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†

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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 in membrane fractions. cps2K-repaired mutants exhibited only modest increases in capsule production due to the effect of the secondary mutation, but capsule was detectable in 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.

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, 58, 63). The 90 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 in gram-negative bacteria expressing group 1 capsules and lipopolysaccharide (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 glucose-1-phosphate (Glc-1-P) to a polyrenol 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 cytoplasm-associated ATPase 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 of 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

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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 GGXXCGXXD, as well as extensive homology to the signature NAD⁺-dependent dehydrogenase, found in other UDP-GlcDHs (14). UDP-GlcDHs play critical roles in the formation of many microbial 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.

expression and purification of cps2K. The open reading frame (ORF) of cps2K, minus the ribosome binding site and GTG start codon, was PCR amplified from S. pneumoniae D39 chromosomal DNA, using the primers Cps2KORF-BglII and Cps2KORF-KpnI, which incorporate a BglII site at the 5' end of the ORF and a KpnI site at the 3' end. The fragment was cloned into the expression vector pQE-40, in which the dihydrofolate reductase region between the His6 tag and the miltocinlating site was excised using BamHI and KpnI. The resulting plasmid, pBX163, was electroporated into the E. coli expression strain M15(pREP4). Transformants were selected on L agar containing both Km and ampicillin.

For expression of recombinant Cps2K, a 100-ml culture of BX163 was grown from an overnight culture diluted 1:100 in L broth containing the appropriate antibiotics at 37°C with shaking to a cell density of ~2 × 10⁶ CFU/ml. Expression of cps2K was induced with isopropyl-thio-galactoside (IPTG) (0.8 mM final concentration) for 4 h at 37°C. Cultures were centrifuged at 20,000 × g for 10 min, and the pellet was stored overnight at ~8°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, and 1 mM phenylmethylsulfonyl fluoride) and treated with 1 mg/ml of lysozyme for 4 to 5 h at 4°C. The lysosome-treated sample was sonicated using three 30-s bursts with a 2-min cooling time on ice in between each burst. Insoluble material was pelleted (20,000 × g for 10 min at 4°C) from this lysate, and the soluble His6-Cps2K was purified from the supernatant using Talon beads as per the manufacturer’s protocol (BD Biosciences). Dithiothreitol (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 blue staining of sodium dodecyl sulfate (SDS)-10% polyacrylamide gels in which the proteins from the lysates, supernants, and His purification had been separated.

A UDP-GlcDH activity assay was done spectrophotometrically by following the accumulation of NADH at 340 nm, which results from the reduction of 2 mol of NAD⁺ for every mole of UDP-Glc oxidized (55). Briefer, 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 MgCl₂, 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 Bio-Rad 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 Cps2-3J1/Cps2-21303F and Cps2-21/P14568R. The two resulting PCR products were cloned separately into pCR 2.1-TOPO (Invitrogen) and transformed into TOP10 cells. Each cloned fragment was excised using EcoRI and KpnI and subcloned together into the S. pneumoniae suicide vector pV4164, resulting in pBX108. The correct orientations of the inserts were confirmed by PCR and sequencing. The ORF of the Km resistance-encoding gene, aphA-3, was amplified from the pneumococcal shuttle vector pSF151 using the primer pair KM151-2/KM151-3, and the resulting PCR product was cloned into pCR 2.1-TOPO vector. pBX108 was partially digested with KpnI and the aphA-3 fragment was excised from TOPO using KpnI and subsequently inserted between the two fragments in pBX108, resulting in pBX113. Correct orientation of all three cloned fragments was then confirmed by PCR and restriction digests. pBX113 was transformed into competent D39, and Δcps2K mutants were selected by Km resistance and confirmed by PCR and sequencing. Constructions of in-frame deletions of cps2H and cps2I were performed as described for deletions ofcps2K except that the primer pairs used were Cps2-Gl/Cps2-G2 and Cps2-I2/
Cps2-K3 for deletion of csp2K and csp2H, and Cps2-I3 for deletion of csp2I.

For the replacement of the Δcsp2K mutants, the csp2K ORF and 500 bp flanking each side were PCR amplified from D39 chromosomal DNA using primer pair Cps2-J1-Cps2-P2. The fragment was cloned into pCR-2.1-TOPO, subsequently excised using EcoRI, and ligated into pY4164. The resulting construct, pBX115, was transformed into Δcsp2K strains BXS11, BXS12, and BXS33. The mixtures were plated on blood agar plates without selection. Strains BXS15, BXS18, and BXS40, which contained repairs of the respective Δcsp2K mutants, were obtained by screening for loss of Km resistance, and the repair of csp2K was confirmed by PCR and sequencing.

For repair of Δcsp2K mutants with csp3D, the UDP-GlcDH gene from serotype 3 S. pneumoniae was PCR amplified from S. pneumoniae WU2 chromosomal DNA using primer pairs Csp3D-F-Csp3D-R and cloned into

<table>
<thead>
<tr>
<th>Strain(s) or plasmid(s)</th>
<th>Properties</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>AM1000</td>
<td>Δ(cps2A to cps2H); type 2 Cps2-</td>
<td>39</td>
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<tr>
<td>BXS3</td>
<td>pBX110 × D39, Δcsp2I, Cps2, pSE572delI (L244T premature stop)</td>
<td>This study</td>
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<td>BXS5</td>
<td>pBX113 × D39, Δcsp2K, Cps2, pSE595delI (G303V)</td>
<td>This study</td>
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<td>BXS12</td>
<td>pBX113 × D39, Δcsp2K, Cps2, pSE629delI (G229R)</td>
<td>This study</td>
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<tr>
<td>BXS13</td>
<td>pBX115 × BX511, csp3D repair of Δcsp2K, Cps2</td>
<td>This study</td>
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<td>BXS15</td>
<td>pBX115 × BX511, csp2I repair, Cps2</td>
<td>This study</td>
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<tr>
<td>BXS16</td>
<td>pJD377 × D39, Em marker insertion downstream of capsule locus, Cps2</td>
<td>This study</td>
</tr>
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<td>BXS18</td>
<td>pBX115 × BX512, csp2K repair, Cps2</td>
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<td>BXS19</td>
<td>BX516 × BX511, Em; capsule replacement in BX511, Cps2+</td>
<td>This study</td>
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<td>BXS22</td>
<td>BX516 × BX512, Em; capsule replacement in BX511, Cps2+</td>
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<td>BXS32, BXS39</td>
<td>pBX113 × D39, independent Δcsp2K derivatives; Cps2, pSE629delI (G229R)</td>
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<td>BXS33</td>
<td>pBX113 × D39, Δcsp2K, Cps2, pSE629delI (G229R)</td>
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<td>BXS35</td>
<td>pBX123 × D39, Em insertion upstream of capsule locus promoter, Cps2+</td>
<td>This study</td>
</tr>
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<td>BXS40</td>
<td>pBX115 × BX533, csp2K repair, Cps2</td>
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<td>BXS44</td>
<td>pBX145 × BX518, csp2E repair in BX518, Cps2+</td>
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<td>BXS45</td>
<td>pBX145 × BX515, csp2E repair in BX515, Cps2+</td>
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<tr>
<td>BXS47, BXS48, BXS49</td>
<td>pBX113 × D39, Δcsp2K, Cps2+; independent derivatives with respective csp2E mutations</td>
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<td>BXS50</td>
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<td>BXS51</td>
<td>pBX113 × D39, Δcsp2K, Cps2; Δ-to-G transition 4 bp downstream of ~10 sequence of capsule promoter</td>
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<td>BXS54, BXS55</td>
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<td>BXS65</td>
<td>pBX113 × D39, Δcsp2K, Cps2, 1-kb vector insertion in csp2L region</td>
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<tr>
<td>BXS665-BXS607, BXS609-BXS612</td>
<td>pBX113 × D39, Δcsp2K, Cps2; independent derivatives with respective csp2E mutations 5632T→G (V196G), 5670insT (E191* premature stop), 6178C→G (T378R), 5904G→C (K312N), 6178C→G (T378R), 6339G→A (W407stop), and 5539T→G (V165G)</td>
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</tr>
<tr>
<td>BXS635</td>
<td>pBX113 × D39, Δcsp2K, Cps2, pSE255delI (F339L)</td>
<td>This study</td>
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<tr>
<td>BXS636</td>
<td>pBX190 × D39, Δcsp2K, Cps2, independent derivatives with respective csp2E mutations 6726G→A (G311R), 5839T→G (I265S), and 6157G→C (G371A)</td>
<td>This study</td>
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<td>D39</td>
<td>Type 2 parent strain, Cps2+</td>
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<tr>
<td>KA1521</td>
<td>Δcsp2E, type 2, Cps2+</td>
<td>15</td>
</tr>
<tr>
<td>WU2</td>
<td>Type 3 parent strain, Cps2-</td>
<td>11</td>
</tr>
</tbody>
</table>

**E. coli strains**

BXS36 | M15(pREP4, pBX163) | This study |
| BXS36 | M15(pREP4, pBX165) | This study |
| DHH5aF | F’ δ89lacZAM15 Δ(uacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK2- mK+)
plus A apE44 λ− thi-1 gyrA96 relA1 | Life Technologies, Inc.
| TOP10 | F’ mcrA Δ(mrr-hsdRMS-mcrBC) δ89lacZAM15 Δ(uacX74 recA1 deoR araD139
mara-ara-lac)7697 galU galK rpsL (Strr) adA1 rpsL | Invitrogen
| M15(pREP4) | F’ Nal Str Rff Thi- Lac’ Ara’ Gal’ Mtl’ RecA’ Uvr’ Lon’ | QIAGEN

**Plasmids**

pCR 2.1 TOPO | PCR cloning vector; Amp’ Km’ | Invitrogen |
| pJY1464 | S. pneumoniae suicide vector; Em’ | This study |
| pJY1464 | pJY1464-type 3 pplaBpnp region | 23 |
| pBX105 | pYJ1464 plus PCR fragments from primer pairs Cps2-G1-Cps2-G2 and Cps2-J12-Cps2-13, for csp2H deletion | This study |
| pBX108 | pYJ1464 plus PCR fragments from primer pairs Cps2-J12303-Cps2-J2 and Cps2-P1-Cps2-P1546, for csp2I deletion | This study |
| pBX110 | Km resistance gene, aphA-3, between two fragments of pBX105 | This study |
| pBX113 | Km resistance gene, aphA-3, between two fragments of pBX108 | This study |
| pBX115, pBX121 | pYJ1464 plus PCR fragment from primer pairs Cps2-J1-Cps2-P2, for repair of csp2I deletion | This study |
| pBX116 | pYJ1464 plus PCR fragment from primer pairs Cps2D-F-Cps3D-R, for csp3D repair of csp2I deletion | This study |
| pBX123 | pYJ1464 plus 900-bp fragment excised with EcoRI from pCVe646 | This study |
| pBX145 | pYJ1464 plus PCR fragment from primer pair Cps2-D3-Cps2-T1, for repair of csp2E | This study |
| pBX163 | parE-40 containing the full-length csp2K lacking GTG start; from primer pair | This study |
| pBX165 | pC2KORF-BglII/Cps2KORF-KpnI | This study |
| pBV190 | pOE-40 lacking dhfr1 reductase region (BamHI-KpnI deletion) | This study |
| pCE646 | pCE-40 lacking dihydrofolate reductase region (BamHI-KpnI deletion) | This study |
| pJE40 | Expression vector, N-terminal His tag; km’ | QIAGEN |
| pSF151 | Streptococcal shuttle vector containing the Km resistance gene, aphA-3 | 56 |

*S. pneumoniae* capsule assembly
tions. In brief, duplicate cultures were grown in THY to a density of
(ELISAs) were performed as previously described (31, 39) with slight modifica-
screening for large, glossy colonies, and repair of the mutations was confirmed by
Repaired
and the reaction mixture was plated on blood agar plates without selection.
/pCR 2.1-TOPO. The cloned fragment was excised using EcoRI and ligated into
/H9004
ugd
accession no. AE007422), or R6
resistance, which was then confirmed by PCR.

coated overnight at 4°C with twofold serial dilutions of the samples. Wells were

indicated by
Forward and reverse primers are indicated by + and −, respectively.
Superscript numbers indicate the positions of the primer start and end in the
homologous sequence of either type 2 capsule (cps2) (GenBank accession no.
AF264741), type 3 capsule (cps3) (GenBank accession no. U15171), pDL276
aphA-3
(TIGR4 sequence) (GenBank accession no. AE047859), or R6 ugp (GenBank accession no. NC003098).

pCR 2.1-TOPO. The forward primer Cps3F-D includes the Shine-Dalgarno sequence and start codon of pC2D. The cloned fragment was excised using KpnI and ligated between the two fragments in pBIX108, resulting in pBIX116. Confer-
rect orientation of all three fragments was confirmed by PCR. The resulting
construct was transfected into BX511, and the reaction mixture was plated on
blood agar plates without selection. Strain BX517, containing the allelic ex-
change of cps3D for aphA-3 in BX511, was obtained by screening for loss of Km
resistance, which was then confirmed by PCR.

Repair of cps2E in the cps2K-deficient strains was conducted essentially as
described above for the repair of Δcpxp2K mutants. cps2E, along with the 500 bp on
either side, was PCR amplified from D39 chromosomal DNA and cloned into
cpC2D 2.1-TOPO. The cloned fragment was excised using EcoRI and ligated into
pY4164, resulting in pBX145. pBX145 was transformed into BX515 and BX518,
and the reaction mixture was plated on blood agar plates without selection.
Repair of 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 enzyme-linked immunosorbent assays
(ELISAs) were performed as previously described (31, 39) with slight modifica-
tions. In brief, duplicate cultures were grown in THY to a density of ~3 × 10^9
CFU/ml, and 5 ml of each was centrifuged at 20,000 × g for 10 min. The pellets
were resuspended in water at a 50× concentration, and the samples were normalized
to the same optical density at 600 nm. Twenty microliters of the cell suspensions
was used for Cps2D blots, and 10 µl was used for Cps2P blots. Samples were
boiled in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer,
and proteins were separated by SDS–10% PAGE. Proteins were transferred to
a nitrocellulose membrane. Cps2D was detected using a polyclonal rabbit
antiserum as described previously (64). Cps2D was detected using a polyclonal rabbit
antiserum as described previously (64). Cps2D

Electron microscopy. Bacteria were prepared for electron microscopy as
described by Kollman (38). Briefly, cultures were grown to a density of ~3 × 10^10
CFU/ml in THY, and 5 ml of each culture was centrifuged at 20,000 × g for
10 min. The pellets were fixed in 500 µl of a 1% glutaraldehyde–4% formalde-
hyde solution for 30 min at 4°C. Fixed samples were further processed by the Uni-
aversity of Alabama at Birmingham electron microscope core facility for micro-
scopy. In general, samples were postfixed in osmium tetroxide, washed in PBS,
and resuspended in 250 µl of PBS. Cps2D-specific antiserum was used at a 1:70,000
dilution to the same optical density at 600 nm. Cps2D and tyrosine-phosphorylated Cps2D [Cps2D

Relative Cps2E protein levels were determined as described previously (15). In
brief, 10 µg of total protein from isolated S. pneumoniae
membranes was separated by SDS–10% PAGE and subsequently transferred to
a nitrocellulose membrane. Cps2E was detected using a polyclonal rabbit antisera directed
against the C-terminal portion of Cps2 and Cps2E and 1,500.

Capsule replacement experiments and linkage analyses to map suppressor
mutations in Δcpxp2K mutants. Emir resistance markers were linked to the capsule
locus by insertion of pJD37 (pJD insertion, downstream) and pBX123 (between
decl and the capsule promoter, upstream) into the D39 chromosome, resulting in
strains BX516 and BX355, respectively. Chromosomal DNAs of BX516 and
BX355 were isolated using a genomic column prep (Qiagen) and used to transform
the Δcpxp2K mutants BX511 and BX512. Em-resistant transformants
were washed three times in PBS containing 0.5% Tween (PBST) and blocked for 1 h
with 200 µl of 1% bovine serum albumin (BSA) in PBS (BSA-PBS) at room temperature. A rabbit polyclonal anti-type 2 antisera (Statens Serum Institute, Denmark)
denatured against a nonencapsulated 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 × 10^9 CFU/ml and heat killed for 45 min at 50°C.
The culture was centrifuged, washed once in PBS, centrifuged, and resuspended in 250 µl of
anti-type 2 antisera diluted 1/10 in PBS. Adsorp-
tion was conducted by rotating the solution overnight at 4°C. AM1000 was
pelleted, and the supernatant containing the adsorbed anti-type 2 antisera was
filtered (0.22-µm-pore-size syringe filter; Millipore) and stored at 4°C
until use. The adsorbed anti-type 2 antisera was diluted 1/5,000 in BSA-PBS, and 100 µl of this solution was added to each well, followed by incubation at
room temperature for 1 h. The wells were washed three times with PBST and
incubated with biotinylated anti-rabbit immunoglobulin conjugated to
streptavidin-alkaline phosphatase for 1 h at room temperature. The wells were
washed three times with PBST, followed by development with 1 mg/ml-nitro-
phenolphosphate 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 anti-
sera was replaced with a nonadsorbed rabbit polyclonal antisera raised
against a type 19 strain (Statens Serum Institute, Denmark). This antisera
contains a high titer of antibody against noncapsular surface antigens and
provides an effective measure of blocking of the surface by the capsule (31). For
competitive-inhibition ELISAs, cultures were grown as described above. Assays
were performed as previously described (13). Briefly, wells of microtiter plates
were coated as described above with heat-killed D39 at a density of 3 × 10^6
CFU/ml in PBS. Cell lysates used as inhibitors were prepared by growing 10-ml cultures of
each strain to a density of 3 × 10^6 CFU/ml in THY. Samples were normalized
to the same optical density at 600 nm and centrifuged at 20,000 × g for 10 min. Pellets
were resuspended in 0.1 ml lysis buffer (0.1% sodium deoxycholate, 0.01% SDS, 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. Twofold serial dilutions of the lysates or culture
supernatants, together with polyclonal anti-type 2 antisera diluted as described
above, were added to the D39-coated microtitre plates. The remainder of the
procedure was as described above for the indirect ELISAs.
were screened for the large-colony phenotype indicative of capsule production and also for loss of Km resistance.

To sequence cps2E, the gene was PCR amplified from chromosomal DNAs of the Δcps2K 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 previously (37), where Cps2E activity is defined as the ability to transfer [3H]Glc from UDP-[3H]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 MgCl2, and 1 µM UDP-[3H]Glc (1 Ci/mmol; Sigma) at 10°C for 10 min. The reaction was stopped by the addition of 1 ml chloroform-methanol (2:1), and the organic phase was extracted using 200 µl pure urea solvent upper phase (1.5 ml chloroform, 25 ml methanol, 23.5 ml H2O, 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 twofold dilutions of RNA samples were used in slot blot analyses to determine the relative amounts of transcripts. Detection of transcripts and densitometry were performed as previously described (6). PCR probes were digoxigenin labeled (Roche), and the amount of labeling was visualized using Pierce SuperSignal chemiluminescent substrate. ImageJ 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. pneumoniae cultures were grown to a density of ~3 × 108 CFU/ml, and cells were sedimented at 20,000 × g for 10 min at 37°C. Pellets were suspended in protoplast buffer (20% sucrose, 50 mM MgSO4, 50 mM Tris [pH 7.4]) at a concentration equal to the cell wall extract. For samples concentrated 2- or 10-fold, pellets were resuspended in 1/200 or 1/500 of the original culture volume. Forty units of mutanolysin (Sigma) was added to each milliliter, 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 × g for 10 min. The supernatant containing the cell wall fraction was filtered (0.22-µm-pore-size syringe filter; Millipore), and the sedimented protoplasts were resuspended in protoplast buffer 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 7.4)] and 1 µM [3H]Glc (1 Ci/mmol; Sigma) at 4°C overnight. Low-molecular-weight contaminants were removed by dialysis at 37°C overnight. Low-molecular-weight contaminants were removed by dialysis (4°C overnight using 6,000- to 8,000-molecular-weight-cutoff dialysis tubing. Additional debris was removed by centrifugation (20,000 × g for 10 min at 4°C), and the supernatant containing the partially purified polysaccharide was collected and stored at 4°C.

The phenol-sulfuric acid method was used to determine total hexose present in the polysaccharide sample (4). A methylpentose assay was used to determine the amount of rhomannose 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 was grown to a density of ~3 × 108 CFU/ml and centrifuged at 20,000 × g for 10 min at 4°C. Pellets were resuspended in 500 µl of water. Serial dilutions of lysates were analyzed for total uronic acid as described previously (9, 26).

RESULTS

Cps2K exhibits UDP-GlcDH activity. To determine whether cps2K encoded an authentic UDP-GlcDH, 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 Materials and Methods. To facilitate purification of the recombinant protein, it was expressed with an N-terminal His tag. UDP-GlcDH activity was assayed spectrophotometrically by following the reduction of NAD+ to NADH during the oxidation of UDP-Glc to UDP-GlcUA, as described in Materials and Methods. The observed activities for bovine Ugd and Cps2K were 0.36 and 0.21 µmol NADH/min/µg purified protein, respectively (activity for the vector control E. coli strain was 0.0032 µmol 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-GlcDH (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 of S. pneumoniae D39 were generated by allelic replacement of cps2K with an aphA-3-containing fragment encoding resistance to Km, as described in 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 × 107 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 defect (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 noncapsulated mutant (AM1000, Δcps2A to Δcps2IF) in ELISAs with a polyclonal antiserum containing a high titer of antibodies to noncapsular surface antigens (Fig. 2C). In this surface accessibility assay, binding of the antibo-
ies is blocked in proportion to the amount of cell-associated capsule (31).

To further assess capsule production in the \( \Delta \text{cps2K} \) 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.

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

To confirm that the small amounts of capsule produced by the \( \Delta \text{cps2K} \) mutants were not due to undetectable levels of GlcUA arising from the activity of a non-Cps2K UDP-GlcDH, we deleted \( \text{ugd} \) in both the parent D39 and the \( \Delta \text{cps2K} \) 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 \( \text{ugd} \) deletion mutants of D39 and BX511 were identical to those of their respective parents (data not shown), indicating that this gene does not contribute to capsule synthesis in these strains.

\( \text{cps2K} \) deletion mutants contain suppressor mutations. The alterations in capsule synthesis following deletion of \( \text{cps2K} \) 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 \( \text{cps2C} \) and \( \text{cps2M} \), which lie upstream and downstream, respectively, of \( \text{cps2K} \) (Fig. 1B). For both independent \( \text{cps2K} \) mutants, transcription was unchanged from that of the parent strain (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 \( \text{cps2LMNQ} \) 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 \( \text{cps2K} \) deletions did not affect translation of the downstream region.

We next undertook repair of the \( \text{cps2K} \) deletions to confirm that the observed phenotype was due to only the mutation we constructed. Clones containing the entire \( \text{cps2K} \) gene and the 500-bp flanking regions were used to transform the \( \text{cps2K} \) mutants. Transformants in which the allelic exchange of \( \text{cps2K} \) was confirmed by PCR. Unexpectedly, repair of the \( \text{cps2K} \) deletion only partially restored capsule production. The colonies of the repaired mutants, though larger than those of the \( \text{cps2K} \) deletion mutants, were 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 \( \text{cps2K} \)-repaired strains (Fig. 4A), and no capsule was detectable in culture supernatants of these strains (Fig. 4B). Consis-
tent 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 nonencapsulated 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 gene on the D39 chromosome. To screen for the parental phenotype, Em-resistant transformants were then screened for the large-colony phenotype.

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 Em resistance marker either upstream or downstream of the capsule locus, and chromosomal 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 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 assayed for both hexose and methylpentose, and cell walls isolated from a 250-ml culture were assayed for methylpentose. The hexose values represent means ± standard errors for two independent cultures. Values in parentheses are after the subtraction of the Cps− value for AM1000.

Polymer isolated from 250-ml cultures was assayed for both hexose and methylpentose, and cell walls isolated from a 250-ml culture were assayed for methylpentose. The hexose values represent means ± standard errors for two independent cultures. Values in parentheses are after the subtraction of the Cps− value for AM1000.

To map the suppressor mutations, we used as recipients the D39 (Cps− parent) 6.7

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hexose, isolated polymer (μg/10^8 CFU)</th>
<th>Methylpentose</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cps2A</td>
</tr>
<tr>
<td>D39 (Cps− parent)</td>
<td>6.7 ± 0.9 (6.7)</td>
<td>0.004 (0.00)</td>
</tr>
<tr>
<td>BX511 (Δcps2K)</td>
<td>0.35 ± 0.01 (0.35)</td>
<td>0.01 (0)</td>
</tr>
<tr>
<td>AM1000 (Cps−)</td>
<td>0.004 ± 0.001 (0)</td>
<td>0.01 (0)</td>
</tr>
<tr>
<td>BX515 (cps2K repair of BX511)</td>
<td>ND</td>
<td>0.0156 (0.0056)</td>
</tr>
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</table>

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

Relative capsule amounts were determined by competitive inhibition ELISA for cell lysates (A) and culture supernatants (B). Results are shown for the Δcps2K mutant BX511 and its derivative BX515 obtained by repair of cps2K only. Results are the means ± standard errors) from two independent cultures assayed in the same experiment and are representative of two experiments. Identical results were obtained for the independent Δcps2K 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) from two independent cultures assayed in the same experiment and are representative of two experiments. (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.
in Fig. 6A, fragments containing the region between cps2D and cps2T could restore the parental phenotype in two independent mutants, suggesting that the suppressor mutations were located in this region. We therefore 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 Δcps2K 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 to E). Consistent with the reduced numbers of bacteria on agar plates, the cps2E mutants consistently contained suppressor mutations, and repair of both the Δcps2K mutants was due to the cps2E suppressor mutations, and repair of both the cps2K deletion and the cps2E point mutation restored the full parental phenotypes. These 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 mutant 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 that of 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, which are 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 those for the original cps2K mutants (data not shown). Sequence analyses of these mutants revealed cps2E mutations in 15 of the strains. All 15 mutations differed from those isolated originally. The locations of the mutations for these 15 strains are given in Table 1 (strains BX532, BX539, BX547 to -549, BX554, BX555, BX605 to -607, BX609 to -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 4 base pairs downstream of the Δ10 sequence in the predicted capsule promoter located upstream of cps2A. A twofold 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 cps2K 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 in the parent strain (Fig. 7B, strain BX556). The location of the suppressor mutants was consistent with the reduced growth of the Δcps2K mutants on agar plates, the reduced capsule levels in these isolates (Fig. 6B to E). Consistent with the reduced numbers of bacteria on agar plates, the Δcps2K mutants were reclassified by Western immunoblot analysis (data not shown). The sec-

**FIG. 5.** Capsule production by cps2K mutants repaired by capsule locus replacement. (A and B) Relative capsule amounts were determined using intact cells (A) and culture supernatants (B) in indirect ELISAs. Results are shown for the Δcps2K mutant BX511 and its derivative BX519, obtained by replacement of the entire capsule locus. Results are the means (± standard errors) from two independent cultures assayed in the same experiment and are representative of three experiments. Identical results were obtained for the independent Δcps2K 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 those for 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 for left CW/P fractions, BX519 (BX511 repair); Cps replacement for right CW/P fractions, BX522 (BX512 repair).
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 isolate 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 they 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 Materials 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 nonencapsulated mutants. Based on the results for the cps2K mutants, we sequenced cps2E in independent mutants from each construction. All contained mutations. The Δcps2H mutants BX522 and BX505 contained a point mutation resulting in an amino acid change (L199F) and a 1-base-pair deletion resulting in a premature stop at residue 244, respectively. The CPS2E alterations in the Δcps2J mutants BX667, BX668, and BX669 were G411R, I265S, and G371A, respectively. For the Δcps2H mutant BX522, 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 C55 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 polypropenyl-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), and 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, and thus this system was not responsible for the observed reduction in capsule levels or 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, cps2I, 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 (cps2I) 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 or 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) (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 either that the linking enzyme cannot efficiently recognize and/or transfer a single, lipid-linked repeat unit or that 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 enterica serovar 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 mu-
tations in cps2E is perhaps surprising considering the other potential targets where mutations could theoretically abolish capsule production. In vitro, Cps2E catalyzes the addition of G1c-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 mutants. Yet, only 3 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 formation with other sugars (8). This domain appears not to be essential for transfer of the \( \text{S. pneumoniae} \) 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 known whether this observation is a direct effect of the cps2E mutation or relates to the low level of capsule produced.

The \( \text{S. enterica} \) Wbp 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 Wbp 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 and 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.

**Acknowledgments**

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**References**


* \( \text{S. pneumoniae} \) capsule assembly 3379

FIG. 8. Predicted topology of Cps2E determined using the TMpred program from the ExPASy Proteomics website (http://www.expasy.org/tools). Numbers denote amino acid number. ▲, locations of mutations in original \( \Delta \text{cps2K} \) mutants. ▼, locations of mutations contained in additional \( \Delta \text{cps2K} \) mutants. Numbers in parentheses represent the numbers of mutants with the same mutation. * and △, amino acid changes found in \( \Delta \text{cps2H} \) and \( \Delta \text{cps2J} \) mutants, respectively. The DXD motif in the cytoplasmic domain is indicated.


