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Effects Of Specific Alterations In Capsule Structure On Streptococcus Pneumoniae Capsule Assembly And Virulence

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EFFECTS OF SPECIFIC ALTERATIONS IN CAPSULE STRUCTURE ON *STREPTOCOCCUS PNEUMONIAE* CAPSULE ASSEMBLY AND VIRULENCE

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MICROBIOLOGY

ABSTRACT

The polysaccharide capsules of *Streptococcus pneumoniae* represent the most important virulence determinant produced by this organism. Ninety-one different serotypes have been identified, but only a small number of these serotypes are responsible for most of the infections caused by *S. pneumoniae*. The reasons why certain serotypes predominate in infections over others are not well understood. One common serotype found in invasive diseases is type 2, however, it is unclear what characteristics of a type 2 capsule makes strains producing it virulent. The type 2 capsule consists of repeat units made of a backbone of Glc-Rha-Rha-Rha, with a side chain Glc-GlcUA linked to the terminal Rha. In type 2 *S. pneumoniae,* neither the role the capsular side chain Glc-UA has in capsule assembly nor the role it has in virulence is known. UDP-GlcUA is synthesized from the oxidation of UDP-Glc by the activity of *cps2K*, a type-specific gene encoded within the type 2 capsule locus containing homology to known UDP-Glc dehydrogenses. Deletion of *cps2K* should eliminate the production of UDP-GlcUA, allowing us to assess the effects of alteration of the side chain on both capsule synthesis and virulence. Deletion of *cps2K* resulted in the production of only low levels of high molecular weight polymer that were retained on the cell membrane but not transferred to the cell wall, demonstrating a requirement for GlcUA in this process. Repair of this mutation only partially restored capsule production, suggestive of secondary mutations selected for during isolation of Δ*cps2K* mutants. Linkage analyses

were used to localize the suppressor mutations to a region containing *cps2E*, the glycosytransferase responsible for the addition of Glc-1-P to a C_{55} polyprenol acceptor (Und-PP) to initiate repeat unit synthesis. Sequence analyses revealed that the original Δ*cps2K* mutants contained different point mutations in *cps2E,* all reducing Cps2E activity. Construction of multiple independent Δ*cps2K* mutants resulted in the same phenotype as the original and a selection for suppressor mutations, the majority of which were located in *cps2E*. Mutants that were reduced in capsule production due to either mutations in *cps2E* or the capsule promoter region, resulting in 0.1% and 60% of the parental levels of capsule, respectively, were unable to efficiently colonize or cause disease in murine models of pnuemococcal infection, demonstrating a requirement for full capsule production by type 2 for these processes. Deletion of *cps2H*, the repeat-unit polymerase, and *cps2J*, the repeat-unit flippase, also selected for suppressor mutations in *cps2E*. Collectively, the data presented here suggests a selection for suppressor mutations may have resulted from the sequestration of undecaprenyl-pyrophosphate due to retention of a repeat-unit on the cytoplasmic side of the cell membrane or an inability to transfer a single repeat-unit or altered polymer to the cell wall. Suppressor mutations which reduced capsule synthesis, such as those in *cps2E*, would help alleviate stress from accumulation of lipid-linked subunits and preclusion of lipid turnover for use in other cellular processes. Thus, in *S. pneumoniae* serotype 2, alterations to the capsule structure or assembly process prevent proper capsule synthesis by interfering with transfer to the cell wall and possibly other steps in the pathway. These alterations also result in reduced virulence of the bacteria, demonstrating a requirement for at least 60% of parental levels of capsule in type 2 to cause disease.

DEDICATION

I dedicate this dissertation to my parents, Davie and Bonnie, and to my husband, Nathan. Thank you for your love and support, and for standing by me. I am truly blessed and none of this would have been possible without you.

This dissertation is also dedicated to Gary Aubrey Styles, whose love of science inspired us all to learn and to question. I know you are looking down and watching over us. Thank you for love and generosity.

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INTRODUCTION

The gram-positive bacterium *Streptococcus pneumoniae* is one of the leading causes of worldwide morbidity and mortality (5, 17, 25, 58). It is one of the most common etiologic agents of pneumonia, otitis media, bacteremia, bacterial meningitis, and sinusitis (5, 17, 25, 58). Children under 2 years of age and the elderly over 60 years of age are the most prevalent groups affected by pneumococcal disease, followed by individuals who are immunocompromised (2, 6). In the United States, *S. pneumoniae* is the leading cause of bacterial pneumonia and the most common cause of community acquired pneumonia, resulting in an estimated 500,000 cases per year (5, 17, 25, 58). Of these cases, approximately 10% result in death. Annually, *S. pneumoniae* causes an estimated 50,000 cases of bacteremia and 3,500 cases of meningitis in the U.S. (5, 17, 25, 58). Although the number of incidences of bacteremia and meningitis are smaller than the number for pneumonia, the fatalities associated with each disease are much higher with an estimated >40% of the bacteremia cases and >55% of the meningitis cases resulting in death. In addition, *S. pneumoniae* is responsible for most recurring cases of middle ear infections (otitis media) in children, causing an estimated 7 million cases per year in the U.S. (25, 54). In the developing world, most cases of pneumonia occurring in children under the age of 5 years are due to *S. pneumoniae*, and at least 2 million of these cases result in deaths worldwide (17, 57).

Highlights of the pneumococcus

Historical Significance. *S. pneumoniae*, also referred to as the pneumococcus, is described as a "lancet shaped" diplococci that was first isolated in 1881 where the description of a substance referred to as "aureole" was observed surrounding the bacterium by light microscopy (60, 82). This "aureole" substance was later determined in 1925 to be what is currently known today as the polysaccharide capsule (8, 82). Many scientific discoveries using the pneumococcus have laid the foundation for techniques still in use today. In 1884, *S. pneumoniae* was the first bacterium designated as gram-positive by Gram who developed this method of staining to differentiate the pneumococcus from *Klebsiella pneumoniae*. Early studies on genetic exchange and transformation in bacteria were conducted in 1928 by Griffith using the pneumococcus, where it was found that live avirulent non-encapsulated pneumococci mixed with heat-killed encapsulated pneumococci could be converted to virulent encapsulated pneumococci during coinfection in mice (38, 82). These early studies by Griffith on transformation later laid the foundation for work done by Avery, MacLeod, and McCarty in their discovery that this "transforming principle" of genetic information was DNA in 1944 (9, 82). Other important discoveries using *S. pneumoniae* include the therapeutic use of penicillin, ability of capsules to induce antibodies and resist phagocytosis in addition to other immune response, and also the use of polysaccharide antigens in vaccines (2).

Pathogenesis of disease. *S. pneumoniae* can be considered a normal part of the human microflora. The nasopharynx serves as the main reservoir for *S. pneumoniae,*

where asymptomatic colonization is established and can occur shortly after birth (6, 17). Children may be colonized simultaneously with as many as four different capsular types, but the persistence of colonization varies with age and also with the serotype (2, 5, 17). In a healthy individual, asymptomatic colonization can occur for a period of a few weeks to a few months (21, 34, 52). However, acquisition of new serotypes during colonization can cause disease in susceptible individuals. Attachment of the pneumococcus in the nasopharynx is mediated by binding to fibronection on glycoconjugate receptors present on human pharyngeal epithelial cells (4, 72). Transmission of *S. pneumoniae* occurs through aerosolized droplets from infected individuals or, more commonly, from individuals carrying the pneumococci asymptomatically (17, 21). From the nasopharynx, the pneumococci can disseminate and cause disease (6, 21). Once dissemination occurs, the pneumococcus encounters numerous host defenses, but has evolved mechanisms to evade the host immune response. Two clinical manifestations can occur, disease caused by invasive infections that result in bacteremia and septicemia where the pneumococcus is found in the bloodstream, and also disease caused by mucosal infections that result in upper respiratory tract infections and pneumonia (21). The mechanisms that lead to the transition from asymptomatic carriage to systemic infection are not well understood.

Prevention of disease. Virulence of this organism is due mainly to the polysaccharide capsule present on its surface, which serves to prevent complement-mediated opsonophagocytosis (discussed in detail below) (1, 18, 81). Although complement does get deposited on the pneumococcal cell surface, the presence of capsule impedes binding of C3b receptors on phagocytes to surface-bound complement

components. Polysaccharide capsules are immunogenic and antibodies to capsules are effective in allowing deposition of complement on the cell surface, therefore increasing accessibility to phagocyte receptors (19). Over 91 different serotypes of capsule have been identified in *S. pneumoniae*, but only a few are responsible for most of the incidences of pneumococcal infections (43, 59, 82). The reasons why certain serotypes predominate in infections over other serotypes are unclear, but the genetic background has been shown to play a role (47). Therefore, the prevalence of a particular serotype in causing disease could be due to a specific structure, a specific residue, the genetic background, or possibly a combination of all three.

Treatment of pneumococcal infections has been increasingly difficult due to the emergence of antibiotic resistance among the bacteria (44, 63). It has been estimated that about ~30% of invasive strains of the pneumococcus isolated in the Unites States are penicillin resistant (44, 63). Polysaccharides are immunogenic, therefore, current prevention of pneumococcal disease is based on the use of polysaccharide-based vaccines, which are directed against the most prevalent serotypes affecting a target population (17, 25). In adults, serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 11A, 12F, 14, 18C, 19A, 19F, 22F, and 23F predominate in infections, but the most prevalent serotypes can vary among age groups and geographical location (17, 25, 65). Of these serotypes, 3, 7F, 9V, 14 and 23F occur most frequently, with types 3, 6B and 19F commonly found in fatal cases (43). In children, types 1, 4, 5, 6A, 6B, 9V, 14, 18C, 19A, 19F, and 23F are the most prevalent types found in invasive disease, with type 3 most commonly isolated from otitis media cases (17, 25).

A multivalent vaccine containing 23 of the most prevalent capsule serotypes found in infections is used for the elderly and those with underlying conditions. The 23 serotypes included in the vaccine are responsible for more than 90% of diseases caused by *S. pneumoniae* (17, 25). This vaccine includes serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C 19A, 19F, 20, 22F, 23F, and 33F. Conjugated protein-polysaccharide vaccines containing 7 to 11 serotypes, including serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, are used for infants and young children, who react poorly to polysaccharide antigens (25, 26, 29). In general, polysaccharides elicit mainly T-independent immune responses, which although are protective, lack strong memory responses (5, 51). Children under the age of two years do not mount adequate T-independent memory responses, therefore, conjugating polysaccharides of serotypes that are prevalent in diseases caused by *S. pneumoniae* in this target population to protein carriers increases the efficacy of the vaccines (17, 26). These vaccines, overall, have been demonstrated to reduce the number of those infected with invasive disease and otitis media, and also the numbers of asymptomatic carriage (17, 29). However, with the use of a vaccine developed to the more prevalent diseases, an increase in the incidence of other serotypes not included in the vaccine has been observed in some areas (35). Therefore, continued research on understanding the pathogenesis of *S. pneumoniae* is needed to develop more effective therapeutic treatments and vaccines for prevention of disease.

Accessory virulence and adherence factors. *S. pneumoniae* produces a number of different virulence factors, both surface-associated and nonsurface-associated. Some

of the main ones are briefly discussed below. Surface-associated virulence factors are attached via the cell membrane or the peptidoglycan cell wall, which is composed of glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked through peptide side chains. The peptidoglycan cell wall itself can serve as a virulence factor by inducing cytokines important for the inflammation process (IL-1 and TNF-α) and activating the alternative complement pathway (2).

The pneumococcus requires choline for growth (69). Choline can be incorporated into some surface polymers and structures such as teichoic acid, lipoteichoic acid, or be an anchor to attach choline-binding proteins (CBP) to the cell surface (69). Some examples of CBP implicated in virulence and/or adherence include autolysin (LytA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), CbpA, CbpD, CbpE, and CbpG (2, 69). Autolysin is an *N*-acetylmuramoyl-L-alanine amidase cell wall degrading enzyme, which under certain conditions, can be activated causing autolysis of the bacterial cell (56). This autolysis can result in the release of bacterial cell wall components and pneumolysin (discussed below) which can activate the inflammation process. PspA and PspC are each required for full virulence of the pnuemococcus in murine models of infection, but the mechanism of action of these two proteins are not fully understood. PspA has been demonstrated to inhibit complement activation and bind lactoferrin (2, 39, 71). PspC appears to bind numerous host proteins, and therefore may have a role in adhesion (64). It has been shown to bind the secretory component of immunoglobulin A, complement C3 component, and complement factor H (30, 40, 67). The remaining choline-binding proteins, CbpA, CbpD, CbpE, and CbpG have all been demonstrated to have roles in adherence and colonization (36, 56, 64).

In addition to surface structures containing choline, the pneumococcus also produces a number of different virulence factors containing LPXTG motifs to anchor them onto the cell surface, including hyaluronidase, neuraminidase, and serine protease PrtA (55). Hyaluronidase is secreted by 99% of clinical isolates and is involved in breakdown of hyaluronic acid of mammalian connective tissue and extracellular matrix, which may aid the bacterium in spreading and colonization (45, 55). Neuraminidase cleaves *N*-acetylneuraminic acid (sialic acid) from host proteins attached on cell surfaces (22, 70). This activity has been shown to have a role in colonization, adherence and the development of otitis media in chinchilla animal models (22, 70). The mechanism of the action of PrtA is unclear, but knock-out mutants have been shown to be reduced in virulence in intraperitoneal models of infection (16).

Some additional virulence factors produced by the pneumococcus include IgA1 protease, pneumolysin (PLY), and a more recently described pilus. *S. pneumoniae* secretes an IgA1 protease that may interfere with host defenses on mucosal surfaces by mediating attachment and adherence (2, 50, 55). PLY is a pore-forming toxin (55) that belongs to the family of thiol-activated toxins, which can lyse cholesterol-containing eukaryotic membranes. Disruption of *ply* results in mutants that are reduced in virulence (15). Release of PLY can trigger the production of proinflammatory mediators such as TNF-α, IL-1β, and IL-6, in addition to activating the classical complement pathway (2, 55). The mechanism of release of PLY is unknown, but cell lysis has been shown to not be required (10). A pilus islet that contains homology to pilus genes described in group A streptococci, group B streptococci and in *Corynebacterium diphtheriae* was discovered in *S. pneumoniae* TIRG4 strain in the *rlrA* pathogenicity island (11). A TIGR4 strain

containing pili was found to be enhanced in adherence, colonization and also in virulence when compared to mutants without pili (11).

The polysaccharide capsule of *Streptococcus pneumoniae*

Role of capsule in virulence. Although the pneumococcus produces many different virulence factors, the main virulence determinant produced by this organism is the polysaccharide capsule. As mentioned earlier, the importance of capsule in virulence was described as early as 1928 with transformation studies by Griffith using the pneumococcus, where it was observed that live avirulent non-encapsulated pneumococci mixed with heat-killed encapsulated pneumococci could be converted to virulent encapsulated pneumococci during coinfection in mice (38, 82). In 1931, a direct role for capsule in virulence was demonstrated in mouse infections where the enzymatic removal of the type 3 capsule resulted in avirulence (7, 82).

Regulation of capsule production in different environments is important for the survival of *S. pneumoniae* in the human host. Reduced capsule amounts may aid the bacterium in colonizing more efficiently by exposing important surface adhesins, whereas the presence of more capsule would protect bacteria in the bloodstream from the potent immune defenses mounted by the host. Animal models have helped in understanding the progression of pneumococcal disease, where similar to what is observed in humans, virulence is associated with capsular type. Generally, serotypes causing disease in human adults are virulent in mice, and serotypes inflicting mainly children colonize but do not cause invasive disease (82).

S. pneumoniae type 3 derivatives, containing defined mutations within the capsule locus or *pgm* (phosphoglucosemutase) resulting in production of only 20% of the parental levels of capsule, were avirulent in mouse models of an i.v. infection in immunologically normal (immunocompetent) mice (41, 53). However, in an i.p. model using immunocompetent mice, the type 3 reduced mutant containing a point mutation in *pgm* was avirulent, but the type 3 reduced mutant containing a mutation in the capsule locus was almost as virulent as the parent in this model (41, 53). It was suggested that the differences observed here in the i.p. model between the two mutants was possibly due to the former containing a mutation in *pgm*, which may affect other cellular pathways resulting in their reduced virulence (53). Differences in infection routes also reflected differences in virulence associated with the reduced capsule mutants. Although, some differences were observed in immunocompetent mice, both type 3 reduced capsule mutants were as virulent as their parent in immunocompromised mice, demonstrating this reduced amount of capsule was still sufficient to cause disease in this environment (41, 53).

S. pneumoniae serotype 2 mutants containing deletions of *cps2C* and *cps2D*, which respectively encode a membrane-associated activation domain and cytoplasmic-associated kinase domain of an autophosphorylating tyrosine kinase encoded within the capsule locus (discussed below), synthesize very short polymers of capsule (12, 13). Both mutants were avirulent in BALB/c mice in both i.p. and i.v. models of infection. A type 2 mutant containing a deletion of *cps2B*, a novel phosphatase and kinase inhibitor encoded within the capsule locus, which produces about 130% of the parental levels of capsule, was found to be avirulent in an i.v. model and but as virulent as the parent in an

i.p. model of infection in BALB/c mice (12). Mutants containing deletions in *cps2A*, which has homology to a transcriptional regulator in *Bacillus subtilis*, were reduced in capsule production and also were found to be essentially avirulent in both i.v. and i.p. models of infection (3). However, since proteins encoded by these genes are involved in forming a phosphoregulatory-relay system, it is possible that other factors involved in virulence are controlled and regulated by these proteins, and therefore are affected by their absence resulting in the observed reduced virulence.

Further evidence supporting the importance of capsule in virulence has been demonstrated by strains exhibiting phase variation, which has been described for some serotypes of *S. pneumoniae* (48, 75, 76). These phase variants are referred to as transparent and opaque, but are not known to contain defined mutations resulting in their observed phenotypes. However, it was determined that opaque variants of serotypes 6A, 6B and 18C contain 1.2- to 5.6– fold more capsule and 2.1- to 3.8 fold less surface-associated teichoic acid than their transparent variants (48). The type 2 D39 transparent variant was found only to contain more teichoic acid than the opaque variant, but the amount of capsule produced by the two variants was not quantified (48). In i.p. models of infection in mice, opaque phase variants of serotypes 2, 6A, and 18C were virulent, whereas all the transparent phase variants were attenuated in virulence.

Capsule switching studies have also been done to determine the roles of different capsular types and the genetic backgrounds in virulence. Here, it was found that replacing the capsule locus of a virulent type 2 strain, highly virulent type 5 strain, and avirulent type 6B strain with the capsule locus from virulent type 3 strain resulted in virulence that was unchanged, a mutant that was essentially avirulent, and an increase in

virulence in i.p. models of infection, respectively (47). Therefore, the above observations suggest that the amount of capsule produced by the bacteria, the capsular type, and also the genetic background are all important factors for the virulence of *S. pneumoniae*.

Role of capsule in colonization. The amount of capsule produced by *S. pneumoniae* has also been shown to be important for the ability of the pneumococcus to adhere and colonize the nasopharynx. There is a requirement for capsule in colonization, but theoretically, reduced amounts on the cell surface would allow the exposure of other surface adhesins involved in the adherence. The requirement for capsule in colonization is supported by studies using serotype 2 and 3 mutants containing defined mutations in the capsule locus and *pgm*, which reduce capsule synthesis (53). In these studies, it was found that serotype 3 mutants producing either no capsule or <0.6 % of the parental levels of capsule did not colonize the nasopharynx, as no bacteria were recovered from the nasal washes collected from mice infected with these mutants. However, mutants synthesizing 20% of the parental levels of capsule colonized as efficiently as their respective parents, demonstrating this amount was sufficient for colonization for serotype 3. The requirement for capsule in colonization was further demonstrated with a non-encapsulated derivative of type 2, and similar to what was observed for a non-encapsulated serotype 3 mutant, this type 2 mutant was unable to colonize. It was also found that *cps2C* and *cps2D* deletion mutants, which synthesize short chains of capsule polymer, were unable to colonize. *cps2B* deletion mutants, which produce about 130% of the parental levels of capsule, and *cps2A* deletion mutants, which synthesize about 50% of the parental levels of capsule, were also unable to colonize. As mentioned above with

regards to virulence, these genes encode proteins involved in a phosphoregulatory system and the deletion of these genes may be affecting other factors involved in colonization.

Phase variation has been shown to play a role in the ability to colonize. Although serotypes 2, 6A, 6B, 9V, and 18C were found to phase vary, only the phase variants of types 9V and 18C were tested for their ability to colonize the nasopharynx, where it was found that transparent variants colonized the nasopharynx of mice more effectively than their opaque counterparts (75). Collectively, these results suggest that not only is there a requirement for capsule in colonization, but also that the amount of capsule produced by the bacteria is an important factor in the ability to efficiently colonize. Other components, in addition to capsule, are involved in colonization and the regulation of these factors are important for this process.

Genetics of the capsule loci. To date, ninety one different serotypes of capsular polysaccharides have been identified in *S. pneumoniae*, but typically only one type is expressed by the bacterium (2, 14, 42, 72). The complete nucleotide sequences for the capsule loci of 90 serotypes have been published (14), and the structures for more than half of them have been determined (72). These polysaccharide capsules are structurally unique and diverse (Fig. 1A). Some capsules contain side chains, whereas others contain unique sugar residues (72). Structurally, some of the capsules can be complex, consisting of multiple different sugars and branches, whereas others can be more simple consisting of only two sugars linked in a repeating unit (Fig 1A).

The capsule loci of almost all serotypes are organized in a similar cassette-like arrangement, where the genes essential for the biosynthesis of type-specific sugars unique

A.

Type	Repeating Unit
2	-3)-α-L-Rha*p*-(1,3)-α-L-Rha*p*-(1,3)-β-L-Rha*p*-(1,4)-β-D-Glc*p*-(1- 2 | 1 α-D-GlcUA*p*-(1,6)-α-D-Glc*p*
3	-3)-β-D-GlcUA-(1,4)-β-D-Glc*p*-(1-
19B	β-D-Glc 1 | 6 4)-β-D-ManNAc-(1,4)-α-L-Rha-(1-PO_4^-,4)-β-D-ManNAc-(1,4)-α-D-Glc*p*-(1- 3 | 1 β-D-Rib-(1,4)-α-L-Rha

B.

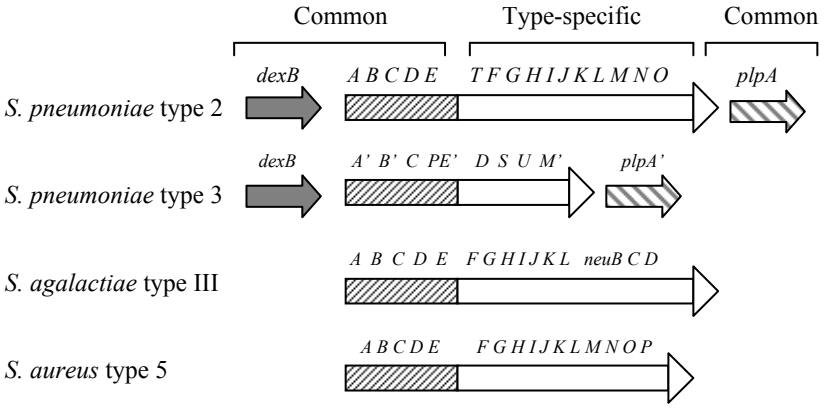

FIG. 1. Capsule structures and genetic loci. A) Structures of repeat units in *S. pneumoniae* types 2, 3, and 19B. Initiation of synthesis in most serotypes begins with the addition of Glc-1-P to a polyprenol lipid acceptor. B) Genetic organization of the capsule loci of *S. pneumoniae* types 2 and 3, *S. agalactiae* type III, and *S. aureus* type 5. *S. pneumoniae* capsule loci are flanked by *dexB* upstream and *plpA* (also referred to as *aliA*) downstream. Arrows indicates putative transcripts of each capsule locus.

to a specific serotype are flanked by homologous sequences found in all of the serotypes (Fig. 1B). This arrangement is similar to the organization of capsule loci found in many other bacteria, including *Streptococcus agalactiae* (Group B streptococcus), *Staphylococcus aureus*, *Lactococcus lactis*, and Group 1 capsules of *Escherichia coli* (Fig. 1B) (14, 28, 49, 79, 82). In *S. pneumoniae, dexB* lies upstream of the capsule ocus and *plpA* lies downstream. Neither has been shown to have a role in capsule synthesis. *S. pneumoniae* is naturally competent for genetic exchange, therefore the homologous sequences flanking the type-specific genes of the capsule locus can allow for homologous recombination to occur between the serotypes, which could result in the production of new serotypes (31, 32, 47).

The capsule locus can be divided into two regions, an upstream common region followed by a type-specific region (Fig 1B). The type 2 capsule locus (used as a model system in these studies) has been sequenced and the genes have been assigned putative roles based on homology, however the functions of most of the genes have not been experimentally determined. The upstream common region may be found in most or all of the serotypes, and genes located here encode proteins that form a phosphoregulatory system that are involved in initiation, chain length regulation and modulation. For serotype 2, the genes located here are *cps2A*, *cps2B*, *cps2C*, *cps2D*, and *cps2E*. The type-specific region contains genes that encode the enzymes involved in synthesizing the unique sugars specific to each serotype. For type 2, these include a UDP-Glucose (Glc) dehydrogenase (Cps2K for synthesizing UDP-GlcUA), repeat-unit polymerase (Cps2H), repeat-unit flippase (Cps2J), glycosyltransferases (Cps2T, Cps2F, Cps2I, Cps2G) and genes for the synthesis TDP-Rhamnose (Rha) (Cps2LMNO). The genes in this region

are expected to be transcribed as an operon from a putative promoter located just upstream of *cps2A* (46).

Mechanisms of capsule synthesis. There are three main mechanisms for synthesis of capsules and exopolysaccharides that have been described for bacteria, Wzy-dependent, synthase-dependent, and ABC-transporter dependent. Wzy-dependent and synthase-dependent mechanisms have been identified in both gram-positive and gram-negative bacteria, but the ABC-transporter dependent mechanism has only been described in gram-negative bacteria. Synthesis of most capsules in *S. pneumoniae* (89 of the 91) is proposed to occur by a Wzy-dependent mechanism, initially described for the Wzy polymerase used in O-antigen synthesis in *Salmonella enterica* (79, 80). This mechanism of synthesis has also been described for Group 1 and 4 capsules in *E. coli* (79). The serotype 2 capsule of *S. pneumoniae* consists of a singly branched hexasaccharide repeating unit (Fig. 1A) (46, 72, 79). This repeat unit contains a backbone of Glc-Rha-Rha-Rha, with a side chain of Glc-GlcUA linked to the terminal Rha. Based on homology of Cps2H to Wzy, synthesis of the type 2 capsule is proposed to occur by the same process (46). In this mechanism, synthesis begins with a pool of nucleotide precursor sugars in the cytoplasm (Fig. 2). Most of these activated sugars are shared in other cellular pathways such as for synthesis of peptidoglycan, teichoic acid, and lipoteichoic acid. Enzymes required for synthesis of these activated sugars are located outside the capsule locus [for example, phosphoglucomutase (PGM), which converts Glc-6-P to Glc-1-P, and Glc-1-P uridlyltransferase (GalU) which converts Glc to UDP-Glc]. Once a sugar (usually glucose for *S. pneumoniae*) has been activated, it is

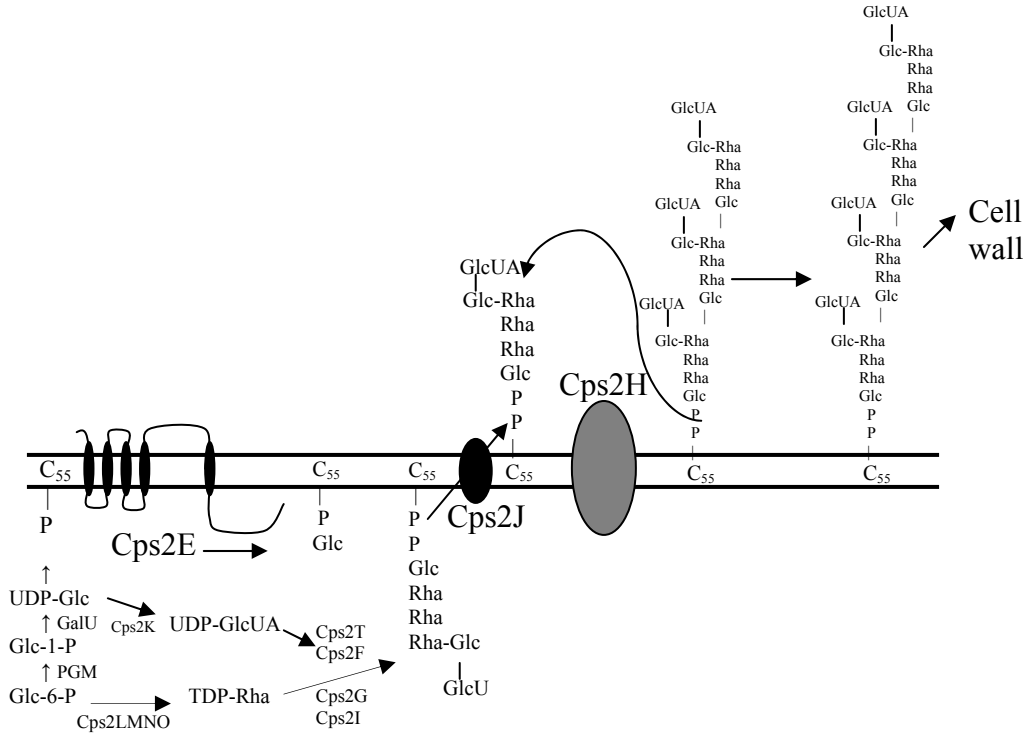

FIG. 2. Wzy-dependent mechanism of capsule synthesis in type 2 *S. pneumoniae*. This mechanism of synthesis was initially described for the Wzy polymerase used in O-antigen synthesis in *S. enterica* and most capsules of *S. pneumoniae* are proposed to occur by this mechanism.

transferred onto a lipid precursor on the cell membrane by the activity of the initiating glycosyltransferase. In serotype 2, Glc-1-P is added onto a C_{55} polyprenyl-phosphate (Und-P) by the activity of Cps2E. This is the same lipid used to initiate peptidoglycan and teichoic acid synthesis (24). Initiation is then followed by addition of the remaining sugars by the glycosyltransferases (Cps2T, Cps2F, Cps2I, and Cps2G). This repeat unit is subsequently flipped to the outside of the membrane, presumably by Cps2J, where it is then polymerized into high molecular weight polysaccharide by the activity of Cps2H. Growth of the polymer occurs at the reducing end by the addition of a preformed polymer on the membrane to the newly formed repeat unit (62). The lipid is then recycled back into the cell to be utilized for other cellular processes. The polymer can then be translocated and linked covalently to the peptidoglycan cell wall (68). Transfer of the polymer to the cell wall is independent of the polymer size (12). The enzyme(s) involved in transfer to the cell wall are not known in any gram-positive system.

Regulation of chain length occurs through the activity of proteins encoded by upstream common genes. Cps2C and Cps2D form membrane-associated activation domain and cytoplasmic-associated kinase domain of an autophosphorylating tyrosine kinase, which was determined to positively influence capsule chain length (12). Cps2B is a phosphotyrosine phosphatase and kinase inhibitor shown to affect the levels of Cps2D phosphorylation (12, 13). Cps2A is a membrane protein containing homology to lytR, a transcriptional regulator in *Bacillus subtilis*. A defined role for Cps2A in capsule regulation and synthesis has not been determined, but mutations in *cps2A* have been demonstrated to reduce capsule amounts (3).

Role of capsule side chain in capsule assembly and virulencce. Little is known about the importance of polysaccharide side chains to capsule assembly, or how side chains contribute to virulence of a bacterium. It has been demonstrated in *Streptococcus agalactiae* (Group B streptococcus) that mutants which fail to make the terminal sialic acid of the type III capsule side chain, due to mutation of either the CMP-sialic acid synthetase or the sialyltransferase, continue to produce an apparently normal capsule (27, 77). However, the amount of capsule produced by the mutants was greatly reduced, demonstrating a requirement for the side chain sialic acid for full capsule production. In the synthesis of *Salmonella typhimurium* LPS O-antigens, polymerization of subunits lacking an abequose branch was not observed *in vivo*, although synthesis of repeat units lacking the side chain abequose branch could be synthesized *in vitro*, further demonstrating a requirement for side chains for proper capsule synthesis (83). The serotype 2 capsule of *S. pneumoniae* does contain GlcUA incorporated into its side chain. However, the effects on capsule assembly and virulence from deletion of a side chain are not known in *S. pneumoniae*.

Role of UDP-Glucose dehydrogenases in capsule synthesis. As mentioned above, type 2 *S. pneumoniae*, contains GlcUA incorporated as part of the side chain of the capsule repeat unit (Fig. 1A). The type-specific gene *cps2K* is predicted to encode a UDP-glucose dehydrogenase (UDP-GlcDH) (46), which catalyzes the NAD^+-dependent oxidation of UDP- Glc to UDP-glucuronic acid (UDP-GlcUA) (Fig. 3A). Cps2K contains the same strictly conserved active site signature sequence of GGXCXXXD, as well as extensive homology to the signature NAD^+- and UDP-sugar binding domains,

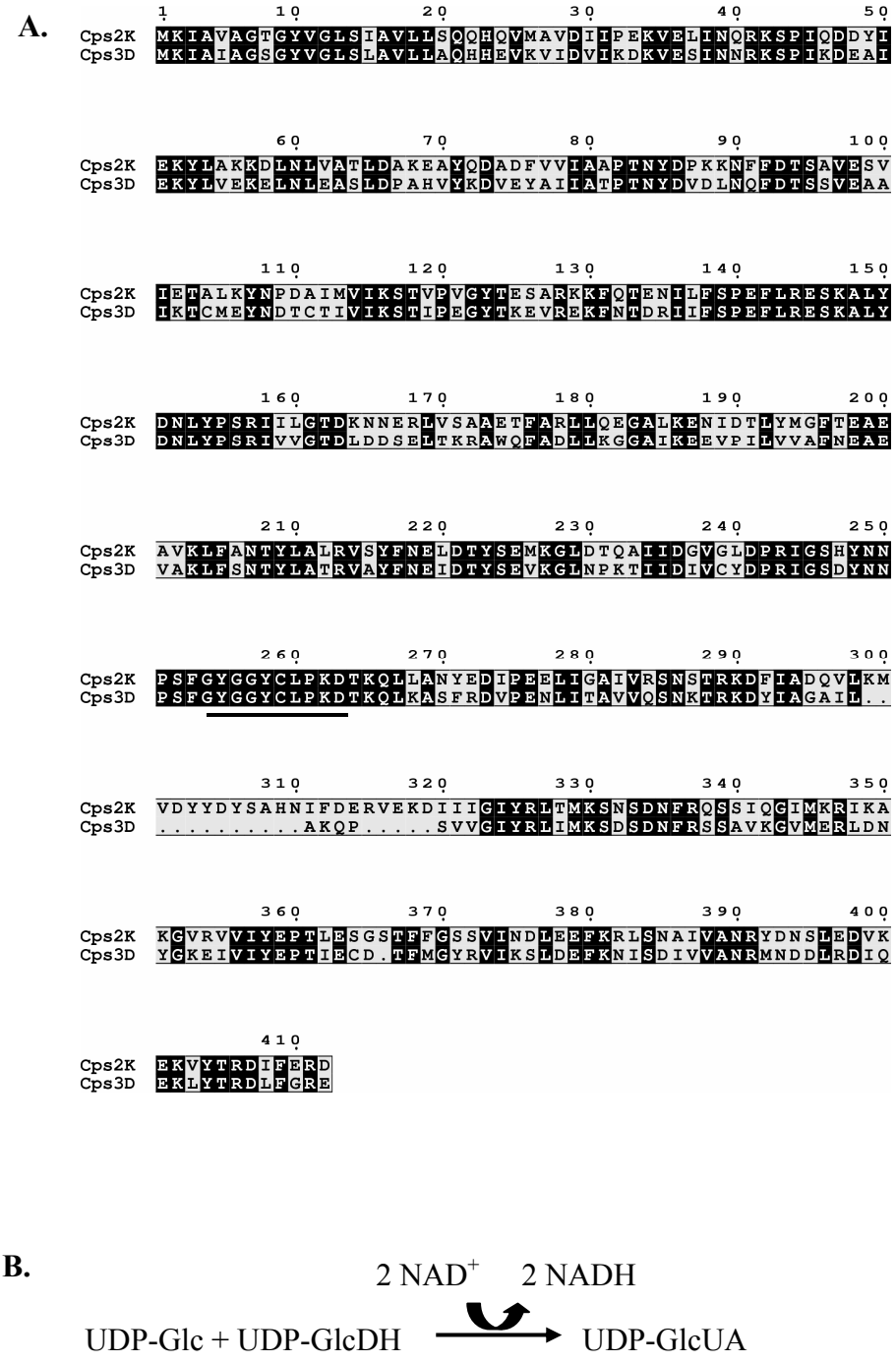

FIG. 3. UDP-Glc dehydrogenase of type 2 *S. pneumoniae*. A) Alignment of Cps2K, the UDP-GlcDH encoded within the type 2 capsule locus, to Cps3D, the UDP-GlcDH encoded within the type 3 capsule locus. Signature GGXCXXXD sequence found in the active site of UDP-GlcDHs is underlined. B) Reaction of the oxidation of UDP-Glc to UDP-GlcUA. For every mole of UDP-Glc that is oxidized to UDP-GlcUA, 2 moles of NAD^+ (nicotinamide) are reduced to NADH.

found in other UDP-GlcDHs (Fig 3B) (23). In higher species of eukaryotes, UDP-GlcUA serves as a substrate for the formation of hyaluron and glycosaminoglycans found in chondroitin sulfate and heparin sulfate, which are important components of synovial fluid, connective tissue and cartilage. UDP-Glc dehydrogenases also play critical roles in the formation of many bacterial capsules, including those of *Streptococcus pyogenes* (33, 78), *Escherichia coli* K5 (66), *Cryptococcus neoformans* (37), and many *S. pneumoniae* serotypes (72). In many of these polymers, GlcUA is part of the backbone structure, and for capsules such as type 3 in *S. pneumoniae*, mutations affecting the synthesis of UDP-GlcUA have severe effects on polysaccharide production and virulence (31, 73). Less is known about the effects of eliminating only GlcUA or other sugars from the side chains of bacterial capsules. The *C. neoformans* capsule contains a side chain of GlcUA and xylose, and in mutants lacking UDP-Glc DH activity, capsule production appears to be completely eliminated (37). However, it is not known what role GlcUA plays in capsule synthesis and virulence of *S. pneumoniae* serotype 2.

Aims of dissertation. *S. pneumoniae* serotype 2 is an invasive strain and is one of the types included in the 23-polyvalent vaccine. Little is known about the specificities of how the structure of a serotype 2 capsule contributes to virulence, nor have the genes involved in synthesizing the type 2 capsule been experimentally determined. The goals of this dissertation are to begin addressing the roles and functions of certain serotype 2 type-specific genes involved in capsule synthesis, and assessing the effects specific alterations to the capsule structure have on capsule assembly and virulence of *S. pneumoniae* in murine models of pneumoccocal infection.

MUTATIONS BLOCKING SIDE CHAIN ASSEMBLY, POLYMERIZATION, OR TRANSPORT OF A WZY-DEPENDENT *STREPTOCOCCUS PNEUMONIAE* CAPSULE ARE LETHAL IN THE ABSENCE OF SUPPRESSOR MUTATIONS AND CAN AFFECT POLYMER TRANSFER TO THE CELL WALL

by

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ABSTRACT

Extracellular polysaccharides of many bacteria are synthesized by the Wzy polymerase-dependent mechanism, where long chain polymers are assembled from undecaprenyl-phosphate-linked repeat units on the outer face of the cytoplasmic membrane. In gram-positive bacteria, Wzy-dependent capsules remain largely cell-associated via membrane- and peptidoglycan-linkages. Like many Wzy-dependent capsules, the *Streptococcus pneumoniae* serotype 2 capsule is branched. In this study, we found that deletions of *cps2K, cps2J,* or *cps2H*, which encode a UDP-glucose dehydrogenase necessary for side chain synthesis, the putative Wzx transporter (flippase), and the putative Wzy polymerase, respectively, were obtained only in the presence of suppressor mutations. Most of the suppressor mutations were in *cps2E*, which encodes the initiating glycosyltransferase for capsule synthesis. The *cps2K* mutants containing the suppressor mutations produced low levels of high molecular weight polymer that was detected only on membrane fractions. *cps2K*-repaired mutants exhibited only modest increases in capsule production due to the effect of the secondary mutation but capsule was detectable on both membrane and cell wall fractions. Lethality of the *cps2K, cps2J*, and *cps2H* mutations was likely due to sequestration of undecaprenyl-phosphate in the capsule pathway and either preclusion of its turnover for utilization in essential pathways or destabilization of the membrane due to an accumulation of lipid-linked intermediates. The results demonstrate that proper polymer assembly requires not only a functional transporter and polymerase but also complete repeat units. A central role for the initiating glycosyltransferase in controlling capsule synthesis is also suggested.

INTRODUCTION

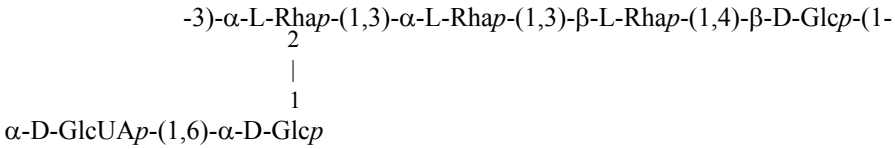
The capsular polysaccharides of *Streptococcus pneumoniae* are essential for virulence of this organism. In systemic infections, such as pneumonia and bacteremia, high levels of capsule are necessary to impede complement-mediated opsonophagocytosis (1, 31, 69), whereas in colonization, reduced amounts of capsule may be sufficient, as surface adhesins must be exposed (20, 39, 51, 59, 63). The ninety described *S. pneumoniae* serotypes vary in their sugar compositions, linkages, and branching patterns (8, 32, 57). Most *S. pneumoniae* capsules consist of repeating subunits that are synthesized by the Wzy-dependent mechanism, which is also used to synthesize capsules and exopolysaccharides in many other streptococci, lactococci, and staphylococci, as well as gram-negative bacteria expressing group 1 capsules and LPS O-antigens (8, 18, 36, 48, 67, 71). In this mechanism, repeat unit synthesis is initiated by transfer of a sugar-phosphate to a lipid acceptor on the cytoplasmic face of the membrane, with subsequent addition of the remaining sugars to complete the subunit. In most *S. pneumoniae* serotypes, CpsE homologues catalyze the initiation step by transferring Glc-1-P to a polyprenol acceptor (15, 37, 47, 60), while unique glycosyltransferases catalyze each subsequent monosaccharide addition. The final subunit is translocated across the cytoplasmic membrane by a Wzx flippase, and the Wzy polymerase then links the repeat units into long chain polymers, with growth occurring at the reducing end of the polysaccharide (50). In gram-negative bacteria, the capsule is ultimately transported and linked to the outer face of the outer membrane (67). In gram-positive bacteria, some or all of the polymer may be linked to the peptidoglycan (6, 17, 21, 54, 65) with the remainder being membrane-associated (6). Modulation of capsule

chain length and amount occurs, at least in part, through the action of a phosphoregulatory system that includes an autophosphorylating tyrosine kinase (6, 7, 44-46, 70). In *S. pneumoniae*, CpsC and CpsD represent the membrane-associated activation domain and cytoplasmic-associated ATP-ase domain, respectively, of this kinase. CpsB is a phosphotyrosine phosphatase and kinase inhibitor that affects the level of CpsD phosphorylation (7, 41).

Although a general picture for capsule synthesis in gram-positive bacteria has emerged, much remains to be learned about specific aspects of this process. As a model system, we have used the *S. pneumoniae* serotype 2 capsule, in which the repeat unit contains a backbone of Glc-Rha-Rha-Rha and a side chain of Glc-GlcUA (Fig. 1A). As for all *S. pneumoniae* capsules assembled by the Wzy-dependent mechanism, the type 2 genetic locus exhibits a cassette-like arrangement, where genes unique to a specific serotype and essential for the biosynthesis of type-specific sugars, polymerases and transporters are flanked by homologous sequences common to all serotypes (Fig. 1B) (3, 23, 30, 34). Putative roles for the type 2-specific genes have been assigned based on homology (34), but their functions have not been experimentally determined.

The type-specific gene *cps2K* is predicted to encode a UDP-glucose dehydrogenase (UDP-GlcDH) (34), which catalyzes the NAD^+-dependent oxidation of UDP- Glc to UDP-glucuronic acid (UDP-GlcUA). Cps2K contains the same strictly conserved active site signature sequence of GGXCXXXD, as well as extensive homology to the signature NAD^+- and UDP-sugar binding domains, found in other UDP-GlcDHs (14). UDP-Glc dehydrogenases play critical roles in the formation of many microbial

A.



B.

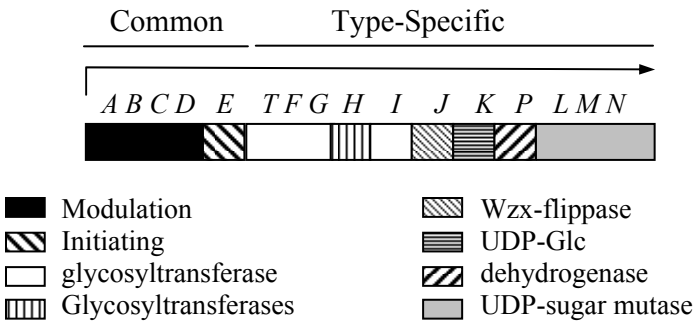


FIG. 1. The type 2 capsule structure and genetic locus. A) Structure of the repeat unit. Synthesis of the backbone initiates by addition of Glc-1-P to a polyprenol acceptor. B) Genetic organization of the type 2 capsule locus (34). *S. pneumoniae* capsule loci are flanked by *dexB* upstream and *aliA* (also referred to as *plpA*) downstream. Arrow indicates the putative transcript containing *cps2A-cps2O*.

capsules, including those of *Streptococcus pyogenes* (25, 66), *Escherichia coli* K5 (52), *Cryptococcus neoformans* (29), and many *S. pneumoniae* serotypes (57), as well as mammalian polymers such as hyaluronan, chondroitin sulfate, and heparan sulfate. In many of these polymers, GlcUA is part of the backbone structure, and for capsules such as type 3 in *S. pneumoniae*, mutations affecting the synthesis of UDP-GlcUA have severe effects on polysaccharide production and virulence (22, 31, 61). Less is known about the effects of eliminating GlcUA or other sugars from the side chains of microbial capsules. The *C. neoformans* capsule contains a side chain of GlcUA and xylose, both of which are derived from UDP-GlcUA. In mutants lacking UDP-Glc DH activity, capsule production appears to be completely eliminated (29). In *Streptococcus agalactiae* (Group B streptococcus), mutants that fail to make the terminal sialic acid of the type III capsule side chain, due to mutation of either the CMP-sialic acid synthetase or the sialyltransferase, continue to produce an apparently normal capsule, albeit at greatly reduced levels (17, 65).

In the present study, we examined the role of the side chain, and specifically the terminal GlcUA residue, in production of the *S. pneumoniae* type 2 capsule. Our results demonstrate that this residue is essential for proper assembly and processing of the capsule, and the inability to synthesize or process a complete repeat unit is detrimental to the cell, due at least in part to failure to transfer the polymer to the cell wall.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Strains and plasmids used are listed in Table 1. *S. pneumoniae* strains were grown at 37^{o}C in THY (Todd-Hewitt

TABLE 1. Strains and plasmids used in this study

Strain(s) or plasmid(s)	Properties[a]	Reference or source
S. pneumoniae		
AM1000	Δ(*cps2A* to *cps2H*), type 2 Cps^-	(39)
BX505	pBX110 x D39, Δ*cps2H*, Cps^-; $cps2E^{5765delT}$ (L244* premature stop)	This study
BX511	pBX113 x D39, Δ*cps2K*, Cps^r; $cps2E^{5953G\rightarrow T}$ (G303V)	This study
BX512	pBX113 x D39, Δ*cps2K*, Cps^r; $cps2E^{5920G\rightarrow A}$ (G292R)	This study
BX513	pBX116 x BX511, *cps3D* repair of Δ*cps2K*, Cps^r	This study
BX515	pBX115 x BX511, *cps2K* repair, Cps^r	This study
BX516	pJD377 x D39, Em marker insertion downstream of capsule locus, Cps^+	This study
BX518	pBX115 x BX512, *cps2K* repair, Cps^r	This study
BX519	BX516 x BX511, Em^R; capsule replacement in BX511, Cps^+	This study
BX522	BX516 x BX512, Em^R; capsule replacement in BX511, Cps^+	
BX532, BX539	pBX113 x D39, independent Δ*cps2K* derivatives, Cps^r; $cps2E^{6132C\rightarrow A}$ (P363T)	This study
BX533	pBX113 x D39, Δ*cps2K*, Cps^r; $cps2E^{6319A\rightarrow G}$ (D425G)	This study
BX535	pBX123 x D39, Em insertion upstream of capsule locus promoter, Cps^+	This study
BX540	pBX115 x BX533, *cps2K* repair, Cps^r	This study
BX544	pBX145 x BX518, *cps2E* repair in BX518, Cps^+	This study
BX545	pBX145 x BX515, *cps2E* repair in BX515, Cps^+	This study
BX547, BX548, BX549	pBX113 x D39, Δ*cps2K*, Cps^r; independent derivatives with respective *cps2E* mutations 5544G→T (D167Y), 6350G→T (W435C), and 6015delA (L369* premature stop)	This study
BX550	pBX113 x D39, Δ*cps2K*, Cps^r; unknown suppressor mutation	This study
BX551	pBX113 x D39, Δ*cps2K*, Cps^r; A to G transition 4 base pairs downstream of -10 sequence of capsule promoter	This study
BX552	pBX110 x D39, Δ*cps2H*, Cps^-; $cps2E^{5539C\rightarrow T}$ (L199F)	This study
BX554, BX555	pBX113 x D39, Δ*cps2K*, Cps^r; independent derivatives with respective *cps2E* mutations 6198A→G (E385G) and 6201T→C (Y386H)	This study

TABLE 1. (Continued)

Strain(s) or plasmid(s)	Properties[a]	Reference or source
BX556	pBX113 x D39, Δ*cps2K*, Cps^r; 1-kb vector insertion in *cps2L* region	This study
BX605-BX607, BX609-BX612	pBX113 x D39, Δ*cps2K*, Cps^r; independent derivatives with respective *cps2E* mutations 5632T→G (V196G), 5607insT (E191* premature stop), 6178C→G (T378R), 5984G→C (K312N), 6178C→G (T378R), 6349G→A (W407*stop), and 5539T→G (V165G)	This study
BX635	pBX113 x D39, Δ*cps2K*, Cps^r; $cps2E^{6060T\rightarrow C}$ (F339L)	This study
BX667-BX669	pBX190 x D39, Δ*cps2J*, Cps^-; independent derivatives with respective *cps2E* mutations 6276G→A (G411R), 5839T→G (I265S), and 6157G→C (G371A)	This study
D39	Type 2 parent strain, Cps^+	(5)
KA1521	Δ*cps2E*, type 2, Cps^-	(15)
WU2	Type 3 parent strain, Cps^+	(11)
E. coli		
BX163	M15 (pREP4, pBX163)	This study
BX165	M15 (pREP4, pBX165)	This study
DH5αF'	F'ø80*lacZ*ΔM15Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsd R17*($r_K^-m_K^+$) *phoA supE44* λ^- *thi*-1 *gyrA96 relA1*	Life Technologies, Inc.
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ø80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
M15 [pREP4]	F^-, Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+	Qiagen
Plasmids		
pCR 2.1 TOPO	PCR cloning vector; Ap^r, Km^r	Invitrogen
pJY4164	*S. pneumoniae* suicide vector; Em^r	(72)
pJD377	pJY4164:: type 3 *plpA/tnpA* region	(23)
pBX105	pJY4164 plus PCR fragments from primer pairs cps2-G0/cps2-G2 and cps2-I2/cps2-I3, for *cps2H* deletion	This study
pBX108	pJY4164 plus PCR fragments from primer pairs cps2-J12303/cps2-J2 and cps2-P1/cps2-P15468, for *cps2K* deletion	This study
pBX110	Km-resistance gene, *aphA-3*, between 2 fragments of pBX105	This study

TABLE 1. (Continued)

Strain(s) or plasmid(s)	Properties[a]	Reference or source
pBX113	Km-resistance gene, *aphA-3*, between 2 fragments of pBX108	This study
pBX115, pBX121	pJY4164 plus PCR fragment from primer pairs cps2-J1/cps2-P2, for repair of *cps2K* deletion	This study
pBX116	pJY4164 plus PCR fragment from primer pairs cps3D-F/cps3D-R, for *cps3D* repair of *cps2K* deletion	This study
pBX123	pJY4164 plus 900 bp fragment excised with *Eco*RI from pCV646	This study
pBX145	pJY4164 plus PCR fragment from primer pairs cps2-D3/cps2-T1, for repair of *cps2E*	This study
pBX163	pQE-40 containing the full length *cps2K* lacking GTG start; from primer pairs Cps2KORF-*Bgl*II/Cps2KORF-*Kpn*I	This study
pBX165	pQE-40 lacking DFHR region (*Bam*HI - *Kpn*I, deletion)	This study
pBX190	pJY4164 plus PCR fragments from primer pairs cps2-I4/cps2-J8 and cps2-J7/cps2-K1 for deletion of *cps2K*; *aphA-3* excised from pBX113 and inserted between 2 fragments	This study
pCV646	pGEM cloning vector plus PCR fragment from c-ups1/c-ups2, Ap^R	This study
pQE40	Expression vector, N-terminal 6xHis tag, Km^R	Qiagen
pSF151	streptococcal shuttle vector containing the Km-resistance gene, *aphA-3*	(56)

[a] Cps^r, reduced capsule levels. *cps2E* superscripts indicate mutations and their locations based on GenBank accession no. AF026471. Amino acid changes are indicated in parentheses. del, deletion; ins, insertion; →, nucleotide change.

broth supplemented with 0.5% yeast extract (Difco)), on BBL plates (Difco), or on blood agar plates (Blood agar base #2, Remel) containing 3% defibrinated sheep blood (Colorado Serum Company). Broth cultures were grown standing in a water bath or incubator; agar plate cultures were incubated in candle jars. *E. coli* DH5αF', TOP10, and M15(pREP4) were grown in L-broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 1 g/L glucose) or L-agar (L-broth containing 15 g/L Bacto agar). Media were

supplemented with the following antibiotics when appropriate: erythromycin (0.3 μg/ml for *S. pneumoniae* and 300 μg/ml for *E. coli*), ampicillin (100 μg/ml), or kanamycin (250 μg/ml for *S. pneumoniae* and 50 μg/ml for *E. coli*).

Expression and purification of Cps2K. The ORF of *cps2K*, minus the ribosome binding site and GTG start codon, was PCR amplified from *S. pneumoniae* D39 chromosomal DNA, using the primer pairs cps2K-*Bgl*II and cps2K-*Kpn*I, which incorporate a *Bg*lII site at the 5' end of the PCR product and a *Kpn*I site at the 3' end, respectively. The fragment was cloned into the expression vector pQE-40 in which the dihydrofolate reductase (DHFR) region between the 6xHis tag and the mutlicloning site was excised using *Bam*HI and *Kpn*I. The resulting plasmid, pBX163, was electroporated into the *E. coli* expression strain M15 (pREP4). Transformants were selected on L-agar containing both kanamycin and ampicillin.

For expression of recombinant Cps2K, a 100 ml culture of BX163 was grown from an overnight culture diluted 1:100 in LB containing the appropriate antibiotics at 37^{o}C with shaking to a cell density of ~2 x 10^{8} CFU/ml. Expression of *cps2K* was induced with isopropyl thio-β-galactoside (IPTG, 0.8 mM final concentration) for 4 hours at 37^{o}C. Cultures were centrifuged at 20,000 x *g* for 10 min and the pellet was stored overnight at -80^{o}C until further purification. The pellet was resuspended in 4 ml of a phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 1 mM dithiothreitol [DTT], and 1mM phenylmethylsulfonylfluoride [PMSF]) and treated with 1 mg/ml of lysozyme for 4-5 hours at 4^{o}C. The lysozyme-treated sample was sonicated using three 30 sec bursts with 2 min cooling time on ice in between each burst. Insoluble material was

pelleted (20,000 x *g* for 10 minutes at 4°C) from this lysate and the soluble 6xHis-Cps2K was purified from the supernatant using Talon Beads as per the manufacture's protocol (BD Biosciences). DTT (1 mM) was present throughout the entire purification procedure in order to stabilize the UDP-GlcDH (14, 53). The presence of the 44 kDa protein was confirmed by Coomassie staining of SDS-10% polyacrylamide gels in which the proteins from the lysates, supernatants, and His-purification had been separated.

UDP-Glc dehydrogenase activity assay was determined spectrophotometrically by following the accumulation of NADH at 340 nm, which results from the reduction of 2 moles of NAD+ for every mole of UDP-Glc oxidized (55). Briefly, 5 µl of the BX163 crude lysate or soluble fraction obtained as described above and containing 50 µg of total protein, or 50 µg of the His-purified protein from BX163, was added to 1 ml of a reaction mixture containing 100 mM Tris-HCl (pH 8.7), 10 mM $MgCl_2$, 0.5 mM UDP-Glc, and 1 mM NAD^+. The accumulation of NADH at room temperature was followed spectrophotometrically at 340 nm. Protein concentrations were determined using the Biorad Bradford Assay method. To determine the amount of NADH produced per minute, a standard curve was extrapolated from the absorbance of NADH standards (concentration range 1 to 500 µM) at 340 nm. Purified bovine UDP-GlcDH (Sigma) was used as a positive control.

Plasmid and mutant constructions. Primers used for the construction of plasmids and mutants are listed in Table 2. For an in-frame deletion of *cps2K*, the flanking regions were PCR amplified from D39 chromosomal DNA using primer pairs J1/J12303 and P1/P14568. The two resulting PCR products were cloned separately into

TABLE 2. Primers used in this study

Primer[a]	Sequence[b]	Description[c]
Cps2-D2 (+)	GGTTCTTATGGAGATTACGGGAA	*cps2D*$^{4996-5018}$
Cps2-D3 (+)	CTCACAGGCAAAATTGGATTTTG	*cps2D*$^{4368-4390}$
Cps2-E10 (+)	ATTTACTTCCTCACATTACATG	*cps2E*$^{5343-5364}$
Cps2-E11 (-)	AAACTACTTCGCTCCATCTCTC	*cps2E*$^{6418-6396}$
Cps2-T1 (-)	CTCATGACCATCTGGATTTAC	*cps2T*$^{6449-6468}$
Cps2-T2 (+)	TTATATCATTGGTTCAAAGGGG	*cps2T*$^{6459-6481}$
Cps2-G0 (+)	CAAGGACATGATGTGGTTTGTTA	*cps2G*$^{8665-8688}$
Cps2-G2 (-)	*ATTATAACTATCCATACTAATAA	*cps2G*$^{9671-9647}$
Cps2-I3 (-)	CACCTGAATTTGTCCCAATAAC	*cps2I*$^{11906-11884}$
Cps2-I2 (+)	*TAAAAATGGATGGGGAAATTCAA	*cps2I*$^{10830-10854}$
Cps2-I4 (+)	TTCGATAGTTGAGGATTCAGACTTT	*cps2I*$^{11039-11065}$
Cps2-J1 (+)	CTTGTAGTAAAATACTTGCTAAG	*cps2J*$^{13034-13058}$
Cps2-J12303F(+)	TTCTGAAGGGGTTCTTCGATTTGCA	*cps2J*$^{12303-12327}$
Cps2-J2 (-)	*ATTTTTCTCCTTTCAATACTCGT	*cps2J*$^{13561-13527}$
Cps2-J7 (+)	*TAAGAACCAATAAGTACGAGTAT	*cps2K*$^{13522-13544}$
Cps2-J8 (-)	*CAATTTTCTAGTTCCTTATATAGT	*cps2J*$^{12116-12094}$
Cps2-K1(-)	AACTACTCTTACTCCCTTAGCTTTTA	*cps2K*$^{14627-14602}$
Cps2KORF-BglII (+)	**AAAATAGCAGTAGCAGG	*cps2K*$^{13564-13581}$
Cps2KORF-KpnI (-)	*TTAATCTCTTTCAAAAATA	*cps2K*$^{14798-14780}$
Cps2-P1 (+)	*GAAAGAGATTAATTTAGTATATT	*cps2P*$^{14787-14809}$
Cps2-P15468R(-)	CTTCCTCTACTACACTAAGTATCC	*cps2P*$^{15468-15445}$
Cps2-P2 (-)	CATGCGTTATGACTGTCTTAGG	*cps2P*$^{15283-15262}$
Cps3D-F (+)	*GAGGACTGTAGTAAAAT	*cps3D*$^{1012-1029}$
Cps3D-R (-)	*CCCTTATTCTCTGCC	*cps3D*$^{2215-2210}$
CpsL-1 (+)	AGGTTATTTCATTATGAAAGG	*cps2L*$^{15496-15517}$
CpsL-2 (-)	CCGAAAAAATTATCTGTCATCTAG	*cps2L*$^{16399-16375}$
c-ups1	GAGCCCATGTTTCTCAATAGG	*cps2*$^{449-470}$
c-ups2	ATCTTAGTAGACTTCCCGCG	*cps2*$^{1729-1946}$
KM151-2 (-)	*GTACTAAAACAATTCATCCA	*aph-3*$^{2543-2523}$
KM151-3 (+)	*GAGGAAGGAAATAATAA	*aph-3*$^{1729-1746}$
LDH-F (+)	GTCGGTGATGGTGCTGTAGGTTCATC	*ldh*$^{164-189}$
LDH-R (-)	GTCGATGTTAGCGTGTGACCAAACAG	*ldh*$^{710-687}$
Ugd-1 (+)	GGGCATTCTTCCATCTAAAAATGA	*ugd*$^{148064-148088}$
Ugd-2 (-)	GCATTTAAACTTCTCCTCTCAGC	*ugd*$^{148627-148604}$

[a] Forward and reverse primers are indicated by plus (+) and minus (-), respectively.
[b] * and **, *Kpn*I (GGTACC) and *Bgl*II (AGATCT) sites present at the 5' end.
[c] Numbers in superscript indicate the positions of the primer start and end in the homologous sequence of either type 2 capsule (*cps2*) (GenBank accession no. AF026471), type 3 capsule (*cps3*) (GenBank accession no. U15171), pDL276 *aphA-3*

sequence (GenBank accession no. AF216803), TIGR4 *ldh* sequence (GenBank accession no. AE007422) or R6 *ugd* sequence (GenBank accession no. NC003098).

pCR 2.1-TOPO (Invitrogen) and transformed into TOP10 cells. Each cloned fragment was excised using *Eco*RI and *Kpn*I and subcloned together into the *S. pneumoniae* suicide vector pJY4164, resulting in pBX108. The correct orientations of the inserts were confirmed by PCR and sequencing. The ORF of the kanamycin resistance-encoding gene, *aphA-3*, was amplified from the pneumococcal shuttle vector pSF151 using the primer pairs KM151-2/KM151-3, and the resulting PCR product was cloned into pCR 2.1-TOPO vector. pBX108 was partially digested with *Kpn*I, and the *aphA-3* fragment was excised from TOPO using *Kpn*I and subsequently inserted in between the two fragments in pBX108, resulting in pBX113. Correct orientation of all three inserted fragments was then confirmed by PCR and restriction digestions. pBX113 was transformed into competent D39, and Δ*cps2K* mutants were selected by kanamycin resistance and confirmed by PCR and sequencing. Construction of in-frame deletions of *cps2H* and *cps2J* were performed as described for deletions of *cps2K*, except primer pairs used were cps2G0/cps2G2 and cps2I2/cps2I3 for deletion of *cps2H* and cps2I4/cps2J8 and cps2J7/cps2K1 for deletion of *cps2J*.

For repair of the Δ*cps2K* mutants, the *cps2K* ORF and 500 bp flanking each side was PCR amplified from D39 chromosomal DNA using primer pairs J1/P2. The fragment was cloned into pCR 2.1-TOPO, subsequently excised using *Eco*RI and ligated into pJY4164. The resulting construct, pBX115, was transformed into Δ*cps2K* strains BX511, BX512, and BX533. The mixtures were plated on blood agar plates without selection. Strains BX515, BX518 and BX540, which contained repairs of the respective

Δ*cps2K* mutants, were obtained by screening for loss of kanamycin resistance, and the repair of *cps2K* was confirmed by PCR and sequencing.

For repair of Δ*cps2K* mutants with *cps3D*, the UDP-Glc dehydrogenase from serotype 3 *S. pneumoniae*, the ORF of *cps3D* was PCR amplified from *S. pneumoniae* WU2 chromosomal DNA using primer pairs cps3D-F/cps3D-R and cloned into pCR 2.1-TOPO. The forward primer cps3D-F includes the Shine-Delgarno sequence and start codon of *cps3D*. The cloned fragment was excised using *Kpn*I and inserted between the two fragments in pBX108, resulting in pBX116. Correct orientation of all three fragments was confirmed by PCR. The resulting construct was transformed into BX511 and the reaction was plated on blood agar plates without selection. Strain BX517, containing the allelic exchange of *cps3D* for *aphA-3* in BX511, was obtained by screening for loss of kanamycin resistance, which was then confirmed by PCR.

Repair of *cps2E* in the *cps2K* repaired strains was conducted essentially as described above for the repair of Δ*cps2K* mutants. c*ps2E,* along with the 500 bp on either side, was PCR amplified from D39 chromosomal DNA and cloned into pCR 2.1-TOPO. The cloned fragment was excised using *Eco*RI and ligated into pJY4164, resulting in pBX145. pBX145 was transformed into BX515 and BX518, and the reaction was plated on blood agar plates without selection. Repaired *cps2E* strains, BX545 and BX546 respectively, were identified by screening for large, glossy colonies, and repair of the mutations was confirmed by sequencing.

Capsule analyses. Indirect capsule ELISAs were performed as previously described (31, 39) with slight modifications. In brief, duplicate cultures were grown in

THY to a density of ~3 x 10^8 CFU/mL, and 5 mL of each was centrifuged at 20,000 x *g* for 10 minutes. The culture supernatant was collected and filtered (0.45 µm-pore-size syringe filter, Corning). The pelleted cells were resuspended in phosphate buffered saline (342.5 mM NaCl, 6.75 mM KCl, 13.5 mM Na_2HPO_4, and 4.5 mM KH_2PO_4) and heat-killed at 56°C for 20 minutes. All samples were normalized to the same OD_{600}. Wells of polystyrene microtiter plates (Corning Inc.) were coated overnight at 4˚C with two-fold serial dilutions of the samples. Wells were washed 3X in PBS containing 0.5% Tween (PBST) and blocked for 1 hour with 200 µl of 1% bovine serum albumin BSA in PBS (BSA-PBS) at room temperature. A rabbit polyclonal anti-type 2 antiserum (Statens Serum Institute, Denmark) adsorbed against a non-encapsulated type 2 derivative, AM1000, was used for detection of capsule on the cell surface. For adsorption, 250 mL of AM1000 was grown in THY to a density of ~3 x 10^8 CFU/mL and heat-killed for 45 min at 56°C. The culture was centrifuged, washed once in PBS, centrifuged and resuspended in 250 µL of anti-type 2 antiserum diluted 1/10 in PBS. Adsorption was conducted by rotating the solution overnight at 4°C. AM1000 was pelleted and the supernatant containing the adsorbed anti-type 2 antiserum was filtered sterilized (0.22 µm-pore-size syringe filter, Millipore) and stored at 4°C until use. The adsorbed anti-type 2 antiserum was diluted 1/5000 in BSA-PBS, and 100 µl of this solution was added to each well followed by incubation at room temperature for 1 hour. The wells were washed 3x with PBST, and incubated with biotinylated goat anti-rabbit Ig conjugated to strepavidin-alkaline phosphatase for 1 hour at RT. The wells were washed 3X with PBST, followed by development with 1 mg/ml *p*-nitrophenolphosphate in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 0.1 M $ZnCl_2$, pH 10.4). Absorbance was measured at 415

nm. Surface accessibility assays were performed in an identical manner except that the adsorbed type 2-specific antiserum was replaced with a non-adsorbed rabbit polyclonal antiserum raised against a type 19 strain (Statens Serum Institute, Denmark). This antiserum contains a high titer of antibodies to non-capsular surface antigens and provides an effective measure of blocking of the surface by the capsule (31). For competitive-inhibition ELISAs, cultures were grown as above. Assays were performed as previously described (13). Briefly, wells of microtiter plates were coated as above with heat-killed D39 at a density of 3×10^8 CFU/ml in PBS. Cell lysates used as inhibitors were prepared by growing 10 ml cultures of each strain to a density of 3×10^8 CFU/ml in THY. Samples were normalized to the same optical density and centrifuged at 20,000 x *g* for 10 min. Pellets were resuspended in 0.1 ml lysis buffer (0.1% sodium deoycholate, 0.01% sodium dodecyl sulfate, 0.15 M sodium citrate) and incubated at 37°C for 10 min. To the lysed bacteria, 0.9 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) was added and the samples were incubated at 65°C for 15 min. Two-fold serial dilutions of the lysates or culture supernatants, together with polyclonal anti-type 2 antiserum diluted as above, were added to the D39-coated microtiter plates. The remainder of the procedure was as described above for the indirect ELISAs.

Electron Microscopy. Bacteria were prepared for electron microscopy as described by Kolkman *et al* (38). Briefly, cultures were grown to a density of ~3×10^8 CFU/mL in THY, and five mL of each culture was centrifuged at 20,000 x *g* for 10 minutes. The pellets were fixed in 500 µL of a 1% glutaraldehyde, 4% formaldehyde solution for 30 minutes at 4°C. Fixed samples were further processed by the UAB EM

Core facility for microscopy. In general, samples were post-fixed in 1% osmium tetroxide, washed in phosphate-buffer (PB), dehydrated with EtOH, embedded with Polybed, dried, sectioned and stained with uranyl acetate.

Cps2D tyrosine phosphorylation (Cps2D~P), Cps2D and Cps2E protein analyses. Western immunoblots of Cps2D and Cps2D~P were performed as previously described (6). In brief, cultures were grown to a density of ~3 x 10^8 CFU/mL and centrifuged at 20,000 x *g* for 10 minutes at 4°C. The pellets were resuspended in water at a 50X concentration, and the samples were normalized to the same OD_{600}. Twenty µL of the cell suspensions were used for Cps2D blots and 10 µL were used for Cps2D~P blots. Samples were boiled in SDS-PAGE loading buffer, and proteins were separated by SDS-10% polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane. Cps2D was detected using a polyclonal rabbit Cps2D-specific antiserum as described (64). Cps2D~P was detected using a monoclonal antibody to phosphotyrosine clone PT-66 conjugated to horseradish peroxidase (Sigma). ImageJ software (http://rsb.info.nih.gov/ij) was used for densitometry analyses.

Relative Cps2E protein levels were determined as described (15) . In brief, 10 µg of total protein from isolated *S. pneumoniae* membranes were separated by SDS-10% polyacrylamide gel electrophoresis, and subsequently transferred to a nitrocellulose membrane. Cps2E was detected using a polyclonal rabbit antiserum directed against the C-terminal portion of Cps2E and diluted 1/5000.

Capsule replacement experiments and linkage analyses to map suppressor mutations in Δ*cps2K* mutants. Erythromycin-resistance markers were linked to the capsule locus by insertion of pJD377 (*plpA* insertion, downstream) and pBX123 (between *dexB* and capsule promoter, upstream) into the D39 chromosome, resulting in strains BX516 and BX535, respectively. Chromosomal DNAs of BX516 and BX535 were isolated using a genomic column prep (Qiagen) and used to transform the Δ*cps2K* mutants BX511 and BX512. Erythromycin-resistant transformants were screened for the large colony phenotype indicative of capsule production and also for loss of kanamycin resistance.

To sequence *cps2E*, the gene was PCR amplified from chromosomal DNA of the Δ*cps2K* mutants using primer pairs Cps2-E10 and Cps2-E11. The fragment was gel extracted (Gel extraction kit, Qiagen) and sequenced at the Sequencing Core Facility of the Helfin Center for Human Genetics at the University of Alabama at Birmingham.

Membrane isolations and Cps2E glycosyltransferase assays. Cps2E glycosyltransferase activity in isolated membranes was tested as described (37), where Cps2E activity is defined as the ability to transfer [^{3}H]-Glc from UDP-[^{3}H]-Glc to an organically soluble product in a reaction conducted at 10˚C. *S. pneumoniae* membranes were isolated as previously described (15, 16). Membranes containing 10 µg of total protein were incubated in a 100 µl reaction mixture of 5 mM Tris-Acetate (pH 7.5), 10 mM $MgCl_2$, and 1 µM UDP-[^{3}H]-Glc (1 Ci/mmol, Sigma) at 10^{o}C for 10 minutes. The reaction was stopped by the addition of 1 ml chloroform:methanol (2:1), and the organic phase was extracted using 200 µl pure solvent upper phase (PSUP) (1.5 ml chloroform,

25 ml methanol, 23.5 ml H_2O, and 0.183 g KCl). The amount of radioactivity incorporated into the organic phase was measured by liquid scintillation counting.

Analysis of capsule transcripts. RNA was isolated from 50 mL *S. pneumoniae* cultures using a previously described hot acid phenol procedure (27). Serial 2-fold dilutions of RNA samples were used in slot blot analyses to determine the relative amounts of transcripts. Detection of transcript and densitometry was performed as previously described (6). PCR probes were digoxigenin-labeled (Roche) and the amount of labeling was visualized using Pierce SuperSignal Chemiluminescent Substrate. Image J software was used for densitometry analyses. The intensity of each band was normalized to lactate dehydrogenase (*ldh*) transcripts, and these ratios were compared for the parent and mutant strains.

Capsule immunoblots. Fractionation of *S. pneumoniae* into cell wall and protoplast fractions was performed as previously described with minor modifications (73). This method results in minimal cross contamination of fractions (6). In brief, *S. pneumonie* cultures were grown to a density of ~3 x 10^8 CFU/mL, and cells were sedimented at 20,000 x *g* for 10 minutes at 4°C. Pellets were suspended in protoplast buffer (PPB) (20% sucrose, 50 mM $MgSO_4$, 50 mM Tris [pH 7.4]) at 1/100 the original culture volume. Forty units of mutanolysin (Sigma) were added to each ml, and the sample was incubated overnight at room temperature (the *S. pneumoniae* autolysin LytA is also active under these conditions). After incubation, the formation of protoplasts was confirmed by light microscopy. Protoplasts were sedimented at 10,000 x *g* for 10

minutes. The supernatant containing the cell wall fraction was filtered (0.22 μm-pore-size syringe filter, Millipore), and the sedimented protoplasts were resuspended in PPB in a volume equal to the cell wall extract. For samples concentrated 2- or 10-fold, pellets were suspended in 1/200 or 1/500 of the original culture volume.

The fractions were further processed and analyzed for capsule and teichoic acids in immunoblots as previously described (6). In brief, 20 μL of sample containing either cell walls or protoplasts was combined with 10 μL of buffer B1 (50 mM EDTA, 0.5% Tween 20, 0.5% Triton X-100, 50 mM Tris [pH 8], [Qiagen]) and 2 μL of Qiaprotease (20 μg/ μL [Qiagen]), and incubated at 37°C for 30 minutes. 10 μL of SDS-PAGE loading dye was added to each sample, followed by heating at 100°C for 8 minutes. The samples were separated by SDS-10% polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. Capsular polysaccharides were detected using a rabbit polyclonal antiserum against the type 2 capsule (Statens Serum Institute, Denmark) that had been adsorbed against the non-encapsulated AM1000 (as described above) and diluted 1/1000. The presence of teichoic acid (C-polysaccharide) in the cell wall fractions was detected using a rabbit polyclonal antiserum diluted 1/5000 (Statens Serum Institute, Denmark).

Isolation and characterization of capsule produced by Δ*cps2K* mutants. 250 mL cultures of D39 and a Δ*cps2K* mutant, BX511, were grown to mid-exponential phase in Chemically Defined Media (CDM) (58) containing 0.0005% choline chloride, 0.25% sodium bicarbonate, and 0.073% cysteine-HCl. The polysaccharide isolation procedure was based on previously described methods (28, 68) with modifications, as described

below. Cultures were centrifuged at 20,000 x *g* for 10 minutes and the pellet was resuspended in 5 mL of water. Water-saturated phenol was added to a final concentration of 1%, and the suspension was incubated overnight at room temperature. Microscopy was used to confirm lysis of the bacteria. Cellular debris was pelleted by centrifugation at 20,000 x *g* for 30 minutes at 4°C. The supernatant was collected, and ethanol and sodium acetate were added to final concentrations of 60% and 7.2%, respectively, to precipitate the polysaccharide. The solution was centrifuged at 20,000 x *g* for 30 minutes at 4°C. The resulting pellet was dissolved in 5 mL of water and the pH was adjusted to 7.5 with 1 M NaOH. Forty units of DNase (Promega) and 40 µg of RNase (Qiagen) were added and the suspension was incubated at 37°C for 4 hours. Fifty Units of proteinase K (Qiagen) was then added, and the sample was incubated at 37°C overnight. Low molecular weight contaminants were removed by dialysis at 4°C overnight using 6000-8000 Da molecular weight cut-off dialysis tubing. Additional debris was removed by centrifugation (20,000 x *g* for 10 minutes at 4°C), and the supernatant containing the partially purified polysaccharide was collected and stored at 4°C.

The phenol/sulfuric method was used to determine total hexose present in the polysaccharide sample (4). A methylpentose assay was used to determine the amount of rhamnose present in the extracted polysaccharide samples (24). Carbazole and *m*-hydroxydiphenyl assays for measurement of total hexuronic acids (9, 26) were used to assess the GlcUA content in extracted polymer and whole cells. For whole cells, 10 mL of *S. pneumoniae* cultures were grown to a density of ~3 x 10^8 CFU/mL and centrifuged at 20,000 x *g* for 10 minutes at 4°C. Pellets were resuspended in 500 µL of water. Serial dilutions of lysates were analyzed for total uronic acid as described (9, 26).

RESULTS

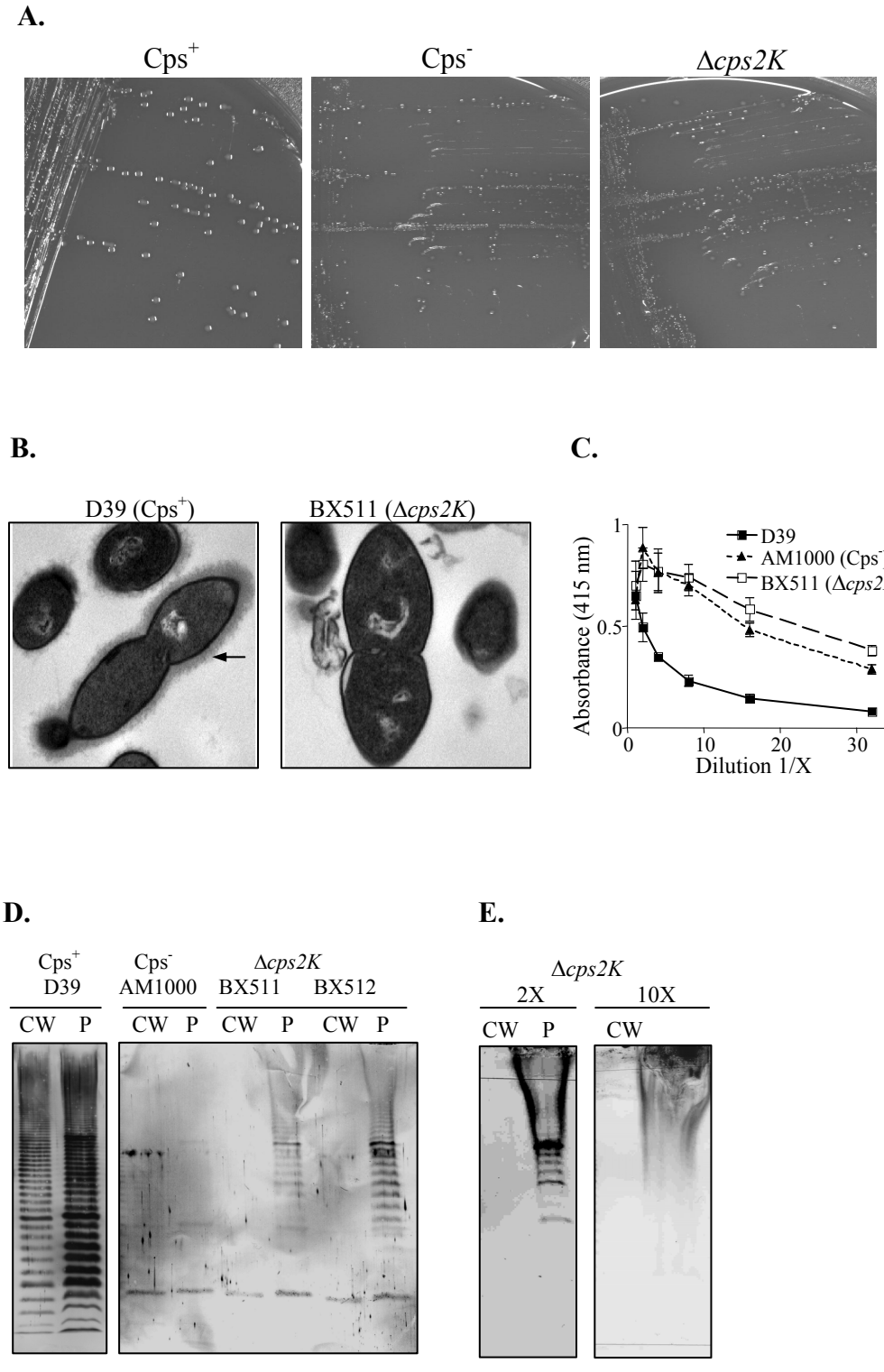
Cps2K exhibits UDP-Glc dehydrogenase activity. To determine whether *cps2K* encoded an authentic UDP-Glc dehydrogenase, the gene was cloned from the *S. pneumoniae* capsule type 2 strain D39 into the expression vector pQE-40 and expressed in *E. coli*, as described in the Materials and Methods. To facilitate purification of the recombinant protein, it was expressed with an N-terminal His_6 tag. UDP-Glc dehydrogenase activity was assayed spectrophotometrically by following the reduction of NAD^+ to NADH during the oxidation of UDP-Glc to UDP-GlcUA, as described in the Materials and Methods. The observed activities for bovine Ugd and Cps2K were 0.36 and 0.21 μmoles NADH/min/μg purified protein, respectively (activity for the vector control *E. coli* strain was 0.0032 μmoles NADH/min/μg total protein). As described below, repair of an *S. pneumoniae* D39 *cps2K* deletion mutant with *cps3D*, the *S. pneumoniae* type 3 UDP-Glc dehydrogenase (2, 23) complemented the defect, further confirming the function of Cps2K.

***cps2K* deletion mutants exhibit severe reductions in capsule synthesis and fail to transfer polymer to the cell wall**. In-frame deletion mutants were generated in *S. pneumoniae* D39 by allelic replacement of *cps2K* with an *aphA-3*-containing fragment encoding resistance to kanamycin (Km), as described in the Materials and Methods. Multiple independent *cps2K* mutants were derived in separate transformation reactions with D39. In contrast to the large, glossy colonies of the encapsulated D39 parent, all of the resulting Km-resistant transformants exhibited a small, rough colony morphology

(Fig. 2A). When individual colonies were plated to determine CFU/colony, the numbers were the same for the parent and mutant strains (~4 x 10^7 CFU/colony). However, microscopic observation revealed fewer bacteria per chain for the mutants. Thus an overall lower number of bacteria were present in each colony, suggestive of a possible growth defective (discussed further below). Using a polyclonal antiserum to the type 2 polysaccharide in indirect and competitive-inhibition ELISAs, no capsule was detectable using intact cells, cell lysates, or culture supernatants from two independent *cps2K* mutants (data not shown). Further, no surface-localized capsule was detectable by electron microscopy (Fig. 2B). Consistent with a severe reduction in capsule synthesis, whole cells of the *cps2K* mutants exhibited the same high reactivity as a non-encapsulated mutant (AM1000, Δ*cps2A-H*) in ELISAs with a polyclonal antiserum containing a high titer of antibodies to non-capsular surface antigens (Fig. 2C). In this surface accessibility assay, binding of the antibodies is blocked in proportion to the amount of cell-associated capsule (31).

To further assess capsule production in the *cps2K* mutants, isolated cell fractions were examined in immunoblot analyses with the type 2-specific antiserum. These analyses revealed the presence of low levels of high molecular weight polymer on the membrane-containing protoplast fractions, but no polymer was detected on cell wall fractions (Fig. 2D), even when the latter were concentrated 2- or 10-fold (Fig. 2E). Teichoic acid was present in similar amounts in the cell wall fractions of the parent and mutant strains (data not shown), confirming that fractionation of the mutants had released the peptidoglycan from the cell, and that synthesis of teichoic acid was not affected by the mutations.

FIG. 2. Phenotypes of Δ*cps2K* mutants. A) *S. pneumoniae* serotype 2 parent strain D39, the non-encapsulated D39 derivative AM1000, and the Δ*cps2K* mutant BX511 streaked for isolation on blood agar. B) Electron micrographs of D39 and the Δ*cps2K* mutant, BX511. The arrow indicates the capsule. C) Indirect ELISA for surface-accessibility using whole cells and a polyclonal antiserum to surface antigens. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of two experiments. D) Capsule immunoblots of cell wall (CW) and protoplast (P) fractions reacted with polyclonal antiserum against type 2 capsule. E) Capsule immunoblots for BX512. Prior to fractionation to yield cell walls and protoplasts, the bacterial samples were concentrated 2- and 10-fold more than in panel D. The loadings in this panel therefore represent 2- (2X) and 10-fold (10X) more sample than panel D. The smear in the protoplast lanes results from the heavily overloaded capsule-containing protoplast sample. The lack of capsule in the similarly overloaded cell walls is evident.


A.
Cps+
Cps-
Δcps2K
B.
D39 (Cps+)
BX511 (Δcps2K)
C.
D39
AM1000 (Cps-)
BX511 (Δcps2K)
Absorbance (415 nm)
Dilution 1/X
D.
Cps+ D39
Cps- AM1000
Δcps2K BX511
BX512
CW P
E.
Δcps2K
2X
10X
CW P
CW

The reduction in capsule was further demonstrated by assaying total hexose and methylpentose (for rhamnose) in polymer extracted from whole cells. In the *cps2K* mutants, the levels of both sugars were approximately 5% of the parental level (Table 3). The presence of capsular polysaccharide in cell wall fractions was examined by using the methylpentose assay to assay for rhamnose. Here, the *cps2K* mutant BX511 was not different from the non-encapsulated strain (Table 3). Using a carbazole or *m*-hydroxydiphenyl assay to measure total uronic acid, GlcUA was undetectable in whole cells or extracted polymer from the Δ*cps2K* mutants (data not shown).

TABLE 3. Hexose and methylpentose levels in *cps2K* mutants.

Strain	Hexose	Methylpentose	
	Isolated polymer[a]	Isolated polymer	cell wall[b]
D39 (Cps^+ parent)	6.7 ± 0.9 (6.7)[c]	0.67 (0.67)	0.736 (0.727)
BX511 (Δ*cps2K*)	0.35 ± 0.01 (0.35)	0.037 (0.034)	0.01 (0)
AM1000 (Cps^-)	0.004 ± 0.001 (0)	0.003 (0)	0.01 (0)
BX515 (*cps2K* repair of BX511)	n.d.[d]	n.d.	0.0156 (0.0056)

[a], Polymers isolated from 250 ml cultures were assayed for both hexose and methylpentose. The hexose values represent means ± standard errors for two independent cultures. The values are μg/10^8 CFU.
[b], Cell walls isolated from a 250 ml culture were assayed for methylpentose. The values are μg/ 10^8 CFU.
[c], Values in parentheses are after the subtraction of the Cps^- value from AM1000.
[d], not determined

To confirm that the small amounts of capsule produced by the Δ*cps2K* mutants were not due to undetectable levels of GlcUA arising from the activity of a non-Cps2K UDP-GlcDH, we deleted *ugd* in both the parent D39 and the Δ*cps2K* mutant BX511. This gene is identified in the genome sequence of strain R6, a derivative of D39 (33). It is located outside the capsule locus and is predicted to encode a UDP-GlcDH with 40%

identity and 61% similarity to Cps2K. The phenotypes of the *ugd* deletion mutants of D39 and BX511 were identical to their respective parents (data not shown), indicating that this gene does not contribute to capsule synthesis in these strains.

***cps2K* deletion mutants contain suppressor mutations**. The alterations in capsule synthesis following deletion of *cps2K* were more severe than what had been anticipated at the outset of this study. To confirm that these effects were not due to any polar or feedback effects on transcription of the capsule locus, RNA slot blot analyses were performed. The probes used were specific for *cps2C* and *cps2M*, which lie upstream and downstream, respectively, of *cps2K* (Fig. 1B). For both independent *cps2K* mutants, transcription was unchanged from the parent D39 (data not shown). In addition, the levels of two capsule proteins, Cps2D and Cps2E, as well as the level of Cps2D tyrosine-phosphorylation, were unchanged in the mutants (shown for Cps2D and Cps2D~P in Fig. 3). The presence of Rha in the mutant polymer (described above) indicated that proteins encoded by the downstream genes *cps2LMNO* and necessary for synthesis of TDP-Rha (a precursor for subunit assembly) were present. The results of experiments described in the next section further indicated that the *cps2K* deletions did not affect translation of the downstream region.

We next undertook repair of the *cps2K* deletions to confirm that the observed phenotype was due to only the mutation we constructed. Clones containing the entire *cps2K* gene and the 500 bp flanking regions were used to transform the *cps2K* mutants.

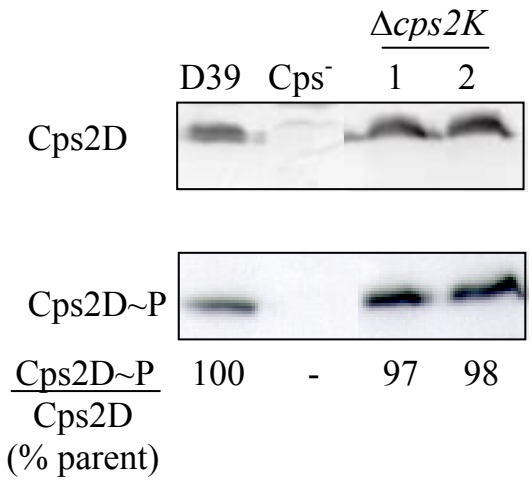


FIG. 3. Cps2D and tyrosine-phosphorylated Cps2D (Cps2D~P) in Δ*cps2K* mutants. Cps2D and Cps2D~P were detected in western immunoblots. Cps2D~P/Cps2D ratios were normalized to those of the parent D39 to obtain percent value. The Cps$^-$ strain was AM1000, in which *cps2A*-*cps2H* is deleted. The independent Δ*cps2K* mutants were BX511 (1) and BX512 (2).

Transformants in which the allelic exchange of *cps2K* and *aphA-3* had occurred were identified by screening for loss of kanamycin resistance and then confirming the presence of *cps2K* by PCR. Unexpectedly, repair of the *cps2K* deletion only partially restored capsule production. The colony size of the repaired mutants, though larger than the *cps2K* deletions, was still extremely small. Using cell lysates in competitive-inhibition ELISAs, only 0.1% of the antibody-reactive capsular material produced by the parent was detectable with the *cps2K*-repaired strains (Fig. 4A), and no capsule was detectable in culture supernatants of these strains (Fig. 4B). Consistent with this low level of capsule, reactivity of the repaired mutants in the surface accessibility assay remained high, although it was less than that of both the non-encapsulated strain AM1000 and the *cps2K* deletion mutant (Fig. 4C). In immunoblot analyses, the full range of high to low molecular weight polymer was observed in both the protoplast and cell wall fractions (Fig. 4D). Analysis by the methylpentose assay demonstrated the presence of rhamnose in the cell wall fraction of the repaired strain (Table 3, BX515). The UDP-GlcDH of *S. pneumoniae* serotype 3, *cps3D*, was also used to repair the *cps2K* deletion. This repair resulted in the same phenotype as that obtained using *cps2K* (data not shown). These results suggested that the *cps2K* deletion mutants contained suppressor mutations that affected proper capsule synthesis.

Suppressor mutations map to *cps2E*. To determine whether the suppressor mutations were linked to the capsule locus, linkage analyses were performed using derivatives of the parent D39 as donors. These strains contained an erythromycin (Em) resistance marker either upstream or downstream of the capsule locus, and chromosomal

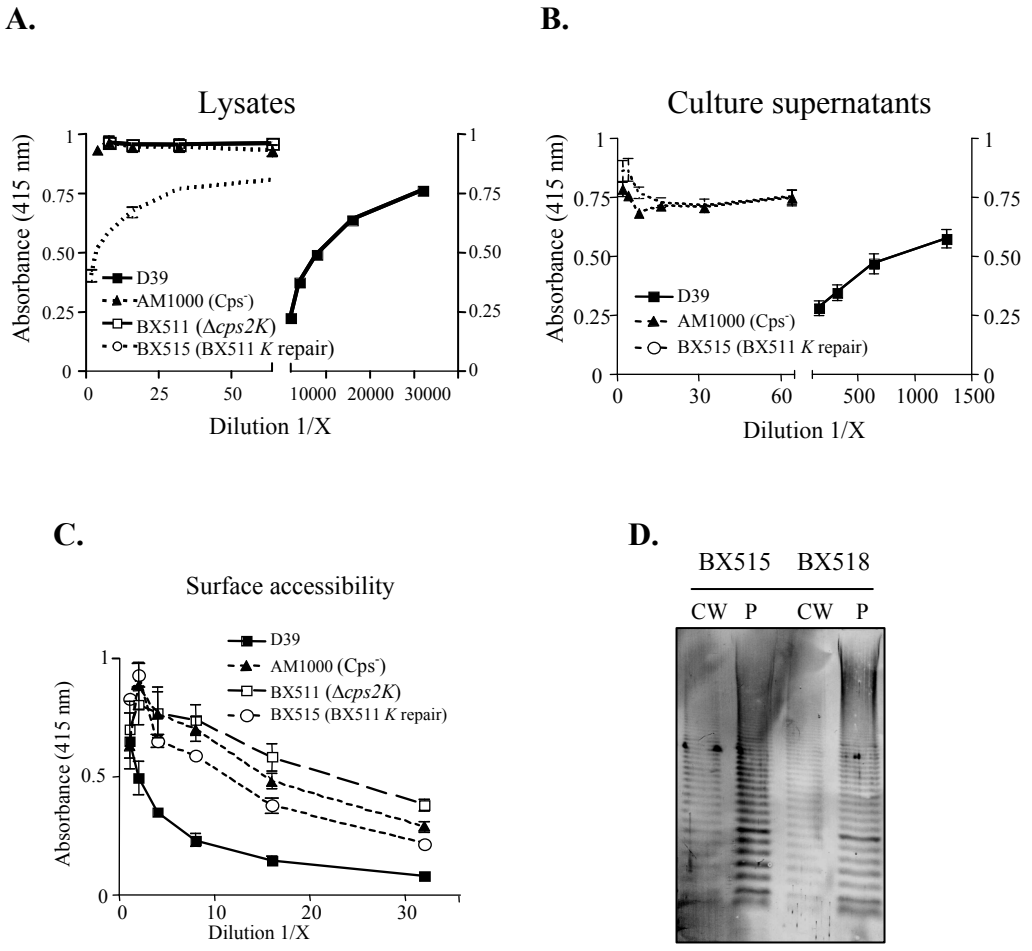


FIG. 4. Capsule production of *cps2K*-repaired mutants. Relative capsule amounts were determined by competitive inhibition ELISA for cell lysates (A) and culture supernatants (B). Results are shown for the Δ*cps2K* mutant BX511 and its derivative BX515 obtained by repair of *cps2K* only. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of two experiments. Identical results were obtained for the independent Δ*cps2K* mutant BX512 and its respective derivative BX518. C) Indirect ELISA for surface-accessibility using a polyclonal antiserum to surface antigens. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of two experiments. BX515 was significantly different from AM1000 and BX511 ($P = 0.002$ and 0.003, respectively, using a paired *t*-test to compare dilutions 1/8 – 1/32). BX511 and AM1000 were not different. D) Capsule immunoblots reacted with type 2-specific polyclonal antiserum. BX515, *cps2K* repair of BX511; BX518, *cps2K* repair of BX512. CW, cell wall fraction; P, protoplast fraction.

DNA from each was used to transform the *cps2K* deletion mutants. Our expectation for these experiments was that transformation of the entire capsule locus would repair the c*ps2K* deletion and any other mutation(s) that might be contained in this region, resulting in a parental capsule phenotype if no other mutations were present elsewhere in the chromosome. To screen for the parental phenotype, Em-resistant transformants were examined for the presence of large, glossy colonies, which are indicative of capsule production. Approximately 2% of the Em-resistant transformants obtained with the upstream insertion and approximately 1% of those obtained with the downstream insertion exhibited large colonies. All of the large colony Em-resistant transformants examined were Km-sensitive, denoting repair of *cps2K*. ELISAs and immunoblot analyses of the Km-sensitive isolates demonstrated that full capsule production, including release of capsule into the culture supernatant and transfer to the cell wall, had been restored (Fig. 5). These results indicated that the suppressor mutations were located in or near the capsule locus.

To map the suppressor mutations, we used as recipients the *cps2K* mutants in which the deletion had been repaired using *cps2K* and the 500 bp flanking regions. These strains should contain only the suppressor mutations, and their repair should result in parental capsule synthesis. Restriction enzyme-digested chromosomal DNAs from the D39 derivatives containing the Em-resistance markers flanking the capsule locus were used to transform the recipients. Em-resistant transformants were then screened for the large colony phenotype. As shown in Figure 6A, fragments containing the region between *cps2D* and *cps2T* could restore the parental phenotype in two independent mutants, suggesting the suppressor mutations were located in this region. We therefore

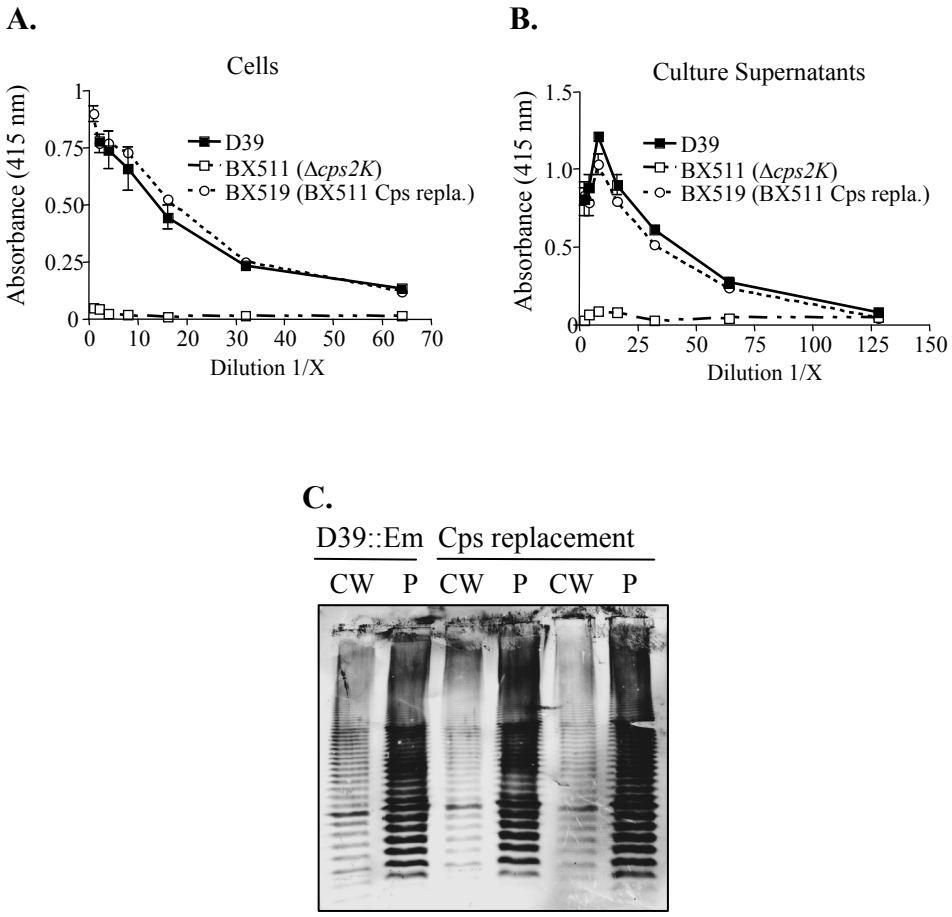
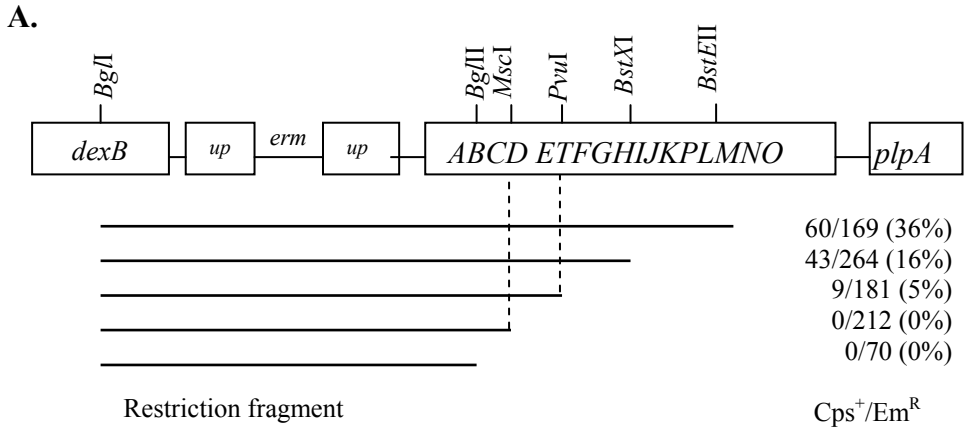
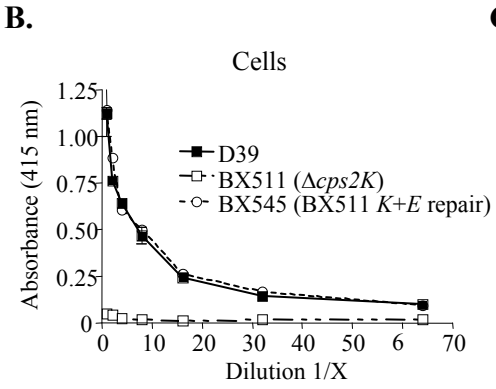
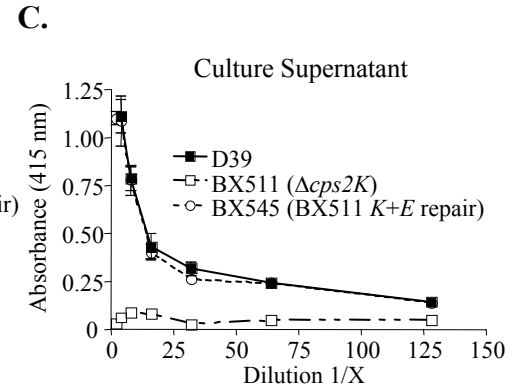


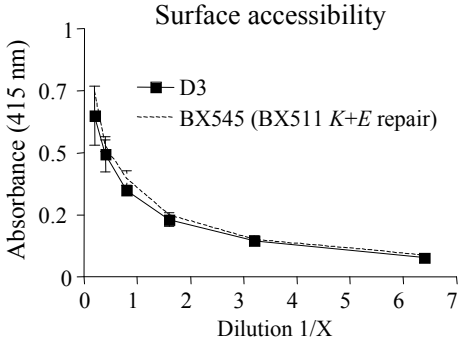
FIG. 5. Capsule production of *cps2K* mutants repaired by capsule locus replacement. Relative capsule amounts were determined using intact cells (A) and culture supernatants (B) in indirect ELISAs. Results are shown for the Δ*cps2K* mutant BX511 and its derivative BX519, obtained by replacement of the entire capsule locus. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of three experiments. Identical results were obtained for the independent Δ*cps2K* mutant BX512 and its respective derivative BX522. Capsule levels for the D39 derivative BX516, containing an Em marker downstream of the capsule locus and used to replace the entire capsule locus, were identical to D39 (data not shown). C) Capsule immunoblots reacted with type 2-specific polyclonal antiserum. CW, cell wall fraction; P, protoplast fraction. D39::Em, BX516 donor for capsule replacements; Cps replacement left CW/P fractions, BX519 (BX511 repair); Cps replacement right CW/P fractions, BX522 (BX512 repair).

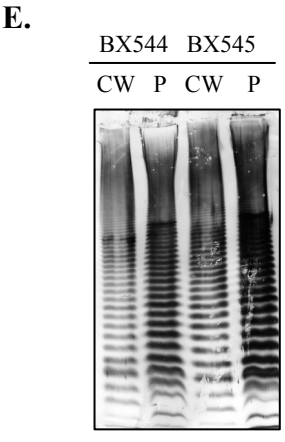
FIG. 6. Localization of suppressor mutations in Δ*cps2K* mutants. A) Restriction fragments of BX535 (D39 containing an Em marker upstream of the capsule locus) were used to transform BX518, a *cps2K*-repaired strain. Em-resistant transformants were screened for large, smooth colonies to denote repair of the suppressor mutation. Vertical lines indicate the region expected to contain the mutation based on the ability of the fragments to restore the parental phenotype. Numbers are Em^R-smooth transformants/total Em^R transformants examined in two independent transformations. B) Indirect capsule ELISAs of intact cells for derivatives in which both *cps2K* and *cps2E* have been repaired. Results are shown for BX545, the *cps2K* and *cps2E* repaired derivative of BX511. Identical results were obtained for BX544, the doubly repaired derivative of BX512. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of three experiments. C) Indirect capsule ELISAs of culture supernatants, as in panel B. D) Indirect ELISAs for surface accessibility using a polyclonal antiserum to surface antigens. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of two experiments. E) Capsule immunoblots reacted with type 2-specific polyclonal antiserum. CW, cell wall fraction; P, protoplast fraction. BX544, *cps2K* + *cps2E* repair of BX512; BX545, *cps2K* + *cps2E* repair of BX511.


A.
BglI
BglII
MscI
PvuI
BstXI
BstEII
dexB
up
erm
up
ABCD ETFGHIJKPLMNO
plpA
60/169 (36%)
43/264 (16%)
9/181 (5%)
0/212 (0%)
0/70 (0%)
Restriction fragment
Cps+/EmR


B.
Cells
Absorbance (415 nm)
1.25
1.00
0.75
0.50
0.25
0
D39
BX511 (Δcps2K)
BX545 (BX511 K+E repair)
0 10 20 30 40 50 6 70
Dilution 1/X


C.
Culture Supernatant
Absorbance (415 nm)
1.25
1.00
0.75
0.50
0.25
0
D39
BX511 (Δcps2K)
BX545 (BX511 K+E repair)
0 25 50 75 100 125 150
Dilution 1/X


D.
Surface accessibility
Absorbance (415 nm)
1
0.7
0.5
0.2
0
D3
BX545 (BX511 K+E repair)
0 1 2 3 4 5 6 7
Dilution 1/X


E.
BX544 BX545
CW P CW P

PCR amplified and sequenced this region from three independent *cps2K* deletion mutants. For each, a different point mutation was identified in *cps2E*. This gene encodes a 455 amino acid protein, previously demonstrated to be the glycosyltransferase responsible for the addition of Glc-1-P onto a polyprenol carrier to initiate repeat unit synthesis (15). The *cps2E* mutations contained in the Δ*cps2K* mutants BX511, BX512, and BX533 were G303V, G292R, and D425R, respectively.

To confirm that the mutations in *cps2E* were responsible for the altered capsule phenotype observed in the repaired *cps2K* mutants, allelic exchange of a parental copy of *cps2E* for the mutated *cps2E* was performed with two of the independent mutants. Clones containing the entire *cps2E* gene and the 500 bp flanking regions were used to transform the repaired *cps2K* strains. Transformations were plated in the absence of selection and colonies were screened for the large, glossy parental phenotype. Approximately 10% of the colonies were large. Sequence analyses confirmed the repair of the *cps2E* mutations in several large colony transformants. ELISA, surface accessibility, and immunoblot analyses demonstrated parental levels of capsule in these isolates (Fig. 6B-6E). Consistent with the reduced numbers of bacteria on agar plates, the *cps2K* mutants and the repaired *cps2K* mutants (both of which contained a *cps2E* mutation) exhibited altered growth patterns (Fig. 7A). In contrast, growth of the *cps2E* deletion mutant KA1521 and the doubly repaired *cps2K* + *cps2E* mutant BX545 was like that of the parent D39 (Fig. 7B). Thus, the capsule and growth phenotypes in the *cps2K*-repaired mutants were due to the *cps2E* suppressor mutations, and repair of both the *cps2K* deletion and the *cps2E* point mutation restored the full parental phenotypes. These

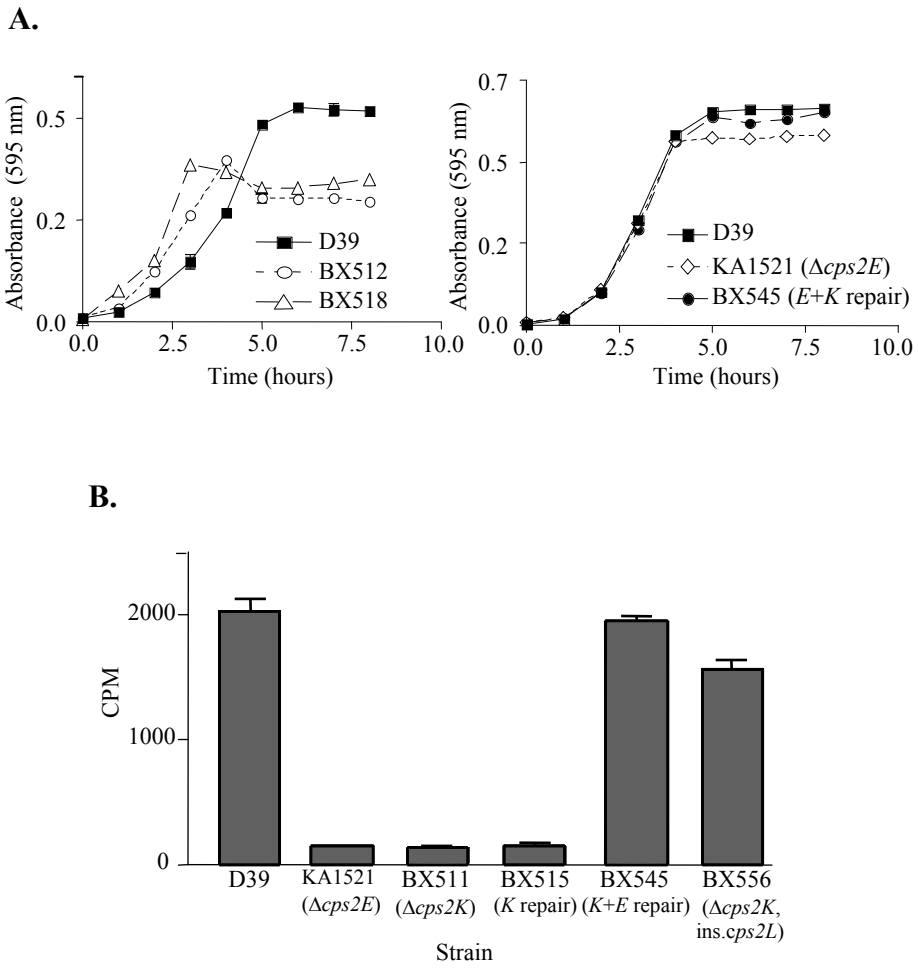


FIG. 7. Growth and Cps2E glycosyltransferase activity. A) and B) Growth curves of BX512 (Δ*cps2K*), BX518 (*cps2K* repair), BX545 (*cps2K* + *cps2E* repair) and KA1521 (Δ*cps2E*) compared to the parent D39. Cultures were diluted 1/10 from a THY culture and grown in THY. Absorbance readings were taken at indicated time points. C) Isolated membranes were used to measure incorporation of [^{3}H]-Glc from UDP-[^{3}H]-Glc to an organically soluble product, indicative of transfer of Glc-1-P to a polyprenol carrier (15). Membranes contained 10 µg of total protein and equivalent levels of Cps2E, as determined by western blotting (not shown). Similar results were obtained for BX512 and its respective repaired derivatives.

results confirmed that no other mutations or downstream effects of the *cps2K* deletions were responsible for the observed phenotypes.

To determine the effects of the suppressor mutations on Cps2E activity, isolated *S. pneumoniae* membranes were used as a source of enzyme activity for *in vitro* assays. As shown in Fig. 7C, Cps2E activity for the *cps2K* and repaired *cps2K* mutants BX511 and BX515, respectively, was not detectable above the background level observed with the *cps2E* deletion strain KA1521. In contrast, parental levels of activity were observed with the doubly repaired *cps2K* + *cps2E* mutant BX545. Cps2E protein levels of the mutant and repaired strains were unchanged from the parent (data not shown). *cps2E* deletion mutants do not make capsular polysaccharide that is detectable by ELISA (15) or immunoblotting (data not shown). Thus, capsule synthesis in the *cps2K* and repaired *cps2K* mutants, each of which contains a *cps2E* mutation, must be due to a low level of *in vivo* Cps2E activity.

***cps2K* deletion mutants consistently contain suppressor mutations, located in *cps2E* or elsewhere within or near the capsule locus**. To determine whether suppressor mutations were necessary for the isolation of *cps2K* deletion mutants, we generated 18 additional *cps2K* mutants in independent reactions. For all, the colony morphologies and capsule ELISA analyses were similar to the original *cps2K* mutants (data not shown). Sequence analyses of these mutants revealed *cps2E* mutations in fifteen of the strains. All fifteen mutations differed from those isolated originally. The locations of the mutations for these 15 strains are given in Table 1 (strains BX532, BX539, BX547-549, BX554, BX555, BX605-607, BX609-612, and BX635). The three remaining *cps2K*

mutants could be restored to the parental phenotype by transformation with the complete capsule locus, indicating the presence of suppressor mutations in this region. The mutations in two of the mutants were localized by linkage and sequence analyses. One of these mutants, BX551, contained a transition mutation located four base pairs downstream of the -10 sequence in the predicted capsule promoter located upstream of *cps2A*. A two-fold reduction in the amount of capsule transcript was observed for this mutant by RNA slot blot analysis (data not shown). The second mutant had a 1-kb insertion located in *cps2L*. This gene encodes the Glc-1-P thymidylyltransferase that converts Glc-1-P to TDP-Glc in the first step of TDP-Rhamnose synthesis (34, 43). The insertion is expected to be polar on *cps2MNO*, the remaining genes in the capsule locus that are required for the final three steps in TDP-Rha synthesis. Cps2E activity in the *cps2L* suppressor mutant was similar to that of the parent strain (Fig. 7B, strain BX556). The location of the suppressor mutation in the third non-*cps2E* mutant has not been determined.

In both the original and subsequent experiments to construct *cps2K* deletions in the parent D39 strain, the number of isolates obtained was small (≤1 Km-resistant isolates per 10^6 recipients). This result was consistent with the necessity to transform the rare spontaneous mutants that contained *cps2E* or other suppressor mutations that allowed for survival in the presence of a *cps2K* deletion. To determine whether the frequency of obtaining *cps2K* deletion mutants could be enhanced, we used as recipients isolates already containing *cps2E* mutations, which had been derived by repair of a *cps2K* deletion. Here, ~ 500 Km-resistant isolates were obtained per 10^6 recipients. Both D39 and the recipients already containing *cps2E* mutations were transformed with donor DNA

containing an Em-resistance marker unlinked to the capsule locus at high efficiency (~ 500 to 800 Em-resistant isolates per 10^6 recipient for each strain), indicating that both were equally competent for transformation. Thus, deletion of *cps2K* is detrimental to the cell, and such mutants can be isolated only in the presence of suppressor mutations that reduce or eliminate capsule synthesis.

Deletion of *cps2H* or *cps2J* also selects for isolates that contain *cps2E* mutations. To determine whether other mutations that affected polymer assembly would be detrimental to the cell, in-frame deletions of *cps2J* and *cps2H*, which encode the putative Wzx flippase and Wzy polymerase, respectively, were constructed as described in the Material and Methods. These mutants should synthesize complete repeat units that are either retained on the cytoplasmic face of the membrane (flippase mutants) or translocated to the outer face of the membrane but not polymerized (polymerase mutants). The Km-resistant transformants obtained exhibited the small, rough colony morphology indicative of non-encapsulated mutants. Based on the results for the *cps2K* mutants, we sequenced *cps2E* in independent mutants from each construction. All contained mutations. The Δ*cps2H* mutants BX552 and BX505 contained a point mutation resulting in an amino acid change (L199F) and a one base pair deletion resulting in a premature stop at residue 244, respectively. The Cps2E alterations in the Δ*cps2J* mutants BX667, BX668, and BX669 were G411R, I265S, and G371A, respectively. For the Δ*cps2H* mutant BX552, the level of Cps2E protein was similar to that of the parent strain whereas Cps2E activity was not detectable in the *in vitro* assay, as observed for the original *cps2K* mutants (data not shown). As discussed below, the suppressor mutation in

the *cps2H* mutant is located in an extracytoplasmic loop of Cps2E whereas the suppressor mutations of the original *cps2K* mutants are located in a cytoplasmic region.

DISCUSSION

In both gram-positive and gram-negative bacteria, capsule synthesis by the Wzy-dependent mechanism likely initiates on the C_{55} lipid undecaprenyl-phosphate (Und-P), the same lipid acceptor that is used to initiate synthesis of peptidoglycan, LPS O-antigen repeat units in gram-negative bacteria, and teichoic acids in gram-positive bacteria. In *S. pneumoniae*, synthesis initiates by transfer of Glc-1-P to a polyprenyl-P whose size and properties are consistent with Und-P (15). By analogy with peptidoglycan synthesis, the Und-P acceptor is expected to be recycled from the outer to the inner face of the cytoplasmic membrane following transfer of the linked polymer to another lipid-linked subunit or acceptor. The cellular levels of Und-P are low (40), thus the amounts and ratios of different polymers on the cell surface may be limited by the pool of available Und-P. The results of the present study lead to several conclusions regarding Wzy-dependent capsule synthesis in *S. pneumoniae*, as discussed below.

Lack of the terminal GlcUA of the side chain alters the ability to transfer the type 2 capsule to the cell wall. The lack of cell wall polymer in the Cps2K mutants could reflect a requirement for recognition of GlcUA by one or more enzymes in the capsule pathway, an alteration in the secondary structure of the polymer such that it no longer serves as a substrate for one or more enzymes, or an insufficient level of polymer substrate for transfer. The shift to predominantly high molecular weight polymer in the

Cps2K mutants is consistent with continued polymerase activity in the absence of chain termination and suggests that both the flippase and polymerase are active in the absence of the GlcUA residue, although we cannot exclude the possibility that their activities are not optimal. Low levels of polymer substrate do not inherently preclude transfer to the cell wall, as we have shown previously that deletion of *cps2C* or *cps2D* results in the synthesis of very small amounts of mainly low molecular weight polymer that is effectively transferred (6). Although it has been reported that Cps2C has a role in transfer of polymer to the cell wall (42), *cps2C* and *cps2D* deletion mutants exhibit parental ratios of cell wall to membrane-associated polymer (6), demonstrating that it is not required for this function. The Cps2K mutants were unchanged with regard to Cps2D production and tyrosine phosphorylation, thus this system was not responsible for the observed reduction in capsule levels, nor the failure to transfer polymer to the cell wall. The absolute requirement for GlcUA may therefore lie with the enzyme or enzymes necessary for transfer of the polymer from Und-P to the cell wall. Such enzymes have not been identified in any gram-positive bacteria, and not enough genes are present in the capsule loci to encode enzymes unique to this function.

Mutations eliminating side chain assembly, transport, or polymerization are obtained only in the presence of suppressor mutations. The lethality of the *cps2K*, *cps2J*, and *cps2H* mutations may have resulted from sequestration of Und-P in the capsule pathway and either preclusion of its turnover for utilization in essential pathways or destabilization of the membrane due to an accumulation of lipid-linked intermediates. This effect is most easily explained for the Wzx-flippase (*cps2J*) mutants, which would

be expected to accumulate single repeat units on the inner face of the cytoplasmic membrane. For the *cps2K* mutants, the effect appears to reflect either directly or indirectly the inability to transfer polymer to the cell wall. It has not been established whether polymer transfer from Und-P to the cell wall occurs directly or via an intermediate acceptor nor whether membrane-bound polymer in the parent strain is retained on Und-P or transferred to another acceptor. The high levels of membrane-bound polymer that accumulate in the parent strain apparently without harm (Fig. 2D and (6)) indicate that either this level of Und-P sequestration is not lethal or the membrane-bound polymer is not linked to Und-P. The lethality of the *cps2K* mutations and the severe reductions in membrane-bound polymer in these mutants is consistent with the latter and a failure to transfer the polymer from Und-P to another membrane acceptor in the mutants. In the Wzy-polymerase (*cps2H*) mutants, lipid-linked intermediates should accumulate only if single repeat units cannot be transferred from Und-P to the cell wall or another acceptor. The fact that isolation of these mutants required suppressor mutations suggests that transfer of single repeat units either did not occur or was very inefficient. Our previous studies demonstrated that short polymers can be transferred to the cell wall (6). The present results therefore suggest that either the linking enzyme cannot efficiently recognize and/or transfer a single, lipid-linked repeat unit, or the missing polymerase is involved in the transfer.

Secondary mutations, some of which were localized to the initiating glycosyltransferase, have similarly been noted in studies examining *Pseudomonas aeruginosa* LPS flippase (*wzx*) mutants (12), *Xanthomonas campestris* xanthan gum mutants (35) and *Salmonella typhimurium* LPS mutants that failed to polymerize O-

antigen subunits due to the lack of an abequose branch (74). Effects on cell viability resulting from the accumulation of lipid-linked subunits were also observed in these studies and in the characterization of *E. coli* LPS mutants (12, 49, 74). In contrast, mutations in *S. agalactiae* that resulted in lack of the side chain terminal sialic acid in the type III capsule led to reductions in capsule amount (~20% of parental levels) that could be fully restored by complementation (17). Thus, either these mutations were not lethal or any secondary mutations that occurred did not have an apparent phenotype in the complemented strain. In contrast to our observations, essentially all of the *S. agalactiae* polymer was transferred to the cell wall for both the parent and mutant strains, possibly precluding the necessity of a secondary mutation.

Cps2E may have functions in addition to the initiation of repeat unit formation. The high frequency of suppressor mutations in *cps2E* is perhaps surprising considering the other potential targets where mutations could theoretically abolish capsule production. *In vitro*, Cps2E catalyzes the addition of Glc-1-P to Und-P as well as the reverse reaction (15). The retention of Cps2E activity in a *cps2L* suppressor mutant, which would lack the ability to synthesize the TDP-Rha precursor and therefore fail to add Rha to Und-P-P-Glc, suggests that either the Cps2E reverse reaction occurs *in vivo* or the accumulation of Und-P-P-Glc is not toxic. Thus, mutations in the glycosyltransferase that catalyzes addition of the first Rha to the repeat unit, as well as mutations affecting TDP-Rha synthesis (*cps2LMNO*), or polar mutations in essentially any part of the locus could be effective in relieving the stress induced by the *cps2K*, *cps2J*, or *cps2H* mutations. Yet, only three of our 26 suppressor mutations occurred outside *cps2E.*

Mutations in other genes may therefore not be sufficient to prevent lethality or Cps2E may provide many effective targets for disrupting capsule synthesis if it has roles beyond that of repeat unit initiation. Consistent with this possibility is the presence of a large extracytoplasmic domain in addition to the cytoplasmic region that contains the glycosyltransferase activity necessary for repeat unit initiation (Fig. 8). Approximately 20% of the suppressor mutations were located in the extracytoplasmic loop, which exhibits no conserved domains suggestive of putative functions. Most of the suppressor mutations were located in the cytoplasmic domain in residues conserved among Cps2E homologues in *S. pneumoniae* and other bacteria. However, none were in residues known to be important in glycosyltransferase activity, such as the DXD motif characteristic of UDP-sugar binding sites (10, 19).

Cps2E belongs to a family of proteins that is structurally similar and includes initiating glycosyltransferases used for the syntheses of LPS O-antigens, xanthan gum exopolysaccharide, and capsules (62). The extracytoplasmic loop is present in the *Salmonella enterica* WbaP, which initiates LPS O-antigen synthesis by the addition of galactose-1-P to Und-P, but is absent in the Cps2E homologues of all *S. pneumoniae* serotypes that lack Glc in their polymers and therefore must initiate repeat unit formation with other sugars (8). This domain appears not to be essential for transfer of the *S. pneumoniae* capsule to the cell wall, as it is lacking in the CpsE homologue of serotype 4, which exhibits cell wall-associated capsule (6, 54). We noted, however, that the repaired *cps2K* mutants that retained *cps2E* suppressor mutations failed to release capsule from the cell. It is not yet know whether this observation is a direct effect of the *cps2E* mutation or relates to the low level of capsule produced.

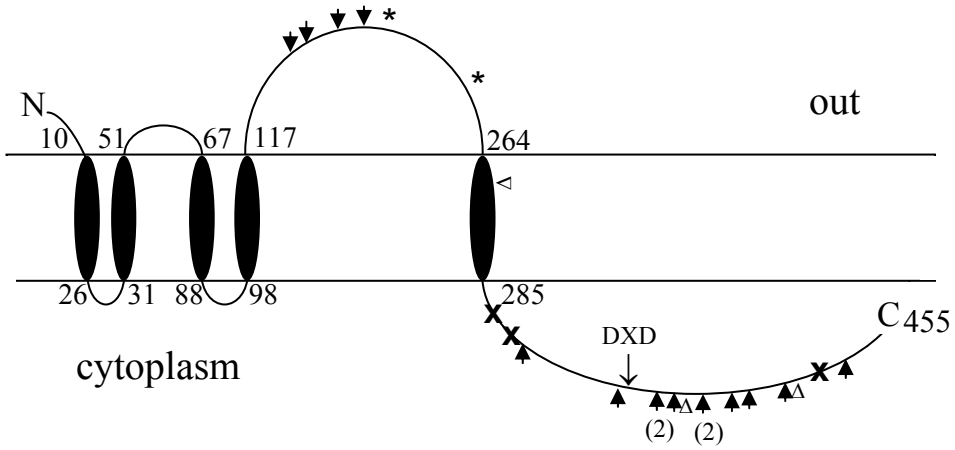


FIG. 8. Predicted topology of Cps2E using TMpred program from the ExPASy Proteomics website (http://www.expasy.org/tools). Numbers denote amino acid number. Locations of mutations in original Δ*cps2K* mutants are indicated by **X.** Locations of mutations contained in additional Δ*cps2K* mutants are indicated by ▲ or ▼. Numbers in parentheses represent the numbers of mutants with the same mutation. Amino acid changes found in Δ*cps2H* and Δ*cps2J* mutants are indicated by * and Δ, respectively. The DXD motif is indicated in the cytoplasmic domain.

The *S. enterica* WbaP protein is bifunctional, with the C-terminal cytoplasmic domain containing the glycosyltransferase activity and the N-terminal domain proposed to be important in releasing Und-P-P-galactose from WbaP and preferentially allowing the release of completed subunits (62). Such a role could fit with the phenotypes observed for the Cps2K mutants and the frequent occurrence of suppressor mutations in Cps2E, i.e., if the repeat unit remains associated with Cps2E until complete, the lack of GlcUA would block synthesis, resulting in the accumulation of lipid-linked repeat units on the inner face of the cytoplasmic membrane. Suppressor mutations in Cps2E that relaxed the requirement for a complete repeat unit could allow some synthesis to continue. As discussed above, however, the lack of GlcUA would still be an impediment to capsule synthesis due to its requirement for transfer to the cell wall.

Conclusions. The results of these studies demonstrate that the inability to properly assemble the capsule can be detrimental to the cell, and mutants affected in the assembly process may carry suppressor mutations that affect the phenotypes observed. Although we began the studies with a focus on the role of the side chain, the results strongly point toward Cps2E, the initiating glycosyltransferase, as a central player in the control of polymer assembly. Identifying the further roles of Cps2E, along with determining the requirements for cell wall association of the polymer are essential to fully understanding the capsule assembly process. The use of *cps2K* and other deletions to readily generate mutations in *cps2E* provides a unique means for potentially identifying proteins with which Cps2E interacts and for characterizing a class of glycosyltransferases that is widespread in nature. In addition, the ability to block capsule

synthesis at intermediate stages by targeting functionally equivalent enzymes present in many bacteria could provide a novel therapeutic approach to bacterial infections that would be effective because of loss of an important virulence factor and detrimental effects on cell viability.

ACKNOWLEGEMENTS

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MUTATIONS AFFECTING CAPSULE SYNTHESIS IN TYPE 2 *STREPTOCOCCUS PNEUMONIAE* RESULT IN A REDUCED ABILITY TO COLONIZE AND CAUSE SYSTEMIC DISEASE

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ABSTRACT

The requirement for capsule for *Streptococcus pneumoniae* to cause systemic infections has been well established, as nonencapsulated derivatives are known to be avirulent. The presence of capsule has also been demonstrated to be required for colonization of the nasopharynx of mice, as demonstrated by serotype 3 reduced capsule mutants. In these studies, we have assessed the role of reduced capsule amounts in type 2 D39 in colonization and in causing systemic infections. Through the construction of Δ*cps2K* mutants containing various suppressor mutations and the repairs of various mutations, we have isolated type 2 derivatives that produce <0.025% to 60% of the parental levels of capsule. Using BALB/c mice, we found that none of the reduced capsule mutants colonized as efficiently as the parent. These results are in contrast to what was observed previously for serotype 3 derivatives, where it was shown that 20% of the parental levels of capsule were sufficient for colonization of the nasopharynx. We also found that type 2 reduced capsule mutants were avirulent in BALB/c mice in i.v. and i.p. models of infection. These reduced capsule mutants were cleared from the blood within 24 hours. In CBA/N mice, the type 2 derivative producing 60% of the parental levels of capsule was attenuated in virulence in pneumonia and i.p. models of infection, and avirulent in an i.v. model of infection. These results are also in contrast to what has been observed for type 3 reduced capsule mutants, which were as virulent as their parent in all models of infection in CBA/N mice. Therefore, our data demonstrate that mutations affecting parental levels of capsule production in serotype 2 interfere with the ability to colonize and cause disease in murine models of pneumococcal infection.

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen and is one of the most common etiologic agents of pneumonia, meningitis, otitis media and bacteremia (4, 10, 16). The polysaccharide capsules of *S. pneumoniae* are the most important virulence determinant produced by this organism (10, 33). At least 91 different serotypes exist, and all share a common function of inhibiting complement-mediated opsonophagocytosis by preventing complement receptors on phagocytes from reaching complement bound on the bacterial surface (1, 9, 11, 30). The main reservoir for *S. pneumoniae* is the nasopharynx, where asymptomatic colonization is established (2, 4). From the nasopharynx, the pneumococci can disseminate and cause disease, which occurs primarily in young children, the elderly and immunocompromised individuals (2, 4). The mechanisms that lead to the transition from an asymptomatic carriage to a systemic infection are not well understood.

There is an absolute requirement for capsule in causing systemic infections and for colonization of the nasopharyngeal cavity (23, 33). A reduced amount of capsule would be advantageous to the bacteria in adhering to the nasopharynx by allowing the exposure of other surface adhesins that aid in the process of colonization. Some of the other factors previously shown to be involved in the colonization include choline binding proteins (CbpD, CbpE, CbpG, Lyt B, and Lyt C), neuraminidase and pyruvate oxidase, where mutants deficient in these proteins were reduced in their ability to colonize in animal models (18, 24, 26). However, efficient colonization does occurs with reduced capsule amounts, as demonstrated with *S. pneumoniae* serotype 3 derivatives containing defined mutations within the capsule locus or *pgm*, encoding the cellular

phosphoglucomutase that converts Glc-6-P to Glc-1-P, a precursor in capsule synthesis. These strains produce approximately 20% of the parental levels of capsule, but still exhibit near parental levels of colonization (23, 28). In addition, transparent phase variants of serotype 9V and 18C, although not containing defined mutations, produce less capsule and more teichoic acid than opaque phase variants, also colonize better (22). Although 20% of the parental levels of capsule are sufficient for type 3 to colonize, the reduced amounts may affect the stability and duration of colonization (unpublished data). In immunocompetent mice, type 3 reduced capsule mutant containing a mutation in the capsule locus was attenuated in virulence in some models of infection, whereas a reduced capsule mutant containing a mutation in *pgm* was avirulent in all models of infection (19, 23). However, both these mutants were as virulent as their parents in causing systemic infections in immunocompromised mice. Transparent phase variants of serotypes 6A and 18C were also shown to be attenuated in virulence compared to opaque variants in an i.p. model of infection, further demonstrating a requirement for capsule in virulence of *S. pneumoniae* (22).

The serotype 2 capsule is comprised of repeating units containing a backbone of glucose and three rhamnoses, with a side chain of glucose and glucuronic acid linked to the terminal rhamnose (14, 20, 27). Most strains producing the type 2 capsule are invasive and are found to be prevalent in diseases caused by *S. pneumoniae* in adults in some geographical areas. Therefore, type 2 is one of the serotypes included in the pneumococcal 23-polyvalent polysaccharide vaccine, which is mainly used for prevention of pneumococcal disease in adults (16). Strains producing a type 2 capsule are not as prevalent in diseases caused by *S. pneumoniae* in infants and young children,

therefore is not included in the conjugated vaccines used for prevention in these age groups (10, 15).

Some studies on colonization and virulence have been done using serotype 2 D39 mutants producing very short chains of polymer or synthesizing more capsule than the parent (7, 8). These mutants were unable to colonize, and the former were avirulent in mice while the latter were virulent in an i.p. model but avirulent in an i.v. model of infection. Previously, we constructed various mutations in the capsule locus of type 2 D39 which resulted in reduced capsule production (32). These are the first studies with type 2 D39 *S. pneumoniae* containing defined mutations resulting in a range of reduced capsule amounts. We have assessed the role of varying amounts of type 2 capsule on colonization and in virulence using both immunocompetent mice and immunocompromised mice. In these studies, we have determined that 60% of the parental levels of type 2 capsule are not sufficient for the ability of D39 to efficiently colonize or cause disease. Only the parent D39 and derivatives producing parental capsule amounts were able to colonize and cause systemic disease in murine models of pneumococcal infection using either immunocompetent or immunocompromised mice.

MATERIALS AND METHODS

Bacteria and growth conditions. The strains used in these studies are listed in Table 1. *S. pneumoniae* strains were grown in Todd-Hewitt broth (BD) supplemented with 0.5% yeast extract (BD) (THY) at 37°C , on blood agar base #2 (Remel) supplemented with 3% sheep red blood cells (Colorado Serum Co., Denver, Co.), or on tryptic soy blood agar plates supplemented with 5% sheep blood (Becton Dickinson).

When appropriate, antibiotics were used at the following concentrations: erythromycin at 0.3 μg/mL, kanamycin at 250 μg/mL, optochin (ethylhydrocupreine-HCl) at 10 μg/mL, and gentamicin at 1 μg/mL. All mutant strains used have been previously described (32).

TABLE 1. Strains used in this study.

Strains	Properties[ab]	Reference
S.pneumoniae		
BX511	Δ*cps2K*, Cps^r; *cps2E*$^{G5953G\rightarrow T}$ (G303V); no detectable surface-associated capsule, no capsule released into culture supernatant	(32)
BX515	BX511 *cps2K* repair, Cps^r; ~0.1% of D39 levels of surface-associated capsule, no capsule released into culture supernatant	(32)
BX546	BX515 *cps2E* repair, Cps$^+$; D39 levels of capsule both surface-associated and released	(32)
BX551	Δ*cps2K*, Cps^r; A to G transition 4 base pairs downstream of -10 sequence of capsule promoter; no detectable surface-associated capsule, no capsule released into culture supernatant	(32)
BX621	BX551 *cps2K* repair, Cps^r; contains Q364* change to premature stop in Cps2E and capsule promoter mutation; ~0.025% of D39 levels of surface-associated capsule, no capsule released into culture supernatant	This study
BX642	BX621 *cps2E* repair, Cps^r; A to G transition 4 base pairs downstream of -10 sequence of capsule promoter; ~60% of D39 levels of surface-associated capsule, similar levels to D39 of capsule released into culture supernatant	This study
BX647	Repair of promoter mutation in BX642, Cps$^+$; D39 levels of capsule both surface-associated and released	(32)
D39	Type 2 parent strain, Cps$^+$	(5)

[a] Cps^r, reduced capsule levels

[b] *cps2E* superscripts indicate mutations and their locations based on GenBank accession no. AF026471. Amino acid changes are indicated in parentheses. →, nucleotide change.

Analysis of capsule transcript of promoter mutant. RNA was isolated from 50 mL *S. pneumoniae* cultures using a hot acid phenol procedure previously described (17). RNA for each sample was diluted 2-fold per slot, and the relative amounts of transcript

were determined using slot-blot analyses. Detection of transcript and densitometry was performed as previously described (7). In brief, PCR probes were digoxigenin-labeled (Roche) and the amount of labeling was visualized using Pierce SuperSignal Chemiluminescent Substrate. Primers used for PCR probes are listed in Table 2. Densitometry was performed using Image J software. The intensity of each band was normalized to lactate dehydrogenase transcripts. The ratio of the mutant was compared to the parent D39 ratio.

TABLE 2. Primers used in this study.

Primer[a]	Sequence	Description[b]
Cps2-D2 (+)	GGTTCTTATGGAGATTACGGGAA	*cps2D*$^{4996\text{-}5018}$
Cps2-E10 (+)	ATTTACTTCCTCACATTACATG	*cps2E*$^{5343\text{-}5364}$
Cps2-E11 (-)	AAACTACTTCGCTCCATCTCTC	*cps2E*$^{6418\text{-}6396}$
Cps2-T1 (-)	CTCATGACCATCTGGATTTAC	*cps2T*$^{6449\text{-}6468}$
CpsL-1 (+)	AGGTTATTTCATTATGAAAGG	*cps2L*$^{15496\text{-}15517}$
CpsL-2 (-)	CCGAAAAAATTATCTGTCATCTAG	*cps2L*$^{16399\text{-}16375}$
C-ups3 (+)	GTCTATCTCTATCAACTTTTCTTGG	*cps2*$^{1019\text{-}1042}$
Cps2-A8 (-)	CGCAGTTACCACTAGATTAAGATATC	*cps2A*$^{1620\text{-}1594}$
LDH-F (+)	GTCGGTGATGGTGCTGTAGGTTCATC	*ldh*$^{164\text{-}189}$
LDH-R (-)	GTCGATGTTAGCGTGTGACCAAACAG	*ldh*$^{710\text{-}687}$

[a] Forward and reverse primers are represented by plus (+) and minus (-), respectively.
[b] Numbers in superscript indicate the positions of the primer start and end in the homologous sequence of either type 2 capsule (*cps2*) (GenBank accession no. AF026471), or TIGR4 *ldh* sequence (GenBank accession no. AE007422).

Capsule analyses. Competitive-inhibition ELISAs to determine relative capsule amounts were performed as previously described (13). Briefly, cultures of D39 were grown in THY to a density of ~3 x 10^8CFU/ml. The cultures were centrifuged at 20,000 x *g* for 10 minutes. The pelleted cells were resuspended in phosphate buffered saline (342.5 mM NaCl, 6.75 mM KCl, 13.5 mM Na_2HPO_4, and 4.5 mM KH_2PO_4) and heat-killed at 56°C for 20 minutes. Wells of microtiter plates were coated with a 100 µL of

heat-killed D39 at a density of ~3 x 10^8CFU/ml in PBS and incubated overnight at 4˚C. Cell lysates used as inhibitors were prepared by growing 10 ml cultures of each inhibitor to a density of 3 x 10^8CFU/ml in THY. Samples were centrifuged at 20,000 x *g* for 10 min and normalized to the same optical density. To 100 µL of normalized samples, 0.1 ml lysis buffer (0.1% sodium deoxycholate, 0.01% sodium dodecyl sulfate, 0.15 M sodium citrate) was added and the solution was incubated at 37°C for 10 min. To the lysed bacteria 0.9 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) was added, then incubated at 65°C for 15 min. Two-fold serial dilutions of the lysates or culture supernatants, together with polyclonal anti-type 2 antiserum diluted 1/5000, were added to the D39-coated microtiter plates. The remainder of the procedure was preformed as described (23) where capsule was detected with biotinylated goat anti-rabbit Ig conjugated to strepavidin-alkaline phosphatase and incubated for 1 hour at RT. The wells were washed 3X with PBST, followed by development with 1 mg/ml *p*-nitrophenolphosphate in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 0.1 M $ZnCl_2$, pH 10.4). Absorbance was measured at 415 nm.

Surface accessibility assays were performed as previously described (32). In brief, cultures were grown and heat-killed as described above. Wells of microtiter plates were coated overnight at 4˚C with 2-fold serial dilutions of the samples. Wells were washed 3X in PBS containing 0.5% Tween (PBST) and blocked for 1 hour with 200 µl of 1% bovine serum albumin BSA in PBS (BSA-PBS) at room temperature. Surface accessibility was determined using a non-adsorbed rabbit polyclonal antiserum raised against a type 19 strain (Statens Serum Institute, Denmark) diluted 1/500 in 1%BSA/PBS. This antiserum contains a high titer of antibodies to non-capsular surface

antigens and provides an effective measure of blocking of the surface by the capsule (19). 100 µl of this solution was added to each well and incubation at room temperature for 1 hour. The wells were washed 3x with PBST, and incubated with biotinylated goat anti-rabbit Ig conjugated to strepavidin-alkaline phosphatase for 1 hour at RT. The wells were washed 3X with PBST, followed by development with 1 mg/ml *p*-nitrophenolphosphate in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 0.1 M $ZnCl_2$, pH 10.4). Absorbance was measured at 415 nm.

Colonization and mouse virulence. Colonization and virulence were assessed in 8- to 12-week-old BALB/cByJ and CBA/N (CBA/CaHN-*Btk*^{xid}) female mice (Jackson Laboratories, Bar Harbor, ME). For colonization, bacterial inoculums and intranasal inoculations were performed as described with slight modifications (23, 31). In brief, bacteria were grown to a density of 3 x 10^8 CFU/mL. The culture was centrifuged at 12,000 x g for 10 minutes at 4°C, and the pellet was resuspended in Lactated Ringer's solution to a final concentration of 10^9 CFU per 10 µL. 10 µL of this bacterial suspension was inoculated into the nares of mice. Ten days postinoculation, mice were sacrificed by asphyxiation in a CO_2 chamber, and nasal washes were collected as previously described. In addition, nasal tissue was also collected by scraping of the nasal cavity. Serial dilutions of both nasal washes and nasal tissue were plated on blood agar containing either no antibiotic, 1 µg/mL of gentimicin, 1 µg/mL of gentimicin and 10 µg/mL of optochin, or 0.3 µg/mL of erythromycin where appropriate and pneumococci were enumerated (pneumococci are capable of growth on gentimicin but not optochin).

PCR of type 2 specific capsule primers was used to further confirm the bacteria recovered were pneumococci.

For pneumonia model of infection, mice were anesthetized with 100 mg/kg ketamine and 15 mg/kg xylazine (University of Alabama Animal Resources Program, Birmingham, AL) and inoculated intranasally with 50 μL of Lactated Ringer's solution containing 10^7 or 10^8 CFU. Mice were kept on their backs until consciousness was regained and were further monitored for 21 days. Hearts and Lungs of mice that had succumbed to infection were homogenized in 500 μL of Lactated Ringer's solution and plated on blood agar to confirm the presence of pneumococci (23).

Intravenous and intraperitoneal infections were performed as previously described (23). In Brief, bacteria were grown in THY at 37°C (to the same density described above), diluted in Lactated Ringer's solution to the desired concentration, and 0.2 mL was injected i.p or i.v. into the tail vein of the mice. For mixed competition experiment, mice were infected i.v. with 1:1 ration of D39 and BX515 so that the total CFUs in the inoculum was ~3 x10^7 CFUs per 0.2 mL. For blood clearance assays, mice infected intravenously were bled retro-orbitally at the various time points indicated and blood samples were serially diluted and plated on blood agar to enumerate pneumococci. All mice were monitored for 21 days. Hearts and lungs of mice that had succumbed to infection were also homogenized as described above, and then serially diluted and plated to enumerate pneumococci.

Complement deposition analyses. Complement assays were performed as previously described (1). In brief, serum from BALB/c mice was pooled and stored at -

85°C. Bacteria were grown in THY to a density of ~3 x10^7 CFUs/mL. Samples were normalized to optical density and 0.5 mL of cells was centrifuged, washed once in Veronal gelatin buffer (Sigma), and resuspended in 100 μL of Veronal gelatin buffer. Serum was added to 10% of the final volume of the bacterial suspension and was incubated at 37°C for 30 min. Bacteria were centrifuged, washed 3X in PBS and resuspended in 50 μL of peroxidase-conjugated goat antiserum to C3 (Cappel Research Products, MP Biomedicals, LLC, Solon, Ohio) diluted 1:61,000 in 1% BSA/PBS and incubated for 1 hr at RT. Samples were centrifuged, washed 3X, and resuspended in 100 μL of water. 10 μL of sample was resuspended in buffer containing the detected substrate [SureBlue Reserve TMB Microwell Peroxidase Substrate (3,3',5,5' tetramethylbenzidine), KPL]. Color development was measured at 450 nm after the addition of 1 N HCl to stop the reaction. Nonspecific binding was determined by incubation of the bacteria with heat-inactivated serum. Results were normalized to OD_{595} of the final suspension. For determination of total C3 bound, 20 μL of the opsonized bacteria was separated by SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose. C3 was detected using by anti-C3 HRP diluted as above and was developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.).

Statistics. A two-tailed Fisher's exact test was used for comparison of the numbers of mice colonized and the number that survived between the reduced capsule mutants and the parent strain. A two-tailed Mann-Whitney test or student *t* test was used to compare median times to death.

RESULTS

Capsule production. Serotype 2 D39 and isogenic derivatives of D39 producing reduced capsule amounts used in the colonization and virulence studies are listed in Table 1. All isogenic derivatives contain defined mutations within the type 2 capsule locus, and have been described previously (32). In brief, BX511 contains an in-frame deletion of *cps2K*, the UDP-Glc dehydrogenase encoded within the capsule locus, and a point mutation in *cps2E*, the glycosyltransferase responsible for the addition of Glc-1-P to a polyprenyl-phosphate to initiate repeat unit synthesis (14). This mutant produces <0.025% of the parental levels of capsule, which is membrane associated only and not transferred to the cell wall. BX515, the *cps2K* repaired derivative of BX511, contains only a point mutation in *cps2E* resulting in the production of ~0.1% of the parental levels of surface-associated capsule. In addition, no capsule from BX515 is released into the culture supernatant when grown in broth culture. The parent D39 releases capsule into the culture supernatant, but the reasons for how and why this occurs are unknown. Of the total amount of capsule produced by D39, about 85% is surface-associated and 10-15% is released into the culture supernatant (unpublished data). BX621 contains a point mutation in *cps2E* and in the capsule promoter region, resulting in the production of only ~0.025% of the parental levels of capsule. BX621 also does not release any capsule into the culture supernatant when grown in broth culture. This strain was derived from BX551, a Δ*cps2K* mutant, which contains a transition mutation of an A to a G located 4 base pairs downstream of the -10 sequence of the capsule promoter region (Fig. 1A). The *cps2K* deletion was repaired in BX551 as previously described (32) to obtain strain BX621. As shown in Figure 1B, compared to the parental levels, BX551 and BX621

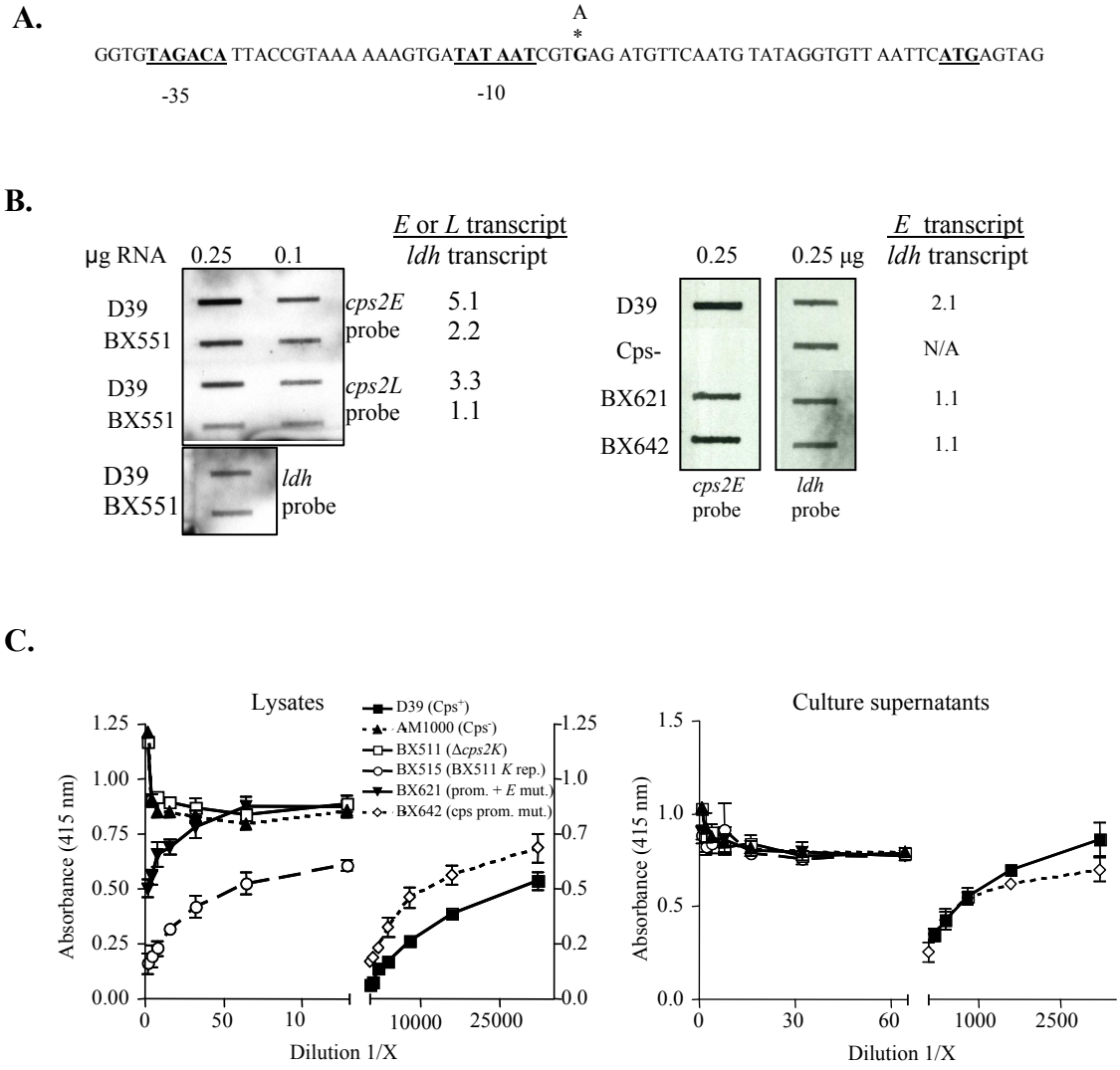


FIG.1. Mutation in the type 2 capsule promoter region reduces capsule transcript and decreases capsule production. A) Promoter region of the type 2 capsule locus. The -10 and -35, and translational start sequences are in bold. Location of a transition mutation 4 base pairs downstream of the -10 sequence in strains BX551, BX621, and BX642 is marked by an *. B) RNA slot blot analysis of capsule transcripts of BX551, BX621 and BX642 compared to the parent D39. Capsule transcripts were visualized with either digoxigenin-labeled *cps2E* or *cps2L* probes and normalized to *ldh*. C) Relative capsule amounts were determined by competitive-inhibition ELISA for cell lysates (left) and culture supernatant (right). Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of three experiments. Symbols for strains are as shown in the left panel.

exhibited approximately a 2-fold reduction in the amount of capsule transcript. BX642, *cps2E* repaired strain of BX621, contains only the mutation in the capsule promoter region, producing about 60% of the parental levels of cell-associated capsule. Release of capsule into the culture supernatant was unaffected in BX642, releasing relative similar amounts to the D39 parental levels (Fig. 1C). Respectively, BX546 and BX647 are full repairs of the *cps2E* mutation in BX515 and the promoter mutation in BX642, both of which synthesize parental levels of surface-associated and released capsule amounts (32).

Ability of D39 derivatives to colonize the nasopharynx. In these studies, the abilities of type 2 derivatives containing defined mutations which resulted in reduced levels of capsule production were assessed for their ability to colonize the nasopharynx as described (23). Under this model, mice are inoculated intranasally with 10^9 CFUs in a low volume inoculum, where stable colonization occurs for at least 2 weeks (31). As shown in Table 3, 7 out of 10 mice were colonized with D39 and the fully repaired strains BX546 and BX647, the same number as obtained by Magee and Yother for D39 (23). However, the log CFU/mL recovered from the nasal washes was slightly less than those obtained previously (2.5 vs. 3.6). In this study, nasal washes were collected from mice that were sacrificed 10 days postinoculation, whereas previously they were sacrificed 7 days postinoculation, which may account for some of the observed differences. The *cps2K* deletion mutant, BX511, did not colonize at all. This mutant was not expected to colonize since it produces <0.025% the parental levels of capsule that is membrane-associated only and not transferred to the cell surface. It was therefore expected to give similar results as those previously observed for the nonencapsulated strain AM1000 (23).

TABLE 3. Nasopharyngeal colonization of BALB/c mice by D39 and its derivatives[a]

Strain	Capsule Production (%)[b]	Colonization[c]	Nasal wash + tissue[d] log CFU/mL (± SEM)
D39	100	7/10	2.51 (0.46)
BX511	<0.025	0/9*[f]	-
BX515	~0.1	2/10*[e]	1.70 (0.24)
BX546	100	7/10	2.29 (0.38)
BX621	~0.025	2/10*[e]	2.37 (1.04)
BX642	~60	2/10*[e]	2.28 (0.89)
BX647	100	7/10	2.75 (0.63)

[a] Mice were inoculated i.n. ~10^9 CFU in a 10 µL inoculum volume.
[b] Cell-associated capsule levels were determined by competitive-inhibition ELISA.
[c] Values represent the number of mice colonized per total number of mice.
[d] Values represent log CFU per milliliter recovered from nasal washes and nasal tissues. The total volume collected was 200 µL.
[e] Significantly different when comparing the total number colonized by reduced capsule mutants to the total number colonized by the parent and the fully repaired strains (*P = 0.0002) using a Fisher's Exact test.
[f] Significantly different from the parent D39 (*P = 0.0031) Fisher's Exact test.

The difference in the number of mice colonized by BX511 was significantly different from the number colonized by the parent D39 (P=0.0031). Unlike what was observed with serotype 3 reduced mutants, the reduced capsule mutants of serotype 2, BX515, BX621, and BX642 all colonized the nasopharynx equally to each other, but less efficiently than the parent. As shown in Table 3, only 2 out of 10 mice were colonized by strains producing either ~0.025%, ~0.1% or ~60% of the parental D39 levels of surface-associated capsule (P=0.0036 for the total colonized by the mutants compared to the total colonized by the fully encapsulated strains). The log CFU/mL of pneumococci recovered from the nasal washes and nasal tissues of the reduced capsule mutants were comparable to those obtained from the parent D39 and the fully repaired mutants (Table 3). These results demonstrate that the presence of some capsule on a type 2 does aid in colonization

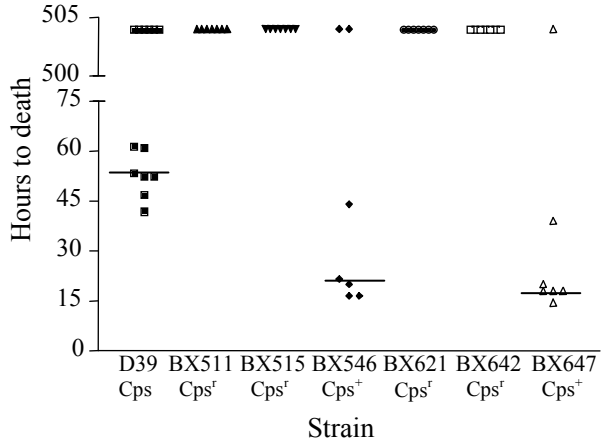
of the bacteria, but 60% of the parental levels are still not sufficient for efficient colonization.

Ability of reduced capsule derivatives of D39 to cause invasive disease.

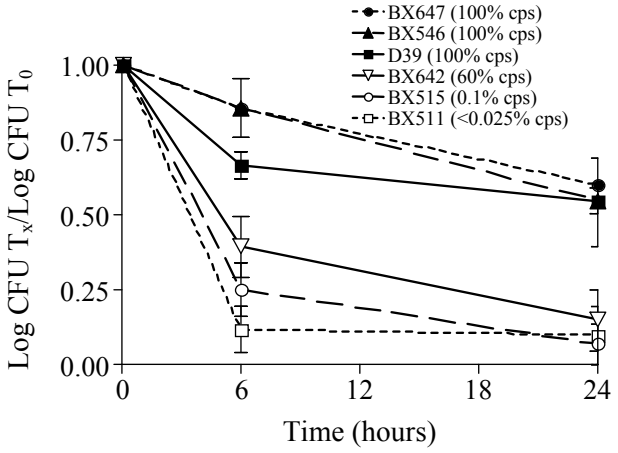
Groups of 7 BALB/c mice were infected i.p or i.v. with 10^6 or 10^7 CFUs, respectively, using the various strains listed in Table 1. As demonstrated in Figure 2A and 2C, the mice were susceptible to infection caused by the parent D39 and the fully repaired strains, BX546 and BX647, in both models of infection. 4 to 6 out of the 7 mice infected intravenously had succumbed to infection caused by the fully encapsulated strains, whereas no mice died when infected with the reduced capsule mutants. A similar trend was observed for mice infected intraperitoneally (Fig. 2C). The Median times to death and the number killed were not significantly different between the fully repaired strains and the parent D39 in either model. The log CFUs/ml recovered from the hearts and lungs of mice that had succumbed to both i.v. and i.p. infections from BX546 and BX647 were comparable to the log CFUs/ml recovered from D39 (Table 4). All reduced capsule mutants were rapidly cleared from the blood within 24 hours as shown in Figure 2B. An inverse correlation was observed between the rates of clearance of each strain in the bloodstream to the amount of capsule produced. No CFUs were recovered from mice infected with the reduced capsule mutants by 24 hours, whereas 5 to 7 logs of CFUs/mL were recovered from the blood of mice which were infected with the parent D39 and the fully repaired strains BX546 and BX647 and that were still alive 24 hours. These results demonstrate that in immunologically normal mice, 60% of the parental levels of capsule in D39 are not sufficient to cause systemic infections.

FIG. 2. Reduced type 2 capsule mutants are avirulent in BALB/c mice. Groups of 7 mice were infected with each strain. A time to death of 504 h indicates survival. Median times to death are indicated by the horizontal bars and were compared using an unpaired student *t* test and the numbers surviving were compared using a Fisher's exact test. A) Mice were inoculated i.v. with 10^7 CFU. The LD_{50} of D39 in BALB/c mice infected intravenously is approximately 5 X 10^6 CFU (8). The median times to death and number killed were not significantly different between D39 and the two fully repaired strains BX546 and BX647. The number surviving following infection with the reduced capsule mutants was significantly different when compared to the parent D39 and also their respective fully repaired derivatives ($P = 0.018$ for D39, $P = 0.02$ for BX546 and $P = 0.0047$ for BX647). B) Blood clearance. Mice that were infected intravenously were retro-orbitally bled at 1 min, 6 h and 24 h. Results are plotted as the log CFU/mL recovered at Time X (6 and 24 hour bleed) over the log CFU/mL recovered at Time 0 (1 min bleed). Reduced capsule mutants were cleared from the blood within 24 h. Only the fully encapsulated strains remained in the blood at this time. The number of bacteria collected at 6 and 24 h were not significantly different between D39 and the fully repaired strains BX546 and BX647. However, the number of bacteria isolated from the mutants at the same time points were significantly different compared to their fully repaired derivatives and also the parent D39, except BX642, which was not significantly different from D39 at the 6 h time point. C) Mice were inoculated i.p. with 10^6 CFU. The LD_{50} of D39 in BALB/c mice infected intraperitoneally is approximately 5 X 10^4 CFU (8). The median times to death and numbers killed were not significantly different between D39 and the two fully repaired strains BX546 and BX647 in both models. The total number survived by the reduced capsule mutants is significantly different from the total number survived by the fully encapsulated strains ($P = 0.0002$).

A.


505
500
75
60
45
30
15
0
Hours to death
D39 Cps
BX511 Cps^r
BX515 Cps^r
BX546 Cps^+
BX621 Cps^r
BX642 Cps^r
BX647 Cps^+
Strain

B.


BX647 (100% cps)
BX546 (100% cps)
D39 (100% cps)
BX642 (60% cps)
BX515 (0.1% cps)
BX511 (<0.025% cps)
1.00
0.75
0.50
0.25
0.00
Log CFU T_x/Log CFU T_0
0
6
12
18
24
Time (hours)

C.

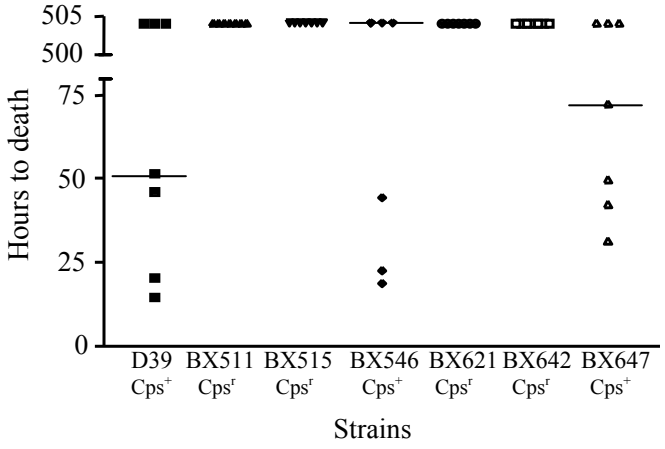

505
500
75
50
25
0
Hours to death
D39 Cps^+
BX511 Cps^r
BX515 Cps^r
BX546 Cps^+
BX621 Cps^r
BX642 Cps^r
BX647 Cps^+
Strains

TABLE 4. Organ burden

Strain	Route	Dose (log CFU)	Log CFU/mL (±SEM)[a] Heart	Lung
BALB/c mice				
D39	i.v.[b]	7.65	7.41 (0.36)	6.56 (0.31)
BX546		7.69	7.0 (0.33)	5.93 (0.37)
BX547		7.85	6.83 (0.37)	6.11 (0.15)
D39	i.p.[b]	6.2	6.35 (0.92)	5.55 (0.64)
BX546		6.65	6.53 (0.14)	5.10 (0.84)
BX647		5.9	6.56 (0.44)	5.81 (0.15)
CBA/N mice				
BX647	i.n.	7.28	7.07 (0.26)	7.60 (0.58)
BX642		8.85	6.94 (0.25)	7.42 (0.60)
BX647	i.v.	2.7	8.32 (0.14)	8.02 (0.12)
BX647	i.p.	2.6	9.25 (0.10)	8.32 (0.09)
BX642		4.1	7.18	7.16
BX642		7.8	7.42 (0.50)	7.25 (0.48)

[a] The phenotypes of the bacterial colonies recovered postmortem were identical to the phenotypes of the bacteria in the original inoculum. Mutants were confirmed by PCR and sequencing.
[b] The LD_{50}s of D39 in BALB/c are approximately 5×10^6 CFU (i.v.) and 5×10^4 CFU (i.p.) (reference 9).

A competition experiment was done on a mixed population of the parent D39 and BX515 to assess the ability of D39 to have a protective affect on the survival of one of the reduced capsule mutants, BX515, in the blood. BALB/c mice were infected i.v. with a 1:1 ratio of D39 and BX515 and CFUs were isolated from blood taken at various time points. 4 of the 7 mice infected with the mixed population had succumbed to infection, the same number which had died from an i.v. infection with D39 alone (Fig. 2A and data not shown). At 24 hours, BX515 was cleared from the blood of all mice, whereas mice that still contained bacteria had an increase in the number of D39 isolated (data not shown). Only D39 was recovered from the hearts and lungs of mice that had succumbed

to infection (data not shown). These results demonstrate only the fully encapsulated parent D39 was able proliferate and cause disease.

Ability of type 2 reduced capsule mutants to cause invasive disease in immunocompromised mice. Two of the reduced capsule mutants, BX515 and BX642, were selected for further studies in examining invasive disease in the immunocompromised mouse strain CBA/N. These mice express an X-linked immunodeficiency causing them to respond poorly to polysaccharide antigens and to phosphocholine, both components of the *S. pneumoniae* cell wall (25, 29). This deficiency renders the mice highly susceptible to pneumococcal infections (23, 25, 29). Therefore, it is possible that in immunocompetent mice, type 2 strains synthesizing reduced amounts of capsule are avirulent, however in immunocompromised mice, reduced amounts of capsule are still sufficient to cause disease. Strains BX515 and BX642 were selected because they produced ~0.1% and ~60% of the parental levels of surface-associated capsule. BX647 was used as the positive control, since it is the fully repaired derivative of BX642 and yielded similar results to those observed for the parent D39 in BALB/c mice (Table 3, Fig. 2 (32)). For a pneumonia model, mice were lightly anesthetized and intranasally inoculated with 10^7 to 10^8 CFUs. Following i.n. inoculation of CBA/N mice, all 7 mice inoculated with 10^7 CFUs of BX647 had succumbed to infection (Figure 3). BX515 was avirulent in this model, using either 10^7 or 10^8 CFUs. BX642 was attenuated in virulence both in the median time to death and the numbers killed when the mice where inoculated with 10^8 CFUs (Figure 3). The log CFU/mL of bacteria recovered from the hearts and lungs of mice that were susceptible to infection

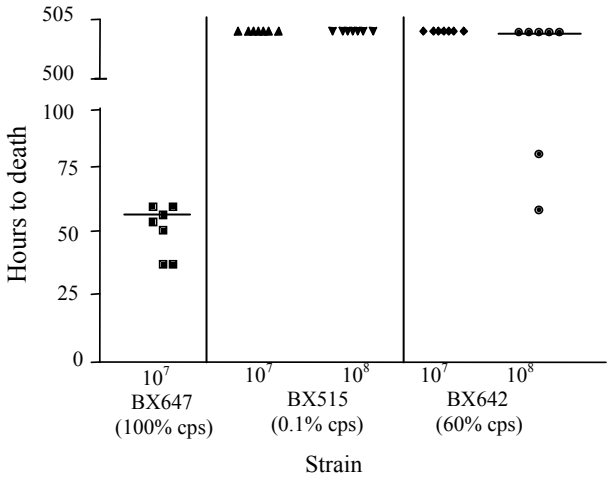


FIG. 3. Reduced type 2 capsule mutants are avirulent or attenuated in virulence in a pneumonia model using CBA/N mice. Groups of 6-7 mice were inoculated for each strain with 10^7 of BX647, BX515 and BX642, and also 10^8 of BX515 or BX642. A time to death of 504 h indicates survival. Median times to death are indicated by the bars and were compared using a Mann-Whitney unpaired test, and were significantly different between the fully encapsulated BX647 and the partially encapsulated BX642 (P = 0.0023). The number of mice that survived infection BX515 and BX642 were also significantly different from the number survived by infection from BX647 (P = 0.0006 and P = 0.021, respectively).

were similar between infection with BX647 and BX642, demonstrating the pneumonia had become bacteremic (Table 4).

Since it is possible that the inability of BX515 and BX642 to cause disease following i.n. inoculation may be due to a reduced ability to survive in the bloodstream (23), they were next examined for their ability to cause systemic disease in CBA/N mice. Mice were inoculated i.v. initially with 10^2 CFUs. All 7 mice inoculated with this dose of BX647 succumbed to infection, whereas all mice inoculated with BX515 or BX642 survived (Table 5). Increasing the inoculum to 10^4 CFUs did not result in killing either (data not shown). The same results were obtained in an i.p. model when mice were inoculated with 10^2 CFUs of BX647 and BX642. However, when infected intraperitoneally with BX642, 1 out of 6 died when infected with 10^4 CFUs and 6 out 6

TABLE 5. Virulence of type 2 reduced capsule mutants following i.v. and i.p. inoculation in CBA/N mice.

Strain	Capsule production (%)	Route	Dose (log CFU)	No. dead/no. total[a]	Median time to death (h)
BX647	100	i.v.	2.7	7/7	36
BX642	~60		4.6	0/7*[b]	-
BX515	~0.1		4.8	0/7*[b]	-
BX647	100	i.p.	2.6	7/7	29
BX642	~60		7.8	6/6	52*[d]
BX642	~60		4.1	1/6**[c]	504**[e]

[a] The phenotypes of the bacterial colonies recovered postmortem were identical to the phenotypes of the bacteria in the original inoculum. Mutants were confirmed by PCR and sequencing.

[b,c] Significantly different from the fully repaired strain using a Fisher's exact test (*P = 0.006 and **P = 0.0047)

[d,e] Significantly different from the fully repaired strain using a Mann-Whitney test (*P = 0.0082 and **P = 0.0012)

died when infected with 10^7 CFUs. These results indicate that in immunocompromised mice, type 2 derivative producing 60% of the parental levels of capsule are attenuated in virulence, and the ability to cause disease is also dependent on the route of infection.

Complement binding and accessibility. The ability of some of the reduced capsule mutants to bind complement was assessed by determining the amount of accessible complement bound to the surface of whole cells and also the total amount of C3 bound. As shown in Figure 4A, about 2-fold more bound C3 was accessible in the non-encapsulated type 2 strain AM1000 compared to the parent D39. BX511 also had a 2-fold increase in the amount of accessible surface-bound C3 compared to D39. However, BX515, which produces about 0.1% of the parental levels of capsule exhibited similar C3 accessibility binding as the parent D39. BX642, which produces about 60% of the parental levels of capsule, exhibited an intermediate level of accessible surface-bound C3. Upon examination of total complement bound, less complement was bound to D39 compared to the lesser encapsulated and nonencapsulated strains, demonstrating the presence of capsule interfered with total C3 binding and accessibility to bound C3 (Fig. 4B). Although complement was bound on BX515, it was not accessible to anti-C3 antibodies (Fig. 4A and 4B).

To determine the overall accessibility of the pnemococcal surfaces, a surface accessibility ELISA was done using a polyclonal antiserum containing a high titer of antibodies to non-capsular surface antigens. In this assay, binding of the antibodies is blocked in proportion to the amount of cell-associated capsule (19). As shown in Figure 4C, both AM1000 and BX511 exhibited high levels of binding, whereas BX515 and

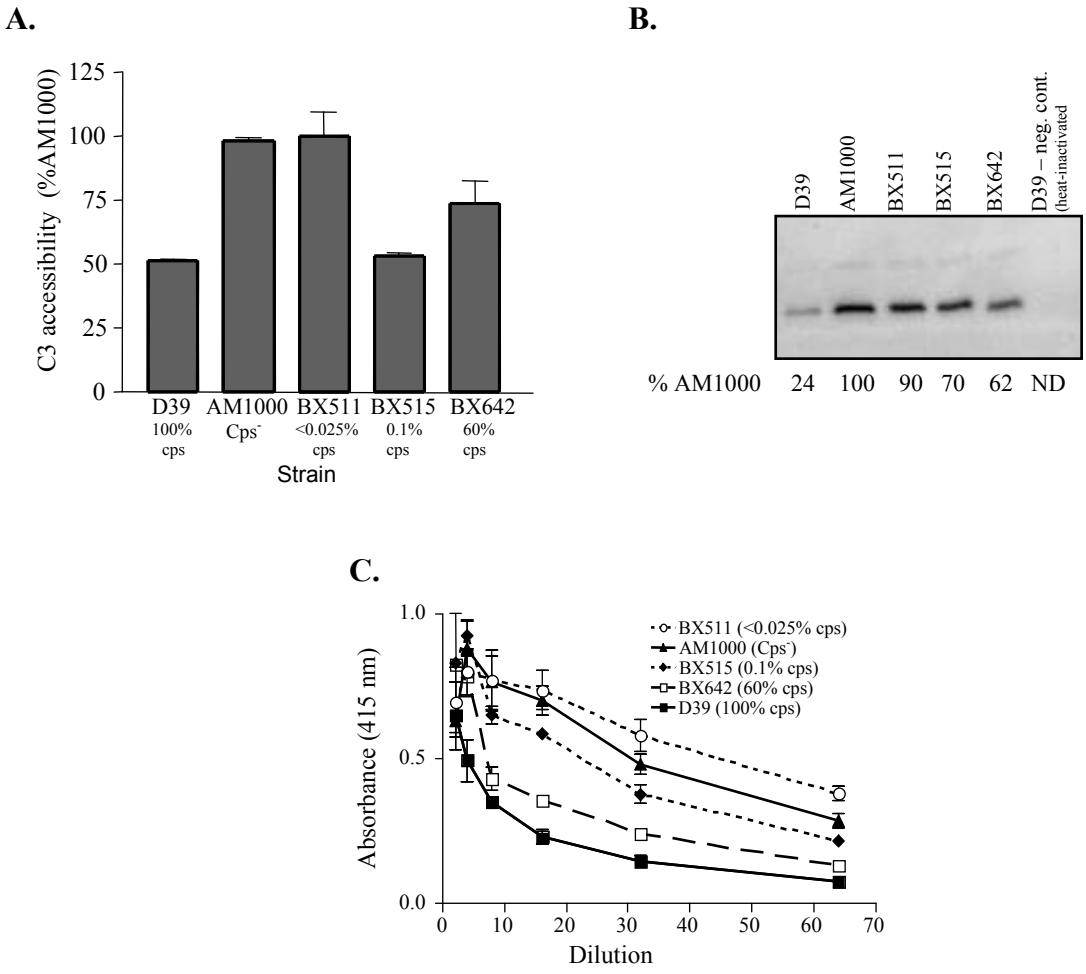


FIG. 4. Complement deposition and surface accessibility of the reduced capsule mutants. A) ELISAs of accessible surface-bound C3 were preformed as described in the Materials and Methods section. Results are reported as % bound of the nonencapsulated strain, AM1000. Results are the means (± standard errors) of 3 independent experiments. The amount of accessible surface-bound C3 was not significantly different between AM1000 and BX511 or BX642, but was significantly different the D39 and BX515 ($P = 0.0036$, and $P = 0.0035$, respectively). B) Western blot of total C3 bound, as detected using peroxidase-conjugated anti-C3. The bands represent the C3b β-chain (65 kDa), and the iC3b α-chain fragments. Labworks software was used for densitometry analyses and reported as % of AM1000. C) Indirect ELISAs for surface accessibility using a polyclonal antiserum to surface antigens. BX515 was significantly different from AM1000 and BX511 ($P = 0.002$ and 0.003, respectively, using a paired *t*-test to compare dilutions 1/8 – 1/32). BX511 and AM1000 were not different. BX642 was significantly different from AM1000 ($P = 0.02$), using an unpaired *t*-test to compare dilutions 1/8 – 1/32. Results are the means (± standard errors) of two independent cultures assayed in the same experiment and are representative of two experiments.

BX642 were intermediate in their reactivity, consistent with amount of capsule produced by the two mutants. These results demonstrate that the surfaces of the reduced capsule mutants should be accessible to antibodies and complement.

DISCUSSION

Regulation of capsule production is important for the pathogenesis of *S. pneumoniae*. The bacteria must be able to sense its surrounding environment and be able to modulate capsule production as needed. In the bloodstream, full capsule production is advantageous to the bacteria by protecting it from numerous host cell defense mechanisms. However, in the nasopharynx where colonization is established, a reduced amount of capsule would allow the exposure of other surface adhesions to aid in this process. Previous studies in our lab have shown that at least 20% of the parental levels of capsule are sufficient for colonization of serotype 3 strains (23). Mutants that produced less than 20% or were nonencapsulated were unable able to colonize the nasopharynx (23). A nonencapsulated derivative of type 2 was also unable to colonize, further demonstrating a requirement for capsule during this process (23). These studies were done using strains that contained defined mutations within *pgm* or the capsule locus which resulted in reduced capsule amounts. Weiser *et al.* have shown that certain serotypes of *S. pneumoniae* are phase variant and that transparent phase variants of serotypes 9V and 18C are able to colonize the nasopharynx more efficiently than the opaque variants (28). It was also shown in these studies that transparent variants were recovered from the nasal washes of infant rats that were originally inoculated with the transparent variant, whereas pneumococci recovered from infant rats that had been

colonized with the opaque variant were more of the transparent phenotype (28). It was later determined that opaque variants of serotypes 6A, 6B and 18C contain1.2- to 5.6–fold more capsule and 2.1- to 3.8- fold less surface-associated teichoic acid than their transparent variants (22). The phase variants of type 2 D39 were not assessed for their ability to colonize, but only for their ability to cause systemic disease in an i.p murine model (22). The type 2 D39 transparent variant was found only to contain more teichoic acid than the opaque variant, but the amount of capsule produced by the two variants was not quantified due to the lack of a good monoclonal antibody for a sandwich ELISA. Only a quelling reaction was utilized to determine that the opaque variant had a larger zone of reactivity than the transparent variant. Unlike the strains used in these studies with serotype 2 D39 and previous studies with serotype 3, the transparent and opaque phase are not known to contain defined mutations resulting in their observed phenotype. Therefore, the ability of the transparent phase variants to colonize more effectively than the opaque variants may be a result of increased amount of teichoic acid and possibly other surface components present on the cell surface, and not solely dependent on reduced capsule amounts.

Here, we have shown that type 2 derivatives containing defined mutations resulting in reduced capsule expression do not yield the same results as those observed for serotype 3 reduced capsule mutants or for transparent phase variants. D39 derivatives, producing between 0.025% to 60% of the parental levels of surface-associated capsule, did not colonize as efficiently as the parent D39. Only the fully repaired strains were able to colonize at the equivalent levels of the parent D39. Here, like what was observed for serotype 3, there is a requirement for capsule in colonization.

However, unlike serotype 3, 60% of the parental levels of capsule were not sufficient for the ability of type 2 D39 to colonize. Other factors, not present or functional in type 2, may be aiding in colonization and adhesion for type 3 and the transparent phase variants described by Weiser *et. al*, i.e. teichoic acid and other surface components in the case of transparent variants. *S. pneumoniae* serotype TIGR4 strain has been shown to contain a pilus islet in the *rlrA* pathogenicity island containing homology to pilus genes described in group A streptococci, group B streptococci and in *Corynebacterium diphtheriae* (6). TIGR4 containing these pilus-like surface structures outcompeted nonpiliated mutants in colonization of mice and also were found to adhere to lung epithelials, whereas the nonpiliated mutants were incapable of adhering to these cells (6). TIGR4 containing the pilus was also shown to be more virulent in an i.p. model of infection and induce a stronger TNF and IL-6 response than the non-piliated mutant. Not all strains contain the pilus islet. D39 does not contain a pilus, but when the pilus from TIGR4 was inserted into D39, there was a gain in virulence, ability to colonize mice, and the ability to adhere to lung epithelial cells (6). If type 3 contains a similar pilus, it is possible that colonization would also be enhanced in the presence of reduced capsule amounts compared to type 2 reduced capsule mutants without a pilus. Some strains of serotypes 6, 19F, and 23F have also been found to contain this pilus islet. These serotypes are also found to be more prevalent than other serotypes in invasive disease in children and adults, leading to a possible correlation between pili and virulence (6).

There is an absolute requirement for capsule in causing systemic infections as nonencapsulated strains are avirulent (23). In these studies, we further demonstrate the essential nature for capsule in causing disease in both immunological normal mice and in

immunocompromised mice. Previously, it was found that a type 3 reduced capsule mutant containing a mutation in the capsule locus was avirulent in an i.v. model of infection, but almost as virulent as the parent in an i.p. model of infection in BALB/c mice (23). The differences observed between the lethality of the mutant in i.p. and i.v. models may reflect the different niches available in the different routes of infection, where the reduced capsule mutants were effectively cleared in the bloodstream in an i.v. model, but localized to a specific area in an i.p. model so that the site of infection could be maintained (23). A type 3 reduced capsule mutant containing a mutation in *pgm* was avirulent in both an i.v. and i.p. model of infection in BALB/c mice (19). It was suggested that the differences between the results of the two type 3 reduced capsule mutants in an i.p. model may be attributed to the former containing a mutation in the capsule locus affecting only capsule amounts, whereas the latter contains a mutation in *pgm*, which may be affecting other cellular pathways (23). However, both type 3 mutants were as virulent as their parent in CBA/N mice (19, 23). Transparent phase variants of serotypes 2, 6A and 18C were also shown to be attenuated in virulence in an i.p. murine model (22). When splenic cultures of these mice were analyzed, the pneumococci recovered had a more opaque phenotype than the original inoculum, whereas mice infected with opaque variants had bacteria recovered of the same phenotype as the original inoculum, further demonstrating the requirement for capsule in causing systemic disease and also the importance of regulating capsule amounts in different environments (22). Here, we have found that derivatives of type 2 D39 producing less than the parental levels of capsule were all avirulent in BALB/c mice in both i.v. and i.p models of infection. Mutants producing less than parental levels of capsule were cleared from the

bloodstream within 24 hours. This rapid clearance can, in part, be attributed to increased complement deposition on the cell surface. The presence of capsule impedes complement-mediated opsonophagocytosis (1, 11, 12), and all the mutants used in these studies exhibited increased amounts of C3 deposited on their surfaces compared to the parent D39 (Fig. 4B). The reduced capsule mutants, except BX515, also exhibited increased accessibility to surface-bound C3 (Fig. 4A). It is possible that for BX515, increased amounts of complement are deposited on the cell surface, yet the capsule that is present is somehow masking the bound complement making it inaccessible to antibodies to C3.

Although BX642 produces more than half the D39 parental levels of capsule, it was attenuated in i.p. and pneumonia models of infection in CBA/N mice. These results demonstrate that for type 2 D39, 60% of capsule is not sufficient to effectively cause systemic disease even in immunocompromised mice. It is not known why 20% of the parental levels of capsule in serotype 3 is sufficient, yet 60% of the parental levels in type 2 is not sufficient, for causing disease in immunocompromised mice and for colonization. The differences observed between the results of the type 2 and 3 reduced capsule mutants may reflect the differences in the types of mutation contained within each strain. One of type 3 mutants analyzed in previous studies contained only a point mutation in *cps3D*, the UDP-Glc dehydrogenase encoded within the capsule locus. This point mutation affected UDP-GlcUA production, one of the sugars incorporated in the type 3 capsule, which resulted in reduced capsule amounts. Type 2 mutants used in these studies contained mutations in or affecting the upstream common genes (*cps2E* in BX515 and the capsule promoter region in BX642). The upstream common genes, *cps2A*, *cps2B*, *cps2C*, *cps2D*,

and a more recently added *cps2E*, are involved in initiation, regulation and modulation of capsule synthesis (9, 14, 33). It has previously been shown that deletion of *cps2C* and *cps2D*, which respectively encode proteins that form a membrane-associated activation domain and cytoplasmic-associated kinase domain of an autophosphorylating tyrosine kinase encoded within the capsule locus, resulted in the synthesis of very short polymers of capsule (7). Both mutants were unable to colonize and were avirulent in BALB/c mice in both i.p. and i.v. models of infection. Deletion of *cps2B,* a novel phosphatase and inhibitor of Cps2D activity, resulted in the production of 130% the parental levels of capsule (7). This mutant was also unable to colonize, cause disease in an i.v. infection, but was able to kill in an i.p. model of infection (7). Deletion of *cps2A*, which contains homology to a transcriptional regulator in *Bacillus subtilis*, resulted in mutants that synthesized about 50% of the parental levels of capsule, and these mutants were also unable to colonize and cause disease (3). It is a possibility that mutations of the upstream common genes, *cps2ABCDE*, may be affecting other colonization or virulence factors controlled or regulated by these proteins. BX642, containing the mutation in the capsule promoter region, would also have similar results due to decreased transcription of these upstream common genes, resulting in reduced protein levels. These additional factors may be located outside the capsule locus as earlier studies in our lab have demonstrated the importance of not only capsular type but also the genetic background in virulence of *S. pneumoniae* in an i.p. model of infection (21). The capsule locus of a virulent type 2 strain, highly virulent type 5 strain, and avirulent type 6B strain were replaced with the capsule locus from a virulent type 3 strain. Expression of the type 3 capsule in the type 2 background did not affect virulence, whereas expression of the type 3 capsule in the type

6B background increased the LD_{50} >100-fold and resulted in a mutant that was essentially avirulent in the type 5 background (21). Thus, reduced virulence observed with the type 2 mutants used in these and previous studies may not solely reflect reduced capsule amounts but affects on other factors involved in these processes.

The above observations further highlight the essential nature of capsule to survival of *S. pneumoniae* inside a host. The results presented in this study and also in previous ones suggest that the amount of capsule produced by the bacteria, the capsular type, and also the genetic background are all important factors that contribute to the virulence of *S. pneumoniae*. Unlike what has previously been observed for type 3, reduced capsule amounts in type 2 D39 are not sufficient colonization and survival in the host from mutations in *cps2E* or the capsule promoter region. In type 2 D39, at least 60% of the parental levels of capsule must be present on the cell surface in order to evade the host immune response and proliferate. Other factors may be aiding in these processes and further investigations are needed to better understand their roles, along with their contributions to the pathogenesis of *S. pneumoniae.*

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CONCLUSIONS

Although many studies have been done on the polysaccharide capsules of *S. pneumoniae*, there is still much to learn about the regulation and synthesis of capsules in this organism. Capsules represent the most important virulence determinant of *S. pneumoniae*, and the regulation of these surface structures is essential to its survival in the human host. Through the construction of defined mutations that alter the capsule structure and also capsule levels, we have demonstrated the importance of proper repeat unit synthesis in capsule assembly, transport, and virulence of serotype 2 *S. pneumoniae*. Initial studies were focused on characterization of Cps2K, the UDP-Glc dehydrogenase encoded within the type 2 capsule locus, to assess the effects removal of the side chain GlcUA from the repeat unit. However, the results of our experiments have, more importantly, led us to investigations on Cps2E and defining its role as a central player in controlling capsule synthesis. The studies presented in this dissertation have not only demonstrated the importance of proper capsule synthesis, but have further highlighted the complexities and elegance of capsule synthesis and regulation in type 2 *S. pneumoniae*.

Little is known about the effects of altering capsule side chains or parts of side chains on polysaccharide synthesis and assembly in any bacterial system. It has been demonstrated that in the synthesis of LPS O-antigens in *S. typhimurium*, subunits of mutants with defects in abequose synthesis could not be polymerized into long O-side chains *in vivo* (83). In *S. agalactiae*, mutations in either the CMP-sialic acid

synthetase or sialyltransferase that resulted in lack of the side chain terminal sialic acid in the type III capsule led to severe reductions in capsule amount (27, 77). Although reduced in capsule amounts, all the polymer produced by the *S. agalactiae* sialic acid mutant was still transferred to the cell wall. In *S. pneumoniae*, the roles capsular side chains in capsule synthesis and also virulence has not been determined. In the first part of this dissertation, we showed that the formation of a complete repeat unit is essential to proper capsule assembly and synthesis in serotype 2 *S. pneumoniae*. Alteration of the capsule structure not only affected the capsule assembly process, but also affected the amounts produced. Mutants lacking the ability to synthesize UDP-GlcUA produced very low levels of polysaccharide that was retained on the cell membranes and not transferred to the cell wall, demonstrating a requirement for GlcUA in the translocation process. Low amounts of polymer do not prevent transfer to the cell wall, as *cps2C* and *cps2D* deletion mutations result in the synthesis of low levels of short polymer but do not affect transfer to the cell wall (12). The retention of an altered polymer on the cell membrane is a unique discovery, and this phenotype is not known to occur in other bacterial systems that have been described. Failure to transport the altered polymer to the cell wall can have lethal affects to the cell, which may have resulted from sequestration of the C_{55} lipid undecaprenyl-phosphate (Und-PP). Sequestration of this lipid would prevent its turnover for use in other cellular pathways such as peptidoglycan synthesis and teichoic acid synthesis, or may have also resulted in the instability of the cell membrane due to an accumulation of lipid-linked subunits.

Currently, it is unclear if all of the polymer produced by the Δ*cps2K* mutants is associated with Und-PP or another unidentified acceptor. Therefore, the detrimental

effects observed in Δ*cps2K* mutants may be directly or indirectly related to failure to transfer the polymer to the cell wall. It is not known if the failure to transfer occurs due to inability to translocate the altered polymer from Und-PP to the cell wall directly, from Und-PP to an unknown acceptor, or from the unknown acceptor to the cell wall. All scenarios would result in the accumulation of lipid-linked subunits. We had speculated that if all of the polymer was attached to Und-PP, mild acid hydrolysis treatments would release capsule bound to the membranes in Δ*cps2K* mutants, and some release would also occur in both the parent D39 and the *cps2K*-repaired strains. However, no release of polymer was observed in Δ*cps2K* mutants, whereas some polymer release was observed in D39 and the *cps2K*-repaired derivatives (unpublished data). These results would suggest that the polymer retained on the membranes in Δ*cps2K* mutants may not all be linked to Und-PP, but to another unidentified acceptor. Further in depth studies are needed to elucidate the actual linkage of this polymer to the cell membrane and cell wall in both the Δ*cps2K* mutants and the parent D39.

Whatever the cause for the detrimental effects observed in Δ*cps2K* mutants, i.e. sequestration of Und-PP or destabilization of the cell membrane resulting from accumulation of lipid-linked subunits, the lethality could be alleviated by the selection of suppressor mutations that reduced capsule synthesis. Effects on cell viability or selection for suppressor mutations resulting from accumulation of lipid-linked subunits has been observed in studies examining mutants affected in LPS O-antigen synthesis in *Pseudomonas aeruginosa* and *S. typhimurium*, and enterobacterial common antigen (ECA) in *E. coli* (20, 61, 83). Surprisingly, the majority of suppressor mutations selected for during isolation of *cps2K* mutants were located in *cps2E*, which encodes the initiating

glycosyltransferase for repeat unit synthesis in type 2 and many other serotypes of *S. pneumoniae*. Little is known about the exact enzymatic mechanisms of this family of glycosyltransferases, but the ability to rapidly generate mutations in Cps2E by deletion of *cps2K* has provided a novel means of identifying important residues involved in activity. Interestingly, mutations affecting polymerization of subunits (Δ*cps2H*) and translocation across the cell membrane (Δ*cps2J*) also selected for suppressor mutations in *cps2E,* presumably also due to sequestration of Und-PP and preventing its turnover for use in other cellular pathways. The consistency of the locations of the suppressor mutations resulting from alterations of the capsule structure and assembly process, suggests a more important role for Cps2E beyond just initiation of capsule synthesis, and the involvement of Cps2E in a tight, regulated relationship between capsule synthesis and other cellular pathways. These pathways are connected and an influence on one can have effects on another and may prevent the lethality that could result from the sequestration of Und-PP, which is shared among these pathways.

If Cps2E is a central player in controlling capsule assembly, it may interact with multiple other proteins involved in capsule biosynthesis, as suggested by the selection for suppressor mutations in *cps2E* from deletions of multiple genes. The identification of important residues in Cps2E may not only be an indication of important residues for activity, but also a reflection of specific areas of interactions of this protein with other proteins that may form a complex to regulate and modulate capsule production. Sixteen of the 23 mutations isolated during deletion of *cps2K*, *cps2H*, and *cps2J* were located in the C-terminal loop of Cps2E, which is expected to contain the glycosyltansferase activity (74). The C-terminal region of this family of

glycosyltransferases is predicted to contain three conserved blocks (74). The first 2 blocks are highly conserved and are speculated to interact with Und-PP, whereas the third block is less conserved and is believed to be involved in sugar specificity, i.e. binding of UDP-Glc to Cps2E. Therefore, it is possible that mutations in this region would result in reduced activity due to an inability of the protein to efficiently bind its substrates. Six of the 23 mutations were located in the large extracellular loop of unknown function. If this extracellular domain is part of a sensing mechanism which coordinates complete repeat unit synthesis, then it is possible that mutations located in this region may indicate possible sites of interactions between Cps2E and other proteins involved in receiving completed subunits for polymerization and/or translocation. For example, two of the mutations generated from deletion of the gene encoding the repeat unit polymerase, *cps2H*, were located in a similar region in the extracellular loop. It is possible, then, that this part of the protein is the site of interaction between Cps2E and Cps2H. However, the generation of more Δ*cps2H* and Δ*cps2J* mutants and identifying the location of suppressor mutations contained in these mutants are needed to further test this hypothesis.

It is unknown if the deletion of other genes in the capsule locus, such as those encoding the other glycosytransferases (*cps2TFGI*), will result in a selection for suppressor mutations, and if these mutations will also be located in *cps2E*. Nor is it known if the other glycosytranferases interact with Cps2E. The proteins encoded by these genes are predicted to form the remaining backbone and also side chain of the type 2 repeat unit. However, it is unclear if one of the glycosyltransferases performs a dual function as not enough enzymes exist for each step in the formation of a complete repeat

unit. Other than Cps2E, the activities of the other glycosyltransferases (Cps2T, Cps2F, Cps2G, and Cps2I) encoded within the capsule locus have yet to be experimentally determined. If the deletions of *cps2K*, *cps2H* and *cps2J* are of any indication, the mutations of the other glycosytransferases should result in similar phenotypes. As we have shown, any mutation which alters the capsule structure should result in inefficient assembly of the polymer and accumulation of lipid-linked subunits. If mutations of these genes also select for suppressor mutations in *cps2E*, it would be interesting to determine if their locations fall into a specific regions of Cps2E, possibly indicating their sites of interaction with this central protein.

The ability to accurately measure glycosyltransferase activity is essential to determining the effects each specific mutation in *cps2E* has on Cps2E activity. Unfortunately, we have been unsuccessful in accurately measuring the affects on Cps2E activity in the *in vitro* assay. In the *in vitro* assay, conditions may not be optimal for accurate measurement of glycosyltransferase activity as other factors or regulatory elements involved in activity may be missing. Some preliminary studies in our lab have shown the importance of reducing agents, such as DTT, to the isolation and assay procedures. The addition of DTT increased the activity of Cps2E in the parent D39 3-fold in the *in vitro* assay (unpublished data). Cps2E, in addition to many of the other proteins encoded by the type 2 capsule, contains a number of cysteine residues. Therefore, if Cps2E forms a complex with other proteins, the lack of reducing agents may result in improper folding or dimerization of the proteins from disulfide bond formations resulting in an inactive or less active protein. Thus, the addition of the reducing agent would allow for stabilization of the protein to a more native form, possibly allowing for

more accurate measurements of activity. Investigations into other factors that may be involved in regulating activity of this protein are currently underway.

In addition to missing regulatory elements, temperature may also be a factor for optimization of the *in vitro* Cps2E glycosyltransferase assay. Our current assay has been optimized for measurement of the incorporation of radiolabeled UDP-Glc at 10°C to minimize the background activity of other non-Cps2E glycosyltransferases. At this temperature, the observed activities of strains containing *cps2E* mutations are the same as a nonencapsulated type 2 derivative AM1000, which contains a deletion of the genes *cps2A-H*. Strains containing *cps2E* suppressor mutations synthesize very low amounts of polymer, whereas Δ*cps2E* mutants synthesize no polymer. Therefore, *cps2E* suppressor mutants must have some level of activity above background. If Cps2E forms a complex with other proteins for repeat unit synthesis, than the mutations in *cps2E* may represent cold-sensitive mutations which affect complex formation at low temperatures but may be functional at higher physiological temperatures. We have observed an increase in the amount of incorporated radiolabeled UDP-Glc with increasing temperatures ranging from 25° C to 35° C in strains containing *cps2E* mutations and also in a mutant containing only a deletion of *cps2E* (Fig.1). This increase in activity was not observed in AM1000, suggestive of increased incorporation due to one of the glycosyltransferases present in a Δ*cps2E* mutant and Δ*cps2K* mutant, but not present in AM1000. The most likely candidates would therefore be Cps2T, Cps2F and Cps2G. Deletion of the genes encoding these glycosyltransferases would allow us to assess which protein is involved in incorporation of UDP-Glc onto the membranes, and also allow us to begin to address the functions of one of the glycosyltransferases.

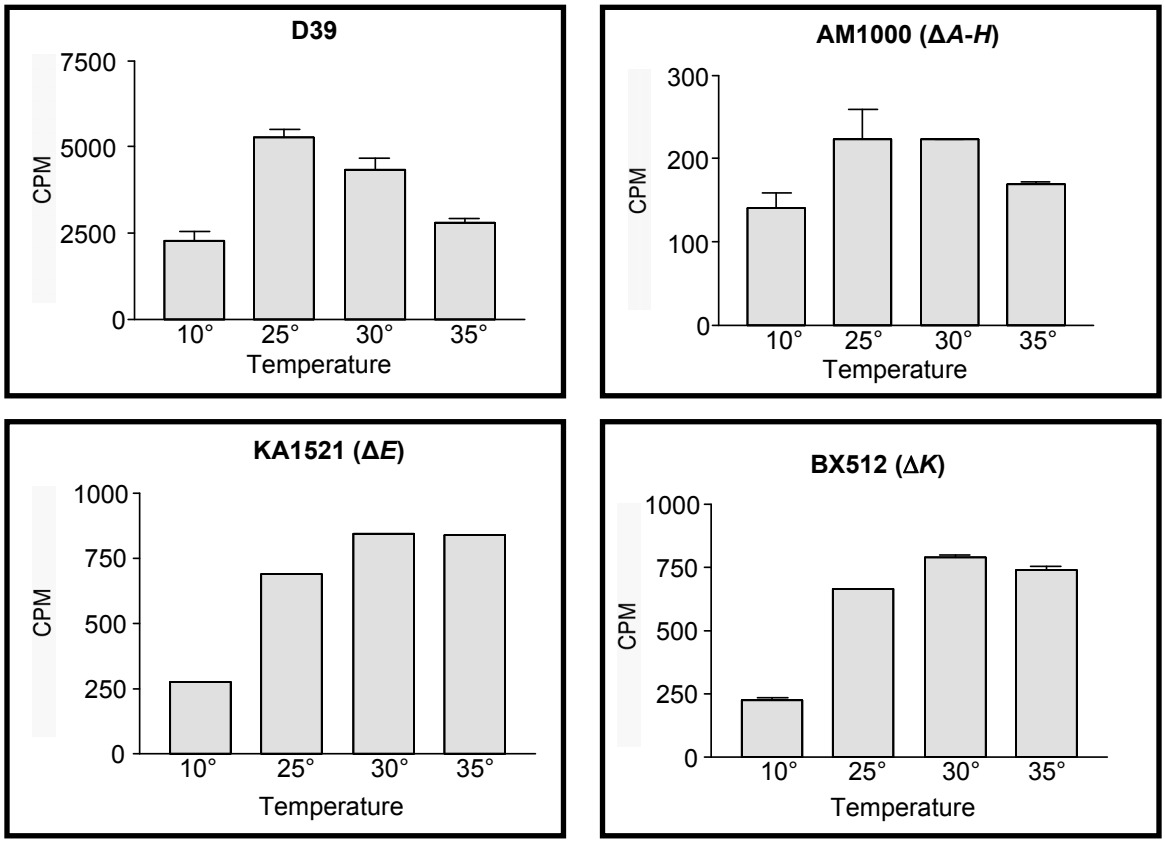

FIG. 1. Glycosyltransferase activity assay at different temperatures. Isolated membranes containing 10 μg of total protein were used to measure incorporation of [^{3}H]-Glc from UDP-[^{3}H]-Glc to an organically soluble product, indicative of transfer of Glc-1-P to a polyprenol carrier.

If the mutations in *cps2E* represent cold-sensitive mutations, higher physiological temperatures could allow mutant Cps2E proteins to interact with other proteins, thus allowing some synthesis to occur *in vivo*. These proteins could still form complexes, thus allowing some synthesis to continue, although at greatly reduced amounts. All Δ*cps2K* mutants examined, independent of the specific type of *cps2E* mutation, produced low levels of high molecular weight polymer that was retained on the cell membrane when the bacteria were grown at physiological temperatures. Higher temperatures may be a requirement for the cold-sensitive *cps2E* mutants to be active in forming complexes with transporters, other glycosyltransferases, and polymerases, thus allowing some capsule synthesis to occur. When the effects of temperature on growth and capsule synthesis were assessed in the parent D39 and a *cps2K*-repaired derivative containing a *cps2E* mutation, it was observed that both strains could grow and synthesize capsule at 30° C and 33° C, with no remarkable differences in growth rates between the two strains in the two different temperatures (unpublished data). D39 could also grow at 22°C, although at a reduced rate. However, BX518, a *cps2K*-repaired derivative containing a *cps2E* mutation, could not grow at this lower temperature. It is possible that in some mutants containing mutated Cps2E proteins, synthesis can still initiate, yet the lower temperatures prevents complex formation with other transporters from receiving the repeat unit for the next step in capsule assembly. This failure to move the repeat unit could result in accumulation of lipid-linked subunits, which would therefore be lethal to the cell. These results are suggestive of defects from the *cps2E* mutation in not only capsule production but also viability at the lower temperatures. The data presented here

further support the importance of temperature on properly regulating Cps2E activity and its role in capsule production.

In type 2 *S. pneumonaie*, the exact mechanism of synthesis of the Glc-GlcUA side chain is unclear, and the genes involved in linkage of the side chain to the backbone have not been identified or experimentally determined. Since it is a possibility that one or more of the enzymes encoded within the capsule locus performs a dual function, one of the glycosyltransferases may incorporate Glc onto a lipid acceptor for initiation of side chain synthesis. This would be consistent with the observations of glycostransferase activity with strains, lacking Cps2E but containing the remaining glycosyltransferases, as described above. The incorporation of Glc into an organically soluble product, by a non-Cps2E glycosyltransferase, was determined by TLC, however the identity of this organically soluble product is currently unclear (unpublished data). If one of the other glycosyltransferases is involved in synthesis of the side chain Glc, then this could explain the increase in activity observed with increasing temperatures in the glycosyltransferase assays in a Δ*cps2E* mutant and in strains containing *cps2E* mutations, but not observed in AM1000. Deletion mutations affecting the other glycosyltransferases, along with *in vitro* synthesis of the type 2 repeat unit are needed to determine the specific roles of the glycosyltransferases in capsule assembly.

Presently, we possess a large collection of Cps2E mutants, but only a few of them have been characterized in this dissertation. Most of the mutations identified in *cps2E* are contained in double mutants lacking *cps2K*, *cps2H,* or *cps2J*. Therefore, the affects of the *cps2E* mutations alone are generally unknown. All mutations are located in either the large cytoplasmic loop or the extracellular loop of Cps2E, with the majority located in

the cytoplasmic loop. The majority of the mutations are unique, and the effects each has on Cps2E activity and capsule synthesis is not known. Repair of all the deletion mutations is needed so that the affects of only the *cps2E* mutation can be assessed to provide insights to their roles in Cps2E activity. We had begun these studies with an examination of the role of the side chain in type 2 capsule of *S. pneumoniae*, but all our results have been centered around Cps2E. We have discovered a novel means to identify important residues involved in glycosyltransferase activity. But more importantly, we have learned there is more to the role of this protein in survival of the bacteria then just in the role of repeat unit initiation. Being able to synthesize the type 2 repeat unit *in vitro* and determine the further roles of Cps2E will provide unique insights into fully understanding capsule synthesis in *S. pneumoniae* and possibly other bacteria.

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APPENDIX A

ANIMAL USE APPROVAL FORM

THE UNIVERSITY OF
ALABAMA AT BIRMINGHAM

Vice President for Research

MEMORANDUM

DATE: January 5, 2007

TO: Janet Yother, Ph.D.
BBRB-661 2170
FAX: 975-6715

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on January 5, 2007.

Title: Mechanisms of Polysaccharide Synthesis by S. Pneumoniae

Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)

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