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Several distinct localization patterns for penicillin-binding proteins in *Bacillus subtilis*

Dirk-Jan Scheffers, Laura J. F. Jones and Jeffery Errington*

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Summary

Bacterial cell shape is determined by a rigid external cell wall. In most non-coccoid bacteria, this shape is also determined by an internal cytoskeleton formed by the actin homologues MreB and/or Mbl. To gain further insights into the topological control of cell wall synthesis in bacteria, we have constructed green fluorescent protein (GFP) fusions to all 11 penicillin-binding proteins (PBPs) expressed during vegetative growth of *Bacillus subtilis*. The localization of these fusions was studied in a wild-type background as well as in strains deficient in FtsZ, MreB or Mbl. PBPs function in both vegetative growth and transverse insertion of septal cell wall material during septation. The recent discovery of an actin-like cytoskeleton in *B. subtilis* (Jones et al., 2001) has altered this view. Two proteins, MreB and Mbl, form filamentous, helical structures just below the cytoplasmic membrane, which are made up of polymers that show a striking resemblance to actin polymers (van den Ent et al., 2001). These structures determine the shape of the cell, and the occurrence of at least one *mreB*-like gene is closely linked to bacteria that have a complex (i.e. non-coccoid) shape (Jones et al., 2001). Recent work from our laboratory has shown that Mbl governs the insertion of new cell wall material in a helical pattern in *B. subtilis* (Daniel and Errington, 2003). However, how the connection between the internal actin-like cytoskeleton and the exoskeleton formed by the cell wall is made remains elusive.

The cell wall of *B. subtilis* consists of peptidoglycan (PG), glycan strands with peptide side-chains that are highly cross-linked, and covalently linked anionic polymers (Foster and Popham, 2001). The polymerization and cross-linking of peptidoglycan is mediated by penicillin-binding proteins (PBPs). The PBPs can be subdivided into three classes (see Table 1): class A high-molecular-weight (high-MW) PBPs with both a transglycosylase and a transpeptidase domain; class B high-MW PBPs, with an N-terminal domain of unknown function and a transpeptidase domain; and a class of low-molecular-weight (low-MW) PBPs with D,D-carboxypeptidase or endopeptidase activity (Goffin and Ghuysen, 1998; Foster and Popham, 2001). *B. subtilis* PBPs function in both vegetative growth and the formation of the primordial cell wall and cortex of endospores. Strikingly, both *B. subtilis* and *Escherichia coli* contain large numbers of genes coding for PBPs, 16 and 12, respectively, of which products have been detected biochemically for 11 (*B. subtilis*) and 12 (*E. coli*) (Foster and Popham, 2001). Studies in *B. subtilis* (Popham and Setlow, 1996; Popham et al., 1999; McPherson et al., 2001; McPherson and Popham, 2003), *E. coli* (Denome et al., 1999) and *Streptococcus pneumoniae* (Hoskins et al., 1999; Paik et al., 1999) of single and multiple inactivations of *pbp* genes suggest a redundancy in function for both high-MW and low-MW class PBPs.

Previous studies on PG synthesis in *E. coli* and *B. subtilis* revealed the dispersed insertion of cell wall material along the cylindrical part of the cell during vegetative cell wall synthesis. Strikingly, both *B. subtilis* and *Escherichia coli* contain large numbers of genes coding for PBPs, 16 and 12, respectively, of which products have been detected biochemically for 11 (*B. subtilis*) and 12 (*E. coli*) (Foster and Popham, 2001). Studies in *B. subtilis* (Popham and Setlow, 1996; Popham et al., 1999; McPherson et al., 2001; McPherson and Popham, 2003), *E. coli* (Denome et al., 1999) and *Streptococcus pneumoniae* (Hoskins et al., 1999; Paik et al., 1999) of single and multiple inactivations of *pbp* genes suggest a redundancy in function for both high-MW and low-MW class PBPs.

Several distinct localization patterns for penicillin-binding proteins in *Bacillus subtilis* during elongation, and transverse insertion of septal cell wall material during septation. The recent discovery of an actin-like cytoskeleton in *B. subtilis* (Jones et al., 2001) has altered this view. Two proteins, MreB and Mbl, form filamentous, helical structures just below the cytoplasmic membrane, which are made up of polymers that show a striking resemblance to actin polymers (van den Ent et al., 2001). These structures determine the shape of the cell, and the occurrence of at least one *mreB*-like gene is closely linked to bacteria that have a complex (i.e. non-coccoid) shape (Jones et al., 2001). Recent work from our laboratory has shown that Mbl governs the insertion of new cell wall material in a helical pattern in *B. subtilis* (Daniel and Errington, 2003). However, how the connection between the internal actin-like cytoskeleton and the exoskeleton formed by the cell wall is made remains elusive.

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growth (Burman et al., 1983; Mobley et al., 1984; Schlaeppi et al., 1985; Woldringh et al., 1987; de Pedro et al., 1997). Biochemical evidence from B. subtilis, E. coli and Haemophilus influenzae points to cell wall synthesis being mediated by a ‘holoenzyme’ composed of murein synthases and hydrolases that track along the peptidoglycan to insert new PG strands into the cell wall (Bhardwaj and Day, 1997; Höltje, 1998; Alaedini and Day, 1999; Simon and Day, 2000). Several PBPs would form part of such a holoenzyme, providing transglycosylase, transpeptidase, carboxypeptidase and endopeptidase activities, together with hydrolases that remove old glycan strands from the cell wall. Given the difference in cell wall synthesis between elongation and division, specific holoenzyme complexes for both processes have been proposed (Höltje, 1998). One difference between the holoenzyme involved in cell wall elongation or invagination during division would be the presence of a cell division-specific transpeptidase. Two multienzyme complexes with different PBP composition have indeed been found in H. influenzae, and one complex contained PBP2, implicated in elongation, whereas the other contained PBP3, implicated in division (Alaedini and Day, 1999). In both B. subtilis (PBP2b; Daniel et al., 2000) and E. coli (PBP3; Weiss et al., 1997), the cell division-specific transpeptidase is localized at the septum. As E. coli only contains one other class B high-MW transpeptidase, PBP2, this was proposed to be a unique component of the elongation complex (Höltje, 1998). Recently, E. coli PBP2 was shown to localize in a spot-like pattern in the lateral cell wall as well as to the septum in dividing cells (Den Blaauwen et al., 2003), showing that this PBP does not localize exclusively to the lateral wall. B. subtilis PBP1 localizes to the cell division septum as well (Pedersen et al., 1999). These are the only PBPs for which localization has been studied. In B. subtilis, PBPs that localize to the lateral cell wall have not yet been found. Two candidates for lateral wall localization in B. subtilis are PBP2a and PbpH (Murray et al., 1998; Wei et al., 2003). Single knock-outs of these genes are indistinguishable from the wild type, but a double mutant is non-viable, and cells of a pbpA mutant expressing pbpH from an inducible promoter became round before lysing, after depletion of the inducer (Wei et al., 2003). Also, germinating, ovoid-shaped, spores lacking PBP2a require more time to attain the cylindrical shape characteristic of vegetative cells (Murray et al., 1998).

We wanted to study the localization of PBPs in B. subtilis to determine where peptidoglycan synthesis occurs and to search for a link between the sites of peptidoglycan synthesis and active cell shape determination by the MreB/Mbl cytoskeleton. N-terminal green fluorescent protein (GFP) fusions to all the vegetatively expressed PBPs of B. subtilis were constructed, and their localization was determined by fluorescence microscopy. The data show that PBPs localize in several distinct patterns. Some PBPs localize specifically to the lateral part of the cell, whereas others are localized specifically to the septum. Other PBPs show localization to both the septum and the lateral

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Expression</th>
<th>Localization during vegetative growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A PBPs (bifunctional transglycosylase/transpeptidase)</td>
<td>ponA</td>
<td>PBP2a/b</td>
<td>veg&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pbpF</td>
<td>PBP2c</td>
<td>veg, late stages of spor&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pbpD</td>
<td>PBP4</td>
<td>veg&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pbgG</td>
<td>PBP2d</td>
<td>spor&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Class B PBPs (transpeptidase)</td>
<td>pbgA</td>
<td>PBP2a</td>
<td>veg&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pbgB</td>
<td>PBP2b</td>
<td>veg, spor&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pbgC</td>
<td>PBP3</td>
<td>veg, low expression during spor&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>spoVD</td>
<td>SpoVD</td>
<td>spor&lt;sup&gt;10&lt;/sup&gt;</td>
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<td></td>
<td>pbpH</td>
<td>PbpH</td>
<td>veg&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>yrrR</td>
<td>YrrR</td>
<td>ND</td>
</tr>
<tr>
<td>Low-MW PBPs (carboxypeptidase)</td>
<td>dacA</td>
<td>PBP5</td>
<td>Septal, distinct spots at periphery</td>
</tr>
<tr>
<td></td>
<td>dacB</td>
<td>PBP5*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>dacC*</td>
<td>PBP4a</td>
<td>Late stationary phase&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>dacF</td>
<td>DacF</td>
<td>Distinct foci and bands at cell periphery</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-MW PBPs (endopeptidase)</td>
<td>pbpE</td>
<td>PBP4*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pbpX</td>
<td>PbpX</td>
<td>Septal, low fluorescence at periphery</td>
</tr>
</tbody>
</table>

<sup>a</sup> veg, vegetative growth; spor, sporulation.
<sup>b</sup> In addition to the full-length GFP fusion protein a cleaved GFP product was present.
<sup>c</sup> ND, not done.

<sup>1</sup>(Popham and Setlow, 1995); <sup>2</sup>(Pedersen et al., 1999); <sup>3</sup>(Popham and Setlow, 1993); <sup>4</sup>(Popham and Setlow, 1994); <sup>5</sup>(McPherson et al., 2001; Pedersen et al., 2000); <sup>6</sup>(Murray et al., 1997); <sup>7</sup>(Yanouri et al., 1993); <sup>8</sup>(Daniel et al., 2000); <sup>9</sup>(Murray et al., 1996); <sup>10</sup>(Daniel et al., 1994); <sup>11</sup>(Wei et al., 2003); <sup>12</sup>(Buchanan and Ling, 1992); <sup>13</sup>(Pedersen et al., 1998); <sup>14</sup>(Wu et al., 1992).
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wall, sometimes with an irregular distribution of fluorescence. The patterns of GFP–PBP localization are not markedly altered in the absence of either MreB or Mbl. The results show that PBPs localize in an organized rather than a dispersed fashion and implicate PBP3, PBP5, PBP4a, and possibly PBP4, in lateral cell wall synthesis.

Results

All class A high-MW PBPs localize at least partially to the septum

Bacillus subtilis contains four genes, ponA, pbpF, pbpD and pbpG, encoding five class A high-MW PBPs (Table 1). PBP1a and 1b are both products of ponA and probably arise through C-terminal processing (Popham and Setlow, 1995). Strikingly, a strain in which all these genes have been knocked out is still viable and contains PG, indicating that B. subtilis must contain at least one other protein that can perform the transglycosylation reaction (Mcperson and Popham, 2003). Of the four genes for class A PBPs, pbpG (previously named ywhE) is expressed only during sporulation (Pedersen et al., 2000; Foster and Popham, 2001) and was therefore omitted from our analysis. Strains expressing N-terminal GFP fusions to PBP1 (the fusion does not discern PBP1a and PBP1b), PBP2c and PBP4 were constructed. In each case, the plasmid insertion replaces the endogenous gene with the gfp fusion gene, so that only the GFP fusion protein should be synthesized. The presence of GFP fusion proteins of the expected size was confirmed by Western blotting (Fig. 1A). For GFP fusions to PBP2c and PBP4, next to the full-length GFP fusion protein, a cross-reacting species of similar intensity (not shown) at a lower MW was detected (Fig. 1A). This indicates that some of the fusion protein was subject to proteolysis, which might influence the localization pattern (see below). Although activity tests were not possible for most of the PBPs because the null mutants have no detectable phenotype, this was possible for PBP1 because a ponA mutant has been reported to grow more slowly than the wild type, with an increase in mean cell length (Popham and Setlow, 1996). A comparison of the doubling times during exponential growth on two different media for wild-type cells (Td: 20 min on 2TY; 39 min on S+) with the PBP1 mutant (Td: 29 min on 2TY; 57 min on S+) and the strain expressing GFP–PBP1 (Td: 22 min on 2TY; 37 min on S+) showed that the strain expressing GFP–PBP1 does not have a growth defect. The length distribution of cells expressing GFP–PBP1

Fig. 1. Detection of GFP fusion protein synthesis and substrate binding.

A. Detection of GFP using a specific anti-GFP antibody. Cells were expressing: no GFP–PBP (wild-type cells, lane 1), GFP–PBP1 (lane 2, MW 126.3 kDa), GFP–PBP2c (lane 3, MW 105.9 kDa), GFP–PBP4 (lane 4, MW 97.3 kDa), GFP–PBP2a (lane 5, MW 106.9 kDa), GFP–PBP2b (lane 6, MW 106.2 kDa), GFP–PBP3 (lane 7, MW 101.1 kDa), GFP–PbpH (lane 8, MW 105.9 kDa), GFP–YffR (lane 9, MW 92.2 kDa), GFP–PBP5 (lane 10, 75.4 kDa), GFP–PBP4a (lane 11, MW 79.6 kDa) and GFP–PbpX (lane 12, MW 70.6 kDa). Top, Western blot from a 7.5% SDS-PAGE gel; bottom, Western blot from a 10% SDS-PAGE gel. Note that there are non-specific cross-reaction species at ≈70 kDa and ≈30 kDa.

B. Detection of PBP2b in cells expressing GFP–PBP2b (lane 1, MW 106.2 kDa) and in wild-type cells (lane 2, MW 79.3 kDa).

C. Detection of penicillin-binding proteins using the fluorescent penicillin analogue Bocillin FL. Top, control; Western blot using a specific anti-GFP antibody; bottom: Bocillin FL-labelled samples. Membranes were prepared and labelled as described in Experimental procedures from cells expressing: no GFP–PBP (wild-type cells, lane 1), GFP–PBP5 (lane 2), GFP–PBP4a (lane 3), GFP–PbpX (lane 4) and GFP–PBP1 (lane 6). Lane 5, MW markers, 175 kDa, 83 kDa, 62 kDa, 48 kDa and 33 kDa respectively. Asterisks indicate unique bands in the Bocillin FL-labelled gel that correspond to GFP fusion proteins.

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was similar to wild type (not shown). Thus, GFP–PBP1, at least, appears to be functional.

PBP1 localized predominantly to the septum (Fig. 2A), as reported previously based on immunofluorescence microscopy (Pedersen et al., 1999). Sometimes GFP–PBP1 was observed to be retained at the new poles of two recently separated cells (Fig. 2B and C, arrowheads). Cells that did not contain a septum showed a faint, uneven peripheral staining (Fig. 2B, arrows). All other PBPs expressed during vegetative growth also showed enriched localization at the septum. GFP–PBP2c fluorescence was relatively weak (Fig. 2D), but extended exposure clearly showed septal fluorescence, as well as unequal fluorescence at the edges of the cell (Fig. 2E). A similar but more evident pattern was observed with PBP4, which localized to the septum in dividing cells (Fig. 2F), but also showed a characteristic distribution of fluorescent foci at the edges of the cell (Fig. 2G). Unfortunately, cleavage of GFP–PBP2c and GFP–PBP4 (Fig. 1A, lanes 3 and 4) makes it difficult to interpret this pattern. Nevertheless, we can conclude that none of the class A PBPs expressed during vegetative growth localizes exclusively to the lateral cell wall: all show at least some localization at the division septum. Away from the septum, especially in non-dividing cells, localization of these PBPs tends to be concentrated in spots that are not evenly dispersed around the membrane.

Most class B high-MW PBPs are dispersed around the membrane

The *B. subtilis* genome encodes six genes for class B PBPs, transpeptidases that form the peptide bonds that link glycan strands (together with the transpeptidase activity of class A high-MW PBPs; Foster and Popham, 2001). Three of the class B PBPs have been partially characterized. The first, PBP2b, is the only known essential PBP in *B. subtilis*. It plays a specific role in the synthesis of septal cell wall material and accordingly localizes to the septum as judged by immunofluorescence microscopy (Daniel et al., 2000). PBP2a and PbpH have been implicated in cylindrical cell wall synthesis (see Introduction; Murray et al., 1998; Wei et al., 2003). GFP fusions to PBP2a, PBP2b, PBP3, PbpH and YrrR were made and checked for expression of the GFP fusion protein (Fig. 1A). SpoVD was omitted from our analysis as it is well known to be specific for spore cortex formation (Daniel et al., 1994).

As PBP2b is an essential PBP in *B. subtilis* (Daniel et al., 2000), transformation of *B. subtilis* 168 with pSG5061 required the addition of xylose to the growth medium to induce the expression of GFP–PBP2b. Western blotting showed that the GFP–PBP2b fusion protein was the only copy of PBP2b being produced (Fig. 1B) and, therefore, that the GFP–PBP2b fusion is functional. All other GFP–PBP-expressing strains could be obtained through transformation of *B. subtilis* 168 with the respective plasmids in the absence of xylose. GFP–PBP2b localized exclusively to the septum (Fig. 3B), as described previously (Daniel et al., 1998; 2000).

To test the functionality of GFP–PBP2a and GFP–PbpH, we made use of the observation that a *pbpA/pbpH* double knock-out is not viable (Wei et al., 2003). *Gfp–pbpA* was transformed into a *pbpH* knock-out strain, and *gfp–pbpH* into a *pbpA* knock-out strain. Both crosses gave viable transformants provided that xylose was added to

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**Fig. 2. Localization of class A high-MW PBPs.** Fluorescence micrographs of cells expressing GFP–PBP1 (A and B), GFP–PBP2c (D and E) and GFP–PBP4 (F and G) are shown, with a phase-contrast image of the cells shown in (B) for comparison (C). Illumination for fluorescence was 1 s (A and B), 2 s (D, F and G) or 5 s (E). Bar (same for all) 5 µm.
Localization of low-molecular-weight PBPs

The low-MW PBPs of *B. subtilis* include \(\text{d,d}\)-carboxypeptidases, which cleave non-cross-linked pentapeptide side-chains to tripeptides during cell wall maturation, and \(\text{d,d}\)-endopeptidases, which cleave peptide cross-links in peptidoglycan (Foster and Popham, 2001). The \(\text{d,d}\)-carboxypeptidases PBPs and \(\text{d,d}\)-endopeptidase PBPs are implicated in spore peptidoglycan synthesis (Foster and Popham, 2001) and were not considered in this study. PBP5 is required for normal cell morphology during stationary phase growth and sporulation (Todd *et al*., 1986) and has been demonstrated to be instrumental in cleavage of non-cross-linked pentapeptides in the vegetative cell wall (Atrih *et al*., 1999), whereas the \(\text{d,d}\)-carboxypeptidase activity of PBP4a and the \(\text{d,d}\)-endopeptidase activity of PbpX have only been inferred from sequence analysis (Foster and Popham, 2001). GFP fusions to PBPs and PbpX were made and checked for expression of the GFP fusion protein (Fig. 1A). As it was not possible to test the functionality of GFP fusions to low-MW PBPs in an *in vivo* screen, we tested whether the fusion proteins were capable of binding the fluorescent penicillin analogue Bocillin FL (Zhao *et al*., 1999). Binding of substrate implies that the proteins are properly folded and correctly inserted into the membrane.

As can be seen in Fig. 1C, unique bands appeared in a Bocillin FL-labelled gel that correspond to GFP fusions to PBPs and PbpX, with wild-type cells and cells expressing GFP–PBP1 as controls. GFP–PBP5 was not resolved in a 12% SDS-PAGE gel, but we chose this type of gel because it gave by far the best results for the rather faint Bocillin FL labelling. Therefore, GFP fusions to PBPs and PbpX, at least, result in proteins that are capable of substrate binding and are therefore likely to be functional.

GFP–PbpX localized predominantly at the septum, but with an even fluorescence around the rest of the cell.
periphery (Fig. 4C). In comparison with a membrane dye, the GFP fluorescence signal at the septum was markedly enhanced with respect to that of the lateral wall (not shown). GFP–PBP5 localized to the septum and in concentrated spots along the lateral wall (Fig. 4A), similar to GFP–PBP4 (compare with Fig. 2F and G). GFP–PBP4a localized in fluorescent foci present around the cell periphery and was not enriched at the site of the septum (Fig. 4B), similar to the localization of GFP–PBP3 (Fig. 3C).

Septal PBP localization depends on cell division

An unexpectedly large proportion of the PBPs (nine out of 11) localized to both the lateral wall and the septum. To establish whether this localization pattern was dependent on the formation of a division site, localization of all of the GFP–PBP fusions was examined in an FtsZ depletion strain. Depletion of FtsZ blocks cell division and leads to the formation of aseptate filaments (Beall and Lutkenhaus, 1991). As can be seen in Fig. 5 for GFP–PBP1, GFP–PBP2b and GFP–PBP5, the transverse bands at the septum were dependent on cell division because, in the absence of IPTG, the fluorescent signals were distributed along the membrane, and transverse bands were almost absent (note the uneven distribution of signal for GFP–PBP5; Fig. 5G). This was also observed for GFP fusions to PBP1, PBP2a, PBP2c, PbpH, YrrR and PbpX (not shown). As a control, the GFP–PBP2b strain was depleted for GFP–PBP2b by removing xylose from the growth medium (but retaining IPTG to allow continued FtsZ synthesis; Fig. 5E). This showed that depletion of GFP–PBP2b leads to filamentation, as described before (Daniel et al., 2000). Interestingly, residual GFP–PBP2b was evident as spots at about mid-cell that represent incomplete cell division sites, providing support for the proposal that, in PBP2b-depleted cells, a limiting amount of PBP2b is sufficient for the initiation of septation but does not support progression of septal ingrowth (Daniel et al., 2000). The GFP fusions to PBP3 and PBP4a, which did not localize to the septum, still displayed a spotty pattern along the membrane (not shown) in FtsZ-depleted filaments. We conclude that the localization of PBPs at the septum is dependent on the formation of an FtsZ ring.

Three-dimensional reconstruction of PBP organization

The fluorescence patterns observed with GFP–PBP3 (Fig. 3C) and GFP–PBP4a (Fig. 4B) were slightly reminiscent of the patterns observed with Mbl and MreB, actin-like bacterial cytoskeletal proteins that are involved in cell shape determination (Jones et al., 2001). This suggested that these PBPs could be recruited to an internal cytoskeleton formed by MreB/Mbl during cell wall synthesis. We determined the three-dimensional organization of PBP4a and PBP3 by looking at the GFP pattern of these proteins in stacks of optical sections. Out of focus light was reduced by a deconvolution algorithm. Optical sections through cells expressing GFP–PBP4a (Fig. 6A) and GFP–PBP3 (Fig. 6B) revealed fluorescent bands that might track laterally along the membrane, but complete helices were generally not detectable.

It was possible that the absence of a complete helix resulted from lack of continuity of the PBPs on the continuous MreB or Mbl ‘tracks’. If so, combination of the PBP3 and PBP4a patterns might show a clearer pattern. A strain was constructed in which both GFP fusion proteins could be co-expressed (strain 3132). The localization of GFP–PBP4a, expressed as an extra copy at the amyE locus (strain 3102), was indistinguishable from that of the Campbell insertion at the dacC locus (strain 3105; not shown). Fluorescence micrographs of cells
expressing both GFP–PBP4a and GFP–PBP3 displayed a pattern similar to that of each of the single GFP–PBP fusions (Fig. 6C). However, in three-dimensional reconstructions of these patterns, some spots resolved into arcs that traversed half to three-quarters of the circumference of the cell (Fig. 6D; see Supplementary material). The arcs were usually tilted relative to the long axis of the cell, reminiscent of the helical cables of MreB and Mbl. The limited amount of GFP–PBP fusion proteins expressed might preclude them from extending to full turns of the cell circumference. Taken together, it seemed possible that the subcellular distribution of PBP3 and PBP4a is directed in part by the MreB/Mbl cytoskeleton.

**PBP localization in the absence of MreB or Mbl**

To test directly whether the localization of PBP3 and PBP4a was dependent on the internal MreB/Mbl cytoskeleton, the GFP–PBP constructs were examined in strains affected in MreB or Mbl synthesis. Derivatives with the mbl gene disrupted were generated directly by transformation of gfp–pbp strains with chromosomal DNA from the Δ(mbl::spc) strain 2505 (Jones et al., 2001). To localize the GFP–PBP in a xylose-dependent MreB depletion strain, vectors containing the GFP fusions under the control of Pspac were constructed (see Experimental procedures). Unfortunately, expression of the fusions from the Pspac promoter was weaker than from Pxyl, with only fusions
to PBP2a, PBP3, PBP4a and PbpX being detectable by fluorescence microscopy in the wild-type background. Despite repeated efforts and the inclusion of up to 1 mM IPTG in the selection medium, transformation of the plasmid containing $P_{spac}$-controlled gfp–pbpB did not give any transformants, consistent with the notion that expression of the GFP–PBP fusion proteins via this particular $P_{spac}$ construct is poor.

Both the depletion of MreB and the absence of Mbl had severe effects on cell morphology, as described previously (Jones et al., 2001). Depletion of MreB gave rise to bloated cells (Fig. 7B and D) that often formed only partial division septa (Fig. 7B), whereas the absence of Mbl led to a slight increase in cell diameter and sometimes to aberrant placement or orientation of the division septum (Fig. 7E and F; the mbl phenotype is not as prominent under these growth conditions as in rich medium). The different localization patterns (i.e. septal or fluorescent foci/bands) were studied in both backgrounds. GFP–PBP2a localized to the septum in both backgrounds, as in wild-type cells (Fig. 7A, B and F). The localization pattern clearly followed the septum when it was incomplete (Fig. 7B) or asymmetric (Fig. 7F). GFP–PBP4a localized in fluorescent foci in both backgrounds, similar to the wild-type background (Fig. 7D and H). Figures 7E and G show the localization patterns of GFP–PBP1 and GFP–PBP3.
respectively, in the mbl knock-out background. Again, there was no detectable difference from the localization in wild type. All the other GFP–PBP tested (not shown) also showed no significant differences in localization in the MreB deletion or mbl knock-out background compared with the wild type.

Finally, the effect of the absence of Mbl on the combination of GFP–PBP3 and GFP–PBP4a was studied using a strain in which mbl expression is controlled by $P_{\text{lac}}$ (strain 3128). In both the presence and the absence of IPTG, these cells displayed a spotty fluorescence pattern, indicating that Mbl depletion does not recognizably affect the localization of GFP–PBP3 or GFP–PBP4a (not shown).

**Discussion**

In this paper, we present the first systematic study of the cellular localization of proteins involved in cell wall synthesis in a bacterium. The localization patterns of two individual PBPs have been studied previously in *B. subtilis* (Pedersen et al., 1999; Daniel et al., 2000) and *E. coli* (Weiss et al., 1997; Den Blaauwen et al., 2003), but a study encompassing more than one PBP has not been performed. This work was prompted by recent developments in the study of bacterial morphogenesis, notably the discovery of the MreB/Mbl cytoskeleton (Jones et al., 2001), which suggested a connection between a cytoplasmic shape-determining mechanism and the external cytoskeleton formed by the cell wall.

*Construction and functionality of GFP–PBP fusion proteins*

All 11 genes coding for PBPs involved in vegetative growth from each of the different PBP classes were used for N-terminal GFP fusion (for review and classification, see Foster and Popham, 2001). Full-length fusion proteins were detected for all 11 constructs (Fig. 1A). Processing of the transmembrane signal sequence is predicted for low-MW PBPs (see Gittins et al., 1994) and has been shown for PBP4a when expressed in *E. coli* (Pedersen et al., 1998). We observed some processing of GFP fusions to two class A high-MW PBPs, PBP2c and PBP4, but not for the other PBPs. Not all GFP fusions were expressed to similar levels as judged by Western blotting (with both cells and membrane preparations that were equilibrated for either cell density or protein concentration), which was reflected by differences in the fluorescence intensities of the GFP fusions. We do not know what caused this variation in protein expression.

Owing to the redundancy of PBPs in *B. subtilis* (Popham and Setlow, 1996; Popham et al., 1999; McPherson et al., 2001; McPherson and Popham, 2003), we could not test the functionality of all GFP–PBP fusions directly. However, PBP2b is essential (Daniel et al., 2000), a double mutant of PBP2a and PbpH is not viable (Wei et al., 2003), and a ponA knock-out strain grows more slowly, with slightly elongated cells, than wild type (Popham and Setlow, 1996). We used these characteristics to study the functionality of our GFP fusions to PBP2b, PBP2a, PbpH and PBP1. GFP–PBP2b was fully functional in the sense that, when present as the only copy of PBP2b in the cell (Fig. 1B), cell viability and particularly cell division were normal. Depletion of GFP–PBP2b led to filamentation as described before (Fig. 5E; Daniel et al., 2000). Also, expression of GFP–PBP2b from $P_{\text{xy}}$ did not lead to a great increase in protein level compared with the wild type (Fig. 1B), and the fluorescence patterns for GFP–PBP1 and GFP–PBP2b were similar to the patterns observed for wild-type PBP1 and PBP2b using immunofluorescence (Pedersen et al., 1999; Daniel et al., 2000).

GFP–PBP2a was essential for growth in a $pbpH$ knock-out strain, as was GFP–PbpH in a $pbpA$ knock-out strain, and depletion of GFP–PbpH from a $pbb4$ knock-out strain resulted in cell swelling and lysis, as described for PbpH from a $pbb4$ knock-out strain (Wei et al., 2003). Finally, cells expressing GFP–PBP1 did not show a marked reduction in growth rate or alteration in cell size as described for a ponA knock-out. Taken together, these results show that our GFP fusions to PBP1, PBP2a, PBP2b and PbpH result in functional proteins. For the low-MW PBPs, we have tested substrate binding by the GFP fusion proteins and showed that GFP–PBP5 and GFP–PBP4a bind the fluorescent penicillin analogue Bocillin FL, which implies that these proteins have a correct membrane orientation and are correctly folded. This gives some confidence that the localization patterns we have observed represent the genuine position of the PBPs in the cells.

*PBP localization and the implications for cell wall synthesis*

We have found that PBP localization in *B. subtilis* is dispersed for some PBPs and occurs in spatially restricted zones, either at the septum or in spots near the lateral wall, for other PBPs. Previous studies on cell wall synthesis in *B. subtilis*, *E. coli* and *H. influenzae* have led to the suggestion that two distinct synthetic multienzyme complexes exist: one for the synthesis of the lateral cell wall, with new cell wall material intercalating in a dispersed manner in the cylindrical part of the cell during growth, and a second activity for the synthesis of the transverse septal cell wall during division (Burman et al., 1983; Mobley et al., 1984; Schlaeppe et al., 1985; Woldringh et al., 1987; de Pedro et al., 1997; Aalaeini and Day, 1999). With nine out of 11 PBPs localizing at least partially to the...
septum, all necessary activities, including the specific transpeptidation catalysed by PBP2b, are provided for the synthesis of the transverse septal wall. Our results implicate the following PBPs, which gave a bright fluorescence at the cell periphery, in lateral wall growth: PBP4 (class A high-MW), PBP3 (class B high-MW), PBP5 and PBP4a (low-MW D,D-carboxypeptidases), although for PBP4, this cannot be stated unequivocally because of the processing of some of the GFP fusion proteins. Notably, PBP2a and PbpH, both implicated in lateral wall growth (Murray et al., 1998; Wei et al., 2003), do not localize specifically to the cell periphery. The absence of PBP4, PBP3 or PBP4a has no major effects on cell morphology (Todd et al., 1986; Popham and Setlow, 1994; Murray et al., 1996; Pedersen et al., 1998), but the occurrence of elongated cells in the absence of PBP1 is aggravated when PBP4 is absent as well (Popham and Setlow, 1996). A double deletion of the genes coding for PBP3 and PBP4 has no effect on cell morphology (Murray et al., 1996).

The PBP localization patterns we observed are in good agreement with results obtained recently by staining PG precursors with fluorescent vancomycin (Van-FL). This revealed a strong fluorescence signal at the division site, together with a fainter, helical signal along the lateral wall (Daniel and Errington, 2003). The strong fluorescence signal at mid-cell is indicative of a high synthetic activity at that location. This is in good agreement with our observation that most PBPs localize to this site. The high synthetic activity at mid-cell was abolished in cells depleted for FtsZ (Daniel and Errington, 2003), which fits our observation that the PBPs that localize to the septum become dispersed along the lateral wall in cells depleted for FtsZ. The helical pattern along the lateral wall was absent in an mbl knock-out, where Van-FL staining was exclusively in transverse bands at mid-cell, showing that the Mbl component of the cytoskeleton is required for targeted insertion of PG precursors at the lateral wall (Daniel and Errington, 2003). Localization of PBPs appeared to be unchanged in an mbl knock-out, showing that the absence of precursors or Mbl does not result in a major shift of PBPs from the cell periphery to mid-cell (see below).

Our results are also in agreement with a recent study that showed that PBP2 of E. coli, a class B high-MW PBP, classically associated with lateral wall synthesis (Spratt, 1975), localizes to both the lateral wall and the division site (Den Blaauwen et al., 2003). The localization pattern of E. coli PBP2 is similar to the pattern of many PBPs from B. subtilis, most notably PBP4, YrrR and PBP5, which showed strong fluorescence both at the septum and in the lateral wall. Apparently, most of the PBPs can function in lateral as well as septal wall synthesis, which would agree with the known redundancy in function for PBPs in both B. subtilis and E. coli (Popham and Setlow, 1996; Denome et al., 1999; Popham et al., 1999; McPherson et al., 2001; McPherson and Popham, 2003).

The localization of PBP2b is dependent on the presence of several cell division proteins (Daniel et al., 2000). The distinct localization patterns observed with two other PBPs, PBP3 and PBP4a, suggest that these PBPs are either dependent on some factor that prevents them from localizing predominantly to the septum or actively recruited to the lateral wall. The localization patterns of PBP3 and PBP4a resembled patterns observed previously for the cytoskeletal proteins MreB and Mbl (Jones et al., 2001), and three-dimensional reconstruction of the fluorescent signals of GFP–PBP3 and GFP–PBP4a, either individually or in combination, revealed short arcs at the cell periphery that did not extend to continuous helices spanning the entire cell circumference. These patterns could represent PBP3 and PBP4a partially co-localizing with the Mbl cytoskeleton, which is required for the helical insertion of PG precursors along the lateral wall (Daniel and Errington, 2003). However, if that was the case, one would expect a disappearance of the specific PBP3 and PBP4a patterns with the disappearance of Mbl, which we did not observe. In an mbl knock-out strain, insertion of PG precursors is restricted to mid-cell (Daniel and Errington, 2003), but PBP3 and PBP4a still localize to the lateral cell wall, and the localization patterns were apparently unchanged. This indicates that the apparent organization of PBP3 and PBP4a is not dependent on Mbl and, combined with the similar result in an MreB depletion strain, this precludes the idea that PBP3 and PBP4a track along the internal cytoskeleton. We also tested whether the GFP–PBP3 and GFP–PBP4a patterns changed when precursor synthesis was inhibited by bacitracin. Bacitracin abolished the helical Van-FL signals (Daniel and Errington, 2003), but had no effect on the localization of PBP3 and PBP4a (not shown), showing that PBP localization is not affected by the availability of PG precursors. If there is indeed some signal that recruits PBP3 and PBP4a to the lateral wall, this would explain why there is no dramatic change in the localization pattern to mid-cell in the absence of either Mbl or PG precursors. Although we deem this unlikely, it may be that overproduction of PBP3 and PBP4a causes the appearance of fluorescent patterns that are not representative of the situation in the cell.

Recently, Wei et al. (2003) showed that PBP2a and PbpH are probably the key players in lateral wall elongation in B. subtilis. As such, they are the most likely candidates for control by the Mbl cytoskeleton. Unfortunately, the fluorescence signals for these proteins were too weak and dispersed to be able to tell whether their localization is affected in the absence of Mbl.

To conclude, this work describes the localization of GFP fusions to 11 PBPs implicated in cell wall synthesis during
vegetative growth of *B. subtilis*. We show that some PBPs localize in distinct rather than dispersed patterns along the lateral cell wall and at the septum. Our results implicate PBP3, PBP5 and PBP4a, and possibly PBP4, in lateral cell wall synthesis. We hypothesize that PBP3 and PBP4a, which appear to localize exclusively to the lateral cell wall, are actively targeted to the lateral wall. Future challenges are to understand the basis of the positional information for PBP3 and PBP4a, and to investigate whether PBPs in *B. subtilis* form multienzyme cell wall synthesis complexes.

**Experimental procedures**

**General methods**

The strains and plasmids used in this study are listed in Table 2 or were derived from these strains as described below. *B. subtilis* cells were made competent for transformation with DNA by the method of either Kunst and Rapoport (1995) or Anagnostopoulos and Spizizen (1961) as modified by Jenkinson (1983). DNA manipulations and *E. coli* DH5α transformations were carried out using standard methods (Sambrook *et al*., 1989). Solid medium used for growing *B. subtilis* was nutrient agar (Oxoid), and liquid medium was either casein hydrolysate (CH) medium (Sterlini and Mandelstam, 1969) or S medium (Sharpe *et al*., 1998) supplemented with 1% (v/v) CH (S+), with antibiotics added as required. Chloramphenicol was used at 5 µg ml⁻¹, spectinomycin at 50 µg ml⁻¹, erythromycin at 1 µg ml⁻¹, kanamycin at 5 µg ml⁻¹ and phleomycin at 0.2 µg ml⁻¹. Media used for growing *E. coli* strains were 2xTY (Sambrook *et al*., 1989) and nutrient agar supplemented with ampicillin (100 µg ml⁻¹) as required.

**Construction of GFP fusions**

Approximately one-third of each of the promoter-proximal parts of the *ponA*, *pbpA*, *pbpB*, *pbpC*, *pbpD*, *pbpE*, *pbpF*, *pbpX*, *dacA*, *dacB*, *dacC*, *pbpH* and *yrrR* genes was amplified using polymerase chain reaction (PCR) and cloned into pSG4902. All primers and restriction endonucleases used are listed in Supplementary material (Table S1). pSG4902 allows a translational fusion of gfp, under the control of *P_yrr*, to be made to the 5’ end of a gene of interest and carries *bla* and *cat* selectable markers (Wu and Errington, 2003). Transformation of the resulting plasmids (Table 2) into *B. subtilis*, with selection for chloramphenicol resistance, resulted in the formation of the resulting plasmids (Table 2) into *B. subtilis* with selection for chloramphenicol resistance, resulted in the formation of the resulting plasmids (Table 2).

The resulting strain, 3102, was transformed to chloramphenicol resistance. This resulted in strain 3128, with *gfp–pbpC* and *gfp–dacC* expression, both under the control of *P_yrr* and *mbl* under the control of *P_spac*.

To study the GFP fusions in an *mreB* deletion background, the various *gfp* fusion constructs had to be put under the control of another promoter. For the *pbpA*, *dacC*, *pbpC* and *pbpX* fusions, an XbaI fragment containing the ribosome binding site (RBS) and the translational fusion of *gfp* to the gene of interest was cut from the pSG4902 derivatives and ligated into XbaI–SmaI-digested pSG1170 (Lewis and Marston, 1999). The remaining XbaI cohesive ends were filled in with the Klencow fragment of DNA polymerase before a second round of ligation. The correct orientation of each insert was confirmed by PCR. The resulting plasmids (Table 2) were transformed into strain 168. Chromosomal DNA samples from strains with a correct integration were isolated, and the integrated plasmid was transformed into strain 2060 to study the localization of the PBP of interest under *mreB* depletion conditions, using the *P_spac* promoter to drive *gfp–pbp* expression. For the *pbpB*, *ponA*, *pbpF*, *pbpD*, *pbpH*, *yrrR* and *dacA* fusions, another strategy was used. PCR products were performed on the pSG4902 constructs using a forward primer designed to amplify the entire fusion of *gfp* to the gene of interest from the pSG4902 derivatives, including the RBS on pSG4902 and introducing *Smal* sites, and reverse primers directed against the parts of the genes included on pSG4902, and introducing *XbaI* sites. This resulted in PCR products of the entire fusion that were ligated into *Smal–XbaI*-digested pSG1170. These constructions were checked by sequencing and transformed into strain 168. Chromosomal DNA samples from strains with a correct integration were isolated, and the integrated plasmids were moved by transformation into strain 2060 to study the localization of the various GFP–PBP fusions under *mreB* depletion conditions using the *P_spac* promoter to drive expression of the GFP fusion protein.

**Microscopy**

Cells from an overnight culture were inoculated into fresh S+ medium containing xylose (0.5%) and grown to exponential phase. Cells from exponentially growing cultures were

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**Table 2.** Bacterial strains and plasmids used in this study.

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**Plasmids**

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a. *gfp:* F64L, S65T variant of GFP (GFPmut1; Cormack et al., 1996).
b. Notation: 168 transformed to chloramphenicol resistance with plasmid pSG5043.

d. Mounted on a thin film of 1% agarose in water, essentially as described previously (Glaser et al., 1997), on microscope slides fitted with GeneFrames (ABgene). Image acquisition was performed as described previously (Lewis and Errington, 1997) using METAMORPH version 4.6 software (Universal Imaging). To determine the distribution of the fluorescence signal, fluorescence signal intensities at the lateral wall and at the septum were measured and compared with the fluo-
rescence signal intensities obtained with the membrane stain FM95.5 (Molecular Probes). Image stack acquisition and deconvolution were done as described previously (Jones et al., 2001) using AUTODEBLUR version 7.0 software (Auto-Quant Imaging). Assembly of micrographs was done using Adobe PHOTOSHOP version 6.0.

Depletion experiments

For MreB depletion experiments, cells were grown on solid medium containing 0.5% xylose, and cells from a single colony were inoculated into S+ medium supplemented with 1 mM IPTG and with (control) or without (depletion) 0.5% xylose. Cells were grown to an OD600 of >1 before diluting to an OD600 of ≈0.1 in the same medium to ensure good MreB depletion. Cultures that had reached an OD600 of ≈0.5 were processed for microscopy as described above.

For Mbl depletion experiments, cells were grown on solid medium containing 0.5 mM IPTG, and cells from a single colony were inoculated into S+ medium with or without 0.5 mM IPTG. This culture was grown overnight, and diluted to an OD600 of ≈0.1 in the morning into fresh medium, again with or without 0.5 mM IPTG and with 0.5% xylose. Cultures that had reached an OD600 of ≈0.5 were processed for microscopy as described above.

For FtsZ depletion experiments, cells were grown on solid medium containing 0.5 mM IPTG, and cells from a single colony were inoculated into S+ medium supplemented with 0.5 mM IPTG and xylose (maximally 0.5%). Cells were grown to an OD600 of ≈0.4, washed twice in medium without IPTG and diluted to an OD600 of ≈0.1 in S+ medium supplemented with xylose (maximally 0.5%) and with (control) or without (depletion) 0.5 mM IPTG. Cultures that had reached OD600 of 0.5 were processed for microscopy as described above. As a control, cells expressing GFP–PBP2b were depleted for GFP–PBP2b as above by dilution into S+ medium supplemented with 0.5 mM IPTG, but without xylose.

Immunodetection of GFP fusion proteins and Bocillin FL labelling

Cells from cultures grown as for microscopy with 0.5% xylose were harvested by centrifugation. Cell pellets were subsequently washed with PBS, resuspended in PBS to 1/20th of their original volume, and diluted to an OD600 of >0.1 in the same medium to ensure good MreB depletion. Cells that had reached an OD600 of ≈0.5 were processed for microscopy as described above.

Immunodetection of GFP fusion proteins and Bocillin FL labelling

Cells from cultures grown as for microscopy with 0.5% xylose were harvested by centrifugation. Cell pellets were subsequently washed with PBS, resuspended in PBS to 1/20th of the original volume and disrupted by sonication. The disrupted cells were diluted 1:1 with 2×SDS-PAGE sample buffer, heated for 5 min at 95°C, and 7.5 μl samples were loaded onto 7.5% and 10% SDS-PAGE gels to resolve both high-MW fusion proteins and low-MW proteolysis products. Proteins were subsequently transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences) by Western blotting. GFP fusion proteins were detected with a GFP-specific polyclonal antiserum (Santa Cruz Biotechnology), and PBP2b was detected with anti-PBP2b antiserum (Daniel et al., 2000). Western blots were developed using an ECL detection kit (Amersham Biosciences). For Bocillin FL (Molecular Probes) labelling, cells were grown as for microscopy with 0.5% xylose, harvested by centrifugation, washed with PBS and resuspended to 1/30th of their original volume in buffer A (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0). Cells were mechanically disrupted using glass beads in a FastPrep 120 cell disrupter (Qbiogene) at 4°C. Debris and non-disrupted cells were removed by centrifugation at 80 000 r.p.m. for 10 min at 4°C. Membranes were isolated by centrifugation at 80 000 r.p.m. for 60 min at 4°C. Membranes were labelled with 10 μM Bocillin FL for 20 min at 30°C at a membrane protein concentration of 1.3 mg ml⁻¹, and the reaction was stopped by adding an equal volume of 2×SDS-PAGE sample buffer. Samples were run on 12% SDS-PAGE gels, and labelled proteins were detected using a Fuji-FLA 5000 imager (Fuji Image). The same samples were used for detection of GFP fusion proteins as described above.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3854/mmi3854sm.htm

Table S1. Primers used in this study.

Video recording. Rotating three-dimensional reconstruction of the GFP fluorescence from cells of a strain containing GFP fusions to both PBP3 and PBP4a (from Fig. 6D).

References


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