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The localization of key *Bacillus subtilis* penicillin binding proteins during cell growth is determined by substrate availability

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Summary

The shape of bacteria is maintained by the cell wall. The main component of the cell wall is peptidoglycan (PG) that 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 LipidII is delocalized by treatment of the cells with nisin. Alteration of this localization is only seen in a LipidII-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. Peptidoglycan allows bacteria to maintain shape, generate turgor and provides protection against osmotic shock (Typas *et al*., 2011). Peptidoglycan is composed of a single molecule formed by strands of repeating disaccharide units that are cross-linked by peptide side chains attached to the disaccharides. Peptidoglycan synthesis is the main target of most commonly used antimicrobials. These antimicrobials work at various stages of PG synthesis: (i) inhibition of the synthesis of the PG precursor LipidII (which consists of the disaccharide-pentapeptide linked to a undecaprenol carrier lipid that facilitates translocation over the membrane); (ii) inhibition of the recycling of the undecaprenol carrier lipid; (iii) binding to LipidII to prevent access of PG synthesizing enzymes, collectively known as penicillin binding proteins (PBPs), to LipidII; and (iv) binding and blocking of the active site of PBPs. Penicillin binding proteins incorporate the disaccharide-pentapeptide moiety of LipidII into a growing PG network through a combination of transglycosylation reactions that attach the disaccharide to glycan strands and transpeptidation reactions that form peptide cross-links 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 *Bacillus subtilis*, these PBPs are PBP2B, which is essential and required for division (Daniel *et al*., 2000), and PBP2A and PbpH, which are required for cell wall synthesis during elongation (Wei *et al*., 2003). 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 (Typas *et al*., 2011). 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 (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011; van Teeffelen *et al*., 2011). Various antimicrobials that block PG synthesis at different steps were shown to block MreB dynamics (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011; van Teeffelen *et al*., 2011). Specifically, PBP2A and PbpH, the PBPs essential for elongation in *B. subtilis* were shown to be drivers for MreB dynamics (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011). 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

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correct localization of PG synthesis (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011). 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 (Scheffers and Pinho, 2005). In *Staphylococcus aureus*, the 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 LipidII by the addition of D-cycloserine, which blocks the addition of the terminal D-Ala-D-Ala residues of the LipidII pentapeptide (Pinho and Errington, 2005). In *Streptococcus pneumonia*e, 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 (Morlot *et al*., 2004), 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 (Barendt *et al*., 2011). 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, and PBP5 accumulates even more at the division site when cell wall synthesis along the lateral wall is inhibited (Potluri *et al*., 2010). Finally, *Caulobacter crescentus* PBP3 delocalizes when its active site is mutated (Costa *et al*., 2008). *Str. pneumoniae* and *Sta. 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 (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011). To do so, we made use of nisin to alter the localization of LipidII, the substrate of PBPs. Nisin is a lantibiotic produced by *Lactococcus lactis* strains that forms pores in the bacterial membrane that kill the cell (Breukink and de Kruijff, 2006). A second activity of nisin is the removal of LipidII away from the septum into clustered patches along the membrane (Hasper *et al*., 2006). This removal, or sequestration, of LipidII 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 (Hasper *et al*., 2006). In this report, we used nisin to delocalize LipidII and tested the substrate availability model in *B. subtilis*.

Results

Bacillus subtilis *PBP2A and PbpH are redistributed upon LipidII delocalization*

LipidII and immature PG can be labelled with fluorescent vancomycin (Van-FL) that binds to the terminal D-Ala-D-Ala of LipidII. In mature PG, the amount of disaccharides with D-Ala-D-Ala pentapeptides, substrates for Van-FL labelling, is only 1.6% (Atrih *et al*., 1999), as the terminal D-Ala residue(s) are removed from PG during the formation of peptide cross-links or processed by D,D-carboxy-peptidases. Thus, Van-FL is an excellent marker for PG synthesis, labelling predominantly unincorporated LipidII as evidenced by severely reduced staining of cells in which LipidII synthesis is blocked (Daniel and Errington, 2003). We used Van-FL to label *B. subtilis* and found that LipidII is present at the septum and along the lateral wall in punctate patterns (Fig. 1A), as previously described (Daniel and Errington, 2003; Tiyanont *et al*., 2006).

To test the substrate availability model for PBP localization, we wanted to delocalize LipidII from its normal localization along the lateral wall. Nisin is a lantibiotic that is capable of sequestering LipidII into patches along the lateral wall (Hasper *et al*., 2006). Importantly, this sequestration of LipidII is not coupled to the formation of membrane pores as nisin mutants that do not form stable membrane pores also show LipidII sequestration (Hasper *et al*., 2006). We used the hinge mutant (N20P/M21P)nisin (PP-nisin) instead of nisin Z (nisin) because PP-nisin (i) is less toxic to bacteria as determined by minimal inhibitory concentration measurements (Wiedemann *et al*., 2001), (ii) is incapable of forming stable pore complexes with LipidII (Hasper *et al*., 2004), and (iii) is still active in LipidII delocalization (Hasper *et al*., 2006) (Fig. 1B).

Bacillus subtilis PBPs PBP2A and PbpH, are Class B transpeptidases involved in elongation and MreB dynamics (Wei *et al*., 2003; Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011), and PBP2B is a Class B PBP essential for division (Daniel *et al*., 2000). PBP1 is a Class A bifunctional transglycosylase/transpeptidase involved in division (Scheffers and Errington, 2004) as well as elongation through an interaction with MreB (Kawai *et al*., 2009). We used fully functional green fluorescent protein (GFP) fusions to these PBPs (Scheffers and Errington, 2004; Scheffers *et al*., 2004) and determined the localization of the PBPs before and after treatment with PP-nisin. Midexponentially growing cells were treated with PP-nisin and were analysed by fluorescence microscopy. As shown in Fig. 1, PP-nisin caused the redistribution of PBP2A, PbpH and PBP1 (Fig. 1E,H,N) to patches in the lateral membrane that are similar to the patches formed by LipidII. Division-site associated PBP1 and division-site-

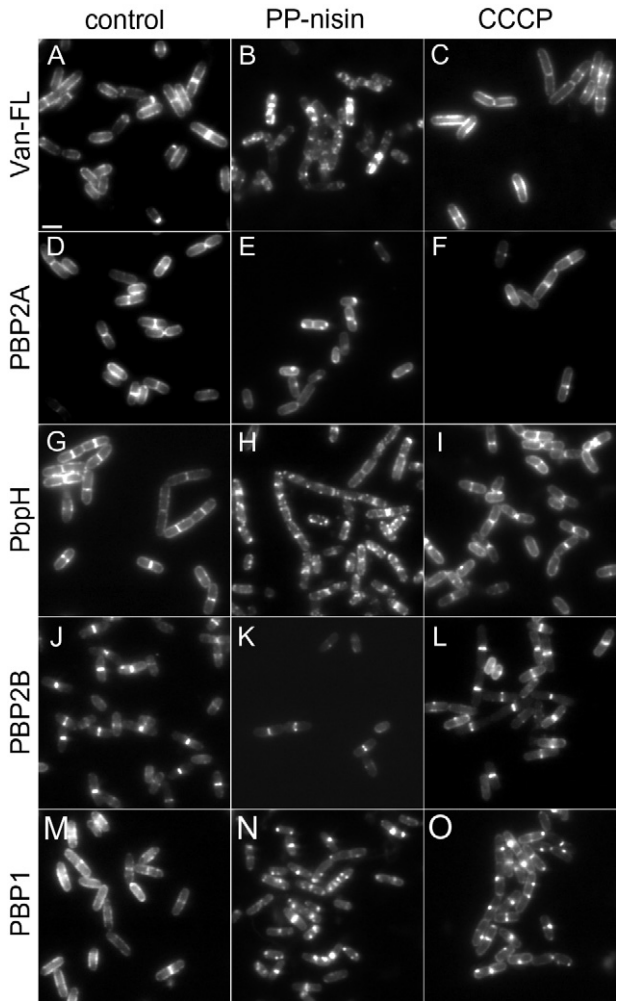

Fig. 1. PP-nisin delocalizes LipidII and elongation specific PBPs in *B. subtilis*. PG synthesis visualized by vancomycin-labelling (A–C) and localization of GFP-PBP2A (D–F), GFP-PbpH (G–I), GFP-PBP2B (J–L) and GFP-PBP1 (M–O) in untreated cells (A,D,G,J,M) or after treatment with PP-nisin (B,E,H,K,N) or CCCP (C,F,I,L,O). Scale bar, same for all, 2 μm.

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). Because the dissipation of the membrane potential results in mislocalization of various proteins, including MreB (Strahl and Hamoen, 2010), 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 LipidII (1.5 μg ml^{-1}) 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 LipidII and the localization of the PBPs was tested by dissipation of the PMF with the ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Addition of CCCP did not change the localization of LipidII, 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 LipidII relocalization, 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, Strahl and Hamoen, 2010). 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 (Johnson *et al*., 2004; Strahl and Hamoen, 2010). 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 LipidII upon treatment of cells with PP-nisin independent of the dissipation of the membrane potential.

Bacillus subtilis *PBP2A colocalizes with LipidII during patch formation*

The patches formed by *B. subtilis* GFP-PBP2A, GFP-PbpH and LipidII after treatment with PP-nisin were very similar, but we could not formally determine whether the PBP and LipidII 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 LipidII. When this strain was labelled with Van-FL, the localization patterns of RFP-PBP2A and LipidII were found to overlap (Fig. 2A–C). These cells were treated with PP-nisin to determine the amount of overlap between spots formed by LipidII 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 LipidII patches that form upon treatment with PP-nisin. To

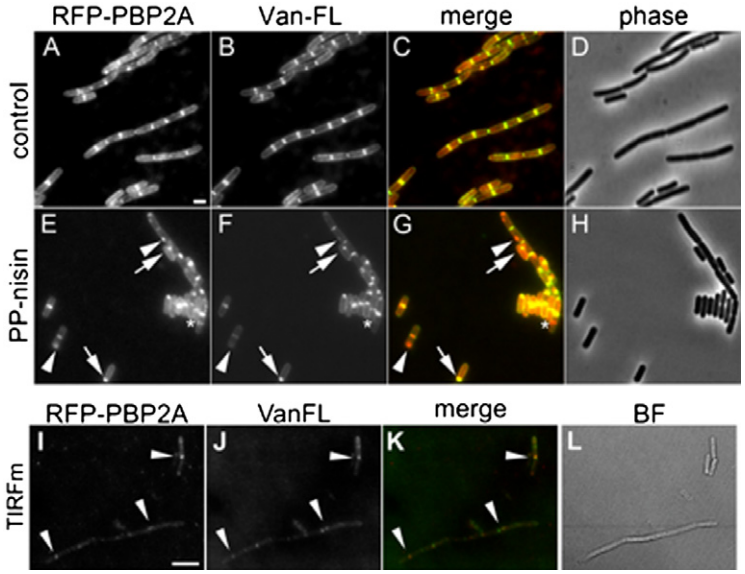

Fig. 2. Colocalization of LipidII and PBP2A in *B. subtilis*.
A–H. Epi-fluorescence microscopy of *B. subtilis* strain 4042 imaged for RFP-PBP2A (A,E, red in overlay) and Van-FL (B,F, green in overlay), an overlay of the images (C,G) and a phase-contrast image of the cells (D,H) in untreated cells (A–D) or after treatment with PP-nisin (E–H). Scale bar, same for all, 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. Two hundred one cells contained patches of both Van-FL and PBP2A, and in 188 cells, these patches overlapped (94%).
I–L. *Bacillus subtilis* strain 4042 labelled 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. Arrow heads indicate the delocalized spots. Scale bar, 2 μm.

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 LipidII staining pattern. Again, PP-nisin addition resulted in RFP-PBP2A delocalization to patches that overlapped with Van-FL-labelled LipidII patches. The resolution of the time lapse microscopy was not high enough to identify whether Van-FL or PBP2A patches formed subsequently or simultaneously.

Redistribution of PBPs is dependent on LipidII 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 LipidII delocalization, the effect of PP-nisin on PBP2A was studied in cells that were depleted for LipidII. Fosfomycin is an antibiotic that blocks the first cytosolic step in LipidII biosynthesis (Kahan *et al.*, 1974) and treatment of cells with fosfomycin blocks the movement of MreB, Mbl and PbpH (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011). Cells expressing RFP-PBP2A were incubated with fosfomycin for 30 min to deplete LipidII 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 LipidII depletion (Fig. 3D). This experiment shows that PBP2A patch formation is dependent on the presence of LipidII and not caused solely by the addition of PP-nisin to cells.

Staphylococcus 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 LipidII substrate is made unavailable for binding (Pinho and Errington, 2005). PBP2A, PbpH and LipidII already occupy most of the cell surface so it would be difficult to

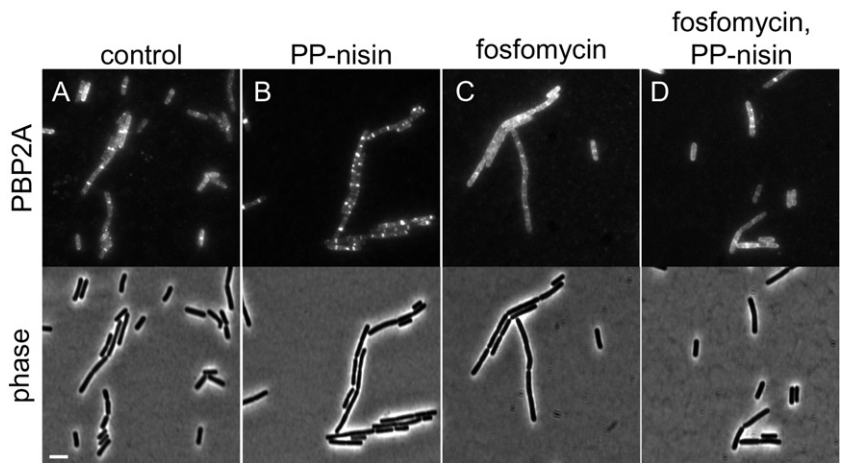

Fig. 3. LipidII is required for PBP2A patch formation. *B. subtilis* strain 4042 imaged for RFP-PBP2A (top) and phase-contrast image of the cells (bottom). Control cells were either not treated (A) or treated with PP-nisin only (B). Fosfomycin was used to block LipidII production, and cells were not treated (C) or treated with PP-nisin (D). Patch formation only occurred in cells treated with PP-nisin in which LipidII production was not blocked. Scale bar, same for A–D, 4 μm.

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 LipidII 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 LipidII, 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 LipidII delocalization into patches and thus that the localization of PBP2A and PbpH is determined by substrate availability.

Discussion

In this study, we have used nisin to alter the location of LipidII 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 (Typas *et al.*, 2011), with an additional role for substrate availability predominantly in bacteria that lack MreB(-like) filaments (Scheffers and Pinho, 2005). 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 (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011; van Teeffelen *et al.*, 2011), 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 (Jones *et al.*, 2001; Kawai *et al.*, 2009; Typas *et al.*, 2011), 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 LipidII using nisin (Hasper *et al.*, 2006) and followed the localization of key PBPs that are known drivers of MreB dynamics in

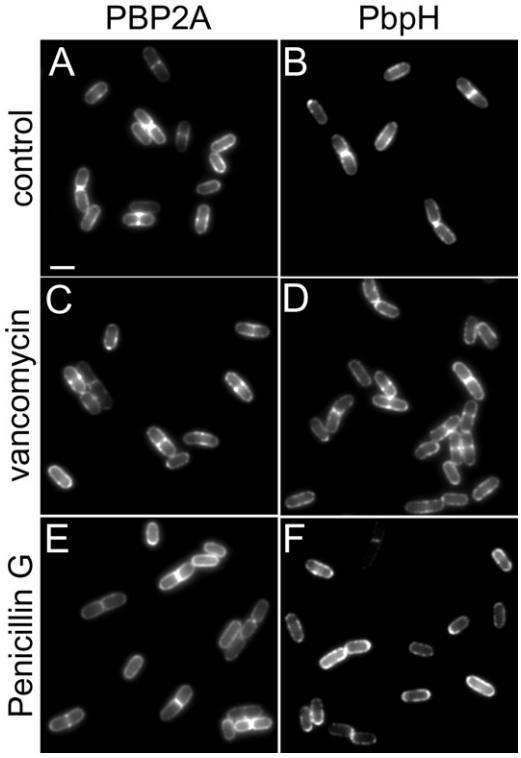

Fig. 4. Blocking substrate binding does not alter the localization of PBPs.
A–F. Localization of GFP-PBP2A (A,C,E) and GFP-PbpH (B,D,F) before (controls, A,B) or after treatment of *B. subtilis* cells with vancomycin (C,D) or penicillin G (E,F). Scale bar, same for all, 2 μm.

B. subtilis. As these PBPs localized to the patches formed by LipidII after nisin treatment, we postulate that substrate availability determines the localization of PG synthesis in MreB containing bacteria as well.

Nisin as a tool to delocalize LipidII

Nisin is a lantibiotic that has two modes of action – it binds to LipidII, or to the precursor of teichoic acid, LipidIV, to form pores in the bacterial membrane, which kills the cell (Breukink and de Kruijff, 2006; Wolf *et al*., 2012). The second mode of action of nisin is the removal of LipidII away from the septum into clustered patches along the membrane (Hasper *et al*., 2006). This removal, or sequestration, of LipidII results in defective PG synthesis, although it has not been shown whether this defect is (i) through a block of synthesis as LipidII is no longer available for PG synthesis complexes that are located elsewhere, or (ii) 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 (Hasper *et al*., 2006), yet our results suggest that the second explanation is (also) true. In addition, it is very likely that the nisin-bound LipidII is no longer available for incorporation into PG through transglycosylation (Bonev *et al*., 2004), 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 LipidII because it is less toxic. Also, initially we had hoped that this non-pore-forming mutant would not affect the membrane potential as the collapse of membrane potential caused by nisin has an effect on the localization of various proteins including MreB (Strahl and Hamoen, 2010). 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 the membrane potential independent proteins AtpA and SdhA was analysed 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 LipidII 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 LipidII 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 (Strahl and Hamoen, 2010) and PBP1 (this study). We are currently investigating other variants of nisin lantibiotics to see if it is possible to delocalize LipidII 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 Scheffers and Pinho, 2005; Typas *et al*., 2011). 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 division (Ouellette *et al*., 2012). Yet although interactions between cytoskeletal proteins, proteins involved in LipidII biosynthesis, and PBPs have been described (e.g. Divakaruni *et al*., 2007; Mohammadi *et al*., 2007; Kawai *et al*., 2009), 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 *Sta. aureus* and *Str. 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 membrane potential-dependent cannot be studied (as mentioned earlier), 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 LipidII 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 LipidII showed that the patches formed in the presence of nisin overlay, indicating that the sequestration of LipidII into patches indeed recruits PBP2A (Fig. 2). The possibility that patch formation by PBPs is an artefact of PP-nisin insertion in the membranes was excluded by showing that PP-nisin does not induce PBP patch formation in cells depleted for LipidII (Fig. 3).

The redistribution of PBP2A and PbpH to patches could only be achieved by delocalization of LipidII. Antibiotic treatments that have altered the localization of various PBPs in other organisms (as mentioned earlier) by either blocking the catalytic site of the PBP (PenG) or blocking the LipidII for binding (vancomycin) or by depleting LipidII (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 reduced (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011). 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 CCCP (Strahl and Hamoen, 2010), 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 MurG (Mohammadi *et al*., 2007) 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 cross-linking transpeptidase in *Sta. aureus*, which is targeted to the division site by wall teichoic acids (Atilano *et al*., 2010). *Streptococcus pneumoniae* StkP, A Ser/Thr kinase, localizes to the division site by recognizing uncross-linked muropeptides through its PASTA domains (Beilharz *et al*., 2012) – these PASTA domains are also found in several PBPs and hypothesized to target these PBPs to sites of PG synthesis, e.g. *Sta. aureus* PBP1 (Pereira *et al*., 2009) and *Str. pneumoniae* PBP2x (Jones and Dyson, 2006). The LipidII 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 proteins (Ganchev *et al*., 2006). Recent molecular dynamics simulations showed that LipidII forms specific amphiphilic patterns on the surface of bacterial membranes that could serve as landing sites for lantibiotics such as nisin (Chugunov *et al*., 2013), as well as for other proteins such as PBPs. The molecular dynamics simulation revealed that most solvent accessible part of the LipidII headgroup is the terminal D-Ala-D-Ala dipeptide, which is the substrate for transpeptidase binding. These findings suggest that LipidII could indeed serve as a targeting molecule for PBPs. It is assumed in the literature that transglycosylation is the first reaction in LipidII 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 LipidII through the peptide moiety first, before carrying out the transglycosylation reaction. The dependency of the *Sta. aureus* class A PBP2 on a transpeptidase substrate is an example of this possibility (Pinho and Errington, 2005). Unfortunately, the dependence of PBP1, the major Class A PBP from *B. subtilis*, on an intact membrane potential, prevented us from investigating the LipidII dependence of its localization.

It is interesting to note that LipidII synthesis is conserved in bacteria that do not contain a detectable cell wall such as *Chlamydia* and *Wolbachia* (Henrichfreise *et al*., 2009) and that PBPs are essential for division in *Chlamydia* (Ouellette *et al*., 2012). It was proposed that the conservation of a LipidII biosynthetic pathway in these organisms is essential, with LipidII functioning as a targeting signal for proteins involved in cell division (Henrichfreise *et al*., 2009). Combined with previous work in *Sta. aureus* (Pinho and Errington, 2005) and *Str. pneumoniae* (Morlot *et al*., 2004), our work provides strong support for the notion that LipidII can act as a determinant for protein localization, independent of cytoskeletal proteins.

Concluding remarks

The use of nisin to alter the location of LipidII 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. This raises important questions as to how Lipid II synthesis and translocation is 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, Utrecht, the Netherlands) and Oscar Kuipers (University of Groningen, Groningen, the Netherlands). Bodipy® FL vancomycin and 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] were from Molecular Probes (Life Technologies Europe BV, Bleiswijk, the Netherlands). 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 (Scheffers *et al*., 2004) was replaced with an *mKate2* cassette resulting in plasmid pDJ84, which was

Table 1. Strains used in this study.

Strain	Relevant characteristics	Source/construction
B. subtilis		
168	*trpC2*	Laboratory collection
2083	*trpC2 ponA*::pSG1492 (*cat* P_{xyl}-*gfp-ponA*$^{1-394}$)	Scheffers *et al*., 2004
3103	*trpC2 pbpA*::pSG5043 (*cat Pxyl-gfp-pbpA*$^{1-804}$)	Scheffers *et al*., 2004
3122	*trpC2 pbpB*::pSG5061 (*cat* P_{xyl}-*gfp-pbpB*$^{1-825}$)	Scheffers *et al*., 2004
3140	*trpC2 pbpH*::pSG5058 (*cat* P_{xyl}-*gfp-pbpH* $^{1-827}$)	Scheffers *et al*., 2004
4042	*trpC2 pbpA*::pDJ84 (*cat* P_{xyl}-*mKate2-pbpA*$^{1-804}$)	this work
BS23	*trpC2 atpA–gfp* P_{xyl}*–'atpA cat*	Johnson *et al*., 2004
BS112	*trpC2 sdhA–gfp* P_{xyl}*–'sdhA cat*	Johnson *et al*., 2004
RWSB1	*amyE::(*P_{xyl}*gfp-mreB spc)*	Dominguez-Escobar *et al*., 2011

transformed to strain 168 resulting in strain 4042. The pDJ84 plasmid was sequenced, and correct integration at the *pbpA* locus was verified by PCR.

Microscopy

Bacillus subtilis strains were cultured as described (Scheffers *et al*., 2004), using S+ medium for all experiments except time-lapse microscopy of strain 4042 and microscopy of strain BS23 (AtpA-GFP), where casein hydrolysate (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. Labelling with Van-FL was done for 10 min with a 1:1 mixture of vancomycin and Bodipy FL vancomycin (Van-FL) at 1 μg ml^{-1} with shaking before processing the cells for microscopy. Cells were treated with PP-nisin at 1.5 μg ml^{-1} for 10 min, CCCP at 0.2 mM for 2 min, vancomycin at 10 μg ml^{-1} or penicillin G at 10 μg ml^{-1} for 10 min. Subsequently, cells were spun down, resuspended in 1/10th volume of PBS and mounted on agarose pads (1% in PBS). The LipidII depletion experiments were done as follows: strain 4042 was grown on CH, and fosfomycin (500 μg ml^{-1}) 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^{-1} 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 on 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 (Nikon Instruments, Tokyo, Japan) equipped with a CFI Plan Achromat DM 100× oil objective (Nikon Instruments), and either a Hamamatsu Orca Flash4.0 sCMOS camera or a Hamamatsu ORCA R2 CCD camera (Hamamatsu Photonics, Hamamatsu City, Shizuoka Pref., Japan). Simultaneous TIRFm and epifluorescence imaging was done as described (Spira *et al*., 2012) using an IX71 Olympus microscope (Tokyo, Japan), equipped with 488 nm and 561 nm lasers, a 100× TIRF (1.49NA) objective and a sCMOS camera, assembled by Applied Precision (GE Healthcare, Issaquah, WA, USA). For time-lapse experiments, cells were treated with PP-nisin and immediately transferred to agarose pads containing Van-FL at 1 μg ml^{-1}, and microscopy was performed as described (de Jong *et al*., 2011) using an IX71 Microscope (Olympus), equipped with a 100× phase-contrast objective (1.3 NA) and a CoolSNAP HQ2 camera (Princeton Instruments, Trenton, NJ, USA), assembled by Imsol (Preston, UK). Autofocus was performed using diascopic light and using the autofocus routine present in Deltavision's Softworx software (Applied Precision, Issaquah, WA, USA). Image analysis was done with the Nikon elements (Nikon Instruments), ImageJ (http://rsb.info.nih.gov/ij/) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) packages. Time-lapse microscopy images were deconvolved using Softworx; other images show original untreated data.

Membrane potential measurements were performed essentially as described (Breeuwer and Abee, 2004). *Bacillus 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. $DiSC_3(5)$ 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.

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Author contributions

MCAL, KB, DMA, JWV and DJS performed experiments and analyzed data. JWV and DJS conceived the experiments and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Fluorimetric measurement of the collapse of membrane potential by nisin and PP-nisin. The $\Delta\Psi$-sensitive fluorescent dye $DiSC_3(5)$ accumulates on polarized membranes of glucose energized *B. subitilis* cells, which results in fluorescence quenching. Dissipation of $\Delta\Psi$ 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 that were performed in duplicate. Addition of nisin or PP-nisin at concentrations used to delocalize LipidII (1.5 $\mu g\ ml^{-1}$) resulted in a significant increase in $DiSC_3(5)$ fluorescence, indicative of a (partial) collapse of the membrane potential.
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). Scale bar, same for all, 2 μm.
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). Scale bar, same for all, 2 μm. To show clear membrane localization, background light was subtracted, and out-of-focus light was removed by two-dimensional-blind deconvolution as described (Johnson *et al*., 2004).
Fig. S4. Time-lapse microscopy showing PG synthesis visualized by vancomycin-labelling (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. Scale bar 2 μm.
Movie S1. A montage of frames from the time-lapse microscopy images shown in Fig. S4.