CHAPTER 3

Pentapeptide-rich peptidoglycan at the
Bacillus subtilis cell-division site

Danae Morales Angeles1, Yun Liu2, Alwin M. Hartman2, Marina Borisova3, Anabela de Sousa Borges3, Niels de Kok1, Katrin Beilharz2, Jan-Willem Veenings3, Christoph Mayer3, Anna K.H. Hirsch2 and Dirk-Jan Scheffers1*

1 Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands.
2 Stratingh Institute for Chemistry, University of Groningen, The Netherlands.
3 Interfaculty Institute of Microbiology and Infection Medicine Tübingen, Department of Biology, University of Tübingen, Tübingen, Germany.
4 Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, The Netherlands.
5 Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, Biophore Building, CH-1015 Lausanne, Switzerland.

Molecular Microbiology 104(2) 319-33

Experiments presented in Figures 1-6, 8, S2-9 were performed by D. Morales Angeles

Experiments presented in Figure 5-6 were performed by K. Beilharz

Experiments presented in Figure 7 were performed by N. de Kok

Experiments presented in Figure 5s were performed by M. Borisova

Synthesis of HADA was performed by Y. Liu and A.M. Hartman
ABSTRACT

Peptidoglycan (PG), the major component of the bacterial cell wall, is one large macromolecule. To allow for the different curvatures of PG at cell poles and division sites, there must be local differences in PG architecture and eventually also chemistry. Here we report such local differences in the Gram-positive rod-shaped model organism *Bacillus subtilis*. Single-cell analysis after antibiotic treatment and labeling of the cell wall with a fluorescent analogue of vancomycin or the fluorescent D-amino acid analogue (FDAA) HCC-amino-D-alanine revealed that PG at the septum contains muropeptides with unprocessed stem peptides (pentapeptides). Whereas these pentapeptides are normally shortened after incorporation into PG, this activity is reduced at division sites indicating either a lower local degree of PG crosslinking or a difference in PG composition, which could be a topological marker for other proteins. The pentapeptides remain partially unprocessed after division when they form the new pole of a cell. The accumulation of unprocessed PG at the division site is not caused by the activity of the cell division specific penicillin-binding protein 2B. To our knowledge, this is the first indication of local differences in the chemical composition of PG in Gram-positive bacteria.

INTRODUCTION

The bacterial cell wall is a structure unique to bacteria, providing shape and protection from environmental challenges to the cell. The main component of the cell wall is peptidoglycan (PG). PG is a large macromolecule composed of glycan strands connected by peptide cross-bridges that form a net-like structure. Isolated PG molecules, so-called sacculi, retain the shape of the cell they surrounded. PG is constantly remodeled to allow the bacterial cell to expand its volume, to divide, and to allow the attachment of other molecules such as teichoic acid, or to allow the incorporation of proteins that cross the PG layer (e.g., pili, flagella). PG grows by the incorporation of Lipid II precursor molecules, composed of a disaccharide with a pentapeptide side chain, connected to a bactoprenol carrier molecule that anchors the building block to the membrane. The disaccharide moiety is coupled to a glycan strand by a glycosyl transferase and released from the carrier, and the pentapeptide moiety can be crosslinked to peptides on different strands by transpeptidases.

The pentapeptide composition differs between bacteria, for instance in *Bacillus subtilis* the pentapeptide is composed of L-Ala-D-Glu-L-meso-diaminopimelic acid-D-Ala-D-Ala, but the method of the primary DD-crosslink formation is very similar: the D-amino acid on the 4th position of one pentapeptide is covalently attached to the crosslinking protein (a transpeptidase), with concurrent release of the 5th D-amino acid on the pentapeptide. The donor peptide is subsequently crosslinked to the 3rd amino acid on the acceptor pentapeptide, generally an amino acid containing a free amino group, such as meso-diaminopimelic acid or L-lysine. Additionally, L,D-crosslinks can be formed between two amino acids at position 3, although these crosslinks are neither universal nor do they appear to be essential. Not all peptides are used in crosslinks, and unlinked pentapeptides are generally modified to tetra- or tripeptides, except in some bacteria such as *Staphylococcus aureus* that contains a very large amount of free pentapeptides. In *S. aureus*, the degree of PG crosslinking is a determinant for antibiotic resistance, with lower crosslinking resulting in higher antibiotic sensitivity through a yet unknown mechanism, which may have to do with easier access to the antibiotic target, or an overall weaker PG structure.

PG is one large molecule, and its synthesis and turnover are controlled by a large group of enzymes that coordinate these activities on various locations along the bacterial cell wall. In rod-shaped bacteria, two complexes for PG synthesis have been identified, the ‘elongasome’ and the ‘divisome’, responsible for PG synthesis along the lateral wall and at the cell division...
site respectively. These two machines are coordinated by the cytoskeletal proteins MreB and FtsZ and consist of Lipid II synthetases, flippases, membrane proteins with unknown coordinating functions such as MreC/D and RodZ, proteins from the Shape, Elongation, Division and Sporulation (SEDS) family and Penicillin Binding Proteins (PBPs). SEDS and PBPs are the proteins that incorporate Lipid II molecules into PG. SEDS proteins, such as FtsW and RodA, have glycosyl transferase activity. PBPs are divided into two groups: high-molecular-weight (HMW) and low-molecular-weight (LMW) PBPs. HMW PBPs are further classified into class A PBPs, whose members show glycosyl transferase and transpeptidation activity, and class B PBPs, whose members only have transpeptidation activity. The LMW PBPs have various activities, such as DD-carboxypeptidase, transpeptidase or endopeptidase activity, but always one activity per enzyme.

The different machineries involved in PG synthesis and the different structural requirements to PG on different locations in the cell, suggest that the composition of PG may vary along the sacculus. Atomic force microscopy showed that there are local differences in PG architecture between division sites and other places along the bacterial wall, which vary in thickness or in patterning. However, this technique does not reveal the underlying chemistry or crosslinking degree. Fluorescent labeling methods have uncovered different PG growth modes used by different bacteria, yet what happens after the insertion of Lipid II is unclear. Currently, local modifications to PG and local changes to the crosslinking degree cannot be identified as the methods to analyze PG composition provide only information on population averages.

In this study, we use a combination of antibiotics treatment and labeling of the cell wall with a fluorescent analogue of vancomycin (Van-FL) or the fluorescent D-amino acid analogue (FDAA) HCC-amino-D-alanine (HADA). Van-FL binds to the terminal D-Ala-D-Ala of the disaccharide pentapeptide subunit, whereas HADA is incorporated at the 5th position in Bacillus subtilis stem-peptides and rapidly processed after incorporation, so both labeling methods only reveal unincorporated Lipid II and newly synthesized PG. We found that septal PG is rich in pentapeptides, and that these pentapeptides remain partially unprocessed after division when they form the new pole of a cell. Mature PG contains only a small number of pentapeptides, 3.7% of total muropeptides of which 1.6% contains a terminal D-Ala and 2.1% a glycine residue at position 5. This is the first indication of local differences in the chemical composition of PG in Gram-positive bacteria. Our findings reveal that there are local differences in PG crosslinking and processing throughout the cell wall.

RESULTS
PG at the division site contains high concentrations of pentapeptides

We used fosfomycin to block Lipid II synthesis, and then stained nascent PG with Van-FL. To our surprise, 20 min treatment with fosfomycin at a concentration that blocks synthesis of new Lipid II and PG, does not completely block Van-FL labeling of cells (Figure 1A), something that would be expected if Van-FL predominantly labels Lipid II. Daniel and Errington also observed continued Van-FL labeling of cells that were treated with various antibiotics that block Lipid II synthesis, and interpreted this as an incomplete block of Lipid II synthesis. Bacitracin treatment had a stronger effect on reduction of Van-FL staining than fosfomycin. We decided to investigate this phenomenon in more detail, using both Van-FL and the newly developed D-Ala

Figure 1. Accumulation of unprocessed pentapeptides at the septum. Exponentially growing B. subtilis (168) was either labeled with Van-FL or HADA after growth for 20 min in the absence (control) or presence of fosfomycin (500 μg/ml) bacitracin (500 μg/ml) or D-cycloserine (500 μg/ml) to block Lipid II synthesis (A). In a reverse experiment, cells were labeled with HADA for 5 min and allowed to continue growth for 20 min in the presence of fosfomycin or bacitracin (B). Arrows indicate labeled septa. Scale bar (same for all): 5 μm.
analogue HADA, which in *B. subtilis* is incorporated exclusively in the 5th position of the stem peptide\(^{15}\) (Supporting Information Figure S1). Van-FL and HADA labeling both report the presence of a pentapeptide, but Van-FL can label pentapeptides that are already present before the label is added, whereas HADA labeling depends on active Lipid II/PG synthesis.

*B. subtilis* grown in the presence of either fosfomycin, bacitracin or D-cycloserine for 20 min prior to labeling with Van-FL, showed strong labeling at the division site (Figure 1A). None of these treatments killed the cells within the 20 min timeframe of the experiment as determined by both membrane permeability measurements as well as dilutions of treated cells, although prolonged exposure (60 min) did have an effect (Supporting Information Table S1, Figure S2). Quantification of the fluorescence signals at the septum revealed that fluorescence was increased when cells were treated with fosfomycin and bacitracin, but not when cells were treated with D-cycloserine (Supporting Information Figure S3). We cannot explain the increase in Van-FL signal but do note that also the background signal was higher in the fosfomycin and bacitracin stained samples. There are various possible explanations for the septal labeling pattern, which could also occur in combinations. First, if the antibiotic-induced block of Lipid II synthesis is incomplete, this could cause accumulation of Lipid II. Second, Lipid II at the division site may no longer be incorporated into PG and/or processed during the 20 min period in which PG synthesis is blocked. Third, bacitracin and fosfomycin inhibit the activity of carboxypeptidases that cleave D-Ala at position 5. Fourth, Van-FL could label existing PG material, not Lipid II, of which the pentapeptide has not been processed as the donor in crosslinking or through cleavage of the 5th D-Ala. To distinguish between these explanations, we repeated the experiment with HADA labeling. Fosfomycin treatment resulted in a near complete block of HADA incorporation, and bacitracin and D-cycloserine completely abolished HADA incorporation (Figure 1A), indicating that, in fact, the antibiotic treatments efficiently block Lipid II synthesis. This result strongly suggests that the Van-FL label observed at the division site is not caused by the staining of residual Lipid II that was synthesized during the antibiotic treatment. Then, in a reverse experiment, cells were labeled with HADA before resuspension in growth medium for 20 min in the presence of fosfomycin, bacitracin or D-cycloserine. Cells treated in this manner showed strong division-site labeling, similar to the Van-FL labeling (Figure 1B), indicating that HADA is incorporated into the cell wall, but then fails to be processed at the division site. HADA labeling followed by 20 min of growth without antibiotics also resulted in septal labeling, indicating that the retention of label is not the result of blocked PG synthesis or processing (Figure 1B top panel). Comparison of the level of HADA fluorescence at the septa indicated that the levels of fluorescence were similar for all cells analyzed (Supporting Information Figure S3).

Kuru *et al.* (2012) used a *B. subtilis dacA* (PBP5) knockout strain to visualize PG along the lateral wall. PBP5 is the major D,D-carboxypeptidase of *B. subtilis* which contributes to PG maturation by removing the majority of terminal D-Ala residues from stem peptides that were not used as donors in crosslinking reactions\(^{15}\). We repeated the labeling experiments in a dacA knockout strain, to see if we could increase the labeling, especially at the lateral wall. As expected, both HADA and Van-FL labeling at the lateral wall increased due to the increase of unprocessed pentapeptides in PG (Figure 2A and D). Van-FL labeled the entire cell circumference, which can be explained by the fact that Van-FL labels all D-Ala-D-Ala residues in the wall. HADA resulted in a patchy pattern on the lateral wall, as it is only labeling material synthesized during the 5 min labeling pulse. Blocking PG synthesis with bacitracin, again almost completely blocked HADA incorporation all over the cells (Figure 2B), whereas Van-FL still labeled the cell circumference (Figure 2E), indicating that this labeling pattern reflected primarily PG, not Lipid II. Again, when cells were labeled with HADA before blocking PG synthesis, label was retained at the division site, indicative of absence of processing at the division site (Figure 2C).

---

**Figure 2.** Accumulation of pentapeptides at the septa of a dacA knockout strain. Exponentially growing *B. subtilis* 4056 was either labeled with HADA (A, B) or Van-FL (D, E) after growth for 20 min in the absence (A,D) or presence of bacitracin (500 μg/ml, B,E) to block Lipid II synthesis. In a reverse experiment, cells were labeled with HADA and allowed to continue growth for 20 min in the presence of bacitracin (C). Scale bar (same for all): 5 μm.
It has been reported that in some bacteria the incorporation of fluorescent D-amino acids occurs primarily through exchange reactions on the outside of the cell, and thus that the FDAA is not incorporated into Lipid II itself\textsuperscript{15,22}. In \textit{B. subtilis}, the major PBP responsible for such an exchange reaction is PBP4, in the absence of which incorporation of various NBD-labeled D-amino acid analogues is reduced but not blocked\textsuperscript{23}. We confirmed that this was the same for HADA-labeling: although overall labeling was somewhat reduced in a PBP4 knockout strain, septal labeling was still clearly detectable (Supporting Information Figure S4). Treatment of cells with D-cycloserine, which blocks incorporation of D-Ala into Lipid II, completely blocked HADA-labeling of cells (Figure 1A). Fosfomycin, which blocks Lipid II synthesis but not transpeptidase activity, causes a block of HADA labeling in \textit{B. subtilis} (Figure 1A). In \textit{Escherichia coli}, where all incorporation occurs via transpeptidation\textsuperscript{15,24}, cells could still be labeled with HADA after a 20 min treatment with fosfomycin (Supporting Information Figure S5). Finally, our results in \textit{B. subtilis} 168 were not strain-specific as HADA labeling of strain PY79 gave similar results (Supporting Information Figure S5). Combined, these controls suggest that at least part of the HADA-labeling in \textit{B. subtilis} occurs via the Lipid II route.

Division-site labeling is caused by accumulated pentapeptides, not unincorporated Lipid II

Although the above results indicate that it is unlikely that the Van-FL/HADA fluorescence observed at the division site is produced by an accumulation of Lipid II, we wanted to confirm that the labeling pattern reflects material that has been incorporated into PG. To do so, we isolated sacculi from HADA-labeled and non-labeled cells. The isolation of sacculi involves boiling the cells repeatedly in SDS and treatment with hydrofluoric acid, which should remove all membrane associated Lipid II and wall-associated polysaccharides. Sacculi isolated from HADA-labeled cells displayed clear HADA labeling in rings at the division sites, indicative of PG with a high concentration of pentapeptides at the division site (Figure 3A). Sacculi isolated from non-labeled cells, which were subsequently labeled with Van-FL displayed a similar labeling pattern (Figure 3B), although in addition to division rings, polar fluorescence was also occasionally detected. Sacculi were also isolated from cells that were grown for 20 min in the presence of bacitracin, fosfomycin or D-cycloserine. When these sacculi were labeled with Van-FL similar patterns were observed as for the cells grown in the absence of antibiotics (Figure 3D, F, and H). Finally, sacculi isolated from cells that were growth in the presence of bacitracin, fosfomycin or D-cycloserine. When these sacculi were labeled with Van-FL similar patterns were observed as for the cells grown in the absence of antibiotics (Figure 3D, F, and H).

Figure 3. Pentapeptides are present at the septum in isolated sacculi. Exponentially growing \textit{B. subtilis} were labeled with HADA and sacculi were isolated from labeled and non-labeled cells either immediately (A–B) or after 20 min of continued growth in the presence of bacitracin (500 μg/ml C–D), fosfomycin (500 μg/ml E–F), D-cycloserine (500 μg/ml G–H) or without antibiotic (I). After isolation, sacculi were stained with Van-FL (B, D, F, H). HADA fluorescence (A, C, E, G, I) Van-FL fluorescence (B, D, F, H). Scale bar (same for all): 1 μm.
HADA-labeled and then grown for an additional 20 min in the presence of bacitracin, fosfomycin or D-cycloserine also still showed rings (Figure 3C, E, G). This result shows that the accumulation of pentapeptide material at the septum is not caused by an accumulation of Lipid II or the result of antibiotic treatment. As a final control for HADA incorporation into PG, HADA-labeled sacculi were incubated with lysozyme and the fluorescence was followed over time (Figure 4). Lysozyme digests the 1,4-β-glycosidic bond between MurNAc and GlcNAc, therefore if the fluorescence is caused by HADA that is incorporated into PG, the signal at the septum should decrease over time as PG fragments are released by lysozyme. Lysozyme digestion was followed for 10 min, with an untreated sample as a control for bleaching. The fluorescence at the division site of the sacculi treated with lysozyme decreased over time, compared to the untreated control (Figure 4, compare signals at arrows between top and bottom rows). Combined, these results show that the HADA-label is incorporated into the PG and that there is an accumulation of unprocessed D-Ala-D-Ala at division sites.

3D structure of the pentapeptide rings

To visualize the 3D structure of the pentapeptide containing PG at the division site in live cells, Z-stack pictures of HADA-labeled B. subtilis in exponential phase were taken, followed by deconvolution and 3D reconstruction. Figure 5 shows that the labeled muropeptides at the division site form a structure similar to a ring, comparable to what was observed with sacculi (Figure 3). This kind of donut-shaped ring structure also looks like the structure of the B. subtilis septum obtained with atomic force microscopy. In addition, Kuru et al. showed that E. coli, Agrobacterium tumefaciens and S. aureus also present a ring at the division site when labeled with a short HADA pulse (2012).
Pentapeptides at the division sites are retained at the poles and eventually processed

We used fluorescence time-lapse microscopy to determine if the pentapeptide-enriched PG at the septum is processed. Exponentially growing *B. subtilis* cells were labeled with a short pulse of HADA, and *B. subtilis* growth was followed by fluorescence time-lapse microscopy (Figure 6). At the initial stage, HADA labeling is localized as a band at the septum and the signal stays strong in agreement with our previous results. As the size of the cells increases and the septum starts to become two new poles, the HADA signal decreases indicating that some HADA-labeled material is processed. Interestingly, after the cells have split, some HADA signal is retained in both new cells at the poles, suggesting that crosslinked muropeptides containing HADA are integrated to the cell wall at the poles. Fluorescence at the poles was also conserved in the next generation. These results are similar to those obtained with *E. coli* by 25, confirming that the material incorporated into the poles is highly stable, and to a recent study from the Winkler lab where retention of HADA at cell poles was observed26.

Next, we investigated whether it was possible to increase polar HADA labeling. Deletion of both *ezrA* and *gpsB* has been reported to lead to polar retention of PBP1 and ongoing polar PG synthesis as reported by Van-FL staining27. Van-FL labeling of *ezrA gpsB* double mutant cells indeed resulted in fluorescent labeling of the cell poles, as expected (Figure 7) and was not observed with the single deletions as reported (not shown). However, with

Figure 6. Time-lapse of HADA labeled cells. Exponentially growing *B. subtilis* were labeled with HADA and applied to an agarose slide containing growth medium for time-lapse microscopy. The HADA signal (B) and growth of the cells (A, overlay of HADA fluorescence and phase contrast images) was imaged every 20 min for 100 min. Each arrow follows a HADA-labeled septum over time.

Figure 7. A *gpsB ezrA* double deletion mutant does not synthesize polar PG. Exponentially growing wild type cells (E 168) and *B. subtilis* *gpsB ezrA* double deletion strain 4144 were labeled for 5 min with Van-FL (A, B) or HADA (C, D) and immediately imaged. Using the ObjectJ plugin for ImageJ, the average fluorescence signal over the width of the cell was calculated for axial positions along the length of the cell. The data was then plotted against the corresponding cell position, as described in Figure 6. The relative fluorescence intensity distribution plots (G, H) show that the HADA label is retained at the poles (H 4144) and not at the poles (E 168) of wild type *B. subtilis*.
HADA no polar labeling was observed (Figure 7). We confirmed this observation by analyzing the fluorescence distribution along the length of the cell which showed that cells labeled with Van-FL have increased labeling at the poles which is completely absent from HADA-labeled cells, whereas increased labeling at division septa and at 1/4 and 3/4 positions along the length of the cells was observed with both labeling methods (Figure 7). As HADA labeling requires active PG synthesis this result strongly suggests that it takes cells longer to process pentapeptides present in polar PG in the absence of ezrA and gpsB, but also that the polar Van-FL labeling is not the result of ongoing synthesis at the poles. In addition, this result (together with those from the dacA mutant, Figure 2) shows that in the absence of antibiotics, it is possible to obtain different labeling patterns for Van-FL and HADA. Again, this indicates that Van-FL labels existing pentapeptides whereas HADA only reports on newly synthesized pentapeptides.

Pentapeptides at the septum are not involved in PBP2B localization

Cell division requires the activity of a specific Class B transpeptidase, PBP2B in \textit{B. subtilis} \cite{28}. Presence of this protein is essential for division and in its absence other cell division proteins, collectively known as the divisome, also lose their localization at the division site \cite{28}. PBP2x, the PBP2B homologue of \textit{Streptococcus pneumoniae}, is required for labeling of the constricting septa with FDAAs \cite{22}. In addition, the localization of PBP2x to midcell is dependent on its PASTA domains \cite{29}. PASTA domains are capable of binding uncross-linked muropeptides albeit with low (millimolar) affinity \cite{29–34}. We wanted to investigate whether the cell-division-specific activity of PBP2B, which contains two C-terminal PASTA domains, is responsible for generating the pentapeptide-enriched PG at the septum, and/or whether the PASTA domains of PBP2B are responsible for the localization of PBP2B to the septum.

GFP-PBP2B localization was not affected by bacitracin treatment, showing that a block in Lipid II synthesis does not affect the assembly of the divisome (Supporting Information Figure S6). We then generated a set of strains in which native PBP2B is produced in the presence of IPTG and GFP-PBP variants are produced in the presence of xylose (Figure 8A). This allowed us to simultaneously check the capacity of the GFP-PBP2B variants to complement the depletion of wild type PBP2B, as well as their localization. In addition to a truncation of the PASTA domains, we also created a transpeptidase-inactive mutant by changing the catalytic serine (Ser312) to alanine, and combined the catalytic site mutant with the PASTA truncation. As a control, we made a ‘supertruncate’ variant in which most of the PBP2B was removed.

As expected, depletion of PBP2B was lethal and expression of the GFP-supertruncate construct did not rescue this phenotype. To our surprise, all other GFP-PBP2B variants allowed cells to grow with growth rates similar to wild type GFP-PBP2B (Supporting Information Figure S7). To make sure that the complementation was not an artifact of the GFP-fusion, a second set of strains with the \textit{pbpB} alleles alone was created. Again, all constructs except the supertruncate complemented the depletion of \textit{pbpB} (Supporting Information Figure S7). The expression level of the GFP-PBP2B constructs was similar in all strains, as evidenced by the levels of in-gel GFP fluorescence. Mutation of the catalytic Ser312 was confirmed, since the active-site
mutants no longer bound Bocillin-FL, which requires the catalytic serine for covalent attachment (Supporting Information Figure S8). When we analyzed the mutant strains for localization of the GFP-PBP2B variants, we noticed that none of the strains showed strong cell-division defects, and that all the GFP-PBP2B variants still preferentially localized to the septum, meaning that neither the PASTA domains, nor the catalytic serine are required for PBP2B localization (Figure 8B). Labeling of the cells with HADA revealed strong septal labeling in all cases, showing that the transpeptidase activity of PBP2B is not required for the accumulation of pentapeptide-containing PG at the septum (Figure 8B).

**DISCUSSION**

Although PG is one large macromolecule, it has long been known that there are local differences in PG architecture and possibly chemistry. The architecture of PG needs to be different to allow for the different curvatures of the sacculus at poles and division sites. Early electron microscopy studies of the *B. subtilis* cell wall have already shown wall bands at the division site (Figure 1), and different PG growth modes at the poles (26). Atomic force microscopy revealed cable-like structures on the *B. subtilis* lateral wall and thick, defined septa (4). It has been well documented that next to regions of high PG turnover, there are regions of so-called inert PG (iPG) in E. coli, which play an important role in keeping the shape of the cells as improperly located iPG can be found at branching points in shape mutants (37, 38). Determining the underlying chemistry of local differences in PG synthesis, e.g., the level of crosslinking and processing of muropeptides at different sites in the sacculus, has so far been difficult as methods addressing these matters rely on bulk analysis of digested PG molecules by HPLC and LC/MS. The exciting development of FDAAs to label PG synthesis has made it possible to start addressing such local differences at the single-cell level.

Using the unique properties of HADA, namely its incorporation into the 5th position of the stem peptide in *B. subtilis* PG and rapid processing at the lateral wall by PBP5 (Figure 2, Supporting Information Figure S1), we show that there is a population of stable, pentapeptide-containing PG at the division site in *B. subtilis*. The labeling patterns observed with HADA are very similar to patterns observed with Van-FL after labeling unmodified PG, and thus these patterns are truly reflective of the presence of D-Ala-D-Ala moieties at the division septum. This also explains why Van-FL labeling still reveals the division site when cell-wall synthesis is blocked with various antibiotics as shown here and by Daniel and Errington (39), or when cell-wall synthesis is blocked by the addition of CCCP (39), which delocalizes various proteins involved in PG synthesis such as MreB and PBP1 (40, 41). Importantly, the presence of pentapeptide-containing septal PG was confirmed by the observation that Van-FL labeled sacculi isolated from untreated cells at the septa. This indicates that the presence of pentapeptides at the septum is not the result of an accumulation of pentapeptides at the septum caused by HADA or Van-FL labeling of live, growing cells. Also, HADA-label was retained upon isolation of sacculi and was released by lysozyme treatment, indicating that the observed HADA-label is incorporated into PG. Some of the unprocessed PG observed at the division site is retained at the cell poles and probably part of iPG in *B. subtilis*, as was recently also noted in time-lapse experiments with *B. subtilis* PY79 where the focus was on PG turnover at the lateral wall (42). However, there must be some processing of the septal material upon splitting of the cells as the label observed at the poles is never as strong as at the septum, and we also do not see consistent labeling of all poles with Van-FL. Our results suggest that PG at the poles is slowly converted into ‘true’ polar, inert PG, and that this processing is affected in a dacA strain (43). This implies that PG chemistry at the pole depends on the age of the pole. Accumulation of pentapeptides at the septum has previously been proposed for *S. pneumoniae*, as its main DD-carboxypeptidase was found to be absent from the septum (44), but to our knowledge, our report is the first to convincingly show the presence and persistence of pentapeptides at the septum. This does not mean that this septal material is not crosslinked, as the D-Ala-D-Ala moiety can remain attached to an acceptor stem peptide. This is also evidenced by the finding of the HADA label in the two major pentapeptide containing muropeptide peaks during HPLC analysis of a dacA mutant (Supporting Information Figure S1).

PG that is incorporated at the lateral wall is processed rapidly, primarily by the major DD-carboxypeptidase PBP5. Retention of D-Ala-D-Ala containing PG at the septum thus suggests that PBP5 is absent from, or not active at the division site. A GFP-fusion to PBP5 is localized both at the septum and at the lateral wall (45), indicating that the activity of PBP5 may somehow be blocked at the division site. In E. coli, PBP5 and FtsZ act together (46) and it seems to be the absence of PBP5 at midcell, and thus the presence of pentapeptides, that allows for a correct placement of the FtsZ-ring ensuring correct cell division and cell shape in the following generations (38). *B. subtilis* does not show dramatic shape defects in the absence of DD-carboxypeptidases, but this may be because the septal PG is thick, splits rather than invaginates, and because septum shape is coordinated by other proteins that are absent...
A candidate protein that could connect unprocessed PG and FtsZ is PBP2B, a Class B transpeptidase that is essential for formation of the septum\(^{28-46}\), as this protein contains two PASTA domains that have been implicated in binding of uncrosslinked muropeptides\(^{29-34}\). Recently, the localization of the essential \(S.\ pneumoniae\) PBP2x (homologue of PBP2B) was reported to be dependent on either its own C-terminal PASTA domains, or the presence of PASTA domains on its interacting partner StkP \(^{29,47}\). Removal of the PASTA domains from PBP2B did not abolish localization of PBP2B nor did it affect its function. To our surprise, replacement of the active-site serine also did not affect localization or function, and even a PBP2B construct lacking both the active site serine and the PASTA domains was functional in that it allowed cells to grow, localized to the division site and showed PG labeling similar to wild type cells. We cannot formally exclude the possibility that a minute amount of wild type PBP2B is produced in the depletion strain, which, in turn, only rescues this strain when other inactive forms of GFP-PBP2B are expressed. However, as growth rates and localization patterns of the catalytically inactive variants are indistinguishable from wild type cells, we deem this unlikely. Also, a construct in which most of the protein was deleted was not capable of complementing a PBP2B depletion strain. It seems that it is not the transpeptidase activity of PBP2B that is essential, but rather that the presence of the protein in the divisome is required and its catalytic activity can be compensated by another, non-essential transpeptidase. In this respect, it is important to note that depletion of any component of the \(B.\ subtilis\) divisome results in destabilization and delocalization of the other components\(^{38}\).

The discovery of the accumulation of pentapeptide-containing PG may seem paradoxical as it was recently reported that proteins containing SPOR domains, that bind to ‘denuded’ PG, which is devoid of stem-peptides, localize to the septal site of sacculi isolated from both \(B.\ subtilis\) and \(E.\ coli\)\(^{48}\). However, it is easy to envisage that ‘denuded’, e.g., extremely processed, PG, and unprocessed PG coexist at the septum — while at the same time noting that we could not observe unprocessed PG in \(E.\ coli\). In \(B.\ subtilis\), D-Ala-D-Ala containing pentapeptides comprise only a small portion of the total PG, and even when all this material is concentrated at the septum, this would mean that a large fraction of the muropeptides at the septum is processed, for example because the stem-peptide served as a donor in a crosslinking reaction.

It remains to be established what the specific function of the pentapeptides at the septum is and how the relative lack of stem-peptide processing at the septum is regulated. We are currently investigating this.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions**

Strains used in this study are listed in Table 1. All \(Bacillus\) strains were grown in casein hydrolysate (CH)-medium at 30°C\(^{30,51}\). Strain 3122 was induced by adding 0.5% (w/v) xylose and strain 4055 with 0.02 mM IPTG. Strain 4056 (\(dacA:\)kan) was constructed by transformation of \(B.\ subtilis\) 168 with chromosomal DNA of \(B.\ subtilis\) DK654 (kind gift from Dan Kearns, Indiana University Bloomington). Deletion of \(dacA\) (PBPS) was confirmed by PCR and labeling with Bocillin-FL (Life-Technologies).

**Construction of GFP-PBP2B mutants and strains**

The coding sequences for full length \(pbpB\) and \(pbpB1-1991\) were amplified from \(B.\ subtilis\) 168 chromosomal DNA using primers djs501 (5’-GTCGGATCCCTATGATTCAAATGCCAAAAAG) and djs502 (5’-GAACCTCGAGTGTTACACC) or djs503 (5’-GAACCTCGAGTTATTCCTCTCATGATCG) respectively (503 introduces a stop codon after position 1991) and subcloned into pGEM-T (Promega). After sequencing, the PCR fragments were cloned using BamHI/\(XhoI\) into pSG1729, generating plasmids pDMA001 (gfp-pbpB) and pDMA002 (gfp-pbpB\(\Delta\)PASTA). pDMA001 and pDMA002 were used as template plasmids in a Quickchange PCR (Stratagene) to introduce mutation \(pbpB7934G\) changing the catalytic Ser312 to Ala using primers djs504 (5’-GATGTAACCCGGGCACATGGAATG) and djs505 (5’-GATCTTATGGGCCCCGAGTTTCTAC) resulting in plasmids pDMA003 (gfp-pbpBSer312Ala) and pDMA004 (gfp-pbpBSer312Ala\(\Delta\)PASTA). All plasmids were sequence verified. Sequence verification of an initial PCR product generated using djs501/502 revealed the introduction of a G676A mutation resulting in a stop codon at position 222. This ‘\(pbpB\)-supertruncate’ construct was subcloned into pSG1729, generating pDMA005, to serve as a negative control in complementation assays. All plasmids were cloned into \(B.\ subtilis\) 3295 and integration into amyE locus was verified by growing the transformants on starch plates. To remove gfp from pDMA001,
Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype</th>
<th>Source/construction</th>
<th>Bacillus subtilis</th>
</tr>
</thead>
</table>
| 168              | pTG1279                         | Laboratory collection                      | pDMA002, pDMA003, pDMA004 and pDMA005, these plasmids were digested using KpnI and BamH I. Plasmid and gfp DNA fragments were separated on agarose gels, plasmid DNA was isolated and the overhangs were filled using DNA Blunt enzyme (Thermo Scientific) and the linear plasmids were religated. All plasmids were sequenced and cloned into B. subtilis 3295 as described above.

**Synthesis of HCC-amino-D-alanine (HADA)**

HADA synthesis was performed according to 24, with details provided in the Supporting Information.

**Inhibition of cell-wall synthesis and PG labeling**

Fosfomycin and bacitracin were used to inhibit cell-wall synthesis. Cells were grown until exponential phase and then incubated in CH medium containing fosfomycin (final concentration 500 μg/ml) or bacitracin (final concentration 500 μg/ml) for 20 min at 30 °C. To check if cell-wall synthesis was inhibited, cells were labeled with Van-FL or HADA and then imaged. Labeling with Van-FL was performed by incubating the cells with a mixture of 1:1 vancomycin (Sigma-Aldrich) and BODIPY® FL Vancomycin (Molecular Probes, Life Technologies) at a final concentration of 1 μg/ml, for 5 min at 30 °C. For HADA labeling, cells were incubated with HADA (Molecular Probes, Life Technologies) at a final concentration 0.5 mM) for 5 min at 30°C. Excess label was removed by three washes with Phosphate-Buffered Saline (PBS, 58 mM NaHPO4; 17 mM NaH2PO4; 68 mM NaCl, pH 7.3). A reverse experiment was performed, in which cells were grown until exponential phase and labeled with HADA (final concentration 0.5 mM) for 5 min at 30°C. Cells were then washed three times in PBS and incubated in CH medium (either with or without bacitracin at 500 μg/ml) for an additional 20 min.

**Sacculi isolation**

Sacculi were purified according to 19 with some modifications. B. subtilis 168 was grown until exponential phase, boiled for 5 min and collected by centrifugation. Cells were resuspended in sodium dodecyl sulfate (SDS, 5% w/v) and boiled for 25 min. The insoluble material was recovered by centrifugation and boiled in SDS (4% w/v) for 15 min. The insoluble material was washed five times in hot water to remove the SDS. Sacculi were digested with 100 μg/ml amylase (Bacillus licheniformis type X1A in 10 mM Tris-HCl
[pH 7.0], 10 mM NaCl, 0.32 M imidazol) for 2 h at 37°C and further resuspended in sodium phosphate buffer (50 mM, pH 7.3) containing 100 μg/mL α-chymotrypsin and incubated overnight at 37°C. Sacculi were washed with water and resuspended in hydrofluoric acid (48% vol/vol) for 24 h at 4°C. Finally, sacculi were washed with Tris-HCl (50 mM, pH7) and cold water until pH was 7.

For HPLC analysis, chymotrypsin treated sacculi were extensively washed with water, resuspended in buffer (50 mM MES, 1 mM MgCl₂, pH 6.0) to 16.6 mg/ml and 200 μl of the sacculi were digested overnight at 37°C with mutanolysin (50 μg/ml) under constantly shaking. Samples were centrifuged for 10 min at 17,000 g and 50 μl of supernatant was analyzed by HPLC on a C18 column (HyperClone™ 5 μm ODS (C18) 120 Å, LC column 250 x 4 mm, 00G-4361-D0 from Phenomenex). The following 290-min-gradient program at a flow rate of 0.5 ml/min was used. Five minutes of washing with 100% buffer A (40 mM sodium phosphate (pH 4.5)) was followed by a linear gradient over 270 min from 0% to 100% buffer B (40 mM sodium phosphate (pH 4) containing 20% (vol/vol) methanol). A 5-min delay and 10 min of re-equilibration with buffer A completed the method. Elution profiles were monitored by detecting UV absorbance at 202 nm and fluorescence with excitation/emission at 405/450 nm. Chromatograms were presented in the GraphPad Prism 6 program.

**Bacterial viability**

Bacterial viability after antibiotic treatment was determined by two different assays: the live/dead bacterial viability assay and 10-fold dilution series.

Live/dead assay (Life technology) discriminates between cells with or without a compromised membrane using two DNA dyes: the membrane permeable SYTO9 dyes and membrane impermeable propidium iodide. Exponentially growing bacteria were mixed with SYTO 9 dye (0.877 μM), propidium iodide (7.5 μM) and the corresponding antibiotic to be tested (final concentration: fosfomycin 500 μg/ml, bacitracin 500 μg/ml and D-cycloserine 500 μg/ml). Cells were incubated at 30 °C or 37 °C (see above). Viability of cells was monitored by microscopy at 20 min and 1 h after antibiotic treatment. Pictures were taken using FITC and TRITC filters.

For the 10-fold dilution series, bacteria were grown as described above and diluted to OD₆₀₀ 0.4. The corresponding antibiotic (final concentration: fosfomycin 500 μg/ml, bacitracin 500 μg/ml and D-cycloserine 500 μg/ml) was added to the bacteria and cells were further grown for 20 min and 1 h. After this time, the bacteria were rapidly diluted in growth medium in a ten-fold dilution series and 2 μl of each dilution was plated on LB agar. Plates were incubated at 37°C overnight.

**Fluorescence microscopy**

Cells or sacculi were spotted on agarose (1% w/v in PBS) pads and imaged using a Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a Hamamatsu Orca Flash4.0 camera. Image analysis was performed using the software packages ImageJ [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/), ObjectJ [https://sils.fiw.uniba.nl/bcb/objectj/index.html](https://sils.fiw.uniba.nl/bcb/objectj/index.html) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

**Time-lapse and 3D reconstruction**

Time-lapse microscopy was performed as described previously using a DV Elite, IX71 (Olympus, Japan) microscope with a sCMOS camera assembled by Applied Precision (GE Healthcare), with laser excitation at 405 nm (laser power 20%) and a CFP filter. Pictures were taken every 5 min.

Z-stacks were captured with steps of 0.08 μm on the same microscope setup. Deconvolution and 3D reconstruction was performed using Deltavision’s Softworx software (Applied Precision).

**ACKNOWLEDGEMENTS**

We would like to thank Michael Van Nieuwenzhe, Yves Brun and Erkin Kuru (Indiana University, Bloomington, USA) for sending us an initial stock of HADA and detailed protocols for synthesis. We would like to thank Avatar Joshi and Daniel Kearns (Indiana University, Bloomington, USA) for sending strain DK654, and Dennis Claessen (Leiden University, NL) for strains 4221 and 4143. We thank Oguz Bolgi and Alexander Schneider for technical assistance.

Work in the Scheffers lab is supported by a VIDI fellowship (864.09.010) from the Netherlands Organisation for Scientific Research. Work in the Veening lab is supported by the EMBO Young Investigator Program, a VIDI fellowship (864.12.001) from the Netherlands Organisation for Scientific Research, and ERC starting grant 337399-PneumoCell. Yun Liu was supported by a PhD fellowship from the Chinese Scholarship Council, and Anabela de Sousa Borges was supported by a doctoral grant (SFRH/BD/78061/2011) from POPH/FSE and FCT (Fundação para a Ciência e
Tecnologia) from Portugal. Work in the Hirsch lab is supported by funding from the Dutch Ministry of Education, Culture and Science (Gravitation program 024.001.035) and a VIDI grant (723.014.008) from the Netherlands Organisation for Scientific Research (NWO-CW).

**AUTHOR CONTRIBUTIONS**

The conception or design of the study: DMA, DJS.

The acquisition, analysis, or interpretation of the data: DMA, YL, AMH, MB, ASB, NdK, KB, JMV, CM, AKHH, DJS

Writing of the manuscript: DMA, ASB, JMV, CM, AKHH, DJS

**REFERENCES**


20. Strahl, H. & Homan, L. W. Membrane potential is important for bacterial cell division. PNAS 107, 12281–12286 (2010).


SUPPLEMENTAL METHODS

HADA synthesis

General

Starting materials and reagents were purchased from Sigma-Aldrich or Acros. Yields refer to analytically pure compounds and have not been optimized. All solvents were reagent-grade. $^1$H and $^{13}$C spectra were recorded at 400 MHz on a Varian AMX400 spectrometer (400 MHz for $^1$H, 101 MHz for $^{13}$C) at 25°C. Chemical shifts ($\delta$) are reported relative to the residual solvent peak. Splitting patterns are indicated as (s) singlet, (d) doublet, (t) triplet, (q) quarted, (m) multiplet, (br) broad.

Synthesis of 7-Hydroxycoumarin-3-carboxylic acid

Preparation of 7-Hydroxycoumarin-3-carboxylic acid was based on a literature procedure [1, 2]. A mixture of 2,4-dihydroxy benzaldehyde (1 g, 10 mmol), Meldrum’s acid (1.445 g, 10 mmol), piperidinium acetate (29 mg, 0.2 mmol) and ethanol (25 mL) was stirred at room temperature for 20 mins, then refluxed for 2 hrs. It was cooled to room temperature and chilled in an ice bath for 1 hr. The crystallized product was filtered, washed with ethanol, and dried in vacuo to get the product (1.45 g, 71%) as an off-white powder. $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.68 (s, 1H), 7.75 (d, J = 8.6 Hz, 1H), 6.84 (d, J = 8.6, 1H), 6.74 (s, 1H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 164.2, 163.9, 157.5, 157.0, 149.4, 132.0, 114.0, 112.5, 110.6, 101.8.

Synthesis of HADA

Preparation of HADA was based on a literature procedure [3]. A mixture of 7-Hydroxycoumarin-3-carboxylic acid (100 mg, 0.485 mmol), carbonyldiimidazole (78.7 mg, 0.485 mmol) and anhydrous DMF (5 mL) was stirred at room temperature for 2 h under an atmosphere of nitrogen. Boc-D-2,3-diaminopropionic acid (99 mg, 0.485 mmol) was added and the mixture was stirred at room temperature for 20 h under an atmosphere of nitrogen. The solvent was removed, the solid was diluted with EtOAc (30 mL) and washed with 1 N HCl (20 mL) and water (20 mL). The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated to dry. The crude product was treated with trifluoroacetic acid/DCM (50:50, 3.5 mL) for 30 mins at room temperature. Then the solvent was removed in vacuo. 47 mg of crude HADA was purified by reversed-phase HPLC (HPLC conditions: column, XTerra® Prep MS C18 10 μm, 7.8 mm x 150 mm; flow rate, 1 mL min⁻¹; wavelength, 254 nm; temperature, 23 °C; isocratic method, water/acetonitrile 30:70 (0.1% TFA) for 30 min). After lyophilization of the solvent, it was dissolved in 1 N HCl/ACN, and lyophilized to get the desired product (22 mg) as a pale yellow solid. $^1$H NMR (400 MHz, dms) $\delta$ 8.93 (t, J = 5.8 Hz, 1H), 8.81 (s, 1H), 7.83 (d, J = 9.6 Hz), 6.94 – 6.86 (dd, J = 2 Hz, 5.3 Hz, 1H), 6.84 (s, 1H), 4.39 (d, J = 5.7 Hz, 1H), 3.89 – 3.78 (m, 1H), 3.74 – 3.63 (m, 1H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 169.2, 164.5, 162.9, 161.2, 156.7, 148.7, 132.5, 115.0, 113.5, 111.2, 102.3, 56.3.

Table S1. Viability of cells upon antibiotic treatments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic</th>
<th>%Live 20 mins</th>
<th>%Dead 20 mins</th>
<th>Total of cells 20 mins</th>
<th>%Live 1 hr</th>
<th>%Dead 1 hr</th>
<th>Total of cells 1 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis 168</td>
<td>control</td>
<td>90.4</td>
<td>9.6</td>
<td>101</td>
<td>83.9</td>
<td>18.1</td>
<td>359</td>
</tr>
<tr>
<td></td>
<td>bacitracin</td>
<td>93.8</td>
<td>6.2</td>
<td>371</td>
<td>67.7</td>
<td>32.3</td>
<td>547</td>
</tr>
<tr>
<td></td>
<td>fosfomycin</td>
<td>87.4</td>
<td>12.5</td>
<td>302</td>
<td>90.7</td>
<td>9.3</td>
<td>485</td>
</tr>
<tr>
<td></td>
<td>D-cycloserine</td>
<td>92.9</td>
<td>7.1</td>
<td>462</td>
<td>81</td>
<td>19</td>
<td>531</td>
</tr>
<tr>
<td>PY79</td>
<td>control</td>
<td>91.9</td>
<td>8.1</td>
<td>335</td>
<td>90.6</td>
<td>9.4</td>
<td>339</td>
</tr>
<tr>
<td></td>
<td>bacitracin</td>
<td>94</td>
<td>6</td>
<td>272</td>
<td>64.3</td>
<td>35.7</td>
<td>563</td>
</tr>
<tr>
<td>Escherichia coli  MC1061</td>
<td>control</td>
<td>99.9</td>
<td>0.1</td>
<td>864</td>
<td>99.6</td>
<td>0.4</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>fosfomycin</td>
<td>95.8</td>
<td>4.1</td>
<td>453</td>
<td>98</td>
<td>2</td>
<td>444</td>
</tr>
<tr>
<td>MG1655</td>
<td>control</td>
<td>99</td>
<td>0.9</td>
<td>330</td>
<td>98.2</td>
<td>1.8</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>fosfomycin</td>
<td>99.2</td>
<td>0.7</td>
<td>378</td>
<td>99.8</td>
<td>0.2</td>
<td>531</td>
</tr>
</tbody>
</table>
Figure S1. HPLC detection of muropeptides from *B. subtilis* wild type (A, C) or ΔdacA (B, D). Samples were monitored using a UV detector (202 nm) and a fluorescence detector with excitation/emission set at 405/450 nm respectively. PG was either non-labeled (black traces) or HADA labeled (orange traces). The UV traces of PG from labeled and non-labeled cells did not vary significantly. However, the UV trace of ΔdacA vs wild type was different, as expected, and revealed two new peaks representative of disaccharide pentapeptide and tetrasaccharide tetrapeptide-pentapeptide muropeptides (arrows), which are identified and in agreement with the previous analysis of Atrih et al. [4]. The fluorescence traces showed various background fluorescence peaks which were identical between labeled and unlabeled PG. However, the two pentapeptide peaks indicated showed a marked increase in fluorescence, indicative of the presence of HADA-labeled material. This is fully in agreement with the analysis of HADA-labeled ΔdacA PG described by Kuru et al. [3].

Figure S2. Plating assays of antibiotic treatments/killing. Exponential growing cells were incubated in growth medium (CH) with antibiotics (500 μg/ml) for 20 mins or 60 mins at 37°C before making 10-fold dilution series in CH and spotting 2μl of each dilution on LB agar plates.
Figure S3. Quantification of HADA and Van-FL fluorescence signals at septa. Signal intensities at septa were determined using ImageJ for 100 cells for each condition and the distribution of intensities was plotted as box plots. Cells from the experiment depicted in Figure 1 were used for these calculations.

Figure S4. HADA labeling of a PBP4 deletion strain, with the fluorescence at septa compared to wild type. Signal intensities were determined as described for Figure S3.
CHAPTER 3: Pentapeptide-rich peptidoglycan at the Bacillus subtilis cell-division site

Figure S5. HADA labeling of B. subtilis PY19, and E. coli strains MG1655 and MC1061. Exponentially growing B. subtilis PY19 (A-D) were labeled with HADA after growth for 20 mins in the absence (A) or presence of bacitracin (500 μg/ml, B) to block Lipid II synthesis. In a reverse experiment, cells were labeled with HADA and allowed to continue growth for 20 mins in the absence (D) or presence of bacitracin (C). A similar experiment was performed with E. coli strains MG1655 (E-H) and MC1061 (I-L), where fosfomycin (500 μg/ml) was used to block Lipid II synthesis instead of bacitracin. Arrows indicate labeled septa. Scale bar (same for all): 5 μm.

Figure S6. Localization of FtsZ-eYFP (strain 4055, A, C) and GFP-PBP2B (strain 3122 B, D) is not affected by incubation with bacitracin for 20 mins.

Figure S7. Growth of pbpB depletion strain 3295. In all experiments, strains were grown in LB in the presence of 0.05 mM IPTG to exponential phase, cells were washed and resuspended to OD600 0.05 in LB with either no additions or 0.05 mM IPTG or 0.2% xylose. Cells were grown at 30°C in 96-well plates for 400 mins with constant shaking and monitoring of OD600. A) PBP2B depletion blocks growth. B) The gfp-pbpB constructs do not support growth in the absence of xylose. C) Expression of gfp-pbpB constructs with 0.2% xylose restores growth to all strains except the strain that expresses gfp-pbpB truncated at amino acid 221. D, E identical to B, C but with pbpB alleles lacking gfp.
Figure S8. Bocillin and GFP in-gel fluorescence of strains expressing GFP-PBP2B constructs. Strain 3295 (lane 5, grown with IPTG to induce pbpB) was transformed with the different GFP-PBP2B constructs which were expressed by the addition of xylose (0.2%) to the growth medium with simultaneous depletion of wild type PBP2B (lanes 1-4). Cells were equalized for OD, labeled with Bocillin FL650-660 (Life Technologies) at 7.5 μM for 15 mins at 23°C with shaking. Subsequently cells were harvested, lysed by sonication and cell-free extracts were prepared in SDS-PAGE buffer. Samples were analyzed on 10% SDS-PAGE. Fluorescent bands were visualized using a Typhoon Trio (GE Healthcare) scanner. A) Bocillin 650-660 fluorescence shows extra bands (arrows) in cells expressing GFP-PBP2b and GFP-PBP2B truncated, but not in cells expressing the catalytic Serine mutants. B) In gel GFP-fluorescence confirms the presence and equal amounts of all GFP-PBP2B constructs. C) Overlay image shows that the Bocillin bands indicated with arrows in A) overlap with the GFP-PBP2B constructs in B).

SUPPLEMENTAL REFERENCES:


