Pneumococcal cell biology in a new light
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Chapter 6

The localization of key *Bacillus subtilis* and *Streptococcus pneumoniae* Penicillin-Binding Proteins during cell growth is determined by substrate availability

This chapter has been published* by Marta Carolina Afonso Lages¹, Katrin Beilharz¹,⁻², Danae Morales Angeles, Jan-Willem Veening and Dirk-Jan Scheffers in Environmental Microbiology


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Abstract

The shape of bacteria is maintained by the cell wall. The main component of the cell wall is peptidoglycan (PG), which is synthesized by Penicillin Binding Proteins (PBPs). The correct positioning of PBPs is essential for the maintenance of cell shape. In the literature, two different models for localization of PBPs have been proposed – localization through interaction with a cytoskeletal structure or localization through the presence of substrate. Here we show that the localization of PBPs critical for the rod shape of *Bacillus subtilis* is altered when the substrate lipid II is delocalized by treatment of the cells with nisin. Lipid II delocalization also alters PBP localization in oval-shaped *Streptococcus pneumoniae*. Alteration of this localization is only seen in a lipid II-dependent manner and is not influenced by dissipation of the membrane potential, a secondary effect of nisin treatment. Our results strongly suggest that the localization of PG synthesis at the periphery of the cell is substrate-driven, even in bacteria that contain actin-like MreB cytoskeletal structures.
Introduction

Peptidoglycan (PG) is the main component of the bacterial cell wall, which is present in almost all bacteria. PG allows bacteria to maintain shape and generate turgor and provides protection against osmotic shock\textsuperscript{78}. PG is composed of a single molecule formed by strands of repeating disaccharide units that are crosslinked by peptide side chains attached to the disaccharides. PG synthesis is the main target of most commonly used antimicrobials. These antimicrobials work at various stages of PG synthesis: (1) inhibition of the synthesis of the PG precursor lipid II (which consists of the disaccharide-pentapeptide linked to a undecaprenol carrier lipid that facilitates translocation over the membrane), (2) inhibition of the recycling of the undecaprenol carrier lipid, (3) binding to lipid II to prevent access of PG synthesizing enzymes, collectively known as Penicillin Binding Proteins (PBPs), to lipid II and (4) binding and blocking of the active site of PBPs. PBPs incorporate the disaccharide-pentapeptide moiety of lipid II into a growing PG network through a combination of transglycosylation reactions that attach the disaccharide to glycan strands and transpeptidation reactions that form peptide crosslinks between the pentapeptide chains that are attached to the glycan strands. In rod-shaped bacteria, specific class B PBPs, that have only transpeptidase activity, are responsible for the architecture of the bacterial cell. In \textit{Bacillus subtilis}, these PBPs are PBP2B, which is essential and required for division\textsuperscript{213}, and PBP2A and PbpH, which are required for cell wall synthesis during elongation\textsuperscript{214}. PBPs interact with other proteins such as the bacterial cytoskeletal proteins MreB and FtsZ that form dynamic, membrane-associated polymeric structures that are thought to guide the localization of PBPs and hence cell wall synthesis\textsuperscript{78}. Recently, it was shown that active PG synthesis during cell wall elongation is required for the dynamics of MreB, rather than MreB dynamics guiding PBPs\textsuperscript{79,80,215}. Various antimicrobials, that block PG synthesis at different steps, were shown to block MreB dynamics\textsuperscript{79,80,215}. Specifically, PBP2A and PbpH, the PBPs essential for elongation in \textit{B. subtilis}, were shown to be drivers for MreB dynamics\textsuperscript{79,80}. This led to the proposition of a model in which MreB(-like) polymers function by restricting the diffusion of PG synthesis complexes within the membrane, rather than actively positioning PBPs along a scaffold to ensure correct localization of PG synthesis\textsuperscript{79,80}. If PG synthesis by PBPs essential for elongation is a requirement for MreB dynamics, the model that MreB actively determines the localization of PBPs in the membrane becomes less likely.

An alternative model that has been proposed for PBP localization is substrate availability, which is supported by various observations\textsuperscript{216}. In \textit{Staphylococcus aureus}, the
 Localization of key PBPs during cell growth

High molecular weight (HMW) PBP2 delocalizes from the septum, when its substrate binding site is blocked by oxacillin, or when the substrate itself is blocked for binding by the antibiotic vancomycin or by the alteration of the structure of lipid II by the addition of D-cycloserine, which blocks the addition of the terminal D-Ala-D-Ala residues of the lipid II pentapeptide. In Streptococcus pneumoniae, the localization of several HMW PBPs to the zone of active PG synthesis is restricted by PBP3, a carboxypeptidase that cleaves the terminal D-Ala from pentapeptide chains, outside of the zone of active synthesis, although it has to be noted that the location of PBP3 exclusively outside the zone of active PG synthesis could not be reproduced by a different laboratory. Although the localization of PBP3 needs to be resolved conclusively, the absence of PBP3 causes an accumulation of pentapeptide substrates over the cell surface and delocalization of PBPs with transpeptidase activity, compatible with the substrate availability model. In Escherichia coli, the carboxypeptidase PBP5 delocalizes from the division site, where it is most active, when its active site is mutated, or it accumulates even more at the division site when cell wall synthesis along the lateral wall is inhibited. Finally, Caulobacter crescentus PBP3 delocalizes when its active site is mutated. S. pneumoniae and S. aureus lack the MreB cytoskeleton, and neither E. coli PBP5 nor C. crescentus PBP3 are specifically associated with elongation. Therefore, we wanted to test the substrate availability model by following B. subtilis PBP2A and PbpH, that are involved in the synthesis of the lateral cell wall and that drive MreB dynamics. To do so, we made use of nisin to alter the localization of lipid II, the substrate of PBPs. Nisin is a lantibiotic, produced by Lactococcus lactis strains, that forms pores in the bacterial membrane, which kill the cell. A second activity of nisin is the removal of lipid II away from the septum into clustered patches along the membrane. This removal, or sequestration, of lipid II results in defective PG synthesis, which also kills bacteria, as shown with nisin variants that do not have the capacity to form pores but still have antibacterial activity. In this report, we used nisin to delocalize lipid II and tested the substrate availability model in B. subtilis and S. pneumoniae.

Results

B. subtilis PBP2A and PbpH are redistributed upon lipid II delocalization

Lipid II and immature PG can be labeled with fluorescent vancomycin (Van-FL) which binds to the terminal D-Ala-D-Ala of lipid II. In mature PG, the amount of disaccharides with D-Ala-D-Ala pentapeptides, substrates for Van-FL labeling, is only 1.6%, as the terminal D-Ala residue(s) are removed from PG during the formation of
peptide crosslinks or processed by D,D-carboxy-peptidases. Thus, Van-FL is an excellent marker for PG synthesis, labeling predominantly unincorporated lipid II as evidenced by severely reduced staining of cells in which lipid II synthesis is blocked\textsuperscript{222}. We used Van-FL to label \textit{B. subtilis} and found that lipid II is present at the septum and along the lateral wall in punctate patterns (Fig. 1A), as previously described\textsuperscript{222,223}.

To test the substrate availability model for PBP localization we wanted to delocalize lipid II from its normal localization along the lateral wall. Nisin is a lantibiotic that is capable of sequestering lipid II into patches along the lateral wall\textsuperscript{220}. Importantly, this sequestration of lipid II is not coupled to the formation of membrane pores since nisin mutants that do not form stable membrane pores also show lipid II sequestration\textsuperscript{220}. We used the hinge mutant \textit{[N20P/M21P]nisin} (PP-nisin) instead of nisin Z (nisin), because PP-nisin \textsuperscript{(1)} is less toxic to bacteria as determined by MIC measurements\textsuperscript{224}, \textsuperscript{(2)} is incapable of forming stable pore complexes with lipid II\textsuperscript{225}, and \textsuperscript{(3)} is still active in lipid II delocalization\textsuperscript{220} (Fig. 1B).

![Fig. 1 PP-nisin delocalizes lipid II and elongation-specific PBPs in \textit{B. subtilis}. PG synthesis visualized by Vancomycin-labeling (A-C) and localization of GFP-PBP2A (D-F), GFP-PbpH (G-I), GFP-PBP2B (J-L) and GFP-PBP1 (M-O) in untreated cells (A, D, G, J, M) or after treatment with PP-nisin (B, E, H, K, N) or CCCP (C, F, I, L, O). The scale bar, same for all, is 2 \textmu m.](image-url)
**Localization of key PBPs during cell growth**

*B. subtilis* PBPs PBP2A, PbpH, are Class B transpeptidases involved in elongation and MreB dynamics, PBP2B is a Class B PBP essential for division, as well as elongation through an interaction with MreB. We used fully functional GFP fusions to these PBPs and determined the localization of the PBPs before and after treatment with PP-nisin. Mid-exponentially growing cells were treated with PP-nisin and analyzed by fluorescence microscopy. As shown in Fig. 1, PP-nisin caused the redistribution of PBP2A, PbpH and PBP1 to patches in the lateral membrane that are similar to the patches formed by lipid II. Division-site-associated PBP1 and division-site-specific PBP2B did not change localization upon PP-nisin treatment (Fig. 1K), suggesting that the patch formation is specific, or only visible, at the lateral membrane.

**PBP2A and PbpH patch formation at the lateral membrane is not caused by a collapse of membrane potential**

Membrane pore formation by nisin results in a collapse of the proton motive force (PMF). Since the dissipation of the membrane potential results in mislocalization of various proteins, including MreB, a control experiment was performed to determine the effect of the addition of PP-nisin on the membrane potential. Addition of nisin resulted in a collapse of the membrane potential as expected from its pore-forming activity (Fig. S1). The addition of PP-nisin at concentrations used to delocalize lipid II (1.5 μg/ml) also resulted in a partial collapse of the membrane potential under our experimental conditions (Fig. S1). The effect of the collapse of the PMF on distribution of lipid II and the localization of the PBPs was tested by dissipation of the PMF with the ionophore CCCP. Addition of CCCP did not change the localization of lipid II, PBP2A, PbpH and PBP2B (Fig. 1C, F, I, L). PBP1 showed similar distribution patterns after PP-nisin and CCCP treatment (Fig. 1N, O). Therefore, PBP1 redistribution can be caused by lipid II relocation, PMF dissipation, or both. Because the distinction between these explanations could not be made for PBP1, we left PBP1 out of our further analysis. A similar observation was made for MreB, as was expected based on previous results (Fig. S2). To test whether the addition of PP-nisin affected the localization of other membrane proteins, the localization of AtpA-GFP and SdhA-GFP were examined. AtpA-GFP and SdhA-GFP are randomly distributed throughout the membrane and their localization patterns are not sensitive to PMF dissipation with CCCP. Treatment with PP-nisin did not result in a patchy accumulation of AtpA or SdhA and the patterns were indistinguishable from the control and CCCP treated samples (Fig. S3).
Combined, these results show that PBP2A and PbpH are redistributed to patches similar to patches formed by lipid II upon treatment of cells with PP-nisin, independent of the dissipation of the membrane potential.

**B. subtilis PBP2A colocalizes with lipid II during patch formation**

The patches formed by *B. subtilis* GFP-PBP2A, GFP-PbpH and lipid II after treatment with PP-nisin were very similar, but we could not formally determine whether the PBP and lipid II patches overlap as the excitation and emission spectra of GFP and Van-FL overlap. Therefore, we constructed a *B. subtilis* strain that expressed a red fluorescent protein fused to PBP2A (RFP-PBP2A) to allow simultaneous visualization of PBP2A and lipid II. When this strain was labeled with Van-FL, the localization patterns of RFP-PBP2A and lipid II were found to overlap (Fig. 2A-C). These cells were treated with PP-nisin to determine the amount of overlap between spots formed by lipid II patches and PBP2A (Fig. 2E-G). Cells were scored for the presence of PBP2A or Van-FL patches, with 40% of the cells containing patches of both (out of a total of 50% cells that contained patches, n = 496). In cells containing patches of both Van-FL and PBP2A, 94% of spots were found to overlap, strongly indicating that PBP2A is redistributed to lipid II patches that form upon treatment with PP-nisin. To confirm that both the overlapping signals originated from the same cell surface we used total internal reflection microscopy (TIRFm). TIRFm measures events at a surface depth of less than 200 nm and excludes background fluorescence originating from the rest of the cell. TIRFm of cells containing PBP2A and Van-FL patches confirmed that these patches overlapped (Fig. 2I-L).

Subsequently, RFP-PBP2A and Van-FL distribution was followed in cells after treatment with PP-nisin in a time-lapse microscopy experiment (Fig. S4, movie S1). Cells were treated with PP-nisin, immediately transferred to agarose pads containing Van-FL, and imaged. The addition of Van-FL to the pads was necessary to reduce the treatment time of the cells before microscopy, but led to a less intense lipid II staining pattern. Again, PP-nisin addition resulted in RFP-PBP2A delocalization to patches that overlapped with Van-FL labeled lipid II patches. The time resolution of the time-lapse microscopy was not high enough to identify whether Van-FL or PBP2A patches formed subsequently or simultaneously.
Localization of key PBPs during cell growth

Fig. 2 Colocalization of lipid II and PBP2A in *B. subtilis*. (A-H) Epifluorescence microscopy of *B. subtilis* strain 4042 imaged for RFP-PBP2A (A, red in overlay) and Van-FL (B, green in overlay), an overlay of the images (C, G) and a phase-contrast image of the cells (D, H) in untreated cells (A-D) or after treatment with PP-nisin (E-H). The scale bar, same for all, is 2 μm. Arrows indicate spots of clear colocalization, arrowheads indicate spots of colocalization with a weaker signal for Van-FL, and the asterisk indicates PBP2A fluorescence without Van-FL staining. In total, 496 cells were analysed, out of which 223 had Van-FL patches and 228 had PBP2A patches. 201 Cells contained patches of both Van-FL and PBP2A and in 188 cells these patches overlapped (94%). (I-L) *B. subtilis* strain 4042 labeled with Van-FL was imaged by TIRF microscopy after PP-Nisin treatment for RFP-PBP2A (I) and Van-FL (J). (K) Merged signals for RFP-PBP2A and Van-FL; (L) bright field image of the cells. Arrowheads indicate the delocalized spots. The scale bar is 2 μm.

Redistribution of PBPs is dependent on lipid II delocalization

The patchy patterns formed by GFP-PBP2A and GFP-PbpH upon treatment with PP-nisin were quite striking. To confirm that redistribution of PBPs is dependent on lipid II delocalization, the effect of PP-nisin on PBP2A was studied in cells that were depleted for lipid II. Fosfomycin is an antibiotic that blocks the first cytosolic step in lipid II biosynthesis\(^{231}\) and treatment of cells with fosfomycin blocks the movement of MreB, Mbl and PbpH\(^{79,80}\). Cells expressing RFP-PBP2A were incubated with fosfomycin for 30 minutes to deplete lipid II and subsequently treated with PP-nisin. Phase contrast microscopy showed that neither treatment killed the cells in the time frame of the experiment (Fig. 3). Control cells showed normal PBP2A localization and PBP2A patch formation upon PP-nisin treatment (Fig. 3A,B). Fosfomycin treatment did not alter the PBP2A localization pattern (Fig. 3C) and importantly, PBP2A patches were absent from cells that were treated with PP-nisin after lipid II depletion (Fig. 3D). This experiment shows that PBP2A patch formation is dependent on the presence of lipid II and not caused solely by the addition of PP-nisin to cells.
**Fig. 3 Lipid II is required for PBP2A patch formation.** *B. subtilis* strain 4042 imaged for RFP-PBP2A (top) and phase-contrast image of the cells (bottom). Control cells were either not treated (A) or treated with PP-nisin only (B). Fosfomycin was used to block lipid II production and cells were not treated (C) or treated with PP-nisin (D). Patch formation only occurred in cells treated with PP-nisin in which lipid II production was not blocked. The scale bar, same for A-D, is 4 μm.

*S. aureus* PBP2 changes its localization from the division site to the entire cell surface when its active site is occupied by a β-lactam antibiotic, or when the lipid II substrate is made unavailable for binding\(^3\). PBP2A, PbpH and lipid II already occupy most of the cell surface so it would be difficult to see a dramatic redistribution of PBP2A/PbpH when substrate binding is disabled. However, if blocking substrate binding would also lead to a marked difference in localization, this would argue against the specific requirement of lipid II binding for localization of PBP2A and PbpH. Thus, we monitored the effect of two antibiotics on the distribution of PBP2A and PbpH. Vancomycin binds to the terminal D-Ala-D-Ala residues on lipid II, making it unavailable for binding by PBPs, and Penicillin G blocks the active site of PBPs. Treatment with these antibiotics, which results in the inability of PBPs to bind substrate, did not lead to a redistribution of PBP2A or PbpH (Fig. 4). This experiment suggests that PBP2A and PbpH redistribution into patches can only be achieved by lipid II delocalization into patches, and thus that the localization of PBP2A and PbpH is determined by substrate availability.
Localization of key PBPs during cell growth

Fig. 4 Blocking substrate binding does not alter the localization of PBPs. A-F: localization of GFP-PBP2A (A, C, E) and GFP-PbpH (B, D, F) before (controls; A, B) or after treatment of *B. subtilis* cells with vancomycin (C, D) or penicillin G (E, F). The scale bar, same for all, is 2 μm.

**Streptococcus pneumoniae** PBPs localize in patches upon lipid II delocalization

To test whether redistribution of PBPs upon lipid II delocalization occurs in other bacteria, *S. pneumoniae* PBPs were investigated. *S. pneumoniae* is an ovaly shaped Gram-positive bacterium which also performs lateral and septal cell wall synthesis, but lacks the MreB cytoskeletal protein. In *S. pneumoniae*, lipid II is localized at midcell and at the equatorial rings (Fig. 5A). Nisin treatment did not cause localization of lipid II to foci but the localization pattern became more uniformly distributed along the periphery of the cells (Fig. 5B). For *S. pneumoniae*, nisin was used instead of PP-nisin since PP-nisin did not have a discernible effect on lipid II localization, probably because of the reduced effect of PP-nisin on *Streptococci* as determined by MIC measurements. GFP fusions to *S. pneumoniae* PBP1A, a Class A bifunctional transglycosylase/transpeptidase, and PBP2x, a Class B transpeptidase, localize to sites of active cell wall synthesis and show patterns similar to the Van-FL labeling (Fig. 5D, G). These patterns are similar to the localization of PBP2x and PBP1a that was previously determined using immunofluorescence. Upon treatment with nisin, PBP1A and PBP2x form patchy spots, similar to the spots formed by *B. subtilis* PBP2A and PbpH. While Van-FL staining clearly demonstrates delocalization of lipid II upon nisin treatment, the localization pattern does not match the spotty PBP pattern. A possible reason for this discrepancy could be the rapid clustering of the GFP fusions after lipid II delocalization. Again, the possibility that the change of localization patterns was caused by
a collapse of the PMF was excluded, as the localization patterns of Van-FL, GFP-PBP1A and GFP-PBP2x did not change upon treatment with CCCP (Fig. 5C, F, I; Fig. S5). This result suggests that substrate availability is a driver for PBP localization in other bacteria as well.

Discussion

In this study we have used nisin to alter the location of lipid II as a direct method to test the substrate availability hypothesis. Active localization of PG synthesis has long been considered to be coordinated by cytoskeletal elements\textsuperscript{78}, with an additional role for substrate availability predominantly in bacteria that lack MreB(-like) filaments\textsuperscript{216}. Three recent papers showing that PG synthesis is required for the dynamics of MreB-like filaments, with key PBPs involved in elongation of the cell wall as drivers\textsuperscript{79,80,215}, prompted our study. We hypothesized that it is unlikely that the MreB architecture determines the placement of the PG synthesis machinery when MreB(-like) filaments require active PG synthesis to be dynamic. MreB plays an important role as a scaffold in organizing the PG synthesis machinery, as evidenced by the loss of shape of MreB mutant cells and the multiple interactions between MreB and proteins involved in PG synthesis\textsuperscript{36,78,228}, but the placement of MreB filaments may not be the determinant for the architecture of PG in MreB containing bacteria. To test whether the localization of substrate can determine the localization of the PG synthesis machinery, we actively delocalized lipid II using nisin\textsuperscript{220} and followed the localization of key PBPs that are known drivers of MreB dynamics in \textit{B. subtilis}. 

![Fig. 5 Nisin delocalizes lipid II and PBPs in \textit{S. pneumoniae}. PG synthesis visualized by Van-FL-labeling (A-C) and localization of GFP-PBP1A (D-F) and GFP-PBP2x (G-I) in untreated cells (A, D, G) or after treatment with nisin (B, E, H) or CCCP (C, F, I). The scale bar, same for all, is 2 μm.](image-url)
As these PBPs localized to the patches formed by lipid II after nisin treatment, we postulate that substrate availability determines the localization of PG synthesis in MreB-containing bacteria as well. In addition we showed that lipid II delocalization also leads to PBP redistribution in *S. pneumoniae*.

**Nisin as a tool to delocalize lipid II**

Nisin is a lantibiotic that has two modes of action. Firstly, it binds to lipid II, or to the precursor of teichoic acid, Lipid IV, to form pores in the bacterial membrane, which kills the cell\(^2\)\(^{219,232}\). The second mode of action of nisin is the removal of lipid II away from the septum into clustered patches along the membrane\(^2\)\(^220\). This removal, or sequestration, of lipid II results in defective PG synthesis, although it has not been shown whether this defect is (1) through a block of synthesis as lipid II is no longer available for PG synthesis complexes that are located elsewhere, or (2) through delocalized but ongoing PG synthesis resulting in formation of defective cell walls. The first explanation was suggested by the authors that originally described the sequestration effect\(^2\)\(^220\), yet our results suggest that the second explanation is (also) true. In addition, it is very likely that the nisin-bound lipid II is no longer available for incorporation into PG through transglycosylation\(^2\)\(^233\), which raises the question whether synthesis is not blocked per se.

We used the non-pore-forming hinge mutant [N20P/M21P]nisin (PP-nisin) to delocalize lipid II because it is less toxic. Also, initially, we had hoped that this non-pore-forming mutant would not affect the membrane potential since the collapse of membrane potential caused by nisin has an effect on the localization of various proteins including MreB\(^2\)\(^229\). As it turned out that the addition of PP-nisin caused a partial collapse of the membrane potential (Fig. S1), control experiments with CCCP were performed to make sure that the localization of the proteins used in this study is not sensitive to a membrane potential collapse (Fig. 1). To verify that PP-nisin had no additional effects on the localization of membrane proteins other than membrane potential collapse, the localization of two proteins independent of membrane potential, AtpA and SdhA, was analyzed in the presence of PP-nisin. AtpA and SdhA retained their random localization throughout the membrane and did not cluster into similar patches as formed by lipid II and PBPs (Fig. S3). This showed that PP-nisin had no other detectable effects on membrane protein organization. Nisin and PP-nisin are useful tools to study the effect of lipid II delocalization as shown in this study, however the effect on the membrane potential makes them unfit to follow various interesting enzymes involved in PG synthesis such as MreB\(^2\)\(^229\) and PBP1 (this
study). We are currently investigating other variants of nisin lantibiotics to see if it is possible to delocalize lipid II without collapsing the membrane potential.

**Substrate availability as a determinant for localization of PBPs**

Bacterial cytoskeletal elements play an important role in the organization of cell wall synthesis. FtsZ has long been known to coordinate cell division and the concomitant synthesis of PG, and when MreB was discovered and found to be conserved in bacteria that are ‘non-coccoid’ a role for MreB in orchestrating PG synthesis during elongation of bacterial cells was proposed (see 78,216). The importance of cytoskeletal proteins and PG synthesis for accurate division has recently been underscored by the finding that *Chlamydia*, which does not contain FtsZ nor detectable amounts of PG, requires MreB and PBPs for accurate division234. Yet although interactions between cytoskeletal proteins, proteins involved in lipid II biosynthesis, and PBPs have been described (e.g. 26,228,235), an active role for cytoskeletal proteins in positioning PBPs has not been established. Findings that indicated that PBPs could be delocalized in the presence of cytoskeletal elements were reported first for the non-MreB-containing bacteria *S. aureus* and *S. pneumoniae* and later also for *E. coli* and *C. crescentus* (see introduction). Combined, these results suggested that PBPs localize to sites where PG precursors are available. However, in none of these studies this hypothesis was tested on PBPs that are specifically involved in cell wall elongation (so thought to be dependent on MreB), nor have any of the authors used methods to actively displace PG precursors to study the effect on PBP localization.

The use of nisin allowed us to address whether PBPs localize to sites where PG precursors are abundant after altering the localization of the precursor. Although the use of nisin has its limitations, as proteins whose localization is dependent on membrane potential cannot be studied (above), it is a powerful tool to analyse the key Class B PBPs involved in division (PBP2B) and elongation (PBP2A/PbpH) in *B. subtilis*. (PP-)nisin sequestered lipid II in patches along the lateral wall and similar patches were observed for PBP2A and PbpH, whereas PBP2B stayed at the division site (Fig. 1). Colocalization of PBP2A and lipid II showed that the patches formed in the presence of nisin overlap, indicating that the sequestration of lipid II into patches indeed recruits PBP2A (Fig. 2). The possibility that patch formation by PBPs is an artifact of PP-nisin insertion in the membranes was excluded by showing that PP-nisin does not induce PBP patch formation in cells depleted for lipid II (Fig. 3).
Localization of key PBPs during cell growth

The redistribution of PBP2A and PbpH to patches could only be achieved by delocalization of lipid II. Antibiotic treatments that have altered the localization of various PBPs in other organisms (above) by either blocking the catalytic site of the PBP (PenG), blocking the lipid II for binding (vancomycin), or depleting lipid II (fosfomycin) did not affect the localization of PBP2a or PbpH. There are two possible explanations for this observation – either PBP2A and PbpH do become more randomly localized along the lateral membrane, or PBP2A and PbpH are part of a larger cell wall synthesis machinery, including cytoskeletal elements, that stops or slows down movement in the absence of substrate. We favour the latter explanation as it corresponds to the observed reduction of movement of MreB(-like proteins), RodA, PbpH, and PBP2A when PG synthesis is blocked or reduced79,80. The limitations of using nisin prevented us from doing an experiment to see whether other components of this machinery move to the same patches. It is important to note that MreB forms patches with CCCP229, whereas PBP2A and PbpH do not, so the patches we observe after nisin treatment are not the result of PBPs passively following MreB or MreB associated proteins such as MurG26, after a collapse of the membrane potential.

There are several examples of protein targeting in bacteria by substrate molecules or recognition of molecular patterns. Examples from cell wall synthesis pathways include PBP4, the major crosslinking transpeptidase in Staphylococcus aureus, which is targeted to the division site by wall teichoic acids202. Streptococcus pneumoniae StkP, a Ser/Thr kinase, localizes to the division site by recognizing uncrosslinked muropeptides through its PASTA domains43 – these PASTA domains are also found in several PBPs and hypothesized to target these PBPs to sites of PG synthesis, e.g. S. aureus PBP1110 and S. pneumoniae PBP2x134. The lipid II headgroup sticks out of the membrane to about 1.9 nm above the membrane surface with the pentapeptide pointing away from the membrane surface, making it accessible for binding by proteins236. Recent molecular dynamics simulations showed that lipid II forms specific amphiphilic patterns on the surface of bacterial membranes that could serve as landing sites for lantibiotics such as nisin237, as well as for other proteins such as PBPs. The molecular dynamics simulations revealed that the most solvent-accessible part of the lipid II headgroup is the terminal D-Ala-D-Ala dipeptide, which is the substrate for transpeptidase binding. These findings suggest that lipid II could indeed serve as a targeting molecule for PBPs. It is assumed in literature that transglycosylation is the first reaction in lipid II incorporation into PG, followed by transpeptidation, however this has never been shown in vivo. It could be that the class A bifunctional transglycosylases/transpeptidases bind lipid II through the peptide moiety first, before carrying out the transglycosylation.
reaction. The dependency of the *S. aureus* class A PBP2 on a transpeptidase substrate is an example of this possibility. Unfortunately, the dependence of PBP1, the major Class A PBP from *B. subtilis*, on an intact membrane potential, prevented us from investigating the lipid II dependence of its localization.

It is interesting to note that lipid II synthesis is conserved in bacteria that do not contain a detectable cell wall such as *Chlamydia* and *Wolbachia* and that PBPs are essential for division in *Chlamydia*. It was proposed that the conservation of a lipid II biosynthetic pathway in these organisms is essential, with lipid II functioning as a targeting signal for proteins involved in cell division. Combined with previous work in *S. aureus* and *S. pneumoniae*, our work provides strong support for the notion that lipid II can act as a determinant for protein localization, independent of cytoskeletal proteins.

**Concluding remarks**

The use of nisin to alter the location of lipid II provided us with a direct method to test the substrate availability hypothesis. We have shown that the localization of PBP2A and PbpH, specifically associated with the growth of the lateral wall in *B. subtilis*, is governed by the localization of substrate. Also, various PBPs from *S. pneumoniae* depend on lipid II positioning for their localization. This raises important questions as to how lipid II synthesis and translocation are controlled in time and space, and how other PBPs that cooperate in a complex with Class B transpeptidases are targeted to sites of PG synthesis.

**Methods**

Nisin and PP-nisin were kind gifts from Eefjan Breukink (University of Utrecht, NL) and Oscar Kuipers (University of Groningen). Bodipy FL Vancomycin and 3,3'-Dipropylthiadicarbocyanine Iodide (DiSC₃(5)) were from Molecular Probes, Invitrogen Life Sciences. All other chemicals were from Sigma Aldrich.

Strains used in this study are listed in Table 1. To construct *B. subtilis* strain 4042, the *gfp* cassette from pSG5043 was replaced with an *mKate2* cassette, resulting in plasmid pDJ84, which was transformed to strain 168 resulting in strain 4042. The pDJ84 plasmid was sequenced and correct integration at the *pbpA* locus was verified by PCR.

To construct *S. pneumoniae* strain JWV505 (*Pz-n-gfp-pbp1A*), a PCR using primers PBP1A-F+Xbal (GCACCTTAGAAACAAACCAACGGATTCTGCGCCTAATC) and PBP1A-R+NotI (CGATGCGGCCGCTTATGGTTGTGCTGGTTGAGGATTCTGA), was performed using chromosomal DNA of *S. pneumoniae* D39 as a template. The amplified fragment was subsequently cleaved with Xbal and NotI and ligated into the SpeI and NotI sites of plasmid pJWV25. The ligation mixture was directly used to transform competent *S. pneumoniae* strain JWV509. To construct *S. pneumoniae* strain JWV509 (*Pz-gfp-pbp2X*), a PCR using primers PBP2X-F+SpeI (GGCCACTAGTAAGTGGACAAAAAGAGTAATCC) and PBP2X-R+NotI (CGATGCGGCCGCTTATGGTTGTGCTGGTTGAGGATTCTGA), was performed using chromosomal DNA of *S. pneumoniae* D39 as a template. The amplified fragment was subsequently cleaved with SpeI and NotI.
Localization of key PBPs during cell growth

and ligated into the corresponding sites of plasmid pJWV25. The ligation mixture was directly used to transform competent *S. pneumoniae* D39 cells. Strains JWV505 and JWV509 were obtained by a double crossover recombination event between the *bgaA* regions located on plasmid pJWV25 and the chromosomal *bgaA* locus. Transformants were selected on Colombia blood agar plates containing tetracycline (1 μg/ml) and correct construction and integration was verified by PCR. All constructs were sequence verified.

**Microscopy.** *B. subtilis* and *S. pneumoniae* strains were cultured as described,[43,227] using S+ medium for *B. subtilis* and C+Y medium for *S. pneumoniae* for all experiments except time-lapse microscopy of strain 4042 and microscopy of strain BS23 (*AtpA*-GFP), where CH-medium was used. GFP-fusions under control of *P_xyl* were induced by the addition of 0.25% (w/v) xylose to the growth medium. Labeling with fluorescent vancomycin was done for 10 min for *B. subtilis* and 5 min for *S. pneumoniae* with a 1:1 mixture of vancomycin and Bodipy® FL Vancomycin (Van-FL) at 1 μg/ml with shaking before processing the cells for microscopy. Cells were treated with PP-nisin at 1.5 μg/ml for 10 min, CCCP at 0.2 mM for 2 min, vancomycin at 10 μg/ml, or Penicillin G at 10 μg/ml for 10 min. Subsequently cells were spun down, resuspended in 1/10th volume of PBS and mounted on agarose pads (1% in PBS). The lipid II depletion experiments were done as follows: strain 4042 was grown on CH, and fosfomycin (500 μg/ml) was added to the culture for 30 min. Cells were spun down, resuspended in 1/10th volume of PBS and treated with PP-nisin 1.5 μg/ml for 5 min. As controls, cells from the same culture were processed in the same way but either fosfomycin and/or PP-nisin were not added to the culture. Subsequently the cells were mounted onto agarose pads (1% in PBS). In the case of fosfomycin-treated cells, fosfomycin was also included in the PBS wash buffer and agarose pads. Cells were imaged by microscopy using a Nikon Ti-E microscope equipped with a CFI Plan Achromat D 100x oil objective and either a Hamamatsu Orca Flash4.0 sCMOS camera or a Hamamatsu ORCA R2 CCD camera. Simultaneous TIRFm and epifluorescence imaging was done as described by Spira et al.[239], using an IX71 Olympus microscope, equipped with 488nm and 561nm lasers, a 100x TIRF (1.49NA) objective and a sCMOS camera, assembled by Applied Precision (GE Healthcare, USA). For time lapse experiments cells were treated with PP-nisin, and immediately transferred to agarse pads containing Van-FL at 1 μg/ml, and microscopy was performed as described[158] using an IX71 Microscope (Olympus), equipped with a 100x phase-contrast objective (1.3 NA) and a CoolSNAP HQ2 camera (Princeton Instruments), assembled by Imrol (UK). Autofocus was performed using diascopic light and using the autofocus routine present in Deltavision’s Softworx software. Image analysis was done with the Nikon Elements, ImageJ and Adobe Photoshop packages. Time-lapse microscopy images were deconvolved using Softworx, other images show original untreated data.

**Membrane potential measurements** were performed essentially as described earlier.[240] *B. subtilis* 168 was grown to mid-exponential phase, washed once and resuspended in an equal volume of 50 mM potassium phosphate buffer (pH 7) with 10 mM glucose. DiSC3(5) was added to a final concentration of 1 μM. Equal volumes of suspension were loaded in wells of a 96-well plate, and the plate was transferred to a Synergy Mx (Biotek) plate reader at 37°C. The fluorescence signal (excitation at 643 nm, emission at 666 nm) was monitored for 15 min before addition of nisin, PP-nisin or buffer (at equal volume but with varying final concentration). The fluorescence was followed for an additional 15 min with shaking and the change in fluorescence signal with respect to the buffer control was determined.

**Acknowledgements**

We thank Eefjan Breukink and Oscar Kuipers for the gift of purified nisin and PP-nisin. We thank Henrik Strahl, Peter Lewis, Julia Domínguez-Escobar and Roland Wedlich-Söldner for strains. Oscar Kuipers and Henrik Strahl are also thanked for valuable discussions.

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Table

Table 1. Strains used in this study

<table>
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<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/construction</th>
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Supplemental figures

Fig. S1 Fluorimetric measurement of the collapse of membrane potential by nisin and PP-nisin. The ΔΨ-sensitive fluorescent dye DiSC<sub>3</sub>(5) accumulates on polarized membranes of glucose-energized B. subtilis cells, which results in fluorescence quenching. Dissipation of ΔΨ by nisin or PP-nisin is measured as release of the dye to the medium resulting in an increase in fluorescence. Various concentrations of nisin and PP-nisin were tested; values represent the mean and standard deviation from three different experiments which were performed in duplicate. Addition of nisin or PP-nisin at concentrations used to delocalize lipid II (1.5 μg/ml) resulted in a significant increase in DiSC<sub>3</sub>(5) fluorescence, indicative of a (partial) collapse of the membrane potential.

![DiSC3(5) fluorescence measurement](image)

Fig. S2 Delocalization of MreB by PP-nisin and CCCP. Localization of GFP-MreB in untreated cells (A) or after treatment with PP-nisin (B) or CCCP (C). The scale bar, same for all, is 2 μm.
Localization of key PBPs during cell growth

Fig. S3 Localization of AtpA-GFP and SdhA-GFP is not affected by PP-nisin or fosfomycin. Localization of AtpA-GFP (A-D) and SdhA-GFP (E-H) was performed in untreated cells (A, E) or after treatment with PP-nisin (B, F), CCCP (C, G), or fosfomycin (D, H). The scale bar, same for all, is 2 μm. To show clear membrane localization, background light was subtracted and out-of-focus light removed by 2D-blind deconvolution.

Fig. S4 Time-lapse microscopy showing PG synthesis visualized by Vancomycin-labeling (Van-FL, green in overlay) and localization of RFP-PBP2A (PBP2A, red in overlay) after treatment with PP-nisin. Time indicated in frames is minutes after the addition of PP-nisin. The images have been deconvolved. The scale bar is 2 μm.

Fig. S5 Localization of lipid II and PBP2A in S. pneumoniae. Fluorescence microscopy of S. pneumoniae strain JWV515 (scpB::PZn-rfp-pbp1a) imaged for RFP-PBP1A (C, G, red in overlay) and Van-FL (B, F, green in overlay), an overlay of the images (D, H) and a phase-contrast image of the cells (A, E) in untreated cells (A–D) or after treatment with nisin (E–H). The scale bar is 2 μm.