Class A PBPs have a distinct and unique role in the construction of the pneumococcal cell wall

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In oval-shaped Streptococcus pneumoniae, septal and longitudinal peptidoglycan syntheses are performed by independent functional complexes: the divisome and the elongasome. Penicillin-binding proteins (PBPs) were long considered the key peptidoglycan-synthesizing enzymes in these complexes. Among these were the bifunctional class A PBPs, which are both glycosyltransferases and transpeptidases, and monofunctional class B PBPs with only transpeptidase activity. Recently, however, it was established that the monofunctional class B PBPs work together with transmembrane glycosyltransferases (FtsW and RodA) from the shape, elongation, division, and sporulation (SEDs) family to make up the core peptidoglycan-synthesizing machinery within the pneumococcal divisome (FtsW/PBP2x) and elongasome (RodA/PBP2b). The function of class A PBPs is therefore now an open question. Here we utilize the peptidoglycan hydrolase CbpD that targets the septum of S. pneumoniae cells to show that class A PBPs have an autonomous role during pneumococcal cell wall synthesis. Using assays to specifically inhibit the function of PBP2x and FtsW, we demonstrate that CbpD attacks nascent peptidoglycan synthesized by the divisome. Notably, class A PBPs could process this nascent peptidoglycan from a CbpD-sensitive to a CbpD-resistant form. The class A PBP-mediated processing was independent of divisome and elongasome activities. Class A PBPs thus constitute an autonomous functional entity which processes recently formed peptidoglycan synthesized by FtsW/PBP2x. Our results support a model in which mature pneumococcal peptidoglycan is synthesized by three functional entities, the divisome, the elongasome, and bifunctional PBPs. The latter modify existing peptidoglycan but are probably not involved in primary peptidoglycan synthesis.

Significance

Peptidoglycan, the main structural component of the bacterial cell wall, is made of glycan strands cross-linked by short peptides. It has long been assumed that class A penicillin-binding proteins (PBPs) are the only enzymes capable of synthesizing glycan strands from lipid II. Recently, however, it was discovered that two non-PBP proteins, FtsW and RodA, constitute the core peptidoglycan polymerizing enzymes of the divisome and elongasome, respectively. What, then, is the role of class A PBPs in the construction of the bacterial cell wall? In contrast to previous assumptions, our results strongly suggest that class A PBPs are not an intrinsic part of the divisome and elongasome but have important autonomous roles in construction of the fully mature bacterial cell wall.

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without the involvement of class A PBPs. If so, the function of class A PBPs is an open question, and their role in peptidoglycan synthesis must be reexamined. Here we have addressed this question by exploiting the unique properties of the peptidoglycan hydrolase CbpD (choline-binding protein D).

CbpD is composed of three domains: an N-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain, one or two Src homology 3b (SH3b) domains, and a C-terminal choline-binding domain (Cbd) consisting of four choline-binding repeats (16). CHAP domains are present in many peptidoglycan hydrolases and function as either N-acytethylmuramoyl-l-alanine amidases or endopeptidases (17, 18). Hence, the CHAP domain of CbpD cleaves somewhere within the peptide bridges of streptococcal peptidoglycan. However, the exact bond cleaved has not been identified. The SH3b domain is essential for the function of CbpD, and experimental evidence indicates that it binds to the peptidoglycan portion of the cell wall (16). The choline-binding repeats of the Cbd domain anchor CbpD to cell wall teichoic acid and possibly also lipoteichoic acid through noncovalent interactions with the choline residues decorating these polymers (19). Similar to the CHAP and SH3b domains, the Cbd domain is essential for the biological function of CbpD (16).

Even though CbpD appears to be a key component of the pneumococcal gene transfer machinery, it is still poorly characterized. In the present study, we were able to purify the CbpD protein from *Streptococcus mitis* B6 (CbpD-B6) and show that it specifically cleaves nascent peptidoglycan formed by the pneumococcal PPB2x/FtsW machinery. We utilized this unique specificity of CbpD to study the functional relationships between different peptidoglycan-synthesizing enzymes in *S. pneumoniae*. Our results strongly indicate that class A PBPs are not part of the core machineries of the divisome and elongosome but have an important autonomous role in the construction of the fully matured peptidoglycan layer.

**Results**

**CbpD-B6 Attacks the Septal Area of the Pneumococcal Cell Wall.** It has previously proved very difficult to express and purify the pneumococcal peptidoglycan hydrolase CbpD from *S. pneumoniae* strain R6 (CbpD-R6). In order to further study the properties of this enzyme, we therefore searched for homologous CbpD variants in other streptococcal species. The CbpD allele from *S. mitis* B6 (CbpD-B6) is highly homologous to CbpD-R6. Their CHAP and Cbd domains are 96% and 95% identical, respectively. The major difference between them is that CbpD-R6 contains an extra SH3b domain (*SI Appendix, Fig. S1*). We were able to successfully purify CbpD-B6 using diethylaminoethyl (DEAE) cellulose affinity chromatography (20) and size exclusion chromatography (*SI Appendix, Fig. S2*). The R6 strain is highly sensitive to CbpD-B6, and a concentration of 0.3 μg/mL lysed 50% of the cells in an R6 culture at an optical density at 550 nm (OD_{550}) of 0.2 (see the titration experiment in *SI Appendix, Fig. S3*). To rule out the possibility that lysins from the *Escherichia coli* expression host contaminated the CbpD-B6 protein preparation, a control experiment was performed in which choline (2% final concentration) was added together with the CbpD-B6 preparation to the pneumococcal culture. Exogenously added choline binds to the Cbd domain of CbpD-B6 and inhibits its function by blocking its binding to the choline residues decorating pneumococcal teichoic acids (20). No lysis was observed in the presence of 2% choline (*SI Appendix, Fig. S4*). As no choline-binding lysins are produced by *E. coli*, this shows that the observed muralytic activity is caused by CbpD-B6. The purified CbpD-B6 protein preparation was therefore used for further studies.

Pneumococci exposed to purified recombinant CbpD-B6 were examined by scanning electron microscopy (SEM) for visualization of changes in their ultrastructure. The SEM microscopy analysis clearly showed that CbpD-B6 attacks only the septal region of the peptidoglycan sacculus, resulting in cells that are split in half along their equators (Fig. 1). Interestingly, the rims of both hemispheres in the split cells are thicker than the rest of the peptidoglycan layer. This suggests that CbpD-B6 cleaves the cells along the middle of the equatorial ring, also called the piecrust.

**CbpD-B6 Specifically Cleaves Nascent Peptidoglycan Formed by PPB2x and FtsW.** Since CbpD-B6 attacks the septal region of the cell, we speculated that the enzyme targets the peptidoglycan formed by PPB2x and FtsW. If so, specific inhibition of the divisome activity might render pneumococci less sensitive or insensitive to CbpD-B6. In a recent profiling of the β-lactam selectivity of pneumococcal PBPs, Kocaoglu et al. (21) showed that PPB2x is more sensitive than PBPa1, PBPa1b, PBPa2a, and PBPa2b to several different β-lactams. Hence, by using the appropriate β-lactam at the right concentration it should be possible to inhibit the transpeptidase activity of PPB2x without significantly affecting the function of the other PBPs. To test this hypothesis, we grew pneumococcal cultures in 96-well plates in a microplate reader at 37 °C. When reaching OD_{550} ~ 0.2, each culture was treated with a different concentration of oxacillin. The oxacillin concentrations used ranged from 0 to 100 μg mL^{-1}, i.e., from sub- to supra-minimum inhibitory concentrations (MICs). Ten minutes after being exposed to oxacillin, each culture received 5 μg mL^{-1} of purified CbpD-B6. Comparison of the lytic responses of the cultures showed that the extent of lysis gradually decreased with increasing oxacillin concentrations until the cells became resistant to CbpD-B6 at concentrations between 0.19 and 6.1 μg mL^{-1} (Fig. 2A). The lowest antibiotic concentration that gave full protection against CbpD-B6 (0.19 μg mL^{-1}) corresponds roughly to the MIC value of oxacillin against the R6 strain (*SI Appendix, Fig. S5A*). However, to our great surprise, the pneumococci started to lyse again when the concentration of oxacillin was increased further, i.e., above 6.1 μg mL^{-1}. At the highest oxacillin concentrations used (50 and 100 μg mL^{-1}), the pneumococci became as sensitive as untreated control cells (Fig. 2A). In sum, the results show that as the oxacillin concentration is gradually increased, the lytic response to CbpD-B6 shifts from decreasing sensitivity (S1 phase) to resistance (R phase) and then back to increasing sensitivity (S2 phase).

In line with the observations above (Fig. 1), GFP-CbpD has previously been shown to mainly bind the septal region of pneumococcal cells, and the binding specificity is determined by the C-terminal choline-binding domain (16). To test whether CbpD resistance during the R phase could be explained by altered binding of CbpD after exposure to oxacillin, we analyzed the binding patterns of sfGFP-CbpD-B6 as previously described (16). The fusion protein was expressed and purified essentially as CbpD-B6 and exposed to RH425 control cells as well as RH425 cells treated with 0.8 μg mL^{-1} oxacillin for 10 min (resulting in R phase cells, Fig. 2A). sfGFP-CbpD-B6 retained the localization to the septal region after oxacillin treatment for cells in all division stages (Fig. 3A), although the fraction of cells without septal sfGFP-CbpD-B6 was slightly higher than in the control cells (6.7% in control cells and 11.8% after oxacillin treatment, Fig. 3B). This shows that the R phase cannot be explained by alterations in the binding pattern of sfGFP-CbpD-B6.

Beta-lactam-resistant pneumococci have acquired so-called low-affinity PBPs, modified PBPs that have much lower affinity for β-lactams than the corresponding PBPs of sensitive strains. To verify that the R phase is due to inhibition of PPB2x by oxacillin, the experiment described above was repeated with an R6 mutant strain (KHB321) expressing a low-affinity version of PPB2x (*SI Appendix, Fig. S5B*). The KHB321 mutant was constructed by replacing the extracytoplasmic part of R6-ppb2x with the corresponding part of the low-affinity ppb2x gene from *S. mitis*.
S. mitis strain B6 (SI Appendix, Fig. S6). The B6 strain is a highly penicillin resistant clinical isolate that produces low-affinity versions of PBP2x, PBP2b, and PBP1a (22). When the oxacillin titration experiment was carried out with the KHB321 strain, no R phase was obtained within the concentration range used (0 to 100 μg mL⁻¹ oxacillin) (Fig. 2B). This result clearly shows that inhibition of the transpeptidase activity of PBP2x by oxacillin causes the R phase.

Fig. 1. CbpD-B6 specifically attacks the septal region. Scanning electron micrographs of untreated pneumococci (A) and pneumococci subjected to 0.5 μg mL⁻¹ CbpD-B6 for 60 s before they were fixed and prepared for electron microscopy (B–D). Arrows indicate areas in the cell wall attacked by the muralytic enzyme.

Fig. 2. Inhibition of the core peptidoglycan-synthesizing machinery of the divisome (PBP2x/FtsW) produces CbpD-B6-resistant peptidoglycan. (A) Increasing concentrations of oxacillin were added to exponentially growing wild-type (WT) cells (RH425) at OD₅₅₀ ∼ 0.25 (black arrow). After 10 min, CbpD-B6 was added (red arrow) to a final concentration of 5 μg mL⁻¹. The cells were susceptible to CbpD-B6 at concentrations ranging from 0 to 0.09 μg mL⁻¹ oxacillin (S1 phase, red curves), resistant from 0.19 to 6.125 μg mL⁻¹ (R phase, green curves), and susceptible from 12.5 to 100 μg mL⁻¹ (S2 phase, blue curves). (B) Pneumococci expressing a PBP2x homolog (LA-PBP2x, strain KHB321) with low affinity for β-lactam antibiotics did not give rise to CbpD resistance when subjected to increasing concentrations of oxacillin. (C and D) Strong depletion (green curves) of FtsW (strain css12) and PBP2x (strain SPH163) results in cells resistant to CbpD-B6 (red arrows indicate addition of 5 μg mL⁻¹ of CbpD-B6).
Moreover, the results above show that CbpD-B6 specifically attacks the peptidoglycan formed by PBP2x/FtsW in the divisome. To further substantiate this conclusion, we investigated whether loss of FtsW activity would give rise to resistance against CbpD-B6. Since FtsW is essential and no specific inhibitor is known, we decided to deplete the expression of this peptidoglycan polymerase using the ComRS system (see Western blot in SI Appendix, Fig. S7) as described before (8, 23). Supporting our conclusion, the results showed that strongly depleted cells became fully resistant to CbpD-B6 (Fig. 2 C). As expected, depletion of PBP2x gave the same result (Fig. 2 D). The morphology of pneumococcal cells strongly depleted of PBP2x (8) or FtsW (SI Appendix, Fig. S7) is the same; both become elongated and somewhat enlarged.

The S2 Phase Results from Inhibition of the PBPs Processing PBP2x/FtsW-Synthesized Peptidoglycan. During the S1 phase the oxacillin concentration increases gradually, resulting in progressively stronger inhibition of PBP2x. This causes a gradual reduction in the number of nascent peptide bridges formed by PBP2x and, eventually, complete inhibition of its transpeptidase activity in the R phase. While this line of reasoning provides an explanation for the S1 and R phases, it does not explain the S2 phase. How can a further increase in oxacillin concentration lead to increased cell lysis when PBP2x is completely inhibited? We observed that the S phase disappears if oxacillin (0.8 μg mL⁻¹) and CbpD-B6 are added simultaneously to pneumococcal cultures. After being exposed to oxacillin it takes about 3.5 min before 95% of the cells develop full resistance against CbpD-B6 (Fig. 4). This shows that the peptidoglycan initially formed by the PBP2x/FtsW machinery must undergo some kind of processing before it becomes resistant to CbpD-B6, an operation that takes several minutes. This finding suggested a plausible explanation for the S2 phase. Although PBP2x is more sensitive than the other pneumococcal PBPs to oxacillin, a further increase in oxacillin concentration will eventually affect the transpeptidase activity of the less sensitive PBPs. Presumably, the activity of one or more of these PBPs is required to modify PBP2x/FtsW-synthesized peptidoglycan into a CbpD-B6-resistant form. Consequently, the cells will not become resistant if their activity is blocked. The reason for this is that newly synthesized CbpD-B6-sensitive peptidoglycan will still be present if the activities of PBP2x and the PBP(s) required for processing this peptidoglycan are blocked simultaneously. In sum, our results indicate that the S2 phase results from inhibition of the PBP(s) required for processing PBP2x/FtsW-synthesized peptidoglycan into a CbpD-B6-resistant form.

Peptidoglycan Synthesized by the FtsW/PBP2x Machinery Is Further Processed by Class A PBPs. To determine whether class A PBPs are required to produce CbpD-B6-resistant peptidoglycan, the oxacillin titration experiment described above was performed in the presence of 10 μg L⁻¹ of the antibiotic moenomycin. Moenomycin inhibits bacterial growth by blocking the transglycosylase activity of class A PBPs. The results show that cells treated with moenomycin are equally sensitive to CbpD-B6 as those treated with oxacillin alone, indicating that class A PBPs are not required to produce CbpD-B6-resistant peptidoglycan. Consequently, the S2 phase results from inhibition of the PBP(s) required for processing PBP2x/FtsW-synthesized peptidoglycan into a CbpD-B6-resistant form.
PBP1b, PBP2a) we wondered whether the

![Diagram of CbpD sensitivity assays (A–E)](image)

**Fig. 5.** CbpD sensitivity assays (A–E) demonstrating that class A PBPs are essential for converting PBP2a/FtsW-synthesized peptidoglycan into a CbpD-B6 resistant form. Resistance to CbpD-B6 was tested for different pneumococcal mutants after treatment with different concentrations of oxacillin alone or in combination with moenomycin. Black arrows indicate the addition of antibiotics, while red arrows indicate the addition of CbpD-B6 (5 μg/mL) 10 min later. (A) RH42S cultures treated with moenomycin (10 μg/mL) in combination with the indicated concentrations of oxacillin before being subjected to CbpD-B6. (B) Cultures of a mutant strain expressing a low-affinity PBP1a (LA-PBP1a, strain KHB332). Individual cultures are treated with one of the indicated concentrations of oxacillin for 10 min before being subjected to CbpD-B6. (C) Same setup as in B, except that strain KHB225 (Δpbp2a/Δpbp1b) was used. (D) Same setup as in B, except that strain KHB224 (Δpbp1a/Δpbp1b) was used. (E) Same setup as in B, except that strain ds789 (Δpbp2b, ΔlytA, mreC Δaa182-272) was used and CbpD-B6 was added 15 min subsequent to oxacillin. (F) Schematic summary of the sensitivity of different strains to CbpD-B6 based on the results presented in Figs. 2 A and B and 5 A–E. Three different phases were observed, namely, sensitivity phase 1 (S1), the resistant phase (R), and sensitivity phase 2 (S2). The oxacillin concentrations that gave rise to the different phases are indicated above the plot. S2b* indicates that the genotype of strain ds789 (Δpbp2b, ΔlytA, mreC Δaa182-272) is more complex than denoted in E and F. All experiments were performed three times or more, with highly similar results.
concerted action of all three is needed for the maturation process. To answer this question the oxacillin titration experiment was performed with a mutant strain expressing a low-affinity PBP1a protein from *S. mitis* B6. Using the same concentration range as before (0 to 100 μg mL⁻¹ oxacillin), we only observed the S1 and R phases in this experiment. The S2 phase had disappeared and was replaced with an extended R phase (Fig. 5 B and F). This result shows that the activity of PBP1a alone is sufficient to transform PBP2x/FtsW-synthesized peptidoglycan into the CbpD-B6-resistant form.

The class A PBPs of *S. pneumoniae* strain R6 can be deleted one at a time and are therefore individually nonessential. PBP1a/PBP1b and PBP2a/PBP1b double mutants can also be constructed, whereas PBP1a/PBP2a double mutants are nonviable (6, 7). The fact that pneumococcal cells need either PBP1a or PBP2a to survive indicates that these PBPs can, at least to a certain extent, substitute for each other. If the observed conversion of PBP2x/FtsW-synthesized peptidoglycan into a CbpD-B6-resistant form represents an important element in the construction of a mature pneumococcal cell wall, it would be expected that this processing step can be carried out also by PBP2a. To address this question, we performed the oxacillin titration experiment with a Δ*pbp2a* strain and a Δ*pbp1b* strain. In both cases we observed the typical S1, R, and S2 phases (Fig. 5 C, D, and F), demonstrating that PBP2a can substitute for PBP1a in the peptidoglycan maturation process.

Finally, to determine whether inhibition of class A PBPs has any effect on pneumococcal morphology, cells were treated with moenomycin for 2 h before they were fixed and prepared for transmission electron microscopy (TEM). The amount of moenomycin used (0.4 μg mL⁻¹, corresponding to 0.5 × MIC) partially inhibits peptidoglycan polymerization by class A PBPs. The TEM micrographs revealed that moenomycin-treated cells had thickened cell walls with low electron density, especially in the division zones. The experiment was performed twice with the same result.

**Discussion**

Recently, it became clear that FtsW/PBP2x and RodA/PBP2b constitute cognate pairs of interacting proteins that make up the core peptidoglycan-synthesizing machineries within the pneumococcal divisome and elongasome, respectively (9–11). Both couples consist of a monofunctional transglycosylase working together with a monofunctional transpeptidase. This discovery has important implications for our understanding of pneumococcal cell wall synthesis and the role played by class A PBPs in this process. Before it was discovered that the SEDS proteins FtsW and RodA have glycosyltransferase activity, class A PBPs...
were considered to be the only peptidoglycan polymerases present in pneumococci. Hence, they were regarded as key components of the divisome and elongasome and indispensable for septal as well as peripheral peptidoglycan synthesis. This way of thinking is no longer valid, and the function of class A PBPs has therefore become an open question.

Using CbpD-B6 as a tool, we show that class A PBPs act downstream of the FtsW/PBP2x machinery to produce alterations in the cell wall. Class A PBPs are able to function, i.e., to convert FtsW/PBP2x-synthesized peptidoglycan into a CbpD-B6-resistant form, even when PBP2x is completely inhibited by oxacillin. Similarly, we show that class A PBPs are able to operate independently of PBP2b and the elongasome in a Δpbp2b, ΔlytA, MreCΔani182-272 mutant. Since the conversion process takes about 3.5 min (Fig. 4), the activity of class A PBPs occurs subsequent to and separate in time from FtsW/PBP2x-mediated peptidoglycan synthesis. These results are in agreement with the observation that the FtsW/PBP2x machinery as well as class A PBPs localize to the division site in *S. pneumoniae* (26). They also fit well with data obtained by high-resolution 3D-SIM microscopy showing that the positions of PBP2x and PBP1a are similar in predivisional stages, while PBP2a lags behind PBP2x during septal constriction (27). Together our findings provide three important insights: 1) Class A PBPs have a distinct and unique role in the construction of the pneumococcal cell wall, 2) there exists a class A–mediated mechanism that remodels nascent FtsW/PBP2x-synthesized peptidoglycan into a more mature CbpD-B6-resistant form, and 3) this maturation mechanism is essential.

It is well established that the divisome and elongasome consist of two separate peptidoglycan-synthesizing machineries (5). Their activities are precisely coordinated during the cell cycle, but experiments have shown that the divisome is able to operate in the absence of the elongasome and vice versa. Pneumococcal cultures treated with oxacillin (0.1 μg mL⁻¹) at a concentration that inhibits PBP2x, but not class A PBPs and PBP2b, give rise to highly elongated cells with no septal cross walls (SI Appendix, Fig. S10 B and C). This demonstrates that the elongasome is active even in the absence of a functional divisome. Similar findings were reported previously by others (5, 27–29). In the opposite case, several studies showed that pneumococci are able to grow and form septal cross walls when PBP2b is depleted or deleted (8, 25, 30, 31). Pneumococci that are strongly depleted in PBP2b form long chains of round cells that are compressed in the direction of the long axis (SI Appendix, Fig. S10 D and E). In the present study, we have obtained evidence that class A PBPs operate independently of the divisome and elongasome and hence function autonomously. An important question is therefore whether PBP1a, PBP2a, and PBP1b operate alone or in multiprotein complexes similar to the divisome and elongasome. It was reported that PBP1a forms a complex with CozE, MrcE, and MrcD (32) and that it coimmunoprecipitates with the cell cycle protein GpsB (33). Interestingly, it was shown that aberrant PBP1a activity can be detected outside the midcell zone in pneumococci lacking MrcE or CozE, supporting the model that PBP1a can function autonomously (32). PBP2a, on the other hand, interacts with and is regulated by MacP, a substrate of the global cell cycle regulator StkP (34). The interplay between the two PBPs and their respective partners appears to be specific, as interactions between CozE/PBP2a and MacP/PBP1a have not been detected (32, 34). Presumably, the specific partners of PBP1a and PBP2a are important for the precise spatiotemporal regulation of their activity. Together the data support a model in which PBP1a, PBP2a, and PBP1b are the key players in three separate and autonomous peptidoglycan-synthesizing machineries with partially overlapping functions.

The fact that class A PBP-mediated remodeling of nascent peptidoglycan is inhibited by oxacillin as well as moenomycin strongly indicates that both catalytic domains of these proteins are actively involved in the remodeling process. Hence, the remodeling mechanism most likely involves the synthesis of new glycan strands and the incorporation of these strands into existing peptidoglycan (Fig. 7). How could peptidoglycan synthesis by class A PBPs make the cell wall resistant to CbpD-B6? The muralytic enzyme consists of three different domains, a catalytic CHAP domain, an SH3b domain, and a choline-binding domain that anchors CbpD-B6 to teichoic acid. The SH3b domain probably acts as an auxiliary module that binds peptidoglycan and facilitates the function of the catalytic CHAP domain (16). Previous research showed that all three domains are required for the enzyme to be active (16). Hence, it would be sufficient to block the function of one of these domains to convert the cell wall into a CbpD-B6-resistant form. Inhibiting the activity of the CHAP domain would require that nascent peptide bridges cross-linked by PBP2x be altered to become resistant to the enzyme. A structural change in these peptide

![Fig. 7. Model illustrating the role of class A PBPs in the synthesis of pneumococcal peptidoglycan. The core peptidoglycan-synthesizing machinery, PBP2x and FtsW, of the divisome (yellow shading) produces the primary CbpD-sensitive peptidoglycan (shown in shades of gray), which is subsequently remodeled by class A PBPs into a CbpD-resistant form (shown in shades of green).](image-url)
bridges might also block the binding of the SH3b domain, as the SH3b domain of lysostaphin was reported to bind to the peptide part of the cell wall of *Staphylococcus aureus* (35). The peptide bridges in pneumococcal peptidoglycan consist of a mixture of branched and unbranched cross-links. The branches are introduced by the aminoacyl ligases MurM and MurN. MurM catalyzes the addition of t-Ala or t-Ser, whereas the addition of the second t-Ala is catalyzed by MurN (36). However, as a strain lacking murMN behaved exactly like the wild type when subjected to the oxacillin titration assay (*SI Appendix, Fig. S11*), alterations in branching are not important for CbpD-B6 resistance. Alternatively, we speculated that the SH3b domain recognizes the glycan part of pneumococcal peptidoglycan instead of the peptide part. Thus, the oxacillin titration assay was performed with ΔpdiA and Δadr mutant strains as well. The pdiA gene encodes a peptidoglycan N-acetylmuramidase acetylasyltransferase, while the adr gene encodes a peptidoglycan O-acetyl transferase (37, 38). The ΔpdiA and Δadr strains displayed an S1–R–S2 pattern similar to the wild-type strain, demonstrating that neither N-acetylation nor O-acetylation significantly affects the ability of CbpD-B6 to cleave pneumococcal peptidoglycan during the S1 and S2 phases (*SI Appendix, Fig. S12 A and B*). Furthermore, it is possible that class A PBP-mediated remodeling of pneumococcal peptidoglycan affects the ability of CbpD-B6 to attach to teichoic acid via its C-terminal choline-binding domain, resulting in CbpD-B6 resistance. However, we could not detect any significant difference between cells treated with 0.8 μg mL\(^{-1}\) oxacillin (R phase cells) and untreated cells with respect to sGFP-CbpD-B6 binding patterns (Fig. 3).

Considering that *S. pneumoniae* must express either PBP1a or PBP2a to be viable, class A PBPs must serve an essential function. PBP1a appears to have the most prominent role among class A PBPs, as highly β-lactam resistant pneumococci always express low-affinity versions of PBP1a in addition to PBP2x and PBP2b. We clearly show that class A PBPs together with their associated auxiliary proteins somehow remodel the primary peptidoglycan synthesized by the PBP2x/FtsW machinery. As discussed above, this remodeling might involve chemical or structural modifications of the primary peptidoglycan that inhibit the function of the CHAP, SH3b, or Cbd domain of CbpD-B6. Alternatively, class A PBPs and their helper proteins might not synthesize peptidoglycan that is qualitatively different from the primary peptidoglycan synthesized by PBP2x/FtsW, but rather might function as a repair machinery that mends imperfections that arise during construction and expansion of the cell wall (10). This idea is in accordance with the findings of a recently published study on *E. coli*. Vigouroux et al. (39) reported that PBP1b, the major class A PBP in this species, contributes to maintain cell wall integrity by actively repairing cell wall defects. It is conceivable that the peptidoglycan layer synthesized by PBP2x/FtsW, i.e., the divisome, is not perfect. It might not be fully homogenous but might contain irregularities in the form of gaps and small holes. We speculate that CbpD-B6 uses these irregularities to penetrate into the peptidoglycan layer. Perhaps CbpD-B6 is not able to digest “tightly woven” peptidoglycan but depends on imperfections to get access to its substrate.

TEM micrographs of pneumococci treated with moenomycin showed that the electron density of their cross walls was strongly reduced (Fig. 6). This supports the idea that PBP2x/FtsW-synthesized peptidoglycan has less wall teichoic acid and/or a more open architecture. The pore size of peptidoglycan has been estimated to be around 2 nm (40). This represents a formidable physical barrier to the assembly of large proteins and cell wall–spanning complexes that are larger than the pores. In the case of peptidoglycan-spanning machineries such as flagella and type III and IV secretion systems, the problem was solved by the recruitment of lytic transglycosylases or other muralytic enzymes that locally rearrange the cell wall (41). By analogy, it was assumed that muralytic enzymes create gaps in the peptidoglycan layer to allow the insertion or penetration of large proteins and pili (42, 43). However, if the peptidoglycan synthesized by PBP2x/FtsW inherently is more open, i.e., has more gaps and/or larger pores, it would facilitate the insertion of larger protein components, and local degradation of peptidoglycan might not be necessary. This idea fits with the fact that most bacterial proteins translocated across the cytoplasmic membrane are exported by the general secretory SecA-YEG pathway which is localized at midcell septa (44, 45).

We propose a model in which class A PBPs further process the peptidoglycan meshwork synthesized by PBP2x and FtsW to remove imperfections and/or make it denser (Fig. 7). A denser peptidoglycan can be obtained by adding peptidoglycan that is more heavily cross-linked or by introducing more wall teichoic acid. Thus, class A PBPs might together constitute a repairosome that repairs gaps and imperfections in the primary peptidoglycan synthesized by PBP2x/FtsW and/or function to strengthen the primary cell wall before it is exposed to turgor pressure and the external milieu. Since there are three different class A PBPs, it is possible that they together serve both functions. Further studies are required to confirm or reject these ideas.

### Materials and Methods

All data, strains, oligonucleotides, and experimental procedures used in this study are provided in the main article and *SI Appendix*.

#### Cultivation and Transformation of Bacteria

All strains used in the present study are listed in *SI Appendix, Table S1*. Escherichia coli was grown in Luria Bertani (LB) broth or on LB agar plates at 37 °C containing ampicillin (100 μg mL\(^{-1}\)) when necessary. Liquid cultures were grown aerobically with shaking. Chemically competent *E. coli* cells were transformed by heat shocking at 42 °C. *S. pneumoniae* was grown in liquid C medium (46) or on Todd-Hewitt (TH) agar plates (BD Difco) at 37 °C. When grown on TH agar the cells were incubated in a sealed container made anaerobically (<1% O\(_2\)) by including AnaeroGen sachets from Oxoid. Transformation of *S. pneumoniae* was done by adding CSP-1 (final concentration of 250 ng mL\(^{-1}\)) and the transforming DNA (50 to 100 ng) to 1 mL of exponentially growing cells at OD\(_{550}\) = 0.05. Following incubation at 37 °C for 2 h, transformants were selected by plating 30 μL of cell culture on TH agar plates containing the appropriate antibiotic kanamycin (400 μg mL\(^{-1}\)), streptomycin (200 μg mL\(^{-1}\)), or spectinomycin (200 μg mL\(^{-1}\)).

#### Depletion of FtsW and PBP2x

During cultivation of strains css12 and SPH163, ectopic expression of FtsW and PBP2x was maintained by the addition of 0.2 μM of ComS to the growth medium. ComS is a peptide pheromone consisting of seven amino acids (LPYPFAGC). Exogenous peptide pheromone is internalized by the native Ami oligopeptide permease. In the cytoplasm it directly interacts with and activates the constitutively expressed ComR transcriptional activator. In the activated state ComR binds to its cognate comX promoter, which has been engineered to drive the ectopic expression of FtsW (strain css12) and PBP2x (strain SPH163). In depleted cells the ComS peptide is removed from the medium by replacing ComS-containing medium with ComS-free medium. The ComRS system originates from Streptococcus thermophilus, where it regulates competence for natural transformation. See Berg et al. for further details (8, 23).

#### Immunodetection of FtsW-3xFlag

A C-terminally 3xFlag-tagged version of FtsW (FtsW-3xFlag) was ectopically expressed using the ComRS system (strain gi1709). Depletion of FtsW-3xFlag was performed as described above in 5 mL cultures. Parallel cultures induced with 0.2 μM ComS were used as controls. When the level of FtsW-3xFlag was reduced to a concentration rendering the cells immune to CbpD-B6, the cells were harvested at 4,000 × g. Cell lysates were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetected as previously described (47). The anti-Flag antibody (F7425, Sigma-Aldrich) used to detect FtsW-3xFlag was diluted 1:4,000.

#### DNA Cloning

All primers used in this study are listed in *SI Appendix, Table S2*. To construct pRSET-cbpD-B6, the cbpD-B6 gene from *S. mitis* B6 was amplified from genomic DNA using the primer pair so1/so2. The gene was amplified by adding CSP-1 (final concentration of 250 ng mL\(^{-1}\)) to the growth medium. ComS is a peptide pheromone consisting of seven amino acids (LPYPFAGC). Exogenous peptide pheromone is internalized by the native Ami oligopeptide permease. In the cytoplasm it directly interacts with and activates the constitutively expressed ComR transcriptional activator. In the activated state ComR binds to its cognate comX promoter, which has been engineered to drive the ectopic expression of FtsW (strain css12) and PBP2x (strain SPH163). In depleted cells the ComS peptide is removed from the medium by replacing ComS-containing medium with ComS-free medium. The ComRS system originates from Streptococcus thermophilus, where it regulates competence for natural transformation. See Berg et al. for further details (8, 23).

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oxacillin was added in concentrations decreasing from 100 μg mL⁻¹ in the cultures with the sfp-gfp gene. The sfp-gfp gene was amplified using the kp116 and kp119 primers and SPH370 genomic DNA as a template, and the cfpB2x-achap gene was amplified from SO7 genomic DNA using the primer pair kp117/kp118. Using overlap extension PCR and the primers kp116 and kp117, sfp-gfp was fused to cfpB2x-achap. The resulting sfp-gfp-cfpB2x-achap amplicon was cleaved with Ndel and HindIII and ligated into pRSET A, giving the pRSET-sfp-gfp-cfpB2x plasmid.

Amplicons used to transform S. pneumoniae were constructed by overlap extension PCR as previously described by Johnsrud et al. (48). We employed the Janus cassette (49) to knock out genes and to introduce recombinant DNA at desired positions in the S. pneumoniae genome. When the native pbp2x gene was ectopically expressed during transformation using the ComR5 system as described by Berg et al. (23).

The spectinomycin-resistant marker aad9 was employed to knock out LytA in strain dt785.

Expression and Purification of CfpB2x. E. coli BL21 containing pRSET-cfpB2x-B6 was grown to OD₅₅₀ = 0.4 to 0.5 at 37 °C. Then production of CfpB2x was induced by adding a final concentration of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 20 °C for 4 h. The cells were harvested at 5,000 × g for 5 min and resuspended in 1/100 volume culture buffer (TSB) at 4 °C. The cells were lysed by using the Fast Prep method with ≤10 μm glass beads at 6.5 m s⁻¹, and insoluble material was removed by centrifugation at 20,000 × g. CfpB2x was purified from the soluble protein fraction by performing DEAE cellulose chromatography as described by Sanchez-Puelles et al. (20), but using TBS (pH 7.4) instead of a phosphate buffer (pH 7.0). To remove chelate from the eluted CfpB2x protein, it was dialyzed against TBS (pH 7.4) for 1 h and then centrifugation at 10,000 × g, the CfpB2x was incubated further for 10 min at 37 °C before formaldehyde was added to a final concentration of 2.5%. Both nontreated and oxacillin-treated cells were incubated further for 10 min at 37°C before formaldehyde was added to a final concentration of 2.5%. Both nontreated and oxacillin-treated cells were fixed on ice for 1 h. The fixed cells were washed three times in 1/5 volume of phosphate-buffered saline (PBS), before sfGFP-CfpB2x (purified as described for CfpB2x) was bound to the cell surface as described by Eldholm et al. (16).

Briefly, cells were aged on a glass slide (inside a hydrophobic frame made with a PAP pen), and cells were immobilized by incubation at room temperature for 5 min. Nonbound cells were rinsed off the glass by PBS. Cells were then incubated in 100 μL PBS containing 0.05% Tween 20 and 15 μg mL⁻¹ sfGFP-CfpB2x for 8 min at room temperature. Nonbound sfGFP-CfpB2x was washed off the cells by rinsing the glass slide by submerging the glass slide in five tubes each containing 1 mL of PBS. Phase contrast pictures and GFP fluorescence pictures were captured using a Zeiss AxioObserver with an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu Photonics) through a 100x PC objective. An HPX 120 illuminator was used as a light source for fluorescence microscopy. Phase contrast pictures of FtsW-depleted cells were captured as described above. Images were prepared in ImageJ.

**CfpB2x Resistance Assay.** Pneumococcal cells were grown in 96-well microtiter plates, and OD₅₅₀ was measured every 5 min. When reaching OD₅₅₀ = 0.2, oxacillin was added in concentrations decreasing from 100 μg mL⁻¹ down to 0.003 μg mL⁻¹ in a two-fold dilution series. Cells with zero antibiotic added were used as controls. In some cases, 10 μg mL⁻¹ of moenomycin were added together with oxacillin. The cells were grown for 10 min in the presence of antibiotics before purified CfpB2x was added to a final concentration of 5 μg mL⁻¹. CfpB2x-sensitive cells were observed as a drop in OD₅₅₀. For the time kinetic experiments, oxacillin (0.8 μg mL⁻¹) was added simultaneously to the soluble protein fraction by performing DEAE-cellulose chromatography as described by Straume et al. (43). Briefly, the amplicons were cleaved with HindIII and ligated into pRSET A, giving the pRSET-sfp-gfp-cfpB2x plasmid.

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