A New Model of Pneumococcal Lipoteichoic Acid Structure Resolves Biochemical, Biosynthetic, and Serologic Inconsistencies of the Current Model[▽]

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Lipoteichoic acid (LTA) is an essential bacterial membrane polysaccharide (cell wall component) that is attached to the membrane via a lipid anchor. According to the currently accepted structure of pneumococcal LTA, the polysaccharide is comprised of several repeating units, each of which starts with glucose and ends with ribitol, with the lipid anchor predicted to be Glc(β1→3)AATGal(β1→3)Glc(α1→3)-acyl₂Gro, where AATGal is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose. However, this lipid anchor has not been detected in pneumococcal membranes. Furthermore, the currently accepted structure does not explain the Forssman antigen properties of LTA and predicts a molecular weight for LTA that is larger than its actual observed molecular weight. To resolve these problems, we used mass spectrometry to analyze the structure of LTA isolated from several pneumococcal strains. Our study found that the R36A pneumococcal strain produces LTA that is more representative of pneumococci than that previously characterized from the R6 strain. Analysis of LTA fragments obtained after hydrofluoric acid and nitrous treatments showed that the fragments were consistent with an LTA nonreducing terminus consisting of GalNAc(α1→3)GalNAc(β1→, which is the minimal structure for the Forssman antigen. Based on these data, we propose a revised model of LTA structure: its polysaccharide repeating unit begins with GalNAc and ends with AATGal, and its lipid anchor is Glc(α1→3)-acyl₂Gro, a common lipid anchor found in pneumococcal membranes. This new model accurately predicts the observed molecular weights. The revised model should facilitate investigation of the relationship between LTA's structure and its function.

As a major component of the membrane (cell wall) of all gram-positive bacteria, lipoteichoic acid (LTA) is important for bacterial survival, growth, and pathogenicity. Along with teichoic acid (TA), LTA forms a polyanionic barrier, which provides protection against many cationic antimicrobial peptides such as bacteriocins (e.g., nisin) or host antimicrobial peptides (e.g., defensin) (1, 8, 31, 32). LTA is also important for bacterial growth since LTA can inhibit autolysin, which is critical for cell wall remodeling and necessary for replication (22). In addition, LTA is critical in pathogenesis as it is involved in bacterial adhesion to host cells (2, 7, 9) and initiates inflammatory cascades by activating complement (24), Toll-like receptor 2 (12, 19, 41), and/or CD36, a C-type lectin (21, 36). In fact, a monoclonal antibody to LTA has been shown to protect animals from experimental infections (46, 49, 51).

As the effectiveness of LTA in mediating these functions is highly dependent on small changes in LTA's structure, such as alanine or phosphocholine (PC) decorations (11, 29, 31, 53), it is important to have an exact model of LTA structure. Fischer and his colleagues extensively studied LTA purified from the R6 strain of *Streptococcus pneumoniae* (14, 15) and showed that pneumococcal LTA is composed of a lipid anchor and a pentameric repeating unit that contains Glc, AATGal (2-acet-

amido-4-amino-2,4,6-trideoxy-D-galactose), two GalNAc, and ribitol-phosphate residues (Fig. 1A) (14, 15). Either one or both of the GalNAc residues is modified with PC. Based on this chemical structure, it is assumed that the polymer is formed by linking two to eight repeating units that begin with glucose and end with ribitol with phosphodiester bonds (Fig. 1A). The polymer would then be linked to a cytoplasmic membrane lipid anchor: Glc(β1→3)AATGal(β1→3)Glc(α1→3)-acyl₂Gro. The lipid anchor is critical for attaching LTA to the cytoplasmic membrane (3, 13, 17, 30).

The currently accepted structure proposed for pneumococcal LTA (Fig. 1, model A) has several problems. For instance, the predicted lipid anchor could not be detected in pneumococcal membranes (14). The accepted structure does not contain the terminal GalNAc(α1→3)GalNAc(β1→, which is necessary to explain the Forssman antigen properties of pneumococcal LTA (3, 20). In addition, when we examined the mass spectra of LTA from the R36A strain, we found its mass to be about 350 atomic mass units (amu) less than the predicted mass (29, 42). To investigate these problems, we hypothesized that the repeating unit begins with AATGal instead of ribitol and that the repeating unit is anchored to Glc(α1→3)-acyl₂Gro (Fig. 1B), which is abundantly present in the pneumococcal membrane (6, 14, 27). Our evaluation of the two proposed models of LTA structure (models A and B) follows.

MATERIALS AND METHODS

Chemicals and bacterial isolates. Hydrofluoric (HF) acid (48%) and sodium nitrite (NaNO₂) were obtained from Sigma-Aldrich (St. Louis, MO). *S. pneu-*

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A. Model A

B. Model B

FIG. 1. Models of the molecular structure of *S. pneumoniae* LTA. Model A depicts the currently accepted structure (14), and model B depicts a newly proposed structure. Models A and B have lipid anchors of 1,105 and 756 amu, respectively, but both models have repeating units of 1,299 amu. For the LTA with six repeating units, models A and B predict 8,922 and 8,573 amu, respectively. *n*, number of repeating units.

moniae strains R6 (ATCC BAA-255) and R36A (ATCC 12214) were obtained from the American Type Culture Collection (Manassas, VA). The parental strain of R6 is R36A, which is a nonencapsulated strain derived from a capsular type 2 clinical isolate (strain D39) (23, 25). A capsular type 3 isolate (TU2) was isolated as described previously (5). A capsular type 4 clinical isolate (TIGR4) (44) and a capsular type 6B clinical isolate (MX-73HIM), which was isolated from the pleural fluid of a pneumonia patient in Mexico, were provided by S. Hollingshead (University of Alabama at Birmingham, Birmingham, AL).

Purification of LTA. Pneumococcal LTA was purified by using Bligh-Dyer organic solvent extraction (4), octyl-Sepharose chromatography, and an ion exchange chromatography method, as described previously (3, 29). Briefly, pneumococci were cultured at 37°C for 10 h in Todd-Hewitt broth (Difco, Detroit, MI) with 0.5% yeast extract (Difco). Pelleted pneumococci were resuspended in 0.05 M sodium acetate (pH 4.0) and lysed by ultrasonication (Sonicator model W-220F; Heat Systems Ultrasonics, Inc., Plainview, NY). After its extraction from the lysate with a chloroform and methanol mixture, the LTA was adsorbed onto an octyl-Sepharose CL-4B column (Sigma-Aldrich) equilibrated in a mixture of 15% *n*-propanol and 0.05 M sodium acetate (pH 4.7). After the LTA was eluted with 35% *n*-propanol, the LTA was further purified by Q-Sepharose ion exchange chromatography (Sigma-Aldrich) equilibrated in 10 mM 2-amino-2-methyl-1-propanol-HCl (pH 9.5) (Sigma-Aldrich) with 25% *n*-propanol. LTA was eluted using a 0 to 0.6 M sodium chloride gradient.

Treatment with 48% HF acid. Five milligrams of purified R36A LTA in a 10-ml polypropylene tube was hydrolyzed with 1 ml of 48% HF at 4°C for 36 h to hydrolyze completely or for 3 h to hydrolyze partially. The samples were dried

under a nitrogen stream for 1 h at room temperature to remove the HF. The samples were adsorbed onto an octyl-Sepharose CL-4B column (0.5 by 5 cm). After the column was washed with a mixture of 15% *n*-propanol and 50 mM sodium acetate (pH 4.7), the acylated LTA fragments were eluted from the column with 35% *n*-propanol.

Deamination with HNO₂. Five milligrams of purified R36A LTA was dissolved in 0.5 ml of 0.2 M sodium acetate (pH 4.0) containing 0.5% sodium nitrite. After 40 min of incubation at room temperature, the sample was lyophilized and dissolved in 0.5 ml of phosphate-buffered saline (pH 7.2).

MALDI-TOF MS. Purified and treated LTA samples were analyzed using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer from PerSeptive Biosystems (Framingham, MA) in the Mass Spectrometry Shared Facility at the University of Alabama at Birmingham. Briefly, a mixture of 1 μ l of sample and 1 μ l of matrix solution (0.5 M 2,5-dihydroxybenzoic acid and 0.1% trifluoroacetic acid in methanol) was applied to a sample plate. After the sample was dried, it was analyzed with a MALDI-TOF mass spectrometer.

MS/MS. The tandem mass spectrometry (MS/MS) analyses of LTA fragments were performed in the Mass Spectrometry Shared Facility at the University of Alabama at Birmingham with a Micromass Q-TOF2 mass spectrometer (Micromass Ltd., Manchester, United Kingdom) equipped with an electrospray ion source. After being dissolved in distilled water, the samples were injected into the mass spectrometer along with running buffer (acetonitrile-water [1:1] containing 0.1% formic acid) at a rate of 1 μ l/min using a Harvard syringe pump. The injected sample was positively ionized with electrospray (needle voltage of 2.8

FIG. 2. Panels A to F show the mass spectra of LTAs isolated from several strains. Strains are identified at the top of each panel. Each major peak is labeled with its own amu. Each peak has at least three satellite peaks that differ from the major peak by 26 to 28 amu. The satellite peaks are shown for the mass spectra of R36A LTA (B, insert). R6 LTA (A) has three groups of peaks (labeled X, Y, and Z), with the peaks within a group being separated by about 165 amu. Panels G and H show for models A and B, respectively, the predicted masses of LTAs with five, six, or seven repeating units and with zero, one, two, or three PC groups missing. The predicted mass was calculated as follows (23): $(1,299 \times \text{number of repeating units}) + \text{mass of the lipid anchor} - (\text{number of missing PC groups} \times 165) + \text{mass of Na}^+$. The mass of the lipid anchor is 1,105 amu for model A and 756 amu for model B.

kV) and detected with a TOF mass spectrometer. For MS/MS, the parent ion was fragmented into daughter ions by energizing it to 34 to 50 eV before colliding it with argon gas. The daughter ions were analyzed with a TOF mass spectrometer. The MS/MS spectra were processed using the Max-Ent3 module of the MassLynx program, version 3.5.

RESULTS

(i) R36A LTA is representative of pneumococcal species. In our previous studies, we observed that the mass of LTA isolated from the R36A strain is about 350 amu less than the mass predicted by the currently accepted structure of LTA (29). Since the accepted model of LTA structure was established with LTA isolated from *S. pneumoniae* strain R6 (3), we wanted to exclude strain differences as the basis for the observed mass discrepancy. To do this, we isolated LTA using a method based on Fischer's classical method (3, 29) and directly compared the mass spectra of LTA preparations from both the R36A and R6 strains. Even though the classical method is known to remove alanine groups from ribitol (11), we used the method to facilitate comparison of Fischer's results with ours. Consistent with our previous report (29), the mass spectrum of R36A LTA showed three major peaks at approximately m/z 7,272, 8,573, and 9,872 (Fig. 2B). The three peaks correspond

to LTA with five, six, and seven repeating units and a lipid anchor of about 756 amu (i.e., $8,573 = 6 \times 1,299 + 756 + \text{mass of Na}^+$). Each major peak was accompanied by several satellite peaks with about 28- or 26-amu differences (Fig. 2B, insert). These satellite peaks reflect the microheterogeneity in acyl groups (3). The mass difference between the major peaks is 1,299 to 1,302 amu, which corresponds to an oligosaccharide repeating unit that contains two PC groups. The currently accepted LTA structure shown as model A in Fig. 1 predicts 8,922 amu for LTA with six repeating units (Fig. 2G). Since model A also has a repeating unit mass of 1,299, the mass discrepancy of 349 amu (8,922 versus 8,573) is likely not in the repeating unit but, rather, in the lipid anchor (Fig. 2G and H).

In contrast, LTA from the R6 strain shows a different mass spectrum (Fig. 2A). Instead of three isolated peaks, it shows three groups (X, Y, and Z) of peaks, with each group containing several distinct peaks that are separated by about 165 amu, which corresponds to the mass of PC. For instance, group Y has seven peaks, three of which are heavier and three of which are lighter than the 8,075 peak. The seven peaks can be explained if they represent LTA with six repeating units but lacking zero to six PC groups. The peak at 8,075 should represent LTA missing three PC groups since the three heavier

peaks should represent LTA without zero, one, or two PC groups, and the three lighter peaks should represent LTA missing four, five, or six PC groups. Furthermore, the masses of these peaks are correctly predicted by model B (Fig. 2H) but not by model A (Fig. 2G).

Groups X and Z have six and eight distinct peaks, respectively, and the mass difference among the fourth peaks (from the right) in each of the three groups (6,774, 8,075, and 9,375) is roughly 1,300 amu. The mass spectra suggest that group X represents LTA with five repeating units and that its peaks represent LTA lacking zero to five PC groups. Similarly, group Z peaks represent LTA with seven repeating units missing zero to seven PC groups. The masses of the peaks in groups X and Z could also be correctly predicted by model B (Fig. 2H). Consistent with our findings, the repeating units of the R6 LTA were reported to have either one or two PC residues (3). Thus, the R6 and R36A LTAs differ in their mass spectra, but this difference is due only to the variable number of PC residues per repeating unit.

Since the R6 and R36A LTAs differ in structure, we determined the typical structure of pneumococcal LTA by studying LTAs purified from several different strains of *S. pneumoniae*, including some clinical isolates (Fig. 2C to F). All of the purified LTAs showed three major peaks and satellite peaks that are almost identical to the peaks of R36A LTA (Fig. 2). Based on these data, we conclude that R36A LTA is more representative than R6 LTA of pneumococcal LTA (Fig. 2).

(ii) Complete hydrolysis of LTA with 48% HF yields molecular products predicted only by model B. To determine if model B correctly predicts the structure of LTA, we subjected LTA purified from R36A to hydrolysis with 48% HF. HF rapidly cleaves phosphodiester and phosphomonoester bonds and then slowly cleaves the linkage between GalNAc and ribitol (3, 14, 26, 35). As a result of these hydrolysis reactions' occurring at different rates, oligosaccharides without any PC (Fig. 3, product B1) would be produced due to the hydrolysis of the phosphodiester bond between the ribitol and Glc residues and due to the loss of the PC residues. Following these reactions, some of the resulting oligosaccharides would slowly lose the ribitol (Fig. 3, product B2). If model B is the correct structure of LTA, then HF treatment would yield hydrolysis products A1 and A2 (Fig. 3B), whereas if model A is correct, then these products would not be observed (Fig. 3A).

Following HF treatment for 36 h at 4°C and analysis of the hydrolysate of R36A LTA by MS, we observed peaks at m/z 581 and 599, which, respectively, correspond to the sodium adducts of product A1 that are anhydrous and hydrated (Fig. 3C). The spectra also showed peaks at m/z 425 and 447, which correspond to product A2 and its sodium adduct, respectively (Fig. 3C). The chemical nature of these four peaks was further confirmed by analyzing their daughter ions by MS/MS (data not shown). Since the peaks representing products A1 and A2 cannot be explained with model A, these data support model B as the correct structure of pneumococcal LTA. Products A1 and A2 were also observed with R6 LTA, indicating that model B is also appropriate for R6 LTA.

The mass spectra of R36A and R6 LTA also showed peaks with masses that were consistent with the hydrolysis products labeled B1, B2, C1, and C2 in Fig. 3B. When the chemical nature of these peaks was investigated by MS/MS, the B1 and

B2 peaks were found to correspond, respectively, to oligosaccharide with or without ribitol, and the C1 and C2 peaks were found to correspond, respectively, to lipid anchors with two or one acyl chains. The satellite peaks with +26 or -28 amu differences associated with C1 and C2 also reflect the microheterogeneity of their acyl chains. Our findings are consistent with a previous report (3) that showed that the primary peak has $C_{18:1}$ and $C_{16:0}$ acyl chains, the +26 amu peak has two $C_{18:1}$ acyl chains, and the -28 amu peak has $C_{16:1}$ and $C_{16:0}$ acyl chains.

(iii) Partial hydrolysis of LTA with HF produces new peaks that have shifted down by 952 amu. Hydrolysis with 48% HF for a very short time (3 h at 4°C) should cleave only the phosphodiester and phosphomonoester bonds of the LTA, either removing PC residue(s) or making a single cut at the phosphodiester bond between the ribitol and Glc (Fig. 4A, dotted lines). If model A is correct, this partial digest would remove one or more complete repeating units. Since each repeating unit has 1,299 amu, the mass of new peaks would be reduced by multiples of 1,299 amu, and all of the new peaks would be "in phase" with original peaks. In contrast, if model B is correct, this partial hydrolysis would remove the terminal GalNAc-GalNAc-ribitol oligosaccharide, and "out-of-phase" peaks that differ by 951 amu would be observed (Fig. 4A). Based on the MALDI-TOF spectroscopy, masses of molecular fragments were calculated for their sodium adducts since phospholipids and LTA generally appear as ions with sodium adducts in MALDI-TOF studies (3, 16, 40).

Following a partial digestion of the R36A LTA and selection of acylated fragments by purification over octyl-Sepharose, peaks of 952 amu less than the original peaks (m/z 8,923, 7,624, and 6,322) were observed by MALDI-TOF MS (Fig. 4C). Additional out-of-phase peaks at m/z 5,020 and 3,718 were also observed. These peaks, which are separated by 1,299 to 1,302 amu, reflect the ions that have lost additional repeating units in addition to the 952-amu loss. In addition to these peaks, new peaks were found at m/z 7,109, 8,244, and 8,410. These peaks have values of about 165 or 330 amu less than the original peaks and should represent the loss of one or two PC residues, which is also expected to occur during HF hydrolysis. Thus, these findings support model B.

(iv) Partial deamination of LTA with HNO does not create new peaks. A deamination reaction with $NaNO_2$ in acid primarily cleaves the glycosidic bond on the reducing end of AATGal, although the reaction can secondarily cleave the glycosidic bond on the nonreducing end of AATGal (3, 15, 34). If deamination reaction conditions can be adjusted to permit only one cut at the primary cleavage site, the partial deamination could also be used to distinguish between the two models of LTA structure. Specifically, model A predicts the production of out-of-phase peaks shifted by 348 amu, while model B does not (Fig. 5A).

Pneumococcal LTA was subjected to a mild deamination and then examined by MALDI-TOF MS. The reaction product, which was not purified over the octyl-Sepharose column, contained both acylated and nonacylated fragments. Reflecting this, MALDI-TOF spectra showed peaks with and without satellite peaks (Fig. 5C). New peaks without satellite peaks were found at m/z 3,914, 5,215, 6,516, 7,816, and 9,117. New peaks with satellite peaks were found at m/z 3,367, 4,668, and

FIG. 3. Panel A shows the currently accepted pneumococcal LTA structure (model A), and panel B shows the newly proposed LTA structure (model B). Dashed lines indicate the cleavage sites resulting from 48% HF hydrolysis. A1 and A2 indicate the fragments from the terminus that are uniquely found in model B. B1 and B2 indicate the repeating units. C1 and C2 indicate lipidated fragments. Panel C shows mass spectra of the hydrolysates of LTA. The major peaks are identified with labels. Na^+ indicates the sodium adducts; these peaks are 22 amu heavier than the mass of the Na^+ -free ion or 23 ($22 + 1$) amu heavier than the mass of their structures alone. n , number of repeating units.

5,970. Those with satellite peaks represent LTA that has lost two to four repeating units but that still has the lipid anchor. The other group without the satellite peaks corresponds to products containing three to seven oligosaccharide repeating units that were released from the lipid anchor. The chemical nature of the repeating unit was further confirmed by analyzing daughter ions of the peak at m/z 1,317, which was produced after a complete deamination reaction (data not shown). None of the out-of-phase peaks predicted by model A was observed

in the spectra; thus, the structure of LTA is consistent with model B.

DISCUSSION

Model A in Fig. 1 represents the currently accepted structure of pneumococcal LTA based on the study by Fischer's group (3, 14). However, model A predicts results that are inconsistent with our actual MS results obtained with LTA

FIG. 4. Panel A shows the fragments predicted by model B in response to a mild HF hydrolysis reaction. Dotted lines indicate the preferential cleavage sites. The molecular weights of the fragments are shown. Panel B shows the mass spectrum of LTA before the hydrolysis reaction. Peaks at m/z 9,874, 8,574, and 7,274 indicate LTA molecules with five, six, and seven repeating units. Peaks at m/z 4,941, 4,292, and 3,640 reflect ions with two positive charges and, respectively, correspond to ions of 9,874, 8,574, and 7,274 amu. Panel C shows the mass spectrum of the acylated LTA fragments after a mild HF hydrolysis reaction. Acylated fragments were obtained by octyl-Sepharose chromatography. The underlined numbers indicate the peaks representing the prehydrolysis LTA. The other numbers indicate the peaks that appeared after hydrolysis. Dotted arrows show the reduction in the mass (-952 amu) between the prehydrolysis and posthydrolysis peaks. For the calculation of the predicted mass, see the legend of Fig. 2. n , number of repeating units.

from various pneumococcal isolates. Model B, which differs from model A only in where the repeating unit biosynthesis begins, does predict results that are consistent with our new MS results, which were obtained following HF hydrolysis and deamination reactions. Model B is also consistent with the previous data reported by Fischer's group. For instance, when these investigators studied LTA after HF hydrolysis, they found the molecular fragments labeled B1, B2, C1, and C2 in Fig. 3B. Their study did not detect the molecular fragments labeled A1 and A2, which represent an incomplete repeating unit. This can also be readily explained because, following the hydrolysis step, Fischer's group purified the intact repeating units before conducting their mass spectrometry studies (3, 14).

In addition to being consistent with all the biochemical study results, model B resolves a serological dilemma that has been associated with model A (3). Pneumococcal LTA has been known to express the Forssman antigen, which is found on oligosaccharides expressing $\text{GalNAc}(\alpha 1 \rightarrow 3)\text{GalNAc}(\beta 1 \rightarrow)$ at their terminus (3, 20). Since model A has $\text{GalNAc}(\alpha 1 \rightarrow 3)$

$\text{GalNAc}(\beta 1 \rightarrow)$ in the middle of the structure and not at the terminus, Forssman antigenicity has been a significant problem for model A (3, 14). On the other hand, model B places $\text{GalNAc}(\alpha 1 \rightarrow 3)\text{GalNAc}(\beta 1 \rightarrow)$ at the terminus of an intact LTA molecule as well as at its repeating units. In fact, model B predicts repeating units with the minimal structure of Forssman antigens [$\text{GalNAc}(\alpha 1 \rightarrow 3)\text{GalNAc}(\beta 1 \rightarrow)$] prior to their decoration with PC.

Model B also explains a conundrum in LTA biosynthesis, which requires the linkage of the polymerized repeating units to a lipid anchor as the last step of LTA biosynthesis. According to model A, the lipid anchor is $\text{Glc-AATGal-Glc-acyl}_2\text{Gro}$. However, this lipid anchor has not been detected in pneumococcal membranes (14), and there are still no explanations as to how it becomes a part of LTA. In contrast, according to model B, the required lipid anchor is $\text{Glc}(\alpha 1 \rightarrow 3)\text{-acyl}_2\text{Gro}$, which is known to be a major glycolipid of pneumococcal membranes (6, 14, 27).

Model B may resolve yet another problem associated with

FIG. 5. Panel A shows the fragments predicted by Model B in response to a mild deamination reaction. Arrows with dotted lines indicate the preferential cleavage sites (34) and the molecular weight of the fragment that becomes dissociated from the acylated fragments. Panel B shows the mass spectrum of LTA before the deamination reaction. Peaks at m/z 4,291 and 4,941 represent ions with two charges. Panel C shows the mass spectrum of Panel B LTA after the deamination reaction. The underlined numbers indicate the peaks found in untreated LTA. Acylated fragments have satellite peaks and are identified with an asterisk (*). The other numbers indicate the peaks that appeared after the deamination reaction. n , number of repeating units.

biosynthesis of LTA. The repeating unit of staphylococcal LTA is a simple glycerol phosphate and is directly polymerized into LTA by a single enzyme, LtaS (17, 18). However, the pneumococcal LTA repeating unit is much more complex (Fig. 1), and the repeating unit assembly would, like the majority of the pneumococcal capsules (52), require an initial transferase to transfer the initial sugar onto a polyprenyl phosphate acceptor in the membrane. According to model A, the initial transferase should transfer ribitol phosphate to the polyprenyl phosphate lipid acceptor; however, to date, no bacterial polysaccharide has been shown to initiate the formation of repeating units by the addition of ribitol phosphate. Even staphylococci with polyribitol phosphate TA do not begin the polymerization of TA with the transfer of ribitol. Instead, their polymerization begins with the initial transfer of GlcNAc-1-phosphate to a polyprenyl phosphate lipid acceptor and is followed by repeated addition of the ribitol (37, 38). In contrast, model B predicts that the initial sugar transferred to the polyprenyl

phosphate lipid acceptor would be AATGal-1-phosphate. PS-A polysaccharide of *Bacteroides fragilis* also has a repeating unit that is initiated by the addition of AATGal, with the enzyme catalyzing this addition being encoded by *wcfS* (10). TIGR4 *S. pneumoniae* genome has SP1838, which is 44% identical to and 66% similar to *wcfS* (10). SP1838, which is presumed to be essential for pneumococcal survival (45), is also found in the pneumococcal genomes of D39 (SPD1619), R6 (Spr1654), and others (23, 33, 44). While additional studies are required, based on the revised structure of LTA (model B), SP1838 could be the initial transferase for LTA synthesis.

Furthermore, since both TA and LTA of *S. pneumoniae* have repeating units with identical structures and are both likely synthesized by the same set of enzymes, SP1838 would also initiate TA repeating unit synthesis. Although LTA is attached to pneumococci by its lipid anchor, TA is attached to the cell wall through the muramic acid of the peptidoglycan (43). As with LTA, it has been thought that the repeating units

of TA are linked together with phosphodiester bonds and that TA is attached to the cell wall by linking its ribitol to an unknown oligosaccharide linker via a phosphodiester bond (43). This view of TA structure is based on chemical analyses of TA and does not reflect the biological steps involved in the synthesis and polymerization of TA repeating units. As with LTA, the biological synthesis of TA repeating units should start with AATGal and end with GalNAc, and AATGal may be involved in the attachment of TA to the peptidoglycan.

We found LTAs from pneumococcal strains R6 and R36A to be different in structure. The mass spectrometry pattern of R36A LTA is similar to that of LTA from several strains, including two recent clinical isolates (TIGR4 and MX-73HIM). Experimental strains expressing fewer PCs are less virulent than are a wild-type strain (53). Also, strain R6 was derived in 1964 from strain R36A, which was, in turn, derived in 1944 from strain D39 (33). These strains are known to have many genetic differences (33). Thus, R6 LTA should be considered a variant, and R36A LTA should be considered representative of pneumococcal LTA. Furthermore, this observation shows the need to use standardized bacterial strains for studying LTA. Mass spectrum data indicated that about half of typical R6 LTA molecules have repeating units with one PC group, and the other half have repeating units with two PC groups. Our findings are consistent with previous findings, which found two PC groups in 78% of R6 LTA repeating units and one PC group in 22% (3). *licD1* and *licD2* gene products are involved in transferring PC onto LTA/TA repeating units (28, 53), and an enzyme, Pce, is involved in removing PC from LTA/TA (48). Pneumococcal strains with a mutation in *licD2* have only one PC group per repeating unit (53). However, inspection of R6 and D39 genome sequences shows identical DNA sequences for the three genes. Additional studies are needed to explain the structural differences between R6 and D39 LTAs.

Many species of gram-positive bacteria can produce LTA with small structural alterations. In addition to the heterogeneity in PC, pneumococcal LTA may have galactose instead of glucose in the repeating units (47) or may have ribitol decorated with alanine (11, 31). Similarly, LTA from other bacterial species can be variably decorated with alanyl or glucosyl groups (39). It is also becoming clear that these small structural changes have a significant impact on LTA functions, such as resistance to a bacteriocin (31), adhesion to host cells (53), or pathogenic potential (1, 50). Yet it is difficult to investigate the impact of the small structural alterations on LTA function using LTA from many bacterial species (e.g., staphylococci) because a structural feature of their LTAs (highly variable but large numbers of small glycerophosphate repeating units) prevents us from delineating their exact structure. With the ability to determine the precise pneumococcal LTA structure, we can use pneumococci as a model for investigating the impact of small alterations in LTA structure on bacterial survival, growth, and pathogenicity.

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