Attachment of phosphorylcholine residues to pneumococcal teichoic acids and modification of substitution patterns by the phosphorylcholine esterase

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The bacterial lung pathogen Streptococcus pneumoniae has a unique nutritional requirement for exogenous choline and attaches phosphorylcholine (P-Cho) residues to the GalpNAc moieties of its teichoic acids (TAs) in its cell wall. Two phosphorylcholine transferases, LicD1 and LicD2, mediate the attachment of P-Cho to the O-6 positions of the two GalpNAc residues present in each repeating unit of pneumococcal TAs (pnTAs), of which only LicD1 has been determined to be essential. At the molecular level, the specificity of the P-Cho attachment to pnTAs by LicD1 and LicD2 remains still elusive. Here, using detailed structural analyses of pnTAs from a LicD2-deficient strain, we confirmed the specificity in the attachment of P-Cho residues to pnTA. LicD1 solely transfers P-Cho to α-β-GalpNAc moieties, whereas LicD2 attaches P-Cho to β-β-GalpNAc. Further, we investigated the role of the pneumococcal phosphorylcholine esterase (Pce) in the modification of the P-Cho substitution pattern of pnTAs. To clarify the specificity of Pce-mediated P-Cho hydrolysis, we evaluated different concentrations and pH conditions for the treatment of pneumococcal lipoteichoic acid with purified Pce. We show that Pce can hydrolyze both P-Cho residues of the terminal repeat of the pnTA chain and almost all P-Cho residues bound to β-β-GalpNAc in vitro. However, hydrolysis in vivo was restricted to the terminal repeat. In summary, our findings indicate that LicD1 and LicD2 specifically transfer P-Cho to α-β-GalpNAc and β-β-GalpNAc moieties, respectively, and that Pce removes distinct P-Cho substituents from pnTAs.

Crucial host interactions of the human lung pathogen Streptococcus pneumoniae are mediated by its bacterial cell wall components. It is well known that lipoproteins (LPs) are the unique nutritional requirement for exogenous choline and attaches phosphorylcholine (P-Cho) residues to the GalpNAc moieties of its teichoic acids (TAs) in its cell wall. Two phosphorylcholine transferases, LicD1 and LicD2, mediate the attachment of P-Cho to the O-6 positions of the two GalpNAc residues present in each repeating unit of pneumococcal TAs (pnTAs), of which only LicD1 has been determined to be essential. At the molecular level, the specificity of the P-Cho attachment to pnTAs by LicD1 and LicD2 remains still elusive. Here, using detailed structural analyses of pnTAs from a LicD2-deficient strain, we confirmed the specificity in the attachment of P-Cho residues to pnTA. LicD1 solely transfers P-Cho to α-β-GalpNAc moieties, whereas LicD2 attaches P-Cho to β-β-GalpNAc. Further, we investigated the role of the pneumococcal phosphorylcholine esterase (Pce) in the modification of the P-Cho substitution pattern of pnTAs. To clarify the specificity of Pce-mediated P-Cho hydrolysis, we evaluated different concentrations and pH conditions for the treatment of pneumococcal lipoteichoic acid with purified Pce. We show that Pce can hydrolyze both P-Cho residues of the terminal repeat of the pnTA chain and almost all P-Cho residues bound to β-β-GalpNAc in vitro. However, hydrolysis in vivo was restricted to the terminal repeat. In summary, our findings indicate that LicD1 and LicD2 specifically transfer P-Cho to α-β-GalpNAc and β-β-GalpNAc moieties, respectively, and that Pce removes distinct P-Cho substituents from pnTAs.

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This article contains Tables S1 and S2 and Figs. S1–S7.

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2 The abbreviations used are: LP, lipoprotein; CBP, choline-binding protein; Glc, glucose; HMBC, heteronuclear multiple bond correlation; HMOC, heteronuclear multiple quantum correlation; HSQC, heteronuclear single quantum correlation; LTA, lipoteichoic acid; P-Cho, phosphorylcholine; PGN, peptidoglycan; TA, teichoic acid; pnTA, pneumococcal TA; RU, repeating unit(s); TLR, Toll-like receptor; TOCSY, total correlation spectroscopy; WTA, wall teichoic acid; AATGal, 2-acetamido-4-amino-2,4,6-trideoxygalactose; Pce, phosphorylcholine esterase.

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mild acidic conditions (1). However, the enzyme(s) involved in this specific modification have not been definitely identified so far. The most likely candidate is the pneumococcal phosphorylcholine esterase (Pce). Pce is a member of the CBP family, and its enzymatic activity was first described in 1974 (16). It has been shown that Pce is able to hydrolyze about 30% of the total P-Cho residues attached to the GalpNAc moieties of WTA and LTA in vitro (17). The crystal structure of Pce revealed the presence of two structural modules, the catalytic module (residues 1–300) and the choline-binding module (residues 313–540). Both are joined by a small linker, which comprises residues 301–312. Analysis of the crystal structure suggested that the removal of P-Cho residues is limited by the configuration of the active site of Pce in such a way that only residues on the end of the TA chains are accessible to the catalytic center (18). Until now, the focus of previous investigations has mainly centered on choline metabolism and its significance for host interactions (19–22). The specificity of the P-Cho hydrolysis by Pce has not been elucidated to date.

In this study, we investigated the structural specificity of Pce-mediated removal of P-Cho residues from pnLTA. Therefore, we isolated the LTA of a Pce-deficient strain in the noncapsulated TIGR4 background (TIGR4/cps/pce). The LTA was de-O-acylated by hydrazine treatment and purified by gel permeation chromatography as described previously (23). The high-mass region of the deconvoluted spectrum is shown in Fig. 2B (top), and the complete spectrum is depicted in Fig. S1. It shows the typical proportional distribution of chain lengths with the predominant presence of molecules with 6 and 7 RUs for LTA isolated from TIGR4 strains (1, 23, 24). Furthermore, the spectrum indicates that all de-O-acylated pnLTA molecules of this preparation are completely P-Cho–substituted. The respective 31P NMR spectrum is depicted in the top panel of Fig. 2A, and the corresponding chemical structure for de-O-acyl pnLTA (1) is shown in Fig. 2C (and in Fig. 2D as a schematic representation), with X = P-Cho at residues H and G for this preparation. In 31P NMR, LTA of the Pce-deficient strain displays P-Cho signals at δp 0.33 ppm for P-Cho at β-D-GalpNAc moieties (residues D and G, Fig. 2C), at δp 0.12 ppm for P-Cho at the terminal α-D-GalpNAc (residue H) and at δp −0.15 ppm for P-Cho at all other α-D-GalpNAc moieties (residue E). Signals for ribitol-P (residues C’/C) occur at δp 1.89/1.80 ppm.

**The specificity and efficiency of the Pce-mediated P-Cho hydrolysis are influenced by the pH value**

To determine the specificity and efficiency of P-Cho hydrolysis mediated by Pce, we used the above described, completely P-Cho–substituted LTA of pneumococcal strain TIGR4ΔcpsΔpce in its native and therefore acylated form. An earlier study showed that Pce has its highest activity against p-nitrophenylphosphorylcholine and cell wall components at
P-Cho attachment and hydrolysis in pneumococcal TAs

A
Native LTA
TIGR4ΔcpsΔpce

Ribitol-5-P_C
1.95
Ribitol-5-P_C
1.80

B
6 RU

5 RU

6746.33
8044.77

Relative Abundance

6889.40
7054.45
7219.50
7384.55
7549.61
7714.67
8044.77

Native LTA
TIGR4ΔcpsΔpce + 80 μg Pce/mg LTA

Ribitol-5-P_C
1.89
Ribitol-5-P_C
1.80

Native LTA
TIGR4ΔcpsΔpce + 160 μg Pce/mg LTA

Ribitol-5-P_C
1.88
Ribitol-5-P_C
1.80

Native LTA
TIGR4ΔcpsΔpce + 240 μg Pce/mg LTA

Ribitol-5-P_C
1.87
Ribitol-5-P_C
1.80

C
X = H or P-Cho

D
X X

terminal RU
RU 2 to n
RU 1

R = H or D-Ala
n = 2-6

Rib-ol
Rib-ol
Rib-ol

Gro

n

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Table 1

| TIGR4 | Rib-Pc / Rib-Pc' | P-CholD | P-CholE | P-CholF |
|-------|-----------------|---------|---------|---------|
| ΔPce | 6.77            | 6.65    | 1.00    | 5.67    |
| ΔPce + 80 μg of Pce/mg of LTA | 6.77    | 4.47 ± 0.14 | 0.58 ± 0.03 | 5.69 ± 0.06 |
| ΔPce + 160 μg of Pce/mg of LTA | 6.77    | 2.73 ± 0.02 | 0.21 ± 0.01 | 5.81 ± 0.01 |
| ΔPce + 240 μg of Pce/mg of LTA | 6.77    | 2.61 ± 0.07 | 0.04 ± 0.04 | 5.81 ± 0.05 |

pH 8.0 (17). To further evaluate this finding, we treated purified pLTA with different concentrations of heterologously expressed Pce and compared the enzyme activity at physiological pH value (pH 7.4) and a more basic pH of 8.0. Subsequently, Pce-treated pLTA was treated with anhydrous hydrazine and purified by gel permeation chromatography, to avoid aggregates or micelle formation and thus to obtain reliable 1H and 31P NMR integral values.

Analysis of the Pce-treated LTA from strain TIGR4ΔPceΔpce (pH 7.4) by 31P and 1H NMR revealed changes in the Pce substitution pattern depending on the Pce concentrations used (80, 160, or 240 μg of Pce/mg of isolated LTA; Fig. 2A and Table 1). At 80 μg of Pce/mg of isolated LTA, partial hydrolysis of the P-CholΔG and P-CholH moieties could be observed. An increase of the Pce concentration to 160 μg/mg showed only an effect on the hydrolysis of these P-Chol moieties, whereas the amount of Rib-ol-Pc/C and P-CholE remained unaltered. At this concentration, almost all P-CholH moieties have been hydrolyzed, and the amount of P-CholΔG was lowered by more than half. An increase of the Pce concentration from 160 to 240 μg/mg had only a marginal effect. In Fig. 2B, the respective MS spectra recorded from these preparations are depicted, focusing on de-O-acetylated LTA molecules with 6 RU's (the full versions of these MS spectra are shown in Figs. S2–S4). Molecular mass differences of 165 Da between two mass peaks are indicated, which corresponds to the loss of one P-Chol moiety each. In total, we observed the loss of up to seven P-Chol residues (7 × ~165 Da). In combination with the 31P NMR results for P-CholH and P-CholΔG, this indicates that both P-Chol residues from the terminal RU and all P-Chol substrates at β-D-GalpNAc moieties within the RU (residue D) can be hydrolyzed. However, mass peak VI (7054.44 Da) is of highest abundance in the preparations after treatment with 160 or 240 μg of Pce/mg of LTA, whereas mass peak VII (6889.39 Da) is only marginally present. Whether this is a specific P-Chol moiety that is not efficiently hydrolyzed (e.g. P-CholD of the first RU) can only be speculated. The observed masses for all LTA molecules are in agreement with their respective calculated masses and are listed in Table 2. A change in the pH value from pH 7.4 to pH 8.0 improved the efficiency of hydrolysis on P-CholH for the concentration of 80 μg/mg LTA (Fig. 3 and Table 3). Nonetheless, pH 8.0 shows a hydrolysis behavior with a lower overall efficiency for P-Chol attached to residues D and G. An increase of the Pce concentration from 80 μg/mg LTA to 160 μg/mg LTA at pH 8.0 resulted in no further significant change of the hydrolysis of moieties P-CholD, G, and P-CholH. In summary, our in vitro analysis clearly demonstrates that Pce hydrolyzes only the moieties P-CholΔG and terminal P-CholH, P-CholE, and Rib-ol-Pc/C moieties are not hydrolyzed by Pce.

LicD2 transfers P-Chol substituents to β-α-GalpNAc moieties in pLTAs

To study the specificity of the attachment of P-Chol residues by LicD2, we isolated and analyzed LTAs of LicD2-deficient strains. The native LTAs of S. pneumoniae TIGR4ΔpslD2 and TIGR4ΔpslD2ΔlicD2 were isolated, purified, and subsequently analyzed by NMR and MS (Fig. 4). The 31P NMR spectra from isolated LTA revealed only a P-Chol substitution at the α-D-GalpNAc residues within the RU's (P-CholE) and the α-D-GalpNAc at the terminus (P-CholD). The respective signal for P-CholG substitution at the β-D-GalpNAc at 0.33 ppm (P-CholΔG), which is present in pLTA isolated from the respective parental strains TIGR4ΔpslD2 (Fig. 2A, top) and TIGR4ΔpslD2 (1), is absent (Fig. 4A). All NMR data for the hydrazine-treated LTA of TIGR4ΔpslD2ΔlicD2 are listed in Table 4, and a section of the respective 1H, 13C HSQC NMR is shown in Fig. S5, including assignment of signals. In the MS analysis of this de-O-acetyl LTA, only the expected masses for pLTA with one P-Chol residue per RU were observed (Fig. 4B, top). The observed mass for such LTA molecules with 6 RU's is 7054.44 Da (calculated monoisotopic mass: 7054.45 Da; Fig. 4B, top; the complete MS spectrum is shown in Fig. S6) and Table 5), which corresponds to a mass difference of 990 Da compared with the respective LTA molecules with complete P-Chol substitution (calculated monoisotopic mass: 8044.78 Da; Fig. 2B, top panel) and Table 2). This is equivalent to 6 P-Chol residues less in these LTA molecules of strain TIGR4ΔpslD2ΔlicD2. In the MS analysis of de-O-acetyl LTA of strain TIGR4ΔpslD2ΔlicD2, the above described Pce-mediated hydrolysis of P-Chol residues at the α-D-GalpNAc of the terminus (P-CholD) is clearly visible (Fig. 4B, middle panel; the complete MS spectrum is shown in Fig. S7) and Table 5). Notably, LTA chains of strain TIGR4ΔpslD2ΔlicD2 tend to be slightly longer, as observed for TIGR4ΔpslD2ΔlicD2. 31P NMR analysis of the correspond-

Figure 2. Specific phosphorylcholine moieties of pLTA are cleaved off in a concentration dependent manner by the pneumococcal Pce. A, sections (δy, δx, z) of 1P NMR spectra (D2O, 300 K, 283.54 MHz) of hydrazine-treated LTA of TIGR4ΔpslD2Δpce and the respective LTA treated with the indicated amounts of Pce at pH 7.4 in 50 mM KH2PO4/KHPO4, B, section (6600 – 8200 Da) of the respective charge-deconvoluted mass spectra of these LTA preparations. Predicted and observed masses for the resulting LTA molecules with 6 RU (I–VII) are listed as examples of the treatment with 80 μg of Pce/mg of LTA in Table 2. C and D, current structural model for de-O-acetyl LTA of S. pneumoniae strain TIGR4 (and mainly all other pneumococcal strains (24)), depicted as a detailed chemical drawing (C) as well as a schematic cartoon (D).
These experiments indicated that Pce is capable of hydrolysing P-Cho substituents at the terminal D-GalNAc moieties in pneumococcal TAs (16, 17). Our study demonstrates now, by combining NMR and MS analyses, the specificity and efficiency of the P-Cho hydrolysis at GalpNAc residues mediated by Pce. With one-dimensional $^{31}$P and $^1$H NMR spectra, a direct assignment of specific P-Cho residues to the respective α- or β-configured GalpNAc residues of pnTA RUs is possible. With this direct assessment, it is further possible to judge and compare the Pce-mediated P-Cho hydrolysis at different pH values. Compared with LTA of S. pneumoniae strain TIGR4cps, LTA of the Pce-deficient strain exhibited a complete P-Cho substitution pattern. Isolated pnLTA of the Pce-deficient strain was treated at different concentrations and different pH values with heterologously expressed Pce. P-Cho substituents at the terminal α- and β-configured GalpNAc residues (P-Choα) and at the β- and GalpNAc moieties (P-Choβ) were efficiently hydrolyzed at a concentration of 80 μg of Pce/mg of LTA at both tested pH values. An increased hydrolysis rate was observed at a pH of 7.4, when the concentration was increased from 80 to 160 μg/mg LTA, which was not the case at pH 8.0. In contrast, at pH 8.0 and a Pce concentration of 160 μg/mg LTA, the P-Choα residue was completely hydrolyzed, which was not observed at pH 7.4. However, P-Cho substituents at β- and GalpNAc residues were less efficiently hydrolyzed by the Pce at pH 8.0 compared with pH 7.4. A possible reason for the altered hydrolysis efficiency at the studied pH values could be conformational changes of the catalytic domain of Pce. In summary, a removal of about 20–30% of P-Cho residues by hydrolysis was observed, which is in good agreement with previously described findings for in vitro studies (17).

The observed specificity for the Pce-mediated hydrolysis indicates that P-Cho residues bound to either α- or β-GalpNAc or β- and GalpNAc have a different biological importance and makes it most likely that different enzymes are responsible for their specific attachment. Previous studies using the monoclonal antibody TEPC-15, which recognizes a P-Cho epitope in S. pneumoniae, showed that LicD2 mutant strains differ in their P-Cho substitution compared with WT strains (14). By applying detailed chemical structural analyses, we showed here that LicD2-deficient S. pneumoniae strains exclusively exhibit a P-Cho decoration on the α- and β-GalpNAc of their LTA as well as on the β- and GalpNAc moieties of their LTA. Previous studies showed that the pneumococcal Pce is capable of removing in vitro about 15–30% of the total P-Cho residues attached to pneumococcal TAs (16, 17). Our study demonstrates now, by combining NMR and MS analyses, the specificity and efficiency of the P-Cho hydrolysis at GalpNAc residues mediated by Pce. With one-dimensional $^{31}$P and $^1$H NMR spectra, a direct assignment of specific P-Cho residues to the respective α- or β-configured GalpNAc residues of pnTA RUs is possible. With this direct assessment, it is further possible to judge and compare the Pce-mediated P-Cho hydrolysis at different pH values. Compared with LTA of S. pneumoniae strain TIGR4cps, LTA of the Pce-deficient strain exhibited a complete P-Cho substitution pattern. Isolated pnLTA of the Pce-deficient strain was treated at different concentrations and different pH values with heterologously expressed Pce. P-Cho substituents at the terminal α- and β-configured GalpNAc residues (P-Choα) and at the β- and GalpNAc moieties (P-Choβ) were efficiently hydrolyzed at a concentration of 80 μg of Pce/mg of LTA at both tested pH values. An increased hydrolysis rate was observed at a pH of 7.4, when the concentration was increased from 80 to 160 μg/mg LTA, which was not the case at pH 8.0. In contrast, at pH 8.0 and a Pce concentration of 160 μg/mg LTA, the P-Choα residue was completely hydrolyzed, which was not observed at pH 7.4. However, P-Cho substituents at β- and GalpNAc residues were less efficiently hydrolyzed by the Pce at pH 8.0 compared with pH 7.4. A possible reason for the altered hydrolysis efficiency at the studied pH values could be conformational changes of the catalytic domain of Pce. In summary, a removal of about 20–30% of P-Cho residues by hydrolysis was observed, which is in good agreement with previously described findings for in vitro studies (17).
and after treatment with Pce ($\Delta P$ce) are shown. For glycan representation symbols see Fig. 1. *, second isotopic peak.

**Figure 4. LicD2-deficient strains lack the P-Cho residues at the $\beta$-d-GalNAc moieties, which does not alter the Pce-mediated P-Cho hydrolysis at the terminal $\alpha$-d-GalNAc (P-Cho$_{\alpha}$).** Sections of $^{31}$P NMR spectra (A, 3–(−1); D$_2$O, 300 K, 283.54 MHz) (A), charge-deconvoluted MS spectra (5000–9350 Da) (B), and the corresponding structures of the hydrazine-treated pnLTA preparations of TIGR4$\Delta$cps$\Delta$licD2 (top) as well as from TIGR4$\Delta$cps$\Delta$licD2 before (middle) and after treatment with Pce (bottom) (C) are shown. For glycan representation symbols see Fig. 1. *, second isotopic peak.

Table 3

Integration values from $^{31}$P NMR spectra from native LTA of TIGR4$\Delta$cps$\Delta$pce before and after treatment with the indicated amounts of Pce at pH 8.0 shown in Fig. 3.

| TIGR4          | Rib-P$_{C_1}$/ Rib-P$_C$ | $P$-Cho$_{\alpha}$ | $P$-Cho$_{\beta}$ | $P$-Cho$_{\gamma}$ |
|----------------|------------------------|---------------------|--------------------|--------------------|
| $\Delta$psPce  | 6.59                   | 6.54                | 1.00               | 5.57               |
| $\Delta$psPce + 80 $\mu$g of Pce/mg of LTA | 6.59                   | 4.82 ± 0.01        | 0.03 ± 0.03        | 5.52 ± 0.02        |
| $\Delta$psPce + 160 $\mu$g of Pce/mg of LTA | 6.59                   | 4.30 ± 0.02        | ND                 | 5.53 ± 0.03        |

of their WTA. This provides clear proof that the P-Cho attachment to the $\beta$-d-GalNAc residues is mediated by LicD2. By implication, LicD1 catalyzes the attachment of P-Cho to $\alpha$-d-GalNAc (Fig. 6), because it is the only P-Cho transferase besides LicD2 that has been identified in the pneumococcal genome (15). A direct validation of the LicD1-mediated P-Cho transfer is not possible due to its essentiality for the pneumococcus (14). Our results further confirm that LTA and WTA in *S. pneumoniae* are synthesized via a shared biosynthesis route (15, 23). Using LTA isolated from TIGR4$\Delta$cps$\Delta$licD2, Pce still hydrolyzes the P-Cho substituents on the terminal $\alpha$-d-GalNAc (P-Cho$_{\alpha}$) residues, whereas P-Cho moieties at $\alpha$-d-GalNAc located within the RU were not affected. This finding is an indication that P-Cho residues on the $\alpha$-d-GalNAc moieties are not required for binding of Pce. Because the P-Cho residues on the $\alpha$-d-GalNAc moieties are essential for binding of CBPs, whereas P-Cho residues on $\beta$-d-GalNAc are not, a structural explanation for the essential nature of LicD1 is given (14, 15). This is also in line with the results of analyzed pneumococcal strains only possessing one P-Cho per RU in their TAs, which all lack only the P-Cho at the $\beta$-d-GalNAc moieties (21, 24, 25).

In summary, our experiments indicate that Pce is the only enzyme responsible for P-Cho removal from pnTAs. Pce only hydrolyzes the P-Cho residues on $\beta$-d-GalNAc residues as well as on the terminal $\alpha$-d-GalNAc. This in vitro removal corresponds to up to 30% of the total P-Cho content. In vivo, Pce is only able to hydrolyze P-Cho residues present in the terminal repeating unit. This finding is in line with the previous observation that the activity of Pce might be limited by more complex constraints related to the topography of the pneumococcal surface (17). Only residues that are located at the end of the TA chains may be accessible to the active site of the catalytic center of Pce in vivo. Moreover, P-Cho residues on the bacterial
surface are a target for components of the host immune response, such as the human C-reactive protein, the platelet-activating factor receptor, or L-ficolin, which leads to different reactions in immune defense, such as activation of the lectin complement pathway (24). A selective modification of the P-Cho pattern on the surface, mediated by Pce activity, could impair targeting of the pneumococcus by these host components and may thus favor infection and colonization by *S. pneumoniae*.
Tom Sections (LicD1 catalyzes the attachment of LicD2 treatment from TIGR4 cps). Our study revealed that LicD2 solely promotes the specificity of LicD2. Furthermore, we could show on the molecular level the P-Cho attachment and hydrolysis in pneumococcal TAs P-Cho residues to the \( \beta \)-d-GalpNAc residues, and LicD1 catalyzes the attachment of P-Cho residues to \( \alpha \)-d-GalpNAc (Fig. 6).

Experimental procedures

Bacterial strains and growth

Pneumococcal strains used in this study are listed in Table S1. Bacteria were grown on Columbia blood agar plates (Oxoid) or in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY; Roth) containing appropriate antibiotics (kanamycin (150 \( \mu \)g/ml), erythromycin (5 \( \mu \)g/ml), and chloramphenicol (5 \( \mu \)g/ml)). Cultivation on plates or in liquid cultures was performed at 37 °C and 5% CO₂.

Table 5

| LTA species |RU| Chemical formula | Calculated mass | Observed mass | Accuracy |
|-------------|--|------------------|-----------------|---------------|----------|
| TIGR4\(\Delta\)cps\(\Delta\)licD2 |4| C\(_{166}\)H\(_{355}\)O\(_{25}\)N\(_{20}\)P\(_{8}\) | 4787.67 | 4787.67 | 0.0 |
| |5| C\(_{166}\)H\(_{355}\)O\(_{25}\)N\(_{20}\)P\(_{8}\) | 5921.06 | 5921.06 | 0.0 |
| |6| C\(_{166}\)H\(_{355}\)O\(_{25}\)N\(_{20}\)P\(_{8}\) | 7054.45 | 7054.44 | -1.4 |
| |7| C\(_{166}\)H\(_{355}\)O\(_{25}\)N\(_{20}\)P\(_{8}\) | 8187.84 | 8187.83 | -1.2 |
| |8| C\(_{166}\)H\(_{355}\)O\(_{25}\)N\(_{20}\)P\(_{8}\) | 9322.24 | 9322.22 | -2.1 |
| TIGR4\(\Delta\)licD2 | | | | | |
| TIGR4\(\Delta\)licD2 + 160 \( \mu \)g of Pce/mg of LTA | | | | | |

**Figure 5.** \( ^{31}P \) NMR spectra of the isolated PGN-WTA complex after LytA treatment from TIGR4\(\Delta\)licD2 (top) and TIGR4\(\Delta\)licD2 (bottom). Sections (\( \delta \), 3–(−1)) of the respective \( ^{31}P \) NMR spectra (D\(_{2}\)O, 300 K, 283.54 MHz) are shown.

**Mutant construction**

All primers used are listed in Table S2. For the construction of the pneumococcal licD2 mutant in \( S. pneumoniae \) TIGR4\(\Delta\)cps, a DNA fragment consisting of the \( S. pneumoniae \) TIGR4 licD2 gene and ~500-base pair up- and downstream flanking regions were amplified by PCR using primers LicD2phIfor and LicD2Sacrev. The resulting PCR product was cloned into plasmid pUC18 (Thermo Fisher Scientific). This plasmid was used as template for an inverse PCR with primer Invrev1130BamHI and Invfor1130SmaI. Afterward, an \( ermB \) gene, amplified by PCR from vector pTP1 using primer InvrevBamHIErm and InforSmaIErm was inserted (26). The final recombinant plasmid was used to transform and mutagenize \( S. pneumoniae \) TIGR4\(\Delta\)cps.

To delete the pce gene in \( S. pneumoniae \) TIGR4, primer CBP1E1 and CBP2E2 were used to amplify the pce gene from chromosomal DNA of \( S. pneumoniae \) R6 (without choline-binding repeats) by PCR. The resulting PCR fragment was cloned into plasmid pQE-30 (Qiagen) via BamHI and HindIII restriction sites. Primer CpgE6fXma and CbpE4rXma were used as template DNA. After digestion with XmaI, an erythromycin resistance cassette (\( ermB \)) was cloned into plasmid pUC18. This plasmid was used as template for an inverse PCR with primer InvrevBamHIErm and InforSmaIErm was inserted (26). The final recombinant plasmid was used to transform and mutagenize \( S. pneumoniae \) TIGR4\(\Delta\)cps.
P-Chol attachment and hydrolysis in pneumococcal TAs

Table 6
Integration values from 31P NMR spectra from native LTA of TIGR4ΔcpsΔpceΔlicD2 as well as TIGR4ΔcpsΔlicD2 before and after treatment with the indicated amount of Pce shown in Fig. 4.

| TIGR4          | Rib-Pc / Rib-Pc | P-CholD1G | P-CholH | P-CholG |
|---------------|----------------|-----------|---------|---------|
| ΔcpsΔpceΔlicD2 | 6.44 ± 0.04    | ND        | 1.00    | 5.44 ± 0.05 |
| ΔcpsΔlicD2    | 6.44           | ND        | 0.79 ± 0.01 | 5.61 ± 0.11 |
| ΔcpsΔlicD2 + 160 µg of Pce/mg of LTA | 6.44 | ND | ND | 5.69 ± 0.08 |

Figure 6. The two phosphorylcholine transferases LicD1 and LicD2 attach P-Chol specifically to teichoic acids in S. pneumoniae.

Extraction, isolation, and chemical treatment of pnTAs

Isolation and purification of pnTAs (LTA and WTA) as well as treatment of purified LTA with anhydrous hydrazine to generate de-O-acylated LTA have been performed as described earlier (23).

Expression and purification of Pce

E. coli BL21 pRGR12 strain was used for heterologous expression and purification of pneumococcal Pce as described (28). Briefly, bacteria were grown in LB broth medium until exponential phase (A600 of 0.7) on an environmental shaker at 30 °C. At this time point, isopropyl-β-D-thio-galactopyranoside (1 mM) was added, and incubation proceeded for 3 h. After centrifugation, bacterial pellet was solubilized by ultrasonic treatment. The heterologously expressed Pce was purified using DEAE-cellulose, as described (29).

Phosphorylcholine esterase treatment of pnLTA

Treatment of pnLTA with Pce was performed basically as described elsewhere but with some modifications in detail (17). Purified pnLTAs were dissolved in 50 mM K2HPO4/KH2PO4 to obtain a concentration of 2.62 mg/ml at pH 7.4 or pH 8.0, respectively. Heterologously expressed Pce was added in different concentrations (80, 160, and 240 µg/mg of pnLTA). The solution was incubated for 24 h at 37 °C. To inactivate the enzyme, the sample was incubated for 5 min at 100 °C and afterward centrifuged at 10,000 × g for 20 min at 4 °C. Finally, the solution was lyophilized. For each Pce treatment condition, two independent experiments were performed.

NMR spectroscopy

NMR spectroscopic measurements were performed in D2O at 300 K on a Bruker Avance III 700-MHz spectrometer (equipped with an inverse 5-mm quadruple-resonance Z-grad cryoprobe). Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). For calibration of 1H (δH = 2.225 ppm) and 13C (δC = 30.89 ppm) NMR spectra, acetone was used as an external standard. The 31P NMR spectra (δp = 0.0 ppm) were calibrated with 85% phosphoric acid in D2O as an external standard. All data were acquired and processed using Bruker TOPSPIN version 3.0 or higher. 1H NMR assignments were confirmed by two-dimensional 1H,1H COSY and TOCSY experiments, and 13C NMR assignments were indicated by two-dimensional 1H,13C HSQC and based on the 1H NMR assignments. From two-dimensional 1H,13C HSQC-TOCSY experiments, interresidue connectivity was obtained. Connectivity of phosphate groups was assigned by two-dimensional 1H,31P HMQC and 1H,31P HMQC-TOCSY.

Mass spectrometry

All samples were measured on a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) using a Tiviersa Nanomate (Advion, Ithaca, NY) as ion source. All measurements were performed in negative-ion mode using a spray voltage of −1.1 kV. Samples were dissolved in a water/propan-2-ol/trimethylamine/acetic acid mixture (50:50:0.06:0.02, v/v/v/v). The mass spectrometer was externally calibrated with glycolipids of known structure. All mass spectra were charge-deconvoluted and given mass values refer to the monoisotopic mass of the neutral molecules, if not indicated otherwise.

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