Lipoteichoic acid deficiency permits normal growth but impairs virulence of *Streptococcus pneumoniae*

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Teichoic acid (TA), a crucial cell wall constituent of the pathobiont *Streptococcus pneumoniae*, is bound to peptidoglycan (wall teichoic acid, WTA) or to membrane glycolipids (lipoteichoic acid, LTA). Both TA polymers share a common precursor synthesis pathway, but differ in the final transfer of the TA chain to either peptidoglycan or a glycolipid. Here, we show that LTA exhibits a different linkage conformation compared to WTA, and identify TacL (previously known as RafX) as a putative lipoteichoic acid ligase required for LTA assembly. Pneumococcal mutants deficient in TacL lack LTA and show attenuated virulence in mouse models of acute pneumonia and systemic infections, although they grow normally in culture. Hence, LTA is important for *S. pneumoniae* to establish systemic infections, and TacL represents a potential target for antimicrobial drug development.

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**S. pneumoniae** (the pneumococcus) is a human pathobiont that not only colonizes asymptomatically the respiratory tract, but is also capable of causing diseases such as otitis media, acute sinusitis, pneumonia, meningitis, and sepsis. The cell surface of this Gram-positive bacterium consists of a peptidoglycan-wall teichoic acid (PGN-WTA) cell wall and is shielded by a capsular polysaccharide (CPS), allowing the cell to evade recognition by the innate immune system of the host. Like most Gram-positive bacteria, pneumococci contain a structurally unique, complex LTA. Similar LTA structures are known and interactions with host factors. In contrast to many other surface proteins involved in peptidoglycan remodeling, streptococcal teichoic acid (STA) bind choline-binding proteins (CBPs), an important class of cell surface proteins involved in peptidoglycan remodeling and interactions with host factors. For example, the LTA of pneumococci is anchored to the cell membrane by a glycolipid moiety. Both types of teichoic acid (TA) bind a lipid anchor, whereas all other RUs are \( \alpha \) substitution. The unique, complex LTA is also known and involves interactions with host factors.

**Results**

**Linkage structure of pnWTA to PGN.** In order to determine the linkage of pnWTA to PGN, we isolated the PGN-WTA complex from *S. pneumoniae* D39ΔcpsΔlgt using a previously published procedure. This strain lacks the CPS and the gene encoding for the lipoprotein diacylglycerol transferase (Lgt) and is therefore deficient in lipidation of preipolysaccharides. Isolated pnLTA from this strain was shown to be structurally identical with that of its parental strain D39Δcps and to be free of Toll-like receptor 2 stimulating activity. Therefore, we considered this strain to be best suitable for the investigation of the PGN-WTA complex and for prospective cell stimulation assays, avoiding possible contamination with lipoproteins. The cell wall was digested with pneumococcal amidase LytA and the resultant peptidase-free PGN glycan chains carrying pnWTA were isolated by gel permeation chromatography (GPC) (Supplementary Fig. 1a). This material was digested with lysozyme and mutanolysin, producing PNTA chains bound to a variety of small PGN fragments. The mixture was further purified by another GPC step (Supplementary Fig. 1b). Figure 1a shows the relevant section of the mass spectrum obtained from this material. The identified molecules correspond to pnWTA chains with five to seven RUs bound by a phosphate moiety to di-, tri-, or tetramers of MurNAc-GlcNAc disaccharides.

Analysis by \(^{31}\)NMR revealed the occurrence of additional signals when compared to de-O-acetylated pnLTA isolated from the same strain in the chemical shift range between \( \delta p = -1.0 \) and \( -1.3 \) (Fig. 1b). Using a \(^1\)H, \(^{31}\)P-correlated heteronuclear multiple quantum correlation (HMQC) NMR experiment (Fig. 1c), this bridging phosphate was determined to be an \( \alpha \)-1-phosphate because of the cross correlation into the broadened signals (caused by the heterogeneity of the PGN sugar fragments) in the \(^1\)H NMR spectrum between \( \delta p = 5.51 \) and 5.47 ppm. Based on further NMR analysis, we assigned this \( \alpha \)-1-phosphate to AATGalp, which verifies the direct linkage of pnWTA to PGN. The respective \(^1\)H, \(^{13}\)C HSQC NMR spectrum is shown in Supplementary Fig. 2; the complete NMR chemical shift data for the pnWTA are listed in Supplementary Table 1. The structural elucidation of this linkage clarified the last remaining question about the chemical structure of pnTAs: in the final model (Fig. 1d), both TA types contain the same pseudo-pentasaccharide building block, whereas the terminal RU can occur with or without \( 6-O-P-Chol 

**TacL mutants lack pnLTA and attach pnWTA to PGN.** Based on the different linkage structures of pnWTA and pnLTA, we hypothesized that the ligase for pnLTA assembly on the glycolipid is not an LCP phosphotransferase enzyme. A candidate gene
**Fig. 1** Structural analysis of pnWTA bound to small PGN saccharides from *S. pneumoniae* D39ΔcpsΔgt and chemical structures of pnTAs. **a** Section of the charge deconvoluted ESI-FT-ICR-MS spectrum (acquired in negative-ion mode). Signals for molecules including assignment of signals. Magnification shows P As NMR signals that are not observable in pnLTA preparations. **b** Section of the cross correlation of the phosphates between PGN-derivatized saccharides; observed and calculated masses are given. *only second isotopic peak was observable.* **c** Section of the 31P NMR spectrum. The important region for the linkage phosphate is magnified. **d** Chemical structures of TAs. RUs of both TAs contain the same pseudo-pentasaccharide building blocks, whereby the terminal RU can occur with or without D-Ala (D-Ala; R). n number of RU; R′′ and R′′′, alkyl or alkenyl residues of fatty acid chains in lipid anchor (summarized in ref. 8).

**Table:**

| No. | WTA RUs | PGN saccharides | MWobs [Da] | MWcalc [Da] |
|-----|---------|-----------------|------------|-------------|
| 1   | 4       | 2 2 0           | 7983.742   | 7983.841    |
| 2   | 4       | 6 2 1           | 8845.019   | 8845.094    |
| 3   | 2       | 7 3 2           | 8923.200   | 8923.245    |
| 4   | 4       | 7 3 2           | 8978.366   | 8978.306    |
| 5   | 1       | 1 7 2           | 10147.467  | 10147.621   |
| 6   | 2       | 3 2 1           | 10579.636  | 10579.570   |
| 7   | 3       | 4 2 1           | 9323.200   | 9323.245    |
| 8   | 2       | 3 2 1           | 9281.189   | 9281.306    |
| 9   | 1       | 1 7 2           | 9239.179   | 9240.370    |
| 10  | 2       | 5 2 1           | 8845.019   | 8845.094    |
| 11  | 3       | 3 2 1           | 9883.009   | 9884.183    |

**Legend:**

- **RU 1:** Terminal RU
- **RU 2 to n+1:** Lipid anchor
- **R′ = H or D-Ala:** Peptidoglycan
- **R′′ = H or P-Cho:** pnWTA
- **β in pnLTA:** Configuration of AATGalp
- **α in pnWTA:** Configuration of AATGalp

DOI: 10.1038/s41467-017-01720-z
encoding the LTA ligase was recently identified as *rnfB*\(^{20}\), hereafter called *tacL*. Hence, we isolated pnLTA from the isogenic *tacL* mutants D39Δ*cps*Δ*tacL* and TIGR4Δ*cps*Δ*tacL*, their parental strains and the complemented mutants (Supplementary Table 2) as described\(^7\). The *tacL* deletion and its complementation were verified in the respective encapsulated and non-encapsulated *S. pneumoniae* D39 strains by real-time quantitative PCR (qRT-PCR; Supplementary Fig. 3).

The chromatogram in Supplementary Fig. 4 is a representative hydrophobic interaction chromatography (HIC) purification of pnLTA from strain D39Δ*cps*; the normalized phosphate content is depicted in the upper panel. The \(^{31}\)P NMR spectra of the corresponding hydrazine-treated pnLTAs are shown in Fig. 2. The *tacL* mutants lacked detectable amounts of pnLTA (Fig. 2b, e), while pnLTA was present in both the complemented (Fig. 2c, f) and the isogenic parental strains (Fig. 2a, d) indicating that TacL is required for LTA synthesis. The MS analysis of hydrazine-treated pnLTA from in trans-complemented and parental strains resulted in nearly identical profiles indicating that the expressed *tacL* gene was functional (Supplementary Fig. 5). Moreover, the \(^{31}\)P NMR spectra of the PGN-WTA complex after LytA digestion (Supplementary Fig. 6) prove that TacL is not needed for the synthesis of the WTA-PGN linkage.

The genomes of the nonencapsulated *tacL* mutants and their isogenic parental strains were sequenced and compared to exclude the possibility of compensatory mutations. For strain D39Δ*cps*Δ*tacL*, we identified four single-nucleotide polymorphisms (SNPs) in comparison to its parental strain D39Δ*cps* (Supplementary Table 3). Two are located in intergenic regions, the two others are placed in *spd_0768* (Asp297Gly) and *spd_1179* (Ala141Thr), respectively. The latter encodes a predicted lanthionine synthetase, whereas Spd_0768 (CozE) has recently been described as a member of the MreCD complex of *S. pneumoniae* that directs the activity of PBP1a to the mid-cell plane, where it promotes zonal cell elongation and normal morphology\(^{23}\). The identified variants in the genome of strain TIGR4Δ*cps*Δ*tacL* are listed in Supplementary Table 4. Beside three variations in intergenic regions, our analysis identified three SNPs in *sp_1894* and two SNPs in the pseudogene *sp_rs12410* directly located in between *tacL* and *sp_1894*, with a small overlap with the end of the ORF of *sp_1894*. However, PCR amplification and DNA sequencing of these specific regions disproved two of these SNPs. The confirmed SNPs in *sp_1894*, encoding GtfA (a transferase involved in glycosylation of pneumococcal serine-rich repeat adhesins with O-linked N-acetyl-D-glucosamine\(^{24}\)), are Ile370Val and a silent one (Glu432Glu). Furthermore, a 1 bp deletion causing a frameshift in *sp_1914*, encoding a putative membrane protein of unknown function, was identified. None of

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**Fig. 2** \(^{31}\)P NMR spectra of pnLTA preparations. Sections (\(\delta_{P}\) 3–(–3)) of \(^{31}\)P NMR spectra measured from LTA isolated from pneumococcal strains *a* D39Δ*cps*, *b* D39Δ*cps*Δ*tacL*, *c* D39Δ*cps*Δ*tacL* pBAV-tacL, *d* TIGR4Δ*cps*, *e* TIGR4Δ*cps*Δ*tacL*, and *f* TIGR4Δ*cps*Δ*tacL* pBAV-tacL. *a*, *c*, *f* Hydrazine-treated LTA was used, for *tacL* knockout mutants (*b*, *e*) the typical LTA-containing fractions from the HIC purifications (compare Supplementary Fig. 4) were measured without prior hydrazine treatment.
these mutations are likely to cause any alteration in the pnTA structure.

In summary, whole-genome sequencing and subsequent analysis of genomic variations in the tacL mutants apparently excluded secondary mutations with expectable impact on pnTA biosynthesis, especially because none of the observed variations and therefore potentially compensatory mutations took place in both serotypes. Taken together, our data support the hypothesis that TacL is involved in pnLTA assembly, and not in pnWTA biosynthesis, especially because none of the observed variations excluded secondary mutations with expectable impact on pnTA biosynthesis. Yet, the dosage effect of tacL by multiple plasmid copies. The tacL mutant was slightly affected in detergent-induced autolysis, increasing the rate of cell lysis in the presence of 0.01% Triton X-100 in the encapsulated strains in comparison to the wild-type strain. In the nonencapsulated strains, the effect was less pronounced (Supplementary Fig. 7b).

We next analyzed by field emission scanning and transmission electron microscopy (FESEM, TEM) how LTA deficiency affects pneumococcal cell morphology and cell division in strain D39Δcps grown in complex medium (Fig. 3a) or in RPMI\textsubscript{modi} (Supplementary Fig. 8). No significant differences in cell size or the localization of division septa between the parental strain, the tacL mutant, and complemented mutant were observed for both growth conditions. Furthermore, the amount of capsule was similar in strains with or without tacL, as determined by a flow cytometric assay with an antiserum directed against the capsule content.

Loss of TacL does not perturb pneumococcal physiology. The loss of LTA is lethal to S. aureus under standard laboratory conditions\textsuperscript{25}. Hence, we tested the impact of tacL deletion on pneumococcal growth in two complex media and a chemically defined medium (RPMI\textsubscript{modi})\textsuperscript{26} (Supplementary Fig. 7a). TacL deficiency slightly affected growth of the encapsulated D39 tacL mutant in RPMI\textsubscript{modi}. However, this effect could not be observed for the nonencapsulated strain or for the growth of the encapsulated and nonencapsulated strains in the two complex media. The supplemented, nonencapsulated mutant revealed reduced growth in RPMI\textsubscript{modi} compared to both the wild-type and the TacL-deficient strain. This is possibly an effect of the plasmid-based in trans complementation of the mutant due to a gene

![Fig. 3](image-url)
All strains contained a similar amount of P-Cho. Unfortunately, pnTA-specific antibodies are not currently available. For further quantification of the TAs, we made use of an anti-Forssman antibody that recognizes the terminal sugar residues ($\alpha$-D-GalpNAc-(1 $\rightarrow$ 3)-$\beta$-D-GalpNAc-(1 $\rightarrow$)) of pnWTA and pnLTA. Comparable amounts of the Forssman antigen were detected by flow cytometry in the tacL mutant, wild-type and complemented mutant. In summary, the loss of LTA in the tacL mutant appears to be compensated either by a higher abundance of WTA on the cell surface or by the accumulation of TA precursor chains. Another possibility could be that the loss of pnLTA is simply not measureable due to its low abundance in comparison to the pnWTA. At any rate, we can exclude a dramatic decrease of the cell wall P-Cho content in tacL mutants.

The amount of cell surface-attached CBPs depends on the amount of P-Cho-loaded TAs, which serve as an anchor for these proteins. We determined the presence and amount of various CBPs in the wild-type and mutant strains.

**Fig. 4** Quantitative analysis of teichoic acids and choline-binding proteins. Bacteria were grown in THY medium to $A_{600} = 0.35$–0.45 (for LytA 0.4 and 0.8), washed and incubated with antibodies. a The amount of P-Cho and the Forssman antigen of teichoic acids were determined using specific primary antibodies (TEPC-15, anti-Forssman) and secondary Alexa488-labeled antibody in a flow cytometry-based assay. b Surface-associated CBPs were analyzed using specific polyclonal mice IgG against the individual CBPs and secondary Alexa488-labeled antibodies in a flow cytometry-based assay (see also Supplementary Fig. 9 for representative histograms). The values are represented as the geometrical mean fluorescence intensity multiplied with percent-gated events. Results are expressed as means ± s.d. (each experiment was performed at least three times). *$p < 0.05$; ns not significant (Student’s unpaired t test)
CBPs on the cell surface of the nonencapsulated strains using specific polyclonal antibodies against different CBPs (Fig. 4b and Supplementary Figs. 9b and 10). The pneumococcal surface proteins PspC and Pce were slightly, but not significantly, enhanced in the tacL mutant, whereas CbpP (a protein involved in host–pathogen interaction)\(^{29}\) was slightly reduced. Several other CBPs such as PspA, the major autolysin LytA (at \(A_{600}\) 0.4 and 0.8) and the CBPs CbpL and CbpG were unaltered in the tacL mutant, consistent with the similar content of P-Cho and TA chain ends as determined with the antibodies. Taken together, these results suggest that the tacL mutant carries largely unaltered amounts of P-Cho in its cell wall despite the absence of LTA.

### Influence of pnLTA on pneumococcal adhesion and phagocytosis

To elucidate the role of pnLTA in bacterial adhesion to human lung epithelial cells, A549 cells were infected with the nonencapsulated D39 strain, its isogenic tacL mutant, or its complemented mutant. Immunofluorescence microscopy revealed that the TacL-deficient strain showed a significant decrease in the ability to adhere to A549 cells after 2 or 4 h of infection, whereas the adherence of the complemented mutant was comparable to that of the parental strain (Supplementary Fig. 11).

The impact of LTA on pneumococcal phagocytosis by PMA-differentiated (phorbol 12-myristate 13-acetate) THP-1 cells was evaluated in antibiotic protection assays and by double immunofluorescence staining. A time-dependent uptake of pneumococci could be monitored for all tested strains with a moderate increase for the complemented mutant in the antibiotic protection assay compared to the parental strain D39Δcps (Supplementary Figs. 12a and 13). The intracellular killing of the TacL-deficient mutant within the phagocytes was moderately but significantly decreased after 1 or 2 additional hours of incubation as determined by antibiotic protection assays (Supplementary Fig. 12b). In conclusion, pneumococcal adherence to epithelial cells is significantly affected by the loss of LTA, whereas bacterial uptake by phagocytes was not affected.

### Loss of TacL attenuates pneumococcal virulence

We used two different mouse models of infection to assess the impact of the TacL deficiency on pneumococcal pathogenesis (Fig. 5a, b and Supplementary Fig. 14). In the acute pneumonia model (Fig. 5a, Supplementary Fig. 14), mice were infected intranasally with \(S.\) pneumoniae D39lux, D39luxΔtacL, or the complemented mutant. In case of the wild type, 90% of the infected mice developed pneumonia, which resulted in severe sepsis and death of the mice. By contrast, only 30% of the mice infected with the tacL mutant showed severe signs of pneumonia and sepsis. The course of disease is clearly prolonged in mice infected with the TacL-deficient strain. Importantly, complementing the tacL mutant in trans restored the phenotype of the wild type. In the systemic infection (sepsis) model (Fig. 5b), mice were infected intraperitoneally with the encapsulated D39lux strain, D39lux-ΔtacL, or the complemented mutant and the severity of disease was monitored over time. Mice infected with the wild-type strain or the complemented mutant showed earlier onset of disease and died significantly sooner than mice infected with the tacL mutant. In summary, loss of TacL resulted in a significantly attenuated virulence, indicating an essential role of LTA in the pathophysiology of pneumococci.

### Discussion

We show in this work that TacL (also known as RafX, SPD_1672 (strain D39) or SP_1893 (in TIGR4)) is required for pnLTA formation in \(S.\) pneumoniae. We propose that TacL is a lipoteichoic acid ligase acting in the final step of pnLTA synthesis (Fig. 6). Our data are not consistent with the previously hypothesized role of SPD_1672 and its homologs in the biosynthesis of pnWTA, which was based on antibody detection methods and did not involve a detailed structural analysis of WTA or LTA. Remarkably, with the exception of few choline-utilization enzymes\(^{30}\), most of the enzymes in the predicted pathway of pnTA synthesis\(^{13}\) remain uncharacterized.

The undecaprenyl-diphosphate (Und-PP)-linked monomeric repeats are polymerized by the RU polymerase, transported through the cytosolic membrane by TacF and are finally transferred onto the PGN or the glycolipid anchor. We show here that the linkage conformation in pnWTA differs from that of pnLTA; the AATGalp moiety within the first RU of pnLTA is \(\beta\)-configured\(^7\), while the respective AATGalp in pnWTA has the \(\alpha\)-configuration. This observation suggests that the final reactions in pnLTA and pnWTA assembly differ mechanistically and that the reactions are likely catalyzed by enzymes from different classes. The transfer of the TA precursor chains onto the PGN is most likely performed by the LCP proteins Psr, LytR, and Cps2A\(^{19}\), which belong to the same family of phosphotransferases. Here the TA chain is transferred with one phosphate, thus retaining the anomic configuration of the AATGalp moiety of the first RU. By contrast, in LTA synthesis, a new glycosidic bond is formed between the glucose moiety of the glycolipid anchor and the first AATGalp. This reaction inverts the stereochemistry of the anomic carbon, leading to the \(\beta\)-configuration of the AATGalp moiety of the first RU in pnLTA. A similar mechanism has been described in the biosynthesis of

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**Fig. 5** Impact of TacL deficiency on pneumococcal virulence. Survival of CD-1 mice after a intranasal infection with \(-2.5 \times 10^7\) bioluminescent \(S.\) pneumoniae D39lux wild type, D39luxΔtacL, or the complemented mutant (D39ΔtacL pBAV-tacL) in the pneumonia model (\(n = 10\)) (see also Supplementary Fig. 14 for bioluminescence images). b Survival of CD-1 mice (\(n = 12\)) after intraperitoneal infection with \(-3 \times 10^9\) pneumococci in the systemic infection model. \(*p < 0.01;\) ns not significant (Log-rank (Mantel–Cox) test).
Fig. 6 Last steps in the TA biosynthesis pathway in *S. pneumoniae*. The Und-PP-linked monomeric repeats are polymerized by the RU polymerase and transported through the cytosolic membrane by TacF. The complete biosynthesis pathway is reviewed and discussed in ref. 15. Finally, the pnTA precursor chain is transferred onto the PGN to form the pnWTA or onto the glycolipid anchor to form the pnLTA, respectively. The transfer to the PGN is performed—most likely in a semi-redundant manner—by the LCP proteins Psr, LytR, and Cps2A. Here the TA chain is transferred including one phosphate, thus retaining the anomeric α-configuration of the AATGal moiety of the first RU. In pnLTA formation, a new glycosidic bond between the glucose moiety of the glycolipid anchor and the first AATGalp is formed. We propose that TacL may catalyze this reaction, which inverts the stereochemistry of the anomeric carbon leading to the β-configuration of the AATGalp moiety of the first RU in pnLTA. From these observations, it can be concluded that the Und-PP-linked pnTA precursor chains are synthesized with all AATGalp moieties in the α-configuration. AATGal 2-acetamido-4-amino-2,4,6-trideoxygalactose, CM cytoplasmic membrane, GalNAc N-acetylglactosamine, Glc glucose, LCP LytR-Cps2A-Psr family protein.
the lipopolysaccharide (LPS) of Gram-negative bacteria when the O-antigen chain is attached to the core oligosaccharide. The O-antigen is synthesized as an Und-PP-linked precursor and is translocated across the cytosolic membrane by different pathways. In several published LPS structures, the first sugar of the O-antigen always has the β-configuration, whereas it has the α-configuration in the Und-PP-linked precursor. This reaction is catalyzed by the O-antigen ligase Waal (RflA).

TacL of *S. pneumoniae* may be located in the cytoplasmic membrane with nine transmembrane helices (as predicted by TMbase; http://embnet.vital-it.ch/software/TMPRED_form.html) and a *WpyC* Pfam domain (STRING database v. 10.0). *WpyC* superfamily domains are characteristic of enzymes involved in the synthesis of O-antigens in Gram-negative bacteria, including O-antigen ligases. Pneumococcal TacL is composed of 397 amino acids, comparable to the length of the RflA. O-antigen ligase of *E. coli* K12 (419 aa) or Waal of *Pseudomonas aeruginosa* PA01 (401 aa), which are predicted by TMbase to have similar secondary structures to TacL. TacL shares significant sequence similarities to a stretch of 208 amino acids of RflA (21.6% identity, 57.7% similarity) and to a 378 amino acid stretch in Waal (18.8% identity, 49.2% similarity). The attachment of carbohydrate polymers to glycolipids to form complex cell wall glycolipopolymers in Gram-positive and Gram-negative bacteria thus appears to share a conserved mechanism.

The repeating units of the wall and lipoteichoic acids of pneumococci comprise identical RUs, which is a special feature that differs from most other Gram-positive bacteria. Most of the Firmicutes, like *S. aureus*, possess WTA built up of ribitol-phosphate RUs, whereas the LTA comprises repeating *mutants*37. Hence, contrary to *Firmicutes*, like *that differs from most other Gram-positive bacteria. Most of the pneumococci comprise identical RUs, which is a special feature thus appears to share a conserved mechanism.

Attachment of carbohydrate polymers to glycolipids to form complex cell wall glycolipopolymers in Gram-positive and Gram-negative bacteria thus appears to share a conserved mechanism.

Methods

**Bacterial strains and growth conditions.** Bacterial strains are listed in Supplementary Table 2. *Escherichia coli* were cultured on Luria Broth (LB) plates or in liquid LB medium, supplemented with 200 μg ml⁻¹ erythromycin at 37 °C. Transformation of *E. coli* with plasmid DNA was carried out using chemically competent cells. *S. pneumoniae* serotype 2 D39 and serotype 4 TIGR4 and their isogenic mutants were grown on Columbia blood agar plates (Oxoid) containing erythromycin (5 μg ml⁻¹) and/or chloramphenicol (5 μg ml⁻¹), or cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY; Roth) or chemically defined medium (RPMI, Invivogen, HE, Healthcare, Bio-Sciences), respectively. Cultivation of pneumococci on blood agar or in liquid cultures was performed at 37 °C and 5% CO₂ without agitation.

**Mutant construction.** For the construction of the pneumococcal *tacL* mutants in D39 and TIGR4, a DNA fragment consisting of the *S. pneumoniae* D39 *spd_1672* gene and its up- and down-stream flanking regions were amplified by PCR from genomic DNA using primer SPD1672_OLuP_for and SPD1672_OLuP_rev (primers are listed in Supplementary Table 2). The purified PCR products were cloned into pUC18 and *E. coli* DH5α chemically competent cells were transformed with the resulting plasmid. The recombinant plasmid pNH1 harboring the desired DNA insert was purified and used as template for an inverse PCR reaction with primer InvnrevKpnISPD1672 and InvforPstISPD1672. The deleted gene sequence was replaced by an *ermB* gene, amplified by PCR from vector pTPI using primer InvnrevKpnISpErm and InvforPstISpErm. The final recombinant plasmid was used to transform and mutate gene pneumococci. Transformation efficiency was evaluated using the final recombinant plasmid vs. another gene deletion plasmid (for deletion of the *cbpL* gene) in *S. pneumoniae* D39Δ*cbpL*44.Remarkably, the transformation efficiency was thereby significantly increased for the *tacL* deletion (2.8 colonies per ng plasmid for cbpL vs. 29.8 colonies per ng plasmid for *tacL*).

Isogenic mutants were complemented by a pBAV1CpE-based in trans system; pBAV1CpE was modified from pBAV1K-T5-gfp48, by exchanging the kanamycin-resistance gene for a chloramphenicol-resistance gene and the T5 promoter for an erythromycin promoter region (pE), which includes the ribosomal-binding site and start codon. The complete *spd_1672* gene was amplified by PCR using primer 1672_com_for and SPD1672_com_rev and the purified fragment was cloned into pBAV1CpE. The resulting plasmid pBAV-*tacL* was used to transform isogenic *tacL* mutants. The deletion of *tacL* as well as the in trans complementation were verified using qRT-PCR (Supplementary Fig. 3).
Real-time quantitative PCR (qRT-PCR). Encapsulated and nonencapsulated D39 wild-type strain, tacL-deficient mutant, and complemented mutant were cultivated in ThF in citrate-buffered F5 (adjusted to pH 6.5 with NaOH) and harvested for RNA isolation using Eurex GeneMatrix Universal RNA purification kit (robokon). The quality of RNA was checked by agarose gel electrophoresis and standard PCR using primer EmoR_F and EmoR_R (see Supplementary Table 2). The synthesis of cDNA was performed using the SuperScript III Reverse Transcriptase (ThermoFisher) and hexamer primers (GE Healthcare) according to the manufacturer’s instructions. The quality of cDNA was controlled by PCR using primer EmoR_F and EmoR_R and the concentration was measured by nanodrop. cDNA was stored at −20 °C until further tests. For the qRT-PCR experiments, StepOnePlus™ Real-Time PCR System (Applied Biosystems) and Green PCR Master Mix (Bio-rad) were used in combination with tacL-specific primers as well as enolase-primers as control (see Supplementary Table 2). The StepOne software (v. 2.3, Life Technologies) was used for data analysis. The final results are presented as mag- nitude of fluorescence (ΔRn) plotted against PCR cycle numbers.

Sequencing and bioinformatics analysis. Sequencing: 1 ng of purified chromosomal DNA from S. pneumoniae strains D39ΔΔps (PN111), D39cpsΔΔtalc (PN601), D39ΔΔpsΔΔtalc PBAY-talc (PN634), TIGR4ΔΔpsΔΔtalc (PN635) and TIGR4ΔΔpsΔΔtalc PBAY-talc (PN636) was used to prepare individual libraries employing and following the Illumina Nextera® XT DNA Library Prep Kit. An Agilent Technology 2100 Bioanalyzer served to verify tag- mentation and the final library fragment size distribution on a high sensitivity DNA chip was used for the final library purification. The Agilent purified pooled library was applied to a MiSeq Reagent v3 600 cycle kit and sequenced on a MiSeq system as 300 cycle paired-end run. The pooled library was spiked with 5% PhiX control library. A cluster density of 847 ± 100,000 cycle per mm (95% confidence interval) was achieved with 96.46 ± 1.48% of clusters passing filter specifications. 20.3 Mio reads (94.7%) of 211 Mio total reads resulted in 48.5 Gbp reads, belonging to 12.52 Gbp reads of sequence data. Index reads were evenly distributed across the six individual samples. Generated FASTQ files were subjected to further bioinformatics analysis as outlined below.

SNP detection and annotation: SNP detection was done for S. pneumoniae D39ΔΔpsΔΔtalc and TIGR4ΔΔpsΔΔtalc individually by a photometric phosphate test47. The phosphate-containing fractions were placed on poly-L-lysine coated coverslips and allowed to settle for 10 min. After washing twice with 10 mM CaCl2 and trypsin (100 µg ml−1) and treated with the pneumococcal LytA amidase as described elsewhere45. Recombinant His-tagged LytA amide (10 µg LytA permg) was washed twice with water. Finally, the pellet was resuspended in 2–4 ml of water and lyophilized, yielding the purified PGN-WTA complex. Depending on the specific research question, further chemical or enzymatic treatments were performed subsequently.

Chemical and enzymatic treatment of LTA: Hydrolysis treatment of LTA: Purified LTA was dissolved at a concentration of 5 µg µl−1 in anhydrous hydrazine (N2H4, ICN Biomedicals) before incubation for 1 h at 37 °C while being stirred. The reaction was quenched by adding the same volume of acetic acid and dried under a stream of nitrogen; the drying step was repeated twice. Subsequently, the crude de-O-acylated LTA was purified by gel permeation chromatography (GPC) on a Bio-Gel P-10 (4 × 100 cm, 1 ml/column, BioRad; column size: 1.5 × 120 cm; buffer: 150 mM ammonium acetate (pH 4.7) column).

Enzymatic digestion of the PGN-WTA complex: To remove all amino acids from the PGN, the PGN-WTA complex was dissolved in 50 mM Tris-HCl (pH 7.0; 10 mg ml−1) and treated with the pneumococcal LytA amide as described elsewhere41. Recombinant His-tagged LytA amide (10 µg LytA permg) was added in three aliquots after 0.24, and 48 h for a total period of incubation of 72 h at 37 °C. Subsequently, the enzyme was inactivated by boiling for 5 min at 100 °C. After centrifugation (25,000g; 15 min, 20 °C), the supernatant was collected and lyophilized. The crude LytA-treated PGN-WTA complex was further purified by GPC on a Bio-Gel P-30 (45–90 µm, BioRad; column size: 1.5 × 120 cm; buffer: 150 mM ammonium acetate (pH 4.7) column). The high molecular weight material obtained (∼12 mg) was further digested with lysozyme (200 µg Sigma) and mutanolysin (200 µg Sigma) in an 800 µl reaction mixture containing sodium phosphate (20 mM; pH 4.8) and sodium azide (0.02%) at 37 °C overnight. The enzyme was inactivated by heating at 100 °C for 5 min. The soluble material was recovered by centrifugation (18,000×g, 10 min, 20 °C) and lyophilized. Isolation of the pmWNTA bound to small PGN fragments was achieved by a final GPC using the conditions mentioned above.

NMR spectroscopy. NMR spectroscopic measurements were performed in D2O at 300 K on a Bruker Avance® 200 MHz (equipped with an inverse 5 mm quadruple-resonance Z-grad cryoprobe). Deuterated solvents were purchased from Deutero GmbH (Ettenheim, Germany). Acetone was used as an external standard to cali- brate 1H (δ 2.225) and 13C (δc 30.89) NMR spectra. 1H NMR spectra (δc 6.0) were calibrated with 85% phosphoric acid in D2O as an external standard. 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 experiments. Isotopically labeled LTA was used initially and this sample was incubated at 37 °C for 60 min, quenched by adding the same volume of acetone and dried under a stream of nitrogen; the drying step was repeated twice. Subsequently, the sample was incubated at 100 °C for 5 min. The solubilized material was recovered by centrifugation (18,000×g, 10 min, 20 °C) and lyophilized. Isolation of the pmWNTA bound to small PGN fragments was achieved by a final GPC using the conditions mentioned above.

Mass spectrometry. To analyze the pmWNTA bound to small PGN fragments, electrospray ionization fourier-transform ion cyclotron resonance mass spectrom- egy (ESI-FT-ICR-MS) was performed on a 7 Tesla APEX Qe instrument (Bruker Daltonics, Bremen, Germany) using negative-ion mode and a water/propan-2-ol/ 7 M triethylamine/acetic acid mixture (50:50:50 v/v/v) as solvent, as described previously5. MS analysis of hydrazine-treated LTA was done on a Q Exactive Plus (Thermo Scientific, Bremen, Germany) in negative-ion mode using the same solvent. A Triversa Nanomate (Advinol, Ithaca, USA) ion source was used with a spray voltage set to −1.1 kV. The mass scale was calibrated externally with glycolipids of known structure, and all spectra were charge deconvoluted. The given mass numbers refer to the monoisotopic mass of the neutral molecules.

Electron microscopy. Field emission scanning electron microscope: bacteria were fixed in the growth medium with 5% formaldehyde and 2.5% glutaraldehyde for 1 h on ice and washed with HEPES buffer pH 7.3 (10 mM HEPES, 10 mM NaCl, 10 mM MgCl2, pH 6.9). An aliquot of 50 µl of the fixed bacterial solution was placed on poly-l-lysine coated coverslips and allowed to settle for 10 min. After fixation with 2% glutaraldehyde in PBS for 5 min at room temperature, the coverslips were washed with TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.9) before dehydration in a graded series of acetone (10, 30, 50, 70, 90, and 100%) on ice for 10 min for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change in 100% acetone before critical-point drying
Immunized intraperitoneally with 20 fl of pneumococci and incubated in infection medium (DMEM (HyClone™) + 1% heat-inactivated fetal bovine serum (FBS)) at 37 °C and 5% CO2. Subsequently, cells were washed three times with phosphate-buffered saline containing 1% FBS (Gibco) to remove unbound bacteria. Afterwards, bacteria were fixed with PBS containing 1% para-formaldehyde (PFA, Roth).

**Immunofluorescence microscopy.** Fixed pneumococci, bound to A549 cells were washed three times with PBS and blocked for 1 h at room temperature using PBS + 10% heat-inactivated fetal bovine serum (FBS, Gibco) supplemented with 1% para-formaldehyde (1:500, Davids Biotechnologie GmbH) against pneumococci (generated in rabbit against heat-inactivated S. pneumoniae TIGR4 and D39). As a secondary antibody, fluorescence-labeled Alexa-Fluor546 goat anti-rabbit antibody (Abcam) was used (1:500, 1 h, room temperature). Bacterial adherence was monitored for at least 20 cells per coverslip using immunofluorescence microscopy. Each experiment was repeated three times in duplicate. All data are reported as means ± s.d. Statistical analysis was performed using the unpaired two-tailed Student’s t test. In all analysis, a p value of <0.05 was considered statistically significant.

**Flow cytometry.** A confluent layer of monolayer THP-1 cells (ATCC™ TIB-202™) grown in 24-well plates (3 × 105 cells per well in RPMI-1640 (HyClone™) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco)) was differentiated to phagocytes with the addition of 200 mmol ml−1 phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and incubated for 48 h at 37 °C and 5% CO2. Afterwards, THP-1 cells were washed with RPMI-1640 supplemented with 10% heat-inactivated FBS and incubated for another 24 h at 37 °C and 5% CO2. Prior infection pneumococci were cultured to mid-log phase (≤0.35–0.45), centrifuged, and washed with infection medium (RPMI-1640 supplemented with 1% heat-inactivated FBS). THP-1 cells were washed and infected with pneumococci in 500 μl infection medium. Infection was synchronized by centrifugation (2 min, 300 g) to initiate a simultaneous contact between bacteria and phagocytes. Afterwards cells were incubated at 37 °C and 5% CO2 for different time periods. After infection, cells were washed with infection medium and incubated with Penicillin G (100 unit ml−1, Sigma-Aldrich) and Gentamicin (0.1 mg ml−1, Sigma-Aldrich) for 1 h at 37 °C and 5% CO2 to kill extracellular bacteria. Then, phagocytes were washed and lysed with 1% saponin (Sigma-Aldrich) to release intracellular pneumococci. Calcofluor white (1 μg ml−1) of intracellular bacteria were determined by plating bacteria in appropriate dilutions on blood agar plates (Oxoid) to quantify the number of intracellular bacteria. Data were normalized in the antibiotic protection assays to the multiplicity of infection (MOI) or in the time-dependent killing to recovered bacteria at time point 0 h. All assays were analyzed using one-way ANOVA with Bonferroni correction.

**Immunoblot analysis.** *S. pneumoniae* D39 wild-type, its isogenic tacL mutant, and the complemented mutant were cultured in THY medium to mid-log phase, harvested by centrifugation at 3270g at 4 °C for 6 min, and resuspended in 1 ml PBS buffer at pH 7.4. A total of 2 × 108 bacteria were washed and cultured on a 12% SDS-PAGE before transferring to a nitrocellulose membrane by semidry transfer. Membranes were washed for 2 h at room temperature using 5% skimmed milk (Roht) and Tris-buffered saline (TBS; pH 7.4) and incubated overnight at 4 °C with mouse polyclonal antibodies against different CBPs (1:500 in PBS), or for the antigen of TAs with antibodies against P-Chp (TEPSCor; Forsman et al. 1993). Rabbit polyclonal antibodies (15 min at 37 °C and 5% CO2) in 96-well plates (U-bottom, Greiner Bio-One) (1:500 in PBS) for 30 min at 37 °C. After washing with PBS, blots were fixed with 1% formaldehyde overnight at 4 °C. The abundance of CBPs as well as the quantity of teichoic acids in nonencapsulated D39 wild-type, mutant and complemented strains were measured by flow cytometry after fixation with 1% formaldehyde for 1 h at 4 °C. Two hundred μl suspensions containing 8 × 108 bacteria were incubated with specific polyclonal antibodies against different CBPs (1:500 in PBS), or for the quantification of TAs with antibodies against P-Chp (TEPSCor; Forsman et al. 1993) for 45 min at 37 °C. PMA-differentiated THP-1 cells (3 × 105 cells per well) were grown on sterile glass coverslips (12 mm, Hartenstein) and infected with pneumococci as described above. After infection, THP-1 cells were washed with infection medium and incubated with Penicillin G (100 unit ml−1, Sigma-Aldrich) and Gentamicin (0.1 mg ml−1, Sigma-Aldrich) for 1 h at 37 °C and 5% CO2 to kill extracellular bacteria. Then, phagocytes were washed and lysed with 1% saponin (Sigma-Aldrich) to release intracellular pneumococci. Calcofluor white (1 μg ml−1) of intracellular bacteria were determined by plating bacteria in appropriate dilutions on blood agar plates (Oxoid) to quantify the number of intracellular bacteria. Data were normalized in the antibiotic protection assays to the multiplicity of infection (MOI) or in the time-dependent killing to recovered bacteria at time point 0 h. All assays were analyzed using one-way ANOVA with Bonferroni correction.

**Double immunofluorescence staining and microscopy.** PMA-differentiated THP-1 cells (3 × 105 cells per well) were grown on sterile glass coverslips (12 mm, Hartenstein) and infected with pneumococci as described above. After infection, THP-1 cells were washed with infection medium and fixed with 4% paraformaldehyde (Roth) overnight at 4 °C. Glass coverslips were washed with PBS and blocked with PBS + 2% heat-inactivated FBS for 1 h. After washing, extracellular bacteria were stained using a polyclonal α-pneumococci IgG (1:500) and an Alexa-Fluor488-labeled secondary goat α-rabbit IgG (1:500, Abcam) for 30 min at room temperature. Glass coverslips were washed thrice with PBS following permeabilization of THP-1 cells with 0.1% Triton X-100 in PBS (10 min, room temperature). After washing, intracellular pneumococci were stained using a polyclonal α-pneumococci IgG (1:500) and a secondary Alexa-Fluor546-labeled goat α-rabbit IgG (1:500, Abcam) for 30 min at room temperature. Experiments were repeated three times as duplicates. For statistical analysis, 50 cells per glass coverslip were analyzed for the number of intracellular bacteria. Data were normalized to the MOI. All statistical analysis was conducted using GraphPad Prism software. In all analysis, a p value of <0.05 was considered statistically significant.

**Cell lines.** All cell lines used in this study were purchased from ATCC (A549, ATCC CCL-185; THP-1: ATCC TIB-202) and have been tested to be mycoplasma-negative by PCR and scanning electron microscopy.

**Acute mouse pneumonia and systemic infection model.** Eight- to 10-week-old female CD1 mice (outbred, Charles River, Sulzdorf, Germany) were intranasally infected with 108 cfu ml−1 of S. pneumoniae or PBS via a tracheal cannula. Intranasal infection, the infection dose (20 μl) was adjusted to ~2.5 × 108 cfu in PBS (pH 7.4). For nasal infection, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (Ketalar® S. Pfizer Pharma, Karlsruhe, Germany; Rompun® Lyphack, Buchs, Switzerland) and bacteria were administered dropped into the nostrils. The cfu of the infection dose was confirmed by plating of serial dilutions of the inoculum on...
blood agar plates. Mice were monitored for survival and imaged for bioluminescence at pre-chosen intervals using the IVIS Spectrum Imaging System (Caliper Life Sciences, Hopkinton, USA). For the systemic infection model, an injection dose of 3 x 10^3 cfu was administered intraperitoneally in a volume of 200 µl PBS (pH 7.4). All animal experiments were conducted according to the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Sciences Associations (FELASA). All experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischer Leckemünde – Vorpommern (LALLEV M-V, Rostock, Germany, permit no. 72213.1-056/16-1).

Data availability. Raw FASTQ files containing S. pneumoniae genomic sequence data were submitted to the EMBL-EBI European Nucleotide Archive (ENA) and stored in the Short Read Archive (SRA) under the study accession number RRB185585. All other relevant data supporting the findings of the study are available in this article and its Supplementary Information files, or from the corresponding authors on request.

Received: 10 February 2017 Accepted: 11 October 2017

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ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-01720-z

NATURE COMMUNICATIONS

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NATURE COMMUNICATIONS

Received: 10 February 2017 Accepted: 11 October 2017

Published online: 12 December 2017

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**Acknowledgements**

We thank Simone Thomsen (TA isolation and derivatisation), Brigitte Kunz (MS), Heiko Käßner (NMR) (all Research Center Borstel), Peggy Stremlow and Kristine Sievert-Giermann (University of Greifswald), Markus Weigel (Justus-Liebig University of Giessen), and Ina Schleicher (Helmholtz Centre for Infection Research, Braunschweig) for excellent technical assistance. Furthermore, we thank Johannes Müthing (University of Münster) and Franziska Voß (University of Greifswald) for providing antibodies. We thank Rick Lewis (Newcastle University) for critical reading of the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft to N.G. (GI 979/1-1), S.H. (HA 3125/5-1 and GRK 1870), and SFB-TR84 project B01 to T.H. as well as the German Center for Infection Research (DZIF). The purchase of the Illumina MiSeq system was kindly supported by the EU-EFRE (European Funds for Regional Development) program and funds from the University Medicine Rostock awarded to B.K. (all included in the grant number: UHROM10).

**Author contributions**

N.H., F.W., T.P.K., N.G., and S.H. designed the experiments; N.H., F.W., T.P.K., M.R., B.K., A.G.-M. and N.G. performed the research; F.W., N.H., T.P.K., M.R., A.G.-M., T.H., D.S., W.V., N.G. and S.H. analyzed the data; F.W., N.H., T.P.K., W.V., N.G. and S.H. wrote the paper. All authors discussed the results and approved of the manuscript.

**Additional information**

Supplementary Information accompanies this paper at 10.1038/s41467-017-01720-z.

**Competing interests:** The authors declare no competing financial interests.

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