENGLER et al. 1988).

3.2.2 Phosphate-Containing Polymers

These capsular antigens are not polysaccharides in the strict sense, but are comparable to the complex teichoic acids of certain gram-positive bacteria (WICKEN and KNOX 1980). The repeating units of these polymers are compiled in Table 5.

The K18, K22, and K100 antigens (RODRIGUEZ et al. 1988b) have the same sequence of constituents as, and a similar structure to, the capsular polysaccharide antigen of *H. influenzae* type b (*Hib*) (ZON and ROBBINS 1983). Since *Hib* causes severe and often lethal infections in small children and since the capsular antigen is an important virulence determinant (see MOXON and KROLL, this volume), attempts at cross-protection were made with the related and serologically cross-reacting *E. coli* K100 polysaccharide (ROBBINS et al. 1975). These endeavors were, however, discontinued after it became known that *E. coli* K100 strains may also be virulent in children.

The K18 antigen differs from the K22 antigen only in partial (ca. 40%) acetylation of the ribose unit. An immunoelectron microscopic study using absorbed anti-K18

Table 5. Repeating units of phosphate-containing group II capsular polysaccharide antigens of *E. coli*[a]

K antigen	Repeating unit	Reference
K2a	4)-α-Gal-(1,2)-Gro-(3-P-	JANN et al. (1980)
K2ab	4)-α-Gal-(1,2)-Gro-(3-P- 2/3-OAc	JANN and SCHMIDT (1980)
K11	4)-β-Glc-(1,4)-α-Glc-(1-P- 3 2 β-Fru	RODRIGUEZ et al. (1989)
K51	3)-α-GlcNAc-(1-P- 4,6-di-OAc 4-OAc	JANN et al. (1985)
K52	3)-α-Gal-(1-P- 2 2 β-Fru-1-OProp	HOFMANN et al. (1985b)
K18	2)-β-Rib-(1-2)-Rit-(5-P- 3-OAc	RODRIGUEZ et al. (1988b)
K22	2)-β-Rib-(1-2)-Rit-(5-P-	RODRIGUEZ et al. (1988b)
K100	3)-β-Rib-(1-2)-Rit-5-P-	RODRIGUEZ et al. (1988b)

[a] For comparison the structure of the capsular antigen of *H. influenzae* b, which is chemically related to the K22 and K100 antigens of *E. coli*, is given as: 3)-β-Rib-(1,1)-Rit-(5-P-)

and anti-K22 antisera revealed that *E. coli* O23:K18:H15, which was used in this work, is in fact a mixture of bacteria expressing exclusively either the acetylated K18 antigen (ca. 40% of the population) or the unacetylated K22 antigen (ca. 60% of the population). Acetylation does not seem to be a stable trait but subject to phase variation (RODRIGUEZ et al., manuscript in preparation). The immunochemical studies on the *E. coli* K18, K22, and K100 and the *H. influenzae* type b capsular antigens were complemented by the elucidation of their secondary structures based on nuclear magnetic resonance analysis and computer-based modeling (RODRIGUEZ et al., manuscript submitted). We found that in spite of their similar primary structures, these antigens formed helices of distinct shapes in which partial structures (epitopes) were expressed differently.

The K11 and K52 antigens contain the rare component D-fructose bound in a very labile linkage to phosphorylated backbones.

3.2.3 Polysaccharides Containing Glucuronic Acid

The repeating units of the five glucuronic acid-containing group II capsular polysaccharides known today are shown in Table 6. The K4 polysaccharide contains the rare constituent D-fructose as a substituent on a main chain, which has the same structure as chondroitin. Fructose is split off from the polysaccharide under very mild

Table 6. Repeating units of hexuronic acid-containing group II capsular polysaccharide antigens of *E. coli*

K antigen	Repeating unit	Reference
K4	4)-β-GlcA-(1,3)-β-GalNAc-(1- 3 2 β-Fru	RODRIGUEZ et al. (1988a)
K5	4)-β-GlcA-(1,4)1α-GlcNAc-(1-	VANN et al. (1981)
K54	3)-β-GlcA-(1,3)-α-Rha-(1- | CO.NH threonine (serine)	HOFMANN et al. (1985a)
K53	4)-β-GlcA-(1,4)-β-Gal(f)-(1- 2-OAc	BAX et al. (1988)
K93	4)-β-GlcA-(1,4)-β-Gal(f)-(1- 5,6-di-OAc	BAX et al. (1988)
K7	3)-β-ManNAcA-(1,4)-β-Glc-(1, 6-O-Ac	TSUI et al. (1982)

acidic conditions with concomitant loss of serologic K4 specificity. Fructose also occurs as a substituent on an acidic polysaccharide in the capsular antigen of *H. influenzae* type e (BRANEFORS-HELANDER et al. 1981). There are variants of *H. influenzae* e which have a capsular polysaccharide lacking the fructosyl substituent. A comparative variation in *E. coli* K4 would lead to bacteria expressing a chondroitin capsule and such variants have not been found (see Sect. 4). Although the sugar composition of the K5 antigen is not at all unusual, this capsular polysaccharide is of special interest with respect to infection immunity, due to its lack in immunogenicity (see below). The K53 and K93 polysaccharides have the same primary structure, the former antigen bearing one and the latter two O-acetyl groups per repeating unit.

In the K7 antigen (Table 6), ManNAcA was found as an acidic component (MAYER 1969). The same constituent is also present in the enterobacterial common antigen (KUHN et al. 1988).

3.2.4 Polysaccharides Containing *N*-Acetylneuraminic Acid

Two group II capsular polysaccharides which contain NeuNAc as the sole constituent are known in *E. coli*. Their repeating units are:

K1 polysaccharide: 8-α-NeuNAc-2 (MCGUIRE and BINKLEY 1964; ØRSKOV et al. 1979);

K92 polysaccharide: 8-α-NeuNAc-2,9-α-NeuNAc-2 (GLODE et al. 1977).

The K1 polysaccharide has the same structure as the capsular antigen of *N. meningitidis* b. A third member of this group of capsular polysaccharides is the capsular antigen of *N. meningitidis* c, which is a poly α-2,9-NeuNAc. Thus the K92 polysaccharide appears as a molecular hybrid between the *E. coli* K1 (*N. meningitidis* type b) and the *N. meningitidis* type c antigens.

Whereas *E. coli* K92 and *N. meningitidis* c are of low virulence, *E. coli* K1 and *N. meningitidis* b, which have the same capsular polysaccharide, are very virulent, causing neonatal meningitis. This may serve as an example of the transgeneric identity of structure function relationships (KASPER et al. 1973).

4 Modification of Capsular Polysaccharides by O-Acetylation

Many capsular polysaccharides are O-acetylated, mostly to a low degree of about one acetyl group per four to eight sugar units. The acetyl groups are in all instances immunodominant parts of serologic epitopes. Although not every repeating group in the polysaccharides is substituted, the acetylation is not random, always the same hydroxyl group of the same sugar constituent being acetylated. Therefore, there are two related types of epitope present in such polysaccharide preparations: one defined by the nonacetylated sugar and the other by its acetylated form. Sometimes the acetyl group is the only chemical difference between otherwise identical structures of distinct polysaccharide antigens. As an example, the *E. coli* K62 antigen is in essence an acetylated K2 antigen (Table 5) with position 2 or 3 of the galactose acetylated (JANN

and SCHMIDT 1980). Because of this close relationship, the two antigens were renamed as K2a and K2ab (LARSEN et al. 1980). Similarly, the *E. coli* K13, K20, and K23 antigens, a group of structurally identical ribosyl-KDO polymers (Table 4), differ in acetylation, with the K13 antigens being substituted in the KDO, the K20 antigen in the ribose, and the K23 antigen not at all. These polysaccharides are, however, considered as distinct K antigens (VANN et al. 1983).

The *E. coli* K1 antigen is a polysialic acid (see Sect. 3.2.4) which in its nonacetylated form is termed the $K1^-$ antigen. Its O-acetylated form (at C7 or C8) is the $K1^+$ antigen (ØRSKOV et al. 1979). The capsular antigen of *N. meningitis*, which is discussed in Sect. 3.2.4, is not acetylated. While the *E. coli* $K1^-$ and the *N. meningitidis* b antigens are practically not immunogenic, the $K1^+$ antigen is slightly immunogenic. Generally, O-acetyl polysaccharides seem to be more immunogenic than their nonacetylated forms.

The *E. coli* K53 and K93 antigens (BAX et al. 1988) differ in degree as well as in position of acetylation. Both polysaccharides are acetylated in every repeating unit, the K53 with one acetyl in position 2 of the galactofuranose unit and the K93 with two acetyl groups in positions 5 and 6 of this sugar unit (Table 6). In both cases the serologic specificities depend entirely on the acetyl groups; the de-O-acetylated polysaccharide reacts only very poorly with the anti-K53 and with the anti-K 93 serum.

The problem of partial acetylation was recently analyzed with the *E. coli* K18 and K22 antigens with immunoelectron microscopy using absorbed sera (RODRIGUEZ et al., in preparation). Both antigens are structurally identical poly-ribosyl-ribitol phosphates, the K18 antigen being 40% acetylated at C3 of the ribose and the K22 not at all (Table 5). We found that all cells of the *E. coli* K22 preparation reacted with an anti-K22 serum but not with an absorbed anti-K18 serum which is specific for the 3-*O*-acetylribose. In contrast, only about 40% of the *E. coli* K18 preparation reacted with the absorbed, acetylribose specific antiserum and about 60% with the K22 serum. This showed clearly that *E. coli* K18 is in fact a mixture of bacteria differing in their capacity to acetylate their capsular antigen. It was not possible to separate the K18 and K22 specific cells such that their selected specificities remained stable. This depicts the situation found in the analysis of *E. coli* K1, with K18 resembling $K1^+$ and K22 resembling $K1^-$ (ØRSKOV et al. 1979). It is assumed that partial acetylation is due to the activity of a specific transacetylase in only a part of the bacterial population. This activity is probably controlled by a genetic element switching the enzyme activity on or off at a constant frequency. The resulting antigenic switch was first analyzed with *Salmonella* LPSs (O antigens) and was termed O antigen form variation, and the chromosomal site responsible for this variation was mapped (see MÄKELÄ and STOCKER 1969).

5 Structural Mimicry and Its Role in Infection

Bacterial surface structures play an important role in the recognition and elimination of virulent bacteria. As pointed out earlier in this chapter, capsules inhibit the bactericidal activity of complement and phagocytes in the initial phase of infection, i.e., before specific anticapsular antibodies are formed which then neutralize the shilding

effect of the respective capsules. Thus, the onset of the immune response normally drastically increases the efficacy of the host defense.

There are, however, situations in which the immune state of the host vis-a-vis an infecting microorganism is virtually never reached and suceptibility to infection is maintained in later stages of an infection. Well-known examples are extraintestinal infections with *E. coli* exhibiting the K1- or K5-specific capsular polysaccharides. These polysaccharides are not or only marginally immunogenic. As a result, the bacteria are still protected by their capsules late in infection and are therefore very virulent.

The structure of the K1 polysaccharide, which is shown in Sect. 3.2.4 is identical with the terminal carbohydrate region (FINNE 1982; HOFFMANN et al. 1982) of the embryonic form of the neural cell adhesion molecule (N-CAM) (EDELMAN 1985). Because of this identity, the immune system is essentially blind to the K1 polysaccharide, letting it pass as a "self-structure" of the body. It is interesting that the adult form of N-CAM does not contain the terminal sequence 2,8-linked α-sialic acids. Thus, the host does not harbor the antigen to which it is tolerant. Rather, its imprint on the immunologic memory prevents antibody formation and thus a specific immune defense.

The nonimmunogenicity of the K5 polysaccharide has a similar reason. Its structure (see Table 6) is identical with that of the first polymeric intermediate in the biosynthesis of heparin (LINDAHL 1972; NAVIA et al. 1983). The tolerance of the host to the K5 polysaccharide is more stringent than that to the K1 polysaccharide, probably because the corresponding host structure is being synthesized in all stages of life. In earlier diagnostic and epidemiologic studies the K5 polysaccharide had escaped detection altogether and *E. coli* K5 strains were classified as K-nontypable. This situation has been improved by the isolation of a K5-specific bacteriophage (GUPTA et al. 1982) and more recently by the production of murine K5-specific hybridoma cells using autoimmune NZB mice (PETERS et al. 1985).

Structural analysis of the capsular K4 antigen (RODRIGUEZ et al. 1988a) revealed that this polysaccharide has the structure of chondroitin, substituted by fructose (see Table 6). The fructose substituent, which was found to be the immunodominant sugar of the K4 polysaccharide, is in such a labile linkage that it is removed at pH 4 at 37 °C. In buffered cultures of *E. coli* O5:K4:H4, the polysaccharide capsule was found to lose its fructose constituent with the conversion to nonimmunogenic chondroitin. Thus, growth of this *E. coli* strain in body compartments of low pH may convert encapsulated bacteria from a form in which they induce and react with specific anticapsular antibodies to a form in which they can no longer do so. Virulence would then depend on different influences which the same bacteria are experiencing in different environments.

The camouflage effect due to capsular structures, which may be considered as a cause of bacterial virulence, was hitherto only found with group II capsular polysaccharides. These capsular polysaccharides are present on *E. coli* strains which cause extraintestinal infections.

6 The Lipid Moiety of Capsular Polysaccharides

The bacterial capsules were thought to consist of free polysaccharides. In the 1970s indications were found for a lipid constituent on A antigens (group I polysaccharides) and in the 1980s the lipid moiety of capsular group II polysaccharides was characterized.

6.1 Lipid Substitution of Group I Polysaccharides

There are several indications that group I polysaccharides are bound to core-lipid A, and that at least a part of the preparation is in fact an LPS with K specificity. From the K40 antigen from *E. coli* 08:K40 (see Table 3), fractions can be obtained which exhibit on SDS-PAGE the ladder-like patterns characteristic of LPSs. This material reacts in immunoblots with anti-K40 serum but not with anti-O8 serum. Chemical analysis showed that the material contained glucuronic acid and *N*-acetylglucosamine (K40 constituents) as well as galactose, glucose, heptose, and KDO (core constituents). Lipid A characteristic fatty acids were also present (JANN and JANN, unpublished). Since after mild acid removal of lipid A the K40 polysaccharide still contained the core constituents, it must be assumed that the linkage of the K polysaccharides to the core is similar to that of the O-specific polysaccharides in the cell wall LPS. *E. coli* with group I capsular polysaccharide may thus have two different LPSs, a neutral one being the O antigen (O8 or O9) and an acidic one being the capsular K antigen. It was found that the same acidic polysaccharide can be the K antigen of one *E. coli* strain and the O-specific polysaccharide moiety of the LPS of another. Thus, the structures of the K87 and O32 antigens, of the K85 and O141 antigens, and of the K9 and O104 antigens are identical (JANN et al. 1971; ØRSKOV et al. 1977; JANN and JANN, unpublished).

6.2 Lipid Substitution of Group II Polysaccharides

As shown in Fig. 2 with the K12 capsular antigen as an example, the hydrophobic moiety of group II polysaccharides is a phosphatidic acid. In all group II polysaccharides which contain KDO in their repeating unit, KDO is the reducing sugar (SCHMIDT and JANN 1982; JANN and JANN, unpublished), whereas in the polysialyl antigens of *E. coli* (K1 and K92) and *N. meningitidis* b and c the reducing end was reported to be *N*-acetylneuraminic acid (GOTSCHLICH et al. 1981). Group II polysaccharides which do not contain KDO in their repeating unit are currently being studied in our laboratory.

The lipid moiety, which can be liberated from the polysaccharides with mild acid, is a 1,2-diacyl-sn3-glycerol phosphate. In the complete antigen its phosphare is engaged in a glycosidic linkage to the reducing sugar (in many cases KDO), which is extremely labile at pH values below 6. The fatty acids may be saturated or unsaturated and represent the normal spectrum of *E. coli* membrane lipids. Interestingly, no β-hydroxymyristic acid (which is characteristic of lipid A) was found in any of the K antigen-bound phospholipids.

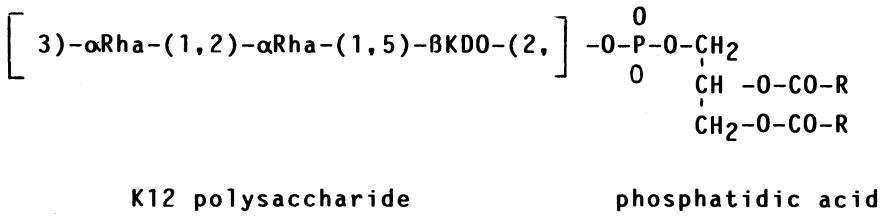


Fig. 2. Structure of the K12 antigen of *E. coli* with the terminal substitution of phosphatidic acid

In all capsular polysaccharides studied, only about 20–50% of the molecules in a preparation are lipid substituted, although this is difficult to evaluate due to the lability of the glycosyl-phosphate linkage. We assume as a working hypothesis that the lipid substituent of the polysaccharide plays a role in the formation and maintenance of the capsule. It is possible that the lipid-substituted polysaccharide fraction is anchored in the bacterial outer membrane by hydrophobic interaction and the unsubstituted polysaccharide is retained by ionic and other interactions. Formation and maintenance of the capsule is then due to the sum of these interactions.

We assume that with all capsular polysaccharides the mode of association to the cell wall and the maintenance of the capsule itself follow the same principle of lipid participation.

7 Biosynthesis of Capsular Polysaccharides

The expression of capsules by the bacterial cell is a very complex process, passing through stages which are associated with different cellular compartments. The activation and interconversion of the sugar constituents take place in the cytoplasm. Subsequent formation of the polysaccharide by transfer of the sugars from their nucleotides to an acceptor is achieved by the coordinated activities of transferases, which are components of or closely associated with the cytoplasmic membrane. The finished polysaccharide is translocated from the cytoplasmic membrane through the periplasmic space and through the outer membrane and then forms the extracellular layer of the capsule.

7.1 Biosynthesis of Capsular Group I Polysaccharides

Little is known about the biosynthesis and surface expression of the *Klebsiella*-like group I *E. coli* capsular polysaccharides. The studies of E. Heath and his colleagues on the biosynthesis of a *Klebsiella* capsular polysaccharide (Troy et al. 1971, 1972) is today still one of the most pertinent and important sources of information. It was shown by these investigators that the capsular polysaccharide is synthesized by polymerization of oligosaccharide repeating units, linked to undecaprenol pyrophosphate. There is evidence that this mechanism is also operative in the biosynthesis of *E. coli* group I capsular polysaccharides (Jann and Jann, unpublished; Whitfield, personal communication).

7.2 Biosynthesis of Capsular Group II Polysaccharides

The studies of F. A. Troy and co-workers (TROY and MCCLOSKEY 1979; WHITFIELD et al. 1984a, b; WHITFIELD and TROY 1984) on the in vitro biosynthesis of the K1 polysaccharide using membrane preparations suggested that undecaprenol phosphate is an essential cofactor in the polymerization, especially as sialylmonophosphoundecaprenol was isolated and evidence was presented for the participation of an oligosialyl lipid in chain elongation (TROY and MCCLOSKEY 1979). Although no such intermediates could be rigorously characterized, the findings were supported by similar results in the biosynthesis of the capsular polysialic acid from *N. meningitidis* b (MASSON and HOLBEIN 1985). Thus, at least with the capsular polysialic acids, participation of undecaprenol-linked oligosaccharides is possible and the mechanism of chain elongation may be the same as with that of a *Klebsiella* capsule and of the LPSs of *Salmonella* of O groups A, B, D, and E (for review see JANN and JANN 1984). The biosynthetic studies on the capsular polysialic acid of *E. coli* concerned only chain elongation. An interesting feature of the polymerization is that the polysialyl chains are terminated to a homogeneous length (about 200 sialyl units) before new chains are initiated. It was possible to use exogenous polysialic acid of relatively low molecular weight (colominic acid) as an external acceptor (WHITFIELD et al. 1984b; WHITFIELD and TROY 1984). Information on the initial reaction and its regulation is not available (see, however, RODRIGUEZ-ASPARICIO et al. 1988).

More recently, we have started in vitro studies on the biosynthesis of the capsular K5 polysaccharide (FINKE and JANN, manuscripts in preparation). As expected from its composition (Table 6), the incorporation of glucuronic acid and of *N*-acetylglucosamine critically depends on the presence of the respective other sugar nucleotide. There is no indication of the intermediary formation of an undecaprenolpyrophospho-derivative of *N*-acetylglucosamine, glucuronic acid, or a disaccharide of both. We therefore assume that the biosynthesis of the K5 polysaccharide proceeds by a stepwise addition of the sugar constituents directly from their nucleotide precursors without the participation of undecaprenol phosphate. This is in contrast to the mechanism of K1 polysaccharide biosynthesis and is reminiscent of the polymerization of the *E. coli* O8 and O9 polysaccharides (JANN and JANN 1984). Also with the biosynthesis of the K5 polysaccharide, the initial reaction is not known. Since the K5 polysaccharide synthesis is not inhibited by tunicamycin in vivo or in vitro (FINKE and JANN, unpublished), the formation of undecaprenolpyrophospho *N*-acetylglucosamine followed by a very rapid elongation of the polysaccharide chain can be ruled out.

The recent finding of KDO as the reducing sugar of the capsular K5 polysaccharide has prompted us to analyze the activity of CMP-KDO synthetase in *E. coli* K5 strains and in an *E. coli* clone expressing the K5 capsule, in comparison to uncapsulated and rough *E. coli*. We found that the activity of this enzyme was significantly elevated in K5 polysaccharide-expressing *E. coli* and even more so in the K5 clone. Temperature regulation, in vitro synthesis, and susceptibility to inhibition were the same for CMP-KDO synthetase and the capsular K5 polysaccharide. This has led us to the assumption that the synthesis and utilization of CMP-KDO are related to the biosynthesis of the capsular K5 polysaccharide, probable by involvement in an initial step (FINKE et al. 1989). The chromosomal site of the gene responsible for enhanced CMP-KDO

synthesis (determining either a second synthetase or a regulator) was located in a region of the K5 expression gene block which directs reactions general for all group II capsular polysaccharides hitherto tested. In accordance with this, elevated CMP-KDO synthetase activity was detected in all *E. coli* bacteria expressing group II capsular polysaccharides, including the polysialic acid K1 antigen (FINKE et al., manuscript submitted). It is possible that KDO is involved in a reaction initiating the biosynthesis of all group II capsular polysaccharides.

8 Surface Expression of Capsular Polysaccharides

The analysis of the translocation of capsular polysaccharides from the cytoplasmic membrane (the site of polymerization) to the bacterial surface has focussed on two major aspects, i.e., its biochemistry and energetics and its topography. Both approaches will be briefly discussed in respect of the capsular group II polysaccharides. The studies on these polysaccharides are most advanced because the temperature control of their expression can be used to great advantage. Growth of the respective bacteria at the capsular restrictive temperature of 18 °C yields phenotypically acapsular forms which start to synthesize and express their capsular polysaccharides upon shift up to the capsular permissive temperature of 37 °C. In addition, the genetics of these polysaccharides has made substantial progress (BOULNOIS and ROBERTS, this volume).

8.1 Biochemistry of Translocation

Using bacteriophage absorption for an analysis of surface-exposed polysaccharide, WHITFIELD et al. (1984a) found that expression of the capsular K1 polysaccharide starts about 20 min after shift from a capsule restrictive to a capsule permissive temperature. Full expression is reached after a further 25–30 min. We have shown that the same is true for the K5 and K12 polysaccharides, with slightly varying expression rates (KRÖNCKE et al., manuscript submitted). The temperature shift experiment has proven to be valuable for the analysis of polysaccharide translocation. Using various inhibitors, it was found that protein synthesis and transmembrane potential are both essential for full expression. Whereas chloramphenicol inhibits the translocation only early after the capsule permissive temperature is reached (induction phase), carbonyl-cyanide-*m*-chlorophenyl hydrazone (CCCP) inhibits the active transport at all times (WHITFIELD et al. 1984a; WHITFIELD and TROY 1984; KRÖNCKE et al., manuscript submitted). Both inhibitions are reversible. Translocation of the K5 and K12 polysaccharides also occurs at pH 8.5, indicating that it is probably not driven by a transmembrane ΔpH.

Recent genetic analysis of the capsular group II polysaccharides (ECHARTI et al. 1983; ROBERTS et al. 1986; SILVER et al. 1984; 1987; BOULNOIS and ROBERTS, this volume) has shown that the genes directing the expression of group II capsules comprise three distinct and adjacent regions: one determining polymerization, flanked by two regions determining modification and surface expression (transport)

functions (BOULNOIS et al. 1987; I. S. ROBERTS et al. 1986, 1988; BOULNOIS and ROBERTS, this volume). Whereas the polymerization genes are characteristic of the respective K polysaccharides and thus different in different *E. coli* strains, the modification and transport genes are identical for many if not all *E. coli* expressing group II capsular polysaccharides. The role of these gene regions in the biosynthesis/surface expression of the capsular polysaccharides was studied with mutagenesis of cloned capsular genes. Mutations in any one region resulted in acapsular mutants. Mutations in the synthesis region abrogated the polymerization and no capsular polysaccharide was found; mutations in the transport region resulted in polysaccharide which could be released from the mutant cells by shock procedures and which contained the phosphatidyl substituent (see GOTSCHLICH et al. 1981; SCHMIDT and JANN 1982); mutations in the modification region resulted in polysaccharide which could only be released from the mutant cells by homogenization and which apparently did not contain the phosphatidyl substituent.

It is possible that modification gene(s) direct a (trans-)lipidation, possibly from a cytoplasmic primary carrier lipid. They may be involved in the translocation of the polysaccharides across the cytoplasmic membrane. The transport genes direct the synthesis of several proteins (SILVER et al. 1987). At least one of these seems to be an outer membrane protein (KRÖNCKE et al., unpublished) not identical with the K protein (PAAKKANEN et al. 1979). It will be interesting to analyze whether the synthesis of any of these proteins is repressed at a capsule restrictive temperature.

Based on these results we postulate as a working hypothesis that the complex process of translocation of the capsular group II polysaccharides includes a (vectorial) modification of the polysaccharides coupled with the concerted action of several proteins, some of which may be periplasmic and at least one of which is an outer membrane protein.

8.2 Topography of Translocation

It is known that capsules cannot be visualized in the elctron microscope directly, because they dehydrate and collapse during preparation. After stabilization with antibody they can, however, be demonstrated well (BAYER, this volume). In our study of the cellular expression of the K5 and K12 polysaccharides (KRÖNCKE et al., manuscript submitted) we have therefore used the respective K specific monoclonal antibodies (PETERS et al. 1985; ABE et al. 1988). The expression kinetics of these polysaccharides in temperature upshift experiments, when measured with this technique, were in principle the same as in the bacteriophage absorption experiments. We observed that the newly exported polysaccharide accumulated in a few distinct patches on the bacterial surface before it distributed laterally and finally formed a continuous capsule (Fig. 3). There was an indication of exit at sites where the cytoplasmic and outer membranes came into close apposition. It is possible that these junction sites mediate transport of many macromolecules across the bacterial cell wall. Nothing is knwon about the transport specificity of these sites.

Electron microscopic analysis of thin sections from modification and transport mutants of clones expressing the K1, K5, and K12 capsules (Sect. 7.2, BOULNOIS and ROBERTS, this volume) revealed that polysaccharide expression was blocked at

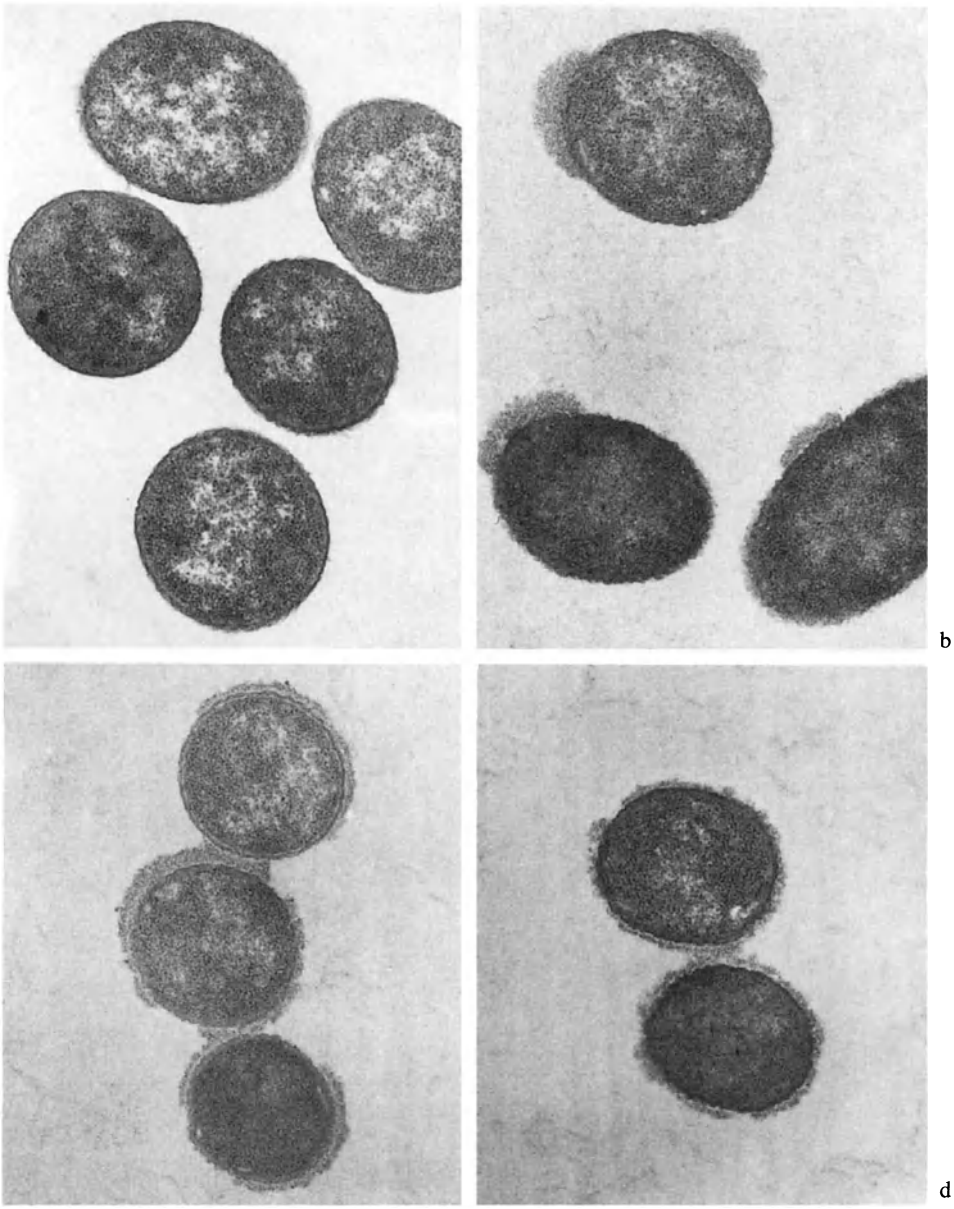
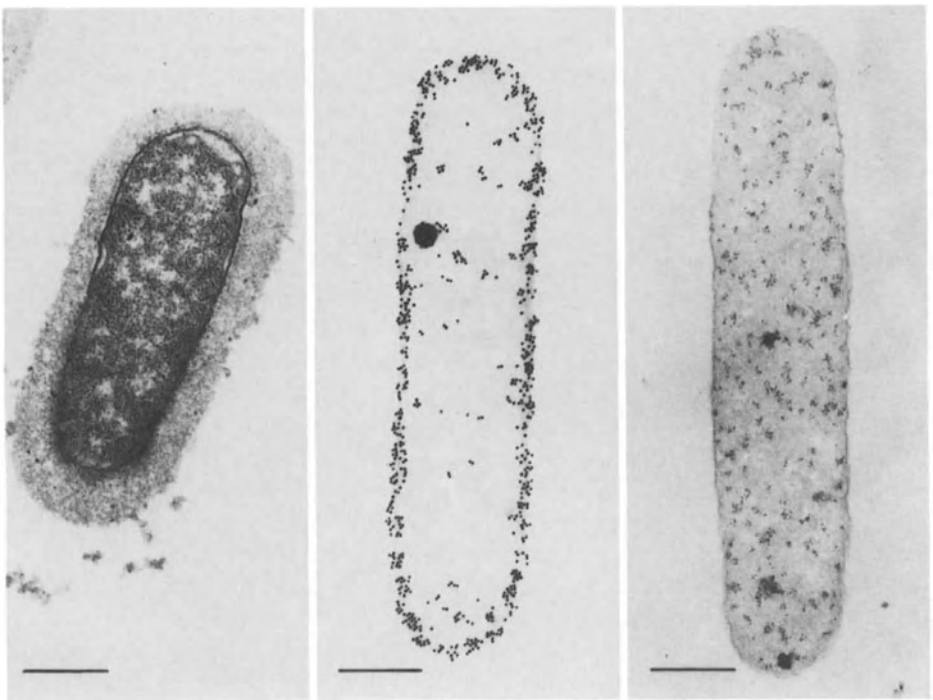


Fig. 3a–d. Electron microscopic analysis, on ultrathin sections from *E. coli* 010:K5, of the surface expression of the capsular K5 polysaccharide after temperature shift from the capsule restrictive temperature of 18 °C to the capsule permissive temperature of 37 °C. Bacteria were taken for sample preparation **a** at the time of temperature shift, **b** 25 min after temperature shift, **c** 30 min after temperature shift, and **d** 50 min after temperature shift. The capsules were stabilized before embedding into Epon with a K5-specific monoclonal antibody



a b c

Fig. 4a–c. Immunogold electron micrographs of ultrathin sections from *E. coli* O10:K5 and from mutants of the K5 clone *E. coli* LE392 (pGB118): *a* wild type *E. coli* O10:K5, **b** transport (block 1) mutant, **c** expression (block 3) mutant. The capsule specific material was demonstrated by stabilization of the capsule before embedding and thin sectioning with a K5-specific monoclonal antibody (**a**), or by treatment of the ultrathin section with a K5-specific monoclonal antibody, followed by incubation with second antibody adsorbed on gold spheres (**b** and **c**). The diameter of the gold spheres was 15 nm in **b** and 5 nm in **c**

different stages (KRÖNCKE et al., manuscript submitted). As shown in Fig. 4 with mutants of a K5 clone as example, in the transport mutant the polysaccharide antigen is associated with the cytoplasmic membrane and in the modification mutant it is present in the cytoplasm. This can be taken as an indication that the polymerization of the group II capsular polysaccharides occurs at the inner face of the cytoplasmic membrane. In the modification mutant of the same K5 clone the translocation of the polysaccharide across the cytoplasmic membrane is apparently blocked, whereas in the transport mutant the polysaccharide can be translocated through the cytoplasmic membrane but subsequent processes of transport through the periplasm and the outer membrane are not operating. Interestingly, electron micrographs of thin sections from *E. coli* wild-type strains in which the translocation of the capsular polysaccharide was inhibited with chloramphenicol or CCCP during temperature shift had the same appearance as shown in Fig. 4c for the modification mutant (KRÖNCKE et al., manuscript submitted). Thus, the cellular location of the K5 and K12 polysaccharides in modification mutants is the same as in bacteria in which the polysaccharide expression was blocked by inhibition of protein synthesis or by

de-energization of the cytoplasmic membrane. This indicates to us that the translocation of capsular polysaccharides is coupled with their modification and that both concerted processes need membrane energy as well as the activity of proteins which seem to be absent in bacteria grown at capsule restrictive temperatures.

9 Conclusions

The capsular polysaccharides (K antigens) of *E. coli* have been extensively studied in several laboratories. These investigations have led to a coherent picture of their general chemical features and to a classification on a biochemical, genetic, and microbiological basis. They have also allowed the definitive structural formulation of most of the *E. coli* capsular polysaccharides known today, as well as the chemical description of intergeneric structure–function relations. The importance of these polysaccharides as virulence determinants could be demonstrated in several instances. Their role in bacterial evasion of host defences is becoming clearer with an intercalation of chemical and biomedical approaches. By the same token, the reduced ability of the host to produce certain capsule specific antibodies (and also immune cells) can be explained on a biochemical basis.

There is still some ambiguity as to the formation and maintenance of a capsule by the acidic polysaccharides. However, the finding that practically all capsular polysaccharides terminate in a lipid has been helpful in the development of strategies to tackle this problem. Today, hypotheses for the explanation of the phenomenon of capsule information and maintenance exist and are used for further study of bacterial capsules.

Although knowledge of the biosynthesis and surface translocation of the capsular polysaccharide is accumulating, many reactions and regulatory processes remain to be elucidated. However, the integration of genetic and biochemical approaches, which has recently become more intense, will ensure an unraveling of the mechanisms of capsule biogenesis.

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The Serology of Capsular Antigens

F. ØRSKOV and I. ØRSKOV

International Escherichia and Klebsiella Centre (WHO), Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark

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1 Introduction

1.1 General Remarks

The serology of capsular antigens will primarily be understood by the present authors as the principles that determine the use of immunologic reactions for the detection of capsular antigens, and the application of such reactions for the identification and subdivision of bacteria. Other aspects of the biology of bacterial capsules will be covered in other chapters of this volume.

Escherichia coli will be mentioned often in this chapter for several obvious reasons; one is that the serology of *E. coli* has been treated extensively, another that many aspects of capsular serology can be described by way of examples from the coli group.

The capsules and capsular antigens covered will to a very large extent chemically belong to the polysaccharides. The few noncarbohydrate capsules described, like the capsular polypeptide in *Bacillus anthracis* (WILLIAMS 1986), have until now had little importance for serology in the above sense.

1.2 When is a Surface Structure a Capsule?

When capsules in bacteria were first reported, e.g., in *Streptococcus pneumoniae*, they were described as structures surrounding the bacterial body that were observed in the light microscope in special preparations, e.g., in Indian ink preparation (DUGUID 1951) or in the quellung test, where addition of antiserum made them easily detectable as haloes around the bacteria (AUSTRIAN 1976; KAUFFMANN 1949; NEUFELD 1902; I. ØRSKOV and ØRSKOV 1984).

When KAUFFMANN and his collaborators in the 1940s investigated surface antigens in *E. coli* they found that one group of this species had typical capsules according to the above definition, i.e., the so-called A antigens (KAUFFMANN 1954). VAHLNE (1945) reported, however, that several varieties or mutants of capsulated strains could be found: some were acapsulate in the true sense of the word, but some had weakly developed capsules that could be detected by serologic methods but were not easily detected in the light microscope. KAUFFMANN showed that not only the above-described A forms had surface structures that interfered with the agglutination reaction of the O antigen, a surface component believed to be a true part of the body of the bacteria, but that other surface antigens, the so-called L antigens and B antigens, could also hinder O agglutination. These last-mentioned antigens were not morphologically detectable as capsules in the light microscope. The many similarities between A, L, and B antigens led KAUFFMANN and VAHLNE (1945) to propose the term K antigens, where K stands for the German word *Kapsel* (capsule). These investigators also used the term envelope antigens for the "noncapsular" K antigens. The term K antigen has since been used by many investigators, also in connection with bacteria outside the Enterobacteriaceae group. The introduction of electron microscopic techniques, and especially of immune electron microscopy, has confirmed that bacteria from many diverse groups show surface material outside the outer membrane of the bacterial body that could be, and sometimes is, called capsule.

In this connection it should also be noted that the lipopolysaccharide (LPS) O antigen also can masquerade as a capsule, and that"O antigen capsule" (GOLDMAN et al. 1984) is now an accepted term (see below).

Thus, there is no simple answer to the question heading this section.

1.3 Capsules and Slime

Many investigators have distinguished between capsular antigens and slime antigens. Slime is understood to be excreted, viscous substances that are not anchored in the bacterial outer membrane. A typical example is the M antigen in Enterobacteriaceae (KAUFFMANN 1954; ØRSKOV et al. 1977). GOEBEL (1963) analyzed the chemistry of the M antigen and gave the substance the name colanic acid. The M antigen can be reckoned as a common antigen in this large group of bacteria. Most Enterobacteriaceae strains have the capacity to produce M antigen, but do not produce easily detectable amounts. Under certain conditions, e.g., growth at low temperatures, many or most Enterobacteriaceae will show variants that grow with production of excessive amounts of M antigen slime. A well-known example is the slime wall formation in *Salmonella paratyphi* B cultures. It is tempting to believe that the slime nature of the M antigen is determined by colanic acid molecules not anchored in the outer membrane.

Our knowledge about the principles of the anchoring of capsular polysaccharide antigens in the outer membrane is not complete. It is known that *E. coli* K antigens of both groups I and II may have anchoring structures on the reducing end of the polysaccharide that bind them to the outer membrane, probably through hydrophobic interaction (see SCHMIDT and JANN 1982; JANN and JANN this volume); it is, however, most likely that not all K molecules in a given strain are bound (see also below under B antigens and O antigen capsule, Sect. 2). KUO et al. (1985) similarly described a phospholipid covalently attached to the capsular polysaccharide of *Haemophilus influenzae* type b. It is also well known that most *Klebsiella* antigens can occur as capsules, i.e., bound to the outer membrane, and/or as slime, i.e., not bound (I. ØRSKOV and ØRSKOV 1984).

Finally it should be noted that even the mildest extraction procedures, like cautious suspension of live *E. coli* organisms in saline, will give a suspension containing considerable amount of not only K but also LPS antigens. Another case showing the very loose binding of some capsular antigens to the bacterial wall is exemplified by the serum agar technique (PETRIE 1932) described below.

Thus we can conclude that it is not possible today to establish precise general definitions that distinguish between slime and capsular antigens.

2 Capsules as Antigens

When injected in animals, or when multiplying in the animal body during disease, capsulated organisms usually give rise to antibody production, and anticapsular antibodies produced in laboratory animals have been widely used for subdivision of bacterial species into groups and types.

We shall here primarily use *E. coli* capsules as a basis for the description of capsular serology, not only because it is the bacterial group with which we are most familiar, but also because different characteristic basic groups of capsules have been found in the *E. coli* group.

SMITH and BRYANT (1927) described certain mucoid *E. coli* strains isolated from diarrheal disease in newborn calves from which nonmucoid varieties could be isolated; antisera produced with the nonmucoid forms would not agglutinate the mucoid cultures, while antisera against the mucoid form agglutinated both mucoid and nonmucoid forms. These strains undoubtedly belonged to the category of capsulated *E. coli* that KAUFFMANN (1944) later described as strains with K antigens of the A antigen variety, now belonging to K antigens of group I (JANN and JANN 1987 and this volume). KAUFFMANN used exclusively an agglutination technique with absorbed and nonabsorbed rabbit antisera and described the following groups of *E. coli* K antigens: L, A, and B. ØRSKOV and ØRSKOV (1978) and ØRSKOV et al. (1977) proposed giving up the L, A, and B labels because the agglutination technique which was used for the assignment would sometimes give equivocal results that were not in agreement with capsular antigen examination using other more precise techniques, primarily immunoprecipitation in gels. ØRSKOV et al. (1977) listed in the above paper 74 polysaccharide K antigens and two proteinaceous K antigens: K88 and K99. The K antigens had numbers between K1 and K103. The authors proposed deleting 29 K numbers, most of them B antigens, that could not be confirmed by chemical or immunochemical methods as serologic entities independent of the LPS O antigen. The number of K antigens could be further reduced because some of them were found to be so closely related that for practical reasons they could be labeled as Ka/Kab or Kab/Kac varieties of one K antigen. In some cases two K antigens were identical and one of the two could be deleted. In several cases of serologic cross-reactions it has been possible by means of chemical analysis to describe the basis for the cross-reaction (see JANN and JANN, this volume).

For K antigen analysis simple saline suspensions of bacterial cultures were heated to 60 °C, centrifuged, and the supernatants run in immunoelectrophoresis. The K antigen containing strains showed an anodic precipitation. One group of K antigen test strains, which contained many of those strains that according to KAUFFMANN had L antigens, contained K molecules that were negatively charged and had great mobility in the agarose gel. Chemical analyses showed that these K antigens had comparatively low molecular weights and that many of them had characters in common with capsular antigens found in the *Neisseria meningitidis* and *H. influenzae* group; the label "group II" was proposed for these K antigens (JANN and JANN 1987 and this volume). Group I (JANN and JANN 1987 and this volume) contains polysaccharide K antigens that did not move far anodically in immunoelectrophoresis. Many of these have chemical traits in common with *Klebsiella* capsular antigens (JANN and JANN 1987). The highly thermostable K antigens, called the A forms by KAUFFMANN (1944), all belong to group I. These K antigens are always found in strains that have the O antigens O8, O9, O20, or O101, while group II K antigens can be found associated with many different O antigens. Genetically the two groups are also different as the genetic locus for group II K antigens is the *kpsA* locus close to the *serA* marker (ØRSKOV and NYMAN 1974; ØRSKOV et al. 1976), far from the *rfb* locus for the polysaccharide structure of group I K antigens and the O antigens.

Most invasive strains from human extraintestinal disease have K antigens from group II; these K antigens are usually not developed at growth temperatures below 20 °C (ØRSKOV et al. 1984).

It has been shown that the chains of group I K polysaccharides may terminate in core lipid A, thus making the differentiation between K antigens of group I and O antigen LPSs difficult (JANN and JANN, this volume). In fact, strains with group I K antigens have two LPSs, one being the respective O antigen and the other the respective K antigen. It should be mentioned that usually O antisera produced with boiled cultures of strains with K antigens of group I do not give high antibody titers against the K antigen, but, as expected, primarily O antibodies against O8, O9, O20, and O101 antigens found in such strains (KNIPSCHILDT 1945). Thus, in those strains with K antigens of group I that have O8 or O9 LPSs, the K antigens do not behave as O antigens even though some molecules are bound to lipid A-core. Future examinations will, hopefully, tell us more about the expression of O and K antigens as immunogenetic entities in these strains. In this connection it is also important to point out that the same polysaccharide may occur as group I K antigen in one strain, in this strain coexpressed with either O8 or O9 LPS O antigen, while in another strain a similar structure is found as a polysaccharide chain of an acidic LPS O antigen, and in this strain it is the O antigen. In Table 1 the results of agglutination tests carried out with such a set of strains are recorded (ØRSKOV and ØRSKOV, in preparation). It is seen that the boiled culture of test strain O46 (O46: K^-) agglutinates to a high titer in an OK serum against the test strain for K49 (O8:K49) but does not react with the O serum of that strain, suggesting that the K49 antigen does not behave like an LPS O antigen but like a K antigen. Thus, even though group I K antigens can be found as polysaccharide moieties of LPSs, as a rule they do not, in boiled cultures, induce high-titered antisera against the K antigens, as already mentioned above. There are few quantitative examinations of the amount of O LPS in group I K strains; however, it is a common experience that bacterial extracts from such strains show only weak or no O precipitates with the appropriate high titered O8 or O9

Table 1. Cross-reaction between O antigen (LPS) O46 and K antigen K49 in bacterial agglutination tests

Antigens	O and OK antisera[a]			
	O — O46	OK — O46	O — K49	OK — K49
Nonheated				
O46[b]	320	2560	< 20	640
K49[c]	80	160	80	640
Heated				
100 °C, 1 h				
O46	1280	1280	< 20	≧ 5120
K49	160	80	≧ 5120	1280
O8	0		≧ 5120	

[a] O and OK antisera were produced by immunization of rabbits with heated (100 °C 2 h) and non-heated culture, respectively;
[b] O antigen test strain O46:H16 (P1c);
[c] K antigen test strain O8:K49:H21 (A180a)

antiserum in immunoelectrophoresis. Thus we do not know much about the quantitative expression of the O antigens (O8, O9, or O20) in strains with well-developed capsules.

Another experimental approach, i.e., silver staining of LPS in sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) according toTSAI and FRASCH (1982), also supports the idea that group I K antigens are not just a second O antigen LPS: Silver stained extracts of group I K strains examined in SDS-PAGE usually contain only one set of rungs corresponding to the O8 or O9 LPS O antigen, indicating that most group I K molecules are not found in the form of classical LPSs. The finding of many similarities between group I K antigen and the acidic O antigens suggests that a close evolutionary connection exists between the two antigenic groups, both determined by genes at the *rfb* locus (JANN and JANN, this volume).

Several K antigens established in the 1950s and 1960s, the so-called B antigens (KNIPSCHILDT 1945), many of them found in strains from diarrheal disease, were cancelled as K antigens by ØRSKOV et al. (1977) because it was not possible to find any independent serologic or biochemical basis for them. The sole basis for their earlier status as K antigens was the inagglutinability of live cultures in the homologous O antiserum; for further details see ØRSKOV et al. (1977). More recent investigations of O111 strains earlier labeled O111:K58 (or O111:B4) have thrown some light on this O inagglutination phenomenon (GOLDMAN et al. 1982, 1984). It was found that O inagglutinable O111 strains produced large amounts of pure polysaccharide chains corresponding to the polysaccharide moiety of the O111 LPS, while mutants or wild-type strains that agglutinated in the homologous O antiserum did not produce such O111 polysaccharide. The two types of O111 molecule with and without the lipid A-core connected could be simply demonstrated in immunoelectrophoresis carried out with detergent in the agarose, and the polysaccharide could be purified by methods that would normally not detach the O-specific polysaccharide from LPS (GOLDMAN et al. 1982). The name O antigen capsule was used for the pure polysaccharide (GOLDMAN et al. 1984).

We do not know how widespread O antigen capsules (free O polysaccharides) are among *E. coli* strains. Preliminary investigations have shown that such polysaccharides are found in several of the strains that were earlier labeled K(B) strains. GOLDMAN et al. (1984) found that O capsules in O111 strains influenced sensitivity to the bactericidal action of normal human serum. Further examination of this phenomenon is in progress. Similar findings of free O antigen side chains were reported earlier by JANN et al. (1975).

We do not know the final explanation for the O inagglutinability phenomenon described above, i.e., why some nonheated cultures are not agglutinated in an antiserum produced by boiled bacteria. By standard serologic techniques it is not possible to show any qualitative difference between anti-LPS antibodies in sera produced with live cultures (or sera) or with boiled cultures. However, the O inagglutinability phenomenon is not an absolute one. Live cultures will agglutinate in O antisera, though with a much reduced titer. Thus, there may be differences in affinity between O antibodies found in O sera and in OK antisera that have not yet been explored. It should also be mentioned here that it has recently been suggested that some difference in O specificity could be detected in LPSs with long and short chains from the same strain (MCCALLUS and NORCROSS 1987). Are O antisera preferentially directed against

short-chained LPSs, while OK sera contain O antibodies which react more effectively with long-chained LPSs?

One conclusion can be drawn even before the answer to this question is known: Inagglutinability in O antiserum should not be used as the sole basis for a general K antigen definition.

3 The Clone Concept and Capsule Serology

When the clone concept was first formulated (F. ØRSKOV et al. 1976), it was primarily based on the observation that certain well-defined *E. coli* serotypes were found frequently among enterotoxigenic strains isolated in widespread geographic locations and that the omnipresence of certain other *E. coli* O:K:H serotypes in extraintestinal diseases could best be explained by a clonal connection between such strains (MYEROWITZ et al. 1977). In these papers it was further stressed that such clones had characteristic O:K:H biotypes, i.e., were characterized by genes situated in many different locations on the chromosome. Since then, several papers have stressed the importance of the clone concept in connection with the examination of different pathogenic bacteria, many of them capsulated: *E. coli* (NIMMICH and ZINGLER 1984; CAUGANT et al. 1985; ACHTMAN and PLUSCHKE 1986), *N. meningitidis* (CAUGANT et al. 1986), *H. influenzae* (PORRAS et al. 1986; ALLAN et al. 1987), and *Staphylococcus aureus* (HOCHKEPPEL et al. 1987). In addition to serotype and biotype, many new phenotypic characters have been introduced for a better clone description, e.g., outer membrane proteins (COMP's) and silver-stained LPS patterns (ACHTMAN et al. 1983) and isoenzyme analysis or electrotyping (ET) (SELANDER and LEVIN 1980). The reader is referred to the original papers for discussions about the best, the most practical, and the most basic foundation for a clone description. From the many deliberations we dare to conclude that today ET (OCHMAN et al. 1983) is probably the optimal starting point for a clone description if the aim is to clarify the evlutionary relationship between bacterial strains. Further, we can conclude from the *E. coli* studies that the overall picture shows a very close connection between ET patterns, OMP patterns, biotypes, and O:K:H serotypes. In this connection it may be necessary to stress that the evaluation of the correlation between antigenic characters and other traits in a given clone must be based upon the optimal description of these traits, i.e., the O:K:H serotype. The precise examination of the capsular antigen is thus a necessity in such studies. It should also be stressed that detailed serologic analyses of varieties within the single K antigens have only been carried out to a very limited extent and that such studies most likely will reveal antigenic subgroups within several K antigens. For clonal studies detailed analysis of antigenic factors within a K antigen may be highly important, as it has been in O antigen analysis (PLUSCHKE et al. 1986).

For studies of pathogenicity and virulence one important fact has emerged from the clonal discussion: only a few highly characteristic phenotypes or clones are pathogenic, and complete serotyping for gram-negative bacteria is one highly reliable method for epidemiologic investigation of such clones and their local and worldwide spread.

4 Methods of Serologic Capsule Detection

4.1 Quellung Test (Neufeld)

The capsule swelling test (quellung) was developed for detection of *S. pneumoniae* (NEUFELD 1902) but has been useful for typing of many other bacteria. The principle is based on the reaction of capsular antibodies and polysaccharide capsules on the surface of bacteria, usually carried out in fluid medium. The capsules, which are not directly visible in the light microscope because of their low refraction, will be visible as a halo upon addition of antiserum. In electron microscopy, where the polysaccharide capsule is not visible without special staining, addition of anticapsular antibody will likewise produce a nice presentation of the capsular substance made noncollapsable by the antigen–antibody reaction (BAYER and THUROW 1977; BAYER, this volume).

The capsular quellung test (Neufeld test) has been used extensively for routine bacterial typing, especially in bacteria where most strains are heavily capsulated, e.g., *S. pneumoniae* (LUND and HENRICHSEN 1978) and *Klebsiella pneumoniae* (I. ØRSKOV and ØRSKOV 1984). The method is cumbersome but gives clear-cut results when high titered antisera are available.

4.2 Immunoprecipitation in Gel

Immunodiffusion techniques in agar or agarose are very useful for capsule antigen determination because of the easy differentiation of the precipitation lines of different surface antigens and their respective antibodies when simple bacterial extracts are examined (HOLMGREN et al. 1969; ØRSKOV and ØRSKOV 1970). For routine typing of capsule antigens the most widespread gelprecipitation technique has probably been the countercurrent technique (FUNG and TILTON 1985). Most capsular polysaccharides are acidic and can be tested by this technique; however, some capsules are neutral, e.g., *S. pneumoniae* capsules of types 7F and 14 (LUND and HENRICHSEN 1978), and will therefore not be moved anodically. Special electrophoretic techniques have been introduced to detect such neutral antigens (LUND and HENRICHSEN 1978).

The serum agar technique first developed by PETRIE (1932) for determination of the capsular polysaccharide in *N. meningitidis* strains has also been used for capsular antigen typing of *E. coli* K1 antigen (BRADSHAW et al. 1971). PETRIE (1932) mentioned in his original article that horse antisera are especially well suited for this technique, and in fact the anti *E. coli* K1 antiserum universally used for this technique has been a horse antiserum from one single horse. KAIJSER (1977) has applied the serum agar technique for general K typing of *E. coli* strains.

Crossed immunoelectrophoresis has been widely used for detailed examination of serologic specificity of capsular antigens and has been especially useful for examinations of cross-reactions between capsular antigens; for examples see the papers by LARSEN et al. (1980) on *E. coli* K2 and VANN et al. (1983) on K13, K20, and K23.

In this context it would be apt to mention the Cetavlon technique (ØRSKOV 1976)

for detection of capsular polysaccharides in bacterial extracts, because it is based on the immunoelectrophoretic principle. In the Cetavlon technique the antiserum used in immunolectrophoresis is replaced by Cetavlon, and any acidic polysaccharide in the examined bacterial extract will precipitate in the agarose. The precipitate looks much like an immunoprecipitate.

This method is very useful for work on species like *E. coli* where (a) established K standard antisera do not cover all existing K antigens and (b) many strains are either acapsulate variants of capsulate forms or clones that do not carry a capsular antigen. When using this method for detection of capsular polysaccharide, it is important to remember that only acidic polysaccharides will be precipitated and that neutral polysaccharides exist (LUND and HENRICHSEN 1978). It is further important to remember that acidic LPSs are common, at least in *E. coli*, and that they will also precipitate with Cetavlon. However, as has been discussed above, it is not easy to draw a sharp line between acidic LPSs and certain acidic *E. coli* K antigens of group I.

4.3 Agglutination

Direct bacterial agglutination is a simple and sensitive technique which is still used for determination of many capsular antigens (see Sect. 9). Results may be difficult to interpret when polyclonal antisera produced with whole bacteria are used, because of the many different antigen–antibody reactions involved.

4.4 Immunofluorescence

Immunofluorescence techniques have been used especially for detection of capsules in clinical specimens like *Neisseriae* (MORELLO et al. 1985) and *H. influenzae* type b (KILIAN 1985).

4.5 Gen Probes

Recently DNA probes derived from the biosynthesis regions of cloned *E. coli* K antigens (K1, K5, and K12) have been used with success for the determination of K antigens; it was suggested that this method for K determination would also be useful in the clinical laboratory in cases where few bacteria are present (ROBERTS et al. 1988).

4.6 Other Methods

It is self-evident that practically any immunologic technique can be used for detection of capsular antigens, e.g., indirect hemagglutination, enzyme-linked immunosorbent assay (ELISA), and complement fixation. The reader is referred to LENETTE et al. (1985).

5 Capsular Antisera

5.1 Polyclonal Antisera

For details about antiserum production the reader is referred to manuals and textbooks and to papers about the different bacterial groups. Briefly it can be said that polysaccharide capsular antigens are stable antigens that most often give rise to useful antisera when injected into laboratory animals. In rabbits, the preferred animal for antiserum production, purified polysaccharides act as haptens, and antisera have therefore usually been produced by injection of whole organisms. The resulting antisera will contain a mixture of IgM and IgG isotypes; investigations of isotypes in generally applied capsular antisera have, however, been carried out in only a few cases.

The phenomenon of immunologic tolerance should be briefly mentioned here because of its special importance for antiserum production with heavily capsulated strains. PERCH (1950) found that variants of heavily capsulated *Klebsiella* strains that produced only moderate-sized capsules were often better immunogens than those that produced large capsules. ØRSKOV (1956) showed that huge capsules or capsular substance from *Klebsiella* type 5 caused immunologic tolerance in rabbits.

Usually a short series of immunizations will give useful antisera (F. ØRSKOV and ØRSKOV 1984). However, experience in recent years in our laboratory has shown that in some cases a prolonged immunization may give rise to better K antisera (unpublished observation). Some capsular antigens are persistently poorly immunogenic in rabbits, e.g., the K1 and K5 antigens of *E. coli*, and useful antisera are not available. An anti-K1 horse antiserum has been used in many laboratories over the past 10 years to remedy this situation (ROBBINS et al. 1973). Capsular phages for K1 (GROSS et al. 1977) and K5 (GUPTA et al. 1982) have been isolated and are widely used for detection of these two capsules.

5.2 Monoclonal Antisera

In recent years monoclonal antibodies have been used for investigations of capsular antigens from a variety of capsulated organisms: *S. aureus* (HOCHKEPPEL et al. 1987), *S. typhi* (TSANG and CHAU 1987), *E. coli* (ABE et al. 1988; FROSCH et al. 1985, 1987; SÖDERSTRÖM et al. 1983), and *N. meningitidis* group B (MORENO et al. 1983).

6 Variation Phenomena

6.1 K^+ to K^- Variation

K^+ to K^- variation, or variation from capsulate no noncapsulate form, is described in many capsulated bacteria and is caused by mutational blocks in capsular poly-

saccharide synthesis. This variation phenomenon was described early because of easily detectable morphologic differences between colonies consisting of capsulated and noncapsulated bacteria. Spontaneous K^+ to K^- mutations are thus often easy to detect on agar plates even though they only occur with a typical low mutation rate frequency. Reversion from K^- to K^+ is rarely observed. KAUFFMANN (1935) described this type of variation in Vi antigen carrying *Salmonella* strains. Some mutant colonies were devoid of Vi antigen while others produced decreased amounts. VAHLNE (1945) described similar mutants in *E. coli* that produced intermediate amounts of K antigen. Seemingly paradoxically such intermediate mutants will often give higher agglutination titers in pure capsular antisera than the original fully capsulated strain, probably because they bind less antibody.

In *S. pneumoniae* the K^+ to K^- variation is called S to R variation (LUND and HENRICHSEN 1978).

6.2 Form Variation

ØRSKOV et al. (1979) described form variation in *E. coli* K1 strains. In the same K1 antigen culture two types of colony were found with different reactivity in a K1 antiserum; one form gave strong agglutination, $K1^+$, the other gave weak agglutination, $K1^-$. Either variant colony would give rise to single colonies among which both $K1^+$ and $K1^-$ forms were represented. The phenomenon can also be observed on serum agar plates where $K1^-$ colonies will give strong haloes and $K1^+$ colonies will show weak haloes when horse antiserum is used. Chemical analysis (see JANN and JANN, this volume) has shown that the $K1^+$ form contains the polyneuraminic capsular polysaccharide in an O-acetylated form (ØRSKOV et al. 1979). Form variation phenomena are most likely more widespread than is commonly recognized.

HOSIETH et al. (1985) have described a high frequency loss of the b capsule in *H. influenzae* which was found to be the consequence of the deletion of part of the chromosome responsible for capsule expression. Even though this type of variation occurred with a frequency of 1×10^{-2} to 3×10^{-2}, similar to the above-described K form variation in *E. coli*, it seems to be one-sided and to lead to an irreversible loss of the b capsule. See also SAUNDERS (1986).

6.3 Influence of Growth Temperature

Vi antigen capsular polysaccharide is not developed in *S. typhi* at 20 °C (FELIX et al. 1934); in contrast, Vi antigen in *Citrobacter freundii* is well developed at 18 °C. ØRSKOV et al. (1984) examined the development of all *E. coli* polysaccharide K antigens at 18 °C and 37 °C. Many K antigens, especially many of those found commonly in extraintestinal disease and belonging to group II (see above and JANN and JANN, this volume), were not developed at 18 °C. Most of the K antigens of group I were expressed at both 18 °C and 37 °C. It should be mentioned that a spontaneous K^- mutant (see above) isolated from the test strain for the K9 antigen (O9:K9:H12 = Bi 316–42), a group I strain, was devoid of K9 antigen at 37 °C but not at 18 °C (unpublished observation).

6.4 Variation with Age of Culture

To our knowledge, variation with age of culture has not been investigated in detail. Overnight cultures are used by most investigators, and it is often assumed that capsules are optimally expressed under such conditions. In *N. gonorrhoeae* the existence of true capsules has been a controversial subject; however, HENDLEY et al. (1981) and recently REIMANN et al. (1988) have been able to detect capsules on *N. gonorrhoeae* bacteria by examination of young cultures. These authors also noted that only a limited number of bacteria in a given sample were capsulated. Future examinations may show whether this phenomenon is related to the age of individual bacteria. EDMONDSON and COOKE (1979), who examined the impact of different growth conditions on the immunogenicity of *Klebsiella* capsules, found that 16-h cultures gave better antisera than 4- to 6-h cultures. This finding does not, however, imply that capsule development is better at 16 h than at 4–6 h; see above on immunologic tolerance.

7 Cross-reacting Capsular Antigens

The capsular polysaccharides are composed of repeating oligosaccharides. As the epitopes are usually determined by narrow areas of such structures, it is not surprising that many cross-reactions between capsular substances have been detected. Understanding of the serologic cross-reactions within the single bacterial groups and between the groups is therefore intimately associated with knowledge about the chemical structure of the antigens. For details see the chapter by JANN and JANN in this volume.

Table 2. Cross-reaction between capsular antigens of different bacterial groups[a]

Cross-reaction	References
Streptococcus pneumoniae — *Streptococci*	AUSTRIAN (1973)
— *E. coli*	HEIDELBERGER et al. (1968, 1985), ROBBINS et al. (1973, 1975)
— *Klebsiella*	HEIDELBERGER et al. (1975, 1978)
Haemophilus influenzae type b — *E. coli* (K100)	ROBBINS et al. (1973), SCHNEERSON et al. (1972)
Neisseria meningitidis group A — *Bacillus pumilus*	ROBBINS et al. (1973)
— *E. coli* (K93)	GUIRGUIS et al. (1985)
Neisseria meningitidis group B — *E. coli* (K1)	GRADOS and EWING (1970)
Neisseria meningitidis group C — *E. coli* (K92)	ROBBINS et al. (1973, 1975)
Neisseria meningitidis group H — *E. coli* (K2)	ADLAM et al. (1985)
Klebsiella — *E. coli*	I. ØRSKOV and F. ØRSKOV (1984)
Salmonella (Vi) — *E. coli*	SZEWCZYK and TAYLOR (1983)

[a] The possible importance for certain infectious diseases of cross-reactions between normally occurring bacterial antigens and the capsular antigens of invasive pathogens is discussed in other chapters of this volume

The reader is further referred to the following papers, where the cross-reactions within certain groups are listed (usually in connection with a presentation of the respective antigenic schemes): *S. pneumoniae* (LUND and HENRICHSEN 1978), *E. coli* (F. ØRSKOV and ØRSKOV 1984) and *Klebsiella* (I. ØRSKOV and ØRSKOV 1984).

Many studies have been published about cross-reactions between capsular antigens of different bacterial groups, and the reader is referred to some of these studies in Table 2. It should, however, be pointed out that systematic studies of cross-reactions between several of these groups have not yet been carried out, e.g., *S. pneumoniae* vs *E. coli*, or even *Klebsiella* vs *E. coli*.

8 Special Capsular Serology

8.1 In the Common Clinical Microbiology Laboratory

Capsular antisera are used in only a few instances for bacterial identification. Pneumococcal antiserum containing antibodies against all 83 capsular types ("Omniserum," Statens Seruminstitut, Denmark) is used in laboratories for identification of *S. pneumoniae* (LUND and HENRICHSEN 1978). *Neisseria meningitidis* isolates are divided into eight groups characterized by capsular polysaccharide antigens of which groups A, B, C, and Y are the most commonly found; they are usually determined by agglutination tests (MORELLO et al. 1985). Grouping sera are commercially available. *Neisseria meningitidis* capsular antigens in body fluids are usually detected by countercurrent immunoelectrophoresis (CIE), latex agglutination, or coagglutination tests. Kits for the two last-mentioned tests are commercially available (MORELLO et al. 1985).

Haemophilus influenzae isolates are divided into six types based on capsular polysaccharide antigens, i.e., groups a, b, c, d, e, and f, which may be determined by agglutination, the capsular quellung test, immunofluorescence microscopy, and CIE. The main use of the last-mentioned three tests is for detection of *H. influenzae* in body fluids and clinical samples. Isolated strains are most easily group determined by slide agglutination (KILIAN 1985).

8.2 In the Specialized Laboratory

Serotyping of capsular antigens is part of the serotyping routine for many bacteria. Space will not allow us to go into detail about typing schemes or how to carry out the antigenic analysis; we shall only refer the reader to the many review papers covering this topic; some are listed below under Sect. 9.

The question how far one should carry the serotyping of capsular antigens is of course highly pertinent and can only be answered in one way: as far as necessary to solve the problem in question. As an example one could mention typing of *E. coli* O group 6 strains from travellers' diarrhea and from urinary tract infections. Entero-

toxic *E. coli* (ETEC) belonging to O6 are commonly found all over the world and very often belong to one serotype, O6:K15:H16. Many investigators will rightly think that O:H typing is sufficient serotyping for such strains, because experience tells us that when ETEC strains are O6:H16 they are also K15 (see Sect. 3). In contrast, O6 strains in urinary tract infections are frequently found in different O:K:H combinations, e.g., O6:K2:H1 and O6:K5:H1, and complete serotyping is therefore necessary for an evaluation of clinical or epidemiologic connections between urinary tract infection and O6 strains.

9 Capsular Antigens of Importance in Human and Veterinary Medicine

9.1 Gram-Positive Cocci

9.1.1 Streptococci

Streptococcus group A (*S. pyogenes*): Most strains produce a capsular substance consisting of hyaluronic acid, which is nonimmunogenic.

Streptococcus group B (*S. agalactiae*): Different polysaccharide capsular antigens, Ia, Ib, II, III, and IV, are the basis for serologic subdivision of this group (HENRICHSEN et al. 1984).

Streptococcus group C: Many strains have hyaluronic acid capsules.

Streptococcus suis, which belongs to group D, has been divided into nine capsular polysaccharide types: 1, 2, 1/2, 3, 4, 5, 6, 7, and 8 (PERCH et al. 1983).

Streptococcus pneumoniae: Serologic subdivision is based on examination in rabbit antisera against 83 capsular polysaccharide antigens. "Omni-serum," which as already mentioned contains antibodies to all 83 types, can be used to make the diagnosis of *S. pneumoniae*. Capsular typing is carried out by the quellung reaction. Most capsules consist of acidic polysaccharides; however, the common disease-causing types 7F and 14 are neutral and cannot be demonstrated by the countercurrent technique unless a special buffer system is used (ANHALT and YU 1975). Two typing schemes, the American and the Danish, were established, but now the Danish has gained universal acceptance (HENRICHSEN 1979). For details on serology and epidemiology see the review by LUND and HENRICHSEN (1978).

Several other streptococcal species, e.g., *S. bovis*, *S. mutans*, and *S. salivarious*, produce extracellular polysaccharides that are immunogenic.

9.1.2 Staphylococci

Polysaccharide capsules are found in clinical isolates of *S. aureus*. A classification scheme based on results from bacterial agglutination and immunoprecipitation of cell extracts by use of polyclonal rabbit sera has been developed by KARAKAWA and VANN (1982) and KARAKAWA et al. (1985). Eleven serologically distinct capsular types have been proposed. Monoclonal antibodies have been used successfully for serologic typing by the latex agglutination technique (FOURNIER et al. 1987).

9.2 Gram-Positive Rods

Bacillus anthracis: The polypeptide (poly-γ-D-glutamate) of *B. anthracis* is the chemical basis for the capsule. Only one serotype has been described. Capsule formation is optimal on special media under 5% CO_2 (DOYLE et al. 1985).

9.3 Gram-Negative Cocci and Coccobacilli

9.3.1 Neisseria meningitidis

The following polysaccharide capsular antigens have been described: A, B, C, W-135, X, Y, Z, 29e, L, H, I, and K. They are well defined chemically (see JENNINGS, this volume). For cross-reactions to other bacterial groups, see below. Medically the capsular types A, B, and C are the most important. For details about diagnostic techniques see MORELLO et al. (1985). Polysaccharide vaccine against groups A and C is available (see JENNINGS, this volume).

9.3.2 Neisseria gonorrhoeae

By electron microscopy HENDLEY et al. (1977) detected capsules in *N. gonorrhoeae* that could be stained with alcian blue, ruthenium red, and hyperimmune serum. They suggested that the capsular substance consisted of polysaccharide. Recently REIMANN et al. (1988) were able to confirm that young cultures of *N. gonorrhoae* in immunoelectromicrographs did show capsular material. Different capsular types have not been described.

9.4 Gram-Negative Rods

9.4.1 Enterobacteriaceae

9.4.1.1 Escherichia coli

A general description of 74 capsular polysaccharide K antigens belonging to two main groups, I and II, has already been given in detail above. The K antigens of *E. coli* are found in many combinations with different O and H antigens and are stable important markers in serologic typing. K typing has been carried out by CIE (SEMJEN et al. 1977; ØRSKOV and ØRSKOV 1978), by coagglutination (DANIELSON et al. 1979), and by the serum agar method (KAIJSER 1977). Monoclonal antisera to some K antigens have been produced — K1 (FROSCH et al. 1987), K5 (BITTER-SUERMANN et al. 1986), K12 (ABE et al. 1988), and K13 (SÖDERSTRÖM et al. 1983) — but they are not yet used for general typing purposes.

9.4.1.2 Salmonella

One capsular antigen, Vi (FELIX et al. 1934), can be found in a few *Salmonella* serotypes: *S. typhi*, *S. paratyphi* C, and *S. dublin*, and is usually determined by slide glutination. Monoclonal diagnostic antisera have been produced (TSANG and CHAU 1987).

9.4.1.3 Citrobacter

Capsular antigens have not been described; it should, however, be remembered that *Citrobacter* strains of O group 29 with Vi antigen have been used for antigen production of antiserum for detection of the Vi antigen of *S. typhi* (EWING 1986).

9.4.1.4 Klebsiella

Seventy-two capsular polysaccharide antigens and the corresponding antisera are the basis for subdivision of *Klebsiella* into serologic types (ØRSKOV 1981; I. ØRSKOV and ØRSKOV 1984). *Klebsiella* K antigens have several traits in common with group I K antigens of *E. coli* (see above). Typing is usually carried out by the capsular quellung test, by the countercurrent technique, or by agglutination (I. ØRSKOV and ØRSKOV 1984). For cross-reactions see below and I. ØRSKOV and ØRSKOV (1984).

9.4.1.5 Enterobacter

Some strains of this group have capsules reacting in *Klebsiella* capsular typing sera (EWING 1986)

9.4.1.6 Serratia

Strains from this group may be capsulated, but capsular typing schemes have not been developed (EWING et al. 1959).

9.4.2 Vibrio

9.4.2.1 Vibrio parahaemolyticus

Fifty-three capsular (K) antigens have been demonstrated (SAKAZAKI et al. 1968).

9.4.3 Haemophilus influenzae

PITTMAN (1931) described six distinct capsular types designated a through f. They are all acidic polysaccharides. For cross-reactions see Table 2 and JANN and JANN (this volume). Medically, strains of capsule type b are most important. Typing is usually carried out by the agglutination, the precipitation, or the quellung test (KILIAN 1985).

9.4.4 Pasteurella

9.4.4.1 Pasteurella multocida

Four capsular polysaccharide antigens, A, B, D, and E, have been described (CARTER 1962).

9.4.4.2 Pasteurella haemolytica

Several capsular types have been described in this group (CARTER 1981). See also ADLAM et al. (1985).

9.4.5 Bacteroides fragilis

A capsular antigen with an unusual structure has been demonstrated in *B. fragilis* (KASPER 1976).

10 Concluding Remarks

Serotyping of capsular antigens is a comparatively simple procedure important for a phenotypic definition of bacterial clones. More detailed serologic examination of capsular antigens is important if we are to gain insight into variation phenomena in these antigens and the influence of such changes on the virulence of the bacteria. More knowledge is needed about the physical state and distribution of the K antigens on the surface, and likewise we lack exact knowledge about the interactions between capsular antigens and LPS O antigens in gram-negative bacteria. The development of monoclonal antibodies and gen probes for the capsular antigens will most likely be helpful in solving these problems and may in the future revolutionize the whole field of capsular serology as defined in this chapter.

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The Role of Bacterial Polysaccharide Capsules as Virulence Factors

E. R. MOXON and J. S. KROLL

1 Introduction

Carbohydrates are universally present on the surface of living cells. On eukaryotic cells, many different carbohydrates are attached as glycoproteins and glycolipids; the oligosaccharide moieties are known to act as receptors and it seems likely that they play an important role in cell-to-cell recognition processes. Polysaccharide capsules, in prokaryotes characteristically composed of repeating oligosaccharides, are found on the surface of many bacteria. These capsules are typically composed of only one polysaccharide and lie outside the outer membrane of gram-negative cells and the peptidoglycan layer of gram-positive cells. In general, individual bacteria do not exhibit variation of these antigens as has been described for the variant glycoproteins of trypanosomes (CROSS 1978). Comprising 99% water, these highly hydrated, polyanionic polysaccharide capsules serve many functions. These include determining access of molecules and ions to the bacterial cell envelope and the cytoplasmic membrane, the promotion of adherence to the surfaces of inanimate objects or living cells and the formation of biofilms and microcolonies (COSTERTON and IRWIN 1981). Among certain gram-positive and gram-negative bacteria, capsules have evolved distinctive structural and functional characteristics which are of cardinal importance in the pathogenesis of infections of animals, plants and insects (SUTHERLAND 1977).

Molecular Infectious Diseases Group, Institute of Molecular Medicine and Oxford University Department of Paediatrics, John Radcliffe Hospital, Oxford, UK

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Capsulate bacteria are responsible for causing some of the most serious invasive infections to which man is susceptible, including septicaemia, meningitis, pneumonia, osteomyelitis, septic arthritis and pyelonephritis. The morbidity and mortality caused by these infections is substantial and the evidence implicating bacterial capsules as major virulence factors is compelling (ROBBINS 1978). Nonetheless, it is important to maintain an appropriate overall perspective concerning the biological role of these polysaccharide antigens. Infectious diseases are essentially an incidental, or perhaps accidental, occurrence in the co-evolution of man and microbe, reflecting the struggle of each to survive (ANDERSON and MAY 1982). Thus, although capsules play a pivotal role in the pathogenesis of invasive infections, it is generally not intuitively obvious how decimation of the host might be of advantage to the infecting bacterium since the outcome of the encounter often threatens to terminate further propagation and dissemination of organisms. For this reason, it seems likely that the fixation of genes for capsule expression within bacterial populations subserves other beneficial functions, pathogenicity presumably representing at least part of the price exacted for this adjunct to bacterial survival. For example, capsules may enhance survival and facilitate spread of bacteria from one host to another by preventing dessication. However, there is a paucity of data which address the many other potential roles of capsules because most of the relevant research has been pragmatic, focussing on their importance in the pathophysiology of disease rather than on ecological principles (COSTERTON et al. 1987). This caveat notwithstanding, it is the aim of this chapter to review some of the general features and mechanisms relevant to the role of capsules in pathogenicity and then to consider in some detail the role of the capsular polysaccharides of *Haemophilus influenzae*. This organism affords a classic example of a bacterium of medical importance in which an understanding of the role of its capsular polysaccharide is of fundamental interest.

2 Mechanisms by Which Capsular Polysaccharides Enhance Virulence

It has been recognised since the early years of this century that bacterial capsules enhance virulence potential. In a classic book on the biology of the pneumococcus, WHITE (1938) writes: ". . . the existence of substances inimical to leucocytic ingestion were apparent to ROSENOW (1907) who by saline extraction or by autolysis of pneumococci obtained substances that inhibited opsonic action. The impedin was without action on the number of migrated leucocytes. The action of the specific capsular polysaccharide is manifested by its enhancing resistance to ingestion by phagocytic cells and complement-mediated killing." This last sentence in particular captures the essence of the phenomenology with which this chapter is concerned.

2.1 Impairment of Phagocytosis

Most capsular polysaccharides are hydrophilic and confer a negative charge on the bacterial cell, characteristics which are intrinsically antiphagocytic in their effect,

though the physical chemistry of this has never been satisfactorily explained. The experimental observations of PONDER (1928) and VAN OSS and GILLMAN (1973) argue that the hydrophilic properties of polysaccharide capsules act by reducing the surface tension at the interface between the phagocytic cell and the bacterium and that this impairs the facility with which phagocytic ingestion occurs. In practice, the measurements needed to test this idea are difficult to make so that this attractive hypothesis has not been further investigated. Considering surface charge, it seems reasonable that contact between capsulate bacteria and phagocytic cells would be compromised since the net negative charge on each would tend to result in mutual repulsion. Whatever mechanisms are involved, it is apparent that phagocytic ingestion of capsulate organisms is very inefficient in the absence of factors which facilitate contact between the bacterium and phagocytic cell and which modify the hydrophilic bacterial surface. In elegant experiments, HORWITZ and SILVERSTEIN (1980) showed that attachment of capsule-deficient *Escherichia coli* organisms to phagocytes, using concanavalin A as a ligand, was sufficient to promote efficient phagocytic ingestion, whereas when capsulate *E. coli* organisms were similarly attached, they were not ingested but multiplied.

2.2 Interactions with Complement

Many host factors can act as opsonins which modify the surface of capsulate bacteria, but C3b – the cleavage product of the third complement component – is of central importance, especially against invasive infections caused by capsulate bacteria (WINKELSTEIN 1981). C3b can be generated by two independent mechanisms, the alternative and classical pathways (Fig. 1), its activation proceeding in three stages: formation or regeneration, amplification and stabilisation. The formation of C3b through the alternative pathway is of particular importance in the non-immune host; in the early phase of invasive infection with capsulate bacteria, specific antibodies are absent and therefore antibody-independent activation of C3 and the deposition of C3b on the bacterial surface is a major source of opsonic activity. There are three potentially significant immune functions which result from the formation of C3b. First, C3b deposited on the bacterial surface can act as a ligand of specific receptors on polymorphonuclear leucocytes or macrophages. Second, activation of C3 triggers a cascade of the biologically active products of the terminal complement sequence, C5–C9, whose fixation on the surface of gram-negative bacteria can cause bacteriolysis. Third, C3b can attach to lymphocyte receptors and promote immunoglobulin secretion, lymphokine production and convert B cells to memory cells.

Although there is normally a steady turnover of C3 to C3b through the alternative pathway, it is insufficient to achieve effective opsonisation. However, when C3b is deposited on the bacterial surface, amplification occurs through the action of additional serum factors (B, D and properdin) which form the alternative pathway amplification convertase C3bBb (Fig. 1). The opsonic potential of C3b depends on how much C3 is activated, how much is fixed to the bacterial surface, the location of C3b on the surface and how accessible the cell-bound C3b is to the phagocytic cell. Capsules have the potential to modulate all of these variables.

Activation of the alternative pathway is triggered by many surface-exposed poly-

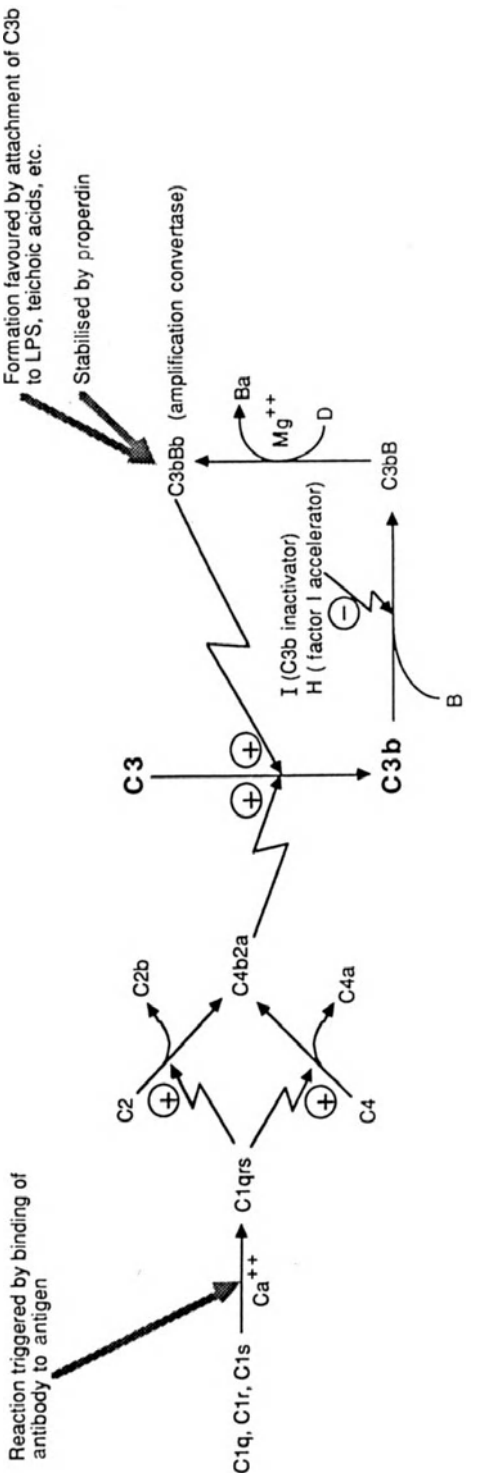

Fig. 1. The complement cascade: early stages of the classical pathway and the alternative pathway of complement activation

saccharides such as the lipopolysaccharides of gram-negative bacteria and the teichoic acids of gram-positive bacteria (MARCUS et al. 1971; WINKELSTEIN and TOMASZ 1978). In contrast, other polysaccharides fail to activate complement because their surfaces favour inactivation of C3b (PANGBURN et al. 1977). The capsules of group B or C meningococci, *E. coli* K1 and group B streptococci containing *N*-acetylneuraminic acid provide a favourable binding site for factor H, a major regulatory protein of the alternative pathway which binds to C3b. The deposition of H-C3b on the bacterial cell surface, instead of the alternative pathway amplification convertase (C3bBb), and the inactivation of C3b by factor I results in breakdown of the amplification loop (Fig. 1 and FEARON 1978). A second mechanism involving structural characteristics of capsular polysaccharides is exemplified by serotypes 7 and 12 pneumococci. These capsules do not bind factor B efficiently and therefore also increase the formation of H-C3b in place of the amplification convertase (BROWN et al. 1983).

Capsular polysaccharides may promote virulence by steric mechanisms. In the case of pneumococci, C3b has been shown to be deposited on the cell wall underneath the capsule such that the capsule may act as a mechanical barrier to recognition of C3b by the phagocytic cells (WINKELSTEIN 1981; BROWN et al. 1983).

Although C3b can be generated via the alternative pathway in the absence of antibodies, activation of C3 through the classical pathway fulfils an additional and cooperative function in host defence against capsulate organisms. The interaction of an immune complex involving bacterial antigens and antibodies results in a conformational change in the Fc portion of antibodies allowing binding and activation of C1 followed sequentially by C4, C2 and C3 so that C3b is deposited on the bacterial cell surface where it can act as an opsonin. The opsonic functions of C3b and immunoglobulins are cooperative since although IgG and IgM possess opsonic activity in their own right, their full potential requires the participation of complement.

The chemical composition of the capsule can be of central importance with respect to the molecular interactions between complement components and the bacterial cell surface in the operation of the classical pathway. Complement proteins, exemplified by C4, are highly polymorphic. Two major loci, A and B, have been described. The product of the C4A gene binds to the mammalian cell surface through the formation of amide bonds whereas that of C4B preferentially establishes an ester linkage (LOW et al. 1984). Up to a quarter of the human population have at least one null A or B gene (HAUPTMANN et al. 1986), and since the chemical structure of the capsular polysaccharide is a determinant of the molecular interactions of this complement component with the bacterial surface, the absence of C4A or C4B genes and the polymorphic variation of C4 molecules are potentially important factors modifying the generation of classical pathway activity (FIELDER et al. 1983).

2.3 Immunogenicity

Many capsular polysaccharides are poor immunogens and are subject to a strong age-related pattern of immune response (ROBBINS 1978). In general, infants aged less than about 2 years possess low or absent concentrations of anticapsular antibodies even following recovery from systemic infection with capsulate bacteria. The reason for the delay in the ability to respond to capsular polysaccharides is not well understood, but it has far-reaching consequences. Young infants — in whom clearance of

invasive bacteria is least efficient — face a period of heightened susceptibility starting from the point at which levels of maternally acquired antibodies decline at about 3 months until the latter half of the 2nd year, when the ability to mount endogenous, type-specific antibodies against polysaccharide antigens matures.

As might be expected, different capsular polysaccharides vary significantly in their capacity to stimulate specific antibody. For example, the capsule polysaccharide of group A meningococcus is exceptional in being a relatively good immunogen (GOLD 1985), while in stark contrast the homopolymer of group B meningococcus possesses characteristics which render it especially inefficient as an immunogen, even in adults (LIFELY et al. 1987). The group B meningococcal polysaccharide is an α- (2–8) linked homopolymer of *N*-acetylneuraminic acid and exemplifies the observation that many polymeric carbohydrate antigens of bacterial origin show structural similarities to cell surface components of the host (FINNE 1982). As a result, immune responses produce antibodies directed against conformational determinants present on the 2° or 3° structure of the polymer, thus providing immunity but preventing autoimmune cross-reactivity (LIFELY et al. 1987). A similar situation also characterises the immune response to type III group B streptococcal polysaccharides (KASPER 1986).

Finally, the shedding of capsule from the bacterial surface is a potential mechanism for jettisoning attached host factors or for nullifying the functional role of circulating host factors such as type-specific antibodies.

3 The Capsular Polysaccharides of *Haemophilus influenzae*

Having reviewed some of the general mechanisms by which bacterial capsules function as virulence factors, the role of the type-specific polysaccharide antigens of *H. in-*

Table 1. Carriage and pathogenicity of *H. influenzae*

Strains	Common nasopharyngeal carriage rates[a]	Principal manifestations of pathogenicity
Capsulated, type b	2%–4%	Meningitis, epiglottitis, pneumonia, suppurative arthritis, osteitis, otitis media, cellulitis, pericarditis. Patients are usually young children. Infections commonly bacteraemic.
Capsulated, other types	1%–2%	Rarely incriminated as pathogens, but all 5 types have sometimes caused diseases as above.
Non-capsulated	50%–80%	Exacerbations of chronic bronchitis, etc. Also otitis media, conjunctivitis, paranasal sinusitis. Patients are commonly adults. Infections rarely bacteraemic.

[a] Carriage rates vary widely between communities and with time. In general they are higher among children than among adults. Probably all humans carry *H. influenzae* in their upper respiratory tracts at least some of the time; and probably most children carry *H. influenzae* type b at some stage during the early years of childhood — though perhaps for only a few months.

fluenzae will now be examined in some detail. A striking feature of the pathogenicity of *H. influenzae* is the singular importance of serotype b strains as a major cause of invasive infections. Table 1 summarises some of the clinically important features relating to carriage and pathogenicity of *H. influenzae*. In 1931, PITTMAN published her seminal paper which reported that *H. influenzae* isolates were either capsulate or capsule deficient, that the former could be divided into six serotypes on the basis of the chemical composition of their polysaccharide capsules (designated a–f), and that most severe infections, including meningitis, were caused by serotype b strains. Subsequently, worldwide clinical experience has consistently shown that type b strains are substantially more pathogenic than any of the five other serotypes in the context of systemic infections and that these invasive infections occur predominantly, although not exclusively, in infants and young children (TURK 1982).

3.1 Structure

The structures of the repeating units of the capsular polysaccharides, established by chemical methods and nuclear magnetic resonance spectroscopy, are shown in Fig. 2.

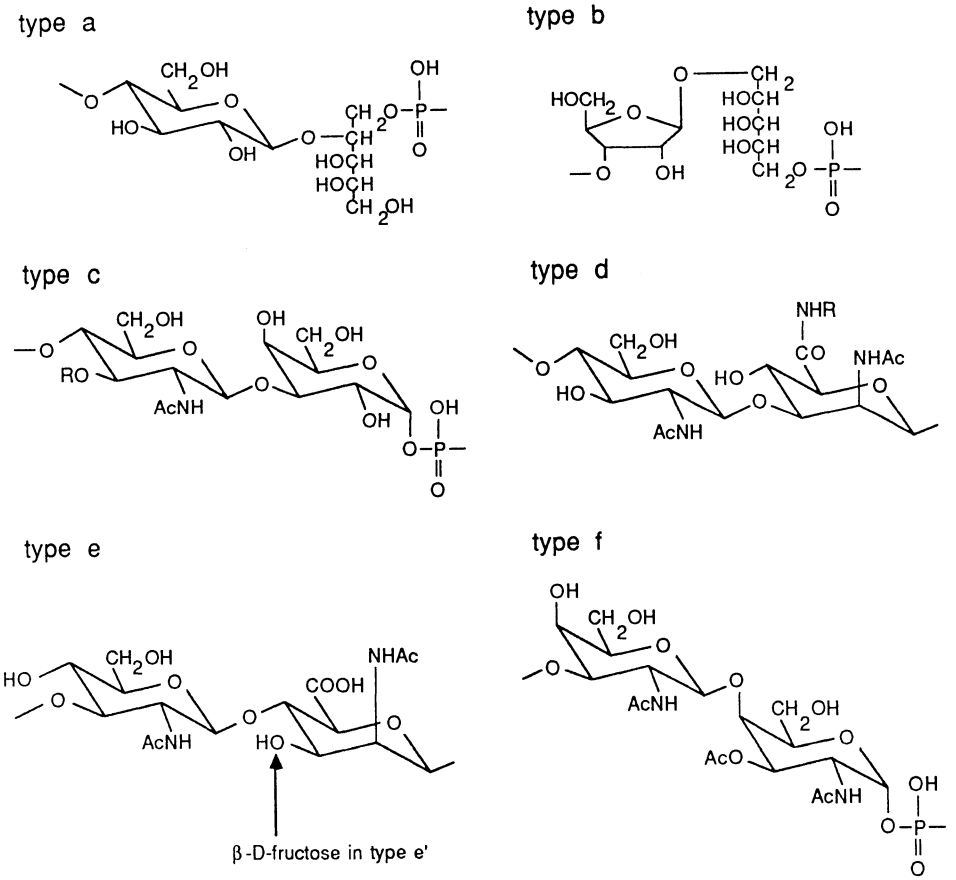

Fig. 2. Structure of capsular polysaccharides of *H. influenzae* (serotypes a through f).

Types d and e contain only glycosidic linkages, but in types a, b, c and f the units are joined through phosphoric diester linkages, i.e. the polymers are strictly teichoic acids rather than polysaccharides. Following common usage, however, all six will continue to be referred to as capsular polysaccharides. Inspection of these structures allows various distinguishing groupings to be made, some of which are reflected in experimental observations of differential pathogenic behaviour of the different serotypes in vitro and in vivo.

All the polysaccharides are acidic — types a, b, c and f because of the phosphoric diester groups, and types d and e through containing carboxylic acid groups. In the first group, types a and b differ from all the rest in containing the five-carbon component ribitol (CRISEL et al. 1975; BYRD et al. 1987). As discussed below, there is marked genetic diversity in the population of type b strains, the capsular type being defined for these purposes by conventional serological means. Such serotypic identity could conceal significant differences in structure (e.g. the different ribosyl–ribitol linkage found in PRP-cross-reactive *E. coli* K100 capsular polysaccharide discussed below). However, structural analysis of the repeating unit from maximally distantly related type b strains shows them to be identical (CRISEL et al. 1975; BRANEFORS-HELANDER 1976).

Type c and f polysaccharides each contain 2-acetamido-2-deoxyhexose, and are O-acetylated (EGAN et al., 1980a, b). In type c strains, the O-acetyl group (R in Fig. 2) is only present in about 80% of the repeating units but appears to be immunodominant (EGAN et al. 1980a). Following O-deacetylation, the polysaccharide does not precipitate with polyclonal antiserum, nor does it apparently inhibit the homologous precipitation of native material. O-acetyl groups are also known to affect immunogenicity of the capsular polysaccharides of *Streptococcus pneumoniae* type 1, *E. coli* K1, and *Neisseria meningitidis* group C (EGAN et al. 1980a).

Type d and e polysaccharides contain 2-acetamido-2-deoxy-D-mannose uronic acid. This is an unusual constituent of bacterial capsular polysaccharide, and its presence in these two serotypes has been proposed to be of phylogenetic significance (TSUI et al. 1981a, b). However, the genetic structure of the *H. influenzae* population revealed by multilocus enzyme electrophoresis (see below) clearly places types d and e at a considerable genetic distance from each other and suggests an alternative explanation of genetic shift for the coincidence of polysaccharide composition. Type d polysaccharide differs from all the rest in carrying amide-linked amino acid substituents (L-serine, L-alanine or L-threonine in non-stoichiometric ratios of approximately 3:3:1) at C6 of the uronic acid. Mild alkaline hydrolysis removes these to leave material which retains substantial cross-reactivity with the native polymer, suggesting a less dominant role for this substitution than for O-acetylation in type c polysaccharide. In about 1% of the repeat units of type e polysaccharide the 3-hydroxyl group is fructosylated. A variant serotype e′ is recognised in which all repeat units carry a side-chain fructose; the biological consequences of this substitution are not known.

Virtually nothing is known of the secondary and tertiary structure of polysaccharide capsules in *H. influenzae*. The type b polysaccharide is apparently assembled within the bacterial cytoplasm at least to a complexity sufficient to gain immunoreactivity to polyclonal antisera, suggested to require at least three subunits (HOOGERHOUT et al. 1987). The site of further polymerisation is not known, nor the process whereby material is transported out of the cell, although this may be energy dependent (KROLL

et al. 1988). The anchoring of polysaccharide to the cell surface is of interest. Studies by KUO et al. (1985) indicate that phospholipid is covalently associated with *H. influenzae* polysaccharide; phospholipid–polysaccharide complexes appear to be generally common among capsulate gram-negative bacteria. GOTSCHLICH et al. (1981) have proposed that a phosphatidic acid residue may be covalently attached to the polysaccharide through a phosphodiester bridge and that this phospholipid moiety may be responsible for anchoring the polysaccharide antigen to the outer membrane of the gram-negative bacterium.

4 Population Biology of Capsulated *Haemophilus influenzae*

The dominance of serotype b strains, amounting to a monopoly in causing invasive systemic infections, is both striking and provocative. How might this remarkable propensity of serotype b strains to cause invasive disease be explained? The simplest explanation would be that the ribosyl–ribitol phosphate polymer (PRP) confers, per se, virulence potential which is denied to strains making any of the other capsular polysaccharides. The most extreme version of other hypotheses argues that the type b capsule is prominent merely as a phenotypic marker which identifies capsulate strains of *H. influenzae* which have evolved a high degree of pathogenicity, in which case the rigidity inherent in serotypic classification of microbes with its implied homogeneity could be very misleading. Stated otherwise, taxonomic classifications tend to obscure the fact that there is extensive variation among natural populations of pathogens; *H. influenzae* is no exception and it is important to know the extent of this variation among natural isolates of capsulate *H. influenzae*. A useful approach is provided by the documentation of polymorphisms characteristic of essential metabolic enzymes which, over time, accumulate selectively neutral mutations which affect charged amino acids. These variant polypeptides can be characterised by gel electrophoresis, and because the genes encoding these enzymes are distributed around the whole chromosome, a study of variation in a sufficiently large group of polypeptides (10–20) constitutes a random sampling of the bacterial genome. Using this technique, known as multilocus enzyme electrophoresis (SELANDER et al. 1986), strains can be ascribed a multilocus enzyme electrotype (ET), an arbitrary numerical label identifying a unique combination of variant alleles at the 10–20 loci under consideration, analogous to a fingerprint (MUSSER et al. 1985). Strains possessing identical ETs are deduced to be of common ancestry (clones) since the likelihood of this arising through convergent evolution is extremely low.

Characterisation of natural isolates of *H. influenzae* by multilocus enzyme electrophoresis has confirmed that the population as a whole exhibits substantial genetic diversity. This diversity can be quantified and a genetic structure to the population defined, as shown pictorially in a dendrogram (Fig. 3). This reveals that the diversity of capsulate *H. influenzae* is as great as that found among *E. coli* (MUSSER et al. 1985), an observation which is perhaps surprising since *H. influenzae* is apparently confined to humans, whereas *E. coli* is ubiquitous in its choice of hosts and is also found in inanimate reservoirs.

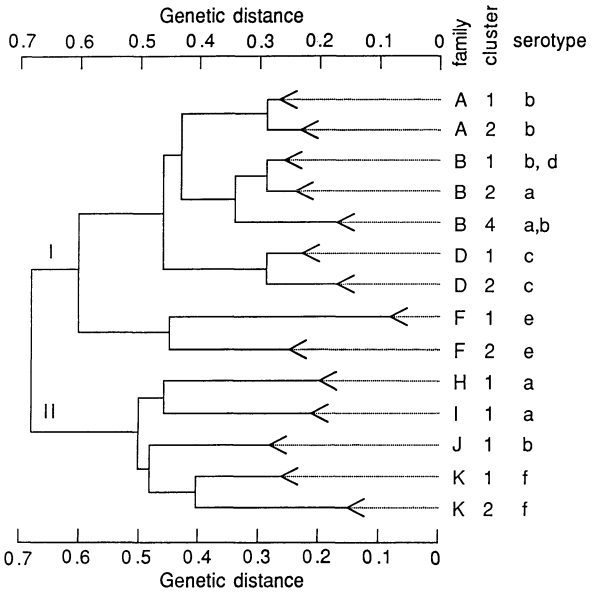

Fig. 3. Dendrogram showing genetic relatedness of different serotypes of capsulate *H. influenzae* based on analysis of 2209 isolates recovered from human hosts worldwid (MUSSER et al. 1988). The scale for genetic distance is given at the *top* and *bottom* of the diagram, and the relatedness of any two clusters is represented by the position of the branch point joining them. Thus, strains in cluster F1 are all related within a genetic distance of 0.08, those in cluster B1 within a genetic distance of 0.25, while strains in these two clusters are only related to each other at a distance of 0.6. The serotypes of *H. influenzae* found in each cluster are shown on the *right* of the dendrogram

Several important facts emerge from these studies. The population structure of natural isolates of capsulate *H. influenzae* of all six serotypes is shown to be clonal (MUSSER et al. 1988). Isolates with the same ET have a worldwide distribution and have been recovered throughout the 40-year period during which serotyping has been practised. This genotypic clustering of strains, as opposed to their more uniform distribution across the spectrum of potential genetic diversity, suggests that there are restraints which limit the extent to which homologous recombination results in the successful introduction of novel genotypes within natural populations of capsulate *H. influenzae* despite the facility with which genetic exchange through DNA transformation can be effected in the laboratory.

The genetic structure apparent in a large collection of capsulate strains of *H. influenzae* is shown in Fig. 3. In this dendrogram, individual isolates with very similar ETs have been grouped into clusters, and the clusters further grouped into families if they lie within a given genetic distance to each other. The 14 numerically dominant clusters are shown. Thus in the A family there are two clusters, A1 and A2, which are common in the population of capsulate strains of *H. influenzae* as a whole, in the B family three common clusters, B1, B2 and B4, and so on.

Table 2 shows the allelic variation at each of the 17 loci studied to generate the ETs for five type b strains in clusters A1, A2, B1, B4 and J1 respectively. These data show that a strain in cluster J1 differs in 11 of 17 alleles when compared with a strain in cluster A1, corresponding to a computed genetic distance of 0.67; in general,

Table 2. Allelic variation among strains of *H. influenzae* serotype b

	Allele at indicated enzyme locus																
	CAK	NSP	PGI	MAE	MDH	G6P	GOT	ADK	6PG	PE1	PE2	LAP	PGM	CAT	GLD	G3P	FUM
A1	9	5	1	5	5	3	5	2	2	4	5	2	4	2	4	2	2
A2	5	5	4	5	5	3	3	2	2	4	5	6	4	2	4	2	2
B1	6	5	5	3	3	6	3	2	2	4	5	3	6	2	4	2	2
B4	6	5	4	3	2	6	1	2	2	4	5	5	4	2	4	2	3
J1	7	7	4	2	5	1	3	1	4	3	5	2	1	3	4	2	2

Allele profiles (ETs) of representative *H. influenzae* type b strains from different clusters. Seventeen enzymes were assayed (MUSSER et al. 1988): carbamylate kinase (CAK), nucleoside phosphorylase (NSP), phosphoglucose isomerase (PGI), malic enzyme (MAE), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), glutamic oxaloacetic transaminase (GOT), adenylate kinase (ADK), 6-phosphogluconate dehydrogenase (6PG), 1 leucyl-alanine peptidase-1 (PE1), leucyl-alanine peptidase-2 (PE2), leucine aminopeptidase (LAP), phosphoglucomutase (PGM), catalase (CAT), glutamate dehydrogenase (GLD), glyceraldehyde-3-phosphate dehydrogenase (G3P) and fumarase (FUM)

strains diverging at a genetic distance of about 0.50–0.70 would show < 70% nucleotide sequence homology and at lower genetic distances the percentage sequence similarity increases in a relatively proportional manner. Evidently, serotype b strains in family J are widely divergent from those in family A. Furthermore, strains in family J can be contrasted with those in family B, which also includes serotype a and serotype d isolates (Table 3).

These data expose the limitations of the taxonomic approach to classifying pathogens. As an example, one can examine the well-established predominance of serotype b strains as a cause of invasive disease. The clonal structure of the population is striking (MUSSER et al. 1988). Clones predict non-random associations between chromosomal genes, including those involved in capsular expression and other determinants of virulence. Such predictions are supported by studies using capsule gene probes to identify informative DNA polymorphisms. Explanations of the greater

Table 3. Allelic variation among selected strains of capsulate *H. influenzae*

	Allele at indicated enzyme locus																
	CAK	NSP	PGI	MAE	MDH	G6P	GOT	ADK	6PG	PE1	PE2	LAP	PGM	CAT	GLD	G3P	FUM
B1 serotype b	6	5	5	3	3	6	3	2	2	4	5	3	6	2	4	2	2
B1 serotype d	6	5	5	3	5	6	3	2	2	4	5	3	6	2	4	2	2
B4 serotype b	6	5	2	3	2	6	1	2	2	4	5	2	4	2	4	2	2
B4 serotype a	6	5	4	3	2	6	1	2	2	4	5	5	4	2	4	2	3
B2 serotype a	6	5	5	3	5	6	3	2	2	4	5	3	1	2	4	2	3
J1 serotype b	7	7	4	2	5	1	3	1	4	3	5	2	1	3	4	2	2

Allele profiles (ETs) of representative *H. influenzae* (serotypes a, b and d) from different clusters. The enzymes assayed are as in Table 2

pathogenicity of serotype b strains must accommodate both the existence of widely divergent serotype b isolates (viz. family A and J) and the close relationship of some serotype b isolates to strains expressing serotype a and d polysaccharides (family B). Since the genes encoding serotype b (or any of the other capsular serotypes) may be in linkage disequilibrium with other virulence determinants, the contribution of capsular polysaccharide to pathogenicity must take into account the clonal characteristics of the population. These arguments emphasize the importance of obtaining a more detailed knowledge of natural variation among the genes for capsule expression and of considering their role in virulence in the context of the extent to which genes for virulence factors other than capsule are important to pathogenicity.

5 The Genetic Basis of Capsule and Virulence Expression in *Haemophilus influenzae* type *b*

The genes for type b capsule are chromosomal and linked in a region designated *cap b* (CATLIN et al. 1972). Cloning of *cap b* (HOISETH et al. 1986) from strain Eagan, a representative, virulent and well-characterised type b isolate from family A (Fig. 3), has shown that it contains a duplication of genes involved in capsule production. Each repeat consists of approximately 17 kb of DNA organised in a directly repeated configuration. An analysis of clinical isolates obtained from all over the world and spanning 40 years has shown that this duplicated configuration of *cap* is characteristic of most (98%) type b isolates examined (ALLAN et al. 1987).

Genetic evidence supporting a crucial role for serotype b capsule in virulence was obtained through experiments involving DNA transformation (MOXON and VAUGHN 1981). Following intranasal inoculation of infant rats with strain Rd, a spontaneous, capsule-deficient derivative of a serotype d nasopharyngeal isolate, invasive infection did not develop; an inoculum of greater than 10^6 of these organisms failed to establish a reproducible and durable bacteraemia following intraperitoneal or intravenous challenge. In contrast, an inoculum of less than ten type b organisms can be sufficient to establish bacteraemia and meningitis. When strain Rd was transformed using whole cell genomic DNA obtained from strain Eagan, type b transformants were obtained which resulted from two distinct recombinational events, the explanation for which is as follows. The organisation of the 17-kb repeats found in most serotype b isolates is not a tandem head-to-tail arrangement but involves their separation by a small (ca. 1 kb) stretch of non-repeated DNA designated the bridge region. The bridge region contains a gene (*bexA*) which is essential for some function which results in the type b capsular polysaccharide reaching the surface of the bacterial cell (KROLL et al. 1988). Type b transformants of Rd were found to have arisen either through acquisition of both 17-kb repeats and the bridge region (as in Rd:b^+:01), or alternatively only one 17-kb repeat plus the bridge region, as in Rd:b^+:02 (Fig. 4).

Analysis of the phenotypes of Rd:b^+:01 and 02 revealed crucial differences. First, Rd:b^+:01 (which has the duplication) makes about twice as much PRP as Rd:b^+:02. Second, Rd:b^+:01 undergoes spontaneous, high frequency loss of capsule production owing to *rec*-dependent recombination between the homologous 17-kb repeats

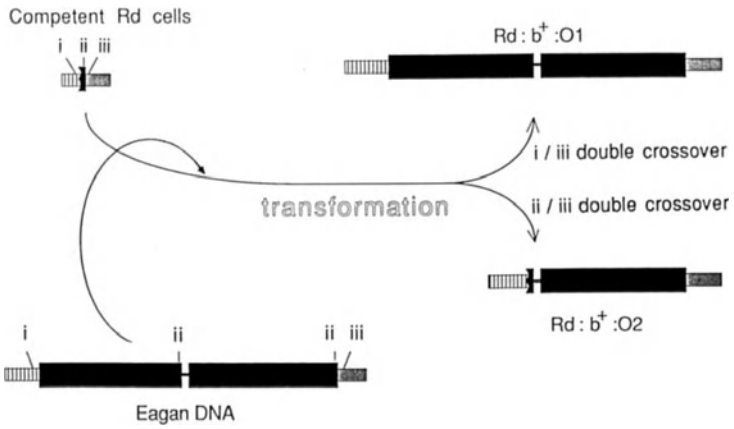

Fig. 4. Schematic representation of the transformation of competent, capsule-deficient strain Rd cells using DNA obtained from serotype b strain Eagan as the donor. The residual *cap* locus found in Rd has three segments of DNA (i, ii and iii) which have sequence homology to DNA in the *cap* locus of the serotype bdonor, strain Eagan, as shown. As segment ii is present in duplicate in Eagan, homologous recombination (double crossover) can result in two outcomes in which *cap* DNA from the donor (Eagan) is integrated into the target locus in Rd to give capsulate transformants. That involving segments i and iii yields the duplicated *cap* locus of Rd:b$^+$:01, while crossover between ii and iii yields the single-copy locus of Rd:b$^+$:02

(HOISETH et al. 1986) which necessarily involves loss of the bridge region (containing the essential *bexA* gene) and reduction of the duplication to a single copy. Rd:b$^+$:02 shows no such instability of the capsular phenotype since it lacks any substantial duplication within *cap b* (KROLL and MOXON 1988). Third, and surprisingly, the lipopolysaccharide (LPS) of Rd:b$^+$:02 is altered, as evidenced by reduction in galactose and loss of an epitope detected by reactivity with monoclonal antibodies (ZWAHLEN et al. 1986). When inoculated into rats, both transformants produced bacteraemia and meningitis, but Rd:b$^+$:02 was decisively more virulent. Since the more virulent Rd:b$^+$:02 produces less capsule than Rd:b$^+$:01, the data are suggestive that LPS modulates virulence expression and that the genetic basis of the altered LPS expression would seem to involve DNA within or linked to *cap b*. The independent contribution of the different LPS molecules was shown by experimental infection of rats using the capsule-deficient 01 and 02 variants. The 02 strain was significantly more virulent than the 01 (ZWAHLEN et al. 1986).

Further analysis of Rd:b$^+$:02 using in vitro mutagenesis to characterise the novel DNA acquired through transformation has revealed the following additional data (Fig. 5). Deletions at positions 1, 2 and 3 result in loss of b capsule production and virulence. In contrast, a deletion at position 4 alters the colonial phenotype (loss of iridescence) but these mutants are fully virulent (ELY et al. 1989). This variant expresses type b capsule as assessed by ELISA and binds a FITC-labelled anti-PRP antibody, although a reduction in the amount of PRP was found. Mutations 3 and 4 mapped to DNA in *cap b* which is found only in serotype b strains. In contrast, mutations 1 and 2 have been mapped to DNA which is found in more than one serotype (HOISETH et al. 1985). Our current thinking is that mutations 1 and 2 lie within a region of *cap b* which is responsible for capsule export or some related function,

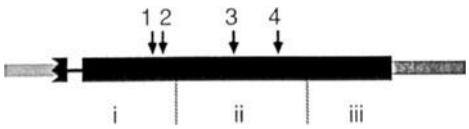

Fig. 5. Diagrammatic representation of the *cap* locus of Rd:b^+:02. Regions i, ii and iii contain DNA hybridising to the chromosome of all serotypes, type b only, and all serotypes respectively. The effects of point mutations or deletions at sites 1, 2, 3 and 4 are described in the text

whereas mutation 3 interrupts one of the genes for the biosynthesis of PRP. Attempts to introduce mutations in the remaining region to the right of mutation 4 have not yet proved successful.

Thus far, the data deal exclusively with the role of genes within or linked to *cap b*. To what extent do these genes mediate the greater virulence potential of type b strains? To investigate this question further, transformants were produced using DNA obtained from strains representative of all six serotypes (ZWAHLEN et al., 1989). These transformants had identical LPS and outer membrane proteins and physical mapping of the serotype a and serotype d transformants has verified that differences within *cap* are confined to a serotype specific region which is flanked by DNA common to all capsulate strains (DHIR et al., unpublished). Following intranasal challenge of rats, only the serotype a and serotype b transformants consistently caused bacteraemia, but the serotype b transformant was significantly more virulent (ZWAHLEN et al. 1989).

In summary, these experiments provide strong evidence that strains differing in their capsular polysaccharides have distinctly different pathogenic potential and that genes within or closely linked to *cap b* confer unique virulence potential upon *H. influenzae*. It is also evident that differences in the structure of LPS can modulate the virulence expression of capsular polysaccharides. Whether or not the genes which mediate the greater virulence of type b strains subserve functions unique to type b capsule expression must await detailed analysis of the genes contained within or closely linked to *cap b*.

6 The Role of Type b Capsule in the Pathogenesis of Meningitis

Haemophilus influenzae meningitis represents a challenging paradigm where the application of genetics to create isogenic strains and the use of a biologically relevant experimental model infection have made it possible to dissect the molecular basis of the host-microbial relationship as it relates to the pathogenesis of a clinically important disease. As described in Sect. 5, using transformants expressing the several different capsules, type b organisms were found to be significantly more likely to result in meningitis. In this section, an attempt will be made to summarise the biological basis for this enhanced virulence of organisms expressing the type b capsular polysaccharide and its implications in the pathogenesis of meningitis.

A simplified summary of the sequential steps in the pathogenesis of meningitis is presented in Fig. 6. The determinants of these events involve complex interactions

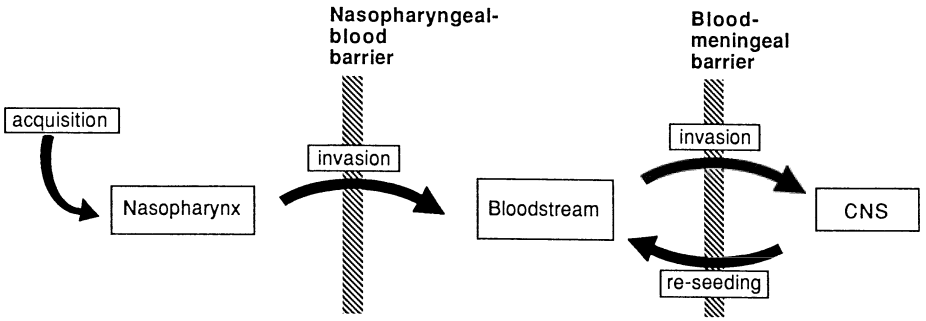

Fig. 6. Pathogenetic sequence for bacterial meningitis caused by *H. influenzae* type b

in which mutuality is of the essence, since both bacterium and host exhibit extensive and varied polymorphisms among the genes that mediate the encounter. As a consequence of this versatile and dynamic relationship, *H. influenzae* may display a spectrum of pathogenic potential for its host which ranges from the negligible to the lethal.

6.1 Colonisation of Respiratory Tract

The extent to which capsule influences the fate of organisms in the respiratory tract (prior to their translocation into the blood) has not been decisively resolved despite extensive clinical, epidemiological and experimental data. Studies on the carriage of *H. influenzae* indicate that its presence in the nasopharynx is extremely common (Table 1) but that most isolates obtained from cultures of the respiratory tract lack capsule. Thus, the prevalence and efficient survival of strains lacking capsule is perhaps the most impressive fact to emerge from such studies (Moxon 1986). Among the approximately 5% of carriers with capsulate strains, half are found to be colonised with type b strains. These findings are consistent with in vitro studies of the adherence of *H. influenzae* to epithelial cells from which no decisive role for capsule in promoting attachment emerges (Anderson et al. 1985; Lipuma and Gilsdorf 1987). The fact that the majority of infections which occur through the contiguous spread of *H. influenzae* (otitis media, sinusitis and pneumonia) are caused by capsule-deficient strains also relegates the relative importance of the role of capsule in survival and pathogenicity within the respiratory tract. In experiments using mice, Toews et al. (1985) showed that pulmonary clearance of a serotype b strain was identical to that of its capsule-deficient variant. An exception to these tentative generalisations may be epiglottitis, where pathogenesis may involve contiguous spread of type b organisms within the respiratory tract. It is virtually always caused by serotype b strains and represents one of the most remarkable examples of tropism found among infections of humans, yet the biological basis for this specificity remains a mystery.

6.2 Cellular Invasion

Does capsule play a role in mediating cellular invasiveness, i.e. the characteristic(s) of *H. influenzae* which endow it with the potential to penetrate a mucosal barrier and

then to survive in the new location? Capsule might be involved in either or both of these events; for example, invasion of the bloodstream involves the penetration of organisms across both the nasopharyngeal epithelium and vascular endothelium and the subsequent survival of the organism within the bloodstream. There are no data which bear upon the role of *H. influenzae* capsule in determining its penetration of nasopharyngeal or endothelial cells. In experimental studies of the rat, the rapidity (a few minutes) with which type b organisms reach the blood following atraumatic intranasal challenge suggests direct entry through vascular endothelium of the nasopharyngeal mucosae (RUBIN and MOXON 1983). How a non-motile organism achieves this rapid translocation is puzzling. *N. meningitidis* becomes internalised by nasopharyngeal epithelial cells (STEPHENS et al. 1983) but no comparable events have been observed for *H. influenzae* type b. Perhaps *H. influenzae* is taken up within phagocytic cells, say tissue macrophages, and by virtue of its capsule is able to survive intracellularly and enter the lumen of blood vessels by diapedesis. Irrespective of the mechanism, the extent to which capsule is or is not involved in translocation of *H. influenzae* across cellular barriers is not understood.

6.3 Intravascular Survival

In contrast, the role of type b capsule in mediating survival of *H. influenzae* in the bloodstream has proved easier to study. In *in vitro* studies, serum complement-mediated bactericidal and opsonic activities were examined using isogenic capsular transformants (a–f) of an unencapsulated *H. influenzae*. The organisms were incubated in pooled sera from adults or infants (age 3 months to 2 years) at a variety of serum concentrations and for varying lengths of time. With respect to serum bactericidal activity in both adult and infant serum pools, both the unencapsulated organism and the type f transformant were relatively susceptible, the type a and e transformants were relatively resistant, and the type b, c and d transformants were intermediate. With respect to serum opsonic activity, the unencapsulated organism and the type f transformant were relatively susceptible, the type a, b and e transformants were relatively resistant and the type c and d transformants were intermediate. Thus, although the type b capsule endows the organism with the ability to resist the bactericidal and opsonic effects of complement, this property is not unique to the type b capsule but rather is shared to some degree with some of the other capsules as well (SWIFT et al. 1988). In vivo, experimental infection of rats showed that capsulate type b strains of *H. influenzae* were cleared much less efficiently than those lacking capsule (WELLER et al. 1977). Taken together, these experiments indicate that type b capsule confers resistance to the opsonophagocytic mechanisms of the host (phagocytic cells, complement components and serum antibodies) but do not identify the specific determinants underlying the greater virulence potential of the serotype b transformant.

In the non-immune host, lacking type-specific serum antibodies, phagocytic ingestion of type b organisms is inefficient. However, *H. influenzae*, whether capsulate or not, is able to activate the alternative pathway. The surface component(s) responsible for complement activation is not known, but type b capsule is neither necessary nor sufficient (QUINN et al. 1977). Whatever the mechanism(s), complement-dependent killing of type b organisms appears to be antibody dependent. Guinea pig serum

deficient in C4 and lacking antibodies to type b capsule did not kill type b organisms although C3 was consumed. However, after addition of human IgG, killing was readily demonstrated (TARR et al. 1982). These experiments suggested an obligatory requirement for type-specific antibodies for killing through the alternative pathway. These observations were confirmed and extended by STEELE et al. (1984) using affinity-purified anticapsular IgG antibodies specific for type b capsule and agammaglobulinaemic serum, both of human origin. Alternative pathway killing occurred only in the presence of antibodies. With C2-deficient human serum or agammaglobulinaemic serum treated with Mg-EGTA (to block the classical pathway), complement was not consumed.

In summary, there is evidence that antibodies to the capsule are required for lysis of *H. influenzae* type b via the alternative pathway in vitro. In contrast, antibodies to either capsule or cell envelope antigens promote killing through the classical pathway. Is complement-mediated bacteriolysis important in vivo? In experimental infection of rats, capsulate strains were cleared much less efficiently than those lacking capsule, but a number of findings (summarised by MOXON and WINKELSTEIN 1988) suggest that phagocytosis – especially by the fixed macrophages of the reticulo-endothelial system — is the crucial mechanism of clearance. In humans, hereditary or acquired deficiencies of the late complement components (C5–9) do not lead to unusual susceptibility to infection with *H. influenzae*, whereas these same deficiencies are associated with predisposition to infection with *Neisseria* (ALPER et al. 1970). However, a critical role of serum antibodies specific for the type b capsule in enhancing bloodstream clearance of *H. influenzae* has been established both in humans (ANDERSON et al. 1972) and through experimental infections of animals (GIGLIOTTI and INSEL 1983).

We can now summarise the clinical and experimental observations on the role of type b capsule in the pathogenesis of meningitis. The capsule confers attributes which are necessary, but not sufficient, to enable *H. influenzae* to survive and replicate within the blood with extreme efficiency. Just how efficiently the organism can accomplish this is highlighted by the following observations. It has been shown that bacteraemia resulting from intranasal challenge with type b organisms may result from the successful survival and proliferation of a single organism (MOXON and MURPHY 1978). In these circumstances, the entire progeny of organisms is clonal and if the magnitude of bacteraemia attained within 15–24 h has reached 10^6 per ml (about 18 generations), a simple calculation indicates that the effective mean generation time in vivo (replication minus clearance) should be in the order of 50–80 min. This figure is consistent with the observations of RUBIN et al. (1985), who made serial measurements of the density of organisms in the blood of rats during the experimental phase of bacterial proliferation. Observations on the time-course and the bacterial numbers which occur in bacteraemic infections of humans indicate that these are consistent with the experimental observations (SANTOSHAM and MOXON 1977). The evidence that bacterial replication in the blood is an essential event in the pathogenesis of invasive disease caused by *H. influenzae* is in contrast with the observations relating to *S. pneumoniae*, as shown in the following experiment. If rats are challenged intravenously with small numbers (< 10) of *H. influenzae* type b or *S. pneumoniae*, sustained bacteraemia develops only in the former case; *S. pneumoniae* is cleared. In contrast, following intraperitoneal inoculation, lethal infection occurs with either organism (RUBIN et

al. 1985). The interpretation of these experiments is simple enough, but far-reaching in its implications. *H. influenzae* replicates in the blood whereas, in contrast, the bacteraemia of *S. pneumoniae* comes about as a result of extravascular replication followed by secondary seeding of the blood. In either case, when the rate of replication exceeds the host's ability to clear the organism, bacteraemia results. Only a select group of organisms have the capacity to cause sustained, high level bacteraemia, the capsulate state being one of the essential requirements. Apparently, a critical product of the numbers of bacteria and duration of bacteraemia is required to damage the structural integrity of the tight junctions which protect against, or limit, the entry of bacteria into the cerebrospinal fluid (CSF) whether via the choroid plexus or by other cellular (e.g. endothelial cell–meningeal) interfaces. Once in the CSF, infection is established if bacterial multiplication exceeds clearance. Contrary to common teaching, clearance of organisms from the CSF is not negligible. PETERSDORF and LUTTRELL (1962) demonstrated this elegantly by intracisternal inoculation of dogs with virulent capsulate pneumococci. Only when the inoculum exceeded 1000 organisms did meningitis occur in the majority. The evidence from experimental and clinical observations is consistent with the following general view of the pathogenesis of bacterial meningitis. The key elements are the capsulate phenotype, which determines the potential for high density bacteraemia, disruption of the blood–CSF barrier (tight junctions) and entry of bacteria into the CSF. The CSF is a propitious and permissive environment for replication of capsulate bacteria owing to the relative absence of host factors mediating efficient opsonophagocytosis. A prediction of the general hypothesis is that there is no essential requirement for meningeal pathogens to exhibit CNS tropism. Any bacterial species capable of causing sustained bacteraemia of a magnitude which disrupts the integrity of the blood–CSF barrier and exceeds the modest capacity of the host to clear bacteria from the CSF is a potential meningeal pathogen. In fact, few organisms possess these attributes.

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The Biologic Significance of Bacterial Encapsulation

A. S. Cross

1 Introduction

Capsules are important determinants of the behavior of bacteria within the animal host. To survive within the host, bacteria must be able to evade a diverse array of defense mechanisms that include complement-mediated bacteriolysis, uptake and killing by phagocytes as well as cell-mediated immune mechanisms. In this chapter the manner in which bacterial capsules, particularly those of *Escherichia coli*, enable the organism to survive in this hostile environment will be detailed.

2 Earlier Observations on the Role of Bacterial Capsules

Capsules have long been associated with virulence properties of bacteria. Although the pathogenicity of *E. coli* to calves was known as long ago as 1892, the basis for this virulence was not known. In 1928 Theobald Smith observed that naturally occurring, unencapsulated mutants of *E. coli* obtained from calves suffering from gastroenteritis and bacteremia were less pathogenic in guinea-pig challenge studies than the encapsulated parents and also were more susceptible to phagocytosis by neutrophils. Similar observations concerning the importance of capsules were made for *Salmonella typhi*, *Haemophilus influenzae*, *Klebsiella*, and *Streptococcus pneumoniae*. Felix

Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.20307-5100, USA

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proposed that the Vi capsule of *S. typhi* masked the bactericidal reaction between the antisomatic (or O antigen) antibodies in host serum and the target of these antibodies on the bacteria. This has been invoked as a general mechanism by which capsules interfere with host defense mechanisms (e.g., HOWARD and GLYNN 1971).

In the intensive study that followed it was shown that:

1. *E. coli* could be serologically typed by a scheme (that included distinct capsular serotypes) similar to that developed for *Salmonella* (for review, see VAHLNE 1945).
2. Serologic typing of clinical isolates (from all sites except blood) revealed that only a small number of K and O serotypes accounted for most of the infections compared with the number in the normal fecal flora, and that K1 and K5 were the predominant capsular serotypes (VAHLNE 1945).
3. The susceptibility of an isolate to serum bacteriolysis was a measure of the virulent properties of an individual strain, and that this property was relatively constant.
4. The presence of a capsule on *E. coli* correlated with serum resistance (SJOSTEDT 1946; MUSCHEL 1960). This observation was strengthened by the exclusion of fecal isolates and by the inclusion of bacteremic specimens in the analysis. This permitted the speculation that a serum-mediated bacteriolytic mechanism prevented bacteria from invading and persisting in the blood (ROANTREE and RANTZ 1960).
5. There was a relationship between a strain's virulence for animals and its susceptibility to bacteriolysis by serum in vitro (ROWLEY 1954).

As was true for *Klebsiella* (EHRENWORTH and BAER 1956), a quantitative relationship was found between the amount of capsule expressed on *E. coli* and its serum sensitivity (SJOSTEDT 1946; GLYNN and HOWARD 1970) as well as the likelihood of its clearance from the bloodstream following intravenous injection (HOWARD and GLYNN 1971). The amount of bacterial cell-associated capsule was greatest during very early phases of growth and this correlated with increased virulence (VERMEULEN et al. 1988). Finally, some capsular polysaccharides were found to be more effective on a weight basis than others in promoting this resistance (GLYNN and HOWARD 1970). These and subsequent studies (e.g., HORWITZ and SILVERSTEIN 1980) led to an updating of FELIX's original hypothesis: capsules may inhibit host bactericidal defenses not only by impeding the binding of antibody (directed toward the O antigen or another component of the cell wall) but also by impeding the efficient fixation of complement on the surface of bacteria (HOWARD and GLYNN 1971). This, in turn, may block the uptake and killing of bacteria by phagocytes via the complement receptors, or the formation of lytic pores by the membrane attack of complement at the cell membrane. With the elucidation of these host immune mechanisms in greater detail, our knowledge of the strategies by which encapsulated bacteria evade these defenses has also been extended (Table 1).

3 Interaction of Complement with Encapsulated Bacteria

More recently, it has been possible to define at the molecular level many of the mechanisms involved in the virulence provided by bacterial capsules. The studies of PILLEMER and WARDLAW in the 1950s, which led to the identification of an alternative pathway (AP) for the activation of complement, revived interest in the bactericidal

properties of serum. In contrast to the classical complement pathway, which usually required the presence of specific, immune antibody, the AP was identified as an important mechanism of natural immunity whereby the host, in the absence of specific, immune antibody, was able to clear the invading microbe by generating membrane pore-forming cylinders, called the membrane attack complex (MAC). The MAC, which is composed of C5b, C6, C7, C8, and multiple molecules of C9, could lead to the lysis of gram-negative bacteria, but, because of the increased thickness of its cell wall, not gram-positive bacteria (for a review of this see JOINER et al. 1984).

Upon exposure to serum, bacteria may activate either the classical or the alternative complement pathway, depending on the availability of antibody and on the surface properties of the bacteria. The lipopolysaccharide (LPS) moiety of gram-negative bacilli and cell wall moieties [such as peptidoglycan (WILKINSON et al. 1979b) or teichoic acid (WINKELSTEIN and TOMASZ 1978)] of gram-positive organisms were found to be important activators of the AP. The small amounts of C3b that are deposited on the bacterial surface may be amplified by the formation of a complex between C3b and factor B of the AP to give C3bBb, which acts as a C3 convertase. Activation of the classical complement pathway leads to the formation of another C3 convertase, C4bC2a.

Factor H is a complement regulatory protein in serum which competes for surface-bound C3b with factor B (FEARON 1978). Factor H, which has no enzymatic activity, is believed to induce a conformational change in C3b that facilitates its degradation by a second regulatory protein, factor I. This disassembly of the C3 convertase terminates the C3b amplification loop and limits the deposition of this important opsonin on the bacterial surface. A C4 binding protein regulates the C4bC2a complex.

Bacterial capsules have evolved to take advantage of this competition between factor B, which promotes amplification, and factor H, which terminates it. This has been best studied in type III, group B streptococci (EDWARDS et al. 1982) and in K1-encapsulated *E. coli* (STEVENS et al. 1978). Both capsules contain sialic acid (the K1 capsule being a homopolymer of sialic acid), which increases the affinity of factor H for cell-bound C3b. Thus although complement can bind to the capsule, the C3 convertase, by which additional attack sites are generated, is inhibited and the complex falls off. Other organisms that contain sialic acid on their surface probably avoid opsonization in a similar manner [e.g., the enveloped Sindbis virus (HIRSCH et al. 1981)]. A similar strategy of limiting complement deposition has been described for capsules that do not contain sialic acid. The capsules of type 7 and type 12 pneumococci have a decreased binding affinity for factor B, which results in a relative increase in its binding of factor H (JOINER et al. 1984). The streptococcal M protein, a surface fibrillar molecule, has a high affinity for factor H and thereby evades killing mediated by the AP (HORSTMANN et al. 1988).

Incubation of bacteria with normal human serum has shown that some capsular types of *E. coli* (K6, 7, 27, 30, 42, 53, 57, and 75) are able to activate the AP efficiently, while others (K1, 3, 5, 12, and 92) that are often associated with extraintestinally invasive infection are poor activators of this pathway (STEVENS et al. 1983). Similar types of data have been generated with intact pneumococci having different capsular types (FINE 1975) and with purified pneumococcal polysaccharide (WINKELSTEIN et al. 1976).

The use of purified complement components has revealed other mechanisms em-

ployed by both encapsulated and unencapsulated organisms to evade lysis by complement. *Campylobacter fetus* has a capsular protein (S protein) which impairs the binding of C3b to its surface (BLASER et al. 1988). In a series of experiments with defined strains of *Salmonella*, the formation of the MAC on the surface of bacteria was observed; however, in contrast to strains with an incomplete LPS, the MAC was sloughed off the surface of strains having a smooth LPS before damage to the organism occurred (for a review of this work as well as studies with *Neisseria gonorrhoeae* and unencapsulated *E. coli*, see JOINER et al. 1984). While there has been little work describing the molecular detail of the modes of interaction between complement components and encapsulated *E. coli*, a similar type of mechanism could conceivably be found with some of its capsular polysaccharides. For example, components could be sloughed from the surface of encapsulated *E. coli*.

4 Functional Relationships Between Capsular Polysaccharides and Lipopolysaccharide

In addition to the role of capsule in the virulence of bacteria for the host, earlier studies of animal virulence and serum resistance had shown an important role for the LPS phenotype. It has become apparent, however, that there is a close functional relationship between the LPS and the capsular surface moieties in these phenomena. For example, the K1 capsule is significantly associated with LPS phenotypes (rough and part-rough) which are poorly able to resist serum bacteriolysis in the absence of capsule (GEMSKI et al. 1980; CROSS et al. 1984). In contrast, K5-unencapsulated mutants of *E. coli* 06:K5, which usually have a smooth LPS phenotype, retain their serum resistance (CROSS et al. 1986), a finding shared by SJOSTEDT (1946) over 40 years ago. In this instance, the capsule does not appear to have an important role in mediating serum resistance. Moreover, when the virulence of K1- and K5-encapsulated strains was compared with unencapsulated mutants in a neonatal rat model of infection, there were significant differences in LD_{50} between the K1-encapsulated parent and mutant that were not observed for the K5-encapsulated parent and its mutant (KIM et al. 1986). The role of the K5 capsule in the virulence of *E. coli* therefore appeared to be different from that of the K1 capsule, and the phenotype of the associated LPS appeared to be an important variable. In addition, when two strains of K1-encapsulated *E. coli* were compared at approximately equivalent levels of K1 expression, the strain with the rough LPS was more easily killed than the one with the smooth LPS (VERMEULEN et al. 1988). These and similar studies (PLUSCHKE et al. 1983) show that the LPS phenotype or presence of a capsule by itself is insufficient to explain a strain's sensitivity to serum bacteriolysis.

The well-known association of specific capsules with only a limited number of O serogroups is believed to be evidence of the existence of bacterial clones of common origin (ØRSKOV et al. 1976). It is possible that many of these clones survived because of unique functional relationships between capsules, LPS, and perhaps other determinants in that clone which enabled the bacterial clone to compete and survive.

5 Interaction of Capsules with Phagocytes

While more than 95% of encapsulated strains retrieved from the blood of patients are resistant to bacteriolysis in serum, most of these strains are susceptible to killing in the presence of serum and neutrophils (CROSS et al. 1984). Here, too, the particular LPS and capsular phenotypes were important: K1-encapsulated bacteria were more resistant to opsonophagocytosis than strains lacking the K1 capsule. Bacteria that had a smooth LPS phenotype were more resistant than those having a rough or part-rough phenotype, among both K1+ and K1− groups. In addition to the K1 capsule, other K phenotypes (K12 and 52) conferred relative resistance to opsonophagocytosis. Thus, while most K serotypes found in clinical cases of bacteremia were associated with serum resistance, only a relatively few K types were associated with resistance to opsonophagocytosis in vitro.

The K5 capsule provides an example of a different interaction with host phagocytic defenses. While encapsulation with K5 does not appear to play a significant role in providing resistance to either serum killing or opsonophagocytosis in vitro, it does appear to protect the organism from uptake and killing by phagocytes in vivo. Both K5+ and K5− strains of an *E. coli* 018 had similar LD50s in a neonatal rat model of infection. A monoclonal antibody directed at the LPS did not provide protection against lethal infection with the encapsulated strain, but it did protect these animals against the unencapsulated mutant. Thus, the K5 capsule prevented anti-LPS antibody from interacting with its target. In contrast, this same monoclonal antibody was able to protect these animals from lethal infection with a strain of 018:K1 (KIM et al. 1986). These data suggest differences between the two capsules in their ability to prevent anti-LPS antibody from mediating the killing of the organisms, and reinforce the observation that morphologic studies of the distribution of different K antigens on bacterial cell walls are needed (HOWARD and GLYNN 1971). Alternatively, capsules may differ in their permeability to opsonins.

The experience with the prototype capsules, K1 and K5, therefore indicates a diversity in the ability of different capsular polysaccharides to interact with the various host defenses. In the case of the K27 capsule of *E. coli*, it is unable to mediate serum resistance at all, and its role in evading host defenses is unclear (TAYLOR and ROBINSON 1980; OPAL et al. 1982). This diversity may be further demonstrated by the requirement for a few select capsular serotypes that are unusually resistant to complement- or neutrophil-mediated attack to be controlled by non-neutrophilic phagocytes. Macrophages and Kupffer cells differ from neutrophils, in part, by the production of higher levels of cytokine mediators. Preliminary evidence suggests that these mediators may enhance bacterial killing in the local cellular environment and, upon their release into the environment, in other phagocytes, including neutrophils. C3H/HeJ mice that are less able to produce interleukin-1 (IL-1) and tumor necrosis factor (TNF) in response to LPS are significantly more susceptible to killing by K1-encapsulated *E. coli* than is the normal-responding C3H/HeN mouse (CROSS et al. 1988; SVANBORG-EDEN et al. 1988; VUOPIO-VARKILA et al. 1988). Exogeneous administration of recombinant IL-1 and TNF to the C3H/HeJ mice provided protection from challenge with up to 20 LD_{50}s (CROSS et al. 1989). This suggests that the ability to produce these and/or other cytokine mediators, perhaps in response to

LPS, may be an important host defense mechanism. Certain capsules may inhibit this induction.

6 Effect of Capsules on Antibody Function

Bacterial capsules may also interfere with phagocytic processes by interfering with antibody function. While capsules of many bacteria that cause bacteremia are immunogenic in man [e.g., many of the polysaccharides of pneumococci and *Klebsiella* (CRYZ et al. 1986) have been incorporated into vaccines], both the K1 and K5 capsules of *E. coli* are poorly immunogenic. Each of these capsular polysaccharides is similar to important moieties of mammals: sialic acid (K1) is found on the surface of mammalian cells and desulfo-heparin (K5) shares a partial identity with host heparin (VANN et al. 1981). Mammalian hosts have developed mechanisms to avoid the production of antibody to their own tissue. The K1 and K5 capsules therefore mimic important host molecules in order to avoid immune recognition. Since these capsules also enable the bacteria to evade antibody-independent opsonization by the AP, it is not surprising that K1- and K5-encapsulated *E. coli* are the most common serotypes found in extraintestinally invasive disease. In the case of *Staphylococcus aureus* and of pneumococci, encapsulated bacteria may promote the efficient generation of C3, but its subcapsular localization at the cell wall may make it physically inaccessible from the complement receptors on phagocytes (WILKINSON et al. 1979a; BROWN et al. 1983).

7 Other Potential Mechanisms of Opsonic Evasion

Capsules may become "antiphagocytic" by mechanisms that have been demonstrated in nonencapsulated organisms but have yet to be demonstrated for encapsulated bacteria. Both *Candida* and herpes simplex virus are able to produce structures that mimic complement receptors of phagocytes. These may limit the effectiveness of complement-mediated host mechanisms by hastening the degradation of bound complement or by diverting C3b away from phagocytes (FRIEDMAN et al. 1984; EDWARDS et al. 1986). Recently a surface protein has been identified which is that binds to surface-bound C3b or C4b bound to the surface of mammalian cells by a phospholipase-sensitive phosphatidylinositol anchor. Like the complement regulatory proteins, this decay accelerating factor (DAF) also inhibits amplification of the complement cascade (NICHOLSON-WELLER et al. 1982). Metacyclic tryptomastigotes of *Trypanosoma cruzi* have a developmentally regulated surface structure that mimics DAF (SHER et al. 1986).

8 Effects of Capsule on Cellular Immune System

One area likely to receive increasing attention is the effect of bacterial capsules on the cellular immune system. The capsule of *Klebsiella* can modulate the ability of B cells to produce antibody to homologous and heterologous antigens (BATSHON et al. 1963;

NAKASHIMA et al. 1971). The purified capsule of *Bacterioides fragilis* can induce abscess formation which can be inhibited by a T cell dependent, antigen-specific mechanism (for review, see KASPER 1986). Finally, bacterial capsules may affect the response of lymphocytes or macrophages by directly mimicking the activity of a cytokine. For example, the capsular material of *Haemophilus actinomycetemcomitans*, a pathogen associated with periodontal disease, was shown to have many biologic activities of IL-1 [bone-resorbing activity, stimulation of collagenase and prostaglandin E_2 activity (HARVEY et al. 1987)]. Hyaluronic acid, which is part of the capsule of *Streptococcus pyogenes*, has also been shown to induce IL-1 production (HIRO et al. 1986). Other capsules may eventually be shown to mimic or induce cytokine mediators. Alternatively, capsules may mask the ability of cell wall constituents to induce inflammatory responses that may come to the aid of the host, as has been suggested for pneumococci (TUOMANEN et al. 1985).

9 Conclusions

The great number of different capsular polysaccharides and the diverse points in the host defenses at which each might act make it increasingly difficult to generalize about the effect of capsules on host defenses. Given the increasing number of mechanisms by which capsules interfere with complement and antibody function, it is now virtually meaningless to refer to capsules as "antiphagocytic." Similarly, the close functional relationship between capsules and other bacterial surface moieties means that one can no longer analyze an effect on the host as being primarily a property of the capsule or the LPS. Finally, some capsules, like the K27 of *E. coli*, have no apparent role in enabling the bacteria to evade host defenses. Perhaps by exploring the mechanisms of evasion already described for nonencapsulated organisms a mechanism for this type of capsule will be found.

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Capsular Polysaccharides as Vaccine Candidates

H. J. JENNINGS

1 Introduction

The discovery of a "specific soluble substance" secreted by pneumococci during growth (DOCHEZ and AVERY 1917) and the identification of this substance as a carbohydrate (HEIDELBERGER and AVERY 1923) were new and important developments in vaccine technology. This became evident when following the initial finding that the isolated capsular polysaccharides of pneumococci were immunogenic in mice (SCHIEMANN and CASPER 1927) it was demonstrated that they were also immunogenic in man (FRANCIS and TILLET 1930). The potential of capsular polysaccharides as vaccines was fully confirmed when it was demonstrated unequivocally that multivalent pneumococcal polysaccharide vaccines were able to provide type-specific protection in humans against the acquisition of pneumococcal infection (MACLEOD et al. 1945). However, at this time the phenomenal success of antibiotic therapy in the treatment of bacterial infections caused a lengthy hiatus in the further development of polysaccharide vaccines.

Two major factors played a role in the renewal of interest in the prophylaxis of bacterial diseases. The first was the expanding incidence of antibiotic-resistant bacterial strains (FINLAND 1979), and the second was that more exacting clinical studies demonstrated that antibiotic treatment of infectious diseases even caused by non-

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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resistant strains of encapsulated bacteria did not always prevent their morbidity or mortality (ROBBINS 1978). For example, "cured" *Haemophilus influenzae* type b meningitis is the leading cause of acquired mental retardation (SELL et al. 1972) and statistics indicate that deaths due to pneumococcal pneumonia still occur at the same rate as they always did despite the use of antibiotic therapy (AUSTRIAN 1976).

Capsular polysaccharides can be readily isolated in their immunogenic high molecular weight form (KABAT and BEZER 1958) and the concept of using pure capsular polysaccharides, devoid of their accompanying complex bacterial mass, is technically elegant. Polysaccharides are with few exceptions immunogenic in man, nontoxic, and free of other deleterious effects associated with whole organism vaccines (ROBBINS 1978; JENNINGS 1983). Another important feature of polysaccharide immunogens is that they can be chemically and physically defined, criteria which add a greater measure of control over their efficacy as biologicals. In fact the group A and C meningococcal polysaccharides were the first vaccines where the standards for their licensure and release depended entirely on physicochemical criteria. As a measure of the success of these vaccines millions of people have now been immunized with capsular polysaccharides resulting in a high degree of protection, with no fatalities or significant adverse effects. Currently polysaccharide vaccines have been licensed for use in the immunoprophylaxis of pneumococcal pneumonia and meningitis caused by *Neisseria meningitis* and *H. influenzae*. Obviously the concept of using capsular polysaccharides is capable of extension to disease caused by other pathogenic bacteria, e.g., group B *Streptococcus*, *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas*, and *Salmonella typhi*; however, the development of these vaccines will depend on their clinical importance and the presence of meaningful epidemiologic data.

Despite the success obtained with the use of polysaccharide vaccines, a number of problems have been identified which severely restrict the further development of this concept. Two of the most important problems are the poor immunogenicity of a few of the purified capsular polysaccharides in all humans, of which the group B meningococcal polysaccharide is the salient example (WYLE et al. 1972), and the poor immunogenicity of all polysaccharides in infants (GOTSCHLICH et al. 1977). Therefore in addition to the capsular polysaccharides this brief review will also be concerned with the delineation of these problems and with attempts to solve them by the chemical manipulation of the above polysaccharides (JENNINGS 1983; JENNINGS et al. 1986). This development has shown great promise in furthering the potential of polysaccharide vaccines.

2 Polysaccharide Vaccines

2.1 Streptococcus pneumoniae

Pneumococci are still among the major causes of death in North America. In adults they are responsible for lower respiratory tract infections and in children they are a major cause of otitis media (middle ear infection). The high mortality associated with pneumococcal pneumoniae prompted the search for an efficacious prophylactic

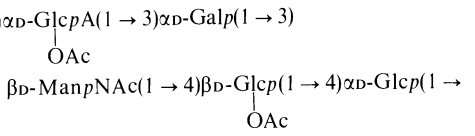
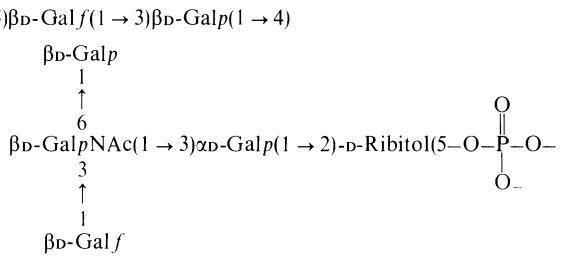
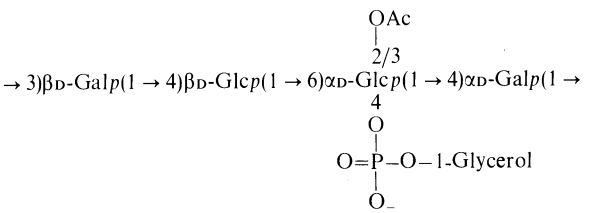
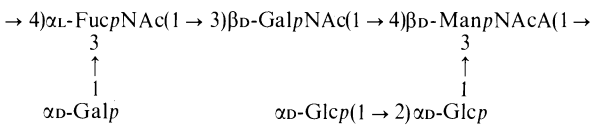
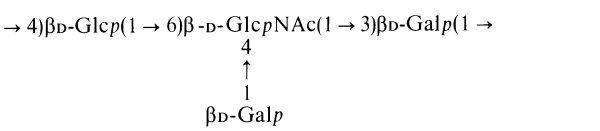
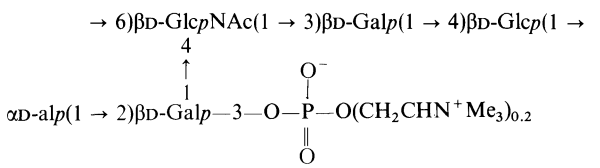
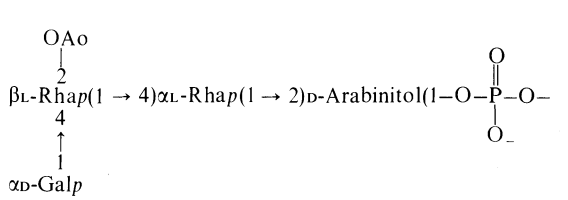
agent and the pneumococcal polysaccharides were the first purified polysaccharides to be used as vaccines (AUSTRIAN 1976; 1985; ROBBINS 1978; JENNINGS 1983). *Streptococcus pneumoniae* are gram-positive organisms, which have in addition to different type-specific capsular polysaccharides, a common group antigen (C-substance) (JENNINGS et al. 1980a). To date, 83 different type specificities have been identified which have been designated 1–83 in the American system. The structures of the pneumococcal polysaccharides have been reviewed (KENNE and LINDBERG 1983). Because of their number and structural complexity, for the purposes of this chapter only the structures of the polysaccharides used in recent pneumococcal vaccines are listed in Table 1. Because of the diversity of pneumococcal capsular types the intent behind the formulation of an initial 14-valent pneumococcal polysaccharide vaccine (JENNINGS 1983) was to limit the number of polysaccharides while maintaining the maximum effective coverage. The 14 polysaccharides chosen are shown in Table 2, and it was estimated that these polysaccharides were involved in 70–80% of all bacteremic infections, based on the frequency of the occurrence of disease isolates (AUSTRIAN 1981). However, recent studies (ROBBINS et al. 1983) have indicated that an increased level of protection (approximately 90%) can be obtained using 23 polysaccharides and this prompted the formulation of the most recent 23-valent polysaccharide vaccine. The increased coverage is largely obtained by the inclusion of type-specific polysaccharides of less frequently isolated pneumococci (Table 1) although other important factors have also influenced the inclusion of additional polysaccharides.

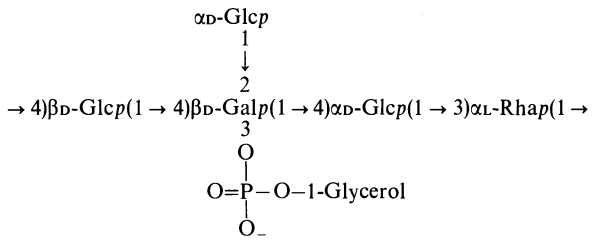
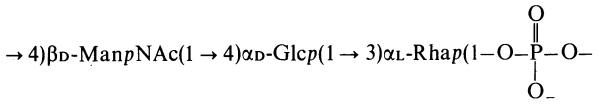
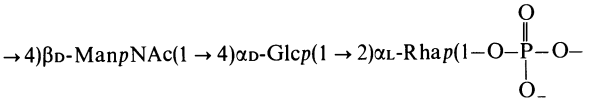
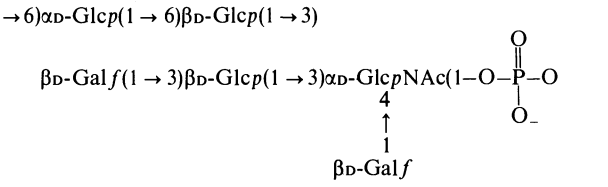
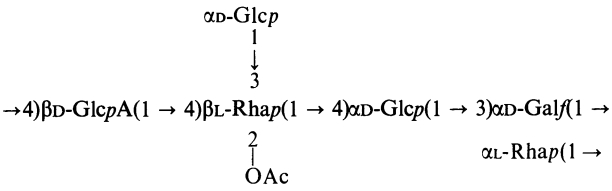
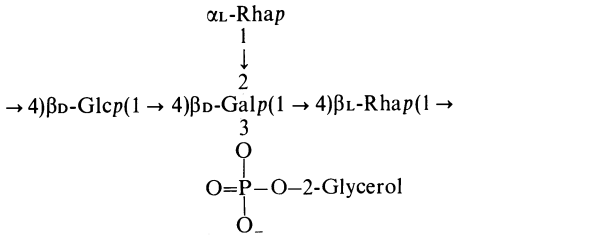
One property of the pneumococcal polysaccharides that appeared favorable for the limitation of their number in the vaccine was their extensive serologic cross-reactivity demonstrated in animal experiments. The origin of this cross-reactivity is the extensive structural homology found in the pneumococcal polysaccharides, which is exemplified in the Danish serotyping system (LUND and HENRICHSEN 1978). This system designates capsular types within groups based on this cross-reactivity (Table 1). More recent studies, however, have demonstrated that some of the above polysaccharides do not provide the same degree of cross-reactivity and thus cross-protection in humans. Therefore the inclusion of additional polysaccharides was proposed to counteract this deficiency (ROBBINS et al. 1983). For example, although the type 19A and 19F polysaccharides differ structurally by only one linkage and are immunologically cross-reactive in animals, the type 19F polysaccharide used previously in the 14-valent pneumococcal vaccine (Table 1) provided inadequate levels of protective antibodies in humans against type 19A pneumococci (ROBBINS et al. 1983). Thus the type 19A polysaccharide, in addition to the type 19F polysaccharide, was included in the 23-valent polysaccharide vaccine (Table 1). A similar argument was used to justify the inclusion of the type 9V polysaccharide in the 23-valent vaccine in addition to the type 9N polysaccharide, which was a component of the original 14-valent polysaccharide vaccine (Table 1).

The choice of the type 6B polysaccharide for the 23-valent vaccine rather than the more frequently encountered type 6A polysaccharide, a component of the 14-valent vaccine, was based on the greater stability of the former (ZON et al. 1982). This is an important factor in the storage of polysaccharide vaccines because their immunogenicity is dependent on their large molecular size (KABAT and BEZER 1958; JENNINGS 1983). Although the structures of the types 6A and 6B pneumococcal polysaccharides

Table 1. Structures of the capsular polysaccharides of *Strept. pneumoniae*

Type	Structure[a]	Reference
1	→3)α-AAT*p*(1→4)αD-Gal*p*A(1→3)αDGal*p*A(1→	LINDBERG et al. (1980)
2	→4)βD-Glc*p*(1→3)αL-Rha*p*(1→3)αL-Rha*p*(1→3)βL-Rha*p*(1→ 2 ↑ 1 αD-Glc*p*A(1→6)αD-Glc*p*	JANSSON et al. (1988)
3	→4)βD-Glc*p*(1→3)βD-Glc*p*A(1→	REEVES and GOEBEL (1941)
4	→4)βD-Man*p*NAc(1→3) αL-Fuc*p*NAc(1→3) αD-Gal*p*NAc(1→4)αD-Gal*p*(1→ 3 2 H_3C CO_2H	JANSSON et al. (1981)
5	→4)βD-Glc*p*(1→4)αL-Fuc*p*NAc(1→3)βD-Sug*p*(1→ 4 ↑ 1 αL-Pne*p*NAc(1→4)βD-Glc*p*A	JANSSON et al. (1985)
6A	→2)αD-Gal*p*(1→3) αD-Glc*p*(1→3)αL-Rha*p*(1→3)Ribitol(5–O–P(=O)(O⁻)–O–	REBERS and HEIDELBERGER (1961)
6B	→2)αD-Gal*p*(1→3) αD-Glc*p*(1→3)αL-Rha*p*(1→4)Ribitol(5–O–P(=O)(O⁻)–O–	KENNE et al. (1979)
7F	→6)αD-Gal*p*(1→3)βL-Rha*p*(1→4)βD-Glc*p*(1→3)βD-Gal*p*NAc(1→ 2 2 4 ↑ | ↑ 1 1 βD-Gal*p* AcO αD-Glc*p*NAc(1→2)αL-Rha*p*	MOREAU et al. (1988)
8	→4)βD-Glc*p*A(1→4)βD-Glc*p*(1→4)αD-Glc*p*(1→4)αD-Gal*p*(1→	JONES and PERRY (1957)
9N	→4)αD-Glc*p*A(1→3)αD-Glc*p*(1→3)βD-Man*p*NAc(1→4)βD-Glc*p*(1→4)αD-Glc*p*NAc(1→	JONES et al. (1985)

Type	Structure[a]	Reference
9V		PERRY et al. (1981)
10A		RICHARDS and PERRY (1989)
11A		RICHARDS et al. (1988)
12F		LEONTEIN et al. (1981)
14		LINDBERG et al. (1977)
15B		JANSSON et al. (1987)
17F		PERRY et al. (1989)

Type	Structure[a]	Reference
18C		Lugowski and Jennings (1984)
19A		Katzenellenbogen and Jennings (1983)
19F		Jennings et al. (1980b), Ohno et al. (1980)
20		Richards et al. (1983)
22F		Richards et al. (1989)
23F		Richards and Perry (1988)

Type	Structure[a]	Reference
25F	Not reported	
33F	$\rightarrow 3)\beta$D-Gal$p(1 \rightarrow 3)$ αD-Gal$p(1 \rightarrow 3)\beta$D-Gal$f(1 \rightarrow 3)\beta$D-Glc$p(1 \rightarrow 5)\beta$D-Gal$f(1 \rightarrow$ 2 2 ↑ | 1 $OAc_{0.4}$ αD-Gal$p$	RICHARDS et al. (1984)

[a] AAT = 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Sug = 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose.

Table 2. Types and cross-reactive types within pneumococcal groups in the current 14-valent and proposed 23-valent polysaccharide vaccine (ROBBINS et al. 1983)

Group	Cross-reactive types		
1[a,b]	None	12	12F[a,b], 12A
2[a,b]	None	14[a,b]	None
3[a,b]	None	15	15F, 15A, 15b[b], 15C
4[a,b]	None	17	17F[b], 17A
5[a,b]	None	18	18F, 18A, 18B, 18C[a,b]
6	6A[a], 6B[b]	19	19F[a,b], 19A[b], 19B, 19C
7	7F[a,b], 7A, 7B, 7C	20[b]	None
8[a,b]	None	22	22F[b], 22A
9	9A, 9L, 9N[a,b], 9V[b]	23	23F[b], 23A, 23B
10	10F, 10A[b]	25	25F[a], 25A
11	11F, 11A[b], 11B, 11C	33	33F[b], 33A, 33B, 33C

[a] Component of 14-valent vaccine;
[b] Component of 23-valent vaccine

differ only in the position of linkage of their α-L-rhamnopyranosyl residues to D-ribitol, this structural feature is critical to their relative stability. ^{31}P NMR studies on the type 6A and 6B polysaccharides (ZON et al. 1982) have shown that the greater instability of the phosphodiester linkages of the type 6A polysaccharide is due to a hydrolytic mechanism involving neighboring group participation of the HO-4 group of D-ribitol. An equivalent mechanism is not possible in the case of the type 6B polysaccharide because the HO-4 group of its D-ribitol residue is glycosidically linked to an α-L-rhamnopyranosyl residue.

Problems remain to be solved in order to perfect the multivalent vaccine for universal use. For instance, formulation of the current 23-valent vaccine was based on the prevalence of pneumococcal types isolated in the United States and Europe,

and it is known that the distribution of pneumococcal disease isolates differs in different parts of the world (LEE 1987). Also, time-related changes in the prevalence of pneumococcal serotypes and age-related differences both in the distribution of pneumococcal types and in immune responses to them (ROBBINS 1978; Austrian 1985) are factors that must be considered. The first of these problems can be solved by the constant surveillance of disease isolates, but the last is particularly serious because infants (age 2 and under) do not respond to polysaccharide vaccines and this age group is particularly vulnerable to purulent otitis media caused by pneumococci. A possible solution to this latter problem lies in the development of polysaccharide–protein conjugate vaccines (see Sect. 3.2).

The complex problems encountered in the formulation of an efficacious pneumococcal polysaccharide vaccine have recently attracted attention to the possibility of formulating an alternate more simple vaccine based on the pneumococcal subcapsular common carbohydrate antigen (C-substance). The structure of the repeating unit of C-substance (JENNINGS et al. 1980a) is shown in Fig. 1, and recent serologic studies have shown that this approach has some merit in that it was demonstrated (BRILES et al. 1982) that both monoclonal and polyclonal antibodies to phosphocholine (a substituent of C-substance) were protective against a lethal challenge with some encapsulated pneumococci in mice. This observation was also largely confirmed by further studies using antiphosphocholine antibodies and a greater variety of pneumococci of different serotypes (SZU et al. 1983). However, in this work it was demonstrated that some serotypes (serotypes 1, 3, and 5) were not susceptible to antiphosphocholine antibodies. On the basis of these results it was predicted that antibodies specific for the complete haptenic structure of C-substance (Fig. 1) would be more effective in mouse protection studies than those specific for phosphocholine alone. Because C-substance is isolated from pneumococci in a small molecular size it is only poorly immunogenic; therefore recently it was conjugated to bovine serum albumin (BSA) prior to being used as an immunogen in rabbits (SZU et al. 1986a). Surprisingly antibodies to the C polysaccharide elicited by the C polysaccharide–BSA conjugate failed to protect mice completely against challenge with type 3 or type 6A pneumococci and interestingly the conjugate did not induce any phosphocholine-specific antibodies in the mice. These results are not encouraging for the development of a C polysaccharide–protein conjugate vaccine. However, they are not definitive, and given its overall simplicity, further exploration of this approach would seem justified.

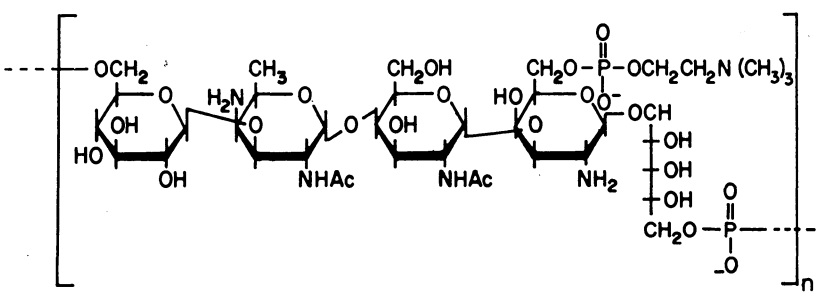


Fig. 1. Repeating unit of the pneumococcal group antigen (C-substance)

2.2 Neisseria meningitidis

Meningococcal meningitis still remains a worldwide problem and occurs in both endemic and epidemic forms (PELTOLA 1983; GOTSCHLICH 1984). Epidemic disease occurs in all parts of the world and incidences as high as 500 per 100000 population have been reported. Without antibiotic treatment the mortality is exceedingly high (85%) and even with this treatment it still remains at approximately 10%. In addition, patients cured by antibiotic therapy can still suffer serious and permanent neurologic deficiencies. These facts together with the emergence of sulfadiazine-resistant strains promoted the rapid development of a commercial vaccine (PELTOLA 1983).

Neisseria meningitidis is a gram-negative organism that has been classified serologically into groups A, B, C, 29e, W135, X, Y, and Z (GOTSCHLICH 1984). Additional groups H, I, and K were isolated in China (DING et al. 1981) and group L was isolated in Canada (ASHTON et al. 1983). The grouping system is based on the organisms' capsular polysaccharides, and the structures of their repeating units are shown in Table 3. It was established (LUI et al. 1971a) that the group A polysaccharide is a partially O-acetylated (1→6)-linked homopolymer of 2-acetamido-2-deoxy-D-manno-pyranosyl phosphate, and that both groups B and C polysaccharides are homopolymers of sialic acid. 3-Deoxy-D-manno-2-octulosonic acid (KDO) has been identified as a component of the group 29e polysaccharide (BHATTACHARJEE et al. 1978) and because of the presence of these unusual and extremely labile 3-deoxy-2-glyculosonic acid components in the polysaccharides, problems were encountered in the application of conventional chemical techniques to their structural elucidation. These problems were largely overcome by the use of ^{13}C NMR spectroscopy. In fact, except for the group A polysaccharide, on which some structural information was already available (LUI et al. 1971a), most of the structures of the other meningococcal polysaccharides

Table 3. Structures of the capsular polysaccharides of *N. meningitidis* and *H. influenzae*

Group	Structure	Reference
N. meningitidis		
A	→6)-αD-ManNAc(1–O–P(=O)(–O⁻)–O–)3-OAc	BUNDLE et al. (1974)
B	→8)αD-Neu*p*NAc(2→	BHATTACHARJEE et al. (1975)
C	→9)αD-Neu*p*NAc(2→ 7/8-OAc	BHATTACHARJEE et al. (1975)
W-135	→6)-αD-Gal*p*(1→4)αD-Neu*p*NAc(2→	BHATTACHARJEE et al. (1976)
Y	→6)-αD-Glc*p*(1→4)αD-Neu*p*NAc(2→(contains OAc groups)→	BHATTACHARJEE et al. (1976)
H. influenzae		
b	→3)βD-Rib*f*(1→1)D-Ribitol(5–O–P(=O)(–O⁻)–O–	CRISEL et al. (1975); BRANEFORS-HELANDER et al. (1976)

were deduced entirely by ^{13}C NMR spectroscopy (JENNINGS 1983). With the advent of high resolution spectrometers, ^{1}H NMR spectroscopy has also recently become a powerful tool in the structural analysis of polysaccharides, where a number of one-dimensional, two-dimensional, and nuclear Overhauser enhancement (nOe) techniques are employed (BAX et al. 1984; BYRD et al. 1987; LERNER and BAX 1987). Some of these techniques, including two-dimensional nOe experiments, were used in the complete structural assignment of the group I and K meningococcal polysaccharides (MICHON et al. 1985a, b).

The capsular polysaccharides of groups A, B, and C *N. meningitidis* can be obtained in high molecular weight immunogenic form by precipitation from the culture medium by Cetavlon (GOTSCHLICH et al. 1969b), and this property is critical to their effectiveness as vaccines (KABAT and BEZER 1958). However, it was established that the large molecular size of these polysaccharides is created by aggregation (micelle formation) of individual polysaccharide chains, and that this aggregation is due to the presence of lipid substituents on these chains (GOTSCHLICH et al. 1981). This was demonstrated when group A, B, and C capsular polysaccharides were treated with sodium dodecyl sulfate or lipases. These reagents, which are not capable of breaking covalent bonds in the polysaccharides, caused their molecular size to be significantly reduced. Di-*O*-palmitoylglycerol and di-*O*-stearoylglycerol were identified as components of the polysaccharides and it was postulated that these di-*O*-acylglycerols were glycosidically attached to the reducing ends of the polysaccharide chains by phosphodiester bonds (GOTSCHLICH et al. 1981). End group di-*O*-acylglycerol phosphate moieties have also been detected in some of the *E. coli* capsular polysaccharides (GOTSCHLICH et al. 1981; SCHMIDT and JANN 1982) and it has been suggested that these substituents could be involved in anchoring the capsular polysaccharides to the outer membranes of their respective bacteria.

In contrast to the pneumococcal vaccine the composition of the meningococcal polysacharide vaccine has been greatly simplified by the fact that fewer polysaccharides are required. In fact groups A, B, and C are responsible for approximately 90% of cases of meningococcal meningitis. Success in the prevention of group A and C meningococcal meningitis was achieved using a bivalent polysaccharide vaccine (GOTSCHLICH et al. 1969a; ARTENSTEIN et al. 1970); this vaccine became a commercial product and has been used successfully in the last decade in the prevention and arrest of major meningities epidemics in many parts of the world. However, there has been a need to augment this vaccine because a significant proportion of cases of meningococcal meningitis are due to groups other than A and C. Group B is of particular epidemiologic importance, but groupy Y and W135 are also significant (CADOZ et al. 1985). The inclusion of the group B polysaccharide in the vaccine remains a special problem (see below); however, a tetravalent vaccine comprising groups A, C, W135, and Y has proven to be safe and immunogenic in humans (CADOZ et al. 1985) and is the currently used meningococcal meningitis vaccine.

Two serious problems remain in the development of a polysaccharide vaccine which would give complete coverage to all serogroup organisms and to all humans. Firstly, it has been established that although the group A and C polysaccharides are efficacious in adults and older children, their effectiveness in infants has only been marginal (GOLDSCHNEIDER et al. 1973; GOTSCHLICH et al. 1977). As in the case of the pneumococcal polysaccharides, the solution to this problem will probably reside in the develop-

ment of polysaccharide–protein conjugate vaccines (see Sect. 3.2). Secondly, the group B meningococcal polysaccharide is only poorly immunogenic in man (WYLE et al. 1972). Two major reasons have been proposed to account for this phenomenon (JENNINGS 1983; LIFELY et al. 1987). One is that the α-(2→8)-linked sialic acid homopolymer (Table 2) is rapidly depolymerized in human tissue because of the action of neuraminidase; the other is that the structure is recognized as "self" by the human immune system and in consequence, the production of antibody specific for this structure is suppressed. The weight of evidence is in favor of the latter explanation because a neuraminidase-sensitive variant of the group C meningococcal polysaccharide [an α-(2→9)-linked sialic acid homopolymer] still proved to be highly immunogenic in man (GLODE et al. 1979). In addition it was demonstrated that conjugation of the group B polysaccharide to a protein carrier (tetanus toxoid) through its terminal nonreducing sialic acid, which stabilizes the polysaccharide to neuraminidase, did not result in any significant enhancement in its immunogenicity (JENNINGS and LUGOWSKI 1981). The above observations are consistent with the fact that the immune mechanism avoids the production of antibody having a specificity for the α-(2→8)-linked sialic acid residues and this was further confirmed by the identification of this structure in the oligosaccharides of human and animal tissue. A novel approach to solving this problem has been to modify the group B polysaccharide chemically (see Sect. 4.3).

2.3 Haemophilus influenza

Type b *H. influenzae* is the major cause of meningitis in children of less than 5 years of age and has an extremely high mortality (approximately 5%–10%). Of equal importance is that survivors of this disease, even those having undergone antibiotic therapy, can suffer severe and permanent neurologic defects (ROBBINS et al. 1978). In consequence an extensive amount of recent work has been dedicated to finding a vaccine against disease caused by this organism. That the capsular polysaccharide was a prime candidate vaccine could be predicted from early studies in which it was demonstrated that protective antibodies found in hyperimmune rabbit antisera could be removed by absorption with the purified type b *H. influenzae* polysaccharide (ALEXANDER et al. 1944).

The *H. influenzae* are gram-negative organisms that can be serologically classified into six types (a through f). Only the structure of the repeating unit of the type b poly- (ROBBINS 1978). This means that any prospective polysaccharide vaccine against saccharide is shown in Table 2 because the most serious disease is caused by type b meningitis caused by *H. influenzae* would have the simplicity of being monovalent. As in the case of the meningococcal polysaccharides, type b *H. influenzae* polysaccharide has a relatively simple structure, being a D-ribofuranosyl-D-ribitol phosphate polymer (Table 2). In addition, end-group phosphoric esters have also been detected in the *H. influenzae* capsular polysaccharides, which could have been part of an original linkage to the outer membrane of the bacteria (EGAN et al. 1982).

The potential of the type b *H. influenzae* polysaccharide as a human vaccine was affirmed when it was demonstrated that it elicited long-lived complement-mediated bactericidal antibodies in adults (ANDERSON et al. 1972). However, because type b

H. influenzae is a disease exclusive to children, more meaningful trials were eventually carried out on this section of the population. One of these was an extensive randomized trial carried out in Finland in 1974 on 100000 children between the ages of 3 months and 5 years. The results confirmed the age dependency of polysaccharide vaccines as previously encountered with the meningococcal and pneumococcal polysaccharides. The *H. influenzae* type b polysaccharide vaccine was shown to be protective in children older than 18 months, whereas no protection was seen in infants vaccinated before 18 months of age (PELTOLA et al. 1977). In accordance with this observation is the fact that serum antibody responses to the *H. influenzae* type b polysaccharide were also found to be low in this latter group of children (KÄYHTY et al. 1984). Obviously the polysaccharide vaccine is unable to protect a rather substantial and vulnerable group of infants. However, because of the crippling nature of this disease and the urgency in finding a vaccine, the *H. influenzae* type b polysaccharide vaccine was licensed in 1985 for use in children over 18 months of age, until a more satisfactory solution could be found. More recently it has been found that efficacy of the type b *H. influenzae* polysaccharide vaccine in children below 18 months of age can be accomplished using *H. influenzae* type b polysaccharide–protein conjugate vaccines (see Sect. 3.2).

2.4 Group B *Streptococcus*

Although human infection due to group B *Streptococcus* was first reported in 1938 (FRY 1938) it is only in the last decade that a significant increase in infections due to this organism has been reported (BAKER and KASPER 1985). During this time group B *Streptococcus* has become the leading cause of neonatal bacterial sepsis and meningitis and is associated with very significant morbidity and mortality (BAKER and EDWARDS 1983). Its prevention is therefore desirable and conceivably this could be achieved using the same principles as are used for the other encapsulated pathogenic bacteria mentioned previously.

Group B *streptococci* are gram-positive organisms which have two distinct polysaccharide antigens (LANCEFIELD 1972). One is a group antigen (C-substance) common to all strains which has recently been demonstrated to be a multiantennary polysaccharide composed of a number of heterogeneous rhamnose-containing oligosaccharides linked together by phosphodiester bonds (MICHON et al. 1987a, 1988). The other is the type-specific capsular polysacccharides that distinguish types Ia, Ib, II, III, and IV, and but for the last mentioned, all of these have been purified and characterized (JENNINGS 1983; WESSELS et al. 1987). While the organisms carrying the type Ia, Ib, II, and III polysaccharides have all been implicated in invasive disease in infants, those carrying the type III polysaccharide are responsible for 66% of all infections. Also, if one considers only infections of the meninges (meningitis) then organisms carrying the type III polysaccharide are even more potent, and are responsible for 80%–90% of all infections (BAKER and KASPER 1985).

The structures of the repeating units of the type Ia, Ib, II, and III polysaccharides are shown in Table 4, and all contain D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and sialic acid. All the polysaccharides have terminal α-linked sialic acid residues linked to O-3 of β-D-galactopyranosyl residues, and an examination of the

Table 4. Structures of the capsular polysaccharides of group B *Streptococcus*

Group	Structure	Reference
Ia	→ 4)βD-Glc*p*-(1 → 4)-βD-Gal*p*-(1 → 3 ↑ 1 βD-Glc*p*NAc 4 ↑ 1 αD-Neu*p*NAc-(2→3)-βD-Gal*p*	JENNINGS et al. (1983a)
Ib	→4)-βD-Glc*p*-(1→4)-βD-Gal*p*-(1 → 3 ↑ 1 βD-Glc*p*NAc 3 ↑ 1 αD-Neu*p*NAc-(2→3)-βD-Gal*p*	JENNINGS et al. (1983a)
II	→4)-βD-Glc*p*NAc-(1 → 3)- βD-Gal*p*(1→4)-βD-Glc*p*-(1→3)-βD-Glc*p*-(1→2)-βD-Gal*p*-(1 → 6 3 ↑ ↑ 1 2 βD-Gal*p* αD-Neu*p*NAc	JENNINGS et al. (1983b)
III	→4)-βD-Glc*p*-(1 → 6)-βD-Glc*p*NAc-(1 → 3)-βD-Gal*p*-(1 → 4 ↑ 1 αD-Neu*p*NAc-(2 → 3)-βD-Gal*p*	WESSELS et al. (1987)

structures of the group B streptococcal polysaccharides (Table 3) reveals considerable structural homology with some important human serum glycoproteins. For example, the 3-*O*-(*N*-acetyl-α-neuraminyl)-β-D-galactopyranosyl group of type Ia, Ib, and III polysaccharides is the end-group oligosaccharide in the human M and N blood group substances (SADLER et al. 1979), and it is reasonable to suppose that common structural features of this type were an evolutionary acquisition by the bacteria to evade the human immune system (CORFIELD and SCHAUER 1982). Thus it is more than coincidental that all bacteria having polysaccharides containing terminal sialic acid are human pathogens.

The asialo-core polysaccharide of the type III group B streptococcal polysaccharide is structurally identical to the capsular polysaccharide of type 14 *S. pneumoniae* and on the basis of serologic experiments in animals it was suggested that antibodies to the type 14 pneumococcal polysaccharide would we protective against type III group B streptococcal organisms (FISCHER et al. 1978). However, confirmatory evidence for the exclusive participation of antibodies to the native type III polysaccharide in the development of human immunity to the disease was obtained when it was demonstrated that in human sera, only antibody directed to the native type III polysaccharide correlated highly with protection (KASPER et al. 1979). This

result is of immunologic interest because although terminal sialic acid is not normally immunogenic, it controls the conformation of the determinants which are responsible for the production of protective antibodies (JENNINGS et al. 1981, 1984).

Because group B *Streptococcus* causes disease in the newborn, the use of immunoprophylaxis as applied to the infant is impractical. Firstly, in the light of experience with the polysaccharides of other pathogenic bacteria, it is probable that the group B streptococcal polysaccharides would be poorly immunogenic in infants. Furthermore, even if they were immunogenic, infants would still remain highly susceptible during the time lag required for effective levels of protective antibodies to be produced. Therefore a different vaccination strategy is envisaged for this disease, one in which the target population for the polysaccharide vaccine would be pregnant women, or because this remains controversial, young women prior to pregnancy. The rationale behind this strategy is that infants could acquire immunity by the placental transfer of poly-saccharide-specific antibodies of the IgG isotype. It has been demonstrated that infants born of mothers having high levels of type III polysaccharide-specific antibodies are less liable to infection (KASPER and JENNINGS 1982).

3 Poor Immunogenicity of Polysaccharides in Infants

3.1 Age-Related Response to Polysaccharides

An important distinction must be made between the humoral response to a pure capsular polysaccharide and the same polysaccharide when it is an integral part of the bacterium (JENNINGS 1983). Thus the immunity received from infection by encapsulated bacteria in terms of the polysaccharide antigen differs from that generated by the purposeful immunization with purified polysaccharide vaccines. For most antigens, including polysaccharides attached to bacteria, the production of antibody is based on the cooperative interaction of two types of lymphocyte called T cells (thymus derived) and B cells (bone marrow derived). This antibody response is associated with its ability to be boosted to higher levels of further exposure to the antigen (anamnestic response) with the production of a wide range of antibodies of the IgG isotype. However, on the basis of studies in mice, pure polysaccharide antigens are considered to be T cell independent, only capable of inducing in mice short-lived immune responses of the IgM isotype with no anamnestic effect (HOWARD et al. 1971). With the exception of infants, the human immune response to polysaccharides is different in that they are able to induce in humans, antibodies of the IgG isotype (YOUNT et al. 1968; KÄYHTY et al. 1983) in addition to those of the IgM and IgA isotypes. Polysaccharides maintain their use in immunoprophylaxis because although there is an absence of a significant anamnestic response, antibody levels induced by polysaccharides in humans are high enough to provide effective protection against bacterial infection and, most importantly, remain at this high level for fairly long periods (HEIDELBERGER et al. 1950).

By comparison, infants respond very poorly to polysaccharide vaccines (GOTSCHLICH et al. 1977; PELTOLA et al. 1977; AUSTRIAN 1985); the antibodies produced

are exclusively of the IgM isotype and the immune response is not boostable. Following the loss of maternally transferred antibodies, infants become very susceptible to encapsulated bacterial infections, because immunity to these infections is due to the presence of circulating antibodies (GOLDSCHNEIDER et al. 1969). Then a maturation process in the immune response of infants to polysaccharides occurs during the next few years of life (GOTSCHLICH et al. 1977). The most promising explanation of this phenomenon is a natural antigenic stimulation caused by exposure to either the infecting bacteria itself or, probably more importantly, to nonpathogenic bacteria having polysaccharides with similar structural features to those of their pathogenic counterparts. Some of these latter organisms have been identified (ROBBINS 1978) and are listed together with the structures of their polysaccharides in Table 5, and obviously cross-reactions among bacteria are probably more widespread than those listed above. One exception to the rule is the polysaccharide of *Escherichia coli* K93 (Table 4), which is unconventional in that although highly cross-reactive with the group A meningococcal polysaccharide (Table 2), it does not share with it one single common glycose residue or linkage (BAX et al. 1988).

Because of the age-related response to polysaccharide vaccines it is extremely important to know precisely when a certain polysaccharide vaccine becomes efficacious in infants. Studies on different pneumococcal polysaccharides have indicated that this is a highly complex phenomenon because it is dependent on polysaccharide structure (DOUGLAS et al. 1983). Surprisingly, the type 3 and 18C polysaccharides were found to be good immunogens in infants as young as 7 months old, both producing antibody responses of the IgG isotype; in contrast the type 6A and 23F polysaccharides

Table 5. Structures of polysaccharides of bacteria, frequently found in human flora, that cross-react with the polysaccharide capsules of human pathogenic bacteria

Pathogen	Cross-reacting organism	Structure	Reference
N. meningitidis			
Group A	*Escherichia coli* K93	$\rightarrow 4)\beta$D-Glc*p*A$(1 \rightarrow 3)\beta$D-Gal$f(1 \rightarrow$ (OAc at 5, OAc at 6)	BAX et al. (1988)
	Bacillus pumilis	(1→6)-linked 2-acetamido-2-deoxamannosyl phosphate residues	ROBBINS (1978)
Group B	*Escherichia coli* K1	→8)αD-Neu*p*NAc(2→ and its OAc^+ variant	ORSKOV et al. (1979)
Group C	*Escherichia coli* K92	→8)αD-Neu*p*NAc(2→9)αD-Neu*p*NAc(2→	EGAN et al. (1977)
H. influenzae			
Type b	*Escherichia coli* K100	$\rightarrow 3)\beta$D-Rib$f(1 \rightarrow 2)$D-Ribitol(5–O–P(=O)(O⁻)–O–	TSUI et al. (1988)

were extremely poor immunogens even in infants 13 months of age. These latter polysaccharides produced in infants only antibodies of the IgM isotype and it has been estimated that infant immunoresponsiveness to the type 6A polysaccharide is delayed until the age of 4 years or more. The immunoresponsiveness to the type 14 and 19F pneumococcal polysaccharides in infants is intermediate between the immunoresponsiveness of the above two groups.

3.2 Polysaccharide–Protein Conjugate Vaccines

The use of capsular polysaccharides as immunoprophylactic agents in human disease caused by encapsulated bacteria is now firmly established. However, despite the many advantages of using capsular polysaccharides as human vaccines, one serious limitation to their general applicability is the poor immune response of these purified polysaccharides in infants (ROBBINS 1978; JENNINGS 1983). A promising area of research to overcome the deficiency of polysaccharide vaccines in infants is the design of a new generation of semisynthetic vaccines based on the conjugation (covalent coupling) of polysaccharides to protein carriers. The capsular polysaccharides of the bacteria primarily responsible for postneonatal meningitis have been conjugated to protein carriers; these include type b *H. influenzae* (SCHNEERSON et al. 1980; ANDERSON 1983; MARBURG et al. 1986), group A (JENNINGS and LUGOWSKI 1981b; BEUVERY et al. 1983a), B (JENNINGS and LUGOWSKI 1981b), and C (JENNINGS and LUGOWSKI 1981b; BEUVERY et al. 1983b) *N. meningitidis*, and type 6A *Strep. pneumoniae* (CHU et al. 1983). For the choice of carrier protein most investigators have used tetanus toxoid or diphtheria toxoid, two proteins currently used as infant vaccines. A recent innovation on this theme has been the use of a mutant-derived diphtheria toxin (CRM 197) (ANDERSON 1983) which is nontoxic. The significance of this protein is that because it does not require detoxifying by treatment with formaldehyde, all its amino groups remain underivatized, which greatly facilitates the conjugation process. The use of other potential bacterial proteins as carriers has not been extensively explored but in one case a serotype outer member protein of *N. meningitidis* was used (MARBURG et al. 1986).

The feasibility of the above approach is well established. More than 50 years ago the type 3 pneumococcal polysaccharide was coupled to horse serum globulin by diazotization of *p*-aminobenzyl ether substituents on the polysaccharide (GOEBEL and AVERY 1931). These workers also demonstrated that this polysaccharide conjugate and a similar conjugate made with the oligosaccharide repeating unit (cellobiouronic acid) of the type III pneumococcal polysaccharide, were able to induce polysaccharide-specific antibody in rabbits unresponsive to the pure polysaccharide (AVERY and GOEBEL 1931). It was also established (GOEBEL 1940) that the cellobiouronic acid conjugate was also able to confer immunity to challenge by live type 3 pneumococci in mice, and all these results have been substantiated by others. The coupling procedures employed in this early work, however, were far too drastic to be used on some of the highly sensitive polysaccharides currently used in human vaccines, and resulted in completely random coupling of the two molecules with the incorporation of highly undesirable structural features into the conjugates. Recently more comprehensive studies on polysaccharide–protein conjugates, specifically directed to their use as human vaccines, have been reported.

Two approaches to the synthesis of these conjugates have been described which involve the use of either random or selective activated sites in the polysaccharide as potential linkage sites (JENNINGS 1985). In effect, the choice of the method employed is largely dictated by the molecular size of the polysaccharide, the larger-sized polysaccharide of necessity requiring random activation. By contrast smaller oligosaccharides or small polysaccharides are adaptable to facile end-group activation. The use of large polysaccharides in conjugates probably originated because of the deliberate development of polysaccharides in this form for use as human vaccines (ROBBINS 1978; JENNINGS 1983). This strategy was based on the knowledge that polysaccharides of large molecular size function as superior immunogens (KABAT and BEZER 1958). Whether this remains true when polysaccharides are coupled to large immunogenic proteins is doubtful, and this is substantiated by evidence from recent comparative immunologic studies. Although these studies were not extensive, they do indicate strongly that both oligosaccharide–protein conjugates and small-sized polysaccharide–protein conjugates are able to perform as immunogens either than, or at least as well as, their larger-sized polysaccharide–protein conjugates (JENNINGS and LUGOWSKI 1981; ANDERSON 1983; MÄKELÄ et al. 1984). This result could be important in the development of polysaccharide–protein conjugate vaccines, because it is obviously advantageous to use as simple and easily defineable immunogen as possible. While this principle is compatible with end-group activation of polysaccharides, random activation interferes with their basic structures, promotes cross-linking, and results in complex ill-defined conjugates.

Some examples of conjugation using the random activation technique are as follows. The high molecular weight *H. influenzae* type b polysaccharide was conjugated to a number of proteins by activating the polysaccharide with cyanogen bromide and functionalizing the protein with an adipic dihydrazide spacer (SCHNEERSON et al. 1980). The coupling occurs through the formation of N-substituted isourea bonds, but both polysaccharide and protein retain unnecessary substituents, i.e., carbonate and underivatized spacer. The larger molecular weight group C polysaccharide from *N. meningitidis* was also conjugated directly to tetanus toxoid using 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (DEC) (BEUVERY et al. 1983b). This method introduces active O-acylisourea groups at many of the polysaccharides, carboxyl groups, some of which, in the presence of protein, form the required amide bonds of the conjugate. However, the introduction of these active groups can cause drastic structural changes in the polysaccharide as a result of internal lactonization (LIFELY et al. 1981), and the quenching of the remainder with ethanolamine introduces permanent unwanted substituents into the polysaccharide (BEUVERY et al. 1983b). Structural modification of the carrier protein is also likely using DEC as reagent.

To develop a more specific approach to the coupling of meningococcal polysaccharides to tetanus toxoid, they were monofunctionalized prior to conjugation (JENNINGS and LUGOWSKI 1981). Controlled periodate oxidation introduced unique terminal free aldehyde groups into the group B and C polysaccharides and also in the group A polysaccharide following the reduction of its terminal 2-acetamido-2-deoxy-D-mannose residue (Fig. 2). The oxidized polysaccharides were then coupled to tetanus toxoid by reductive amination. The advantages of this method over those previously described are that it minimizes the possibility of polysaccharide or protein modifica-

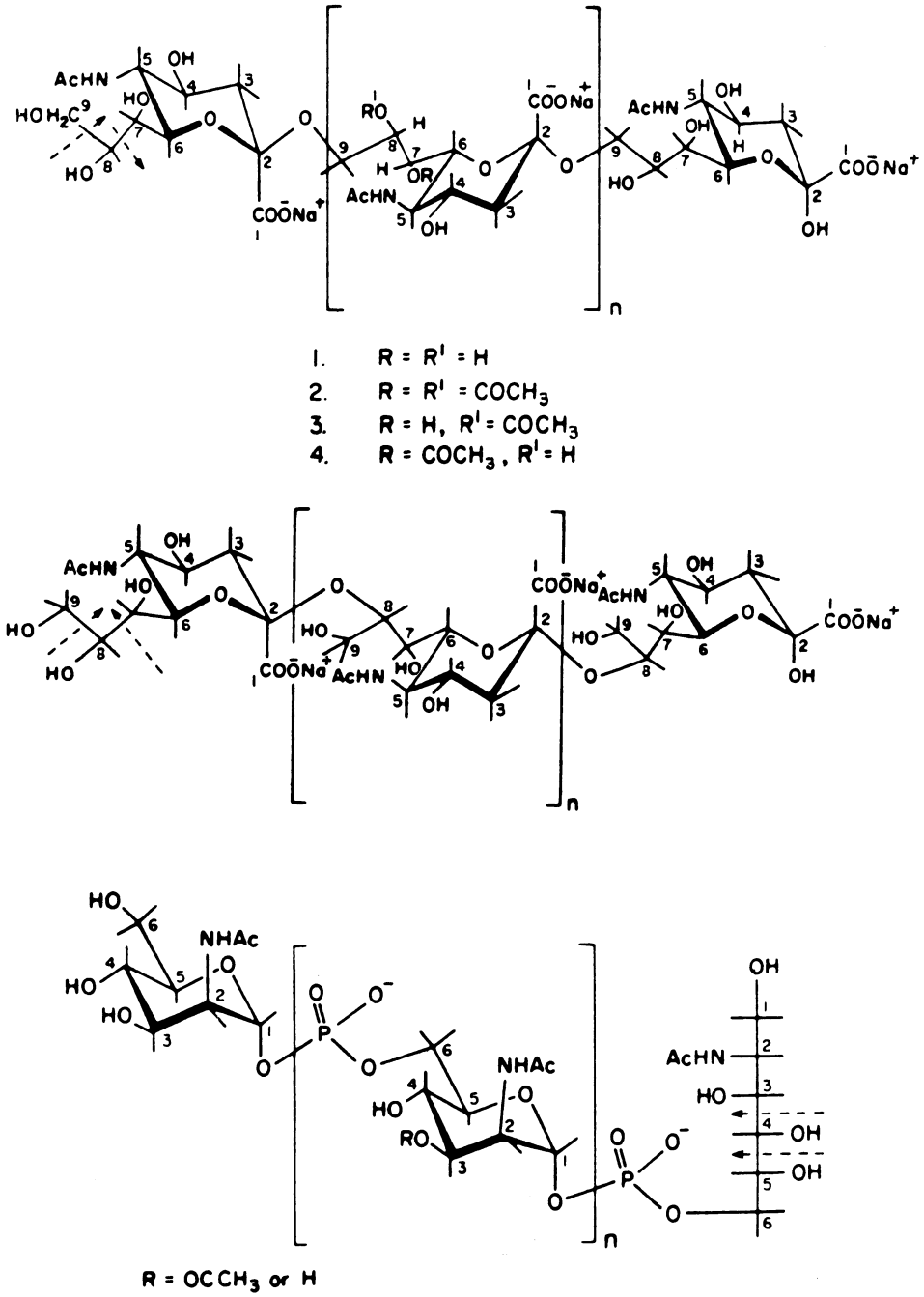


Fig. 2. Structures of meningococcal group C (*upper*), B (*middle*), and end-group reduced A (*lower*) capsular polysaccharides, depicting their periodate-sensitive terminal residues

tion, and eliminates cross-linking. An even more simple method of conjugation would be the direct coupling of the unoxidized polysaccharides to protein through their end-group reducing residues, as has been accomplished with small oligosaccharides (SCHWARTZ and GRAY 1977). Although this has been accomplished using the depolymerized type b *H. influenzae* polysaccharide through its terminal ribofuranosyl residue (ANDERSON 1983), the method proved ineffective using the group B and C meningococcal polysaccharides (JENNINGS and LUGOWSKI 1981). This lack of activity could be associated with the larger molecular size of these latter polysaccharides, but it has also been established that 2-keto-3-deoxy-glyculosonic acid residues, which include the sialic acid end-group of the group B and C meningococcal polysaccharides, are extremely ineffective residues through which to carry out reductively aminated coupling to protein (ROY et al. 1984).

A requirement for using the monofunctional approach to couple polysaccharides to proteins is that the former are first obtained in an appropriate molecular size. This can be achieved by depolymerizing the native polysaccharide and subsequently fractionating the component fragments by gel filtration. Depolymerization methods employed have included autohydrolysis (JENNINGS and LUGOWSKI 1981), acid hydrolysis (ANDERSON 1983), and controlled periodate oxidation (ANDERSON et al. 1986). While the latter method still conforms to the principle of selective end-group activation, the bifunctional nature of the resultant saccharide fragments invokes the distinct possibility of the occurrence of some cross-linking during the coupling procedures. Recently, ultrasonic irradiation has been used to depolymerize a number of bacterial polysaccharides (SZU et al. 1986b). This method is independent of structure and, unlike other methods of depolymerization, has the distinct advantage of producing fragments of a finite and similar size (ca. 50000 kd).

The group A and C meningococcal polysaccharide conjugates were able to induce in mice the production of polysaccharide-specific IgG antibodies indicative of the conversion of the polysaccharide to T cell dependent antigens (JENNINGS and LUGOWSKI 1981). This is demonstrated in Fig. 3, which depicts ELISA assays of the antisera

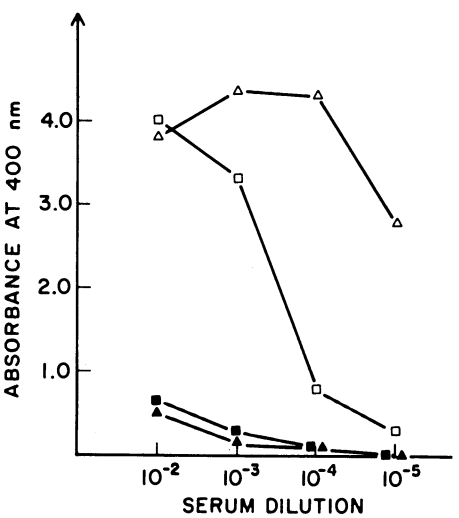


Fig. 3. Titration of the anti-group C polysaccharide–tetanus toxoid conjugate mouse IgG antibodies in ELISA against different antigens. The wells of the ELISA plates were coated with homologous conjugate (△) and the homologous group C polysaccharide (□). Titrations of the preimmune sera with the same antigens are marked with identical but solid symbols (▲, ■)

from mice previously injected with the group C polysaccharide alone and in the form of its tetanus toxoid conjugate. In contrast to the pure group C polysaccharide, which elicited a very weak immune response in mice, the conjugate was able to induce high levels of polysaccharide-specific IgG antibodies. This phenomenon had been previously reported for mice immunized with the type b *H. influenzae* polysaccharide–tetanus toxoid conjugate (SCHNEERSON et al. 1980) and appears to be quite general as it has also been observed in similar animal experiments using other polysaccharide–protein conjugates produced by many diverse procedures (ANDERSON 1983; BEUVERY et al. 1983a, b; MÄKELÄ et al. 1984).

From the immunologic properties of the above conjugates in animals one could predict that conjugate vaccines would have obvious potential in the formulation of future infant vaccines against bacterial meningitis, and this has been amply verified in recent highly successful field trials. Two-month-old infants were immunized with protein-coupled oligosaccharides derived from the capsule of *H. influenzae* type b, and following a second immunization with the same conjugate, a distinct booster effect (anamnestic response) accompanied by the production of polysaccharide-specific IgG antibodies was detected (ANDERSON et al. 1985). Enhancements of polysaccharide-specific responses in infants have also been obtained using an *H. influenzae* type b–diphtheria toxoid conjugate (ESKOLA et al. 1985). The above studies indicate that polysaccharide–protein conjugate vaccines will be valuable immunoprophylactic agents in the prevention of infant meningitis and that this technology is obviously capable of extension into other areas of immunoprophylaxis. However, the final optimal configuration of these conjugate vaccines will depend on criteria of their acceptance as biologicals for human use, as yet undefined, and on maximizing their immunologic performance by varying the many structural parameters involved in their synthesis. This latter aspect will probably be of great importance in the future development of the technology, and already there is some evidence to suggest that the priming responses in infants are dependent on the length of the polysaccharide chains (ANDERSON et al. 1986).

4 Group B Meningococcal Polysaccharide

4.1 Poor Immunogenicity of the Group B Meningococcal Polysaccharide

The poor immunogenicity of the group B meningococcal polysaccharide and the structurally identical *E. coli* K1 capsular polysaccharide (WYLE et al. 1972) precludes their use as vaccines against meningitis caused by group B meningococcal and *E. coli* K1 organisms. Although group B meningococcal organisms are able to produce low levels of group B polysaccharide antibodies in animals and humans, these antibodies are, with one notable exception, almost exclusively of the IgM isotype, and of relatively low affinity (MANDRELL and ZOLLINGER 1982). The exception to the rule was identified in a recent observation that group B polysaccharide-specific monoclonal antibodies of the IgG isotype could be produced by injecting a specialized strain of autoimmune NZB mice with whole group B meningococcal organisms (FROSCH et al. 1985).

The poor immunogenicity of the group B meningococcal polysaccharide is probably attributable to immune tolerance induced by the close structural similarity exhibited by the polysaccharide and cross-reacting oligosaccharides found in the glycopeptides of human and animal fetal brain (FINNE et al. 1983a, b). The polysialosyl chains are carried by a glycoprotein involved in neural cell adhesion, which is termed a neural cell adhesion molecule (N-CAM) (EDELMAN 1983) and which has also been identified in other human and animal tissue (RIEGER et al. 1985; RUTISHAUSER et al. 1985; LYLES et al. 1984; JAMES and AGNEW 1987). For the most part the polysialosyl chains on N-CAM are developmental (fetal) antigens, and their length is reduced rapidly during maturation; however, there is some evidence that they may even persist in specific adult eel tissues (JAMES and AGNEW 1987). The sialooligosaccharides from human and rat fetal brain have been demonstrated to bind to group B meningococcal polysaccharide-specific antibodies and to inhibit the homologous serologic reaction of these antibodies (FINNE et al. 1983b). These sialooligosaccharides are large, consisting of up to 12 linear α-(2 $\rightarrow$ 8)-linked sialic acid residues (FINNE and MÄKELÄ 1985), and it has been estimated that the common epitope on the group B meningococcal polysaccharide and N-CAM is only contained in an oligosaccharide of at least ten sialic acid residues (JENNINGS et al. 1985b; FINNE and MÄKELÄ 1985). The fact that such an oligosaccharide is much larger than the maximum size of an antibody site (KABAT 1966) is probably due to the fact that the common epitope is conformationally controlled (JENNINGS et al. 1984, 1985). This has now been confirmed by ^{13}C and $^{1}HNMR$ studies on oligosaccharide fragments from the group B meningococcal polysaccharide. These studies demonstrate that only the five or six inner residues of a decamer $(NeuAc)_{10}$ are in the precise conformation to bind to group B polysaccharide-specific antibodies (MICHON et al. 1987b).

4.2 Group B Polysaccharide – Outer Membrane Protein Complexes

Noncovalent group B meningococcal polysaccharide complexes with meningococcal outer membrane proteins have been prepared in several laboratories (ZOLLINGER et al. 1979; FRASCH and PEPPLER 1982; MORENO et al. 1985a). An initial clinical evaluation of two of the above preparations (ZOLLINGER et al. 1979) demonstrated that the human response to the group B polysaccharide was enhanced when it was injected in association with the serotype 2 outer membrane protein. However, the enhancement, although encouraging, was only meagre, and the antibodies produced were mostly of the IgM isotype. More recent experiments in mice have largely confirmed the above immunologic properties of this type of complex. In these studies the complexes were prepared by different methods using different serotype proteins (FRASCH and PEPPLER 1982; MORENO et al. 1985a). Again, in all cases a modest enhancement in the production of group B polysaccharide-specific antibodies was observed and the antibodies were mostly of the IgM isotype. There is evidence to suggest that the group B meningococcal polysaccharide and serotype proteins do form true complexes (ZOLLINGER et al. 1979; MORENO et al. 1985a) and that these complexes are probably based on hydrophobic interactions between the protein and lipid components on the polysaccharide (GOTSCHLICH et al. 1981; LIFELY et al. 1987). There is also some evidence to suggest that synergism exists between the group B polysaccharide and serotype

proteins and that it is group specific. This is because when similar complexes were made using the group C meningococcal polysaccharide, they failed to induce in mice a similar enhancement in the immune response to the group C polysaccharide (MORENO et al. 1985a).

It has been hypothesized that the enhanced immunogenicity of the group B polysaccharide in its complexed form is due to the stabilization of its conformation and/or the maintenance of its structural integrity (LIFELY et al. 1987). This latter aspect concerns the tendency of the group B polysaccharide to form interresidue lactones and to be readily cleaved by neuraminidase. However, although it was demonstrated that the stability of the group B polysaccharide could be substantially increased when it was in the form of its Al^{3+} salt, the immunogenicity of the B polysaccharide in this form was not enhanced until it was again complexed with meningococcal outer membrane protein (MORENO et al. 1985b). In summary, while it has been clearly demonstrated that the above group B polysaccharide complexes do induce enhanced responses to the polysaccharide, these responses are meagre, and the rapid decay of these responses and the virtually exclusive production of B polysaccharide-specific antibodies of the IgM isotype are not properties normally associated with an effective human polysaccharide vaccine.

4.3 Chemical Modification of the Group B Meningococcal Polysaccharide

The direct covalent coupling of the group B polysaccharide to tetanus toxoid yielded a conjugate which failed to induce a significant polysaccharide-specific response in either rabbits (JENNINGS and LUGOWSKI 1981) or mice (JENNINGS et al. 1986). This, together with the inability to detect bactericidal activity in the mouse antisera, suggests that the direct coupling of the B polysaccharide to a protein carrier will probably be of little importance in the production of a future human vaccine against group B meningococcal meningitis. This failure prompted interest in the direct chemical modification of the group B polysaccharide. This was done with the idea of creating synthetic epitopes capable of modulating the immune response in such a way as to produce enhanced levels of cross-reactive B polysaccharide-specific antibodies (JENNINGS et al. 1986). There is only one previous report (ØRSKOV et al. 1979) of a similar approach but instead of a synthetic antigen a form variant of *E. coli* K1 was used as the vaccine. The *E. coli* K1 polysaccharide is structurally identical to the group B meningococcal polysaccharide (Tables 2 and 4), but the above variant of the *E. coli* K1 organism produces a capsular polysaccharide randomly O-acetylated at O-7 and OL-9 of its sialic acid residues. While this variant (OAC^+) of the *E. coli* K1 organisms proved to be relatively more immunogenic in rabbits than the non-O-acetylated (OAc^-) variant, producing antibodies having specificities for both the O-acetylated and the non-O-acetylated polysaccharides, the enhancement in immunogenicity observed by ØRSKOV et al. was only marginal.

In selecting possible chemical modifications of the group B polysaccharide (JENNINGS et al. 1986), two major requirements had to be met. First, the chemical modification had to be accomplished with facility and with the minimum of degradation of the polysaccharide. Secondly, in order to produce cross-reactive B polysaccharide-specific antibodies, the antigenicity of the modified polysaccharide to B polysaccharide-

specific antibodies had to be preserved, and this was shown to be dependent on the retention of both carboxylate and N-carbonyl groups (JENNINGS et al. 1986). The most successful modification which satisfied the above criteria was that in which the N-acetyl groups of the sialic acid residues of the B polysaccharide were removed by strong base and replaced by N-propionyl groups (Fig. 4). Finally, due to the poor immunogenicity of the N-propionylated B polysaccharide itself in mice, it was subsequently conjugated to tenatus toxoid, thus yielding a synthetic vaccine.

The potential of the N-propionylated B polysaccharide to enhance the induction of group B polysaccharide-specific antibodies was demonstrated by comparison of the B polysaccharide-specific antibody response induced in mice by the N-propionylated- and N-acetylated B polysaccharides and their respective tetanus toxoid conjugates (Fig. 5) (JENNINGS et al. 1986). While antisera from mice immunized repeatedly with the polysaccharides alone showed no significant binding to the tritium-labeled group B polysaccharide, binding was detected in antisera obtained from mice immunized with each respective tetanus toxoid conjugate. However, the N-propionylated conjugate produced much higher levels of B polysaccharide-specific antibody than the homologous N-acetylated B polysaccharide conjugate, a pronounced booster effect being particularly noticeable following three injections. Interestingly, while the N-acetylated B polysaccharide–tetanus toxoid conjugate gave only a small booster effect in terms of B polysaccharide-specific antibody, it was able to boost this same response significantly in mice which had been previously primed with two previous injections of the N-propionylated B polysaccharide conjugate. This booster effect is indicative of a memory effect based on the participation of T cells, and this is also substantiated by the large proportion of B polysaccharide-specific antibodies of the IgG isotype produced in this immune response (Fig. 5).

Because the N-propionylated B polysaccharide–tetanus toxoid conjugate is capable of inducing B polysaccharide-specific IgG antibodies in mice (JENNINGS et al. 1986) it must be considered as a prototype vaccine against meningitis caused by both group B *N. meningitidis* and *E. coli* K1. Experience would indicate that an immunogenic

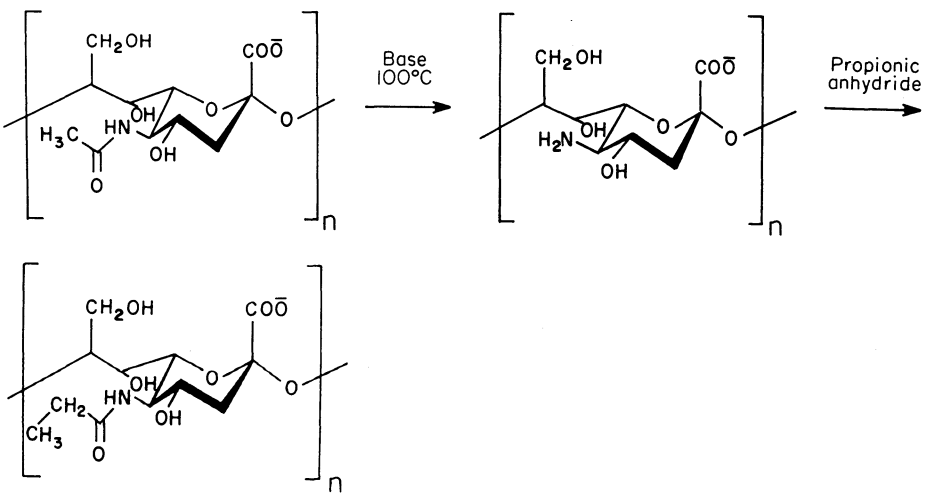


Fig. 4. Reaction sequence leading to the formation of the N-propionylated group B polysaccharide from the native group B polysaccharide

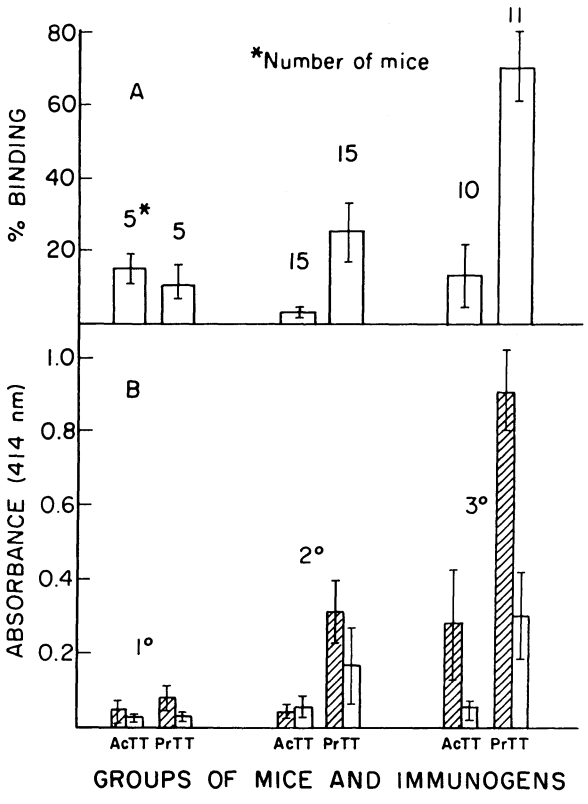


Fig. 5. A Binding of the group B meningococcal polysaccharide to antibodies induced in mice by the group B polysaccharide–tetanus toxoid conjugate (*AcTT*) and the saccharide–tetanus toxoid conjugate (*PrTT*). Mean values ± standard deviation are shown. **B** Corresponding ratio of IgG (*hatched bars*) to IgM (*open bars*) antibodies in the above antibody responses. Means of the optical densities at 414 nm ± standard deviations are shown. The mice are grouped according to the immunogen used and whether they received one (*1°*), two (*2°*), or three (*3°*) injections

form of this capsular polysaccharide would be the ideal vaccine candidate although on the basis of structural homology between the group B polysaccharide and the oligosaccharides of glycopeptides associated with fetal brain (FINNE et al. 1983b) it might be inferred that its success as a vaccine could only be achieved at the risk of breaking tolerance. Certainly the production of B polysaccharide-specific IgG antibodies in mice indicates that the immunogen is capable of breaking tolerance although it must be emphasized that the precise consequences of this have not yet been determined.

Of significance to the above dilemma was a series of further observations made on the N-propionylated group B polysaccharide-specific mouse antiserum (JENNINGS et al. 1987). The antiserum was demonstrated to be highly bactericidal for group B meningococcal organisms and to contain two populations of N-propionylated group B polysaccharide-specific antibodies, only one of which cross-reacted with the group B meningococcal polysaccharide. Of particular significance was the fact that all the bactericidal activity was associated with the antibody population that did not cross-react with the group B polysaccharide and presumably therefore would not cross-react with human tissue antigens. From this evidence it can be inferred that the N-propionylated group B polysaccharide mimics a unique epitope on the surface of group B meningococcal organisms not expressed on the exogenous group B meningococcal polysaccharide. These observations could be of importance in the eventual production of a vaccine against meningitis caused by group B *N. meningitis* and *E. coli* K1.

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Visualization of the Bacterial Polysaccharide Capsule

M. E. BAYER

1 Introduction

The capsule covers the surface of many species of bacteria living in oceans, fresh water, and soil. Capsules are also characteristically present in symbiotic and parasitic bacteria which grow in association with plants and animals (DUDMAN et al. 1977; COSTERTON et al. 1981). The term capsule is used here for the material composed of large molecular weight polysaccharide which is, in contrast to slimes, attached to the cell surface. Other terms for capsular material are exopolymers, glycocalyx (GEESEY 1982), and extracellular polymeric substances (BOWLES and MARSH 1982). Capsules can be characterized by their chemical composition, their physical properties, their physiologic activities affecting the cell's growth and survival, their antigenicity, and, in molecular terms, by their expression

Fox Chase Cancer Center, Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

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(KAUFFMANN 1966; ORSKOV et al. 1977; ROBERTS et al. 1988) and gene structure (ROBERTS et al. 1986; SUTHERLAND 1977; TROY 1979; JANN and JANN 1985).

Capsules and also the proteinaceous S-layers (KOVAL and MURRAY 1986; SMIT 1986; SLEYTR and MESSNER 1983) establish the outermost contact zone of many bacteria. Capsules reveal a wide spectrum of activities such as interactions with ions, macromolecules, viruses, and solid surfaces of man-made and natural, inorganic and organic material (ALLISON and SUTHERLAND 1987; FLETCHER and FLOODGATE 1973; KRELL and BEVERIDGE 1987). The study of capsular synthesis, regulation (TORRES-CABASSA and GOTTESMAN 1987), assembly, and export (WHITFIELD et al. 1984a, b; SUTHERLAND 1985) is of direct relevance to immune biology as well as to clinical microbiology (see BROWN and WILLIAMS 1985). Much work has been focused on the interactions of polysaccharide and specific antibody (VAN DER LEY et al. 1986b), on the capsule's barrier functions toward viruses (BERNHEIMER and TIRABY 1976; VAN DER LEY et al. 1986a) and antibiotics (see BROWN et al. 1980), and on the role of the capsule as mitogen (LUCAS and ASSER 1986) and interleukin-like substance (HARVEY et al. 1987); furthermore, the studies on the functions of capsular polysaccharides during invasion of eukaryotic cells (WOOD 1960; ANDERSON and WILLIAMS 1985) and during microbial destruction of tissues (KAMIN et al. 1986) are of the utmost importance for the clinical microbiologist, especially in the context of the immuno compromised host. Some of these functions can be correlated to the capsular charge and to steric hindrance by the capsular material. However, not only do capsules serve as shields protecting the bacterium, but also the lipopolysaccharides (LPSs) have been shown to play a major role in the resistance of the bacterium, e.g., to killing by serum (TOMAS et al. 1986). Capsulated microorganisms are found in abundance in clinical and natural isolates, and the importance of the capsule for the attachment of cells to natural surfaces and to man-made surfaces used in medicine and industry cannot be overemphasized (COSTERTON et al. 1981). The physicochemical features of the capsule appear to constitute a significant advantage for survival of the capsulated cell in the natural environment. In contrast to the wealth of information available on the chemical composition and biologic activities of capsules, little is known about the formation and structural organization of the assembled macromolecules as they constitute the capsular domain of the living bacterium. As will be demonstrated later, this apparent lack of knowledge is to a large degree due to the biophysical features which generate substantial technical obstacles to their study. The capability of the capsules to function in a variety of roles makes the close examination of the structural organization of capsules essential. Furthermore, the physical properties of bacterial capsules are often very similar to hydration shells of other biologic systems, such as macromolecular assemblies of eukaryotic cell surfaces and matrices of the intercellular space. Study of the structural organization of capsules requires joint efforts from fields of microscopy, NMR, X-ray, and other biophysical techniques. In this report both in vitro and in vivo aspects of capsules will be addressed, with the focus being on the ultrastructural and conformational arrangements of the capsular macromolecules.

Capsules have been reported to consist to more than 95% of water (SUTHERLAND 1972; WILKINSON 1959). Obviously such a high water content is apt to cause

difficulties in the attempts to preserve capsular structures whenever fixation, dehydration, or staining is required in light and electron microscopy (KELLENBERGER 1987, 1980; BAYER and THUROW 1977).

The concept of a bacterial capsule was derived largely from the light microscopic studies of earlier years. However, it was not always possible to demonstrate capsules in the light microscope and to differentiate between capsule and the membranes of the cell envelope. Uncertainties remained in the preparation techniques as well as in interpretation of the data (see DUGUID 1951). Some of the staining procedures were typically plagued by the fact that the charged capsules acted as a barrier for dye molecules of identical polarity. The ambiguities in light microscopic interpretation made the classification of bacterial strains by approaches other than microscopy necessary. Serologic methods were indeed much more attractive and practical. But here again, the presence of the capsule interfered with the immunologic identification of those cell surface antigens that were not directly accessible to immune reactions. As a consequence of their localization, such antigens were shielded from the formation of identifying products. Labeling of cells in thin sections makes it possible nowadays to identify normally inaccessible antigens of cell envelope and cytoplasm without the need to permeate the protective layer (see KELLENBERGER et al. 1980; BAYER et al. 1986).

In the following section the size, shape, and ultrastructure of polysaccharide capsules will be described. We will present data mostly, but not exclusively, from gram-negative organisms, especially from *Escherichia coli* species. We will deal with preservation procedures developed for the study of the capsular organization. The contribution of these methods to the interpretation of the capsular structure will be demonstrated and correlated to recent findings relating to capsular synthesis and export.

The Tools

The presence of a capsule can be established in a number of ways, usually with a combination of physical/chemical and biological approaches. Currently, most feasible methods consist of: 1) specific and fast serological reactions such as ELISA, microsphere agglutination and radioimmune assays; 2) methods of molecular genetics which permit the construction of DNA probes used for recognition of capsular gene(s), an approach that has been reported as useful and rapid (ROBERTS et al. 1986); 3) biochemical and biophysical analysis; 4) virological identification by capsule specific bacteriophage; 5) light microscopy and electron microscopy. The light microscope is useful as diagnostic as well as research tool, since it allows the direct visualization of bacterial capsules and their interaction with antisera and other natural or synthetic macromolecules. The advantage of light microscopy is its rapidity and the fact that unfixed fully hydrated cell preparations can be used. Higher resolution, however, requires the use of electron microscopy, which makes in most instances a stabilization before dehydration of the specimen necessary.

2 General Features of Capsules

The surface of a noncapsulated gram-negative microorganism is represented by LPSs, outer membrane (OM) proteins (LUGTENBERG and VAN ALPHEN 1983), and (under some conditions) phospholipids (INOUYE 1979). The diameter of a rapidly growing *E. coli* K29, excluding the capsule, is about 0.7 μm, as determined from freeze-fractured preparations of unfixed *E. coli* K29. Under well-expressed conditions the capsule of *E. coli* K29 extends the cell surface boundary by more than 1 μm into the medium, much beyond the domain of the O-antigenic chains of LPSs, which can measure up to 1/50 of a micrometer in length. Capsules surround the microorganisms totally and in a more or less even layer of equal thickness. Asymmetric capsular expression has been reported for *Rhizobium* species (VASSE et al. 1984).

In physical terms, capsules are viscous, pliable structures which survive moderate shaking of suspension cultures. In suspension cultures, small amounts of capsular material are released into the growth medium, similar to the release of LPSs into the growth medium (ROTHFIELD and PEARLMAN-KOTHENCZ 1969). Increased mechanical forces such as high pressure or shearing by strong turbulence, as well as exposure to alkaline conditions, will distort and eventually disintegrate the capsule, releasing into the medium large amounts of the polymer. Controlled shearing forces, in combination with detergents, are being used for isolation of capsular material. Purified capsular material consists of fibrous elements which, due to their tendency to associate with each other, form fibers of considerable length (we measured fibers several hundred micrometers long).

Characteristic biophysical features of in vivo capsules are their high water content (SUTHERLAND 1972) and their degree of negative charge. In a given environment (buffer, growth medium) the charge of the capsule depends on the composition of the polysaccharide, e.g., its content of pyruvates and carboxyl groups. Measurement of electrical charge of a bacterium can be achieved by electrophoresis of suspended bacteria. The data show that absence of capsular polysaccharide (in capsule minus mutants) permits a reduced negative charge of the LPSs to take effect. The presence of uncharged O-antigenic polysaccharide does not seem to affect the surface charge (BAYER and SLOYER 1988). Due to their charge, polysaccharides may bind large quantities of water and ions. This water causes a major problem to the microcopist: In order to obtain a faithful microscope image and to prevent the collapse of the polysaccharide, the water has either to be retained in the preparation ("wet" preparation) or to be substituted with other types of molecule during the dehydration and embedding procedures. Both approaches are feasible for a morphologic study of capsules. Although the electron microscope may be considered the tool of choice, we found the light microscope irreplaceable in studies concerning dimensions and shape of hydrated capsules, particularly in those cases in which established procedures of stabilization of the capsule failed and new methods had to be developed. In the subsequent text a variety of approaches to structural studies will be described, and the results will help to form a picture of the in vivo capsule.

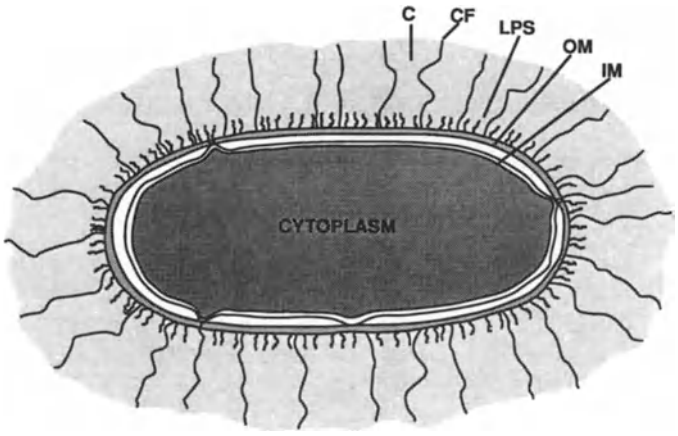


Fig. 1. Diagram of capsulated *E. coli* shown as a thin section. A slight plasmolysis separates inner membrane (*IM*) from outer membrane (*OM*). The capsular domain consists of polysaccharide fibers (*CF*) which originate from the OM. Only a few fibers are shown here; a capsule consists of more than 3000 fibers. Also lipopolysaccharide (*LPS*) molecules, depicted as short fibers, are anchored in the OM. OM and IM are held together during plasmolysis at the areas of adhesion, some of which are shown as sites of export of CF and LPS. The chromosome and ribosomes are not shown

3 The Capsule as Seen Under the Light Microscope

Capsules have been difficult to study using the light mircoscope owing to the fact that procedures of classic histology and bacteriology are often both unreliable and inadequate to stain the capsules. DUGUID (1951), in a critical evaluation of these procedures, found that a variety of capsule stains and mordants not only had a poor affinity to the capsule polysaccharides, but also caused shrinkage and gross distortion during fixation and drying which resulted in destruction of the capsule and disruption of its relationship to the cell membranes. DUGUID also recognized that a number of the "wet film" methods were successful in outlining a capsule, with the India ink treatment being the most reliable.

3.1 Visualization with India Ink

This method requires the exposure of the bacteria to India ink (HAMM 1907; PREISZ 1911; DUGUID 1951). The high optical density of the ink particles reduces drastically the light transmission in the area around the cell, whereas the more transparent bacterium permits the light to pass through. Under the microscope the bacterial capsules therefore appears to the viewer as a bright area sharply offset against a dark background, providing a typical "negative stain" (Fig. 2). This procedure will work only if the carbon particles are large enough to be hindered from penetrating the internal portions of the capsule. If the ink particles are sufficiently small, they will penetrate the capsular space, rendering the capsule invisible under the microscope.

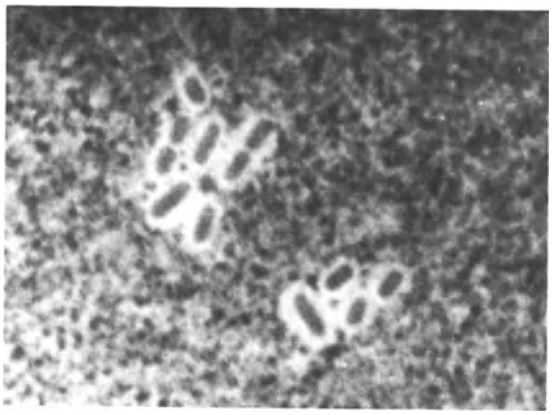


Fig. 2. India ink ("negative") staining reveals the capsule of *E. coli* K29 as a white halo around each cell. Unfixed, "wet" preparation. × 2000

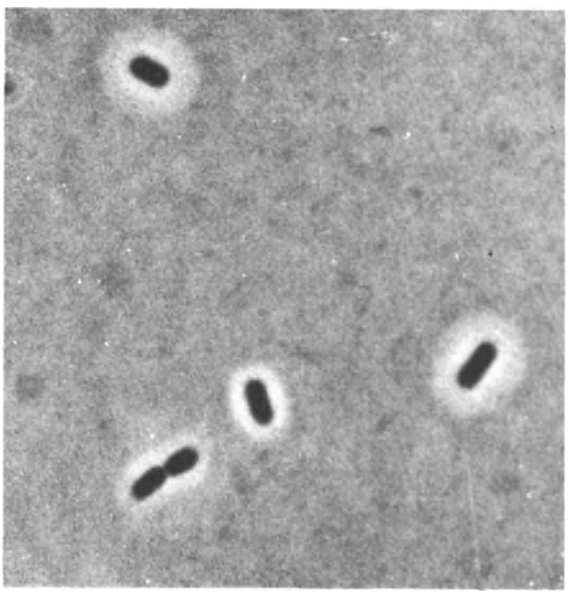


Fig. 3. Capsulated *E. coli* K29 suspended with polystyrene latex beads (0.1 μm) and observed in phase contrast. A relatively large capsule (approximately 1.5 μm thick) is visible in some of the cells. × 2000

Instead of carbon, particles of other composition can be used, such as colloidal silver (HAMM 1907) and latex beads (Fig. 3) (BAYER, unpublished). Simplicity and speed make these "negative staining" techniques very feasible for the evaluation of capsules. Occasionally, the capsule engulfs more than one cell, an effect often observed in dividing cells and also in older suspension cultures. Cells attached to surface often share the capsule with their neighbors. Observations of suspension cultures of *E. coli* K29, K30, and K1 (during the growing phase and the stationary phase) reveals considerable variation in the degree of capsulation of the individual cell. This variation is seen in suspension cultures as well as in cells taken from

colonies grown on agar plates. The India ink procedure allows one to estimate in cultures the number of cells with a well-expressed capsule relative to uncapsulated organisms. However, one should be aware that the presence of small amounts of capsular antigen cannot be recognized by this procedure and other tests, serologic or virologic, have to be included in a screening procedure.

3.2 Visualization with Antibodies

Of great value to the investigation of capsules has been the "swelling" reaction. NEUFELD (1902) reported that addition of homologous agglutinating antibody to pneumococcus cultures caused, within minutes, an apparent swelling (German: "QUELLUNG") of the cells to twice or three times the size of untreated pneumococci. He did not observe this reaction with highly diluted antiserum or nonagglutinating control serum. It is noteworthy that an analogous reaction had already been observed in 1896, when ROGER reported that upon addition of vaccine serum to cultures of the fungus *Oidium (candida) albicans*, a hyaline colorless mass accumulated around the cell walls, whereas normal serum did not have such an effect. NEUFELD also described the "quellung" zone around the capsulated pneumococci as structureless and glassy. Furthermore, he reported that the reaction product interfered with subsequent bacterial staining procedures. When he removed the glassy material by heating of the specimen, the stainability of the bacteria returned.

The product of the "swelling" reaction is a complex between homologous immunoglobulins and the capsular polysaccharide. Its increased refractive index makes the capsular domain recognizable under the light microscope. DUGUID (1951) and others reported that the size and the general shape of the capsule remained the same in India ink and in antibody-treated cells. Thus, significant swelling does not seem to take place during the interaction with antibody. With the use of the phase contrast microscope TOMCSIK (1956) was able to observe substructures such as polar condensations and striations in the capsules of *Bacillus megaterium*. The *B. megaterium* capsule contains not only polysaccharide but also a peptide, and the contribution of either component to the observed substructures remains to be established.

More recently, other methods have been developed to establish the presence of bacterial capsules. For diagnostic purposes, automatic cell counting and cell sorting as well as immunofluorescene methods are being employed with the primary goal of obtaining and quantitating a specific signal while the preservation of the capsular organization is not emphasized in these techniques.

3.3 Visualization with Lectins

Capsular exopolysaccharides can also be identified with the aid of carbohydrate-specific plant lectins. These proteins have been described as useful tools for light and electron microscopy in diagnostic applications and in high resolution studies

(MOBLEY et al. 1984). The specific interaction of carbohydrate and lectin provides information both on the chemical composition of the exopolysaccharide and on conformational and steric aspects of the epitope within the macromolecule. Using lectins specific for *Rhizobium* spp., VASSE et al. (1984) and TSIEN and SCHMIDT (1981) established the presence of an asymmetric capsule covering one portion of the bacterium only; BIRDSELL et al. (1975) used concanavalin A to localize teidsoic acid in the cell wall of *Bacillus subtilis.*

3.4 Visualization with Cationic Proteins

Capsules can also be visualized by making use of the charge attraction between the capsular (anionic) polysaccharides and (cationic) proteins. Electrophoresis revealed that most capsular and cell surface polysaccharides are negatively charged in the pH range and ionic environment of conventional growth media (BAYER and SLOYER 1988). Adsorption of positively charged molecules would increase mass and density of the organelle. When our laboratory employed cationic proteins to improve capsular stabilization, we found the approach very useful for both light and electron microscopy. After addition of polycationized ferritin to capsulated *E. coli* strains, the capsule becomes visible with both the phase contrast microscope and the differential interference contrast microscope (Fig. 4). Capsules delineated by either India ink or antibody treatment show a size distribution very similar to that of capsules treated with cationized ferritin. The charge attraction between capsular polysaccharide and a variety of proteins was studied by TOMCSIK and GUEX-HOLZER (1954). These

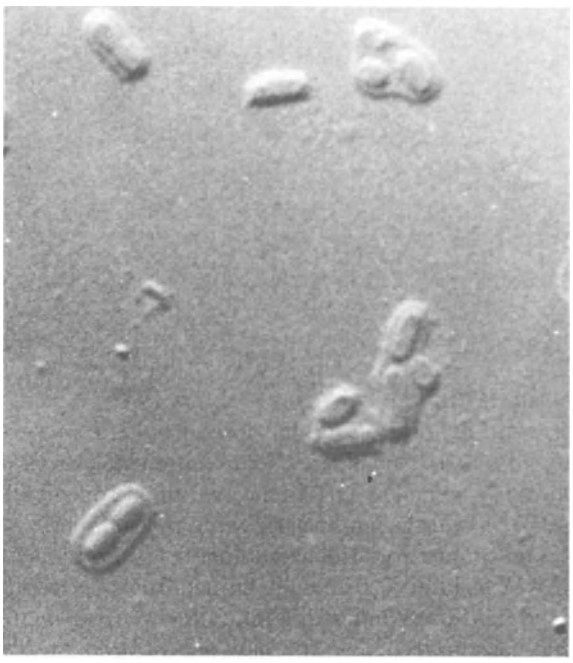


Fig. 4. Capsulated *E. coli* K1 after interaction with cationized ferritin. Note the capsulated and noncapsulated cells. Some of the cells share a capsule. Nomarski contrast. × 2000

authors proposed that a pH-dependent, salt-like, reversible complex of protein and capsule can be established at a small range of pH, mostly between pH 2 and 5, in a reaction representing essentially a precipitation. The authors also noted a potentially deleterious side-effect: The capsules in *Bacillus* species were seen to shrink in the presence of basic proteins, while they swelled in other instances. The advantage of using cationized ferritin is that the interaction of protein and capsule takes place at physiologic pH (6.5–7.2). This procedure enabled us to prevent collapse of those capsules which had previously been difficult to preserve, for example capsules of *E. coli* K1 strains.

In conclusion: Light microscopy of negatively stained or antibody-treated microorganisms define the capsular domain. The capsule excludes comparatively large particles (India ink or Latex) from entering, whereas homologous antibody is able to diffuse into the polysaccharide structure and to form crossbridges. The charge-dependent interaction of cationized ferritin with the anionic charges of the capsular polysaccharide reveals an ultrastructural aspect similar to that of antibody treated capsules. These light microscopic methods are most useful for: 1) the observation of unfixed cell suspensions, 2) circumvention of the potentially deleterious dehydration and embedding steps. One can expect that the use of charged markers in combination with advanced optical microscopy techniques will provide the investigators with new tools to study hydrated domains of bacterial surfaces and microbial colonies as well as glycocalyces of eukaryotic cells and tissues.

4 Electron Microscopy of the Capsule

Early attempts to employ electron microscopy in the study of bacterial capsules revealed clearly some of the problems inherent in the preservation of highly hydrated structures: In vivo, capsular polysaccharides are present only in low concentration and their low electron scattering capability practically eliminates their indirect visibility under the conventional microscope. However, when the cells were reacted with homologous antiserum, sufficient contrast was generated to provide a glimpse of the capsular domain in what was probably the first visualization of a capsule by means of electron microscopy (MUDD et al. 1943). The micrographs showed a zone of slightly increased contrast around the surface of antiserum-treated and air-dried pneumococcus cells. At that time, methods for contrast enhancement such as shadow casting with heavy metals (WYCKOFF 1949) had not been developed and contrast was provided only by the mass density of the object. Since neither the untreated bacteria nor the cells treated with heterologous serum revealed the hazy material, the reaction with the homologous serum was assumed to involve an "increase in thickness and density of the capsular gel." With the development of contrast-enhancing methods such as shadow casting and replica techniques (LABAW and MOSLEY 1954) as well as with the availability of ultrathin sectioning, the resolution of the electron microscopic image increased significantly.

As a consequence, also the artifacts of preservation became increasingly obvious. Such artifacts are especially severe in cells and tissues with high water content. Except for freeze-fracturing methods and the currently still immature cryosectioning techniques, all of today's preparation methods require that after fixation, most of the water of the specimen be removed and replaced by an embedding agent. Since the procedural steps are important for the successful preservation of capsular structures, the effects of fixation and dehydration on capsules will be outlined below.

4.1 Fixation

The use of conventional means of fixation for the stabilization of polysaccharide capsules of a number of *E. coli* strains is of questionable value (BAYER and THUROW 1977). Chemical fixation (aldehyde and osmium tetroxide) as employed for conventional preparations of cells failed, and reaction rates of the adsorption of capsule-specific bacteriophage or agglutination with capsule-specific antibody remained practically unchanged. A combination of ruthenium red with aldehyde or osmium fixation (LUFT 1971a, b) increased the contrast of anionic polysaccharides (EAGLE 1974). However, the reagent failed to exert a stabilizing effect on the capsules of *E. coli* K26 and K29, allowing the capsules to collapse (Fig. 5) (BAYER and THUROW 1977). On the other hand, ruthenium red has been reported to stabilize capsules of some gram-negative (COSTERTON et al. 1981) and gram-positive

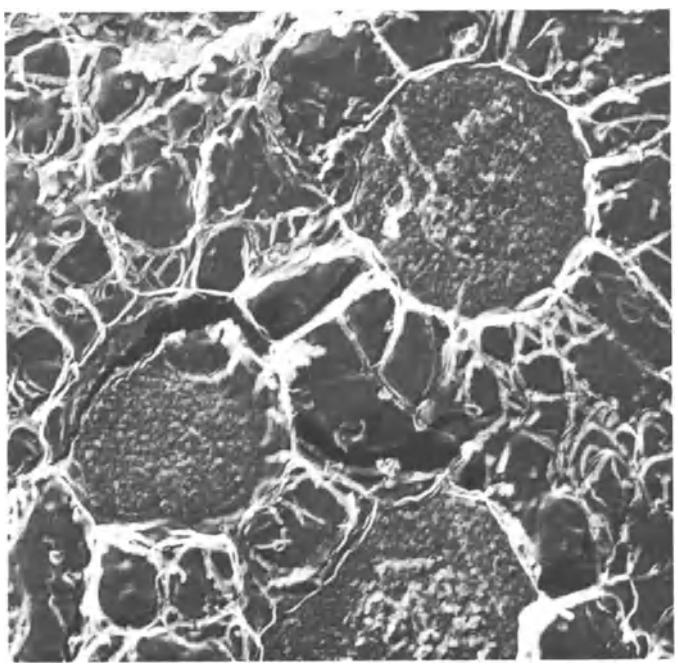


Fig. 5. Effect of dehydration on the capsule of *E. coli* K29. After exposure to 50% acetone, collapse of the capsule into thick strands is observed. Freeze-fracture/freeze-etch preparation. × 27000

organisms (MACKIE et al. 1979) and of *Diplococcus pneumoniae* and *Klebsiella pneumoniae* (SPRINGER and ROTH 1973). Alcian Blue, another cationic dye, has been described as a contrast-enhancing substance suitable for staining the capsule of *Bordatella bronchiseptica* (KLUDAS and RUDOLPH 1984).

Fixed and dehydrated capsules may reveal fine structure. SPRINGER and ROTH (1973) reported that depending on the type of dehydration agent, thin filaments, thick strands, and globular clusters were observed: After alcohol dehydration, the *K. pneumoniae* capsule appeared to be composed of fibrous elements (40 nm in diameter and about 150 nm in length) forming thorny structures on the cell surface, whereas after dehydration and embedding in Epon 812, thinner (7–8 nm), flexible, and twisted fibers about 260 nm long became visible. Fibers were also present on the surface of *Megasphaera elsdenii* (COSTERTON et al. 1981), and extensive networks of fibers were described for the capsules of rumen and other bacteria (PATTERSON et al. 1975; CAGLE 1975).

The above-mentioned data indicate that the capsules of different strains exhibit different sensitivities toward dehydration procedures. Capsules of *E. coli* strains K26, K29, and K1 are destroyed by conventional embedding procedures. To find out at which procedural step the collapse occurs, we studied the effect of dehydration (without fixation) on the capsulated *E. coli* K29 in *freeze-etch* preparations. After stepwise (partial or complete) dehydration in either acetone or ethyl alcohol, the cells were rapidly rehydrated in water and subsequently subjected to the freeze-fracture procedure. The results indicated that the capsules started to collapse during the early dehydration steps and were extensively collapsed in either 50% acetone or 50% ethyl alcohol. In the initial stage of collapse thick strands were formed (Fig. 5). Exposure of collapsed capsules to distilled water for 20 min did not redisperse the collapsed fibers. In contrast to these observations, no special pretreatment appeared to be necessary to stabilize the capsules of a number of gram-positive species. HOCHKEPPEL et al. (1987) described that clinical isolates of types 5 and 8 of *Staph. aureus* revealed extended capsules after standard procedures such as double fixation, dehydration in acetone, and Epon embedding; immunolabeling of ultrathin sections of these capsules was possible. Furthermore, GÜNTHER et al. (1986) reported that the capsule of *Pasteurella multocida* was not stabilized with specific antibody, but that stabilization was achieved with either ruthenium red, diamine platinum II chloride, or alcian blue.

Although the behaviour of the capsule of a particular cell strain during fixation and dehydration is not predictable, most capsules of gram-negative organisms collapse. Therefore, it appears necessary in each case to search for suitable stabilization procedures. For this, the light microscope provides valuable help, allowing one to monitor the overall condition of the capsule during preparative procedures. The visibility of fine structure of capsules depends on the method of dehydration and, for some capsules, on the presence of cationic molecules such as ruthenium red. A structural element of capsules, namely a filament of ~ 2 nm width, has been repeatedly described, and shows a tendency to associate with neighboring fibers and to form thicker strands or entangled networks. The thicker elements appear to be artifacts of dehydration, since they are predominant after dehydration of unstabilized capsules.

4.2 The Capsule After Freeze-Fracture and Freeze-Etching

The freeze-fracture method involves a number of preparative steps: (1) fast freezing of the unfixed or fixed cells at liquid nitrogen temperature, (2) fracturing of the low temperature specimen in a high vacuum environment, (3) shadow casting with a metal of high electron scattering capacity (platinum for example), (4) deposition of a carbon film on the specimen surface (replica formation), and (5) melting of the frozen specimen and chemical removal of organic material from the carbon replica. The cleaned carbon replica represents the surface contour of the fractured specimen and can be viewed in the electron microscope.

For the freeze-etching process, the freeze-fractured specimen is slightly warmed (to $-100°C$) subsequent to step 2. At this temperature, ice sublimates at a controlled rate, whereas cell structures release water to a lesser degree. Therefore, the ice plateau around structures retreats and subsequent shadow casting reveals the differences between higher structural elements and the surrounding ice plane.

The major advantage of freeze-fracture (and freeze-etching) is that chemical fixation and dehydration are omitted. The ultrafast freezing process replaces chemical fixation and is designed to prevent crystallization of the water in and around the cell. Often cryoprotective agents (sucrose, glycerol) are used to prevent ice crystal formation. However, cryoprotection is undesirable for capsule preparations, since the addition of any concentrated solution of salts or sugars will reduce the hydration of the polysaccharide and may cause partial or total collapse of the capsule. For this reason, our laboratory has rarely made use of cryoprotective agents and has preferred to rely on high-speed freezing procedures. Freeze-fractures of *E. coli* reveal fracture faces within the membranes of the cell enevelope (BAYER and REMSEN 1970b; NANNINGA 1970). However, the capsules of *E. coli* (strains K26 and K29) are often not clearly recognizable after freeze-fracturing. Most likely, this is due to an insufficient difference between the structure of the ice surrounding the cell and that of ice contained in the capsular domain (SPRINGER and ROTH 1976). However, ROTH (personal communication) observed that freeze-fractures of unfixed and aldehyde- or OsO_4-fixed *K. pneumoniae* may reveal the capsular domains and also fibers radiating from the cell surface. The light microscope shows that tightly packed capsulated cells retain a relatively wide space between neighboring cells (Figs. 3; 4), as was also observed in freeze-fractures of antibody-pretreated cells and in Lowicryl-embedded organisms (BAYER et al. 1986).

Freeze-etching might have been expected to be the method of choice to reveal the surface of a capsule. However, instead of the expected visualization of a delicate capsular surface, a relatively rough surface composed of conical spines and long strands was observed (Fig. 6). More extensive etching (deep etching) revealed a meshwork of thick strands visible mainly at areas which had just emerged from the receding ice plateau. Absent from the surface were the expected very thin fibers. We hypothesize that these fibers are mechanically unstable in the vacuum and cannot withstand removal of the intermolecular water (MOORHOUSE et al. 1977) following sublimation of the ice. The fibers apparently break off and escape into the vacuum of the freeze-etcher. Such an escape has been observed for much larger macromolecules (bacteriophage) during freeze-etching (BAYER and REMSEN 1978a). The escape of the

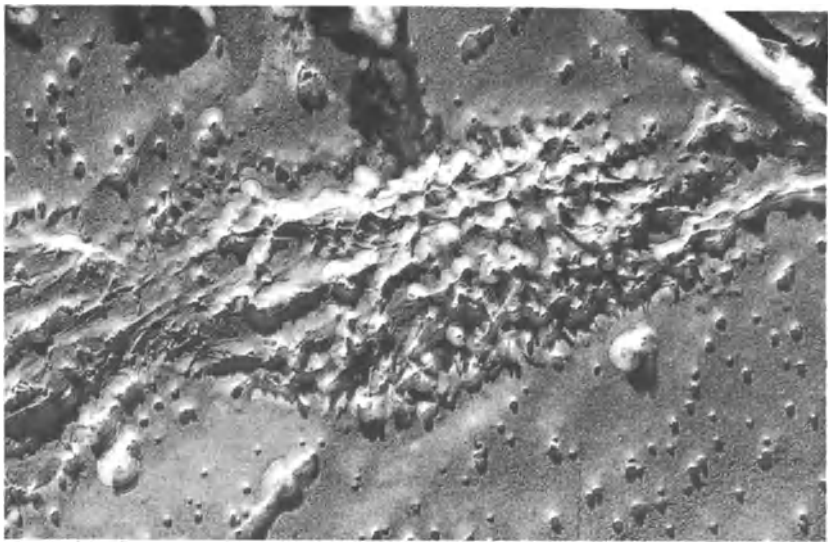


Fig. 6. Freeze-etched cell surface of untreated *E. coli* K29. Many conical elements and fibrils protrude from the cell surface. The main mass of the cell is still buried under the plane of the ice. × 40000

molecules seems to lend support to a theoretical prediction of ANDERSON (1954). It is also possible that the disappearance of the thin fibers could, in part, be an effect of radiation damage during shadow casting.

Freeze-etching was also employed in the search for special membrane structures of "pores" which could be correlated with export sites of capsular material. Until now, no such structures have been found in *E. coli* strains. However, pores were described by ZAAR (1979) and BROWN et al. (1976) in the inner membrane of cellulose-producing *Acetobacter xylinum.*

In conclusion: Freeze fracturing and etching, both of which keep biological specimen in a close to in vivo condition, has generated a number of unanswered questions which focus on the fate of the thin fiber, the proposed delicate structural element of the capsule. These fibers seem to either escape during sublimation of ice, or (less likely) be damaged by the shadow casting process.

5 Approaches to Stabilize the Capsule for Electron Microscopy

These methods have been developed to prevent structural collapse during fixation and dehydration. They make use of antibody cross-linking, of Lowicryl embedding, and of stabilization by cationic ferritin.

5.1 Antibody Treatment

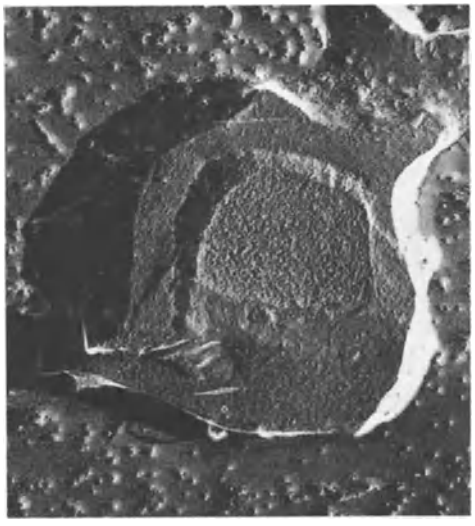
As shown in Sect. 3, exposure of the capsulated bacterium to homologous antibody increases the mass density of the hydrated capsule to such an extent that it can be discerned under the light microscope (NEUFELD 1902) and as an air-dried specimen in the electron microscope (MUDD et al. 1943). Since the resolving power of the microscope is a function of specimen contrast, the resolution obtained in these unstained preparations is low. However, the formation of an immunocomplex in the capsule produces dramatic results in freeze-etched preparations of unfixed cells as well as in ultrathin sections of dehydrated, plastic-embedded specimens.

5.1.1 Freeze-Etching of Antibody-Treated Cells

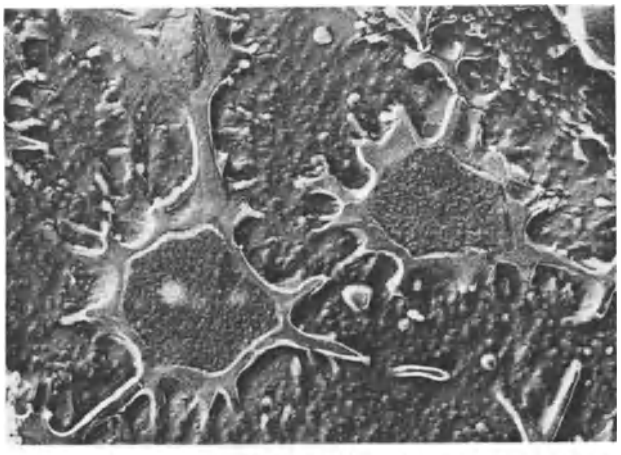
Differences in the sublimation rate of ice during freeze-etching cause the capsule of antibody-treated cells to be retained as a solid plateau which protrudes over the level of faster sublimating ice around the cell. Cytoplasm, cell envelope, and capsule are seen to share the elevated plateau (Fig. 7a) (BAYER and THUROW 1977). The freeze-etched capsules revealed neither detailed fine structure nor fibers nor spike formations. This result is in striking contrast to the data obtained with freeze-etched capsules of cells which had not been pretreated with IgG (Fig. 6). We assume that the lack of features in the antibody-treated capsule is due to the overwhelming amount of IgG in which the relatively low concentration of capsular polysaccharide fibers are embedded, and extensive cross-bridge formation between neighboring polysaccharide strands results in an "averaged" thermodynamically stable smooth surface plateau. Unexpected was the finding that a reduction in the amount of stabilizing antibody to one-fifth or one-tenth of the saturating concentration causes a partial collapse of the capsule, with the agglutinating cells forming a layer of uneven thickness with large projections protruding over deep valleys of the surface (BAYER and THUROW 1977) (Fig. 7b). It appears, therefore, that sufficient concentrations of specific antibody need to be used to avoid creation of such gross distortions.

5.1.2 Ultrathin Sections of Antibody-Treated Capsules

Ultrathin sections of plastic-embedded, antibody-treated cells reveal the capsule as a solid mass (Fig. 7c) whose thickness agrees well with that of similarly pretreated freeze-etched capsules (compare to Fig. 7a). Organized substructures (WHITFIELD et al. 1984b; MACKIE et al. 1979) were not observed in the capsules of the *E. coli* strains K26 and K29 except for occasional radial arrangements of fibers (BAYER and THUROW 1977). In contrast, group B streptococci revealed mosaic pattern of the filamentous proteins of the otherwise concentrically layered cell wall (WAGNER and WAGNER 1985). Differences in the effect of antibody on capsular material are to be expected among capsules of various bacterial species. Successful stabilization may depend on antibody type and its avidity and concentration, as well as on the concentration and molecular conformation of the capsular antigen. Therefore, exposure to homologous antibody alone may not be sufficient for



a



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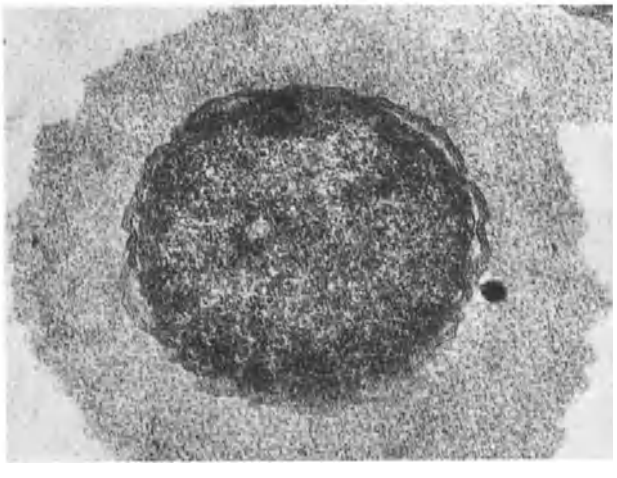

c

Fig. 7a–c. *E. coli* K29 treated with homologous antibody to capsule. **a** After freeze-fracture and freeze-etching, the capsular surface protrudes over the ice, forming an elevated plateau together with the cytoplasm plus cell membrane. × 24000. **b** Treatment with suboptimal concentration of antibody reveals in freeze-etched preparations a highly uneven capsular shape. × 17600. **c** Ultrathin section of the capsule–IgG complex shows a rather smooth surface, analogous to that in **a**. The high contrast particle is bacteriophage K29 that has penetrated the capsule. The penetration path is not visible in this preparation. × 47600

stabilization of the capsule, and additional treatment is needed. Often, ruthenium red is employed as a stabilizer in tandem wih antibody treatment (SPRINGER and ROTH 1973; COSTERTON et al. 1981). Two parameters may affect the outcome of stabilization with antibody: (1) a rapid attachment of antibody, which will increase the diffusion barrier of the cell surface and may therefore block deeper capsule penetration preventing complete stabilization; (2) a more gradual build-up of the antibody–capsule complex, which would leave sufficient time for deep penetration by the antibody. Electron microscopy has shown that *E. coli* capsules and many capsules of other species are fully penetrated by IgG molecules. Since the space between the polysaccharides is wide enough to allow ferritin (mol. wt. ~ 760 000) to permeate the capsules of *E. coli* strains K1, K29, and K30 (see below), the immunoglobulin does not seem to block itself from deeper penetration.

However, stabilization with antibody is not always achievable in cases in which tight spatial arrangements of the polysaccharides exist or extensive cross-linking with immunoglobulin has taken place. Furthermore, antiserum-treated capsules often reveal considerable size variations which constitute a characteristic feature of a cell population (ADLAM et al. 1985) and are not due to insufficient degrees of stabilization. Capsule production responds to environmental factors and has been reported to be inversely proportional to the quality of growth conditions. It has been proposed that increased capsular thickness is caused by a better nutrient supply due to an increased distance between neighboring cells. According to EAGLE (1975) the capsule expression depends on the position of the cells within a cell cluster on solid media. Cells of the interior of a cell cluster showed capsules of more than 600 nm thickness, whereas capsules of only 300 nm thickness were observed in the perimeter of the clusters.

Electron micrographs showed unambiguously that immunoglobulin has access to the deepest parts of an *E. coli* capsule. The speed of IgG diffusion into the capsule can be estimated from an experiment in which antibody was used to block penetration of the capsule by capsule-specific phages. Capsule-degrading phages (FEHMEL et al. 1975) form a penetration path which has been revealed with electron mocroscopy (BAYER et al. 1979). Addition of the anticapsular IgG shortly before adding K29 phage to the host *E. coli* K29 prevented adsorption of the phage. Adding the IgG 10 s after addition of phage gave the virus sufficient time to penetrate the entire capsule and to arrive at the surface of the outer membrane of the envelope. Simultaneous addition of phage K29 and capsular antibody allowed some of the phages to enter the capsule; however, they stopped on their path "halfway through" (BAYER et al. 1979). Fluorescence studies showed that the plasma membrane is affected (de-energized) about 40 s after addition of phage K29 to the host cell (BAYER and BAYER 1981). If most of the 40 s is expended by the phage for full penetration of the capsule, the simultaneously added IgG has overtaken and stopped the phage, indicating that IgG molecules diffuse through the K29 capsule in less than 40 s.

The increasing use of specific antibody for the stabilization of capsules demonstrates its value for ultrastructural microbiology of laboratory strains and bacterial isolates from clinical cases and natural habitats (COSTERTON et al. 1981; HOCHKEPPEL et al. 1987). The disadvantage of the method is the lack of structural detail in the complex of polysaccharide and immunoglobulin.

5.2 Capsule Stabilization by Means Other than Antibody

5.2.1 Dimethylformamide Dehydration and Lowicryl Embedding

A set of embedding media has been developed by KELLENBERGER (KELLENBERGER et al. 1980) and his associates (CARLEMALM et al. 1982) which showed improved properties in terms of decreased denaturation of proteins and preservation of antigenic activity. These media appeared to be promising tools for a faithful localization of epitopes by immunoglobulins. One of the embedding media, Lowicryl K4M, has been used with success in the localization of O antigens and capsular antigen of *E. coli* strains. In the case of rather delicate capsules such as those of *E. coli* K29, additional steps were developed to preserve this capsule in an uncollapsed state without the use of homologous antibody (BAYER et al. 1986). One of the factors for the success of this preparation method was the employment of dimethylformamide as dehydration agent. Ultrathin sections of Lowicryl-embedded *E. coli* K29 showed the capsule as a very faint structure (Fig. 8). Its dimensions appeared to be equal to those of

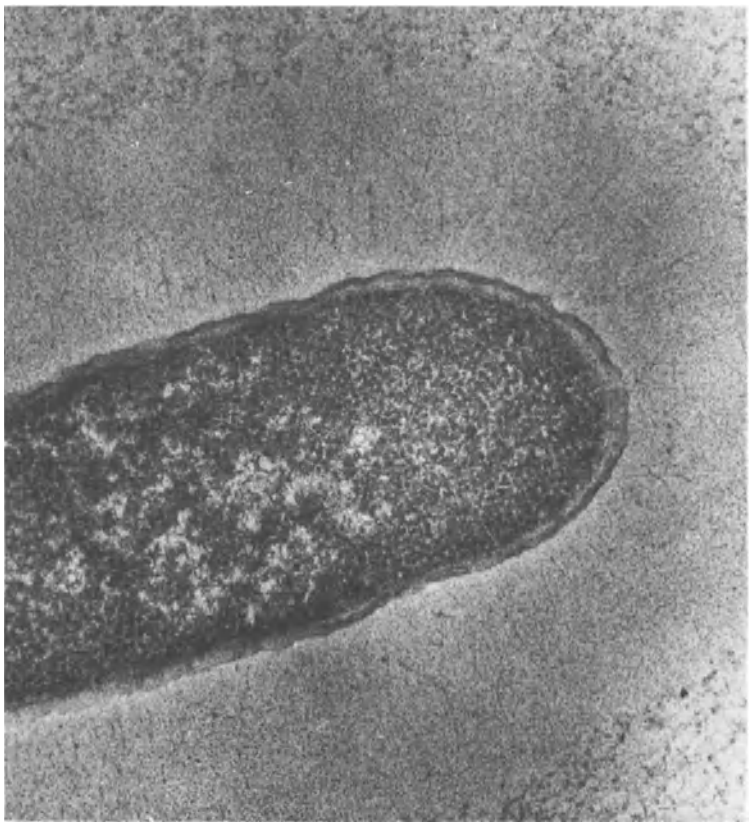

Fig. 8. *E. coli* K29, aldehyde fixed and low temperature embedded in Lowicryl K4M. The ultrathin section shows an uncollapsed capsule and reveals thin fibers originating from the OM and spanning the depth of the capsular domain. × 52000

antibody-stabilized capsules or India ink preparations. The outer edge of the Lowicryl-embedded capsule was found to be slightly wavy and, in general, well defined. The more or less uniform thickness of the capsule suggested a tight control of the cell over the length of the exopolysaccharide. The capsular domain was seen to be traversed by thin fibers 700–800 mm in length and 2–4 nm in thickness. Thicker strands seemed to be composed of the thin fibers. The number of thin fibers of the capsules can be counted from such sections. For *E. coli* K29 we estimated 3500 capsular fibers per cell. Except for these fibers, the capsular area is lacking recognizable structural elements. Exposure of the sections to homologous antibody protein A–gold complexes showed fibers labeled with the capsular antigen (Fig. 9).

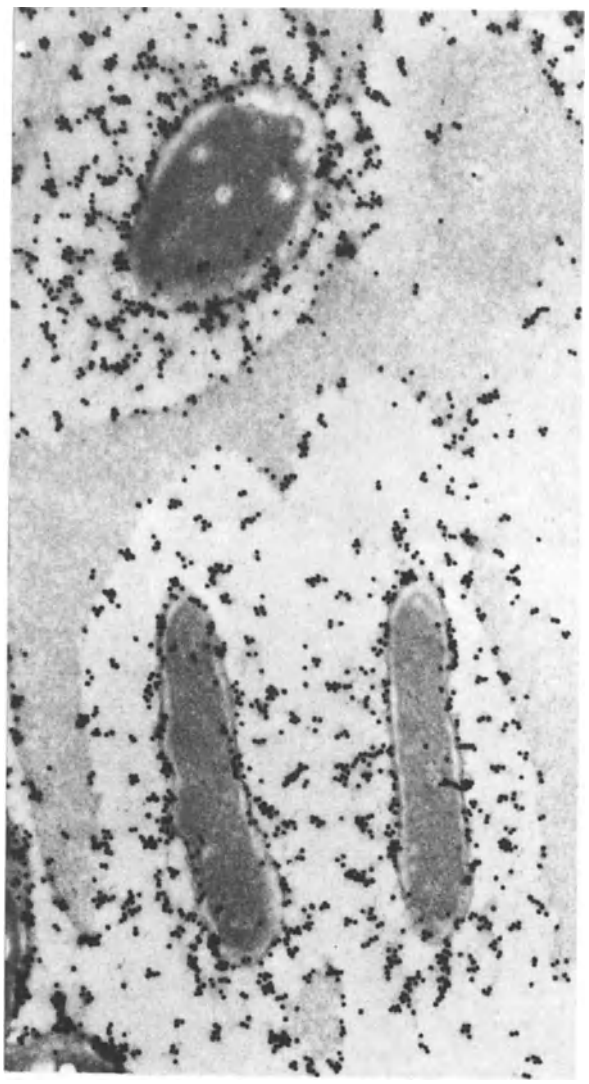

Fig. 9. Immunoreaction on a Lowicryl section of capsulated *E. coli* K29 using gold-labeled anticapsule IgG. Labeled are: strands in he capsular domain, many of which are arranged in a more or less radial orientation, the OM, and a few zones bridging the space (periplasm) between OM and IM (upper cell). × 18 000

Although the contrast in the Lowicryl-embedded cells is low [especially in the inner membrane (IM)], the resolution is sufficient to look for the distribution of the capsular antigen in the cell envelope. We observed that the antigen was present on the outer face of the outer membrane (OM) and at the inner membrane (IM). At the IM it was clustered at a few sites (see upper cell in Fig. 9). At these sites the label crossed the periplasmic space by following the membrane into a junction of IM to OM. These areas may be tentatively identified as the sites of assembly and transfer of K29 antigen. The existence of such antigenic clusters in the IM argues against a high mobility of proteins in the IM, and excludes rapid mixing of these components. Employment of the same methods also allowed us to examine the O antigen distribution within the capsule (BAYER et al. 1986). The LPS was found to be arranged evenly over the entire surface of the OM and was seen to be well shielded by the much larger capsular domain (Fig. 10). Double-labelling was used to inspect the assembly and transport sites of both the capsular antigen and the O antigen. Figure 11 shows the LPS label (large gold particles) and the capsular label (small gold particles). The labels are indeed visible at zones which can be interpreted as areas of IM/OM contact. Therefore both the capsular (K29) and LPS (09) antigens can be localized at one individual membrane contact site. The data suggest to us

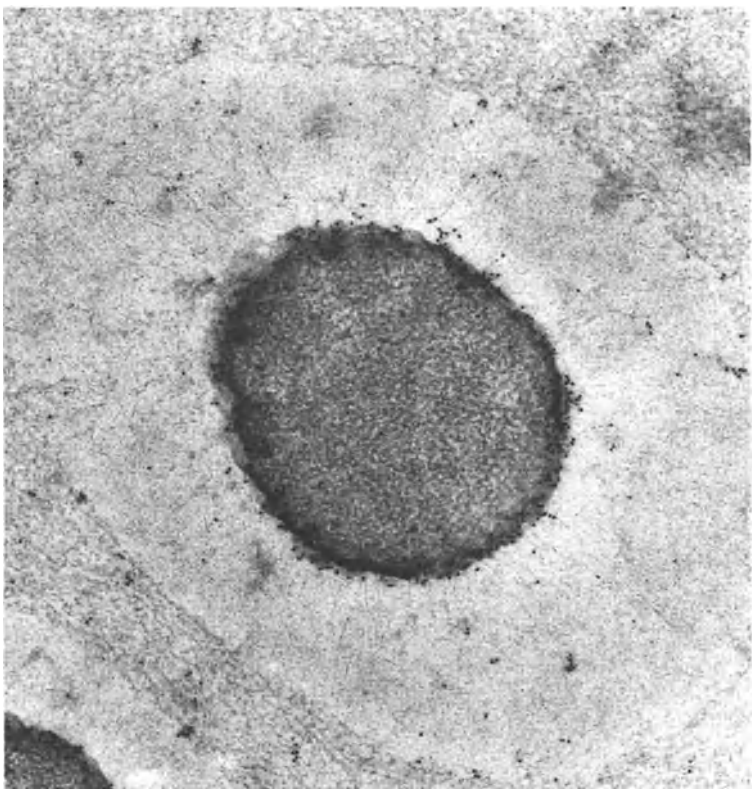

Fig. 10. Ultrathin section of capsulated *E. coli* K29. Immunogold label was directed against 09 antigen. The lable is covering the OM owing to the relatively short chain length of the O antigen. × 42000

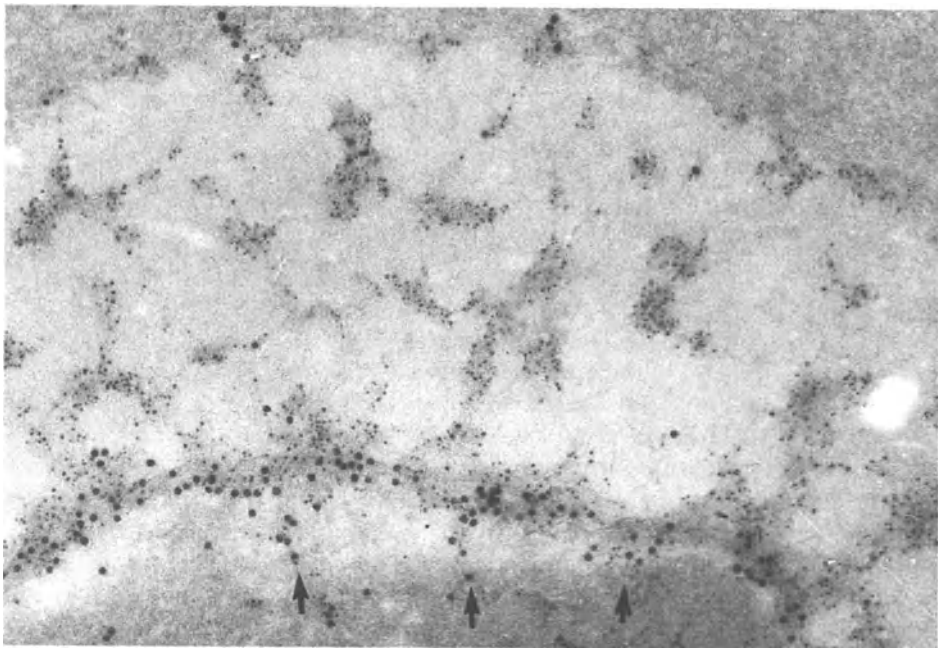

Fig. 11. Ultrathin section of plasmolized *E. coli* K29 after double labeling with capsule antibody [small (4-nm) gold beads] and 09 antibody [large (11-nm) gold beads]. One-half of the cell is shown. The OM contains both labels. The IM is not sharply defined due to an oblique section plane. This orientation allows an enlarged view of the periplasmic space, which is bridged at a few sites by label of both specificities (probably adhesion sites). The label distribution suggests the two antigens are present in three of the bridges (*arrows*). × 64 700

the existence of multifunctional export domains at the IM/OM adhesions. However, a cautious approach to the interpretations is needed, since some loss of capsule-specific label from the thin sections may occur during the prolonged washing procedures. We propose tentatively that the capsular synthesis and LPS synthesis take place either at the same adhesion sites or at separate sites in accordance with our earlier model describing the cell surface as functional mosaic. Since export of filamentous bacteriophage also occurs at the membrane adhesion site (BAYER and BAYER 1986; LOPEZ and WEBSTER 1985), we speculate that elements of transmembrane transport machinery, especially the elements involved in of the cell surface growth, may generate the sites of membrane adhesion.

Other approaches in the study of the capsular structures reveal different aspects of the bacterial surface, for example its charge, and serve as useful tools in the localization and stabilization of polysaccharide assemblies.

5.2.2 Stabilization of the Capsule by Cationic Charges

There are three reasons to employ cationized ferritin for electron microscopy of capsules: the protein serves as marker for negative charges, its adsorption stabilizes the capsule, and it provides high electron contrast. The idea of the use of

countercharges to identify cell surface layers has been employed in the past for the staining of O antigen and for the identification of localized surface charges of bacteria (MAGNUSSON and BAYER 1982). Cationized ferritin was employed as a capsule stain by GRUND et al. (1983) and by WEISS et al. (1979), who reported that *Klebsiella* capsules accumulated a considerable amount of the protein. However, structural elements of the capsular polysaccharide were not discerned. Our laboratory employed cationized ferritin mainly for the purpose of stabilization of the capsules of a variety of strains such as *E. coli* K26, K29, K30, and K1. In all strains tested, cationized ferritin prevented the capsules from collapse during dehydration. Figure 12a shows the capsule of *E. coli* K1, Fig. 12b the capsule of *E. coli* K29. The size of the K29 capsule is similar to the antibody-treated freeze-etched

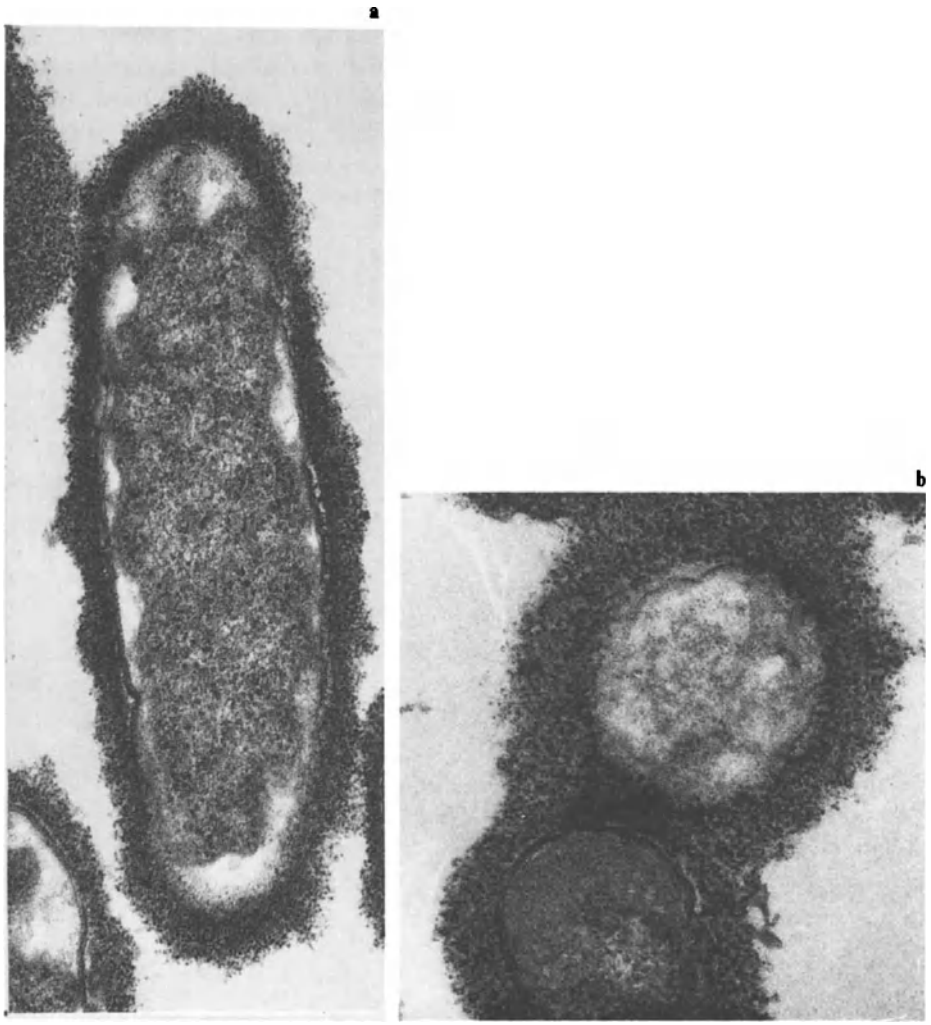

Fig. 12a, b. Cationized ferritin, applied before fixation, attaches to the anionic charges of **a** the *E. coli* K1 capsule (× 36 700) and **b** the *E. coli* K29 capsule (× 33 300). Ultrathin sections, aldehyde and OsO_4 fixation

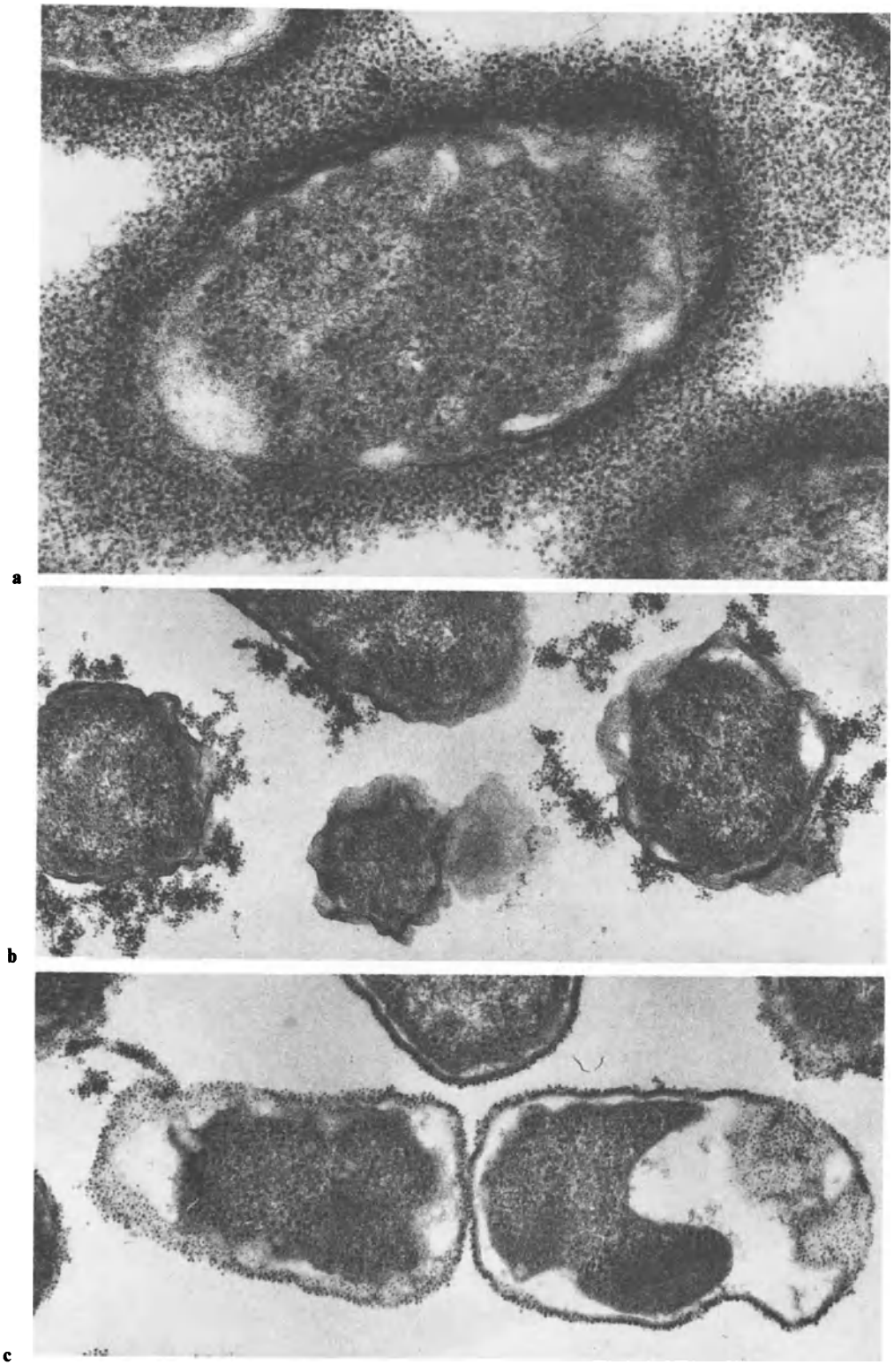

Fig. 13. Cationized ferritin reacts with the capsule as well as with the core LPS and the O antigen: **a** The capsule of wild-type *E. coli* K30. **b** The O antigen (LPS) of K30 capsule-minus mutant. Note the cloud-like appearance of the LPS. **c** The rough LPS of a capsule-minus K30 mutant. Here, a thin, uniform layer of ferritin covers the entire surface. **a** × 63100; **b** and **c** × 36400

capsule (see Fig. 7a). We observed that cell suspensions treated with the protein tended to form aggregates. Cationized ferritin seems to serve as a marker for anionic charges of both capsular polysaccharides and LPSs. An example is shown for *E. coli* K30. A dense mass of ferritin occupies the capsular area of the wild-type cell (Fig. 13a). A K30 mutant lacking capsule but containing smooth type O antigen shows the ferritin at irregular protrusions (Fig. 13b). The rough mutant exhibits a very thin layer of ferritin (Fig. 13c), which is probably formed as a consequence of the free negative charge of the keto-deoxy-octonate in the LPS's inner core region (FERRIS and BEVERIDGE 1986).

5.2.3 Cryofixation and Freeze-Substitution

Cryofixation consists of a very rapid freezing, with optimal freezing rates of 10^5–10^6 K/s (KNOLL et al. 1987). In the freeze-substitution process the ice of the frozen specimen is replaced at low temperature by alcohols or acetone, which are substituted by a polymerizable monomeric resin such as Lowicryl. The resin is polymerized at low temperature, and the material can be sectioned at room temperature. Cryofixation should be an ideal approach to the preservation of bacterial capsules, since chemical fixation as well as interactions with charged marker molecules are circumvented. Cryofixation, followed by freeze-substitution, can be expected not only to maintain the fine structure but also to reduce the extent of conformational changes of the macromolecules. However, as pointed out by KELLENBERGER (1987), with decreasing temperature an increasing hydrophilicity of macromolecules has been predicted.

According to this assumption, the removal of hydration shells will cause aggregation of macromolecules. A network of increasingly fine detail will be formed as the freezing process rapidly achieves the low temperatures. Cryofixation methods on *E. coli* capsules have not been totally successful. While micrographs of capsules, for example of *E. coli* K30 (BEVERIDGE 1987), revealed a layer of entangled fibrils emerging from the surface of the enevelope, the degree of entanglement and the size of many of the fibers did not correspond to the entanglement and size of the thinnest elements observed with other techniques. Furthermore, after cryofixation and cryosubstitution other strains such as *E. coli* K29 and K1 showed a rather amorphous capsule without well-defined fiber structures (BAYER, unpublished). Obviously more data will be available in the near future, involving a greater number of cell strains and a variety of procedural modifications so that it will be possible to assess with confidence the validity of cryofixation methods in the study of capsular structures.

5.3 The Dimensions of Capsule Fibers

Thin polysaccharide fibers, seen for example in *E. coli* K29 (BAYER et al. 1985), and to some extent in *E. coli* K30 (BEVERIDGE 1987), appear to constitute a class of small elements of the capsular domain. Thin fibers have been described in the capsules of a variety of organisms, and measurements revealed diameters of 2–4 nm for *E. coli*

K29 (BAYER et al. 1985) and of 2.5–3 nm and 3–5 nm for *K. pneumoniae* (CASSONE and GARACI 1977). The fibers appear to be stretched out from their basis at the OM to the periphery of the capsule. This would suggest that the length of single fibers represents the thickness of the capsule (see Fig. 1). Low temperature embedded *E. coli* K29 showed fibers measuring about 2.0 nm in width (Fig. 8) and 500–800 nm in length, spanning the entire thickness of the capsule (BAYER et al. 1985). We also observed that temperature sensitive mutants of *E. coli* K29 that failed to produce a well defined capsule generated instead fibers of considerable and variable length, often of more than 50 nm.

When purified and freeze-dried K29 polysaccharide was rehydrated and spread on water–air interfaces, the metal-shadowed specimen showed very long cable-like fibers of varying thickness, with much finer filaments branching off. These fibers maintained their biologic activities, such as their receptor activity for capsule-specific phages, as well as their antibody-binding capacity. Figure 14 shows phage particles attached to fibers of the purified polysaccharide. While concentrated polysaccharide preparations consisted mainly of thick fibers, alkali treatment disentangled them gradually, producing thin fibrils of 3–6 nm thickness and 250–300 nm length [Fig. 15; BAYER and THUROW (1977)]. However, data derived from purified polysaccharides do not necessarily reflect the organization of the in

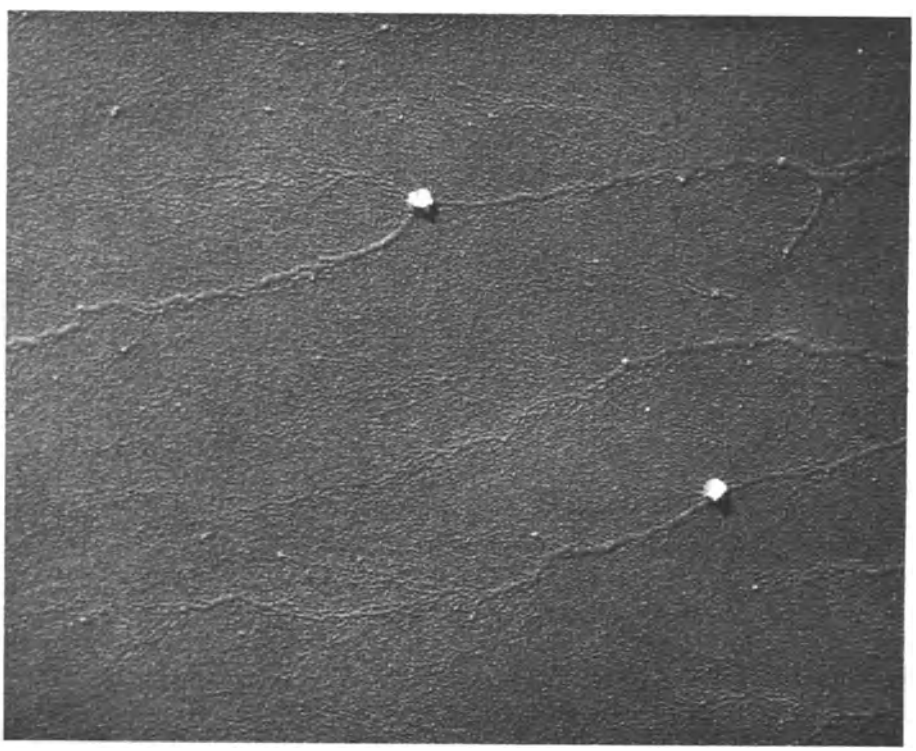

Fig. 14. Fiber bundles of purified K29 capsular polysaccharide after spreading on an air–water interface and Pt-shadow casting. The spherical particles are K29 specific phage, which has been absorbed to the polysaccharide before spreading. × 40000

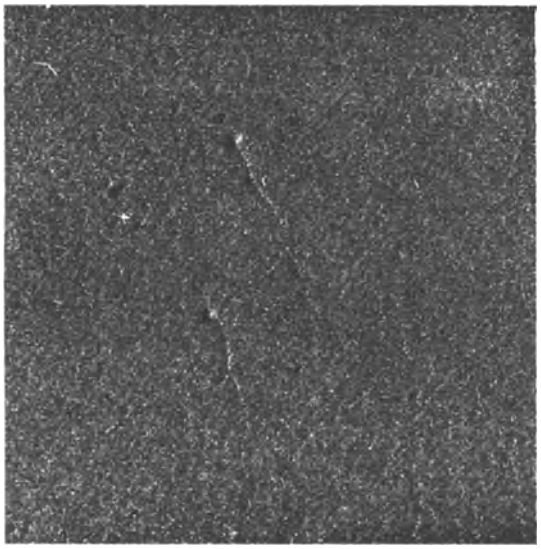

Fig. 15. The smallest fibers found in purified and alkali-treated K29 polysaccharide. Platinum-shadowed specimen. × 65000

vivo capsules, since additional intermolecular parameters, hydrogen and salt bridges, and water molecules will affect the conformation and charge of an in vivo capsule, its degree of hydration, and the presence of other charged and uncharged macromolecules.

Although the capsule appears to be a uniform structural entity, a mixture with LPS is very likely: Micrographs showed that IgG specific for O antigen labeled not only the surface of the OM, but also small clusters within the capsular domain. Furthermore, we observed during purification of capsular material that a tight association had formed between polysaccharide and nucleic acid. It therefore appears likely that an in vivo capsule is heterogeneous due to adsorption of a variety of macromolecules and ions. While the polysaccharide capsules of *E. coli* are comparatively simple in their composition, much more complex macromolecular assemblies are to be expected in a variety of other strains. For example, group B Streptococci exhibit group specific and type specific carbohydrates plus several proteins, all of which appear to be arranged in a mosaic pattern on the capsular surface (WAGNER and WAGNER 1985).

6 Summary

The highly hydrated capsule of *E. coli* strains is composed of a large number of polysaccharide fibers of which the thinnest measure about 2nm in width. The fibers may span the entire distance from the outer membrane to the outer rim of the capsule and show a propensity to associate with each other to form thicker filaments. Presence of thick filaments may also indicate a partial collapse of the

capsular organization due to removal of water. The in vivo capsule represents a relatively open structure with the negatively charged polysaccharide fibers permitting the binding of large quantities of water and ions, and providing intracellular space for diffusing molecules to access the envelope membranes even in conditions of high cell density. Negative charge and steric hindrance of the polysaccharide strands protect the cells against attack by a large variety of harmful macromolecules and against infection by most bacteriophages.

Two types of procedure have been most successful in maintaining the size and overall structure of the capsule: (a) the interaction of cationic molecules with the in vivo capsule, and (b) the use of antibody to stabilize capsules for subsequent dehydration and plastic embedding. A further type of potentially useful procedure, cryofixation and cryosubstitution, has shown interesting results in a number of cases. These techniques are expected to play a significant role in structural studies in the near future. The sites of export of capsular antigen have been described in earlier conventional electron microscopic studies. Data obtained from the recent technique of "on-section" labeling support the model that both the capsular antigen and the O antigen are assembled at junctions of the inner and outer membrane. It is anticipated that one will be able to discern in greater ultrastructural detail the sites of synthesis and transmembrane export, and to identify the sites of the membranes at which the antigen is translocated. Novel membrane fixation and isolation techniques will have to be established and employed in a combination of sensitive microscopic techniques and immuno- and enzyme localization methods. These developments will make it possible to explore questions pertaining to the maintenance and structural organization of microbial capsules and the functional interaction of polysaccharides with natural surfaces, man-made substances and drugs.

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