Streptococcus pneumoniae capsular polysaccharide is linked to peptidoglycan via a direct glycosidic bond to β-D-N-acetylglucosamine

Thomas R. Larson and Janet Yother

For many bacteria, including those important in pathogenesis, expression of a surface-localized capsular polysaccharide (CPS) can be critical for survival in host environments. In Gram-positive bacteria, CPS linkage is to either the cytoplasmic membrane or the cell wall. Despite the frequent occurrence and essentiality of these polymers, the exact nature of the cell wall linkage has not been described in any bacterial species. Using the Streptococcus pneumoniae serotype 2 CPS, which is synthesized by the widespread Wzy mechanism, we found that linkage occurs via the reducing end glucose of CPS and the β-D-N-acetylglucosamine (GlcNAc) residues of peptidoglycan (PG). Hydrofluoric acid resistance, $^{31}$P-NMR, and $^{32}$P labeling demonstrated the lack of phosphodiester bonds, which typically occur in PG-polysaccharide linkages. Component sugar analysis of purified CPS–PG identified only CPS and PG sugars in the appropriate ratios, suggesting the absence of an oligosaccharide linker. Time of flight mass spectrometry confirmed a direct glycosidic linkage between CPS and PG and showed that a single CPS repeat unit can be transferred to PG. The linkage was acetolysis susceptible, indicative of a 1,6 glycosidic bond between CPS and the GlcNAc C-6. The acetylation state of GlcNAc did not affect linkage. A direct glycosidic linkage to PG was also demonstrated for serotypes 8 and 31, whose reducing end sugars are glucose and galactose, respectively. These results provide the most detailed descriptions of CPS–PG linkages for any microorganism. Identification of the linkage is a first step toward identifying the linking enzyme and potential inhibitors of its activity.

Capsular polysaccharide (CPS) assembly and localization in bacteria is a complex, multi-enzyme process leading to anchoring of the CPS polymer on the outer surface of the cell. For pathogens, the protective layer of the CPS can be important for adhesion, biofilm formation, and resistance to complement-mediated opsonophagocytosis and lysis (1, 2). Although substantial information regarding the syntheses of these polymers has accumulated, less is known about the critical steps involved in their attachment to the bacterial surface.

A large number of diverse CPS structures can be elaborated, as exemplified in the Gram-positive pathogen Streptococcus pneumoniae, where 98 CPS serotypes differing in sugar composition and linkages are known (3, 4). S. pneumoniae infections, occurring primarily as pneumonia and sepsis in young children and the elderly, are a significant global disease burden (5). Elaboration of a CPS is essential for these bacteria to cause disease and to colonize the nasopharyngeal cavity (6–8). Despite their structural diversity, all but two of the 98 S. pneumoniae CPS serotypes are synthesized by the Wzy polymerase-dependent mechanism, which is the most common mechanism of CPS synthesis in Gram-positive and Gram-negative bacteria, and is also used in synthesis of many lipopolysaccharide O-antigens in Gram-negative bacteria (9, 10). Polymer synthesis in the Wzy pathway begins with transfer of a sugar-1-phosphate from a nucleotide diphosphosugar donor to the C55 lipid undecaprenyl-phosphate (Und-P). In S. pneumoniae serotype 2 CPS, GlcNAc-1-P is transferred from UDP-GlcNAc (11), and this is followed by addition of the remaining sugars (12, 13) to form the complete repeat unit (Fig. 1). Und-P-P-oligosaccharide repeat units are translocated to the outer face of the cytoplasmic membrane by Wzx and polymerized into high molecular weight (MW) polysaccharide by Wzy. Growth occurs at the reducing end, with single or multiple repeat units being transferred en bloc from Und-P-P to an acceptor Und-P-oligosaccharide repeat unit. Hydrolysis of the donor Und-P-P that remains after transfer yields Und-P, which is recycled back into the cell. In Gram-negative bacteria, CPS is ultimately transported across, and assembled on, the outer membrane (9, 14). For Gram-positive bacteria, CPS is covalently linked to the cell wall and cytoplasmic membrane (15–17); however, the mechanisms involved in ligation are unknown. Failure to transfer CPS from Und-P may result in lethality due to lipid intermediate accumulation and/or reduced turnover of Und-P for essential pathways such as peptidoglycan (PG) (12, 18).

A partial description of a CPS cell wall linkage in a Gram-positive bacterium has been reported for the Wzy-dependent Streptococcus agalactiae type III CPS, in which an oligosaccharide linker and phosphodiester bond are proposed to mediate linkage to the N-acetylgalactosamine (GlcNAc) of PG (16). Exactly how these constituents are linked together or to GlcNAc has not been described. Other PG-linked polysaccharides include wall teichoic and teichuronic acids and the mycobacterial arabino-galactans. The linkage for each of these structures is to the N-acetylmuramic acid (MurNAc) C-6 via a phosphodiester bond. For teichoic acids and arabinogalactans, an oligosaccharide linker is also present (19–24). In this study, we provide a detailed description of a CPS linkage to PG, demonstrating that the S. pneumoniae type capsule | Wzy | Gram positive | pneumococcus | glycobiology

Significance

Bacterial surface polysaccharides form the interface between the bacterium and its environment. In Gram-positive bacteria, the intricate architecture of the peptidoglycan (PG) serves as the scaffolding to which other surface polysaccharides, including capsules, can be anchored. For pathogenic bacteria, proper localization of capsular polysaccharide (CPS) is essential to cause disease. In Streptococcus pneumoniae, CPS is also a primary target for vaccines. Here, we identify a structure for polysaccharide attachment to PG that involves a direct glycosidic linkage. Knowledge of this linkage lays the groundwork for identifying the enzyme(s) involved in its synthesis and for developing new targets for therapeutic interventions.

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1To whom correspondence should be addressed. Email: jyother@uab.edu.

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2 CPS is linked via its reducing end Glc to the GlcNAc of PG by a direct 1,6 glycosidic bond.

**Results**

**CPS Is Linked to PG.** A cell wall fraction containing GlcNAc–MurNAc disaccharides and any linked components was generated by treatment of *S. pneumoniae* with mutanolysin, a β-1,4-N-acetylmuraminidase that cleaves the PG backbone, and LytA, the pneumococcal N-acetylmuramoyl-L-alanine amidase that removes the peptide side chain from MurNAc. The negatively charged CPS was purified from the digest using anion exchange chromatography. CPS eluted in broad fractions, consistent with a range of polysaccharide sizes of varying numbers of repeat units. Gas chromatography and mass spectrometry (GC/MS) analysis identified the sugars present in CPS (Rha, Glc, and GlcUA) and PG (GlcNAc and MurNAc) (Fig. 2). Ribitol was detected in only small amounts, possibly indicative of teichoic acid and CPS on the same PG disaccharide or incomplete PG hydrolysis resulting in copurification of distantly linked CPS and teichoic acid. The molar ratios of Glc, Rha, and GlcUA were those expected from the published serotype 2 structure (11, 25). The GlcNAc and MurNAc ratio of Glc, Rha, and GlcUA were those expected from the published serotype 2 structure (11, 25).

**CPS Is Linked to GlcNAc.** Purified CPS linked to PG disaccharides (which also contains low amounts of teichoic acid, as noted above, but henceforth referred to as CPS–PG) was subjected to β-N-acetylgalactosaminidase to hydrolyze β-D-GlcNAc-1,4-β-D-MurNAc bonds. After ultrafiltration (3,000 Da cutoff), MurNAc was not detected by GlcNAc was identified in the low MW fraction, indicating that the latter remained linked to high MW CPS (Fig. 3). Sodium borodeuteride (NaBD₃) treatment of the high MW fraction resulted in reduction of GlcNAc to deuterated N-acetylgalactosaminol (GlcNAc-ol), indicating that MurNAc was released by the digestion and GlcNAc had remained at the reducing end of the high MW CPS (Fig. 4, A, C, and E). Similar treatment of a non–β-N-acetylgalactosaminidase-digested control sample demonstrated no reduction of GlcNAc (Fig. 4, B and D). Sodium borodeuteride-reduced products were not detected for Glc, Rha, or GlcUA, consistent with linkage of their anomeric C-1 carbons to other sugars. Taken together, these data indicate that CPS and PG are covalently linked, and GlcNAc is located at the reducing end of CPS.

**CPS–PG Linkage Does Not Involve Phosphate Bonds.** To test for phosphodiester bonds, CPS–PG was subjected to digestion by 48% hydrofluoric acid (HF). After ultrafiltration (3,000 Da cutoff), all CPS sugars were identified in the high MW fraction in a ratio consistent with the serotype 2 CPS structure (Fig. 5, A, a). GlcNAc and MurNAc were also present in this fraction (Fig. 5, a–c). Consistent with the known phosphodiester bonds in *S. pneumoniae* teichoic acid repeat units (19, 22), HF treatment resulted in loss of the teichoic acid-specific ribitol and N-acetylglactosamine from the high MW fraction (Fig. 5, A, a) and their appearance in the low MW fraction, where neither CPS nor PG sugars were detected (Fig. 5, A, d). The CPS–PG linkage is thus resistant to HF and likely not mediated by a phosphodiester bond.

To further test for phosphate-mediated linkages, CPS–PG was analyzed using 31P-NMR. Several distinct chemical shifts indicating phosphate and phosphate-mediated bonds were observed (Fig. 5B, A). After treatment with HF, phosphate was removed from the CPS-containing fraction (Fig. 5B, b) and identified in only the lower MW fraction (Fig. 5B, c), consistent with its association with teichoic acid. The complete removal of phosphate without hydrolyzing the CPS–PG bond demonstrates the lack of phosphodiester bond involvement.

To test for any phosphate in the CPS–PG structure, cells were grown in the presence of [32P]phosphate and then separated into cell wall- and membrane-containing fractions. Following SDS/PAGE, 32P incorporation was observed in low MW products only, consistent with the small number of repeat units present in teichoic acid (26) and the detection of teichoic acid-specific bands by immunoblotting (Fig. 5C, a and b). The greater 32P incorporation observed in the membrane fraction reflects the higher amount of membrane-linked lipoteichoic acid, which also contains phosphodiester bonds (19). No 32P was detected in higher MW bands, indicative of the absence of phosphate in the CPS–PG linkage (Fig. 5C, a and c). The lack of 32P signal and CPS overlap also indicates that single repeat units of PG disaccharides rarely contain both CPS and teichoic acid (an outcome previously suggested by failure of antisera to teichoic acid to react with higher MW bands) (15, 27).

**Linkage of CPS to PG Involves a Direct Glycosidic Bond.** Component sugar analysis of CPS–PG with or without HF treatment identified the CPS and PG sugars in their expected ratios, showing no evidence of a linker (see above). To further test for a linkage unit, low MW

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**Fig. 1.** Proposed structure of serotype 2 CPS linked to PG, as determined in this study. The CPS structure is from refs. 11 and 25. PG residues are indicated in blue. Asterisk indicates 80% of the GlcNAc residues are deacetylated in *S. pneumoniae* (28). GlcUA, glucuronic acid; Rha, rhamnose.

**Fig. 2.** CPS is linked to PG. GC/MS total ion chromatogram of cell wall-linked CPS. The molar ratios of Glc, Rha, and GlcUA are consistent with those of serotype 2 CPS. The ratios of the GlcNAc and MurNAc peaks reflect equimolar concentrations, as determined using purified sugar standards. Rib-ol, ribitol. MCounts, megacounts.
CPS was purified by isolation and ultrafiltration of early elutions of CPS–PG from anion exchange chromatography. Triple Q-time-of-flight mass spectrometry (Q-TOF MS) demonstrated two major peaks (Fig. 6). The larger peak at m/z 1,361.99 corresponded to the mass expected for a single repeat unit of CPS linked by a glycosidic bond to a PG disaccharide with the loss of m/z 73, which is likely that of the lactate group on MurNAc. The smaller peak at 1,435.02 with a shift of m/z 73 represents the intact MurNAc on the PG. Taken together, these results show that CPS is directly linked to PG via a glycosidic bond, and a single repeat unit of CPS can be transferred to PG.

**Linkage of CPS to PG Does Not Require Deacetylation of GlcNAc.** Approximately 80% of the S. pneumoniae PG GlcNAc is deacetylated at the 2-acetamino position, resulting in resistance to lysozyme (28). Inactivation of the deacetylase-encoding gene (pgdA) did not alter CPS localization to the cell wall (Fig. S1). The acetylation state is thus not relevant, and there is likely no role for Pgda or the 2-acetylaminomalposition of GlcNAc in the attachment of CPS to PG.

**CPS Linkage Does Not Occur Through the GlcNAc C-2 or C-3.** Smith degradation (29) was used to determine whether the GlcNAc C-2 and C-3 are protected from periodate oxidation, as would be expected if CPS linkage occurred at either of these sites. GC/MS of the reaction products demonstrated degradation of Glc but not Rha (Fig. 7A), consistent with the presence and absence, respectively, of vicinal hydroxyls in these sugars in CPS (Fig. 1). For the PG sugars, MurNAc was not degraded but GlcNAc was reduced to 16.7% of the amount contained in a nonperiodate-treated sample (Fig. 8A). No GlcUA was identified in this fraction (Fig. 8A), demonstrating that all 1,6 bonds had been cleaved. Likewise, no GlcNAc was present in the high MW fraction, demonstrating that it was linked to CPS by a 1,6 bond (Fig. 8B). The lower MW fraction contained the majority of the Glc and Rha (Fig. 8C), and all of the GlcUA (Fig. 8C) and GlcNAc (Fig. 8D). The acetylation labile nature of the CPS–PG linkage is indicative of a 1,6 glycosidic bond. As detailed in Discussion, the collective results provide strong evidence for linkage occurring between the C-6 of GlcNAc in the PG and C-1 of Glc in the CPS backbone.

**Direct Glycosidic Bonds Mediate CPS–PG Linkages of Other S. pneumoniae Serotypes.** More than 75% of the 96 known S. pneumoniae Wzy serotypes, including serotype 2, initiate CPS synthesis with Glcp (3, 4). To examine the CPS–PG linkages in serotype 8, which also initiates with Glcp (Fig. S2), and serotype 31, which initiates with galactose (Galp) (Fig. S3A), isolated CPS–PG was treated with HF and purified by ultrafiltration. GC/MS analysis of the high MW fractions demonstrated only the

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**Fig. 3.** CPS is linked to the GlcNAc residue of PG. GC/MS analysis of low MW products following β-N-acetylgalactosaminidase digestion of CPS–PG. (A) Selective ion monitoring for MurNAc at m/z 187. (B) Selective ion monitoring for GlcNAc at m/z 173.

**Fig. 4.** GlcNAc is at the reducing end of CPS. CPS–PG was either treated with NaBD4 or first digested with β-N-acetylgalactosaminidase followed by isolation of high MW product and subsequent NaBD4 treatment. (A) GC/MS total ion chromatogram of enzyme plus NaBD4-treated CPS–PG. (B) Total ion chromatogram of NaBD4-treated CPS–PG. (C) Selective ion monitoring at m/z 173 (GlcNAc) and 174 (deuterated GlcNAc-ol) of enzyme plus NaBD4-treated CPS–PG. The arrow is the reduced GlcNAc-ol. (D) Selective ion monitoring at m/z 173 and 174 of NaBD4-treated CPS–PG. (E) Electron impact mass spectrum of the GlcNAc-ol peak in C demonstrates the mass spectral fragmentation pattern of deuterated GlcNAc-ol, which resulted from reduction of GlcNAc after release of MurNAc by β-N-acetylgalactosaminidase. kCounts, kilocounts; McCounts, megacounts.
The CPS-PG linkage does not contain phosphate bonds. (A) Resistance to HF. GC/MS analysis of HF-treated CPS–PG. (a) Total ion chromatogram of high MW fraction. (b) Selective ion monitoring of high MW fraction for GlcNAc at m/z 173. (c) Selective ion monitoring of high MW fraction for MurNAc at m/z 187. (d) Total ion chromatogram of low MW fraction demonstrating teichoic acid sugars ribitol (Rib-ol) and N-acetylgalactosamine (GalNAc). (B) Absence of phosphodiester bonds. $31^P$-NMR spectra of CPS–PG and HF-treated products. (a) CPS–PG before HF treatment. (b) High MW CPS–PG fraction obtained after HF treatment. (c) Low MW teichoic acid-containing fraction obtained after HF treatment. (C) Absence of phosphate. Cell wall (CW) and membrane (M) fractions from bacteria grown with $^{32}P$-phosphate metabolic labeling. Likewise, there is no evidence for an oligosaccharide linker as: (i) GC/MS analyses demonstrated only CPS- and PG-specific sugars in the expected ratios, and (ii) the molecular mass of a single CPS repeat unit linked to a PG disaccharide was consistent with the presence of only these sugars.

Susceptibility of the CPS–GlcNAc linkage to acetylation is indicative of a 1,6 glycosidic bond, and is consistent with observations making linkage to carbons other than the GlcNAc C-6 unlikely, as discussed above. During CPS synthesis, Glcp is the reducing sugar following addition of Glc-1-P to Und-P to form β-D-Glc-1-P-P-Und (11). As demonstrated here, the Glc anomer C-1 remains bonded

CPS and PG sugars in the expected ratios (Figs. S2 and S3A). The absence of ribitol in this fraction was indicative of HF cleavage of the phosphodiester bonds of teichoic acid repeat units. Q-TOF MS of CPS–PG from serotype 31 revealed a peak corresponding to the mass expected for a single repeat unit of CPS linked by a direct glycosidic bond to a PG disaccharide (Fig. S3B). These results demonstrate the lack of phosphodiester bonds and oligosaccharide linkers in these CPS structures.

**Discussion**

Bacterial CPS synthesis occurs by three mechanisms: ABC transporter dependent (Gram negatives), synthase dependent (Gram positives), and Wzy dependent (Gram negatives and Gram positives) (9, 10). Cell surface attachment is the final step in each of these processes. In Gram-negative bacteria, linkage is to the outer membrane, either via a linker and a phosphatidylglycerol anchor (ABC transporter pathway) (32) or as a result of ionic interactions with an outer membrane protein that serves as a nucleation point (Wzy pathway) (14). In Gram-positive bacteria, cytoplasmic membrane linkage via phosphatidylglycerol occurs in the synthase pathway (33). In the Wzy pathway, CPS synthesized on Und-P is transferred to PG, and there is also evidence for transfer to an alternate membrane acceptor (18).

The most detailed descriptions of PG–polysaccharide linkages are for the teichoic acids, teichuronic acids, and mycobacterial arabinogalactans. The reducing ends of these polymers are linked to the MurNAc C-6 through a phosphodiester bond and, for teichoic acids and arabinogalactan, an oligosaccharide linker (polysaccharide–oligosaccharide–phosphodiester–MurNAc) (19–24). Similar components may comprise the *S. agalactiae* type III CPS–PG structure, except that this linkage is proposed to be CPS–phosphodiester–oligosaccharide–GlcNAc (16). However, complete analyses to confirm such a structure have not been reported. It was suggested that, like teichoic acid, the *S. aureus* CPS may be linked to the MurNAc C-6 (34); however, no experimental evidence has been presented for this possibility. Additional information regarding any aspect of CPS linkages to the cell wall is sparse.

The present study provides a detailed description of a CPS–PG linkage and demonstrates that, unlike other surface polysaccharide–PG linkages, it is mediated by a direct glycosidic bond. The results provide a clear demonstration that the phosphodiester bonds and oligosaccharide linkers common to other polysaccharide–PG linkages are missing here. Evidence for the lack of a phosphodiester bond came from resistance of the linkage to HF and absence of detectable phosphorous by either $^{31}P$ NMR or $^{32}P$-phosphate metabolic labeling. Likewise, there is no evidence for an oligosaccharide linker as: (i) GC/MS analyses demonstrated only CPS- and PG-specific sugars in the expected ratios, and (ii) the molecular mass of a single CPS repeat unit linked to a PG disaccharide was consistent with the presence of only these sugars.

Susceptibility of the CPS–GlcNAc linkage to acetylation is indicative of a 1,6 glycosidic bond, and is consistent with observations making linkage to carbons other than the GlcNAc C-6 unlikely, as discussed above. During CPS synthesis, Glcp is the reducing sugar following addition of Glc-1-P to Und-P to form β-D-Glc-1-P-P-Und (11). As demonstrated here, the Glc anomer C-1 remains bonded
to another molecule in CPS–PG, as sodium borodeuteride reduction did not produce a deuterated glucitol. The observation of a single, complete CPS repeat unit linked to GlcNAc is consistent with a glycosyltransferase that recognizes the reducing end of CPS and forms a Glc-1,6-GlcNAc linkage; any other mechanism would likely require two enzymes to cleave and transfer an oligosaccharide and forms a Glc-1,6-GlcNAc linkage; any other mechanism would likely require two enzymes to cleave and transfer an oligosaccharide donor (i.e., a non-Leloir enzyme) and possesses an extracellulose linkage to a single Und-P-P linkage. The 96 Wzy CPS serotypes comprise 44 serogroups, wherein the serogroups contain immunologically and chemically related serotypes (3, 4). Wzy proteins exhibit high diversity, with 40 homology groups that are largely serogroup specific (3). These enzymes could potentially provide the specificity necessary for the recognition of such diverse polymers, and the generation of a similar linkage to PG across serotypes.

CPS transfer to PG appears to occur essentially at random, as the polymer sizes on the membrane and cell wall are similar and range from very small to very large (15) (Fig. S1). Indeed, the results of the present study demonstrate that a single CPS repeat unit can be transferred to PG. Simultaneous modification of adjacent MurNAc and GlcNAc residues with teichoic acid and CPS, respectively, appears rare, as neither 32P nor immunoreactive teichoic acid colocalizes with CPS on PG disaccharides, and only low levels of teichoic acid sugars copurify with CPS–PG.

The structure of the S. pneumoniae CPS linkage is distinct among PG-linked polysaccharides. Its identification opens the way for comparison with other CPS–PG linkages and provides the foundation for identifying the linking enzyme and potential inhibitors of its activity.

Materials and Methods

S. pneumoniae serotype 2 strain D39 was used for CPS isolation. Other strains, growth conditions, procedures for 32P incorporation, and immunoblotting are described in SI Materials and Methods. Monosaccharide analyses by GC/MS were performed essentially as described previously (39) and are detailed in SI Materials and Methods.

CPS–PG Preparation and Carbohydrate Treatments. Protoplast and cell wall fractions were generated by incubation of bacteria with mutanolysin and autolysin (LytA). CPS–PG was purified from the cell wall fractions using DEAE cellulose anion exchange chromatography. Details for enzyme digestions, HF treatment, sodium borodeuteride reduction, Smith degradation (29), and acetylation (as in ref. 40 with minor modifications) are provided in SI Materials and Methods. High and low MW products were separated using ultrafiltration (Amicon; 3,000 Da cutoff).

![Fig. 7](image-url) The GlcNAc C-2 and C-3 are not involved in CPS linkage. GC/MS analysis following Smith degradation of CPS–PG. (A) Total ion chromatogram. The degradation product (blue) is overlaid with a nontreated control (red). Glc, but not Rha, is largely degraded. (B) Selective ion monitoring for PG sugars at m/z 173 and 187 following degradation (blue) overlaid with the untreated control (red). (Inset) MurNAc at higher magnification. kCounts, kilocounts.

![Fig. 8](image-url) The CPS–PG linkage is susceptible to acetylation. GC/MS analysis of CPS–PG following acetylation. (A) Total ion chromatogram of the high MW fraction. (B) Selective ion monitoring of the high MW fraction at m/z 173 revealed no GlcNAc. (C) Total ion chromatogram of the low MW fraction. (D) Selective ion monitoring of the low MW fraction at m/z 173. kCounts, kilocounts.

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