1996

Characterization of sequences associated with the type 3 capsule locus in Streptococcus pneumoniae.

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CHARACTERIZATION OF SEQUENCES ASSOCIATED WITH THE TYPE 3 CAPSULE LOCUS IN *STREPTOCOCCUS PNEUMONIAE*

by

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(mentor Janet Yother)

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in the Department of Microbiology in the Graduate School,
The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1996

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph. D. Program Microbiology

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Committee Chair(s) Dr. Janet Yother

Title Characterization of Sequences Associated With The Type 3 Capsule Locus In *Streptococcus pneumoniae*

The type-specific genes of *Streptococcus pneumoniae* serotype 3, *cps3DSUM*, have been identified and encode a UDP-glucose dehydrogenase, type 3 polysaccharide synthase, a UTP:glucose-1-phosphate uridylyltransferase and a protein with homology to phosphomutases, respectively. Analysis of sequence flanking the type 3-specific locus identified genes common to all capsular serotypes. The *cps3* locus is distinguished from those in other serotypes by the presence of deletions and sequences associated with genetic rearrangements. The resulting genetic structure may contain important clues as to the origins of not only the type 3-specific locus but also that of other pneumococcal serotypes as well.

The transcriptional organization of the *cps3* locus was examined by the construction of strains containing *cat* gene fusions. These studies determined the levels of transcription within each of the type 3-specific genes and flanking sequences. Northern blot analyses identified at least two type-specific transcripts within this locus. Northern analyses also suggest that transcription within the *cps3* locus may be growth dependent and that the *cps3* transcripts may be subject to post-transcriptional regulation. The characterization of novel type 3 strain suggest that *cps* expression in *S. pneumoniae* may be regulated by a locus unlinked to the *cps* cassette. This locus was also present in heterologous serotypes.

To access the role of each of the type 3-specific genes in virulence, strains containing insertions within the *cps3* locus were examined in animal studies. Results from these studies determined that *cps3D* and *cps3S* are the only type-specific genes required for

virulence. In addition, a novel type 3 strain was isolated which produces a normal level of capsule in culture but was avirulent in mice. This strain was shown to produce elevated levels of capsule within the *in vivo* environment.

ACKNOWLEDGMENTS

I wish to thank my mentor, Dr. Janet Yother, for her support and encouragement. From the beginning of my graduate school education, she has provided an environment in which independent thought is not only encouraged but nurtured. I would like to also acknowledge the contribution of my thesis committee to the development of my research and education. Their comments, suggestions, and support have helped me to develop a better sense of what is expected of an investigator by his/her peers and have encouraged me to aspire to the highest level of academic and scientific excellence. In particular, Dr. Bill Benjamin is to be thanked for each time he has listened to one of my notions without prejudice. Also, he is to be thanked for providing helpful insights and technical assistance with this project at all levels of its development. It has truly been a pleasure to work in the same field with such a person.

I would also like to recognize the contribution of each member of Dr. Yother's research group, both past and present, for their support, assistance, patience, and friendship. I do not feel that I shall soon encounter such a unique and caring group of individuals.

And finally, I would like to thank my parents, Shirley and Sal, as well as my sisters, Kim and Heidi, for their understanding, patience, and encouragement throughout my graduate school career.

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INTRODUCTION

Background and significance of polysaccharide capsule

The gram-positive coccus *Streptococcus pneumoniae* continues to be a significant cause of infectious disease in the United States. In children, it is the leading agent of otitis media (middle ear infection) and the leading cause of pediatrician visits after well-child care. In both children and adults, infections due to *S. pneumoniae* include pneumonia, septicemia, and meningitis. Despite the use of anti-microbial therapy, the number of deaths due to pneumococcal infections remains high. Virulence factors of the pneumococcus include a polysaccharide capsule, pneumococcal surface protein A (PspA), neuraminidase, pneumolysin, and the cell wall components teichoic acid and lipoteichoic acid. The capsule, however, serves as the major virulence factor for the bacterium, and, unlike the other virulence factors mentioned above, pneumococci which lack a capsule are avirulent (Stryker, 1916). *S. pneumoniae* may be differentiated serologically using antibodies directed against the polysaccharide capsule. Although 90 different capsular serotypes are known (Henrichsen, 1995), only a small fraction of these are routinely isolated clinically, with some being more frequently seen in children than in adults (Finland and Barnes, 1977; Gray and Dillon, 1986). The current vaccine is composed of purified polysaccharide from 23 of the most commonly isolated pneumococcal serotypes. Although this vaccine has been shown to work successfully in healthy adults, it is ineffective in children due to their inability to produce antibodies in response to polysaccharides (Gotschlich *et al.*, 1977; Austrian, 1984). Individuals, such as the elderly and the immunocompromised, who have weakened immune systems are not effectively protected by this vaccine and are also particularly susceptible to pneumococcal infections.

The capsule serves as a virulence factor by providing protection from the host's immune defense system (reviewed in van Dam *et al.*, 1990), primarily by preventing phagocytosis. The cell wall components, peptidoglycan and teichoic acid, are potent activators of complement and the alternative pathway. In the absence of capsule, these cell wall components are susceptible to binding by the complement factors, C3, C3b, and Factor B. The binding of these factors initiates a cascade of reactions within the alternative complement pathway and results in the recruitment of polymorphonuclear monocytes (PMNs) and macrophages, followed by phagocytosis. These cell wall components are also able to induce an intense inflammatory response, and, in the case of meningitis, it is this inflammatory response that is thought to be responsible for much of the resulting pathology (Tuomanen *et al.*, 1985). In the non-immune host and in the absence of α-capsular antibodies, the cell wall components of encapsulated bacteria are shielded underneath the capsule, making them significantly less accessible to binding by complement. In cases where complement is able to bind to the cell wall, it is often hidden deep within the capsule and therefore blocked from participation in activation of the complement cascade. Without the effective binding of complement factors, especially C3b and iC3b, the alternative complement cascade can not be initiated (Fine, 1975). Some capsular serotypes have been shown to bind some complement factors better than other serotypes; those serotypes that bind factor iC3b are more readily phagocytized as this complement factor serves as the ligand for receptors on PMNs. In contrast, other serotypes have been shown to have only limited deposition of complement on the surface and have a decreased ability to proteolyticly degrade bound complement to forms which are unable to stimulate phagocytosis. These serotypes are less susceptible to phagocytosis. It is not clear, however, how a particular capsule structure influences this process. Recent work with serotypes 3, 4, and 14 has suggested that the ability to degrade complement is cell wall associated and is independent of the presence of capsule (Angel *et al.*, 1994). In the presence of type-specific antibodies, pneumococci activate the classical pathway by the

interaction between antibody bound to the capsule and complement components (opsonins). The coating of the bacteria with opsonins serves to stimulate phagocytosis by the recruitment of PMNs and macrophages. As with non-encapsulated pneumococci, opsonophagocytosis serves as the primary line of defense against encapsulated pneumococci (Knecht, 1970; Giebink *et al.*, 1977; Branconier and Odenberg, 1982). Although the presence of a capsule is required for virulence, the degree to which different capsule serotypes are able prevent complement deposition and degradation, and opsonophagocytosis is variable. Serotypes which have only weak antiphagocytic activity may be able to evade immune detection due to the poor immunogenicity of their capsule structure (Wood and Smith, 1949; MacLeod and Krauss, 1950; Knecht, 1970). Studies using 76 different strains representing eight different capsule serotypes have demonstrated a strong association between capsule type and virulence (Briles *et al.*, 1992). Recent work by Kelly *et al.* using near-isogenic strains has demonstrated that the genetic background in which a particular capsule serotype is expressed also plays a role in the determination of pathogenicity (Kelly *et al.*, 1994). This work suggests that, while the capsule is a major determinant of virulence, it is not the sole determinant and, more than likely, it is the combined influences of capsule and other factors within a genetic background which determine the overall virulence of a strain.

Biochemistry and genetics of pneumococcal capsule

The pneumococcus was first isolated independently in 1881 by Sternberg (1881) and Pasteur (1881) as the causative agent of lobar pneumonia. It has since had a long and distinguished history of scientific contribution to the fields of both medicine and biology. Klemperer, in 1891, demonstrated that antisera directed against a specific pneumococcal serotype was able to provide passive protection against infection with that serotype in both animals and humans (Klemperer and Klemperer, 1891a; Klemperer and Klemperer, 1891b). Pneumococcal polysaccharide capsule was first identified in the serum and urine of patients suffering from lobar pneumonia and was shown to be precipitable with immune

serum from those patients (Dochez and Avery, 1917), making this polysaccharide the first non-protein substance shown to be immunogenic (Heidelberger and Avery, 1923). These early observations led, in part, to the discovery of opsonophagocytosis and the demonstration of the ability of encapsulated bacteria to survive within the host while non-encapsulated bacteria were readily cleared by phagocytic cells (Neufeld and Rimpau, 1904; Avery and Dubos, 1931; Dubos and Avery, 1931). The existence of antigenically variable forms (serotypes) of *S. pneumoniae* was soon discovered (Dochez and Gillespie, 1913).

Pioneering work in 1928 by Griffith with non-encapsulated or rough (R) and encapsulated or smooth (S) strains of *S. pneumoniae* in mice demonstrated the phenomena of natural transformation and resulted in the proposed existence of a "transforming principle" (Griffith, 1928). In these experiments, Griffith reported that the simultaneous injection of live R and heat-killed S pneumococci was able to induce infection and death in mice; in contrast, the injection of either live R or heat-killed S alone did not. Similar experiments were later performed using an *in vitro* system of transformation, including transformations involving only crude extracts and in the absence of any cellular factors (Dawson and Sia, 1931; Alloway, 1932). These findings helped establish the existence of a "transforming principle" responsible for genetic inheritance. Later, in 1944, Avery, MacLeod, and McCarty demonstrated that this long sought after "transforming principle" was deoxyribonucleic acid, DNA (Avery *et al.*, 1944). Avery, MacLeod, and McCarty did not, however, state explicitly in their original publication that DNA was the genetic material but stated that fractions containing primarily DNA were able to successfully transform rough pneumococci to encapsulation. The genetic significance of this work did not escape them and was implied in their report that distinct DNAs were capable of transferring the ability to produce capsular substances which in turn were chemically distinct and that these changes were "predictable, type-specific, and heritable" (Avery *et al.*, 1944). Their report was further supported by the work of McCarty which demonstrated the ability of purified DNase to inactivate the transforming property of the DNA-containing fraction (McCarty

and Avery, 1946). In 1949, Ephrussi-Taylor was one of the earliest investigators to perform in depth heredity studies on the transforming agent in *S. pneumoniae* and also to state explicitly that this agent represented the genetic material responsible for the production of distinct capsular polysaccharide (Taylor, 1949). In 1951, Hotchkiss was able to transform resistance to penicillin in pneumococci; the transformation of a non-polysaccharide-related phenotype helped to dispel some skepticism that it was perhaps contaminating polysaccharide in the DNA fractions that acted as the transforming principle (Hotchkiss, 1951). Ephrussi-Taylor later went on to propose the hypothesis that capsule serotype switching occurs through the exchange of genetic cassettes. This hypothesis was based on the premise that the genes responsible for the production of a particular serotype are linked on the chromosome and that they occupy homologous sites on the chromosome (Ephrussi-Taylor, 1951). In support of this proposed model, Ravin was able to demonstrate, using strains containing independent spontaneous mutations, that genes involved in the biosynthesis of type 3 capsule were closely linked on the chromosome (Ravin, 1960). Also, transformations between capsule types were shown to result in the loss of biochemical functions associated with synthesis of the type-specific polysaccharide of the recipient and in the apparent replacement with those associated with the donor (Austrian *et al.*, 1959). Recently, the cassette-recombination mechanism of capsule type switching was demonstrated definitively using insertions linked to the capsule loci of types 2, 3, and 5 and was shown to involve flanking sequences common to all capsule serotypes examined (Dillard and Yother, 1994; Dillard *et al.*, 1995). Dillard *et al.* (1995) were also able to demonstrate that the acquisition of the type-specific genes of a donor strain resulted in a concomitant loss of the type-specific genes of the recipient (Fig. 1).

While cassette-recombination is the primary mechanism by which capsular serotypes are exchanged between pneumococci, some exceptions which can not be fully explained by this model have been noted. For example, transformants producing intermediate levels of polysaccharide capsule have been described through the years. Blake

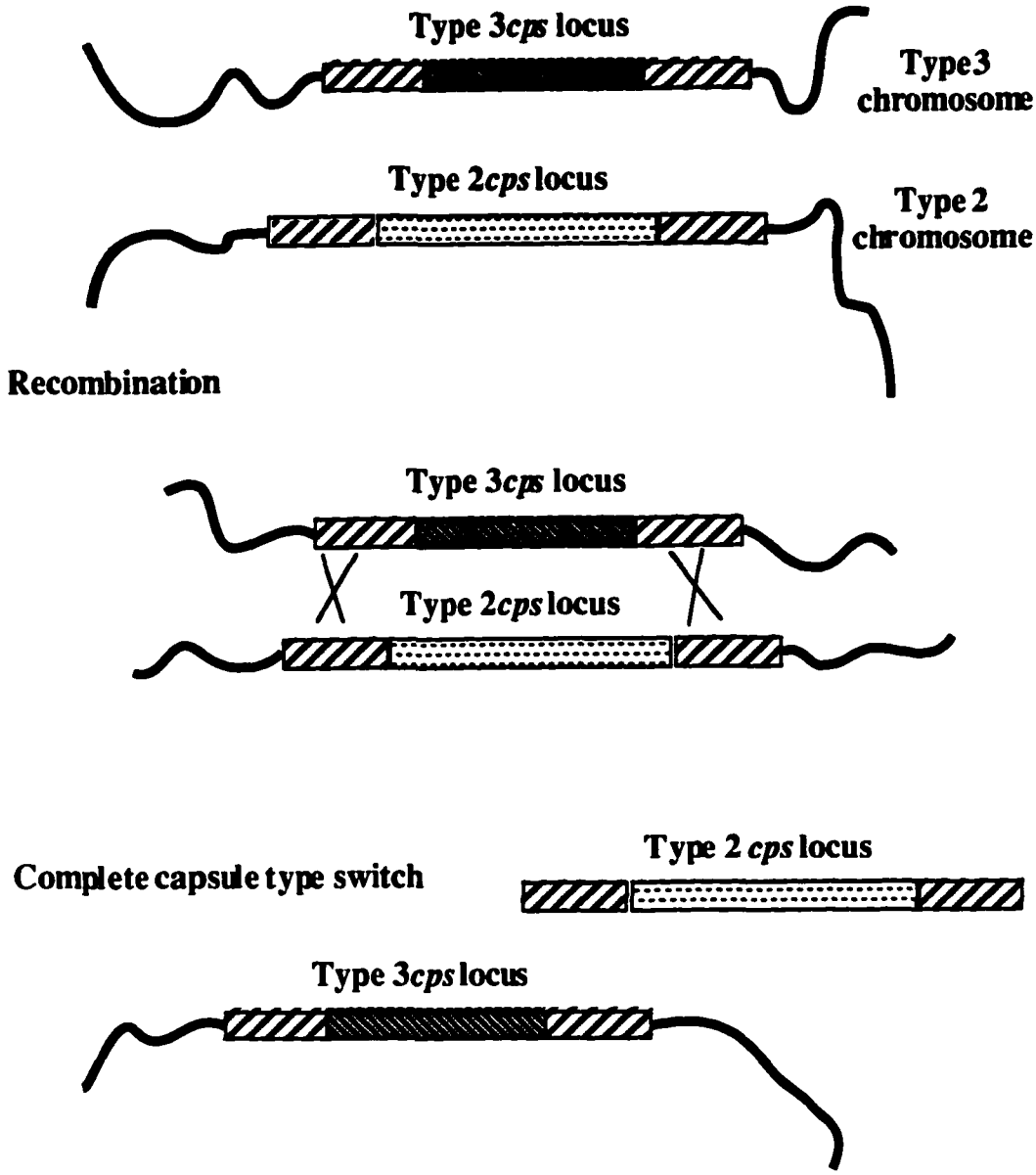


Fig. 1. Cassette recombination mechanism

and Trask reported the identification of distinct variants of serotypes 1 and 2. These strains, although reactive with type-specific antisera, differed in their ability to agglutinate in the presence of immune sera, in their colony appearance, and in their virulence properties (Blake and Trask, 1923, 1933). MacLeod and Krauss made a similar observation with variants of serotypes 2 and 3 (MacLeod and Krauss, 1947, 1956). One such type 3 variant was shown to produce only one-third the normal amount of capsular polysaccharide, despite the ability of this strain to transfer the normal type 3 phenotype in transformation reactions (MacLeod and Krauss, 1956). These data suggest that some factor(s) within the background of the recipient strain resulted in a reduction in type 3 polysaccharide. A strain isolated by our group was shown to produce approximately half the amount of type 3 capsule as the parent type 3 strain. This defect in capsule production could be repaired as a result of reversion or by transformation with DNA from type 2 *S. pneumoniae*, suggesting that the original defect may have resulted from a spon-taneous mutation within the type 3 background. The locus responsible for the repair of this defect was not, however, closely linked to the type 3-specific capsule locus (Caimano and Yother, unpublished data). Insertions within this strain which were used to measure transcription throughout the type 3 capsule locus suggest that this defect may be the result of a form of transcriptional regulation.

The phenomenon of binary encapsulation also serves as an exception that cannot be fully explained by the cassette recombination mechanism. *S. pneumoniae* strains capable of expressing two capsule polysaccharides simultaneously were identified by Austrian and Bernheimer in 1959 (Austrian and Bernheimer, 1959; Austrian *et al.*, 1959). The poly-saccharides produced were essentially identical to those of the parent strains and were cross-reactive with antisera against both the donor and recipient serotypes. Characteriza-tion of these binary encapsulated strains determined that they were the result of a rare event during transformations between certain heterologous capsule types (Austrian *et al.*, 1959). Transformations using donor DNA from types 1, 5, 9, 25, and 33 were shown to be able

to form binary encapsulated strains when type 3 strains were used as recipients. As the type 3 capsule is highly mucoid and would often mask the presence of a second type-specific polysaccharide in these binary strains, these studies were made easier by the isolation of type 3 non-encapsulated spontaneous mutants. These type 3 mutants were known to be deficient in a required UDP-glucose dehydrogenase activity and therefore phenotypically type 3 negative. The non-encapsulated phenotype of the type 3 mutants allowed encapsulated transformants to be quickly distinguished. Also, the loss of capsule resulted in a higher transformation efficiency and an increased isolation of rare transformants. In one such transformation, non-encapsulated type 3 recipients were transformed with donor DNA from a type 1 strain and restored to encapsulation. Analysis of the polysaccharide produced by these strains demonstrated the ability of these transformants to synthesize both type 1 and type 3 capsular polysaccharides (Austrian and Bernheimer, 1959). Earlier studies had demonstrated that the synthesis of both the type 1 and type 3 polysaccharides required the activity of a UDP-glucose dehydrogenase (Taylor, 1949). The ability of these binary strains to produce type 3 capsule was attributed to comple-mentation of UDP-glucose dehydrogenase activity by the type 1 enzyme. Investigators could not, however, detect any evidence of repair of the type 3 dehydro-genase mutation. Also, the DNA isolated from these binary strains did not act in a manner consistent with the presence of a single capsule locus (Bernheimer *et al.*, 1967). It was therefore proposed that binary encapsulation resulted from the maintenance of two distinct genetic units (Austrian *et al.*, 1959).

When used in transformation reactions, donor DNA from binary strains resulted in two classes of transformants. The first class was similar to the binary donor strain. These transformants were shown to contain the genetic material of both capsule types and these loci were shown to be linked on the chromosome. When used in transformations, donor DNA from this class was able to transfer the ability to produce either the linked 1-3 binary capsule or type 1 alone. This binary phenotype was highly unstable and this class of

transformants soon lost the ability to produce both polysaccharides. Based on linkage between the two capsule loci in binary strains, it was proposed that this first class of transformants was the result of homologous recombination between gene(s) within or immediately adjacent to the type 1 and type 3 capsule loci. This homology was proposed to be present at only one end of the capsule loci (Bernheimer *et al.*, 1967; Dillard *et al.*, 1995) (Fig. 2A.)

The second class of binary encapsulated transformants was unable to transform recipient strains to either binary or type 3 encapsulation but retained the ability to transform recipient strains to type 1 encapsulation. In genetic analyses of these strains, the type 1 and type 3 capsule loci were shown to be unlinked. This result suggested the insertion of the type 1 donor DNA into a locus distinct from that normally occupied by the type-specific genes. Unlike the first class, this second class of transformants was stable (Bernheimer *et al.*, 1967). The insertion of donor DNA at an unlinked site on the chromosome has been proposed to occur by either illegitimate recombination (Bernheimer *et al.*, 1967; Dillard *et al.*, 1995) or by a transposition-like event (Dillard *et al.*, 1995) (Fig. 2B).

Through biochemical analyses, the sugar compositions and chemical structures of many of the pneumococcal capsular polysaccharides have been determined (van Dam *et al.*, 1990). Examination of these capsule structures reveals a variety of forms which range from relatively simple structures with only two sugars arranged as a linear polymer (e.g., serotype 3) to highly complex structures containing several different sugars, including modified sugars such as pneumosamine (e.g., serotype 5) arranged in multiply branched chains. Studies on the pneumococcal capsule have often focused on serotype 3, in part, because of its highly mucoid appearance, its established virulence, and its historical participation in early genetics experiments. The chemical structure of the type 3 capsule was reported by Reeves and Gloebel in 1941 to be a linear polysaccharide composed of repeating units of cellobiuronic acid (i.e., glucuronic acid-β(1-4) glucose) (Fig. 3).

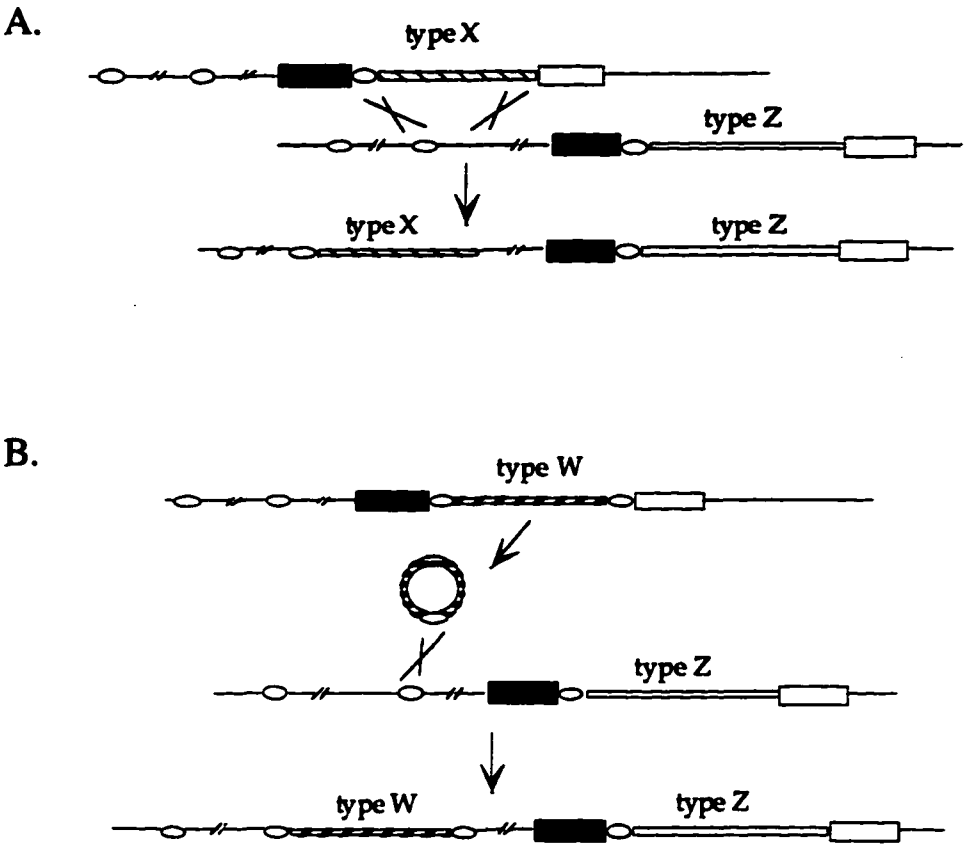
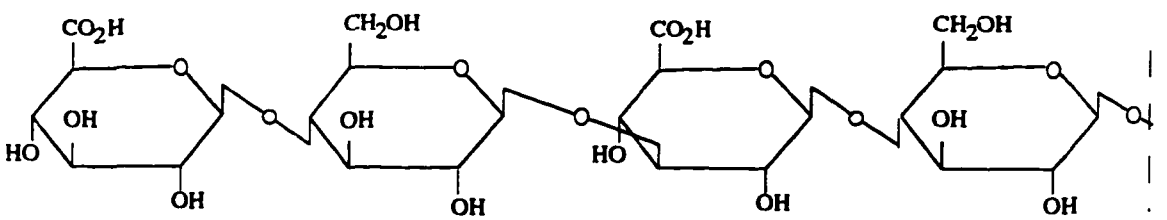


Fig. 2. Proposed mechanism for the generation of binary encapsulated strains
A. Binary encapsulation via homologous and illegitimate recombinations.
B. Binary encapsulation via a transposition-like event.



[β-D-glucuronic acid (1->4) -β-D-glucose (1->3)]n

Fig. 3. Structure of the pneumococcal type 3 capsular polysaccharide.

The discovery of an enzyme from the soil bacterium *Bacillus palustris* which could degrade type 3 polysaccharide (Dubos and Avery, 1931) made it possible to perform reconstitution studies for the regeneration of capsule in live (resting) cell suspensions. In these early studies, investigators determined that the biosynthesis of type 3 polysaccharide required glucose, magnesium, potassium, and phosphate, with little or no capsule produced in the absence of glucose. Other fermentable sugars, such as fructose, galactose, maltose, and lactose, were able to substitute for glucose, but not with the same efficiency (Bernheimer, 1953). Capsule synthesis was also shown to be greater under aerobic conditions. By the use of cell-free extracts to study uridine pyrophosphoglycosyl metabolism, investigators were able to demonstrate the involvement of the nucleotide sugars UDP-glucose and UDP-glucuronic acid in the formation of a type-specific capsule (Smith *et al.*, 1957). The presence of uridylyl transferase activity and a low level of phosphoglucomutase activity was also demonstrated using cell-free extracts from a non-encapsulated type 2 *S. pneumoniae.* When these same studies were attempted with type 3 *S. pneumoniae,* however, the presence of contaminating polysaccharide often interfered with the preparation of extracts. As a result, enzymatic assays were carried out using intact organisms. As with the type 2 cell-free extracts, uridylyl transferase activity was also demonstrated with type 3 *S. pneumoniae* (Smith *et al.*, 1957). These studies did not, however, report the presence of phosphoglucomutase activity in the type 3 cell-free extracts. The finding that both encapsulated and non-encapsulated pneumococci contained identical enzyme systems for uridine nucleotide metabolism led investigators to postulate that the production of type 3-specific capsule was in part due to the presence of specific activities involved in subsequent steps of conjugation and polymerization (Smith *et al.*, 1957).

The ability of resting cell extracts to synthesize type 3 capsule by the addition of glucose alone suggested the presence of a dehydrogenase activity for the conversion of glucose or UDP-glucose to UDP-glucuronic acid. The demonstration of this activity

eluded investigators for some time until the identification of a strong NADH oxidase which inhibited the activity of the dehydrogenase. This oxidase was later shown to co-purify with the dehydrogenase (Smith *et al.*, 1958). The demonstration of direct incorporation of radiolabeled UDP-glucuronic acid into polysaccharide argued against the post-polymerization modification of a glucose polymer to the type 3 glucose-glucuronic acid structure (Smith *et al.*, 1961). Also, the isolation of mutants which were unable to form type 3 capsule when given only UDP-glucose as a precursor but which were able to form intact polysaccharide when supplied with both UDP-glucose and UDP-glucuronic acid supports the involvement of such a dehydrogenase activity (Dillard *et al.*, 1995). Recently, this activity was demonstrated using extracts of an *E. coli* strain overexpressing the type 3-specific UDP-glucose dehydrogenase described below (Arrecubieta *et al.*, 1996).

These studies allowed investigators to propose a mechanism for the synthesis of the type 3 polysaccharide (Mills and Smith, 1962) (Fig. 4). Investigators were unable, however, to distinguish between the activities necessary to form the disaccharide unit and the linear polymer. At the time, two independent activities were proposed. As described below, these functions are most likely performed by the same enzyme (Dillard *et al.*, 1995).

Identification of type-specific capsule loci of S. pneumoniae

Early progress in the field of pneumococcal polysaccharide biochemistry was made during a time when relatively little was known about the genetics of capsule biosynthesis. However, the identification of the enzyme activities associated with capsule biosynthesis later became invaluable in the characterization of non-encapsulated mutants, in transformations involving heterologous capsule types, and in the elucidation of the genetic organization of the capsule loci of different serotypes. Recent work has identified genes involved in the biosynthesis of *S. pneumoniae* serotypes 3, 14, and 19F (Arrecubieta *et al.*, 1994; Dillard and Yother, 1994; Guidolin *et al.*, 1994; Arrecubieta *et al.*, 1995; Dillard *et*

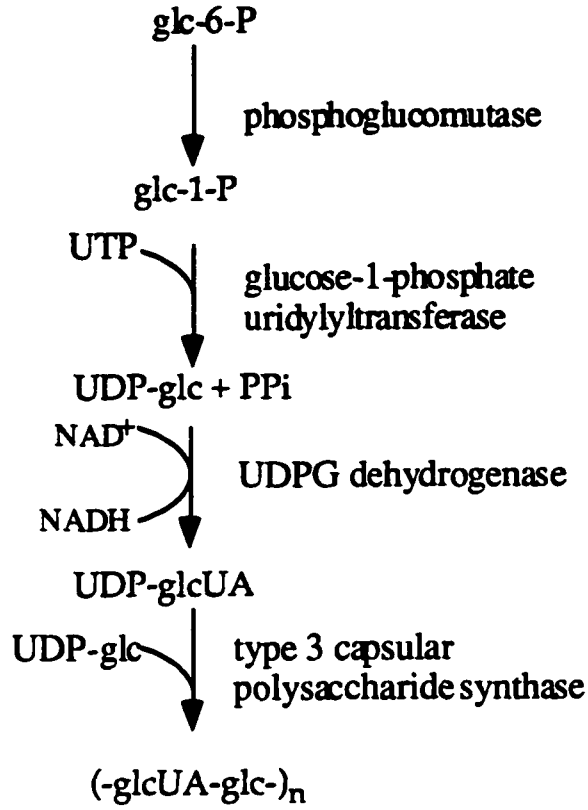


Fig.4. Proposed biosynthetic pathway for type 3 polysaccharide capsule in *S. pneumoniae*

al., 1995; Kolkman *et al.*, 1996). In the case of type 14, Kolkman *et al.* identified the type-specific gene *cps14E* through characterization of a non-encapsulated mutant constructed by transposon mutagenesis; *cps14E* encodes a protein with homology to glycosyl transferases. Analysis of membrane preparations of a serotype 14 strain in which *cps14E* was insertionally inactivated showed a significant reduction in glycosyl transferase activity (Kolkman *et al.*, 1996). Glycosyl transferases have been shown in *Salmonella typhimurium*, *Streptococcus agalactiae*, *Xanthomonas campestris*, and two species of *Rhizobium* to catalyze the addition of a sugar residue to a membrane-bound lipid molecule in the first step of polysaccharide biosynthesis (Osborn and Tze-Yuen, 1968; Jiang *et al.*, 1991; Ielpi *et al.*, 1993; Reuber and Walker, 1993; Rubens *et al.*, 1993) (see below).

In the case of serotype 19, Guidolin *et al.* have identified seven genes, *cps19fA-G*, associated with the synthesis of serotype 19F polysaccharide (Guidolin *et al.*, 1994). Insertions within any of these genes result in a non-encapsulated phenotype. These loci are apparently transcribed as a single operon and it has not been determined whether the insertions within each gene lead to non-encapsulation as a result of loss of function essential for capsule biosynthesis or a polar effect on one or more downstream gene(s). While the genes within the *cps19f* capsule locus are predicted to encode proteins with homology to enzymes involved in the biosynthesis of bacterial polysaccharides, their protein products have not been characterized. Cps19fE, however, has 96% amino acid identity to Cps14E and most likely performs a very similar or identical function (Kolkman *et al.*, 1996).

The work presented within this dissertation involves almost exclusively studies with serotype 3 (Fig. 3). From the simple nature of this structure, one would predict that the number of genes involved in its synthesis would be few in number. Studies on the biosynthesis of capsule provided an extensive background on the enzyme activities expected to be involved in production of type 3-specific polysaccharide. In addition, the highly mucoid appearance of type 3 strains on solid media allows for colonies of this

serotype to be easily distinguished from both non-encapsulated mutants and encapsulated strains of other serotypes. Type 3 strains were also demonstrated to be highly virulent in mice and are often isolated from humans diagnosed with pneumococcal infections.

Recently, the type-specific genes of serotype 3 have been identified as *cps3D*, *cps3S*, *cps3U*, and *cps3M* (Arrecubieta *et al.*, 1994; Dillard and Yother, 1994; Dillard *et al.*, 1995; Caimano *et al.*, 1996). The first two genes encode proteins, demonstrated in biochemical assays, which are active as a UDP-glucose dehydrogenase (Cps3D) and a type 3 capsular polysaccharide synthase (Cps3S) (Dillard *et al.*, 1995; Arrecubieta *et al.*, 1996). The product of the third gene, *cps3U*, has been shown to complement a *galU* deficient strain of *Escherichia coli*; GalU has been shown to be a UTP:glucose-1-phosphate uridylyl-transferase (Giaever *et al.*, 1988; Arrecubieta *et al.*, 1995). The fourth gene, *cps3M*, is described in this dissertation.

Analysis of the type-specific capsule loci from serotypes 3, 14, and 19F reveals a similar genetic organization. Analysis of the regions both upstream and downstream of the type-3 specific locus identified sequences which were shown to be common to all capsular serotypes examined (Guidolin *et al.*, 1994; Arrecubieta *et al.*, 1995; Dillard *et al.*, 1995; Caimano *et al.*, 1996; Kolkman *et al.*, 1996). While the genes within the type-specific loci exhibit very little overall homology, flanking sequences have been shown to be essentially identical between heterologous serotypes. These findings lend further support to the cassette-recombination mechanism previously proposed by early genetic studies and later demonstrated by Dillard *et al.* (Dillard *et al.*, 1995)

Polysaccharide assembly and transport

While the studies described above have greatly enhanced our understanding of the genetic organization of the *S. pneumoniae* type-specific loci and the functions of genes contained within, the mechanism by which pneumococcal capsule is exported to the bacterial cell surface remains unclear. Studies on the biosynthesis of lipopolysaccharide

(LPS) and polysaccharide capsules from gram-negative bacteria have led to a model for the assembly and export of these structures. LPS consists of a lipid A core and an antigenically variable oligosaccharide side chain (O-antigen). The assembly of LPS, first described in *Salmonella enterica*, has been shown to occur by essentially identical mechanisms in many gram-negative bacteria, including *S. typhimurium*, *E. coli*, and *Klebsiella pneumoniae* (reviewed in Whitfield, 1995). In the model for LPS biosynthesis, the O-antigen polysaccharide is assembled on a lipid carrier, undecaprenol phosphate (und-P), by the monomeric addition of sugar residues to one end of the growing oligosaccharide-lipid carrier molecule (und-P-P-sugar) in reactions catalyzed by glycosyl transferases. Once assembled, the O-antigen is moved to a ligation site within the plasma membrane by a transporter molecule, translocated across the membrane and transferred from the und-P carrier to the membrane-associated lipid A core on the periplasmic face of the membrane. While differences have been noted for O-antigens composed of heterologous sugars versus those which are homopolymers, the mechanism of assembly is similar regardless of O-antigen complexity. In addition, a peptide motif ($PX_2PX_4SPK\ X_{11}GXMXG$) has been identified within a series of homologous proteins in *Neisseria meningitidis* (CtrB), *Haemophilus influenzae* (BexC), *R. meliloti* (ExoP), *S.* enterica (CLD), and *E. coli* (CLD, Rol). While each has been implicated in the biosynthesis of polysaccharides, only the *E. coli* and *S. enterica* CLDs and the *R. meliloti* ExoP proteins have been characterized by genetic and biochemical analyses and have been shown to be involved in the determination of polysaccharide chain length and polymerization of oligosaccharides (Bastin *et al.*, 1993; Glucksmann *et al.*, 1993; Becker *et al.*, 1995). The BexC protein from *H. influenzae* type b has been shown to be essential for export of the capsular polysaccharide, but a regulatory role in biosynthesis has not been demonstrated (Kroll *et al.*, 1988).

A similar mechanism has been proposed for the assembly of exopolysaccharides in *E. coli* K1 (polysialic acid capsule), *N. meningitidis* (polysialic acid capsule), and *X. camepstris* (xantham gum) (Frosch and Müller, 1993; Ielpi *et al.*, 1993; Bliss and Silver,

1996). These processes, however, are not as well understood as LPS assembly. In *E. coli*, the polymerization of polysialic acid is thought to involve a lipid intermediate, NeuNAc-P-undecaprenol. Although the role of this lipid molecule is not fully understood, there is an apparent link between assembly and transport of the polysialic capsule (Bliss and Silver, 1996). In *X. campestris*, xantham gum is a complex polysaccharide composed of a β(1-4) glucan chain with a trisaccharide branch (mannose-β(1-4)-glucuronic acid-α(1-2)-mannose) linked α-(1-3) at every two glucose residues. Ielpi *et al.* proposed a mechanism in which the pentasaccharide subunits are assembled on an endogenous polyprenol-P-P lipid intermediate and then transferred as a group to the growing xantham gum polysaccharide (Ielpi *et al.*, 1993). The mechanism by which these polysaccharides are exported to the cell surface is presently unknown.

In *N. meningitidis*, two gene clusters, Region B and Region C, have been identified and shown to be involved in the translocation of the polysialic acid capsule to the bacterial cell surface (Frosch and Müller, 1993). Region B has been shown to encode enzymes responsible for the phospholipid substitution of the polysialic acid capsule. This phospholipid modification is a requirement for capsular translocation to the cell surface. Region C, encoding proteins with homology to ABC transporter proteins, has been implicated in the actual translocation of the polysialic capsule (Frosch and Müller, 1993).

Our understanding of the assembly and transport of polysaccharides in gram-positive bacteria is not nearly as advanced as that for gram-negative bacteria. Sequences have been identified in association with the polysaccharide capsule genes of several gram-positive bacteria, including *S. pneumoniae* (Guidolin *et al.*, 1994; Caimano *et al.*, 1996; Kolkman *et al.*, 1996), *S. thermophilus* (Stingele *et al.*, 1996), *S. agalactiae* (Rubens *et al.*, 1993), and *S. aureus* (Lin *et al.*, 1994). These sequences have homology to genes known to be involved in regulation, assembly, and transport of polysaccharides in gram-negative bacteria. The functions of most of these gram-positive bacterial gene products, however, have not been determined and their role in polysaccharide biosynthesis is

presently only speculative. One exception is *cpsD* from the group B streptococcus. A non-encapsulated derivative of *S. agalactiae* was isolated by transposon mutagenesis and the locus responsible for the capsule-deficient phenotype was identified as *cpsD*; CpsD was shown to have limited homology (31% identity/57% similarity) to RfbP from *S. typhi-murium*. RfbP functions as a galactosyltransferase and is responsible for the transfer of galactose-1-phosphate to the lipid precursor undecaprenol phosphate in the first step of O-antigen biosynthesis (Osborn and Tze-Yuen, 1968; Jiang *et al.*, 1991). In enzyme assays using cell lysates, galactosyltransferase activity was greatly reduced in a *cpsD* negative strain. Also, fractionation studies suggested that CpsD is localized to the plasma membrane (Rubens *et al.*, 1993). This finding is in agreement with a function homologous to that of RfbP, which is also a membrane-associated protein. *S. pneumoniae* Cps14E and Cps19fE have a similar level of homology (31% amino acid identity) to RfbP as *S. agalactiae* CpsD. Functional assays using membrane preparations of *S. pneumoniae* serotype 14 demonstrated direct incorporation of radiolabeled C^{14}-sugar into a glycolipid fraction. Membrane preparations from an insertionally-inactivated *cps14E* strain did not contain glycosyltransferase activity (Kolkman *et al.*, 1996). As stated above, *S. pneumoniae* Cps19fE exhibits 98% amino acid identity to Cps14E and, although an enzymatic activity associated with Cps19fE has not been demonstrated, is likely to perform the same function.

With the exception of the above mentioned studies, very little else is known about the assembly and transport of capsule in *S. pneumoniae*. Studies involving immunochemical and *in situ* assays suggest that the capsules in serotypes 2, 4, 6A, 6B, 7F, 8, 14, 19F, and 23F are covalently linked to the cell wall peptidoglycan. Immunochemical studies with serotype 3 suggest, however, that this polysaccharide is not attached by a covalent association to the cell peptidoglycan (Sørensen *et al.*, 1990). Recent work has advanced our understanding of the assembly of *S. pneumoniae* serotype 3 polysaccharide. Studies with Cps3S, the type 3 synthase, suggest that this enzyme possesses a dual function and is able to catalyze the stepwise addition of both glucose and glucuronic acid by monomer

addition as well as catalyze the formation of both glucuronic acid-β(1-4)-glucose and glucose-β(1-3)-glucuronic acid linkages. Cell preparations from strains containing mutations with *cps3S* are unable to synthesize immunoprecipitable type 3 polysaccharide when given both UDP-sugar precursors in an *in vitro* assay. HasA from *S. pyogenes*, with which Cps3S has homology (23% amino acid identity/50% similarity), has been shown to possess dual activity and is able to catalyze the formation of hyaluronic acid capsule (-glucuronic acid-β(1-3)-*N*-acetylglucosamine-β(1-4)-) from nucleotide sugar precursors in the absence of any other factor (Dougherty and Rijn, 1992). This group A streptococcal enzyme has also been shown to be able to catalyze the synthesis of hyaluronic acid when expressed in *E. coli* and *Enterococcus faecalis* (DeAngelis *et al.*, 1993). Similar studies were attempted with the type 3 synthase; however, the overexpression of *cps3S* in *E. coli* was shown to be toxic to the cells (Dillard *et al.*, 1995). Although biochemical assays using partially-purified membrane preparations from *S. pneumoniae* serotype 3 are able to catalyze the incorporation of radiolabeled UDP-sugar into polysaccharide (Cartee, Yother, and Schutzbach, unpublished results), the activity of purified Cps3S has yet to be demonstrated. In addition, it is unknown if the initiation of biosynthesis of type 3 polysaccharide involves a lipid-carrier or "initiator" molecule. Early studies using crude cell preparations reported that the presence of endogenous type 3 polysaccharide had an enhancing effect on *de novo* synthesis; the addition of preformed type 3 polysaccharide had no such effect. The model proposed for the biosynthesis of acid in group A streptococci by the HasA synthase does not include the use of an activated glycosyl-lipid intermediate. This model instead proposes that synthesis occurs by the monosaccharide addition of the activated glucuronic acid and N-acetylglucosamine subunits to the reducing end of the growing polysaccharide chain (Prehm, 1983). Based on the homology between the group A synthase (HasA) and the *S. pneumoniae* type 3 synthase (Cps3S), and the similarities between the structure of the hyaluronic acid and the type 3 capsule polysaccharides, the model proposed for the synthesis of hyaluronic acid is likely to be applicable to the

synthesis of type 3 capsule. While this model may be used as a basis for the study of type 3 capsule, the synthesis of the other more complex capsule structures will require further studies.

Present studies

The studies presented within this dissertation were designed to advance our understanding of the type 3-specific capsule locus of *S. pneumoniae* by examining both type-specific genes and associated flanking sequences. The specific goals were as follows: 1) to complete the sequence of the most 3' type-specific gene, *cps3M*, and flanking sequences; 2) to characterize the genetic organization of the *cps3* locus; 3) to assess the involvement of *cps3* type-specific genes and flanking sequences in capsule biosynthesis; 4) to examine the transcriptional organization of the type specific locus; 5) to examine the requirement for genes within and adjacent to the *cps3* locus in virulence.

In studies described in the first manuscript, analysis of sequence within the type 3-specific locus identified a novel genetic organization between the type-specific loci and the flanking non-type specific regions when compared to heterologous serotypes. Sequence analysis of the downstream type-specific region identified *cps3M*. Although homologous to known phosphomutases, Cps3M was shown to be significantly smaller in size. This apparent truncation is proposed to have resulted from a deletion within the Cps3M C-terminus. Analysis of sequences downstream of *cps3M* identified two additional genes, *tnpA* and *plpA*, with homology to bacterial insertion sequence (IS) element transposases and peptide permeases, respectively. Both of these genes were shown to be truncated or deleted when compared to their respective homologues. In the *cps3M-tnpA-plpA* structure, the points of truncation of *cps3M* and *plpA* are directly adjacent to the internal *tnpA* truncations. These findings suggest that this structure may be the result of an aberrant recombination or transposition event. Also within the first manuscript is an examination of the upstream flanking region of the type 3-specific locus. Sequence analysis within this region identified genes which are common to all capsular serotypes. A comparison of the Cps3P

sequence present within the upstream region with its respective homologues, however, revealed a deletion similar to those observed within the downstream sequences. Also contained within the upstream region was a sequence with homology to the IS-like *Hinc*-repeat (H-rpt) elements of *E. coli*; this sequence was shown by hybridization to be repeated on the *S. pneumoniae* chromosome. *orf5*, identified adjacent to the H-rpt, was shown to be duplicated on the type 3 chromosome but present in single copy in other serotypes. In *E. coli*, the H-rpt sequences have been shown to be associated with the Rearrangement hot spot or *Rhs* elements. Five such *Rhs* elements (*RhsA-E*) have been identified in *E. coli* K12, as well as other, but not all, *E. coli* strains. Comparison of the *Rhs* elements identified has revealed a common organization and the placement of more closely related *Rhs* sequences into family subgroups (*RhsA-B-C*, *RhsD-E*, *RhsG*). A common element maintained with the *Rhs* subfamilies is the H-rpt sequences (Zhao *et al.*, 1993). Highly conserved copies of this sequences have been identified in *RhsB*, *RhsC*, and *RhsE*. The H-rpt from *RhsB* and *RhsE* are 1291 bp long and contain a 1134 bp ORF. Three tandem copies of H-rpts have been found in *RhsC* but, due to deletion and/or base substitutions, none of these copies is thought to be functional. Partial copies of H-rpt sequences have also been found on the *E. coli* chromosome, but it is not known whether this is an indication that the H-rpt may exist independently of the *Rhs* element or if these partial copies are the result of a deletion event (Zhao *et al.*, 1993). H-rpt sequences have features strongly suggestive of an IS origin, including 11 bp inverted repeats at their termini. Although no direct experimental evidence demonstrating transposition activity has been reported in *E. coli*, a sequence with homology to the H-rpts (57% similarity over 335 amino acids) has been identified in *Aeromonas salmonicida*. The H-rpt homologue in *A. salmonicida*, ISAS2, has been shown to possess transposition activity (Gustafson *et al.*, 1994). The function of the H-rpt elements has been proposed to be one of rearrangement. The presence of a repetitive peptide motif, core duplications, and sequence conservation within the *Rhs* elements suggest a high degree of inter-family recombination (Hill *et al.*, 1994).

Recent studies have identified sequences homologous to H-rpts within the *S. enterica rfb* locus, the *V. cholerae* O-antigen gene cluster, the *A. salmonicida* paracrystalline surface protein array (A-layer) structural gene, *vapA*, and the *S. pneumoniae cps3* locus. The H-rpt sequences present within each of these organisms is associated with an apparent rearrangement or deletion event. These rearrangements, in the case of *S. enterica* serovar Strasbourg and *V. cholerae* serotype O139, resulted in the formation of a novel antigenic structure (Xiang *et al.*, 1994; Bik *et al.*, 1995; Stroeher *et al.*, 1995). In the case of *A. salmonicida*, the insertion of an H-rpt-like sequence resulted in a deletion within the *vapA* gene and loss of the A-layer (Gustafson *et al.*, 1994). The genes flanking the *S. pneumoniae* type 3-specific loci are distinguished from those found in other pneumococcal serotypes by the presence of deletions and truncations. As with the *S. enterica rfb* locus and the *V. cholerae* O-antigen gene cluster, the resulting genetic structure of the *cps3* locus may contain important clues as to the origins of not only this locus but also that of other serotypes as well.

In the second manuscript, virulence studies were performed using strains containing insertions within *cps3S*, *cps3U*, *cps3M*, and the flanking gene, *plpA*. A strain containing a point mutation within *cps3D* was also used. These studies demonstrate a requirement for *cps3D* and *cps3S* for full virulence of *S. pneumoniae* serotype. Insertions within *cps3U*, *cps3M*, and *plpA* did not demonstrate a similar requirement for these genes, as strains containing insertions within either of these genes exhibited virulence properties essentially equal to that of a "normal" type 3 strain. A novel type 3 strain, MC1056, was also identified. Initial characterization of this strain suggests that the production of type 3 capsule is regulated *in vivo*.

ASSOCIATION OF TRUNCATED GENES AND A PARTIAL H-RPT ELEMENT WITH THE TYPE 3 CAPSULE LOCUS OF *STREPTOCOCCUS PNEUMONIAE*

by

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Submitted to Molecular Microbiology

Format adapted for dissertation

Summary

The capsule genes of *Streptococcus pneumoniae* have a cassette-like organization in which the type-specific biosynthetic genes are flanked by genes shared among the different capsular serotypes. Analysis of the type 3 locus has revealed its most striking feature to be a preponderance of partial genes that have homology to sequences involved in polysaccharide biosynthesis and transposition. The sequence of the most downstream type 3-specific gene, *cps3M*, predicts a protein with homology to phosphomutases. The predicted proteins of *tnpA* and *plpA*, the non-type-specific genes downstream of *cps3M*, have homologies with transposases and peptide permeases, respectively. All three of these sequences are truncated when compared to their respective homologs. Partial sequences were also identified in the region upstream of the type 3-specific genes. One of these sequences has homology to the IS-like H-rpt (Hinc repeat) element of *E. coli* and is present in multiple copies on the *S. pneumoniae* chromosome. An open reading frame of unknown function, also located in the upstream region, is duplicated on the type 3 chromosome but is present only in single copy in other capsule types, where it is not linked to the capsule locus. Mutation and transcription analyses of each of the sequences identified in this study showed that none is required for type 3 polysaccharide synthesis but that all except the unknown open reading frame are transcribed. The type 3 locus structure is conserved among independent type 3 pneumococcal isolates but similar deletions are not apparent in other capsular types. A role for transposition-mediated events in the generation of the type 3 locus, and possibly other pneumococcal capsule loci, is suggested by these findings.

Introduction

The polysaccharide capsule of *Streptococcus pneumoniae* is significant for both its role in pathogenesis and its role in the development of molecular genetics. The results of recent studies in our and other laboratories have begun to define the molecular basis for capsule expression in *S. pneumoniae* (Arrecubieta *et al.*, 1994; Dillard and Yother, 1994; Dillard *et al.*, 1995; Guidolin *et al.*, 1994). Among the conclusions of these studies is that

genes shared by apparently all capsular types flank genes that are specific for the type of capsule expressed. As a result of this common organization, we were able to provide direct evidence for a cassette-like transfer of the capsule loci between strains of different capsular types (Dillard and Yother, 1994). In the laboratory, the inter-strain transformation of pneumococcal capsular polysaccharide biosynthetic genes was a central step in demonstrating that the "transforming principle", and hence the genetic material, is DNA (Avery *et al.*, 1944). In nature, the genetic exchange of DNA among *S. pneumoniae* strains is likely to have played a major role in the generation of new strains and in the evolution of capsular serotypes with varying virulence properties. Although 90 serologic capsular types have been recognized (Henrichsen, 1995), strains representing only 23 serotypes account for more than 90% of all pneumococcal infections. The reason for the predominance of these serotypes is unclear, but it appears to reflect the genetic background of the strains and not simply the type of capsule expressed (Kelly *et al.*, 1994). Likewise, the basis for the emergence of new capsule types is unknown. Mutation, recombination, immune selection, and natural transformation could all be expected to contribute to the process.

The type 3 capsule locus contains the type-specific biosynthetic genes *cps3D* and *cps3S* which are required for type 3 capsule production (Dillard and Yother, 1994; Dillard *et al.*, 1995). *cps3D* encodes a UDP-glucose (UDP-Glc) dehydrogenase which converts UDP-Glc to UDP-glucuronic acid (UDP-GlcUA). *cps3S* encodes the type 3 synthase that catalyzes formation of the linkages required to form the $(GlcUA\text{-}Glc)_n$ type 3 polysaccharide. The functions of the two gene products were demonstrated by using *cps3D* fragments to restore mutants defective in UDP-Glc dehydrogenase activity to normal type 3 encapsulation (Dillard and Yother, 1994) and by using an *in vitro* polysaccharide synthesis assay to demonstrate loss of the dehydrogenase and synthase functions in defined type 3 mutants (Dillard *et al.*, 1995). UDP-Glc dehydrogenase activity has recently been confirmed from expression of the cloned gene in *E. coli* (Arrecubieta *et al.*, 1996). Sequence and mutation analyses indicate that *cps3D* and *cps3S* are transcribed as part of the

same operon that utilizes a promoter located directly upstream of *cps3D* (Dillard *et al.*, 1995). This assignment has been confirmed in Northern and primer extension analyses (Arrecubieta *et al.*, 1995 and this communication). Two additional type 3-specific genes, *cps3U* and, as further described here, *cps3M*, are present and are predicted to encode proteins with homology to UDP-Glc-1-P uridylyltransferases (Glc-1-P → UDP-Glc) and phospho-mutases (Glc-6-P → Glc-1-P), respectively (Dillard *et al.*, 1995). The UDP-Glc-1-P uridylyltransferase function has been demonstrated through complementation of an *E. coli galU* mutant (Arrecubieta *et al.*, 1995).

The type 3-specific genes are flanked downstream by sequences shared with other capsule types (Dillard and Yother, 1994; Dillard *et al.*, 1995). The upstream flanking region contains sequences that are repeated in the *S. pneumoniae* chromosome and that are also present in other capsule types but that are not always linked to the type-specific genes (Dillard *et al.*, 1995). Based on the discovery of this repeated element, we previously proposed a transposition-like event to explain the occurrence of binary encapsulated strains and as a possible mechanism for novel capsule type formation (Dillard *et al.*, 1995). In binary encapsulated strains, which occur only rarely, the inter-strain transfer of type-specific capsule gene cassettes results in the maintenance and expression of both the donor and recipient type-specific genes, rather than in the replacement of the recipient's type-specific genes with those of the donor (Austrian *et al.*, 1959; Bernheimer *et al.*, 1967; Bernheimer and Wermundsen, 1969). As described in this communication, characterization of the region flanking the type 3-specific genes has identified sequences with homologies to genes involved in transposition, further suggesting that this process may play a role in either capsule gene transfers or the evolution of the capsule loci.

The region containing the repetitive element separates the type 3-specific genes from common capsule genes that have also been identified adjacent to the type 19F- and type 14-specific genes (Guidolin *et al.*, 1994; Arrecubieta *et al.*, 1995; Kolkman *et al.*, 1996), as well as the capsule locus of *Streptococcus thermophilus* (Stingele *et al.*, 1996). The

predicted products of these common genes have homology with proteins involved in polysaccharide export and, with the exception of the type 3 locus, they are located immediately upstream of the type-specific biosynthetic genes. In type 19F, both the common and the type-specific capsule genes appear to be contained in a single operon (Guidolin *et al.*, 1994). In contrast, the type 3-specific genes can be transcribed independently of the upstream common region (Dillard *et al.*, 1995).

Further characterization of sequences within and flanking the type 3-specific genes has now provided additional evidence for a conserved organization of *S. pneumoniae* capsule loci. Yet, despite the apparent high degree of similarity between loci of different capsular types, important differences that may help explain the emergence of new capsular types and the correlation between serotype and virulence are apparent. Most interestingly, characterization of the type 3 genes has revealed a locus that is distinguished by the presence of partial gene sequences.

Results

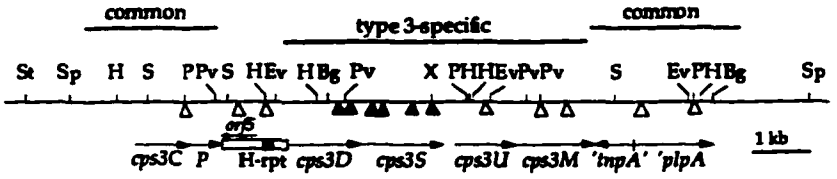
The loci of different capsular serotypes are designated by the locus name followed by the number of the serotype, e.g., type 3 is indicated as *cps3* (Guidolin *et al.*, 1994; Dillard *et al.*, 1995). For type 3, the type-specific genes and the genes downstream are named based on expected function (Dillard and Yother, 1994). Common sequences that are located upstream of the type 3-specific genes have been given the designation of their type 19F homolog when the two are identical, e.g., *cps3C* is equivalent to *cps19fC*. The type 3 locus is shown in Fig. 1A.

Identification of truncated sequences

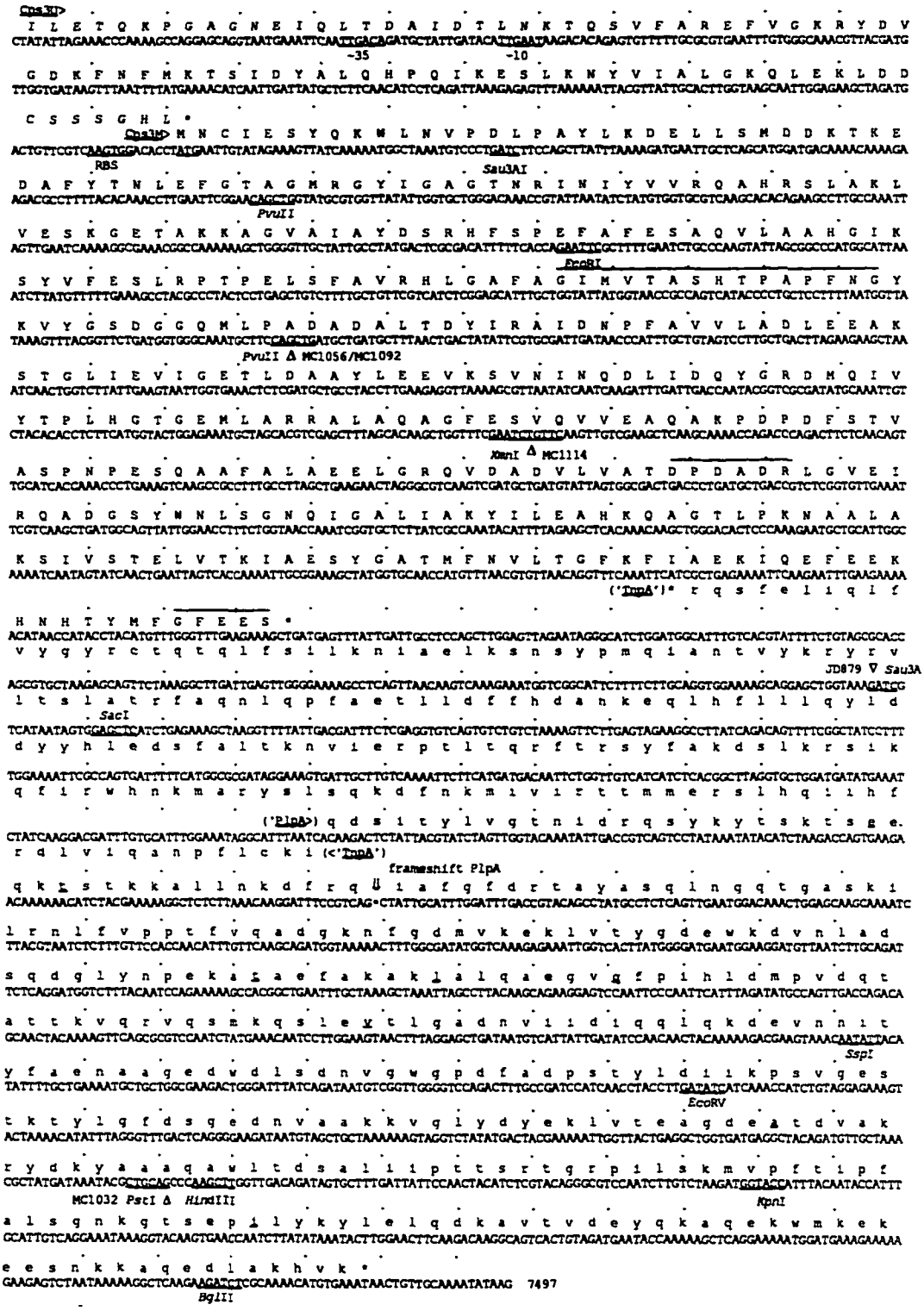
Previous work in our lab identified a 3.1 kb *Hin*dIII fragment from *S. pneumoniae* WU2 which contains *cps3U* and the downstream portion of the type 3 locus. This region also includes the DNA that flanks the type-specific region and is common to all capsular serotypes tested thus far. Partial analysis of this clone indicated, in addition to *cps3U*, the presence of sequences predicted to encode proteins homologous to phosphomutases and

Fig. 1. Type 3 capsule locus.
(*A*) Map of type 3 capsule locus. Restriction sites: Bg, *Bgl*II; Ev, *Eco*RV; H, *Hin*dIII; P, *Pst*I, Pv, *Pvu*II; S, *Sac*I; Sp, *Sph*I; St, *Stu*I; X, *Xba*I. Triangles, with strain names denoted in Fig. 1B, indicate the points of insertion mutations: Δ, insertions which do not affect capsule production; ▲, insertions which result in a capsule-negative phenotype. Descriptions of bacterial strains and plasmids are in Table 1. The insertions in *cps3DSU* have been described (Dillard *et al.*, 1995).
(B) DNA sequence of the region containing the 3' end of *cps3U*, *cps3M*, *tnpA*, and *plpA*. Numbering of nucleotide sequence is based on the previously reported *cps3DSU* sequence (Dillard *et al.*, 1995). The putative *cps3M* -35 and -10 sequences and the putative Cps3M ribosome binding site (RBS) are indicated. Lower case letters are used to denote amino acid sequence which is not expected to be expressed due to lack of translational signals and/or a frameshift mutation. Underlined amino acids in PlpA indicate differences from the type 2 PlpA sequence (Pearce *et al.*, 1994). Overlined amino acids indicate sequences conserved in phosphomutases. Symbols: •, 1 bp deletion in *plpA* sequence; <, >, direction of translation.

A



B



peptide permeases (Dillard *et al.*, 1995). Completion of the characterization of this clone and regions downstream has confirmed that such sequences are present but has also shown that they are not complete genes.

(i) Cps3M. The putative translational start codon of Cps3M, the most downstream of the type 3-specific open reading frames, overlaps the putative stop codon of Cps3U (Fig. 1B). Cps3M has homology to phosphoglucomutases (PGM) and phosphomannomutases (PMM) from both gram-positive and gram-negative bacteria, as well as phosphomutases from rabbit and yeast. Several regions conserved among PGMs and PMNs, and expected to be important in their function are present within Cps3M (Fig. 2). However, a stop codon located immediately after the last amino acid in the putative substrate binding site of Cps3M results in a truncated molecule that has lost over 100 amino acids when compared to the other proteins (Fig. 1B and Fig. 2). The alteration is apparently due to a deletion rather than to a frameshift mutation as sequences homologous to phosphomutases were not detected downstream of the Cps3M stop codon.

(ii) 'TnpA'. Overlapping the C-terminus of Cps3M, but of opposite orientation, is 'TnpA' (Fig. 1B). Although *'tnpA'* extends for 555 bp and has the potential to encode a polypeptide of 22,169 Da, it lacks any apparent transcriptional or translational signals, including a methionine start codon. 'TnpA' has homology with transposases of insertion sequences (IS) from several gram-positive bacteria and one gram-negative bacterium. Compared to the other transposases, however, 'TnpA' represents only an internal portion of a larger ORF (Fig. 3). No inverted or direct nucleotide repeat sequences were identified in *'tnpA'*. A 23 amino acid region of 'TnpA' which overlaps Cps3M is most likely coincidental, as it includes the potential sugar substrate binding site of the latter molecule (Fig. 1B) and has no homology to other transposases.

(iii) 'PlpA. The region adjacent to *'tnpA'* contains sequence that is essentially identical to that found in *plpA* from derivatives of the *S. pneumoniae* serotype 2 strain D39 (permease-like protein A [Pearce *et al.*, 1994], also named *aliA* [Alloing *et al.*, 1994]).

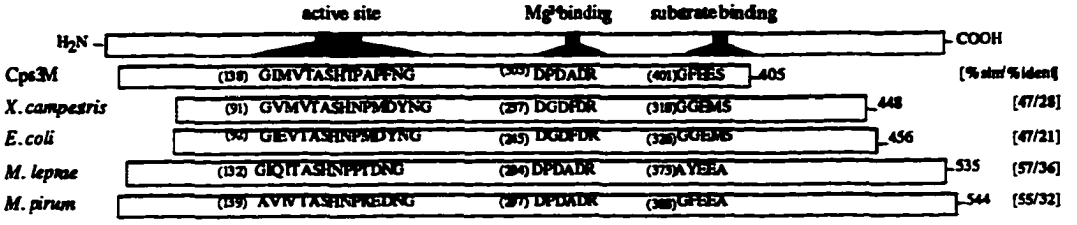


Fig. 2. Comparison of Cps3M with phosphomutases. Amino acid positions are given in parentheses with the total number of amino acids given at the end. The proteins shown are *Xanthomonas campestris* XanA (PGM/PMM) (Koplin *et al.*, 1992), *E. coli* CpsG (PMM) (Aoyama *et al.*, 1994), *Mycobacterium leprae* cosmid clone L308 ORF (GenBank accession # U00022), and *Mycoplasma pirum* ORF5 (Tham *et al.*, 1993). The putative active site regions contain a serine which is thought to be phosphorylated to form the active enzyme (Ray *et al.*, 1983). The putative Cps3M Mg^{2+} binding site is identical to that found in both the mycobacterial and the mycoplasmal ORFs but is variant from the reported Mg^{2+} binding site sequence, DGDGDR (Dai *et al.*, 1992), present in yeast and rabbit muscle PGMs, and in gram-negative bacterial PGMs and PMMs. The putative substrate binding site is homologous to that reported for PGMs and PMMs (Dai *et al.*, 1992).

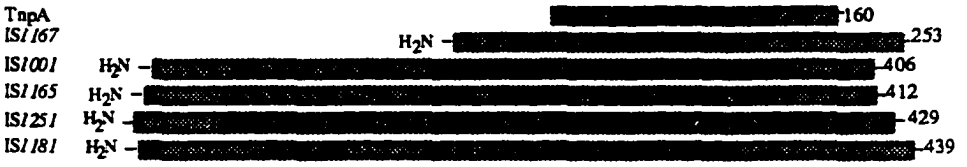
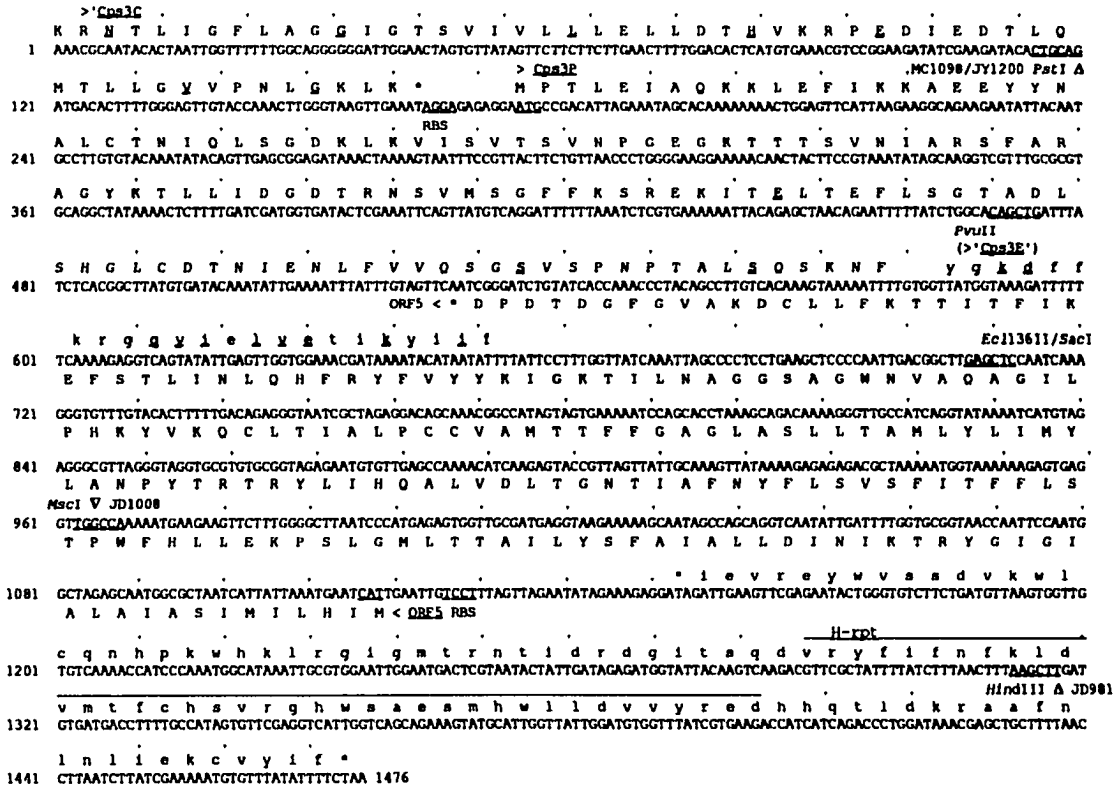


Fig. 3. Comparison of 'TnpA' with transposases. Sizes of the transposases are indicated in amino acids at the C-terminal ends. The sequences shown and their homologies (% identity/% similarity) with 'TnpA' (over the region present in each sequence) are as follows: IS*1167*, *S. pneumoniae* (48/71 [Zhou *et al.*, 1995]); IS*1001*, *Bordetella parapertussis* (19/41 [Zee *et al.*, 1993]); IS*1165*, *Leuconostoc mesenteroides* (31/56 [Johansen and Kibenich, 1992]); IS*1251*, *Enterococcus faecium* (34/61 GenBank accession # L34675); IS*1181*, *Staphylococcus aureus* (29/53 [Derbise *et al.*, 1994]).

PlpA has homology with several bacterial permeases involved in the transport of oligo-peptides, including AmiA, the substrate binding protein member of the Ami ABC transporter in *S. pneumoniae* (Alloing *et al.*, 1994; Pearce *et al.*, 1994). In both gram-positive and gram-negative organisms, proteins belonging to the ABC transporter family of ATP-dependent membrane transport proteins have been found to be involved in the transport of exopolysaccharides (reviewed in Fath and Kolter, 1993). Completion of the type 3 *plpA* sequence has revealed 98% DNA identity with bp 843 to 1949 of the type 2 *plpA*. However, two striking differences between the *plpA*s are apparent. First, the type 3 *plpA* has undergone a deletion at the 5' terminus that resulted in loss of the first 281 amino acids present in the type 2 sequence. The site of the deletion is immediately adjacent to the beginning of *'tnpA'*. Second, a 1 bp deletion in the type 3 *plpA* (Fig. 1B, position 6519) results in a frameshift mutation at amino acid position 43 of the type 3 PlpA. Correcting for the frameshift, the predicted amino acid sequence of the type 3 PlpA differs from that of the type 2 PlpA by only eight amino acids (Fig. 1B).

(iv) ORF5 and H-rpt homology. The region upstream of *cps3D* contains sequence that is present in multiple copies in the chromosomes of strains of types 2, 3, 5, 6B, 8, 9, and 22 (Dillard *et al.*, 1995). Additional sequencing of this region, along with a correction in the previously reported nucleotide sequence, identified an open reading frame (ORF5) of 197 amino acids that reads in the direction opposite to Cps3DSUM (Fig. 4). No extensive homologies with known protein sequences are apparent in this ORF. The protein predicted from the *orf5* sequence is highly hydrophobic, with at least three potential membrane spanning regions present (data not shown). The region between ORF5 and Cps3D contains 39 amino acids that have 46% identity to the H-rpt (Hinc repeat) elements of *E. coli* and 41% identity to the homologous sequence from the *Vibrio cholerae rfbQRS* gene product (Fig. 4) . The H-rpt sequences contain features strongly suggestive of IS elements (Zhao *et al.*, 1993).

Fig. 4. Sequence of the flanking region upstream of the type 3-specific genes. The sequence begins in the 3' end of *cps3C*. The promoter for *cps3D*, the first type 3-specific gene, begins 166 bp downstream of the last nucleotide shown here. Underlined amino acids in the Cps3C, Cps3P, and Cps3E sequences indicate differences from the homologous type 19F sequences (Guidolin *et al.*, 1994). The *cps3C*, *cps3P*, and *cps3E* sequences shown are 95%, 99%, and 65% identical to their type 19F homologs over the regions present in both sequences. The overlined amino acid sequence beginning at bp 1288 is homologous to an internal region of the *E. coli* H-rpt unit (bp 986 to 1104 [Zhao *et al.*, 1993]). Underlined amino acids are different from those in the *E. coli* sequence. Amino acids in lower-case may not be translated. Symbols are as in Fig. 1. The 3' end of the sequence contains four corrections from the previously reported sequence (Dillard *et al.*, 1995): an additional "A" at bp 972 and three "G-A" transpositions at bp 1318-19, 1323-24, and 1326-27.



(v) Upstream common sequences. Further upstream of the type 3-specific locus are sequences that have also been identified upstream of the type-specific genes in a type 19F isolate (Guidolin *et al.*, 1994) and in another type 3 isolate (Arrecubieta *et al.*, 1995). Sequences homologous to the type 19F genes *cps19fC* and *cps19fD* were identified in the type 3 isolate 406 by Arrecubieta *et al.* (Arrecubieta *et al.*, 1995) and are also present in the type 3 WU2 (Fig. 4). The predicted protein sequences of these genes have homology to proteins involved in polysaccharide export (Guidolin *et al.*, 1994; Becker *et al.*, 1995). Comparison of the two type 3 sequences revealed only two nucleotide differences and a single amino acid difference in a 639 bp region containing the *cps19fD* homolog and the 3' end of the *cps19fC* homolog. However, the type 3 WU2 *cps19fD* homolog, *cps3P*, lacks 279 bp found at the 3' end of the type 19F gene. The Cps3P ORF is 154 amino acids long but the last 19 amino acids are not homologous to Cps19fD, whereas the first 135 amino acids are 98% identical (Fig. 4). A shift in reading frame 5 bp past the end of the homology identifies a 23 amino acid peptide ('Cps3E', Fig. 4) that has 57% identity with a peptide encoded by the 5' end of *cps19fE*, the next downstream type 19F capsule gene. The predicted Cps19fE sequence is homologous to glycosyltransferases from several organisms including *Salmonella enterica* group D2 (*RfbP*) and *Streptococcus agalactiae* (*CpsD*). The region deleted in the type 3 strain extends 453 bp from the point of truncation in *cps19fD* to the start of the 23 amino acid peptide encoded by *cps19fE*. The remaining 373 amino acids of Cps19fE have not been identified in the type 3 sequence. The complete Cps19fE homolog has been identified in a type 14 strain, where glycosyltransferase activity has been demonstrated (Kolkman *et al.*, 1996). Although not noted by Arrecubieta *et al.* (Arrecubieta *et al.*, 1995), the type 3 strain 406 sequence (GenBank accession number Z47210) contains the same deletion and partial sequence structures identified in type 3 strain WU2.

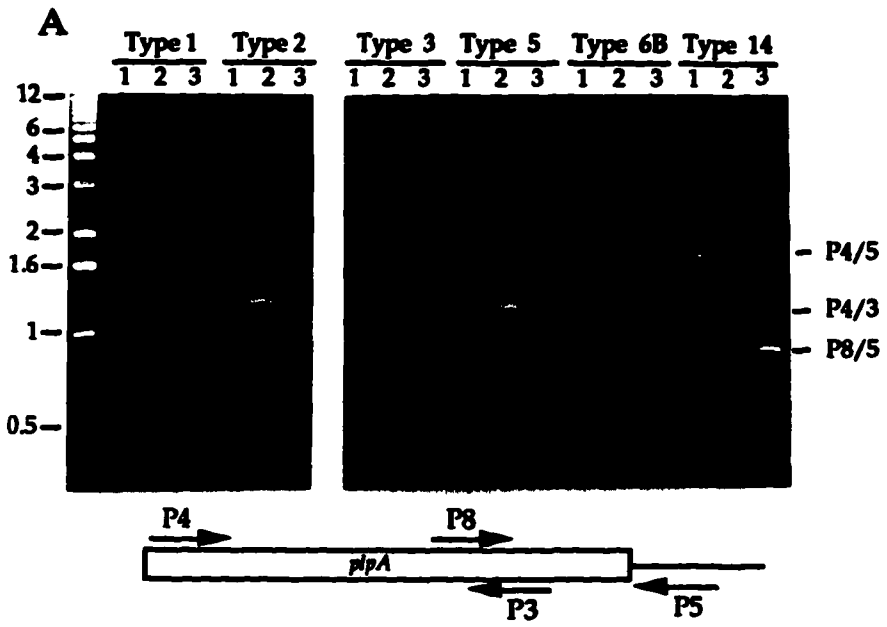
Confirmation of plpA *deletion in the type 3 WU2 chromosome and identification of full length* plpA *in other capsular serotypes*

In contrast to the type 3 *plpA*, the *plpA* identified in derivatives of the serotype 2 D39 strain is a complete gene (Alloing *et al.*, 1994; Pearce *et al.*, 1994). To confirm the deletion of *plpA* in the type 3 WU2 chromosome and to determine whether this gene is intact in strains of other capsular types, PCR analyses using primers expected to permit amplification of the 5' end, the 3' end, and the full length *plpA* were performed (Fig. 5A). A 997 bp PCR product corresponding to the 3' end of *plpA* was obtained from strains of capsular types 1, 2, 3, 5, 6B, and 14. A 1,272 bp product corresponding to the 5' end and a 1,959 bp product corresponding to the full length *plpA* were obtained from strains of types 1, 2, 5, 6B, and 14. However, neither the 1,272 bp or 1,959 bp products were obtained from the type 3 strain. Using the 5' end *plpA* PCR product from the type 6B strain as a probe in Southern blot analyses, we did not detect the 5' end of *plpA* in type 3 WU2 (Fig. 5B). Thus, the 5' end of *plpA* has been lost from the type 3 chromosome, and the deletion may be unique to type 3 strains.

Conservation of the type 3 locus structure among different type 3 strains

To determine whether the deletions observed in strain WU2 are conserved among type 3 strains, we examined the capsule loci of four additional independent clinical isolates by RFLP and PCR analyses. The type 3 strains used - WU2, A66, L8 1995, ATCC 6303, and EF3113 - differ with respect to PspA serotypes, virulence properties, and sites of isolation (Briles *et al.*, 1992). In Southern blot analyses using 14 restriction enzymes, the fragment sizes obtained with 12 enzymes were identical for all strains (Fig. 6A). A *Pvu*II polymorphism was noted for one strain and two strains exhibited the same *Stu*I polymorphism (data not shown). The area examined extends >14 kb from the *Stu*I site upstream of *cps3C* to the *Sph*I site downstream of *plpA* (Fig. 1A). To more precisely determine whether the *cps3M-tnpA-plpA* deletions are characteristic of all type 3 chromosomes, PCR analysis was performed using primers located within *cps3M* and

Fig. 5. *plpA* and *tnpA* in strains of different capsular types.
(A) PCR analysis of *plpA*. Chromosomal DNA was amplified using primers at the 5' (P4) and 3' (P5) extremes and internal (P3, P8) to *plpA*. See Table 2 for primer descriptions. Lanes: 1, P4/P5; 2, P4/P3; 3, P8/P5.
(B) Hybridization with *plpA*. Southern blots were hybridized with the 5' end of *plpA* obtained from DBL1 (type 6B) by PCR amplification using primer combination P4/P3 and followed by digestion of the product with *Bam*HI. Lanes: 1, *Bgl*II; 2, *Hin*dIII; 3, *Sac*I; 4, *Sph*I. Strains: WU2 (type 3), D39 (type 2), DBL1 (type 6B).
(C) Hybridization with *tnpA*. The 1.6 kb band which appears in lane 1 of type 6B is the result of contamination from an adjacent lane containing the molecular size standards. Lanes and strains are as in (B).



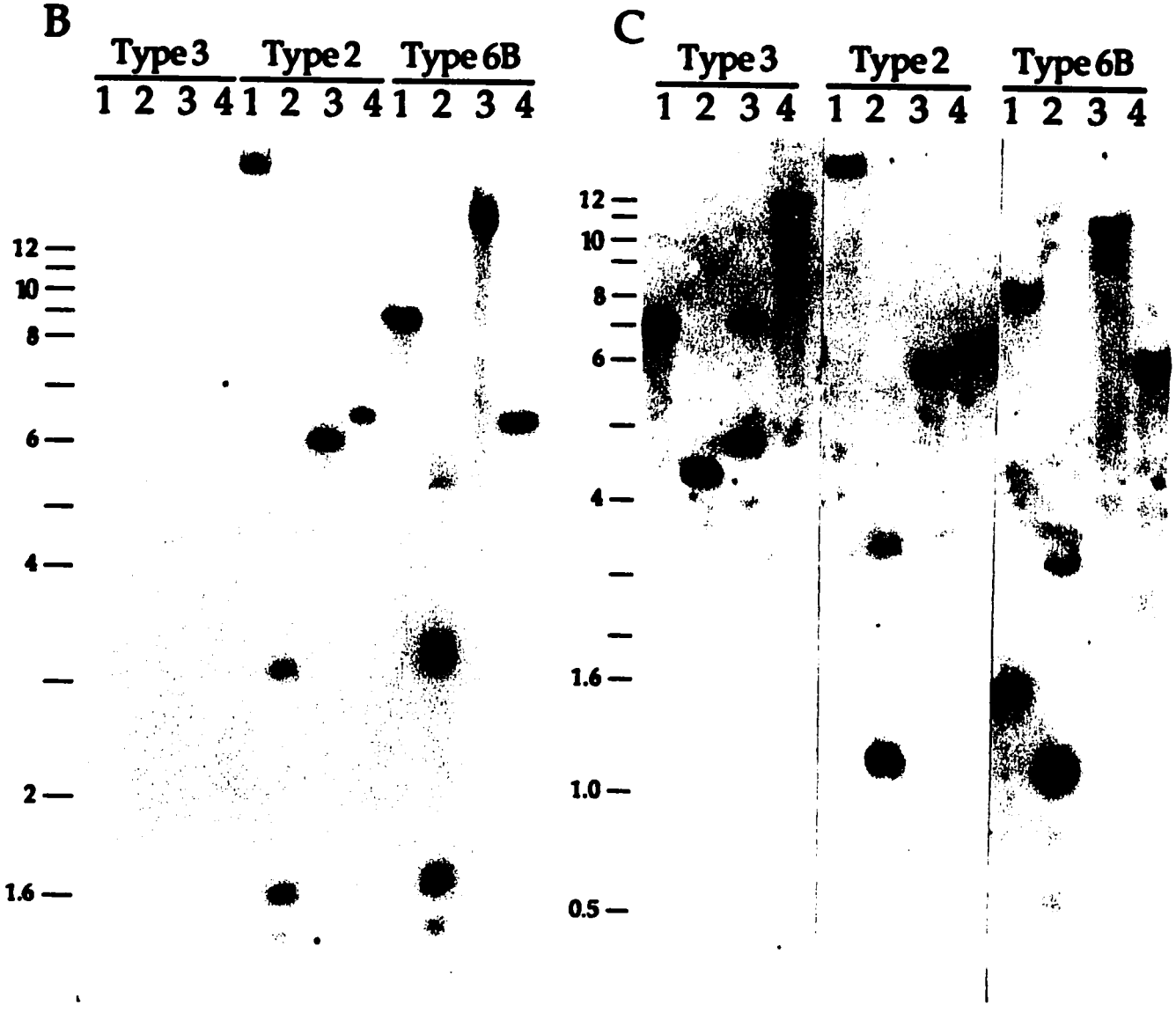
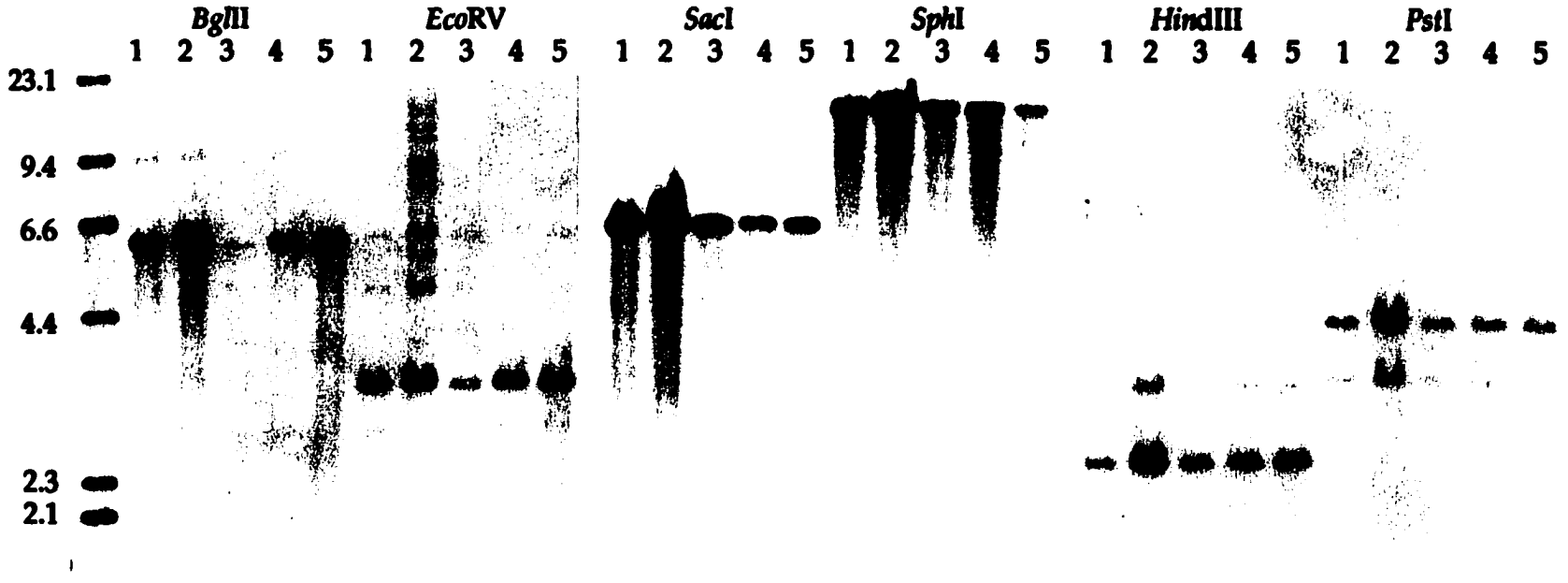
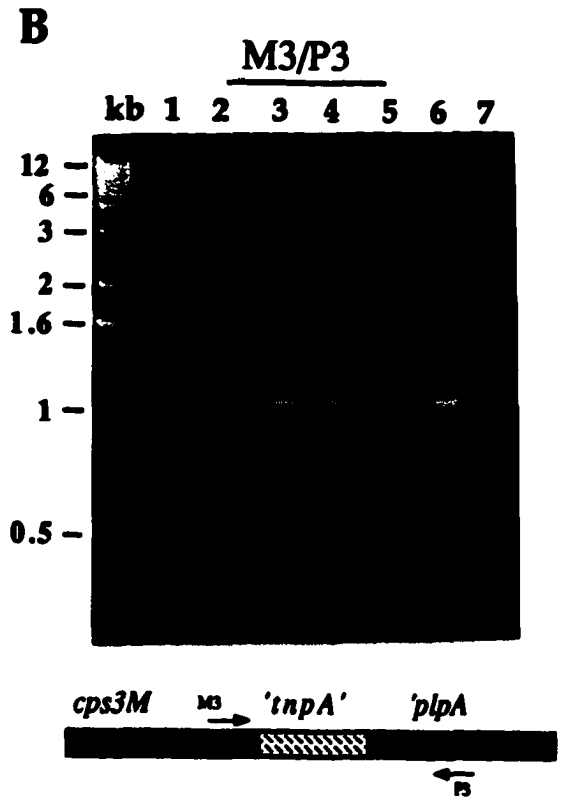


Fig. 6. Conservation of type 3 locus structure among independent type 3 isolates.
(A) RFLP analysis. Restriction digests were probed with pJD351 (Dillard and Yother, 1994) which contains a 2.4 kb *Sau*3AI fragment extending from the 3' end of *cps3D* through the middle of *cps3U*. Results from 6 of the 14 tested enzymes are shown. Not shown are the results with *Bst*XI, *Cla*I, *Hha*I, *Pvu*II, *Sca*I, *Stu*I, *Sty*I, and *Xcm*I. Lanes: 1, WU2; 2, A66; 3, EF 3113; 4, L8 1995; 5, ATCC 6303.
(B) PCR analysis. Chromosomal DNA was amplified using primers within the 3' end of *cps3M* (M3) and internal to *plpA* (P3). See Table 2 for primer descriptions. Lanes: 1, No DNA; 2, WU2; 3, A66; 4, ATCC 6303; 5, L8 1995; 6, EF3113; 7, DBL1 (type 6B).





plpA. The amplified products were identical for all five type 3 strains (Fig. 6B). No amplification product was detected using a type 6B strain as a negative control.

tnpA *is present in single copy and is linked to* plpA *in other capsule types*

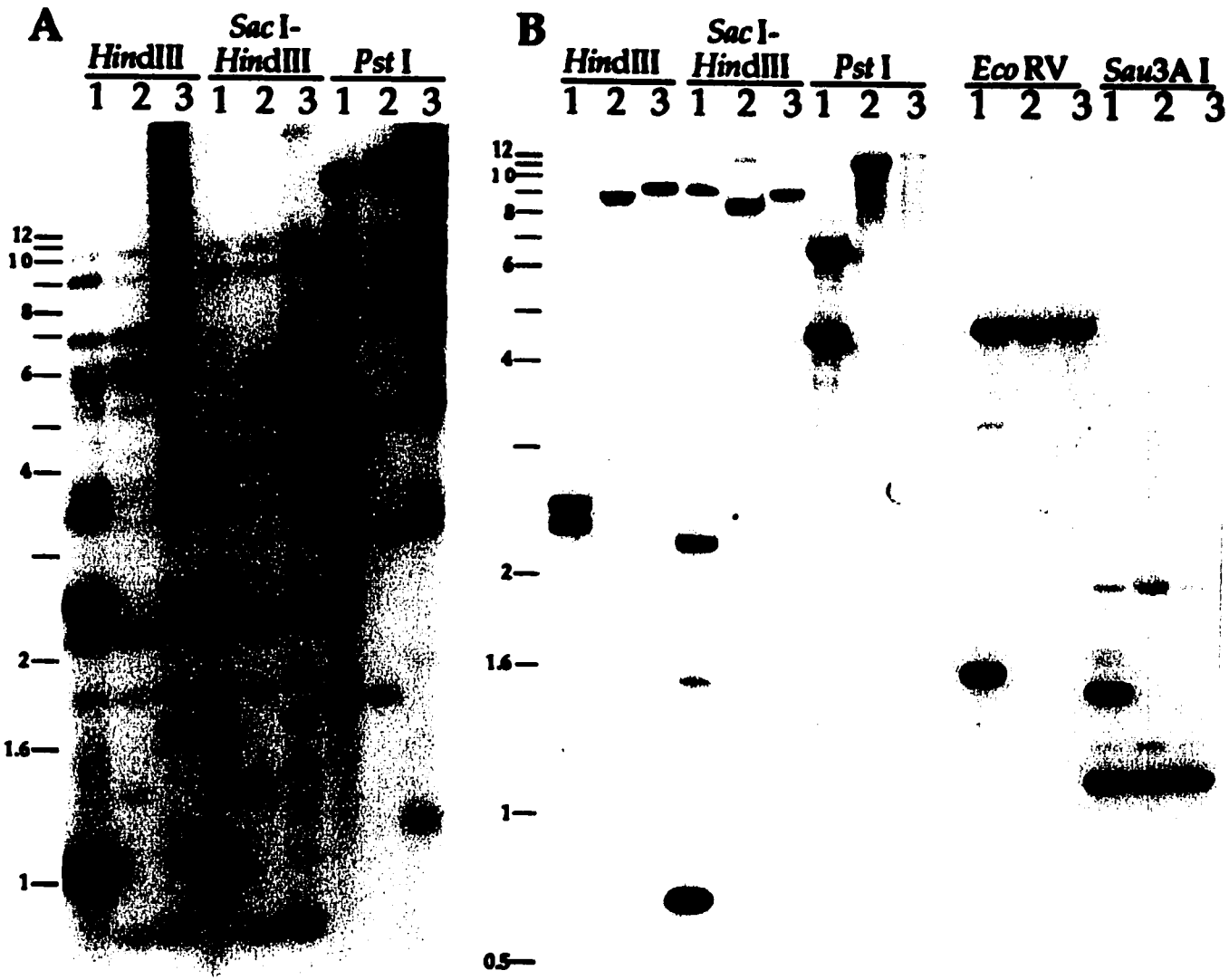
IS*1167*, with which *tnpA* has homology (Fig. 3), is present in multiple copies in the pneumococcal chromosome (Zhou *et al.*, 1995). However, only a single copy of *tnpA* was detected in strains of types 2, 3 and 6B (Fig. 5C). The sequences detected using *tnpA* as a probe are not likely to be copies of IS*1167*, as the two sequences are only 53% homologous and hybridizations were done at >95% stringency. Thus, these two transposases represent distinct members of a family of transposases.

Probes specific for *plpA* also revealed only a single copy of this gene (Fig. 5B). Restriction mapping with either *tnpA*- or *plpA*-specific probes yielded maps identical to those determined using a single probe containing both sequences (Dillard *et al.*, 1995, Fig. 5B, Fig. 5C, and data not shown). Thus, *tnpA* is present in strains of other capsule types and both it and *plpA* are located on the same restriction fragments.

Identification of the upstream repetitive element

The sequence upstream of the type 3-specific genes that is repeated on the *S. pneumoniae* chromosome was identified as a result of hybridization analyses that utilized a plasmid now known to contain part of both the *orf5* and H-rpt-like sequences. Results from Southern blot analyses using probes specific for either the H-rpt or *orf5* determined that the repetitive element is the H-rpt (Fig. 7A). Only a single copy of *orf5* was found in strains of capsule types 2 and 6B. However, in the type 3 strain, two copies were present (Fig. 7B). One of these copies appears to be the same nucleotide sequence detected in the type 2 and 6B chromosomes. Based on restriction fragment sizes, the unique copy is the one upstream of *cps3DSUM* (Fig. 7B). From the present data, it is clear that our previous analyses of linkage between type-specific genes and the *orf5*/H-rpt region were based on insertions in *orf5* (Dillard *et al.*, 1995). Thus, the type 2 and type 6B copies of this gene are not linked to their respective capsule loci.

Fig. 7. H-rpt and ORF5 Southern analyses.
(A) Hybridization with H-rpt-specific probe generated by PCR amplification of type 3 chromosomal DNA using primers Hinc1 and Hinc2.
(B) Hybridization with ORF5-specific probe generated using primers Orf1 and Orf2. See Table 2 for primer descriptions. Lanes 1, WU2 (type 3); 2, D39 (type 2); 3, DBL1 (type 6B).



Transcription of the type 3 locus

Insertion-duplication mutations using a vector that yields transcriptional *cat* fusions were constructed in the genes of the type 3 locus. The insertions were confirmed in Southern blot analyses (data not shown). As shown in Fig. 8, *cps3D* and *cps3S* are transcribed at approximately equivalent levels. *cps3U* is transcribed at approximately one-half the level of *cps3DS*, whereas transcription through *cps3M* and *plpA* is about one-sixth of the *cps3DS* level. A low but detectable level of transcription was apparent through *cps3C*, *tnpA*, *plpA*, and the H-rpt region (Fig. 8). Further analysis of the *cps3S-U* intergenic region identified sequence with the potential to form a secondary structure within the mRNA that overlaps the putative Cps3U ribosome binding site (bp 3732 to 3771 in the sequence presented in Dillard *et al.*, 1995). Since stem-loop structures are often associated with regulation, such as transcription termination and attenuation control, insertions were made immediately upstream and downstream of this potential stem-loop structure. The levels of transcription were similar for both constructs (Fig. 8).

Northern blot analyses using probes for the type 3-specific genes showed that *cps3D*, *S*, *U*, and *M* are present on the same transcripts (Fig. 9A). Although multiple transcripts are apparent, all four probes detected an approximate 6,300 nt transcript. The remaining transcripts may result from transcription initiations at additional promoters or they may be the result of processing of the larger transcript. Utilization of the promoter identified upstream of *cps3D* (Dillard *et al.*, 1995; Arrecubieta *et al.*, 1996) and of a potential transcription termination sequence identified downstream of *plpA* (Pearce *et al.*, 1994) would result in an approximate 6,500 nt transcript that would contain *cps3DSUM-tnpA-plpA*. Hybridization with *tnpA*- and *plpA*-specific probes showed that these sequences are contained on the same transcripts as *cps3M* (Fig. 9B). Hybridization with an *orf5* probe did not detect any specific transcripts, thus the 6,300 nt transcript does not result from promoter activity in the distal upstream region and neither copy of the type 3 *orf5*

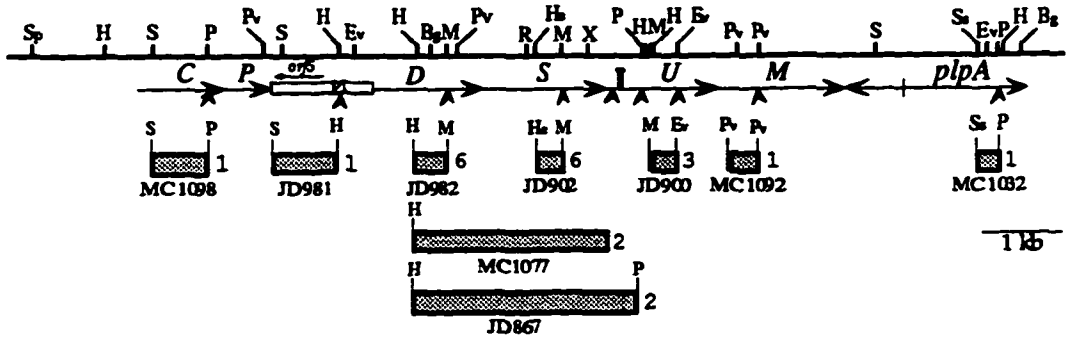
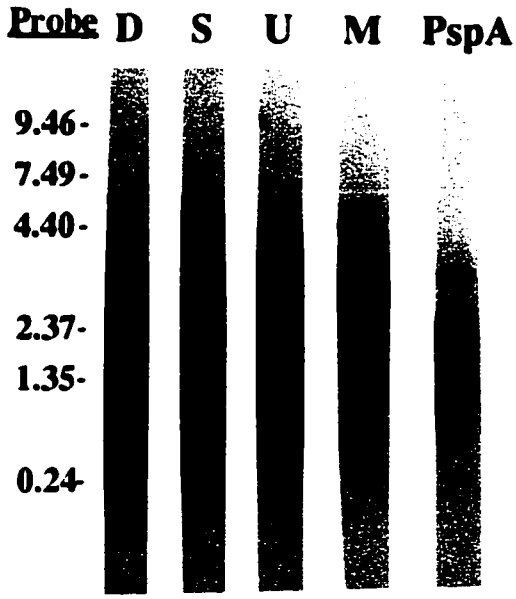


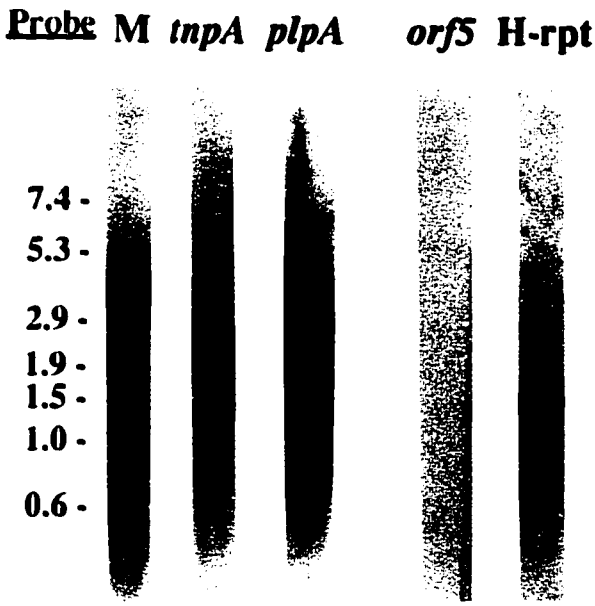
Fig. 8. Transcriptional analysis of the type 3 locus using *cat* fusions. Restriction sites and positions of fragments used to construct gene fusions with *cat* are indicated. Levels of resistance to chloramphenicol (μg/ml) are given to the right of each fragment to indicate the direction of transcription. Insertion of *cat* fusions in the orientation opposite to that indicated for transcription results in resistance to 0.5 μg/ml. Symbols: ▲, points of *cat* fusions; ▮, potential stem-loop structure. Strain descriptions are given in Table 1. Restriction enzymes (in addition to those indicated in Fig. 1): M, *Mun*I; He, *Hae*III; Ss, *Ssp*I.

Fig. 9. Northern analysis of the type 3 locus. Each lane contains 20 μg denatured RNA isolated from strain WU2. The probe used is indicated above each lane.
(A) Hybridization with probes from each of the type 3-specific genes (*cps3D*, *S*, *U*, or *M*). The *pspA* probe recognizes the transcript for pneumococcal surface protein A (major transcript expected to be ~2 kb (Yother and Briles, 1992 and unpublished data) and was used to confirm the integrity of the RNA preparation.
(B) Hybridization with probes specific for the flanking sequences (*tnpA*, *plpA*, *orf5*, and H-rpt) compared with a *cps3M* probe.
(C) Map of the type 3 capsule locus indicating the locations of the probes.

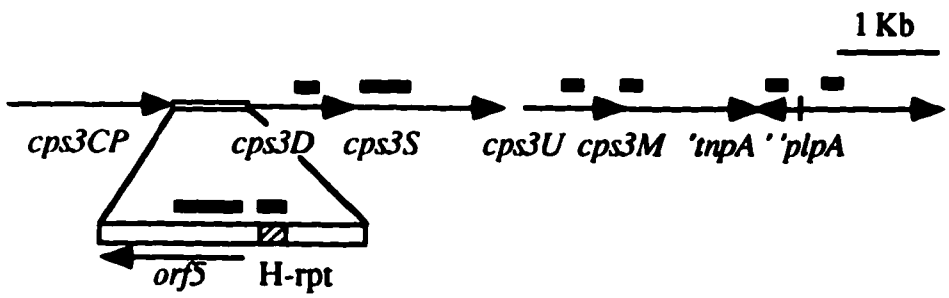
A.



B.



C.



sequences is transcribed under the conditions examined. Consistent with this result, no potential promoter sequences are apparent in the region immediately upstream of *orf5* (Fig. 4). Hybridization with the H-rpt-like sequence identified several transcripts, the most prominent being approximately 1,500 nt in size (Fig. 9B). Although processing cannot be ruled out as the source of these multiple transcripts, they may instead be the result of transcription from the multiple copies of H-rpt identified on the *S. pneumoniae* chromosome. In *E. coli*, a complete H-rpt sequence is approximately 1,300 bp (Zhao *et al.*, 1993).

Effect of mutations on type 3 capsule production

Effects of insertion mutations within the type 3 locus on capsule production were determined by colony morphology on blood agar medium and in ELISA assays. As reported previously, strains containing mutations in either *cps3D* or *cps3S* do not make detectable type 3 polysaccharide (Dillard and Yother, 1994). With these exceptions, no differences between the type 3 parent and the mutants were apparent by inspection of colony morphology (data not shown). Further, ELISA assays indicated no differences in the amounts of polysaccharide produced by the parent and mutant strains (Fig. 10). Thus, of the genes currently identified, only *cps3D* and *cps3S* are required for type 3 synthesis under laboratory culture conditions.

Discussion

The type 3 capsule locus presents a structure distinct from that of other pneumococcal capsule types thus far described. The presence of functional genes involved in polysaccharide production, along with sequences that represent truncations, mutations, and remnants of genes associated with transposition and polysaccharide biosynthesis, is analogous to the situation observed with the *rfb* O-antigen gene clusters in *Vibrio cholerae* O139 (Bik *et al.*, 1995) and *Salmonella enterica* group D2 (Xiang *et al.*, 1994). In the case of *V. cholerae* O139, genes involved in capsule and O-antigen synthesis from a non-O1 strain

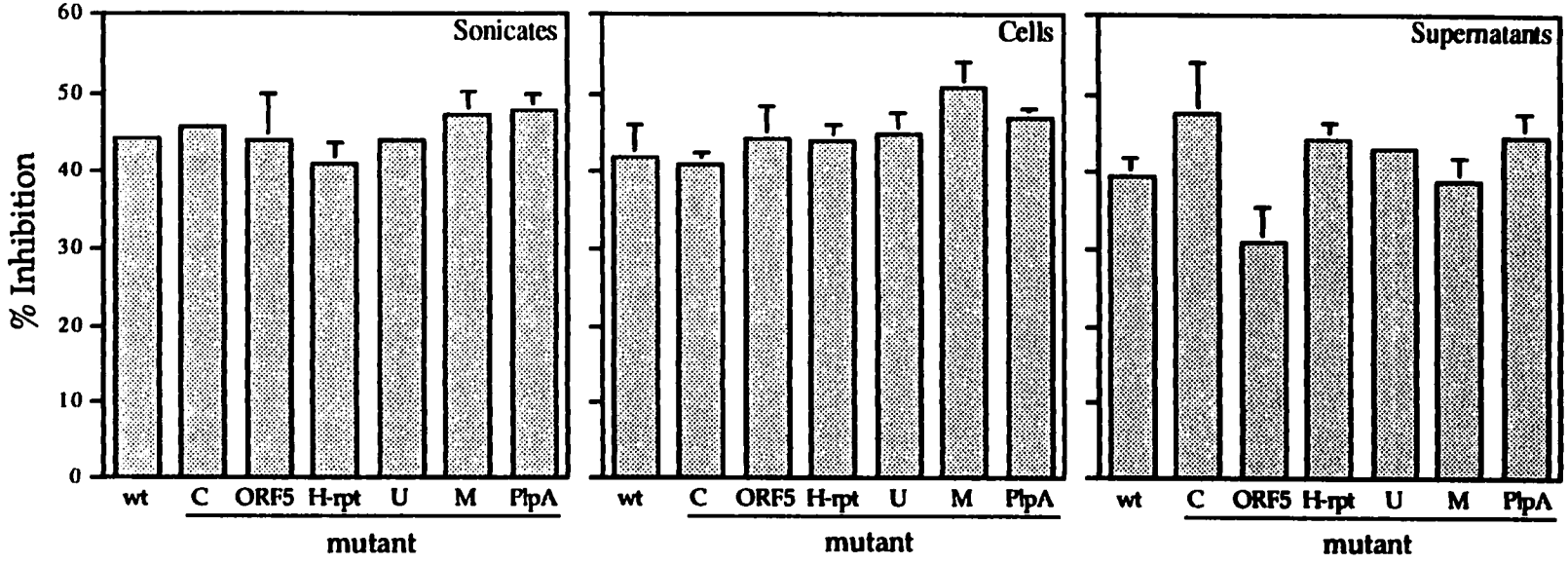


Fig. 10. Production of type 3 polysaccharide. Polysaccharide was measured in competitive-inhibition ELISA assays using a mAb against type 3 capsule. Strains: wt, JD770; Cps3C$^-$, JY1200; ORF5$^-$, JD1008; H-rpt, JD981; Cps3U$^-$, JD900; Cps3M$^-$, MC1056; PlpA$^-$, MC1032. Strain descriptions are given in Table 1. The H-rpt mutation of JD981 separates the upstream region from the type 3-specific genes but does not result in inactivation of H-rpt. The normal production of type 3 capsule in this mutant confirms that transcription of the type 3-specific genes is independent of the upstream region. Strains JD982 (Cps3D$^-$) and JD902 (Cps3S$^-$) were negative for inhibition (data not shown).

have replaced part of the *rfb* locus in a recipient O1 strain. *rfbQRS*, a putative IS element with homology to the H-rpt of *E. coli*, is located in the region flanking the inserted DNA, and *rfb* sequences on either side of the element are deleted (Bik *et al.*, 1995; Stroeher *et al.*, 1995). The *S. enterica* group D2 O-antigen is a hybrid of groups D1 and E1. A sequence with homology to H-rpt, as well as truncated and mutated fragments of genes, are also present in this locus (Xiang *et al.*, 1994). In both of these loci, transposition and recombination events have been central to their evolution.

Based on the finding of a repetitive sequence located upstream of the type 3-specific genes and elsewhere on the pneumococcal chromosome, we previously proposed that transposition may play a role in the formation of binary encapsulated strains and that the presence of two sets of type-specific genes could provide a mechanism for the generation of novel capsule types (Dillard *et al.*, 1995). The demonstration that the repetitive sequence is homologous to the IS-like H-rpt, along with the identification of a partial transposase sequence immediately downstream of the type 3-specific genes, lends support to the involvement of transposition in the formation of this locus, although its exact role is not easily discerned. The H-rpt sequences of *E. coli* are usually found in association with the *Rhs* (rearrangement hot spot) loci and are present in multiple copies on the chromosome. They have features suggestive of insertion sequences but neither the functions nor the origins of H-rpt have been defined (Zhao *et al.*, 1993; Hill *et al.*, 1994). It has been proposed that the H-rpt may be involved in the rearrangement of adjacent sequences and may have contributed to the formation of complex loci such as those of the *rfb* gene clusters (Hill *et al.*, 1994; Xiang *et al.*, 1994; Bik *et al.*, 1995). In addition to these examples, H-rpt homologs have been identified in *Xanthobacter autotrophicus* (Janssen *et al.*, 1989) and in *Aeromonas salmonicida*, where a transposition function has been demonstrated as a result of insertions in the gene encoding a paracrystalline surface protein array subunit (Gustafson *et al.*, 1994). In all cases, the H-rpt GC content is lower than that of the organism in which it is found (~40% vs. ~50% for *E. coli* [Zhao *et al.*, 1993] and *S. enterica*,

~43% vs. ~48% for *V. cholerae*, ~50% vs. ~60% for *A. salmonicida*, and ~63% vs. 68% for *X. autotrophicus* [Krieg and Holt, 1984]), suggesting that the element originated in another organism. The homologous sequence in type 3 *S. pneumoniae* is repeated in the pneumococcal chromosome but the copy identified in the type 3 locus is only 117 bp in length, compared to 1,291 bp for the complete *E. coli* H-rpt sequence. However, partial copies of H-rpt have been identified in *E. coli* (Zhao *et al.*, 1993), and other copies of the *S. pneumoniae* sequence may be complete. The GC content of the pneumococcal H-rpt-like sequence (38.5%) is similar to that of the complete *E. coli* element and is consistent with the GC content of other genes in the type 3 locus (38 to 40%) and of the *S. pneumoniae* chromosome as a whole (~39% [Deibel and Seeley, 1974]). To our knowledge, this is the first gram-positive organism in which an H-rpt-like element has been identified.

The type 3 capsule locus may have resulted from deletions and rearrangements that occurred during an insertion event that placed these genes in their present position or that altered a more complex capsule locus. Whatever its origins, the structure of the locus is highly conserved among independent type 3 isolates and has thus either evolved only recently or holds importance for functions presumably associated with the type 3 capsule genes. Intact versions of several of the deleted genes (*cps3P*, *cps3E*, and *plpA*) are expected to encode proteins potentially involved in polysaccharide synthesis. While these common functions may be necessary for synthesis of more complex structures, the simplicity of the type 3 polysaccharide argues for an equally simple mechanism of synthesis that may not, for example, involve lipid intermediates or require additional transport proteins. Type 3 synthesis would, however, be expected to require the functions of a phosphoglucomutase and a UDP-Glc-1-P uridylyltransferase for conversion of Glc-6-P to Glc-1-P, and Glc-1-P to UDP-Glc, respectively. Clearly, the type 3-specific genes expected to encode these functions are not required under laboratory culture conditions and these roles must be fulfilled by essential cellular proteins such as those involved in cell wall and teichoic acid synthesis. Furthermore, it is not clear that Cps3M actually functions as a phosphomutase,

as it lacks a domain expected to be important for this activity (Dai *et al.*, 1992; Sandlin and Stein, 1994) and we have not been able to demonstrate such a function (unpublished data). Nonetheless, the fact that *cps3U* and *cps3M* are both maintained as open reading frames and are transcribed suggests that they do have important functions. Likewise, *tnpA* and *plpA*, which are not expected to encode proteins, are transcribed and are contained on the same transcript with *cps3DSUM*. No transcription termination sequences are apparent in either the type 3-specific genes or at the junction with downstream flanking sequences. Possibly, such sequences were deleted as a result of rearrangements in the type 3 locus and the co-transcribed flanking sequences may be required to stabilize the transcript that does not end until a termination sequence is encountered following *plpA*.

The pneumococcal capsular polysaccharides represent a diverse group of structures which have the common function of protecting the bacterium against phagocytosis. As with other surface molecules with essential roles in bacterial pathogenesis, the ability to vary antigenically provides a selective advantage to strains expressing capsular polysaccharides associated with enhanced virulence. Until now, the mechanisms underlying pneumococcal capsule diversity have remained largely unknown, with the descriptions of binary encapsulated strains providing the only insights into the possible origins of new capsular types (Austrian *et al.*, 1959; Bernheimer *et al.*, 1967; Bernheimer and Wermundsen, 1969). Characterization of the type 3 locus now suggests that genetic rearrangements, possibly mediated by an H-rpt-like element, were important in the evolution of this locus. This scenario appears to be a common theme among many polysaccharide-associated genetic loci (Xiang *et al.*, 1994; Bik *et al.*, 1995, 1996). Whether such rearrangements have occurred in the capsule loci of other pneumococcal serotypes remains to be determined, yet it is apparent that the diversity of pneumococcal capsular structures is the result of multiple genetic mechanisms that, in addition to the exchange of capsule gene cassettes via natural transformation and recombination, likely include transposition-mediated events.

Experimental Procedures

Bacterial strains and plasmids

The strains and plasmids used in these studies are described in Table 1. *S. pneumoniae* strains were grown in Todd-Hewitt broth (Difco, Detroit, MI) supplemented with 0.5% yeast extract (THY) or on Blood Agar Base #2 (Difco) supplemented with 3% sheep red blood cells. *E. coli* derivatives were grown in L-broth or on L-agar. Erythromycin was used at 0.3 μg/ml for *S. pneumoniae* and 250 μg/ml for *E. coli*. Kanamycin was used at 50 μg/ml for *E. coli* and 10 μg/ml for *S. pneumoniae*. Ampicillin was used at 100 μg/ml for *E. coli*. The insertion-duplication vectors pJY4163, pJY4164 (Yother *et al.*, 1992), and pSF151 (Tao *et al.*, 1992) were used to construct insertion mutations at targeted sites on the *S. pneumoniae* chromosome. Insertions were confirmed in Southern blot analyses.

Isolation and cloning of the type 3 locus DNA

Cloning of the 3.1 kb *Hin*dIII fragment containing the downstream type 3-specific genes has been described (Dillard *et al.*, 1995). The 3' end of the type 3 *plpA* was obtained as a PCR product using primers P5 and P6 (Table 2). The region upstream of *cps3D* was obtained from JD1008 by cloning the 2.2 kb *Hin*dIII fragment flanking the pJD396 insertion (Table 1).

DNA techniques and sequence analysis

Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) and purified by CsCl centrifugation (Radloff *et al.*, 1967) when necessary. Chromosomal DNA from *S. pneumoniae* was prepared using a modification of Hotchkiss (1957). Briefly, cultures of *S. pneumoniae* (10 ml) were grown to stationary phase in the presence of 1% choline chloride to prevent autolysis (Briese and Hakenbeck, 1983). Cultures were centrifuged at 12,000 x g for 10 min and were then washed with 1 ml 0.1 M EDTA/0.15 M NaCl. Washed cell pellets were resuspended in 0.475 ml 0.1 M EDTA/0.15 M NaCl. SDS was added to 0.25%, and the suspensions were incubated at 37°C for 5 min and were

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Relevant genotype/characteristics	Reference
Strain		
S. pneumoniae		
WU2	Type 3 encapsulated; parent strain for sequencing and mutation analyses	(Briles *et al.*, 1981)
JD770	Type 3 encapsulated derivative of WU2 containing insertion-duplication of *'cps3DSU'*,identical to WU2 in capsule production and virulence, Em^R	(Dillard and Yother, 1994), (Kelly *et al.*, 1994)
L82006	Type 1 encapsulated	(McDaniel *et al.*, 1992)
D39	Type 2 encapsulated	(Avery *et al.*, 1944)
A66	Type 3 encapsulated	(Briles *et al.*, 1992)
ATCC 6303	Type 3 encapsulated	(Briles *et al.*, 1992)
EF3113	Type 3 encapsulated	(Briles *et al.*, 1992)
L8 1995	Type 3 encapsulated	(Briles *et al.*, 1992)
DBL5	Type 5 encapsulated	(Yother *et al.*, 1982)
DBL1	Type 6B encapsulated	(Briles *et al.*, 1992)
L82231	Type 14 encapsulated	(Briles *et al.*, 1992)
JD867	pJD355 × WU2, insertion-duplication of *'cps3DSU'*, type 3 encapsulated, Em^R	(Dillard *et al.*, 1995)
JD900	pJD357 × WU2, $Cps3U^-$, type 3 encapsulated, Em^R	(Dillard *et al.*, 1995)
JD902	pJD362 × WU2, $Cps3S^-$, non-encapsulated, Em^R	(Dillard *et al.*, 1995)
JD981	pJD392 × WU2, insertion duplication of *'orf5*-H-rpt', type 3 encapsulated, Em^R	(Dillard *et al.*, 1995)
JD982	pJD390 × WU2, $Cps3DS^-$, non-encapsulated, Em^R	(Dillard *et al.*, 1995)
JD1008	pJD396 × WU2, $ORF5^-$, type 3 encapsulated, Km^R	(Dillard *et al.*, 1995)
JY1200	pJY5006 × WU2, $Cps3C^-$, type 3 encapsulated, Em^R	This work
MC1032	pMC107 × WU2, $PlpA^-$, type 3 encapsulated, Em^R	This work
MC1056	pMC135 × WU2, $Cps3M^-$, type 3 encapsulated, Em^R	This work
MC1077	pMC159 × WU2, insertion-duplication of *'cps3DS* terminating in *S-U* intergenic region, type 3 encapsulated, Em^R	This work
MC1092	pMC123 × WU2, $Cps3M^-$, type 3 encapsulated, Em^R	This work

Table 1 (Continued)

Strain/Plasmid	Relevant genotype/characteristics	Reference
MC1098	pMC186 × WU2, Cps3C$^-$, type 3 encapsulated, Em^R	This work
MC1114	pMC205 × WU2, Cps3M$^-$, type 3 encapsulated, Em^R	This work
MC1119	pMC180 × WU2, insertion-duplication of *'tnpA-plpA'*, type 3 encapsulated, Em^R	This work
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r$_K^-$m$_K^+$) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* ϕ80Δ*lac*Δ(*lacZ*)*M15*)	(Ausubel *et al.*, 1987)
LE392	F$^-$ *hsdR514* (r$_K^-$m$_K^+$) *supE44 supF58* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* l$^-$	(Tilghman *et al.*, 1977)
Plasmids*		
pJY4163 and pJY4164	Lack origin of replication for *S. pneumoniae*; promoterless *cat* gene downstream of multiple cloning site (opposite orientations in pJY4163 and pJY4164), Em^R	(Yother *et al.*, 1992)
pSF151	Lacks origin of replication for *S. pneumoniae*; Km^R	(Tao *et al.*, 1992)
pJD355	pJY4164::2.0 kb *Sau*3A1-*Pst*I (*'cps3D-cps3S-cps3U'*)	(Dillard *et al.*, 1995)
pJD357	pJY4164::0.275 kb *Mfe*I-*Eco*RV (*'cps3U'*)	(Dillard and Yother, 1994)
pJD362	pJY4164::0.4 kb *Hae*III-*Mun*I (*'cps3S'*)	(Dillard and Yother, 1994)
pJD364	pJY4164::3.2 kb *Hin*dIII (*'cps3UM-tnpA-plpA'*, bp 3905-7220, Fig. 1B)	(Dillard and Yother, 1994)
pJD374	pJY4163::1.4 kb *Sau*3A1 (*'cps3M-tnpA'*, bp 4734-6108, Fig. 1B)	(Dillard and Yother, 1994)
pJD377	pJY4164::1.2 kb *Sac*I-*Hin*dIII (*'tnpA-plpA'*,bp 6121-7310, Fig. 1B)	(Dillard and Yother, 1994)
pJD390	pJY4164::0.35 kb *Hin*dIII-*Mun*I (*'cps3D'*)	(Dillard *et al.*, 1995)
pJD392	pJY4164::0.6 kb *Ecl*136II-*Hin*dIII (*'orf5*-H-rpt', bp 709-1317,Fig. 4)	(Dillard *et al.*, 1995)
pJD396	pSF151::0.26 kb *Ecl*136II-*Msc*I (*'orf5'*, bp 709-965, Fig. 4)	(Dillard *et al.*, 1995)
pJY5006	pJY4163::0.8 kb *Sac*I-*Pst*I (*'cps3C'*, upstream *Sac*I to bp 120, Fig. 4)	This work
pMC107	pJY4163::0.27 kb *Ssp*I-*Pst*I (*'plpA'*, bp 6941-7211, Fig. 1B)	This work
pMC123	pJY4164::0.36 kb *Pvu*II (*'cps3M'*, bp 4822-5190, Fig. 1B)	This work

Table 1 (Continued)

Strain/Plasmid	Relevant genotype/characteristics	Reference
pMC135	pJY4163::0.36 kb *Pvu*II (*'cps3M'*, bp 4822-5190, Fig. 1B)	This work
pMC159	pJY4164::1.9 kb PCR product (*'cps3D-cps3S*; terminates in *cps3S-cps3U* intergenic region, Fig. 8)	This work
pMC180	pJY4164::0.3 *Sac*I-*Rsa*I (*'tnpA-plpA'*, bp 6121-6419, Fig. 1B)	This work
pMC186	pJY4164::0.8 kb *Sac*I-*Pst*I (*'cps3C'*, upstream *Sac*I to bp 120, Fig. 4)	This work
pMC205	pJY4164::0.48 kb *Eco*RI-*Xmn*I (*'cps3M'*, bp 4983-5458, Fig. 1B)	This work

* Most of the restriction sites are shown in Figs. 1, 4, and 8. The gene(s), or part(s) thereof, contained in the clone and the location of the fragment (for sequence presented here) are given in parentheses.

then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Chromosomal DNA was precipitated by the addition of 2 vols of 95% ethanol and reprecipitated twice by the addition of 1 vol 0.1 M EDTA/0.15 M NaCl and 2 vols of ethanol. The DNA was then washed with 70% ethanol, air dried, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

S. pneumoniae was transformed as previously described (Yother *et al.*, 1986). *E. coli*, washed in water and resuspended in 10% glycerol, was electroporated using a BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, CA).

PCR amplifications were performed using *Taq* polymerase (Fisher Scientific, Pittsburgh, PA). The Genius System (Boehringer Mannheim, Indianapolis, IN) was used for DIG-dUTP labeling of probes and chemiluminescent detection in Southern blotting.

Templates for DNA sequencing were prepared using either PCR amplification products or plasmid DNA. Sanger dideoxy sequencing of plasmid DNA was performed using the Sequenase 2.0 kit (US Biochemicals, Cleveland, OH). Sequencing of PCR products was performed using the Sequenase PCR Product Sequencing kit (US

Biochemicals). Primers for sequencing were purchased from Gibco/BRL (Grand Island, NY). Greater than 98% of the sequence was obtained for each strand of the *cps3U-cps3M-tnpA-plpA* and the *orf5*/H-rpt regions. Sequence from the *cps3CPE* region (80% single-stranded) was compared to the published sequence for type 19F (Guidolin *et al.*, 1994). Differences between the two were verified in the type 3 sequence.

Sequence analyses and data base searches were performed using The University of Wisconsin Genetics Computer Group (GCG) programs FASTA and TFASTA (Genetics Computer Group, 1991) and the NCBI BLAST server (Altschul *et al.*, 1990). Sequence alignments and consensus assignments were performed using the GCG PILEUP and PRETTY programs. Amino acid sequence similarities were determined using the GAP program. The sequence has been submitted to GenBank for assignment of an accession number.

Transcription analyses

Gene fusions with the *cat* reporter gene were constructed using the insertion-duplication vectors pJY4163 and pJY4164 (Table 1) to target insertions into the *S. pneumoniae* chromosome. Sites of the insertions were confirmed in Southern blot analyses. Due to low levels of chloramphenicol acetyltransferase activity in the standard enzyme assay (Shaw, 1975), levels of resistance to chloramphenicol (μg/ml) were used to assess *cat* expression. Levels of resistance were determined by growth on blood agar medium con-taining erythromycin (0.3 μg/ml) and chloramphenicol (1 to 8 μg/ml in 1 μg/ml increments). The level of resistance was defined as the highest concentration permitting the formation of single colonies.

For Northern analyses, cultures of *S. pneumoniae* (500 ml) were grown in THY to an OD_{620} = 0.05 and then placed on ice. Cells were pelleted by centrifugation at 4000 x g for 15 min at 4°C. RNA was then prepared according to the procedure of Pearce *et al.* (1994). Any remaining chromosomal DNA was removed by RQ1 DNase digestion (Promega Corporation, Madison, WI). RNA was then extracted two times with

phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) and precipitated overnight in 70% ethanol. The pellet was resuspended in 50 µl DEPC-treated water containing 10 mM Ribonucleoside-Vanadyl Complex (New England Biolabs, Beverly, MA) and stored at -20°C. The yield and purity were determined by spectrophotometry and agarose gel electrophoresis. For blotting experiments, RNA was denatured at 65°C for 10 min and electrophoresed on a 1.0% agarose/2.2 M formaldehyde gel with DIG-labeled and unlabeled RNA markers (Boehringer-Mannheim or Gibco-BRL). The *cps3D*, *S*, and *U* probes were derived by PCR amplification of the cloned inserts from pJD390, pJD362, and pJD357, respectively (Table 1). The *cps3M* probe was derived by PCR amplification of an internal fragment from pJD364 using the M-specific primers M1 and M5 (Table 2). The *orf5* and H-rpt probes were derived by PCR amplifications from WU2 genomic DNA using the Orf1/Orf2 and Hinc1/Hinc2 primers, respectively. The *tnpA* and *plpA* probes were produced by PCR amplifications of internal fragments from pJD377 using the IS2/P1 and P8/P3 primers, respectively (Table 2). The Genius System (Boehringer-Mannheim) was used for labeling of probes and chemiluminescent detection.

Capsule determinations

Capsular serotypes were confirmed by slide agglutination using type-specific antisera (Statens Seruminstitut, Copenhagen, Denmark). For use in ELISA determinations, cultures of *S. pneumoniae* were grown to mid-exponential phase (OD_{600} = 0.5) in THY and were then heat killed (65°C, 20 min). Cells from two 1-ml aliquots of culture were pelleted by centrifugation at 12,000 x g for 10 min and culture supernatant fluids were saved. Cell pellets were washed and resuspended in 1 ml PBS (50 mM sodium phosphate pH 7.4, 100 mM NaCl). To determine surface-associated type 3 polysaccharide, washed cells were used without further treatment. To determine total cell-associated type 3 polysaccharide, washed cells were sonicated at 4°C for 5 min in a Branson Model 2210 sonicating water bath (Branson, Danbury, CT). Type 3 capsule was quantitated in competitive-inhibition ELISA assays using microtiter plates coated with

Table 2. Primer sequences

Sequence		Source[a]	Position[b]	Reference
Hinc1	5'-GACGTTCGCTATTTTATC-3'	WU2	1285-1302	Fig. 4
Hinc2	5'-GTCTTCACGATAAACCAC-3'	WU2	1404-1387	Fig. 4
IS2	5'-GCCTCAGTTAACAAGTCAAA-3'	WU2	6035-6054	Fig. 1B
M1	5'-GTGGACACCTATGAATTGTATAG-3'	WU2	4682-4704	Fig. 1B
M3	5'-GTCACCAAAATTGCGGAAAG-3'	WU2	5775-5794	Fig. 1B
M5	5'-GGCAGATTCAAAAGCGAA-3'	WU2	5003-4986	Fig. 1B
Orf1	5'-ATCAAAAGGGCGTTAGGGTA-3'	WU2	834-854	Fig. 4
Orf2	5'-AATAATTGATTAGCGCCATT-3'	WU2	1107-1088	Fig. 4
P1	5'-GCCGTAGATGATGACAACCA-3'	WU2	6326-6307	Fig. 1B
P3	5'-TTGCTGTCTGGTCAACTGGC-3'	WU2	6833-6814	Fig. 1B
P4	5'-GCATGCTCTGGATCAGGTTC-3'	R6x	13-32	(Pearce *et al.*, 1994)
P5	5'-CAAGAGAAATACTAAATC-3'	R6x	1971-1953	(Pearce *et al.*, 1994)
P6	5'-GTTGCTAAACGATATGAT-3'	WU2	7181-7198	Fig. 1B
P8	5'-TGCATTTGGATTTGACCG-3'	WU2	6523-6540	Fig. 1B

a Denotes strain from which primer sequence was taken.
b Positions of primers are numbered according to nucleotide sequence published here for WU2 in Fig. 1 or 4, as indicated, and previously for R6x (Pearce *et al.*, 1994).

purified type 3 polysaccharide (ATCC, Rockville, MD) coupled to poly-L-lysine (Gray, 1979) and diluted in PBS to a concentration of 0.25 mg polysaccharide/ml. Ascites fluid containing the type 3-specific monoclonal antibody 16.3 (Briles *et al.*, 1981) was used to detect polysaccharide. Purified type 3 polysaccharide was used as the standard. In preliminary experiments, the dilutions of the parent type 3 culture and of the mAb required to give approximately 50% inhibition were determined. Accordingly, the mAb was used at a dilution of 1/5,000, whole cell and sonicate bacterial samples were each diluted 125-fold, and the bacterial culture supernatant fluids were used at a dilution of 625-fold. Samples, diluted in PBS, were incubated 30 min at room temperature with an equal volume (50 μl) of mAb diluted in 1% BSA/PBS. The mixtures were then transferred to type 3-coated microtiter plates, incubated 2 h at room temperature, and then developed using goat anti-mouse Ig-biotin and strepavidin-alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL) by standard technique (Ausubel *et al.*, 1987). Percent inhibition was determined as (1 - sample OD_{415}/uninhibited OD_{415}) x 100 and was standardized to culture OD. The uninhibited control is the value obtained following incubation of the mAb with either 1% BSA/PBS (cells and sonicates control) or with THY diluted in 1% BSA/ PBS (supernatant fluids control) but without polysaccharide in either case.

The percent inhibition for each sample was determined as the mean of at least four replicates. For each strain, the percent inhibition shown in Fig. 10 is the mean obtained from at least two independent cultures, each of which was assayed in at least two separate experiments. Statistical significance was determined using Student's t-test.

Acknowledgments

This work was supported by Public Health Service grants AI28457 and T32AI07041 from the National Institutes of Health. Access to the Genetics Computer Group sequence analysis programs was made available through the University of Alabama at Birmingham Center for Aids Research (P30 AI27767). We thank Tracy Hampton and Shannon Acton

for their help in cloning the upstream region and in RFLP analyses, David Briles for providing *S. pneumoniae* strains, and Joseph Dillard, Bill Benjamin, Alexis Brooks-Walter, and Rebecca Tart for their assistance and helpful discussions with these studies.

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DETERMINATION OF A REQUIREMENT FOR THE TYPE 3-SPECIFIC CAPSULE GENES AND FLANKING SEQUENCES IN THE VIRULENCE OF *STREPTOCOCCUS PNEUMONIAE*

by

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Prepared for submission to Infection and Immunity

Format adapted for Dissertation

Abstract

The requirement for a polysaccharide capsule in the virulence of *Streptococcus pneumoniae* has been well established. The contribution of the genes contained within the type-specific capsule loci, however, has not been determined. A series of mutations within the *S. pneumoniae* type 3 *cps* locus was examined for their effect on virulence. As reported in this communication, virulence studies using non-encapsulated strains containing either a point mutation within *cps3D* or an insertion within *cps3S* demonstrate a requirement for both of these genes in virulence. Also, the non-encapsulated *cps3D* and *cps3S* mutant strains were rapidly cleared from the blood of infected mice. In contrast, the two other type 3-specific genes, *cps3U* and *cps3M*, which are not required for production of type 3 capsule under laboratory conditions, also were not required for virulence. Insertions within non-type specific flanking sequences also had no effect on the virulence of type 3 *S. pneumoniae*. Although a novel encapsulated strain was shown to be avirulent, the altered virulence of this strain is due to a second mutation and not a result of the insertion within the *cps3* locus. Results from ELISA assays performed on blood from mice infected with MC1056 suggest that the synthesis of capsule may be regulated and that this regulation in the *in vivo* environment is required for the survival of *S. pneumoniae in vivo*.

Introduction

The polysaccharide capsule of the gram-positive bacterium *Streptococcus pneumoniae* serves as a major virulence factor. To date, capsule genes from three pneumococcal capsular serotypes, types 3, 14 and 19F, have been characterized (Arrecubieta *et al.*, 1994; Dillard and Yother, 1994; Guidolin *et al.*, 1994; Kelly *et al.*, 1994; Arrecubieta *et al.*, 1995; Dillard et al., 1995; Caimano *et al.*, 1996). These genes have a conserved organization in which genes common to all capsule types flank genes present only within the locus of the serotype expressed. The type 3 locus contains four type-specific genes, *cps3D*, *cps3S*, *cps3U*, and *cps3M*. The functions encoded by three of these genes have been determined: a UDP-glucose dehydrogenase (*cps3D*), a type 3 polysaccharide synthase

(*cps3S*), and a glucose-1-phosphate uridylyl-transferase (*cps3U*) (Arrecubieta *et al.*, 1994; Arrecubieta *et al.*, 1995; Dillard *et al.*, 1995). The deduced amino acid sequence of the fourth type 3-specific gene, *cps3M*, has homology to phosphoglucomutases (PGM) and phosphomanno-mutases (PMM) from a variety of organisms, including gram-positive and gram-negative bacteria, yeast, and rabbit muscle but this function has not been demonstrated (Dillard *et al.*, 1995; Caimano *et al.*, 1996). The presence of these four genes in the type 3 capsule locus suggests that they are involved in synthesis of the type 3 polysaccharide, a linear molecule that is composed of repeating units of glucuronic acid and glucose $(GlcUA\text{-}Glc)_n$. The predicted pathway for type 3 synthesis would involve conversion of Glc-6-P to Glc-1-P by a phosphoglucomutase, Glc-1-P to UDP-Glc by a Glc-1-P uridylyltransferase, and UDP-Glc to UDP-GlcUA by a UDP-Glc dehydrogenase and polymerization of UDP-GlcUA and UDP-Glc by a polysaccharide synthase to form the repeating structure. However, of the genes contained in the type 3 locus, only those encoding a UDP-Glc dehydrogenase activity (*cps3D*) and a polysaccharide synthase activity (*cps3S*) have been shown to be essential for type 3 capsule production. Presumably, essential cellular enzymatic activities which are involved in the formation of nucleotide sugars and the biosynthesis of cell walls and teichoic acids can provide the Glc-1-P and UDP-Glc necessary for type 3 production.

Although a uridylyl transferase function has been demonstrated for *cps3U* (Arrecubieta *et al.*, 1995), the Cps3M function has not been demonstrated. Analysis of the predicted Cps3M sequence and the amino acid sequences of known phosphomutases identified three conserved sites - an active site, a Mg^{2+} binding site, and a substrate binding site. However, the size of the predicted Cps3M protein (~44 kDa) is significantly smaller than that reported for other phosphomutases, which range from 49 to 69 kDa (Caimano *et al.*, 1996). The difference in size is largely accounted for by a deletion at the C-terminus of Cps3M that results in the loss of as many as 140 amino acids when compared to the other phosphomutases. From the crystal structure of the rabbit muscle PGM, four domains

(I-IV), which together form the floor and sides of the enzymatic active site crevice, were identified (Dai *et al.*, 1992). As a result of the C-terminal deletion in Cps3M, this protein lacks sequences which are expected to be involved in the formation of structural domain IV. An important role for the C-terminus in phosphomutase activity was demonstrated in studies involving *Neisseria meningitidis* in which the deletion of only 15 amino acids from the C-terminus resulted in loss of PGM activity (Zhou *et al.*, 1994).

The deletion observed in the cloned *cps3M* sequence has been confirmed in the *S. pneumoniae* chromosome and is conserved among independent type 3 isolates. In addition, deletions have also been identified in sequences immediately adjacent to *cps3M*, which is the most downstream type 3-specific gene, as well as in sequences located upstream of the type 3-specific genes. As a result, the type 3 locus contains what appear to be remnants of genes involved in polysaccharide biosynthesis and transposition (Caimano *et al.*, 1996).

The reasons why *cps3U* and *cps3M* are present within the type 3 locus but are not required for type 3 synthesis are not known. Under conditions of laboratory culture, both *cps3U* and *cps3M* are transcribed, although at significantly lower levels than either *cps3D* or *cps3S* (Caimano *et al.*, 1996). Northern blot analyses indicate that, under these conditions, *cps3D*, *cps3S*, *cps3U*, and *cps3M* are present on the same transcript, which also includes the downstream sequences *tnpA* and *plpA* (Caimano *et al.*, 1996). The promoter for this transcript is located upstream of *cps3D* (Arrecubieta *et al.*, 1995; Dillard *et al.*, 1995). In a previous study, a type 3 derivative that contains an insertion, which separates *cps3U*, *cps3M*, *tnpA*, and *plpA* from the more upstream genes *cps3D* and *cps3S*, was found to be identical to the parent type 3 strain in terms of capsule production and mouse virulence (Kelly *et al.*, 1994). Thus, in these assays, either transcription of the downstream genes does not require activity from the promoter located upstream of *cps3D* or the activity of the downstream gene products is not essential. Here, we describe the

effects on virulence of specific mutations in the genes of the type 3 locus and the examination of the *cps3M* gene product.

Methods

Bacterial strains and plasmids

The strains and plamsids used in these studies are described in Table 1. *S. pneumoniae* strains were grown in Todd-Hewitt broth (Difco, Detroit, MI) supplemented with 0.5% yeast extract (THY) or on Blood Agar Base #2 (Difco) supplemented with 3% sheep red blood cells. *E. coli* derivatives were grown in L-broth or on L-agar. Erythromycin was used at 0.3 μg/ml for *S. pneumoniae* and 250 μg/ml for *E. coli*. Ampicillin was used at 100 μg/ml for *E. coli*. The insertion-duplication vectors pJY4163 and pJY4164 were used to construct insertion mutations at targeted sites on the *S. pneumoniae* chromosome. Insertions were confirmed by Southern blot analyses. *cps3M* was cloned and expressed in pET-21a (Novagen, Milwaukee, WI) for enzyme assays and immunizations.

DNA techniques

Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) and purified by CsCl centrifugation (Radloff *et al.*, 1967) when necessary. Chromosomal DNA from *S. pneumoniae* was prepared as previously described (Caimano *et al.*, 1996). *S. pneumoniae* was transformed as previously described (Yother *et al.*, 1986). *E. coli*, washed in water and resuspended in 10% glycerol, was electroporated using a BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, CA).

PCR amplifications were performed using *Taq* polymerase (Fisher Scientific, Pittsburgh, PA). The Genius System (Boehringer Mannheim, Indianapolis, IN) was used for DIG-dUTP labeling of probes and chemiluminescent detection in Southern blotting. The *cps3M* probe used in Southern blots was a 0.36 kb fragment containing the region encoding the putative Cps3M active site and flanking sequences derived from PCR amplification of pMC111 in reactions containing DIG-dUTP and primers within the pUC19 vector polylinker.

Table 1 Bacterial strains and plasmids.

Strain/Plasmid	Relevant genotype/characteristics	Reference
Strain		
Streptococcus pneumoniae		
WU2	Type 3 encapsulated, parent strain	(Briles *et al.*, 1981)
JD770	Type 3 encapsulated derivative of WU2, identical to WU2 in capsule production and virulence properties	(Dillard and Yother, 1994) (Kelly *et al.*, 1994)
JD619	Cps3D$^-$, point mutation, non-encapsulated	(Dillard *et al.*, 1995)
JD982	pJD390 x WU2, Cps3D$^-$, type 3 non-encapsulated, Em^R	(Dillard *et al.*, 1995)
JD900	pJD357 x WU2, Cps3U$^-$, type 3 encapsulated, Em^R	(Dillard *et al.*, 1995)
JD902	pJD362 x WU2, Cps3S$^-$, non-encapsulated, Em^R	(Dillard *et al.*, 1995)
MC1032	pMC107 x WU2, PlpA$^-$, type 3 encapsulated, Em^R	(Caimano *et al.*, 1996)
MC1056	pMC135 x WU2, Cps3M$^-$, type 3 encapsulated, Em^R	(Caimano *et al.*, 1996)
MC1057	pMC135 x WU2, Cps3M$^-$, type 3 encapsulated, Em^R	(Caimano *et al.*, 1996)
MC1092	pMC123 x WU2, Cps3M$^-$, type 3 encapsulated, Em^R	This work
MC1094	pMC123 x WU2, Cps3M$^-$, type 3 encapsulated, Em^R	This work
MC1095	pMC173 x WU2, *cps3M* polar mutation, type 3 encapsulated, Em^R	This work
MC1109	pMC205 x WU2, Cps3M$^-$, type 3 encapsulated, Em^R	(Caimano *et al.*, 1996)
MC1119	pMC180 x WU2, '*tnpA-plpA*', type 3 encapsulated, Em^R	(Caimano *et al.*, 1996)
MC1134	pMC176 x WU2, *cps3M* polar mutation, type 3 encapsulated, Em^R	This work
E. coli		
DH5α	F$^-$ *endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR* (ϕ80*ΔlacΔ(lacZ)M15*)	(Ausubel *et al.*, 1987)
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)	(Ausubel *et al.*, 1987)
W1485 (*pgmΔ*::tet)	PGM mutant of W1485	(Lu and Kleckner, 1994)
MC4033	pMC126 x BL21(DE3)	This work
MC4034	pMC126 x BL21(DE3)	This work
Plasmids		
pJY4163 and pJY4164	Lack origin of replication for *S. pneumoniae*; promoterless *cat* gene downstream of multiple cloning site (opposite orientations in pJY4163 and pJY4164), Em^R	(Yother *et al.*, 1992)
pUC19	Broad-host range plasmid, Amp^R	(Ausubel *et al.*, 1987)
pET-21a	T7-based expression plasmid, Amp^R	Novagen
pSF151	Lacks origin of replication for *S. pneumoniae*, Km^R	(Tao *et al.*, 1992)
pJD378	pJY4164::2.2 kb *Hin*dIII-*Sac*I, '*cps3U-cps3M-tnpA*', Em^R	(Dillard and Yother, 1994)

Table 1 (Continued)

Strain/Plasmid	Relevant genotype/characteristics	Reference
pJD396	pSF151::0.26 kb *Ecl*136II-*Hin*dIII, '*orf5*', Km^R	(Dillard *et al.*, 1995
pMC107	pJY4163::0.27 kb *Sac*I-*Pst*I, '*plpA*', Em^R	(Caimano *et al.*, 1996)
pMC111	pUC19::0.36 kb *Pvu*II-*Pvu*II, '*cps3M*', Amp^R	(Caimano *et al.*, 1996)
pMC123	pJY4164::0.36 kb *Pvu*II-*Pvu*II, '*cps3M*', Em^R	This work
pMC135	pJY4163::0.36 kb *Pvu*II-*Pvu*II, '*cps3M*', Em^R	(Caimano *et al.*, 1996)
pMC166	pUC19::0.77 kb *Hpa*I-*Eco*RI, '*cps3U-cps3M*', Amp^R	This work
pMC173	pMC166 *Dra*III::1.95 kb *Eco*RV-*Cla*I, '*cps3U-erm-cps3M*, Amp^R, Em^R	This work
pMC176	pMC166 *Dra*III::1.95 kb *Cla*I-*Eco*RV, '*cps3U-erm-cps3M*, orientation of *erm* gene is opposite of *erm* in pMC173, Amp^R, Em^R	This work
pMC205	pJY4164::0.47 kb *Eco*RI-*Xmn*I, '*cps3M*', Em^R	(Caimano *et al.*, 1996)

Em^R, erythromycin resistant; Km^R, kanamycin resistant.

Templates for DNA sequencing were prepared using plasmid DNA. Sequencing of plasmid DNA was performed by the UAB Automated DNA Sequencing Facility using dye-terminator chemistry on an ABI Model 377 automated DNA sequencer (ABI-Perkin Elmer, Foster City, CA). Primers for sequencing were purchased from Gibco/BRL (Grand Island, NY).

Virulence assays

Virulence of type 3 derivatives was determined in BALB/ByJ female mice (Jackson Laboratory, Bar Harbor, ME). Bacterial cultures were grown to mid-exponential phase (OD_{600} = 0.45) in THY containing erythromycin. Samples were then serially diluted in sterile lactated Ringer's solution, and 0.2 ml was used to infect mice intraperitoneally (i.p.) or intravenously (i.v.) with indicated doses. For protection studies, mice (n = 3) were passively immunized (i.p.) with 0.2 ml α-rCps3M polyclonal serum, diluted 1/10 in sterile lactated Ringer's. At 1 h following immunization, mice were challenged i.v. with a dose of 10^6 colony forming units (CFU).

Bacterial clearance and ELISA assay

Blood samples were taken retro-orbitally from mice following intravenous infection with *S. pneumoniae* strains JD770, MC1056, and MC1092. Samples were taken from three mice for each strain, then at 1 min, 2 h, 4 h, 6 h and 28 h post-infection. Immediately following collection at each time point, samples were diluted and plated on selective and non-selective plates to determine CFUs. For ELISA assays, blood samples from each of three mice per time point were combined. Type 3 capsule contained within each sample was quantitated in competitive-inhibition ELISA assays as described previously (Caimano *et al.*, 1996). In preliminary experiments, the dilutions of each blood sample required to give approximately 50% inhibition were determined. Accordingly, samples were each diluted 125-fold. Percent inhibition was determined as (1 - sample OD_{415}/uninhibited OD_{415}) x 100 and was standardized to the number of CFUs. The uninhibited control is the value obtained following incubation of the mAb with blood from uninfected mice diluted 1/125 but without polysaccharide.

The percent inhibition for each sample was determined as the mean of six replicates. Statistical significance was determined using Student's t-test.

Overexpression of Cps3M

A recombinant clone of *cps3M* capable of being overexpressed in *E. coli* was constructed by using a PCR amplification product obtained from *S. pneumoniae* type 3 strain WU2 and cloned into pET-21a. Primer M1 (5'-GTGGACACATATGAATTG TATAG-3') was used to create a unique *Nde*I site overlapping the putative Cps3M start codon. The 3' primer (P1, 5'-GCCGTAGATGATGACAACCA-3') was located downstream of the Cps3M stop codon. An *Nde*I-*Xho*I fragment of the amplification product was ligated into pET-21a to give an in-frame fusion between the vector ribosome binding site and the putative Cps3M start codon. Initially, ligations were transformed into *E. coli* DH5α. Plasmids containing the appropriate insertion, as determined by sequencing, were transformed into *E. coli* BL21(DE3) to permit induction (Ausubel *et al.*, 1987). For

induction experiments, overnight cultures grown in L-broth and containing ampicillin were diluted 1:100 into 10 ml L-broth plus ampicillin, grown to mid-exponential phase and induced by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) at a final concentration of 2 mM. Following induction for 2 h at 37°C, cultures were centrifuged at 12,000 x *g* for 10 min, washed with 1 vol of phosphate buffer (67 mM $NaPO_4$ [pH 6.6], 3 mM EDTA, 1 mM DTT), and resuspended in 1/10 vol phosphate buffer. An uninduced duplicate culture was used as a negative control.

Enzyme assays

Sonicates were prepared from 10 ml *S. pneumoniae* cultures grown in THY to mid-exponential phase. Cultures were centrifuged at 12,000 x *g* for 10 min, washed with 1 vol sonication buffer, and resuspended in 1/10 vol sonication buffer (50 mM MOPS [pH 7.0], 3 mM EDTA, 1 mM DTT). Samples were then sonicated three times on ice using a Sonic Dismembrator Model 300 (Fisher Scientific, Pittsburgh, PA) at a power setting of 30 with 30 sec pulses followed by 1 min incubations on ice. Following induction as described above, uninduced and induced *E. coli* culture samples were sonicated as described above and used directly in indirect enzyme assays for PGM and PMM activities. PGM activity was assessed according to the method of Joshi (1982) and Smith *et al.* (1957) using 1 ml reaction mixtures that contained 0.1 M Tris-HCl [pH 7.8], 2 mM glucose-1-phosphate, 7.9 mM glucose-1,6-diphosphate, 0.5 mM $NADP^+$, 1 U glucose-6-phosphate dehydrogense, and 5 mM $MgCl_2$. Reactions were started by the addition of 10 to 50 µl sonicate. Commercially obtained rabbit muscle PGM (Sigma Chemical Co., St. Louis, MO) was used as a control. Activity was measured at room temperature and determined by the rate of increase in absorbance at 340 nm. PMM activity was assessed according to the method of Koplin *et al.* (1992) in 1 ml reaction mixtures containing 100 mM MOPS [pH 7.6], 10 mM $MgCl_2$, 0.075 mM glucose-1,6-diphosphate, 1 mM $NADP^+$, 1 mM mannose-1-phosphate, 0.7 U glucose-6-phosphate dehydrogenase, 1.1 U phosphoglucose isomerase, and 0.9 U phosphomannose isomerase. Reactions were started by the addition of 5 to 25

ml crude lysate. In both the PGM and PMM assays, $MnCl_2$, $ZnCl_2$, and $RbCl_2$ at 1.7 mM and 5.1 mM were substituted for $MgCl_2$ in certain reactions. Conversion of $NADP^+$ to NADPH at room temperature was determined by the increase in absorbance at 340 nm for 5 min. The amount of NADPH produced was calculated by the formula $c_{NADPH} = A_{340}/(a_m)(P_l)$ where a_m = molar absorbance coefficient of NADPH (= 6,220) and P_l = path length of cuvette (= 1 cm). One unit is defined as the amount of enzyme required to reduce 1 mol $NADP^+$ to NADPH per min. Each extract was tested in duplicate and at least two independent sonicates were tested for each strain.

Solubilization of rCps3M

E. coli cultures expressing rCps3M were grown in L-broth containing ampicillin and induced and sonicated as described above. Sonicates were then centrifuged at 18,000 x *g* for 20 min. Pelleted material was solubilized by resuspension in 8 M urea followed by dialysis overnight at 4°C in phosphate-buffered saline (PBS). Dialyzed samples were centrifuged at 18,000 x *g* for 25 min and supernatant fluids were saved for assays of PGM activity.

Production of rCps3M antiserum

To obtain rCps3M to generate antiserum, sample bacteria were concentrated from a 5 ml induced culture in which Cps3M was overexpressed. The concentrate was electrophoresed on a 10% preparative SDS-PAGE gel, the protein band containing rCps3M was excised, and the gel slice was ground in PBS using a glass mortar and pestle to form a slurry. For initial immunizations, 1.2 ml of the gel slurry was mixed 1:1 with Freund's Incomplete adjuvant. Ten BALB/ByJ female mice were then injected subcutaneously with two injections of 0.1 ml on each flank. On day 8, mice were boosted by i.p. injection of 0.2 ml of a rCps3M gel slurry prepared as described above, but with lactated Ringer's solution substituting for PBS. Mice were bled 10 days following the boost. Prior to use, antiserum was adsorbed with *E. coli* BL21(DE3) containing pET-21a and diluted 1:200 in PBS containing 0.005% Tween.

Protein and Western blot analyses

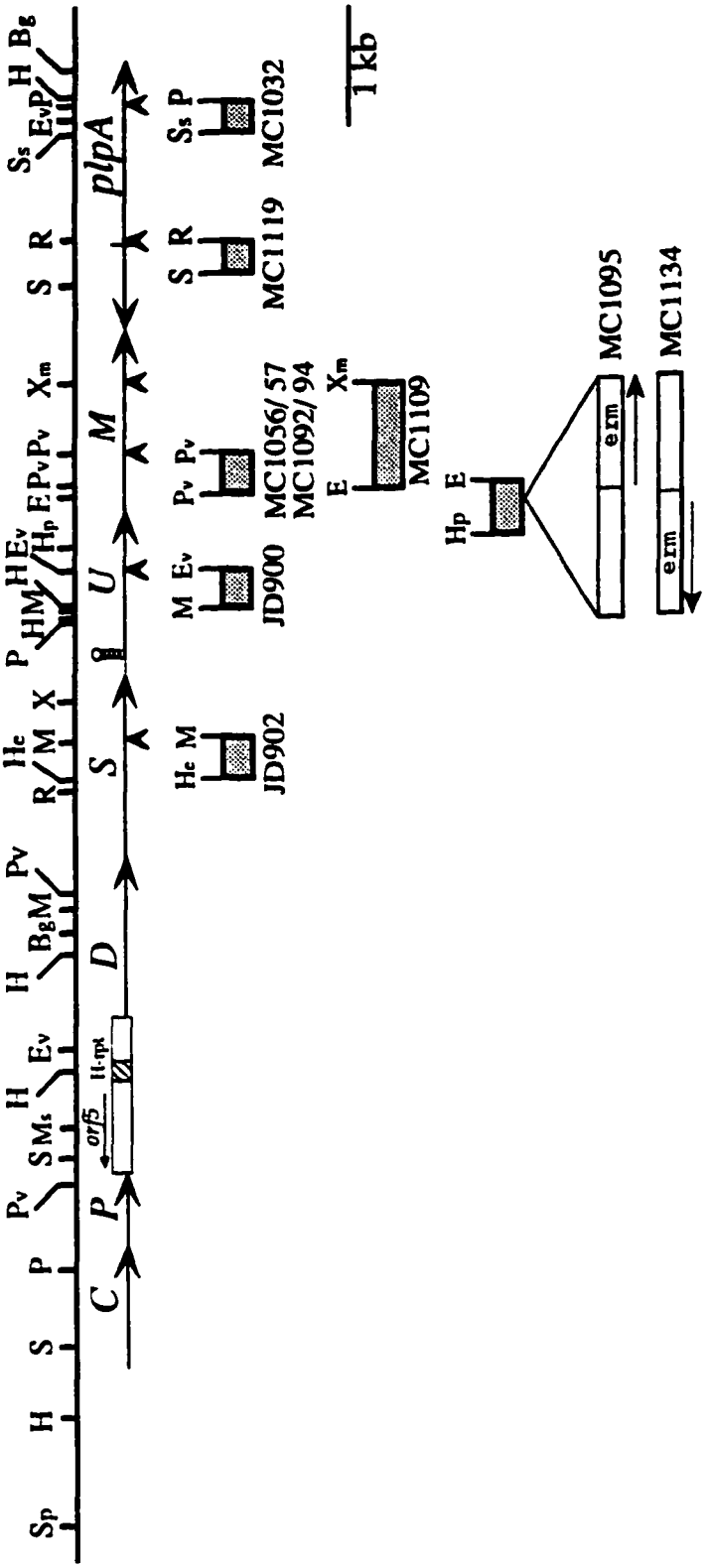
For *E. coli*, cultures were grown to mid-exponential phase and induced when required. Cells were then centrifuged at 12,000 x *g* for 10 min and resuspended in 1/10th vol sonication buffer. A 5 µl volume of concentrated culture was mixed with loading buffer (12.5 mM Tris-Cl pH [6.8], 0.4% SDS, 2% glycerol, 0.5% bromphenol blue, 0.28 M β-mercaptoethanol), boiled for 10 min and then electrophoresed on SDS-10% polyacrylamide gels. For *S. pneumoniae*, cultures were grown in THY to mid-exponential phase, and centrifuged, and the pellets were resuspended in 1/10th vol PBS. Five to 20 µl was mixed with loading buffer, boiled for 10 min and then electrophoresed on SDS-10% polyacrylamide gels. Gels were either stained with Coomassie blue or electrotransferred to nitrocellulose by using the TransBlot transfer system (Bio-Rad, Richmond, CA). Blots were processed as previously described (Yother *et al.*, 1992) using polyclonal mouse α-rCps3M serum. Rainbow marker protein standards were obtained from Amersham/Life Sciences (Arlington Heights, IL) .

Results

Only cps3D and cps3S are required for virulence

Specific mutations were generated in the type 3 locus by using cloned restriction fragments to target insertion-duplication events (Fig. 1). The Cps3D mutant contains a point mutation in *cps3D* that results in loss of UDP-Glc dehydrogenase activity but that is not polar on *cps3S* (Dillard *et al.*, 1995). The Cps3D and Cps3S mutants are non-encapsulated under laboratory culture conditions, whereas all other mutants are of normal type 3 encapsulation (Dillard and Yother, 1994; Caimano *et al.*, 1996; data not shown). Strain JD770 contains an insertion-duplication that separates *cps3D* and *cps3S* from *cps3U*, *cps3M*, *tnpA* and *plpA* (Dillard *et al.*, 1995). This strain is identical to its type 3 parent in terms of capsule production and mouse virulence (Kelly *et al.*, 1994) and is used here as the control parent strain for comparison to other derivatives containing insertion-duplications.

Fig. 1. Location of insertions within *cps3* locus. Restriction sites and position of fragments used to construct insertions are indicated. Triangles, with strain names given below, indicate the points of insertion. The position and orientation of the antibiotic cassettes inserted between *cps3U* and *cps3M* are also indicated. Strains MC1095 and MC1134 were constructed by allelic exchange; all other strains were constructed by insertion-duplication. Restriction enzymes: Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; Hp, *Hpa*I; H, *Hin*dIII; M, *Mun*I; Ms, *Msc*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sac*I; Sp, *Sph*I; St, *Stu*I; X, *Xba*I; Xm, *Xmn*I.



As anticipated, the non-encapsulated Cps3D and Cps3S mutants were not, in the absence of reversion of the mutations, able to cause death when mice were infected by either the i.p. or i.v. routes (Tables 2 and 3). Revertants of both mutants were identified as encapsulated isolates that resulted in death following i.p. infection with the non-encapsulated strains (Table 2).

In contrast to *cps3D* and *cps3S*, none of the other genes examined were required for virulence (Tables 2 and 3). The mutants tested contained insertions within either *cps3U*, *cps3M*, or *plpA*. Two of the *cps3U* insertion mutations (contained in strains MC1095 and MC1134) were designed to block transcription originating upstream of *cps3M*, as previous studies had identified a potential promoter in the 3' region of *cps3U* (Caimano *et al.*, 1996). The results indicate that transcription from the region upstream of *cps3M* is not required for virulence. Likewise, no requirement for the *cps3U* or *cps3M* structural genes was apparent (strains JD900, MC1057, MC1092, and MC1094). The lack of effect of a mutation in *plpA* confirmed that this gene, which is inherently mutant in type 3 strains (Caimano *et al.*, 1996), is not required for virulence. For each strain used in these studies, with the exception of the *cps3D* and *cps3S* revertants, the bacteria recovered from the heart blood of dead mice were encapsulated and erythromycin resistant.

An alteration in virulence was noted for one strain (MC1056) when it was used in infections by either the i.p. or i.v. routes (Tables 2 and 3). This strain, which is essentially avirulent, contains an insertion-duplication mutation in *cps3M* (Fig. 1). However, this mutation is not the cause of the loss of virulence, as three other strains (MC1057, MC1092, and MC1094) containing the same mutation remained virulent (Tables 2 and 3).

Alterations in blood clearance as a result of mutations

To determine the time course of bacterial clearance from the blood, mice were infected i.v. at a dose of 10^7 bacteria with either the parent strain JD770, the non-encapsulated strains JD619 and JD902, or the Cps3M mutants MC1056 and MC1092 which had shown different levels of virulence. In contrast to the parent strain, the non-encapsulated

Table 2. Virulence of *cps3* mutant strains in BALB/ByJ mice by an intraperitoneal route of infection.

Gene containing mutation	Strain	Dose	Alive/Total	Percent survival
Type 3^+	JD770	10^2	2/8	25
		10^3	2/11	18
		10^4	1/5	20
cps3D	JD619	10^7	2/3 [a]	66
cps3S	JD902	10^7	0/2 [a]	0
cps3U	JD900	10^2	2/8	25
		10^3	0/8	0
cps3U	MC1095 [e]	10^1	5/10	50
		10^2	0/3	0
		10^3	0/5	0
cps3M [b, c]	MC1057	10^4	0/3	0
	MC1092	10^4	0/3	0
	MC1094	10^4	0/3	0
cps3M [d]	MC1109	10^1	2/5	40
		10^3	1/5	20
cps3M	MC1056	10^3	5/5 [f]	100
		10^4	8/8 [f]	100
		10^6	6/6 [f]	100
tnpA	MC1119	10^1	5/5	100
		10^2	0/5	0
plpA	MC1032	10^2	0/5	0
		10^3	0/5	0

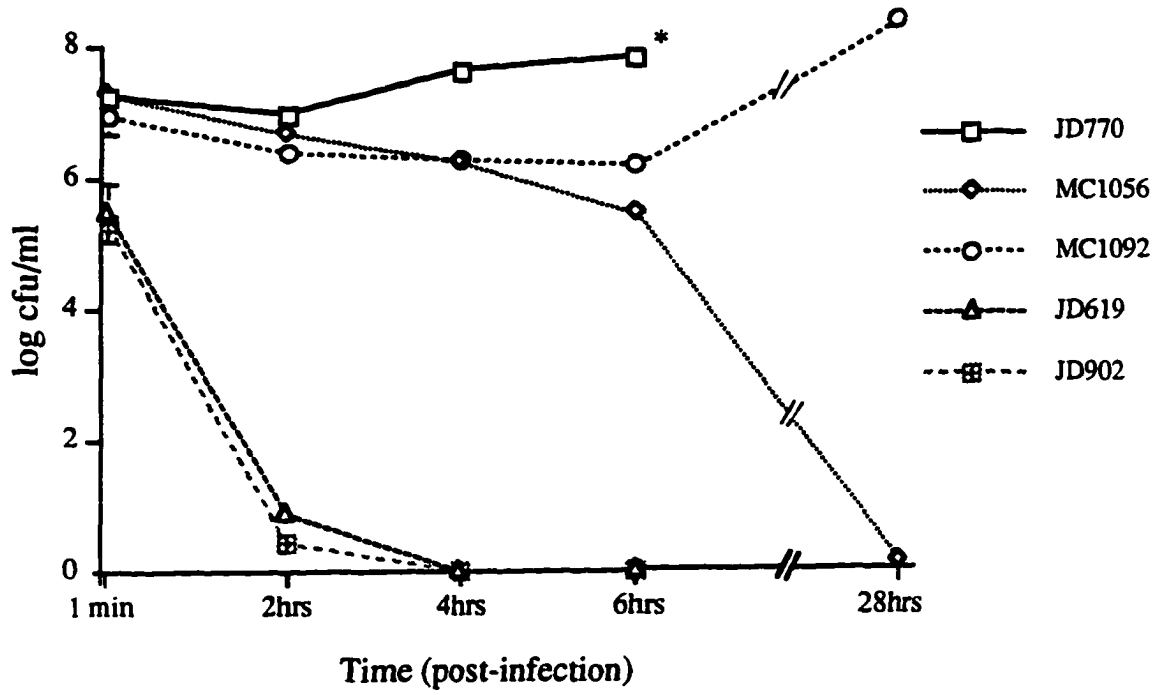
a. *S. pneumoniae* recovered were encapsulated. Therefore, virulence of the Cps3D mutant is due to reversion or suppression of the *cps3D* point mutation. Bacteria recovered from mice infected with the Cps3S mutant were both encapsulated and erythromycin-sensative indicating a loss of the insertion within *cps3S* and restoration of the intact gene.
b. Insertions result in a deletion of 239 amino acids from the Cps3M C-terminus.
c. Strains contain the same insert in oppposite orientations.
d. Insertion results in a deletion of 148 amino acids from the Cps3M C-terminus
e. Strains MC1095 and MC1134 results combined; strains contain *erm* antibiotic cassette in opposite orientations.
f. Significantly different from the 10^3 dose of JD770, $p < 0.005$.
g. Insertion results in a loss of 61 amino acids from the Cps3U C-terminus.

Table 3. Virulence of *cps3* mutant strains in BALB/ByJ mice by an intravenous route of infection.

Gene containing mutation	Strain	Dose	Alive/Total	Percent survival
Type 3^+	JD770	10^6	3/8	38
		10^7	0/8	0
cps3D	JD619	10^7	3/3	100
cps3S	JD902	10^7	3/3	100
cps3U	JD900	10^6	6/13	46
		10^7	1/5	20
cps3U [g]	MC1095[e]	10^6	3/8	38
		10^7	0/3	0
cps3M	MC1092[b]	10^6	0/3	0
		10^7	0/3	0
cps3M	MC1056	10^6	8/8[f]	100
		10^7	11/11[f]	100
plpA	MC1032	10^4	3/5	60
		10^5	0/5	0

b. Insertions result in a deletion of 239 amino acids from the Cps3M C-terminus.
e. Strains MC1095 and MC1134 results combined; strains contain *erm* antibiotic cassette in opposite orientations.
f. Significantly different from the 10^6 dose of JD770, $p < 0.025$.
g. Insertion results in a loss of 61 amino acids from the Cps3U C-terminus.

mutants decreased in number by approximately 100-fold within one min of injection and were readily cleared from the bloodstream (Fig. 2). The Cps3M mutants exhibited patterns of clearance different from each other and from either the parent or non-encapsulated strains. By 4 h post-infection, the numbers of both mutants were reduced as compared to the parent strain. For MC1056, the number of CFUs continued to decline and, at 28 h, bacteria were not detectable. For MC1092, however, the number of bacteria was unchanged at 6 h compared to 4 h and increased when measured at 28 h. These mice died from the infection but the time to death was increased when compared to that of the parent strain (36 ± 5 h vs. < 18 h, $p = 0.02$). Similar results were observed using a 10^6 dose



* Mice (n=3) dead at 18 hours post-infection

Fig. 2. Time course of clearance of *S. pneumoniae* from mouse blood. *S. pneumoniae* parent (JD770) and *cps3M* mutant strains (MC1056, MC1092) were used to infect BALB/ByJ female mice (n = 3 per strain) at a dose of $1x10^7$ CFU. Blood samples were taken retro-orbitally at t = 1 min, 2, 4, 6, and 28 h post-infection. Results shown are the average of CFU/ml from three mice plated in duplicate. Except where given, the standard errors for each sample were ≤ 10%.

for the blood clearance assays; no effect on time to death was noted in i.p. infections or with strains containing other mutations (data not shown).

Alterations in capsule production during i.v. infections as a result of mutations

The Cps3M mutant strains MC1056 and MC1092, which differ in their virulence properties, do not differ from the parent type 3 strain in the amount of capsule produced under laboratory culture conditions, as judged by colony morphology on blood agar plates and/or by ELISA assay (Caimano *et al.*, 1996; data not shown). To determine whether these strains produce altered levels of type 3 capsule *in vivo*, blood samples obtained in the clearance assays described above were used to quantitate the type 3 polysaccharide in competitive-inhibition ELISA assays (Fig. 3). Results from these assays demonstrated a significant increase in the amount of type 3 capsule per CFU present in the blood of mice infected with MC1056, as compared to mice infected with the parent strain, JD770. Strain MC1092 also showed an increase in the amount of capsule per CFU; the increase was not, however, as striking as that seen with MC1056 (Fig. 3).

Overexpression of Cps3M in E. coli and generation of α-Cps3M

cps3M remains the only type-3 specific gene for which an activity has not been demonstrated. As reported previously, the predicted amino acid sequence of Cps3M has homology to phosphomutases (Caimnao *et al.*, 1996). In assays using extracts from *S. pneumoniae* type 3 strain WU2, neither PGM nor PMM activity was detectable (data not shown). This result was consistent with the low level of *cps3M* expression that had previously been indicated by transcription analysis (Caimano *et al.*, 1996); this finding is supported by early biochemical studies which were also unable to demonstrate detectable levels of phosphoglucomutase activity using intact cells from serotype 3 strain A66 (Smith *et al.*, 1957). Thus, a recombinant clone containing *cps3M* under the control of an inducible promoter which could be used to overexpress Cps3M in *E. coli* was constructed in the pET-21a vector containing the T7 promoter. The DNA sequence of the cloned gene was the same as that previously reported for *cps3M* (Caimano *et al.*, 1996). By SDS-PAGE

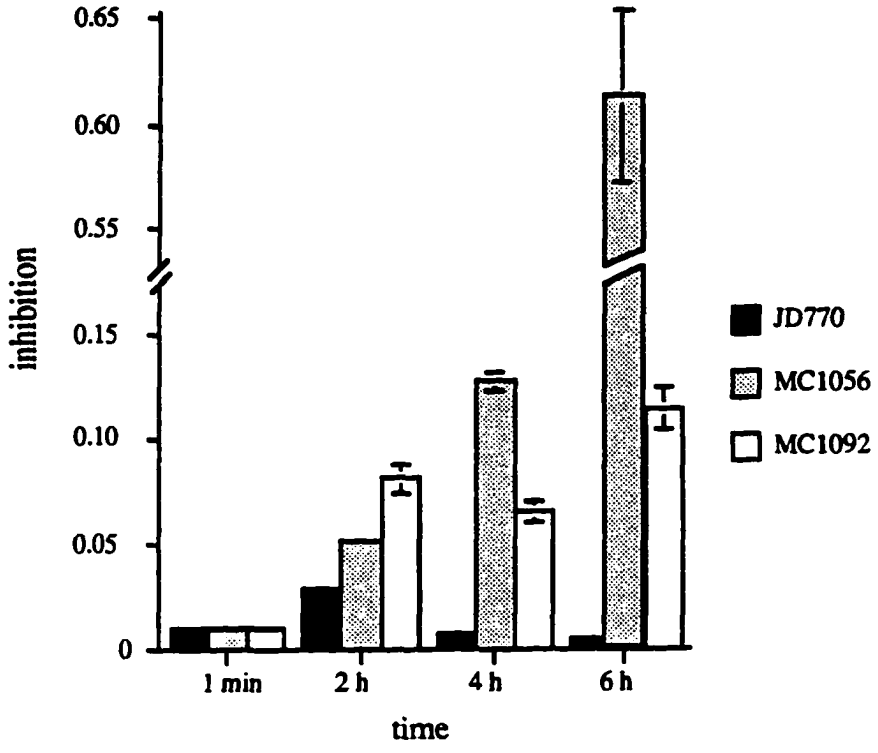


Fig. 3. Quantitation of type 3 capsule in blood of mice infected with *S. pneumoniae*. Following use in clearance studies, blood samples taken over time from mice infected with *S. pneumoniae* parent (JD770) and *cps3M* mutant strains (MC1056, MC1092) were used in competitive-inhibition ELISA assays. Type 3 capsule was detected by using a monoclonal antibody against the type 3 capsule. Inhibition is the % inhibition (as defined in Materials and Methods) divided by the number of CFUs assayed. Standard errors for each sample are ≤ 10%. Both mutants were different from the parent strain at the 2h, 4 h and 6 h time points ($p < 0.0001$). Strains MC1056 and MC1092 were different from each other at those same time points ($p < 0.001$).

analysis, a protein of the expected size of Cps3M (~44 kDa) was identified in induced cultures (Fig. 4). From densitometry measurements, the percentage of induced protein was estimated to be ~25% of total cell protein.

Recombinant Cps3M was used to generate Cps3M-specific antiserum in mice. In Western blot analyses, an immunoreactive protein corresponding to rCps3M was detected in samples from induced cultures expressing rCps3M. No immunoreactive proteins were detected in uninduced cultures containing *cps3M* or in the background strain containing the expression vector (Fig. 5A). The antiserum also detected purified rabbit muscle PGM (Fig. 5B). However, α-rCps3M-specific bands were not detected in type 3 *S. pneumoniae* grown under laboratory culture conditions (data not shown). In addition, no protective effect of this antiserum against i.v. infection with type 3 *S. pneumoniae* was observed (data not shown).

Lack of PGM and PMM activity using recombinant Cps3M

Sonicated samples of *E. coli* expressing recombinant Cps3M (rCps3M) were used to perform both PGM and PMM assays (Fig. 6). Under standard assay conditions, no significant activity was detected for either enzyme (Fig. 7). Control assays containing purified PGM from rabbit muscle were used to verify assay conditions. Previous studies involving a yeast phosphoglucomutase indicate that, while Mg^{2+} is the most potent activator of PGM activity, other divalent cations are capable of substituting for Mg^{2+} in enzyme reactions (Sutherland, 1949). To examine whether Cps3M is activated by metals other than Mg^{2+}, assay conditions were adjusted to include either Mn^{2+}, Zn^{2+}, or Rb^{2+}. The addition of a sonicated sample containing rCps3M to control reactions containing purified rabbit muscle PGM did not alter PGM activity of the latter enzyme.

Microscopic examination of induced cultures overexpressing rCps3M indicated the presence of inclusion bodies. Examination of fractionated induced cultures by SDS-PAGE indicated that rCps3M was localized to the fraction containing insoluble material. To determine whether Cps3M was inactive due to insolubility, sonicates containing rCps3M

Fig. 4. Expression of recombinant Cps3M in *E. coli*. Samples from IPTG-induced cultures containing *cps3M* (MC4033 and MC4034) or expression vector (pET-21a) alone were electrophoresed on a 10% SDS-polyacrylamide gel and stained using Coomassie Blue. Lanes: 1, BL21(DE3) pET-21a; 2, MC4033; 3, MC4034.

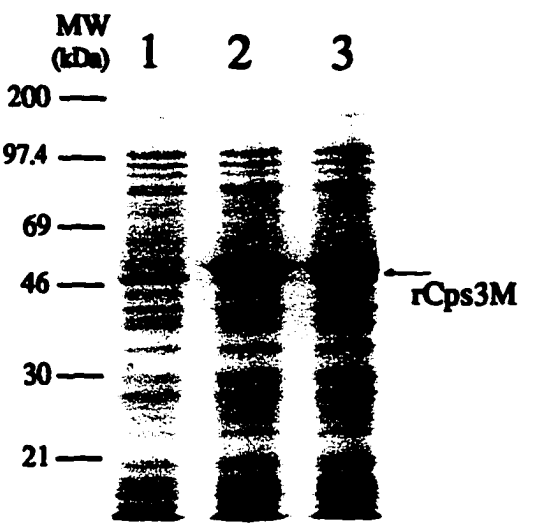


Fig. 5. Western immunoblots using rCps3M antiserum. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to nylon membrane before reacting with rCps3M antiserum (α-rCps3M).
A. Induced *E. coli* cultures expressing rCps3M or vector alone. Lanes: 1, BL21(DE3) pET-21a; 2, MC4033.
B. Cross-reactivity between α-rCps3M and rabbit muscle PGM. Lanes: 1, MC4033 (induced); 2, Rabbit muscle PGM.

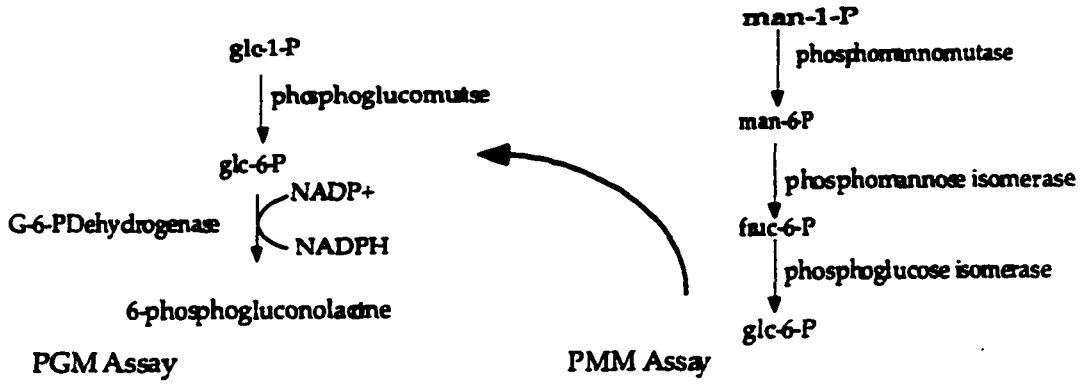
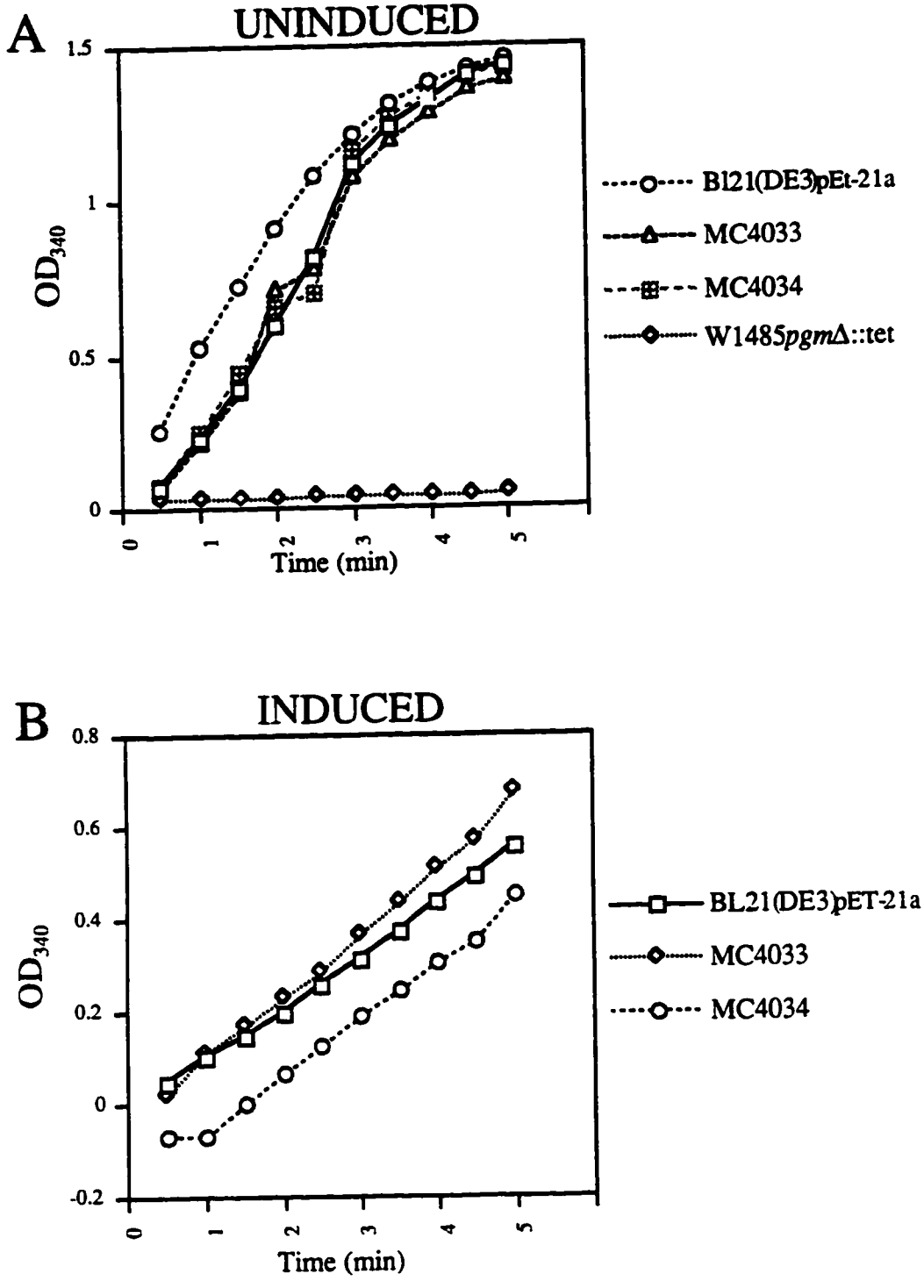


Fig. 6. Diagram of PGM and PMM assays. The above illustration depicts biochemical pathways involved in the indirect assay conditions used to measure PGM and PMM activity.

Fig. 7. Time course of standard PGM enzymatic assays in *E. coli.*
A. Assays of sonicates from uninduced cultures.
B. Assays of sonicates from induced cultures.
Strains shown: BL21 (DE3) pET-21a (expression vector alone); MC4033 and MC4034 (*cps3M*:pET-21a); and W1485*pgm*Δ::tet (*E. coli* PGM negative strain). Assay conditions are as described in Methods and Materials using $MgCl_2$ at 5 mM. Fifty ml of each sonicate was used per reaction. Assays were performed in duplicate using at least two independently grown cultures. Control reactions were performed using purified rabbit muscle PGM. Negative control reactions were set up minus $NADP^+$ or minus sonicate. Representative results from one experiment are shown. Results from PMM assays were similar those illustrated above (data not shown).



were solublized in urea then dialyzed. Following solubilization, approximately 80% of rCps3M was contained within the supernatant fluid compared to pelleted dialyzed material. Assays were performed using solublized samples from induced cultures. As in the previous assays, no PGM activity was detected (data not shown).

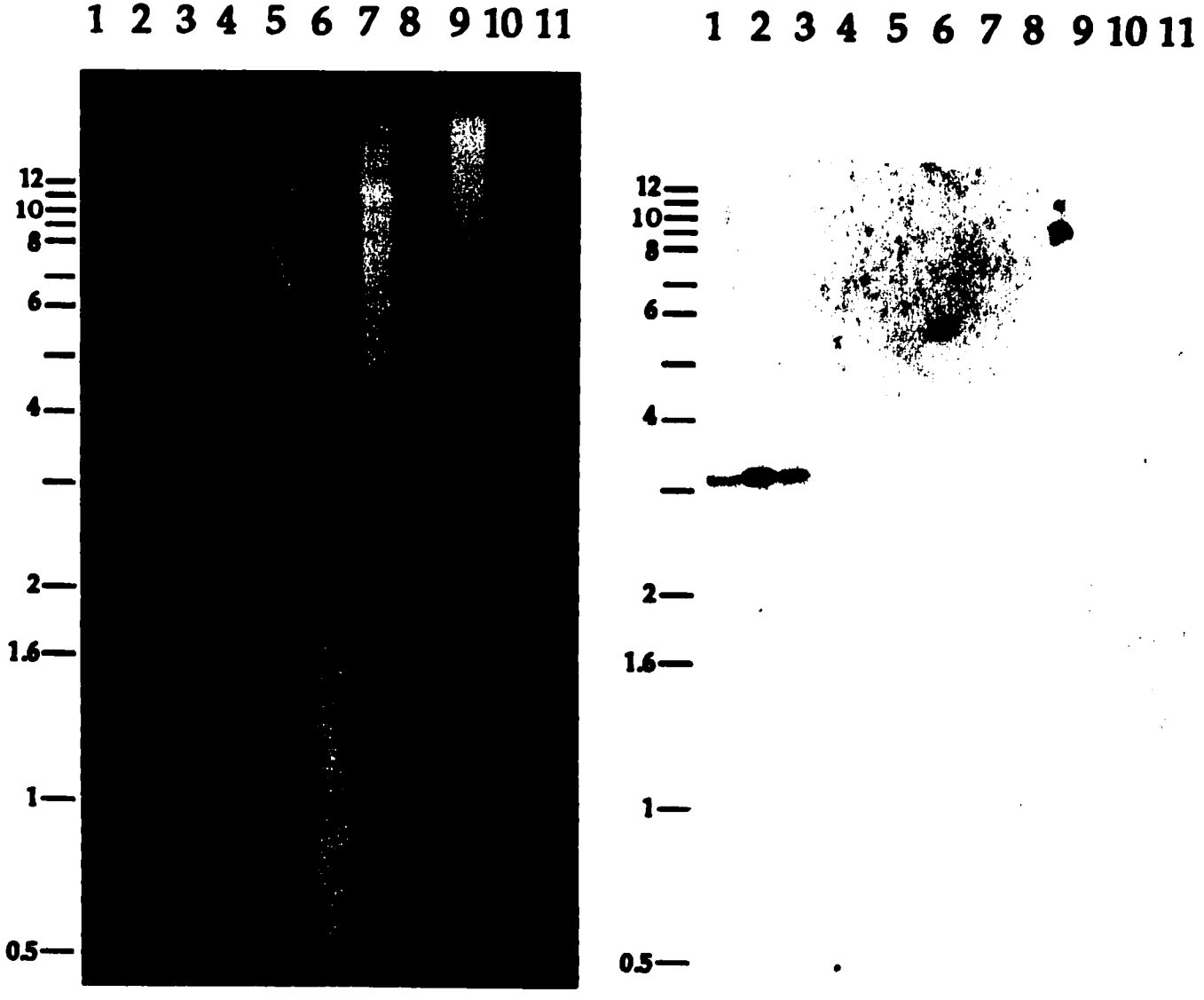
Probing for sequences homologous to cps3M in other pneumococcal serotypes

Because Glc-1-P, the product of PGM activity, is an essential component of normal cellular functions, a gene encoding this activity is expected to be present in *S. pneumoniae* chromosome. Although Cps3M, based on sequence homology to phosphomutases, was initially proposed to perform this function, the lack of a requirement for *cps3M* in the biosynthesis of type 3 capsule under laboratory conditions and in virulence argues for an alternate means of forming glucose-1-phosphate, perhaps by a complementary enzyme activity. To determine if sequences homologous to *cps3M* are present in *S. pneumoniae*, genomic DNA from strains of different serotypes, including type 3, were examined in Southern blot analyses. Using a probe derived from PCR-amplification of sequence overlapping the putative Cps3M active site, which is also present and conserved within known phosphomutases, sequences homologous to *cps3M* were detected only in strains producing the type 3 polysaccharide. A representative Southern blot, performed at 66% stringency, is shown in Fig. 8. In type 3 strains, only a single copy of *cps3M* was identified and mapped to within the *cps3* locus. A reduction in stringency to levels as low as 55% did not identify additional sequences (data not shown).

Discussion

A requirement for capsule in virulence of *S. pneumoniae* has been established for greater than 80 years. Studies on the virulence of serotype 3 *S. pneumoniae* have, however, used almost exclusively non-encapsulated strains which have resulted from spontaneous mutation. Many of these strains have been characterized biochemically and shown to be deficient in either the UDP-glucose dehydrogenase or polysaccharide synthase activities involved in type 3 capsule biosynthesis. One transposon-mediated insertion within type 3

Fig. 8. Hybridization of *S. pneumoniae* with *cps3M* probe. Chromosomal DNAs from eight different *S. pneumoniae* serotypes were digested with *Hin*dIII and probed in Southern blot hybridizations using a fragment overlapping the region containing the Cps3M putative active site. Hybridizations were performed at room temperature; blots were washed at either high or low stringencies with similar results. Results shown are at a stringency level of 65%. Lanes:1, WU2 (3); 2, A66 (3); 3, Rx1 (3/2); 4, *E. coli* DH5α; 5, D39 (2); 6, DBL5 (5); 7, ATCC8 (8); 8, L8 2233 (23); 9, BG 5668 (14); 10, BG 58C (19); 11, BG 7428 (9). Capsular serotypes are indicated in parenthesis. *S. pneumoniae* strain Rx1 is a type 2 derivative containing the type 3-specific *cps* locus (7).



S. pneumoniae has been described which resulted in non-encapsulation; this non-encapsulated type 3 *S. pneumoniae* was avirulent (Watson and Musher, 1990). The location of the transposon-insertion has not been determined and it is unknown whether the loss of capsule and the reduction in virulence of this strain is due to an insertion within the *cps3* locus. The findings reported herein are, to our knowledge, the first studies of type 3 *S. pneumoniae* which utilize site-directed insertions within each of the type 3-specific genes, as well as flanking sequences. Results from these studies using strains containing mutations within either *cps3D* or *cps3S*, which resulted in a non-encapsulated phenotype, are in agreement with previous studies of non-encapsulated strains resulting from either spontaneous mutation or transposon-mediated insertion. When these non-encapsulated strains were used to infect mice (i.v.) they were shown to be avirulent. When used in virulence assays by the i.p. route of infection, however, encapsulated revertants of both strains were isolated. This result is most probably a reflection of the spontanous rate of either mutation, in the case of the *cps3D* strain, or the resolution of the insertion vector, in the case of *cps3S*. The dose required for death due to type 3 *S. pneumoniae* is extremely small ($\leq 10^2$ CFU, i.p.); therefore, even a low frequency of reversion within an infection dose of 1×10^7 could result in a lethal dose. The dose required for death by an i.v. route of infection is significantly higher ($\geq 10^5$); therefore, a low rate of spontaneous reversion would not provide a lethal dose.

Insertions within the two downstream type-3 specific genes, *cps3U* and *cps3M*, do not result in the non-encapsulated phenotype when grown under laboratory conditions as judged by colony morphology and ELISA assay (Caimnao *et al.*, 1996). As these two genes have been maintained within the *cps3* loci of all type 3 strains examined, their role in virulence was examined. The encapsulated *cps3U* mutant strain, JD900, was shown to exhibit a level of virulence identical to that of the parent strain, JD770. Although strains containing insertions within *cps3M* exhibited some reduction in virulence, *cps3M* is not required for virulence of type 3 *S. pneumoniae*. One strain, MC1056, containing an

insertion within *cps3M* was shown to be avirulent; however, the observations made with this strain are likely due to a second mutation and not due to the insertion within *cps3M*. Therefore, *cps3M*, along with *cps3U*, is distinguished from *cps3D* and *cps3S* as being present within the type 3-specific locus but is not required for either the production of type 3 capsule or virulence.

The novel virulence properties of MC1056 are further supported by results from capsule ELISA assays performed on blood samples taken during the time course of infection of mice with this strain MC1056 as compared to the parent strain, JD770, and MC1092. This difference in capsule production is apparent only within the *in vivo* environment, as MC1056 and JD770 were previously shown to produce similar levels of type 3 polysaccharide when grown under laboratory conditions (Caimano *et al.*, 1996). The observation that a strain which produces increased levels of polysaccharide is avirulent could suggest that the amount of capsule produced affects the virulence of *S. pneumoniae* and that the ability to alter the level of capsule produced during infection is advantageous to the bacteria. Studies with group B streptococcus (GBS) have demonstrated that the ability of this bacteria to invade cultured endothelial cells is inhibited by the presence of a polysialic acid capsule and results in attenuated virulence (Rubens *et al.*, 1993). The ability to adhere to and invade lung endothelial cells is thought to provide a mechanism for entry into circulation. As with *S. pneumoniae*, however, a loss of capsule in GBS results in a complete loss of virulence in animal models (Rubens *et al.*, 1991; Gibson *et al.*, 1993). Therefore, the ability of GBS to selectively regulate the amount of capsule *in vivo* could provide a means of allowing for efficient invasion as well as provide protection from host defenses.

The lack of a requirement for either *cps3U* or *cps3M* in virulence or capsule production calls into to question the functions of these genes. Recent studies have demonstrated glucose-1-P uridylyltransferase activity associated with Cps3U (Arrecubieta *et al.*, 1995). Although the predicted amino acid sequence of Cps3M suggested that this protein

may possess phosphomutase activity, enzyme assays using culture sonicates from either *S. pneumoniae* or from *E. coli* strains overexpressing a recombinant form of Cps3M did not demonstrate a detectable level of phosphomutase activity. Several explanations are possible for the lack of detectable activity. One explanation could lie in the apparent C-terminal deletion within Cps3M. Studies involving the crystal structure of the rabbit muscle PGM (Dai *et al.*, 1992) and an *N. gonorrhoeae* PGM mutant strain (Zhou *et al.*, 1994) indicate that residues important for the formation of the active enzyme are present within the C-terminus of these proteins and potentially all phosphomutases. The absence of such sequences could suggest that Cps3M is a mutant form of what was once a larger, and perhaps functional, phosphomutase.

An alternative explanation could lie in the enzyme reaction conditions used. Although Cps3M contains three conserved sites present in phosphomutases, the putative Mg^{2+} binding site is not in complete agreement with the amino acid consensus sequence found in the gram-negative bacterial, yeast, and rabbit muscle PGMs. The amino acid changes within the Cps3M Mg^{2+} binding site were also identified in homologous ORFs found in *Mycoplasma pirum* and *Mycobacterium leprae* (Caimano *et al.*, 1996). However, the protein products of these ORFs have not been demonstrated by biochemical analyses to possess phosphomutase activity. Two phosphoglucomutases (α-PGM and β-PGM) from the gram-positive bacteria *Lactococcus lactis* subs. *lactis* have been identified and have been demonstrated to contain phosphoglucomutase activity (Qian *et al.*, 1994) but their sequence has not been reported and it is unknown whether they share homology with Cps3M. In earlier biochemical studies of pneumococcal polysaccharide biosynthesis, investigators, although able to demonstrate a low level of phosphoglucomutase activity using intact cell extract from type 2 *S. pneumoniae*, were not able to detect similar activity from a type 3 strain (Smith *et al.*, 1957). Reaction conditions used in the PGM assays of both the *S. pneumoniae* resting cells and *L. lactis* were similar to those used in this and other reports; therefore, it is likely that conditions used here should permit detection of

PGM activity associated with Cps3M if such an activity were present or could be inactive in inclusion bodies.

Although we did demonstrate a requirement for *cps3U* and *cps3M* in specific models of pneumococcal virulence, a role for these genes in other models or in human infections cannot be ruled out. The retention of both genes in apparently all type 3 strains argues for their having a necessary function. As these genes potentially encode functions analogous to those of normal cellular enzymes, their presence may suggest a role in altering the levels of available susbstrates and, thus, controlling the amount of capsule synthesized. The increased level of capsule produced by a *cps3M* mutant following i.v. infection hints at a regulatory role for this gene product. The examination of other models of virulence will be necessary to better define what, if any, role these genes have in the virulence of type 3 *S. pneumoniae*.

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DISCUSSION

The work presented within this dissertation contributes to the understanding of the type-specific capsule loci of *S. pneumoniae* serotype 3 and represents an initial step toward our understanding of not only the potential origins of pneumococcal serotypes but also the regulation of type 3 capsule. The major findings of this work include the following:

1. The type 3 capsule locus is distinguishable from the other type-specific loci which have been characterized by the presence of deletions, truncations, and duplicated elements. Analysis of the upstream region flanking the *cps3* locus identified sequences common to all capsule serotypes examined. Within the upstream region, *cps3C* and *cps3P* were identified and compared to their respective homologues from a second type 3 strain and pneumococcal serotypes 14 and 19F. *cps3C* was shown to be 98% identical to *cps14C* and *cps19fC*. Cps19fC has homology to the N-terminus of ExoP from *R. meliloti*. ExoP has been shown to be involved in the export and polymerization of succinoglycan and can be divided into two functional domains, with the N-terminal domain encoding the chain-length determination functions. The C-terminal end of ExoP was shown to contain a potential ATP/GTP nucleotide binding motif, but the function of this portion of the protein has not been determined. Analyses of mutants within the N-terminus of ExoP demonstrate that these strains produce predominantly the low molecular weight form over the high molecular weight form of the polysaccharide. Based on these findings and on the homology between ExoP and a family of proteins involved in O-antigen chain length determination, a similar function was suggested for ExoP in *R. meliloti* (Becker *et al.*, 1995). Although Cps19fC has homology to ExoP, its function has not been demonstrated. The next downstream gene, *cps3P*, was shown to be identical to homologous sequences, *cps14D* and *cps19fD*,

found in types 14 and 19F *S. pneumoniae*. *cps3P*, however, contains a 3' truncation when compared to these homologues. The functions of the Cps3P homologues in types 14 and 19f have not been demonstrated. Cps3P, Cps14D, and Cps19fD were shown to have homology to the C-terminal end of ExoP. These findings could suggest that the dual functions performed by the ExoP protein in *R. meliloti* are performed by two proteins in *S. pneumoniae*.

Two sequences, *orf5* and H-rpt, were also identified downstream of *cps3P*. *orf5* was shown to be duplicated on the chromosome of the type 3 strain WU2 but present in single copy on the chromosomes of types 2 and 6B. The duplicated or "unique" copy of *orf5* on the type 3 chromosome is that copy which is present upstream of the *cps3* locus. ORF5 does not exhibit homology to reported protein sequences contained in GenBank. The H-rpt sequence of *S. pneumoniae*, which exhibits homology to the IS-like H-rpt elements of *E. coli*, was shown to be present in multiple copies on the chromosomes of serotypes 2, 3, and 6B. The copy present upstream of the *cps3* locus represents what is most likely a partial copy of a larger sequence. Data from Northern blot analyses using a probe specific for the H-rpt of *S. pneumoniae* were able to detect a transcript of approximately 1.3 kb, suggesting the possible existence of a larger, and perhaps functional, copy of the H-rpt elsewhere on the chromosome.

Analysis of the *cps3* locus flanking region downstream of *cps3M* identified *tnpA* and *plpA*. Both sequences were shown to be common to all capsular serotypes examined. *tnpA* has homology with transposases from several bacterial IS elements. When compared to these transposases, TnpA is deleted at both the N- and C-termini and most likely represents only a partial copy of a larger protein. *tnpA* was shown to be present in single copy on the chromosomes of *S. pneumoniae* types 2, 3, and 6B. Downstream of *tnpA*, in the opposite orientation, is *plpA*. PlpA has homology to the substrate binding protein from a family of bacterial peptide permeases. The *plpA* from serotype 3 was shown to contain a 5' deletion when compared to the homologous gene from a serotype 2 strain. The type 3

plpA also contains a 1 bp deletion which causes a frameshift mutation early in the coding region of PlpA. With the exception of the 5' deletion, the region of *plpA* present within both type 2 and type 3 is essentially identical (98% nucleotide identity). The deletions within the *cps3M-tnpA-plpA* region were also present within five type 3 *S. pneumoniae* strains examined. Analysis of insertions within both upstream and downstream flanking sequences determined that none of these sequences are required for the production of type 3 capsule under laboratory conditions.

The cassette-recombination mechanism of capsule type switching is greatly supported by these studies. As demonstrated previously by Dillard *et al.* (1995), this form of recombination utilizes flanking sequences common to pneumococcal capsule types to exchange the linked type-specific capsule biosynthetic genes by homologous recombination. This mechanism, however, cannot be used to fully explain stable binary encapsulated transformants. As stated previously, this class of transformants was shown to contain type-specific genes at a site unlinked to the normal chromosomal locus. Previous studies on the then undefined region containing *orf5* and the H-rpt noted that when a fragment within this region was used to construct insertions in types 2 and 6B, the point of insertion was unlinked to the capsule type-specific locus. In the present work, it was determined that the fragment used to construct the types 2 and 6B insertions contained almost exclusively *orf5*. This sequence has now been shown to be present in single copy in types 2 and 6B and not closely linked to the *cps3* locus. The identification of sequences associated with the type 3-specific capsule locus that are duplicated on the chromosome (i.e., *tnpA*, H-rpt, and *orf5*) may provide a possible mechanism for the insertion of type-specific genes at an unlinked site to generate stable binary encapsulated strains.

The characterization of the unique genetic organization of the *cps3* locus has also allowed for speculation as to the origin of this capsule serotype. A similar form of genomic organization has been reported for the polysaccharide biosynthetic loci of other organisms, including *V. cholerae* and *S. enterica*. The polysaccharides produced by these organisms

are involved in the formation of O-antigens. Like the type-specific capsules of *S. pneumoniae*, these O-antigens serve as a source of antigenic variability for these organisms. The mechanism of generating genetic variability had until recently been purely speculative. The identification of sequences associated with recombination and rearrangements and the presence of deletions within these genomic structures have established what appears to be a common mechanism for the generation of genetic diversity within polysaccharide biosynthetic gene clusters. In contrast to the *S. pneumoniae* type 3 *cps* locus, the genetic origins of acquired sequences present in the novel O-antigen gene clusters of *S. enterica* and *V. cholerae* have been identified. In the case of *V. cholerae*, the strain expressing the novel O-antigen, O139, has been associated with the most recent pandemics of cholera, whereas all previous pandemics had been due to O1 strains. Although it is not clear what role the O-antigen plays in pathogenicity, the emergence of the O139 as a dominant serotype suggests that this strain posseses some selective advantage over the O1 serotype. At present, it is not clear whether the novel O-antigen structure of *S. enterica* and the type 3 *cps* locus have afforded these strains any selective advantage. The progenitor structure of the type 3 *cps* locus is not known and it would therefore be difficult to speculate as to what, if any, alterations in virulence may have resulted following the formation of this locus.

It is also tempting to speculate as to what, if any, forces are driving these rearrangements within the O-antigen and capsule loci. In each case, the gene clusters involved are responsible for the production of an antigenically-variable surface exposed structure. In the case of *S. enterica* and *V. cholerae*, the rearrangements within these loci have resulted in a novel antigenic structure. Although at present there is no direct experimental evidence, it would be interesting to explore the role that immune selection has played in this form of genetic evolution.

2. Transcription studies were performed using *cat* gene fusions and Northern blot analyses to determine the level of transcription throughout the *cps3* locus. Results from these studies suggest that the type 3-specific genes are contained on at least two transcriptional units, *cps3DSUM-tnpA-plpA* and *cps3M-tnpA-plpA*. *cps3M* was shown to be transcribed at the lowest level of the four type-specific genes. Transcriptional analyses of the upstream region suggest that the genes contained within this region are also transcribed at lower levels than the type 3-specific genes, *cps3D*, *cps3S*, and *cps3U*. Studies with type 19F suggest that the *cps19f* loci are co-transcribed. Results presented within this dissertation distinguish the type 3 locus from 19F as being composed of at least three transcriptional units: the two type 3-specific transcripts mentioned above and a third transcript containing one or more of the upstream genes.

The inability to obtain intact or detectable *cps3* mRNA later in exponential or in stationary phases and the relatively unstable nature of the *cps* mRNA detected in Northern blot analyses could suggest that the *cps* transcripts are regulated or particularly sensitive to processing or degradation. Northern analyses of a control transcript (*pspA*) did not reveal a dependence on growth phase and this transcript was shown to be stable, as compared to the *cps3* transcripts. The amount of capsule produced in type 3 strains as determined by ELISA is constant throughout the exponential phase but decreases during the later part of logarithmic growth and into stationary phase (Caimano and Yother, unpublished results). Recent studies with the *hasAB* operon, responsible for the production of hyaluronic acid capsule in group A streptococci, demonstrated that, as cultures entered stationary phase, the level of *hasAB* transcription was dramatically reduced (Crater and van der Rijn, 1995). This loss of *hasAB* expression is followed shortly by a loss of hyaluronic acid capsule. Although studies with type 3 may identify a similar growth related correlation between transcription and capsule production, these findings suggest that type 3 *S. pneumoniae* remain encapsulated in the absence of detectable *cps3* mRNA.

A potential stem-loop structure overlapping the translational start codon for Cps3U was detected within the *cps3S-cps3U* intergenic region. As stem-loop structures are often associated with forms of regulation, including both the enhancement and the reduction of mRNA stability, and translation attenuation control mechanisms, the identification of such a structure within the *cps3* locus may suggest a form of regulation. The level of transcription immediately upstream of the structure did not differ from the level detected downstream within *cps3U*. This finding, however, does not rule out other forms of regulation, such as attenuation. It is also possible that appropriate conditions under which the *cps3* transcript is regulated are not achieved when *S. pneumoniae* are grown on solid media or in laboratory culture.

3. Virulence studies using a series of insertions within and adjacent to the *cps3* locus demonstrated an essential role for *cps3D and cps3S* in the infection process. This finding was not unexpected as insertions within *cps3D* and *cps3S* result in a non-encapsulated phenotype. The studies presented within this dissertation are, however, the first demonstration of avirulence of *S. pneumoniae* resulting from site-directed insertions within a type 3-specific locus. The effects of insertions within the two downstream type-3 specific genes, *cps3U* and *cps3M*, were also examined in these studies. Strains containing insertions within either *cps3U* or *cps3M* exhibited virulence properties similar to that of the parent strain when given by either the intraperitoneal (i.p.) or intravenous (i.v.) routes of infection.

4. Two novel type 3 strains, JY1060 and MC1056, have been identified and shown to exhibit unique capsule phenotypes compared to the parent type 3 strain. The first strain, JY1060, as described in the introduction portion of this dissertation, produces a reduced amount of type 3 capsule. The normal capsule phenotype may be restored in this strain by transformation with DNA from serotype 2 *S. pneumoniae*, and the locus responsible for this repair was shown not to be closely linked to the capsule cassette locus. Transcription studies comparing this strain to the parent strain determined that JY1060 is reduced in the

expression of each of the type 3-specific genes. When used in virulence studies (Hardy and Yother, unpublished results), JY1060 was also shown to be reduced in virulence. While the region of the type 2 *S. pneumoniae* chromosome able to repair the defect in JY1060 has been localized to a unique restriction fragment, the locus responsible for this repair has not as yet been identified.

These observations could suggest, however, that the expression of the type 3 capsule is regulated at the transcriptional level by an unlinked factor or factors. Although this is only one of many possible explanations for the observations made with JY1060, examples of trans-activation of capsule genes have been reported for the *cps* genes of *E. coli*, *K. pneumoniae*, and *B. anthracis* (Stout and Gottesman, 1990; Wacharotayankun *et al.*, 1992; Wacharotayankun *et al.*, 1993; Vietri *et al.*, 1995). Of these, the regulation of the group 1 colonic acid capsule in *E. coli* is the best understood. In *E. coli* K-12, the expression of the group 1 capsule genes is enhanced by the action of two factors, RscA and RscB; RscB interacts with a third factor, RscC, to form a two-component regulatory element with RscC acting as the sensor (Stout and Gottesman, 1990). RscA is thought to interact with RscB to enhance transcription of the *E. coli* capsule genes. The low level constitutive production of capsule is due to the inactivation of RscA by the ATP-dependent Lon protease. Studies with *E. coli* group 2 serotypes have identified another positive regulatory factor, RfaH (Stevens *et al.*, 1994), and have demonstrated that RscA and RscB can have a negative regulatory effect on the synthesis group 2 capsule (Russo and Singh, 1993). Although homologues of these factors have not been identified in *S. pneumoniae*, it is not possible to exclude such a system of regulation from the realm of possibilities. Indeed, one could speculate as to whether the defect in JY1060 could be related to a RscA- or RscB-like protein with reduced transactivation ability. Or, in contrast, could this strain contain increased levels or increased activity of a Lon-like protease. It is also possible that the JY1060 *cps* transcript is regulated at the post-transcriptional level. The observation that this strain may be restored to a normal level of type 3 encapsulation by a type 2 DNA

unlinked to the *cps2* locus suggests that an understanding of the mechanism by which transcription is reduced in JY1060 may be applicable to heterologous capsule types.

In addition to reduced *cps* transcription, a second observation was made with JY1060. Although this strain is reduced in the total amount of capsule produced, it was shown to localize that capsule differently than the parent strain, WU2, when grown in liquid culture or on solid media. The capsule produced by WU2 is normally contained in equal amounts as cell-associated and within supernatant fluids. The type 3 mutant strain JY1060, however, retains approximately 70% of the total capsule material as cell-associated (Caimano and Yother, unpublished data). A better understanding of the factor(s) involved in the reduction of capsule in JY1060, along with these observations, could offer insight into the mechanism by which type 3 capsule export is regulated in *S. pneumoniae*.

In addition to JY1060, a second novel type 3 strain, MC1056, was described. This strain was originally constructed to examine the contribution of *cps3M* to virulence. Characterization of MC1056 in virulence assays, however, distinguished it from other encapsulated type 3 strains containing insertions within *cps3M* as being the only strain which resulted in a complete loss of virulence. This observation prompted us to propose that strain MC1056 contains a second mutation which is not closely linked to the *cps* locus. MC1056 produces a level of type 3 capsule which is indistinguishable from the parent strain and also from other encapsulated strains containing insertions within the *cps3* locus. A time course of blood clearance demonstrated that MC1056 was cleared from the blood of infected mice more rapidly than either the parent strain or a strain containing an identical insertion within *cps3M*. The mice infected with MC1056 went on to clear this strain from the blood and survive infection. Mice infected with the parent strain and the other *cps3M* insertions were unable to clear the bacteria and their infection resulted in death.

The requirement for capsule in virulence of *S. pneumoniae* has been well established. In our studies, site-directed insertions within the type 3-specific genes *cps3D* and

cps3S, which result in a loss of capsule, also resulted in a loss of virulence. Although no alteration of capsule was evident in studies used to characterize laboratory grown cultures of MC1056, the rapid clearance from blood and the loss of virulence of this strain in mice suggested that perhaps this strain was altered in capsule production within the *in vivo* environment. Capsule ELISA assays, performed on blood and without further growth in artificial media, demonstrated that samples from mice infected with MC1056 contained significantly higher levels of type 3 capsule per colony forming unit (cfu) compared to the parent strain or the strain containing an essentially identical insertion within *cps3M*. This increased level of capsule is apparently a result of exposure to the *in vivo* environment.

These observations with MC1056 are significant for several reasons. The most obvious is that one would expect that the loss in virulence associated with MC1056 would be accompanied by a reduction in capsule, in contrast to the increase in capsule observed. All non-encapsulated type 3 strains have been shown to be avirulent. Although several encapsulated strains, including those reported within this dissertation, have been shown to be reduced in virulence, MC1056 is the first type 3 encapsulated derivative of *S. pneumoniae* WU2 which is avirulent. Although the observations on MC1056 reported here are preliminary and further characterization of this strain will be required, this strain nonetheless has identified a potential differential regulation of the *cps3* locus within the *in vivo* and laboratory culture environments.

The identification of MC1056, an avirulent encapsulated derivative of *S. pneumoniae* strain WU2, and the observation that this strain is distinguished from the parent strain by the production of increased capsule per cfu leads one to speculate as to whether similar observations would be made when mice are infected with other avirulent serotype 3 strains. Although type 3 *S. pneumoniae* are often isolated clinically and the virulence of different type 3 strains has been well established, several type 3 strains have been isolated from human clinical samples which are avirulent in animal models (Briles *et al.*, 1992). These strains produce phenotypically normal type 3 capsules and have been shown, by

PCR-amplification and RFLP analyses, to contain a type 3 locus similar to the *S. pneumoniae* WU2 type 3 *cps* locus described within this dissertation. Nonetheless, these strains are avirulent at doses identical to those used in the studies involving MC1056. While only speculative, it is possible that an understanding of the factor(s) involved in the loss of virulence of MC1056 may also help to explain the lack of virulence associated with other encapsulated type 3 *S. pneumoniae* strains.

5. Studies have addressed the characterization of *cps3M*, the most downstream type 3 specific gene. While its predicted protein product was found to have homology to phosphomutases, sonicates from cultures of either *S. pneumoniae* or an *E. coli* strain over-expressing a recombinant form of Cps3M did not contain detectable activity in phosphomutase assays. The lack of phosphomutase activity associated with Cps3M was attributed to the deletion within the C-terminus. Insertions within *cps3M* did not eliminate the ability to produce type 3 capsule as judged by colony morphology and by ELISA immunoassay. This data is supported by carbohydrate studies which demonstrate that an insertionally-inactivated Cps3M strain did not differ significantly from the parent strain in assays for total carbohydrate. In addition, this gene is not required for virulence of type 3 *S. pneumoniae*.

In summary, the work presented within this dissertation has significantly advanced our understanding of the *S. pneumoniae* type 3-specific capsule. A better understanding of the 90 different serotypes of *S. pneumoniae* and their origins may help to elucidate the properties conferred by each. The work presented within this dissertation may hold clues as to the origins of at least type 3 and perhaps other *S. pneumoniae* serotypes as well. In addition, the identification of novel type 3 strains has allowed for the initiation of studies on the regulation of type 3-specific capsule both under laboratory growth conditions and in pathogenesis.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

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Major Subject Microbiology

Title of Dissertation Characterization Of Sequences Associated With The Type 3 Capsule Locus In Streptococcus pneumoniae

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Date 12/8/96

PS-1428