Midcell localization of PBP4 of *Escherichia coli* is essential for the timing of divisome assembly

Jolanda Verheul^{1#}, Adam Lodge^{2,7#}, Hamish C.L. Yau^{2,8#}, Xiaolong Liu^{1,9#}, Alexandra S. Solovyova³, Athanasios Typas^{4,5}, Manuel Banzhaf^{6*}, Waldemar Vollmer^{2**}, and Tanneke den Blaauwen^{1***}

¹Bacterial Cell Biology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam; Science Park 904, 1098 XH Amsterdam, The Netherlands.

²Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, NE2 4AX, United Kingdom.

³NUPPA, Leech Building, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

⁴European Molecular Biology Laboratory, Genome Biology Unit, Heidelberg, Germany

⁵European Molecular Biology Laboratory, Structural & Computational Unit, Heidelberg, Germany

⁶Institute of Microbiology & Infection and School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK. B15 2TT

⁷Present address: Iksuda Therapeutics, The Biosphere, Draymans Way, Newcastle Helix, Newcastle upon Tyne, NE4 5BX, UK

⁸Present address: Procter & Gamble, Newcastle Innovation Centre, Whitley Road, Newcastle-Upon Tyne, NE12 9TS, UK.

⁹Department of Biochemistry, University of Oxford, OX1 3QU, Oxford, United Kingdom.

#contributed equally.

*Corresponding author. Tel: +44 121 4145586; Email: m.banzhaf@bham.ac.uk

**Corresponding author. Tel: +44 191 208 3216; Email: w.vollmer@ncl.ac.uk

***Corresponding author. Tel: +31 20 525 3852; Email: t.denblaauwen@uva.nl

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ABSTRACT (203 words)

Insertion of new material into the *Escherichia coli* peptidoglycan (PG) sacculus between the cytoplasmic membrane and the outer membrane requires a well-organized balance between synthetic and hydrolytic activities to maintain cell shape and avoid lysis. The hydrolytic enzymes outnumber the enzymes that insert new PG by far and very little is known about their specific function. Here we show that the DD-carboxy/endopeptidase PBP4 localizes in a PBP1A/LpoA and FtsEX dependent fashion at midcell during septal PG synthesis. Midcell localization of PBP4 requires its non-catalytic domain 3 of unknown function, but not the activity of PBP4 or FtsE. Domain 3 is also needed for the interaction of PBP4 with Nlpl, but not for its interactions with PBP1A or LpoA. Microscale thermophoresis with isolated proteins

shows that domain 3 is needed for the interaction with Nlpl, but not PBP1A or LpoA. *In vivo* crosslinking experiments confirm the interaction of PBP4 with PBP1A and LpoA. We propose that PBP4 functions together with the amidases AmiA and B to create denuded glycan strands to attract the initiator of septal PG synthesis, FtsN. Consistent with this model, we found that the arrival of FtsN and other cell division proteins at midcell was significantly delayed in cells lacking PBP4.

IMPORTANCE (140 words)

Peptidoglycan biosynthesis is a major target for antibacterials. The covalently closed peptidoglycan mesh, called sacculus, protects the bacterium from lysis due to its turgor. Sacculus growth is facilitated by the balanced activities of synthases and hydrolases, and disturbing this balance leads to cell lysis and bacterial death. Because of the large number and possible redundant functions of peptidoglycan hydrolases, it has been difficult to decipher their individual functions. In this paper we show that the DD-endopeptidase PBP4 localizes at midcell during septal peptidoglycan synthesis in *Escherichia coli* and is important for the timing of the assembly of the division machinery. This shows that inhibition of certain hydrolases could weaken the cells and might enhance antibiotic action.

KEYWORDS:, bacterial cell division, endopeptidase, immunolabeling, LpoA, Nlpl, PBP1A, PBP4, peptidoglycan, preseptal, protein interactions

INTRODUCTION

The peptidoglycan (PG) layer is sandwiched between the cytoplasmic membrane (CM) and the outer membrane (OM) of the Gram-negative bacterium *Escherichia coli* forming a

covalently closed network of glycan strands that are interconnected by short peptides (1). The PG layer maintains the shape of the bacterium by stabilizing the cell against its high internal osmotic pressure. To proliferate, the rod-shaped bacterium grows in length and then divides by binary fission into two equally sized daughter cells. The balanced activities of PG synthases and hydrolases ensures a safe extension of the PG layer without defects that would cause cell lysis (2). Beta-lactam antibiotics disturb this balance by inactivating the transpeptidase (TPase) activity of the penicillin binding proteins (PBP)s causing lysis of growing cells. We currently know more than twenty PG hydrolases that can cleave either in the glycan chains or peptides within the PG layer, but how their activities are regulated is poorly understood. This is particularly true for the PG endopeptidases (2). Recently, the OM bound lipoprotein Nlpl was shown to interact with similar affinity with several endopeptidases, suggesting that Nlpl acts as an interaction hub for endopeptidases to regulate their activities by competing protein-protein interactions. (3).

The basic precursor (lipid II) for PG synthesis is the β -(1,4) linked disaccharide *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc) with an undecaprenol-pyrophosphate linked to C1 of MurNAc and a stem peptide with the sequence L-alanine-D-*iso*-glutamate-diaminopimelic acid (Dap)-D-alanine-D-alanine coupled to the C3 of MurNAc. Lipid II is used by glycosyltransferases (GTases), which polymerize the glycan chains, and TPase, which crosslink the stem peptides. The main PG synthases in *E. coli* are the class A PBPs (PBP1A and PBP1B) with GTase and TPase activities (4-7), the integral membrane proteins RodA and FtsW that have GTase activity (8-10) and monofunctional Class B PBPs (PBP2 and PBP3) that have TPase activity (11-13).

The hydrolases are present in greater redundancy than the synthases (2, 14). The carboxypeptidases (CPase) PBP5, 6A and 6B remove the terminal D-Ala of the nascent

pentapeptide (15-Popham:2003uc; 19). The endopeptidases (EPases) PBP4, PBP7, MepA, MepM, MepH, and MepS cleave the crosslinks in the PG (19-21). The amidases AmiA-D hydrolyze the bond between MurNAc and L-alanine at position 1 of the stem peptide (22-25). Finally, the lytic transglycosylases Slt70 and MltA-G cleave the glycan strands to release 1,6-anhydroMurNAc containing turnover products (26-28). The large number of hydrolases reflects the adaptability to various environmental conditions but begs the question of how their potentially dangerous activities are controlled to avoid cell lysis.

Binary fission in *E. coli* is initiated by the assembly of the Z-ring at midcell, which consist of a network of dynamic FtsZ filaments that are anchored to the cytoplasmic membrane by FtsA and ZipA (29). The Z-ring also recruits the PG synthases PBP1A and PBP1B to midcell (30). A number of Z-associated proteins play a role in the organization and dynamics of the ring (31-35). Of these, the integral membrane protein FtsX interacts with FtsA (36) and its cytoplasmic ATPase partner protein FtsE interacts with FtsZ (37). Both localize early to midcell, together with the Z-ring (22). As a complex FtsEX recruits EnvC, which activates AmiA and AmiB that are needed for septum cleavage (24, 38). FtsE versions without ATPase activity still localize at midcell and recruit as FtsEX-EnvC complex but the latter is not able to activate the amidases (39). Presumably an ATPase dependent conformational change in FtsEX induces a conformational change in EnvC, which is then able to relieve an autoinhibitory alpha-helix from the active site of AmiA and AmiB, activating them (40). The third amidase AmiC is activated by the OM-anchored lipoprotein NlpD (22, 41). The recruitment of the other cell division proteins occurs with a time delay of about 20% of the cell division cycle, depending on the growth condition (42-45). FtsK, FtsBLQ, FtsW, PBP3 and FtsN localize in an interdependent fashion. FtsBLQ inhibit the PG synthase activity of PBP3,

FtsW and PBP1B until they are outcompeted by the accumulation of FtsN that relieves this inhibition and initiates septum synthesis (46-50).

While investigating the specific function of various PG hydrolases, we found that PBP4 localizes specifically at midcell as part of the division machinery. PBP4 is a periplasmic endopeptidase (51) with a C-terminal amphipathic alpha-helix that associates with membranes (52). Deletion of *dacB*, which encodes PBP4, does not have morphological consequences but causes a decrease in the percentage of PG crosslinks and of PG-attached lipoprotein (53) and an increased sensitivity to bile salts (54). Overproduction of PBP4 is toxic (55) presumably because the enhanced cleavage of peptide cross-links weakens the PG mesh and eventually causes lysis. PBP4 has three domains that are assembled in an unusual way (56) (Fig. 1A). A non-catalytical domain of unknown function (domain 2) is inserted into the transpeptidase domain 1, and a third domain (domain 3) is inserted into domain 2. Domain 3 is positioned above the active site with its catalytic serine 62, which resides in domain 1, and might be involved in substrate binding or regulation (56, 57) (Fig. 1A). Site directed mutagenesis suggested that residues from domain 1 (R361) and 2 (D155) are important for the endopeptidase activity (58).

Here we determined the localization of PBP4 and different variants during the cell cycle of *E. coli*. The timing of localization at midcell and interactions of PBP4 support a role in remodeling of PG synthesized by the divisome.

RESULTS

PBP4 localizes to the lateral wall and at midcell. To determine the localization of PBP4 in *E. coli* cells as a function of the bacterial cell cycle, we generated an antiserum against PBP4 and removed unspecific antibodies by adsorption to cells of a *dacB* mutant strain that lacks PBP4.

The purified antibodies were specific for PBP4 (Fig. S1) and used to immunolabel the wild-type strain MC4100 grown in rich (TY) medium at 37°C. PBP4 localized strongly at midcell and to a lesser extend in the lateral wall in wild-type cells (Fig. 1B).

PBP4 localizes between the peptidoglycan layer and the outer membrane. Although it was reported that PBP4 has a stretch of weakly amphipathic amino acids at its C-terminus (52), it was not known whether PBP4 is freely diffusing in the periplasm or associates with the membrane or other proteins. It has been previously observed that chemically fixing freely diffusing periplasmic proteins can shock them into the cell poles (11, 59). The fact that we did not observe any polar localization of PBP4 after fixation (Fig. 1B and S1), suggests that it is either associated with the membrane or other proteins. Next, we investigated whether PBP4 was accessible to anti-PBP4 in cells with an intact PG layer. For this, wild-type cells were grown in TY at 37°C and immunolabeled without Triton X-100 permeabilization of the OM or without increasing the pore size of the PG mesh with lysozyme. PBP4 was not accessible without permeabilization of the OM, but was fully accessible in cells with an intact PG (Fig. 1C). In contrast, proteins like FtsN and PBP3 were not accessible without degradation of the PG layer by lysozyme (Fig. 1C). This suggests that PBP4 resides between the OM and the PG layer.

Activity of PBP4 is not needed for midcell localization. Substrate binding can be a key determinant in the localization of proteins (16, 60, 61). To test whether PBP4 substrate binding is needed to localize at division sites we expressed the active site mutant PBP4S62G or A (57) that fails to bind β -lactams and therefore is not able to hydrolyze the PG stem peptide (Fig. S2 and S3). A plasmid encoding the mutant protein was constitutively expressed in a $\Delta dacB$ background by the leakage of a weakened *pTrc99A* promoter. Interestingly, the wild-type and active site mutant when expressed from plasmid were both able to localize at midcell (Fig. 2). This indicates that although PBP4(S62G/A) cannot hydrolyze its substrate or

bind β -lactams covalently (Fig. S2C Fig. S3), it is either still able to interact with its substrate or it localizes through protein interactions.

Since PBP4(S62G/A) likely still interacts with its substrate, we mutagenized the gene to replace two residues (D155 and R361; (58)) involved in the binding of the DAP residue in the stem peptide by alanine. The genes encoding PBP4(D155A) or PBP4(R361A) were expressed by promoter leakage from plasmid in a $\Delta dacB$ background, and both PBP4 versions localized at midcell (Fig. 2). An immunoblot analysis showed that all mutants were expressed to similar levels and that PBP4(R361A) and PBP4(D155A) were able to bind the fluorescent β -lactam Bocilin-FL (Fig. S2). Therefore, all three active site protein variants are potentially able to still interact with substrate as an inactive protein.

A PBP4 version lacking domain 3 (PBP4 Δ D3) was still able to bind β -lactams (Fig. S2B and S3) but did not have DD-carboxypeptidase or DD-endopeptidase activity (Fig. S3). Analytical ultracentrifugation (Fig. S4A) and circular dichroism analysis (Fig. S4B) of isolated PBP4 Δ D3, showed that the removal of domain 3 did not affect the structure, the dimerization of the rest of the protein or its interaction with PG sacculi (Fig. S4C), which suggests a folded active site domain. However, the inactive but substrate binding PBP4 Δ D3 was not localizing at midcell (Fig. 2). Hence, we conclude that PBP4 localization could be largely driven by protein interactions and to a lesser extent by substrate interaction but is independent from its own activity.

PBP4 localizes weakly at inactive divisomes. To further dissect whether the localization of PBP4 at the division site is dependent on the availability of its substrate or on the physical presence of other proteins at the division site, cell division was inhibited through inactivation of PBP3 by its specific inhibitor aztreonam (62). Aztreonam treatment for about two mass doublings stalls the division machinery at midcell resulting in filamentous growth.

In the longer filaments new division machineries are formed and localize at future division sites (5). In these filaments PBP4 localized weakly at all possible division sites (Fig. S5), indicating that the activity of PBP3 was not required for the localization of at least some PBP4 molecules. While PBP3 is inhibited, preseptal PG synthesis (63) still occurs at the potential division sites in the filamenting cells (30, 64, 65). The localization of PBP4 could therefore be associated with the presence of PBP1A or PBP1B, which were previously shown to be recruited to preseptal PG synthesis sites by early cell division proteins (Pazos:2018fj).

PBP1A/LpoA assist in midcell localization of PBP4. PBP4 was recently reported to interact with PBP1A, LpoA and the EPase adaptor protein Nlpl (3). To investigate whether PBP1A and/or LpoA proteins were needed for midcell localization of PBP4, strains deleted for *mrcA* (PBP1A), *lpoA*, *mrcB* (PBP1B) or *lpoB* were grown in rich medium at 37°C and immunolabeled with anti-PBP4. The amount of PBP4 that localized at midcell was identical for the parental wild-type strain BW25113, $\Delta mrcB$ and $\Delta lpoB$, but reduced in the $\Delta mrcA$ and $\Delta lpoA$ strains, whereas the PBP4 concentration was similar for all strains (FIG. 3). This could indicate that PBP1A/LpoA assist in the initial localization of PBP4 at midcell.

Nlpl seems not to be involved in midcell localization of PBP4. We next tested if the interaction with Nlpl affected the localization of PBP4. In the $\Delta nlpl$ cells the amount of PBP4 at midcell per μm average cell circumference was reduced with $42 \pm 6.7\%$ (n=3) compared to wild type and the protein localized later at midcell than in the wild-type cells (Fig. S6). The PBP4 concentration in the $\Delta nlpl$ strain was also lower than in the wild-type cells ($64.8 \pm 17.6\%$, n= 3). This could indicate that the cells down-regulate PBP4 expression or that PBP4 becomes unstable in the $\Delta nlpl$ strain. Interestingly, labeling of PBP4 in a $\Delta nlpl$ strain also did not require lysozyme (Fig. S6), suggesting that one or more additional interactions maintains PBP4 localized outside the PG layer.

PBP4 domain 3 facilitates interaction with Nlpl but not PBP1A/LpoA.

Microscale thermophoresis (MST) in which one protein is labelled with a fluorophore and titrated with an unlabeled partner protein was used to analyze the interactions between PBP4 and its known interaction partners further. The difference in thermophoresis of the complex versus the unbound fluorescently labeled proteins is then used to calculate the affinity of the binding partners. Different versions of PBP4 and its interaction partners were purified and assayed for direct protein-protein interactions. Wild-type PBP4 interacted with PBP1A, LpoA and Nlpl (3). PBP4 lacking domain 3 (PBP4 Δ D3), which did not localize at midcell (Fig. 2), interacted with PBP1A and LpoA but not with Nlpl (Fig. 4). These results suggest that domain 3 of PBP4 facilitates the interaction with Nlpl, but not PBP1A.

Nlpl and PBP1A/LpoA have different binding sites in PBP4.

PBP4 can form a multi-enzyme complex with Nlpl and PBP1A/LpoA (3). PBP1A interacted with PBP4 and could be cross-linked to PBP4 in cells (Fig. 4C). We found that full length LpoA interacted with both PBP4 and PBP4 Δ D3, with apparent K_D values of 315 ± 38 nM and 153 ± 31 nM, respectively (Fig. 4A; Fig. S7). This suggests that domains 1 or 2 of PBP4 are sufficient for the interaction with LpoA. MST assays of PBP4 or PBP4 Δ D3 with LpoA's C-terminal (residues N257-S678; LpoA^C) or N-terminal (LpoA residues G28-T256; LpoA^N) domains also yielded positive binding curves (Fig. 4A; Fig. S7) (66-68). We found that LpoA^C and the full length LpoA version had comparable affinities for both PBP4 versions, suggesting that the C-terminal domain of LpoA is sufficient for interaction with PBP4 (Fig. 4A). Interestingly, the removal of domain 3 from PBP4 resulted in an increased affinity of LpoA^N for PBP4 (app. K_D decreased by ~50 fold) (Fig. 4A Fig. S7). Hence, the interaction between the N-terminal domain of LpoA and PBP4 possibly occurs via a different mechanism dependent on a conformational rearrangement of domain 3 of PBP4. This suggests that the two domains of LpoA, which likely serve different primary

functions, are both sufficient for an interaction with PBP4, albeit with varying affinities. In conclusion PBP4 robustly interacts with PBP1A/LpoA independent of its domain 3.

LpoA has a small effect on PBP4 activity. We found previously that Nlpl does not affect PBP4 activity (3) but considered it possible that PBP4 interaction with PBP1A/LpoA may affect its activity. We first verified that Nlpl or PBP4 did not affect the activity of PBP1A in the presence or absence of LpoA using an *in vitro* glycosyltransferase assay (Fig. 5). We next tested if PBP1A or LpoA affected the activity of PBP4 using *in vitro* PG degradation assays. LpoA decreased the activity of PBP4 by about 30% in a continuous DD-CPase assay with soluble substrate and an endpoint PG digestion assay (Fig 5B and C). To assess whether LpoA also affects PBP4 activity *in vivo*, a $\Delta dacB \Delta lpoA$ strain and $\Delta dacB$ strain were transformed with the plasmids expressing PBP4 or the active site mutant PBP4(S62G) and the toxicity of their expression was compared. Although the double mutant was less fit than the single mutant, it was equally sensitive to the overexpression of PBP4 (Fig. 5D), leaving no indication that LpoA also inhibits the activity of PBP4 *in vivo*.

PBP4 localizes as early and late divisome protein. Since PBP4 localizes at midcell it could be part of or associated with the divisome. The division machinery is assembled in two in time separated steps (42, 69). First, FtsZ and its membrane bound partners ZipA and FtsA in conjunction with other Z-ring associated proteins form the proto-ring that localizes at midcell (70). Second, after approximately 20% of the cell division cycle the remaining proteins assemble subsequently at the division site. To obtain more details on PBP4 localization in relation to other cell division proteins, MC4100 cells were grown to steady state in minimal glucose medium at 28°C. The cells were immunolabeled with antibodies against PBP4 and against FtsZ, PBP3, and FtsN that localize early, late and last, respectively. PBP4 localized strongly at midcell and hardly in the lateral cell wall (Fig. 6). To determine whether PBP4

belongs to the early or late divisome localizing proteins, we analyzed the timing of its midcell localization. To this end, we plotted the extra fluorescence (FCPlus) present at midcell in comparison to the amount of fluorescence in the rest of the cell (Fig. 6B and C). PBP4 localized together with the late localizing proteins like PBP3 and FtsN, but close inspection of its localization timing shows that it arrives in a small amount at 30% of the cell cycle in between FtsZ and PBP3 (Fig. 6C). After 40% it shows about the same timing as PBP3. This could indicate that PBP4 localizes relatively early and dependent on the amount of substrate produced.

Midcell localization of PBP4 depends on FtsE. To investigate if PBP4 localization is dependent on division associated proteins we localized PBP4 in different temperature sensitive strains. Strains were grown in minimal glucose medium at 28°C to an OD₄₅₀ of 0.2, diluted 1:4 in prewarmed medium of 28°C or 42°C and grown for two mass doublings before PBP4 immunolabelling. PBP4 localized in filamentous cells with thermo-labile FtsQ, FtsW or PBP3 indicating that these proteins are not involved in the recruitment of PBP4 (Fig. 7). In filamentous *ftsA(ts)* cells PBP4 localized mostly at one or two division sites, although these cells had 4 to 8 potential division sites. We suspected that the level of remaining FtsA was sufficient to assemble only one or two Z-rings at the non-permissive temperature. Labeling of the filamentous *ftsA(ts)* cells with antibodies against FtsZ showed indeed a similar localization frequency of FtsZ and PBP4 (Fig. 7). This shows that PBP4 needs FtsZ and FtsA for localization in agreement with our previous finding of localizing after FtsZ (Fig 6 C).

PBP4 localized only weakly at division sites in the filamentous *ftsE(ts)* cells (Fig. 7). This suggests that a functional FtsE is important for PBP4 localization. The FtsEX/EnvC complex assists in the assembly of the divisome (71, 72) and is essential for amidase function to cleave PG for cell separation (39). To investigate a possible link between PBP4 and this complex we further dissected PBP4's localization pattern. PBP4 localized at division sites in a $\Delta envC$ strain,

indicating that EnvC is not needed for PBP4 localization. However, PBP4 failed to localize in a $\Delta ftsEX$ strain (Fig. 8). To verify whether FtsE and FtsX were required for PBP4 localization we constructed a $\Delta ftsE$ strain. The *ftsE* and *ftsX* genes reside in an operon and the expression of *ftsX* requires the *ftsE* gene (73). We therefore constructed a $\Delta ftsE$ strain with a weakened pTrc99A promoter placed upstream of *ftsX* to ensure sufficient expression of FtsX. The resulting strain, XL36 grew with a mild filamentous phenotype in rich medium that could be complemented by the expression of FtsE(wt) (Fig S9). PBP4 localized weakly at septal positions in the $\Delta ftsE$ strain XL36 but localized normally when FtsE was expressed to complement this strain (Fig 8B). This suggests that FtsE and FtsX enhance the septal localization of PBP4. Interestingly, PBP4 localized at division sites in a strain lacking the three amidases AmiA, AmiB and AmiC (Fig. S10) and in a strain lacking essential components of the Tol-Pal system, which is involved in the coordination of OM constriction and PG invagination during cell division (Fig. S10).

Activity of FtsE is not required for midcell localization of PBP4. The failure of PBP4 to localize in the FtsE(ts) variant could be due to unstable or inactive FtsE localizing at the division site. Sequencing of the gene revealed a C to T base pair change that is predicted to cause Pro135 to be replaced by Ser in the FtsE protein. To determine the role of this mutation, the sequence of the ABC transporter Mi0796 of *Methanococcus jannashii* for which a crystal structure is available (PDB code 1L2T (74)) was aligned with the amino acid sequence of *E. coli* FtsE using Clustal W (75). Pro135 is part of a loop preceding a small alpha-helix adjacent to the walker B motif that is needed for ATP binding and hydrolysis. We therefore assumed that the mutation affected the ATP binding or hydrolysis but not the cellular localization of the protein. To validate this hypothesis, a wild-type parental strain LMC500 and the $\Delta ftsE$ strain XL36 were transformed with the plasmids expressing FtsE(wt) or the thermosensitive FtsE(ts)

fused to the fluorescent protein (FP) mNeonGreen (mNG), and were grown at both permissive and non-permissive temperatures. mNG-FtsE localized at midcell in wild-type and $\Delta ftsE$ strains and was able to restore wild-type morphology apart from few cells that filamented. FP-FtsE(ts) was not able to localize in the $\Delta ftsE$ XL36 at non-permissive temperature. Even at the permissive temperature localization was not restored implying that localization is further weakened by the FP and the absence of an endogenous FtsE. Localization of PBP4 was restored in the XL36 ($\Delta ftsE$) expressing mNG-FtsE(wt) background (Fig. S9). As it is unclear whether FtsE(wt) lacks stability or activity or both, we expressed three inactive mutants that had been shown to localize to midcell (73). We tested PBP4 localization in those strains to discriminate whether or not FtsE activity is needed for its localization. The K41Q and D162A versions of FtsE cannot bind ATP and FtsE(E163A) binds ATP but cannot hydrolyze it. In contrast to what was shown previously, we found that mNG-FtsE versions unable to bind ATP did not localize to midcell. FtsE(E163A) is able to bind ATP and localized at division sites in filamentous $\Delta ftsE$ cells. PBP4 followed the localization pattern of FtsE versions suggesting that ATP hydrolysis by FtsE is not a requirement for localization of PBP4. Because the mNG seemed to affect the localization of FtsE somewhat, the experiments were repeated with published untagged FtsE versions (73). PBP4 localized in all four FtsE active site mutants (Fig. 8B) showing that the activity of FtsE is indeed not required for the localization of PBP4.

PBP4 affects the timing of the divisome assembly. To measure the timing of divisome in various strain backgrounds, we grew $\Delta dacB$ cells carrying either an empty plasmid or plasmids constitutively expressing PBP4 or PBP4 versions to steady state in minimal glucose medium at 28°C. Cells were fixed, divided into three aliquots and were labeled with anti FtsZ, anti PBP4 or anti FtsN. We determined the timing of the arrival of these proteins at midcell by analyzing fluorescence signals of several thousands of cells (76). In the parental strain FtsZ

and FtsN started to localize at midcell at about 29% and 50% of the cell cycle age, respectively. Similarly, in the $\Delta dacB$ strain expressing wild-type PBP4 from plasmid FtsZ and FtsN started to localize at midcell at about 20% and 49% of the cell division cycle age, respectively (Fig. 9D). Strikingly, all three proteins localized simultaneously and very late at 45% of the cell division cycle time in the $\Delta dacB$ strain (Fig. 9D). Expression of inactive PBP4 versions affected the timing of the divisome assembly as well. Especially $\Delta dacB$ cells expressing PBP4(D155A) from plasmid, which was slightly filamentous (Fig. 9B), assembled the divisome very early and showed a longer cell division period. These results show that PBP4 profoundly affects the timing of divisome assembly.

DISCUSSION

Escherichia coli contains a large number of seemingly redundant PG hydrolases, which possibly fine tune peptidoglycan biogenesis and remodelling in response to environmental parameters. Apart from their enzymatic activities, little is known about the actual function of most PG hydrolases. Here we report that the DD-endopeptidase PBP4 localizes at midcell during septal peptidoglycan synthesis in *E. coli* and is important for the timing of the assembly of the division machinery.

PBP4 localises at midcell during cell division in an FtsEX dependent manner. PBP4 localizes predominantly at midcell during cell division in an FtsEX dependent manner. Inactive PBP4 variants were able to localize at midcell indicating that substrate hydrolysis is not a requirement for localization. However, a version of PBP4 lacking the non-catalytic domain 3 (PBP4 Δ D3), which is required for activity but not β -lactam binding, did not localize to mid-cell. From these results we infer that PBP4 localizes to midcell either by recognizing its substrate

(without the need to hydrolyse it) and/or by protein-protein interactions. Our data also suggest that the bulk of PBP4 localizes late at the division site. Nevertheless, some PBP4 molecules appear to arrive early and may associate to preseptal PG synthesis, which is consistent with the observation that PBP4 is required for the timing of divisome assembly and cell division (Fig 1).

PBP4 might be involved in preseptal PG synthesis. PBP4 interacts with Nlpl, PBP1A and LpoA and these proteins are able to form a ternary complex ((3) and (Fig. 4). PBP4 Δ D3 interacted with PBP1A and LpoA but not with Nlpl, confirming differential PBP4 binding sites of PBP1A, LpoA and Nlpl. The interaction with PBP1A and/or LpoA may contribute to the recruitment of PBP4 at midcell, as the amount of PBP4 at midcell was slightly reduced in Δ mrcA and Δ lpoA strains. PBP1A and its partner LpoA are involved in preseptal PG synthesis (30, 76). PBP4 weakly localizes at preseptal sites in cell filaments generated with aztreonam that have lost septal synthesis but not preseptal synthesis activity. Possibility, some PBP4 enriches at preseptal PG synthesis sites together with PBP1A/LpoA, which localize through the interaction of PBP1A with ZipA (30), and preseptal PG synthesis provide substrates for PBP4. The interaction between Nlpl and PBP4 could function to sequester PBP4 in the lateral wall to prevent its interaction with potential substrate (Fig. 10).

Why does the loss of PBP4 affects the timing of divisome assembly? The absence of PBP4 severely interferes with the timing of the divisome assembly. In most bacteria studied thus far, the Z-ring with its associated early division proteins localizes first to prepare the future cell division site, perhaps by generating the border between the side wall and the future septum (30, 63, 65). After a time delay, the proteins that synthesize the bulk of the septal PG arrive and septation starts (42-45). In the absence of PBP4, there is no time delay between the early and late cell division proteins and a late arrival of FtsZ coincides with the

arrival of FtsN and the initiation of septal PG synthesis. This change of divisome assembly could be caused by direct or indirect effects. PBP4 could be directly needed to open the PG layer to allow insertion of new septal material or to remodel the nascent PG to allow new insertions. Alternatively, the absence of PBP4 might enable Nlpl to bind other EPases, coordinating their activity for remodelling the PG layer during septation. However, the robust localization of PBP4 at midcell suggests a direct function for PBP4 in PG remodelling. We hypothesise that during elongation sufficient PBP4 molecules are retained by Nlpl in the lateral wall above the PG layer to avoid promiscuous PBP4 activity (Fig. 10). This is consistent with the observations that the absence of Nlpl resulted in a reduced amount of PBP4 present in the cells (Fig. S6 and (3)). Nlpl is not enhanced at midcell (3) and therefore we do not anticipate a role for Nlpl in the retention of PBP4 at midcell. PBP4 might be initially recruited to midcell through interactions with PBP1A and LpoA, and possibly the FtsEX-EnvC complex, likely directing PBP4's activity already to preseptal sites (Fig. 10). Once septal peptidoglycan synthesis starts by the mature divisome, more substrate should become available for PBP4 facilitating its accumulation at midcell, which we observed in the immunolocalization experiments. Hence, PBP4 could initially remove crosslinks to help AmiA and AmiB to generate denuded glycan strands. These would facilitate the accumulation of FtsN, which is required to initiate septal PG synthesis. Alternatively, PBP4 could simply remove old glycan strands during preseptal PG synthesis. Because of its impact on the timing of the divisome assembly, the first option seems to be more likely. Whether the function of PBP4 remains the same during septation will need further investigation.

MATERIALS AND METHODS

Culturing conditions. *E. coli* K12 cells were grown to steady state (76) in glucose minimal medium containing 6.33 g of $K_2HPO_4 \cdot 3H_2O$, 2.95 g of KH_2PO_4 , 1.05 g of $(NH_4)_2$, 0.10 g of $MgSO_4 \cdot 7 H_2O$, 0.28 mg of $FeSO_4 \cdot 7 H_2O$, 7.1 mg of $Ca(NO_3)_2 \cdot 4 H_2O$, 4 mg of thiamine and 4 g of glucose per L. For strain MC4100 and its derivatives 50 μ g lysine (Gb1) and for BW25114 and MG1655 derivatives 2 μ g uracil, 20 μ g thymidine, 50 μ g arginine and glutamine (Gb4) per liter pH 7.0 at 28°C or 37°C were added. Absorbance was measured at or 450 nm with a 300-T-1 spectrophotometer (Gilford Instrument Laboratories Inc.). Alternatively, cells were grown in TY (5g yeast extract, 10 g bacto trypton and 85 mM NaCl, pH 7.0), or LB, which is TY with 170 mM NaCl and absorbance was measured at 600 nm.

Strain construction. Strain XL35, the *FtsE* partial deletion strain, was constructed as follows: Primers priXL221 and priXL222 was used to amplify the upstream homologous sequences (*UHS*) from the *ftsE* start codon, and primers priXL223 and priXL224 were used to amplify the downstream homologous sequence after the *FtsE* 151th amino acid (the potential promoter of *ftsX*), from the MC4100 genome. Primers priXL51 and priXL54 were used to amplify the kanamycin resistance cassette from plasmid pKD4 (77). The amplified overlap PCR product (*UHS-FRT-KAN-FRT-DHS*) with primers priXL221 and priXL224 was used to construct the recombinant strain XL34 in which the first 150 amino acids encoding sequencing of *ftsE* was deleted (77). Subsequently, the kanamycin selection cassette was removed with plasmid pCP20 (77) yielding XL35.

Strain XL36, the *ftsE* clean deletion strain, was constructed similarly as XL35. Primers priXL221 and priXL222 were used to amplify the upstream homologous sequences from the *ftsE* start codon, and primers priXL225 and priXL226 were used to amplify the downstream homologous sequences after the *ftsE* stop codon, from the MC4100 genome. In order to restore the *FtsX* expression, a *pTrc99A*down promoter (78) was amplified from plasmid

pSAV057 (79) with primers priXL77 and priXL227 and a chloramphenicol selection cassette was amplified from plasmid pKD3 with primers priXL52 and priXL54. The amplified overlap PCR product (*UHS-FRT-CAM-FRT-DHS*) with primers priXL221 and priXL226 was used to construct the recombinant strain XL33 in which the entire *ftsE* coding sequence was deleted and FtsX was expressed from the chromosomally encoded *pTrc99A* promoter. Subsequently, the kanamycin selection cassette was removed with plasmid pCP20 yielding XL36 (Table 1).

Plasmid construction. The *ftsE* 1181(ts) mutation was amplified from the chromosome of LMC515 (80) using the forward primer priXL187 and the reverse primer priXL188, and sequenced to characterize the mutation. The mutated and the wild-type *ftsE* genes were cloned into pSAV057, p15A origin and Cam^R (79) to produce the plasmids pXL135 and pXL136, respectively. *FtsE* was expressed under the control of the *pTrc99A* down promoter. QuickChange site directed mutagenesis (Agilent technologies, Santa Clara, CA) and Gibson assembly (81) approaches were applied afterwards to construct the inactive *ftsE* mutant plasmids pXL137, pXL138 and pXL139, using the primers listed in Table 2.

To construct the PBP4 expression plasmids, the wild-type *dacB* gene was firstly amplified from the *E. coli* MG1655 genome using primer priXL244 and priXL245, and subsequently cloned into plasmid pSAV057 and pSAV057-dsba^{ss}-mCherry-PBP5 (pNM010 (18)) with *EcoRI* and *HindIII*, to generate the PBP4 plasmids without and with mCherry fusion. Similarly, QuickChange site directed mutagenesis and Gibson assembly approaches were applied afterwards to construct the inactive *dacB* mutant plasmids from these two wild-type plasmids, using the primers listed in Table 2.

Immunolabeling. After reaching steady state for minimal glucose grown cells or the desired OD for rich medium grown cells, the cells were fixed for 15 min by addition of a

mixture of formaldehyde (f. c. 2.8%) and glutaraldehyde (f. c. 0.04%) to the cultures in the shaking water bath and immunolabeled as described (82) with Rabbit polyclonal antibodies against PBP4 or Nlpl (3) preabsorbed against $\Delta dacB$ or $\Delta nlpl$, strains, respectively. As secondary antibody, donkey anti-rabbit conjugated to Cy3 or to Alexa488 (Jackson Immunochemistry, USA) diluted 1:300 in blocking buffer (0.5% (wt/vol) blocking reagents (Boehringer, Mannheim, Germany) in PBS) was used, and the samples were incubated for 30 minutes at 37°C. For immunolocalization, cells were immobilized on 1% agarose in water slabs coated object glasses as described (83) and photographed with an Orca Flash 4.0 (Hamamatsu, Japan) CCD camera mounted on an Olympus BX-60 (Japan) fluorescence microscope through a 100x/N.A. 1.35 oil objective. Images were taken using the program ImageJ with MicroManager (<https://www.micro-manager.org>).

SIM images were obtained with a Nikon Ti Eclipse microscope (Japan) and captured using a Hamamatsu Orca-Flash 4.0 LT camera. Phase contrast images were acquired with a Plan APO 100x/1.45 Ph3 oil objective. SIM images were obtained with a SR APO TIRF 100x/1.49 oil objective, using 3D-SIM illumination with a 561nm laser, and were reconstructed with Nikon-SIM software using the values 0.20-0.25-0.20 for the parameters Illumination Modulation Contrast (IMC), High Resolution Noise suppression (HNS) and Out of focus Blur Suppression (OBS), respectively.

Image analysis. Phase contrast and fluorescence images were combined into hyperstacks using ImageJ (<http://imagej.nih.gov/ij/>) and these were linked to the project file of Coli-Inspector running in combination with the plugin ObjectJ (<https://sils.fnwi.uva.nl/bcb/objectj/>). The images were scaled to 15.28 pixel per μm . The fluorescence background has been subtracted using the modal values from the fluorescence images before analysis. Slight misalignment of fluorescence with respect to the cell contours

as found in phase contrast was corrected using Fast-Fourier techniques as described (76). Data analysis was performed as described (76). In brief, midcell was defined as the central part of the cell comprising 0.8 μm of the axis. From either cell part, midcell and remaining cell, the volume, the integrated fluorescence, and thus the concentration of fluorophores was calculated. The difference of the two concentrations is multiplied with the volume of midcell. It yields FCPlus (surplus of fluorescence) at midcell. For age calculation, all cell lengths are sorted in ascending order. Then the equation

$$\text{age} = \ln(1 - 0.5 * \text{rank} / (n\text{Cells} - 1)) / \ln(0.5)$$

is used, where *rank* is a cell's index in the sorted array, *nCells* is the total number of cells, and *age* is the cell's age expressed in the range 0 .. 1.

Ni²⁺-NTA pulldown assays. Nickel-Nitrilotriacetic acid (Ni-NTA) coupled agarose beads (Qiagen) were used in this assay to retain His-tagged proteins from protein mixtures. Similar to affinity chromatography techniques, the His-tagged protein then becomes a secondary bait protein to bind untagged interaction partners.

Ni-NTA beads (100 μl) were pre-equilibrated with dH₂O and binding buffer (10 mM HEPES/NaOH, 10 mM MgCl₂, 150 mM NaCl, 0.05% Triton X-100, pH 7.5) by centrifugation at 4000 $\times g$, 4 min at 4°C, and then incubated with proteins of interest. Equimolar amounts (1-2 μM) of His₆-tagged and untagged proteins were incubated alone (control) and in combination, for 10 min at 4°C in a 200 μl reaction volume, containing 10 mM binding buffer. An aliquot of this mixture is taken as an 'applied' sample. The protein samples were then added to pre-equilibrated Ni-NTA beads and incubated overnight on a spinning plate at 4°C with 1.3 ml of binding buffer.

Beads were centrifuged at 4000 $\times g$, 4 min, 4°C and washed a further 4-6 times with 1 ml of washing buffer (binding buffer with 30 mM Imidazole) before re-suspending in 250 μl

washing buffer and transferring beads to Proteus spin columns (Generon), centrifuging as described above. Bound proteins were eluted by the addition of 50 µl of SDS-PAGE loading buffer and boiling at 100°C for 5 min. Spin columns were centrifuged a final time at 1500 × *g*, 5 min, RT, to collect ‘eluted’ protein before separation by SDS-PAGE. Eluted samples were run alongside applied samples for comparison.

Microscale thermophoresis. Microscale thermophoresis (MST) is an immobilisation-free method which allows the detection of biomolecular interactions in solution. This technique is based on the specific directed movement of a protein along a heat gradient (thermophoresis), an observation first reported by Carl Ludwig in 1856 (84). The thermophoresis of a protein changes upon ligand binding due to one or more changes to size, charge and/or hydration shell. In MST, a localised heat gradient is initiated by an IR-laser (wavelength 1470 nm). A protein of interest is fluorescently labelled and the change in its thermophoretic mobility, in the presence of an unlabelled ligand, is measured and expressed as a change in the normalised fluorescence (FNorm).

Proteins of interest (10-20 µM) were fluorescently labelled with an amine reactive dye (NT-647- N-hydroxysuccinimide (NHS)), cysteine reactive dye (NT-647 maleimide) or a histidine reactive dye (NT-647-Tris-NTA), according to manufacturer’s instructions (Nanotemper). Fluorescently labelled proteins were diluted to an appropriate concentration and a fixed concentration was titrated against a two-fold serial dilution of unlabelled ligand, across 16 samples, in MST running buffer (25 mM HEPES/NaOH, 150 mM NaCl, 0.05% Triton X-100, pH 7.5).

MST measurements were carried out as described in (85), using standard or premium capillaries on a Monolith NT.115™ MST machine (Nanotemper). Binding curves and Kinetic parameters were plotted and estimated using manufacturer provided software (NT Analysis

1.5.41 and MO. Affinity Analysis (x64)). Capillary scans were carried out prior to all measurements to check for consistent fluorescence counts, confirming that any subsequent change in fluorescence was due to ligand binding and not due to inaccurate pipetting or adsorption and dilution effects.

SDS-denaturation (SD) test. In instances where capillary scans showed a ligand concentration dependant change in raw fluorescence, we investigated whether this was a property of the binding interaction by carrying out an SD-test.

Samples (10 μ L) with the highest and lowest concentration of unlabelled ligand were centrifuged 10 000 $\times g$, 5 min, RT and mixed 1:1 volume ratio with SD-test buffer (40 mM DTT, 4% SDS). Mixtures were boiled at 100 $^{\circ}$ C for 10 min, to abolish ligand binding, before being spun down and subjected to another capillary scan. If the fluorescence between samples that contained the highest and lowest concentration of ligand, after SDS treatment, were now back to $\pm$ 10% of each other, the initial observations were a property of ligand binding and a binding curve was plotted from the raw fluorescence data. If the ligand concentration dependent change in fluorescence was still observed, then this indicated that the fluorescently labelled protein was aggregating and assay and buffer conditions were optimised accordingly

In vivo cross-linking / co-immunoprecipitation assays. Method is described in, and adapted from (86). An overnight culture of *E. coli* BW25113 cells and an appropriate mutant strain was used to inoculate 150 ml of Lennox LB (Fisher Scientific) and was cultivated to an OD₅₇₈ of 0.5-0.6 at 37 $^{\circ}$ C before harvesting by centrifugation (4500 $\times g$, 4 $^{\circ}$ C, 25 min). Cells were resuspended in 6 ml of CL buffer 1 (50 mM NaH₂PO₄, 20% sucrose, pH 7.4). The amine reactive cross-linker, DTSSP (3,3'-dithiobis (sulfosuccinimidyl)propionate) (ThermoFisher), was freshly dissolved (20 mg/ml in dH₂O) and added to the isolated cell suspension and incubated at 4 $^{\circ}$ C

with agitation for 1 h. Cross-linked cells were then harvested by centrifugation ($4500 \times g$, 4°C , 25 min) and resuspended in 6 ml CL buffer 2 (100 mM Tris/HCl, 10 mM MgCl_2 , 1 M NaCl, pH 7.5). DNase, protease inhibitor cocktail and phenylmethylsulfonyl fluoride were added prior to sonication at low levels before ultracentrifugation of the lysate ($140,000 \times g$, 4°C , 1 h). The membrane pellet was resuspended in 2.5 ml of CL buffer 3 (25 mM Tris/HCl, 10 mM MgCl_2 , 1 M NaCl, 1% Triton X-100, 20% glycerol, pH 7.5) and the solubilised membrane extracted o/n with stirring at 4°C .

Samples were ultracentrifuged ($140,000 \times g$, 4°C , 1 h) to remove debris, before removing 2×1.2 ml of each supernatant to be subsequently diluted with 0.6 ml of CL buffer 4 (75 mM Tris/HCl, 10 mM MgCl_2 , 1 M NaCl, pH 7.5). One sample was incubated with an optimised concentration of specific antibody with the other used as a negative control. Both samples were incubated at 4°C with agitation for 5 h. For the isolation of antibodies, and thus cross-linked interaction partners, 100 μl of protein G-coupled agarose bead resin (Roche) were washed ($2 \times$ CL buffer 4, $2 \times$ CL wash buffer [2:1 CL buffer 3 and CL buffer 4]) and added to each sample, and incubated o/n at 4°C with agitation.

Samples were centrifuged and the supernatant retained before washing the beads 10×1 ml with CL wash buffer. After the final wash, beads were resuspended in 250 μl CL wash buffer and transferred to 2 ml spin dry columns and centrifuged to isolate the beads. These were then resuspended in 50 μl of fresh SDS-loading buffer and boiled to elute bound proteins, and reverse cross-linkage, and were collected by centrifugation ($10,000 \times g$, RT, 5 min). Supernatant and elution samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane by Western blotting to detect for specific interaction partners using purified antibodies. The secondary antibody used here is Trueblot Anti-Rabbit IgG-HRP specific for native antibodies.

Peptidoglycan digestion assays. To test for hydrolase activity on PG, 10 µl of sacculi isolated from strains of interest (usually *E. coli* strains MC1061, BW25113 or D456) were incubated with 1-10 µM of respective enzymes, 37°C for between 1 h and overnight, as indicated for respective proteins. The standard reaction conditions were 10 mM HEPES/NaOH, 10 mM MgCl₂, 150 mM NaCl, 0.05% Triton X-100, pH 7.5, in 100 µl reaction volume. Following incubation, samples were boiled at 100°C, 10 min, to terminate reactions before digesting remaining PG overnight at 37°C, with 1 µM cellosyl. The samples were centrifuged at 10,000 × *g* for 5 min, RT, to obtain digested muropeptide products in the supernatant.

To test for activity against soluble muropeptides; first, 100 µl of intact sacculi were incubated overnight at 37°C with 1 µM cellosyl and cellosyl digestion buffer (20 mM NaPO₄, pH 4.8). Next, samples were boiled at 100 °C, 10 min, before centrifugation at 10,000 × *g* for 5 min, RT, to obtain the soluble muropeptides in the supernatant (87). Ten µl of the supernatant was then used as muropeptide substrate for incubating with enzymes of interest, for the appropriate incubation time, at 37°C. Reactions were then terminated by boiling at 100°C for 10 min.

After digestion of respective substrates, products were reduced with NaBH₄, adjusted to pH 4-5 and separated for analysis by reversed-phase HPLC as described below in HPLC analysis.

Reduction of muropeptides with sodium borohydride. PG digestion samples were transferred to 2 ml vials following centrifugation. Muropeptides were reduced in a 1:1 volume ratio of sodium borohydride buffer (0.5 M sodium borate, pH 9.0) and a small spatula of sodium borohydride pellets, centrifuging at 3000 × *g*, 30 min, RT as described in (87). The samples were adjusted to pH 4-5 using HPLC grade phosphoric acid before separation and analysis by reversed-phase HPLC.

Reversed-phase HPLC analysis of mucopeptides.

Following the protocol of (87), reduced mucopeptides were separated for analysis on reversed-phase HPLC systems with a ProntoSil 120-3-C18-AQ 3 µm reversed-phase HPLC column (Bischoff). A linear gradient of solvent A (50 mM sodium phosphate, pH 4.31 supplemented with 0.2% NaN₃) to 100 % solvent B (75 mM sodium phosphate, pH 4.95, 15% methanol) over 90 or 180 min was used to separate mucopeptides at 52°C. Unlabelled mucopeptides were detected at UV absorbance 205 nm. In assays where ¹⁴C-radiolabelled mucopeptides were used, detection was achieved by flowing scintillation cocktail along with standard buffers to give a radioisotope scintillation count (radioactivity CPM). Mucopeptide profiles were recorded and analysed using Laura V4.2.11.129 (LabLogic Systems Ltd.).

Spectrophotometric D-alanine release assay (DD-carboxypeptidase assay).

This protocol was adapted from (58). The carboxypeptidase (CPase) activity of PG hydrolases results in the release of the terminal D-Ala residue from the pentapeptide stem of PG precursors. Using UDP-MurNAc pentapeptide as a substrate, and in this case PBP4, it was possible to spectrophotometrically measure the release of D-Ala.

Each reaction sample consisted of 200 µl of CPase buffer (50 mM HEPES/NaOH, 10 mM MgCl₂, pH 7.6), 3 units of D-amino acid oxidase (Sigma), 6 units of horseradish peroxidase (HRP) (Sigma), Amplex Red (Sigma), and an optimised concentration of protein.

All constituents of the reaction were added and mixed directly in a quartz cuvette (Hellma, 10 mM light path, 15 mM centre), before the addition, and brief mixing by pipette, of purified UDP-MurNAc pentapeptide (BACWAN, Warwick University) to begin the reaction. The released D-Ala residues from the CPase activity of PBP4 are oxidatively deaminated by the action of D-amino acid oxidase to produce pyruvate and hydrogen peroxide (H₂O₂). The released H₂O₂ is reduced to H₂O by HRP using Amplex Red as an electron donor. Oxidised

Amplex Red produces resorufin, which has an intense pink colour and the production of which was measured spectrophotometrically using a Cary 100 Bio UV-visible spectrophotometer (wavelength 555 nm). The change in absorption over 10 min was measured and analysed using the complementing software.

Circular dichroism. Proteins were dialysed overnight against 10 mM NaPO₄, pH 7.5 and concentrated/diluted to 0.4 mg/ml. CD measurements were taken using a Jasco J-810 spectropolarimeter using a wavelength range of 180-250 nm. The average of 10 runs was taken for each protein with a buffer control subtraction. For a direct comparison, correcting for the differing amino acid sequences, the collected data were converted to molecular CD and plotted against wavelength (nm). The resulting CD spectra are compared in Fig. S4 and show that PBP4 lacking domain 3 is folded, consisting of both α -helices (~190, 208 and 222 nm) and β -sheets (~210 nm).

Analytical ultracentrifugation. Purified PBP4 and PBP4 Δ D3 were dialyzed over night against 25 mM HEPES/NaOH, 150 mM NaCl, pH 7.5, in preparation for AUC experiments. AUC sedimentation velocity (SV) experiments were carried out in a Beckman Coulter (Palo Alto, CA, USA) ProteomeLab XL-I analytical ultracentrifuge using absorbance at 280 nm and interference detection. The AUC runs were executed at the rotation speed of 45,000 rpm and the temperature of 20°C using an 8-hole AnTi50 rotor and double-sector aluminium-Epon centerpieces. The sample volume was 400 μ l and the sample concentrations ranged between approximately 0.25 and 1.4 mg/ml. The partial specific volumes ($\bar{v}$) of the proteins were calculated from their amino acid sequence, using the program SEDNTERP (88). Sedimentation velocity boundaries were analyzed using the size-distribution c(s) model implemented in the program SEDFIT <http://www.analyticalultracentrifugation.com> (89). The experimental values of the sedimentation coefficient were converted to the standard conditions (s_{20,w}), which is

the value of sedimentation coefficient in water at 20°C. The size-distribution peaks were integrated to obtain the weight-averaged values for sedimentation coefficient and molecular mass.

The atomic coordinates from the published high-resolution structure of (56) (pdb accession code 2EX2) were used to calculate the sedimentation coefficient values for the monomeric and dimeric forms of PBP4 /PBP4ΔD3 using the program SoMo (90). PBP4 /PBP4ΔD3 crystallographic dimer was built using program PyMol (<https://pymol.org/2/>).

PG binding assay. To assay for interactions of respective proteins with purified PG, we used a PG binding protocol as described in (91). Briefly, ~100 µg purified PG was pelleted by centrifugation (10,000 × *g*, 10 min, 4°C) and resuspended in binding buffer (10 mM Tris/Maleate, 10 mM MgCl₂, 50 mM NaCl, pH 6.8). PG was incubated with 10 µg of desired protein before incubating on ice for 30 min and centrifuging at 10,000 × *g*, 10 min, 4°C. An aliquot of the supernatant (S) was taken for SDS-PAGE. The pelleted PG was washed with 200 µl binding buffer by centrifugation as before and the wash supernatant was taken as sample W for SDS-PAGE. The pelleted PG was then resuspended in 100 µl of 2% SDS and incubated for 1 h on a stirring plate, 4°C. Mixtures were centrifuged a final time at 10,000 × *g*, 10 min, 4°C, with the supernatant, now containing any initially bound protein, taken as the pellet sample (P). A negative control sample with no PG was assayed in parallel to determine that binding of protein was specific. Samples, along with corresponding controls, were analysed by SDS-PAGE and visualised by Coomassie staining. Presence of protein band in pellet sample (P) indicated binding to PG, whilst presence in supernatant sample (S) indicated no binding.

Bocillin binding assay. A fluorescent-bocillin binding assay was used to determine whether a PBP was purified with a correctly folded active site. Bocillin FL is a commercially available dye-β-lactam conjugate, synthesised from penicillin V and BODIPY (Molecular

Probes, Inc., Eugene, Oreg.). Ten µg of purified PBP was incubated for 10 min at 37°C, with 20 ng of Bocillin FL in a 50 µl volume with 10 mM HEPES/NaOH, 10 mM MgCl₂, pH 7.5. A negative control sample was pre-incubated with 1 mM ampicillin for 30 min, 37°C, to block the PBP active site, prior to incubation with Bocillin FL. After incubation, samples were boiled at 100 °C for 10 min with 40 µl of SDS-PAGE loading buffer and resolved by SDS-PAGE. Fluorescent signal was observed using Typhoon Fluorescence-imager (Excitation; 488 nm, emission; 520 nm, BP20 PMT 400-600 V), followed by visualisation of gels by Coomassie staining. Proteins incubated with ampicillin should have no detectable binding of Bocillin FL. For whole cells, a culture was grown in 5 mL TY and induced with 10 µM IPTG for 3 mass doublings to an OD₆₀₀ of 0.5 and the cells were pelleted and washed once with PBS (8 g NaCl, 1.8 g Na₂HPO₄, 0.24 g KH₂PO₄ per liter, adjusted to pH 7.4) at 4°C and resuspended in 100 µl PBS with 5µg/ml Bocillin FL and incubated for 20 min at RT. To remove the excess Bocillin FL, the samples were pelleted and resuspended in 100 µl PBS with cOmplete™ Mini Protease Inhibitor Cocktail (Sigma) and 2 U DNase I (NEB). Then sonicated on ice for 10 sec and boiled 15' at 99°C after adding 25 µL of SDS-PAGE 5x loading buffer and resolved by SDS-PAGE. Fluorescent signal was observed using a home-made imager for Midori green, followed by visualisation of gels by Coomassie staining.

***In vitro* PG synthesis assay with radiolabelled lipid II.** The assay was published by Bertsche et al., (92). Lipid II (1.2 nmol, 11,000 dpm) was vacuum dried and dissolved in 5 µl of methanol. The reaction was performed in a total volume of 50 µl in 25 mM Tris/HCl, 10 mM MgCl₂, 100 mM NaCl, 0.05% Triton X-100, pH 7.5 (buffer B), with 0.5 µM PBP1A, 1 µM LpoA, 1 µM PBP4(S62A), 2 µM Nlpl. The reaction was performed for 1 h at 30°C. The pH of the samples was adjusted to 4.8 prior to boiling for 5 min followed by a digestion with 50 g/ml cellosyl for 1 h at 37°C and boiling for 5 min.

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J.V., A.L., H.C.L.Y, X.L., A.S.S., and M.B did most experiments. A.T., M.B., V.W., and T.B. designed most experiments. M.B, W.V and TdB analyzed the data and wrote the manuscript. All authors critically read the manuscript and provided feedback.

TABLE 1 Strain and plasmids used in this study.

Strain	Mutation	Genotype	Source
LMC500 (MC4100)	Wild-type	<i>F⁻, araD139, Δ(argF-lac)U169, deoC1, flbB5301, lysA1, ptsF25, rbsR, relA1, rpsL150</i>	(80)
BL21(DE3)	Overexpression strain	<i>F⁻ ompT, dcm lon hsdS hsdSB(rB-mB-) λ(DE3)</i>	Novagen
BW25113	Keio background strain	<i>F⁻, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB)568, hsdR514</i>	(77)
DH5α	Storage strain	<i>F⁻ endA1 glnV44 thi-1 recA1 φ80ΔlacZΔM15, λ⁻</i>	Invitrogen
MC1061	Laboratory strain	<i>Δ(araA-leu)7697 Δ(lac)X74 galk16 galE15(GalS) λ⁻e14- mcrA0 relA1 rpsL150(str^R) spoT1 mcrB1 hsdR2</i>	(93)
LMC509	FtsZ (ts)	MC4100 <i>ftsZ184</i>	(80)
LMC511	FtsA (ts)	MC4100 <i>ftsA10</i>	(80)
LMC512	FtsA (ts)	MC4100 <i>ftsA1882</i>	(80)
LMC515	FtsE (ts)	MC4100 <i>ftsE1181</i>	(80)
LMC531	FtsQ (ts)	MC4100 <i>ftsQ(ts)</i>	(80)

LMC2487	FtsW (ts)	JLB17; F ⁻ <i>thr, trp, his, thy, ara, lac, gal, xyl, mtl, rspL, tonA, ftsW</i> amino acid replacement G311D	(94)
LMC510	PBP3 (ts)	MC4100 <i>ftsI</i> 2158	(80)
BCB677	ΔPBP1A	BW25113Δ <i>mrcA</i>	(95)
BCB678	ΔPBP1B	BW25113Δ <i>mrcB</i>	(95)
BCB192	ΔLpoA	BW25113Δ <i>lpoA</i>	(95)
LMC2217	ΔLpoB	BW25113Δ <i>lpoA</i>	(95)
BCB655	ΔPBP4	BW25113Δ <i>dacB</i>	(95)
BCB904	ΔNlpl	BW25113Δ <i>nlpl</i>	(95)
BCB1363	ΔPBP4ΔLpoA	BW25113Δ <i>dacB::kan^R, ΔlpoA</i>	This work
BCB744	ΔFtsE	BW25113Δ <i>ftsE kan^R CAG60397</i>	This work
BCB746	ΔFtsEX	BW25113Δ <i>ftsEX cat^R CAG60399</i>	This work
BCB1081	ΔFtsE 1-150	XL34 LMC500Δ <i>ftsE 1-150::kan-ftsX</i>	This work
BCB849	ΔFtsE 1-150	XL35 LMC500Δ <i>ftsE 1-150::ftsX</i>	This work
BCB1082	ΔFtsE	XL33 LMC500Δ <i>ftsE::cam-pTrc99A</i> down <i>ftsX</i>	This work
BCB850	ΔFtsE	XL36 LMC500Δ <i>ftsE::pTrc99A</i> down <i>ftsX</i>	This work

Plasmid	Name	Characteristics	Source
pET21b	pET21b-PBP4(S62A)Δ1-60	Inactive PBP4 lacking residues 1-60 (Amp ^R)	(3)
pET21b	pET21b-His-PBP4(S62A)Δ1-60	For purification of His-PBP4 (active site mutant) (Amp ^R)	(3)
pET21b	pET21b-PBP4Δ1-60	Native PBP4 lacking residues 1-60 (Amp ^R)	(3)
pET28a	pET28a-His-LpoA(sol)	Soluble LpoA (LpoAΔ1-27) construct, N-terminal His-tag, Kan ^R	(66)
pET28a	pET28-His-LpoA ^N	Purification of His-LpoA ^N	(66)
pET28a	pET28-His-LpoA ^C	Purification of His-LpoA ^C	(66)
pTK1A-His	pTK1A-His	Full length His-PBP1A, Kan ^R	(66)
pET28a-	pET28a-His-Nlpl	Soluble Nlpl construct, N-terminal His-tag, Kan ^R	(6)
pBAD18	pBAD18-His-PBP4ΔD3	for purification of His- PBP4 lacking domain 3 (Amp ^R)	This work
pXL86	pTHV mCh-FtsE wt	pTrc 99A down expressing mCh-FtsE(wt) colE1 ori, Amp ^R	This work
pXL89	pTHV mCh-FtsE ts	pTrc 99A down expressing mCh-FtsE(ts) (P135S) colE1 ori, Amp ^R	This work
pXL110	pSAV mNG-NNN-FtsE	pTrc 99A down expressing mNG-FtsE, p15A origin, Cam ^R	This work
pXL133	pSAV-PBP4	pTrc 99A down expressing PBP4, p15A origin, Cam ^R	This work

pXL134	pSAV-PBP4 S62G	pTrc 99A down expressing PBP4 S62G, p15A origin, Cam ^R	This work
pXL135	pSAV-FtsE	pTrc 99A down expressing FtsE, p15A origin, Cam ^R	This work
pXL136	pSAV-FtsE(ts)	pTrc 99A down expressing FtsE(ts), p15A origin, Cam ^R	This work
pXL137	pSAV-FtsE K41Q	pTrc 99A down expressing FtsE K41Q, p15A origin, Cam ^R	This work
pXL138	pSAV-FtsE D162A	pTrc 99A down expressing FtsE D162A, p15A origin, Cam ^R	This work
pXL139	pSAV-FtsE E163A	pTrc 99A down expressing FtsE E163A, p15A origin, Cam ^R	This work
pXL140	pSAV-PBP4-ΔD3	pTrc 99A down expressing PBP4Δ173-247 (domain 3), p15A origin, Cam ^R	This work
pXL150	pSAV-PBP4-D155A	pTrc 99A down expressing PBP4 D155A, p15A origin, Cam ^R	This work
pXL151	pSAV-PBP4-R361A	pTrc 99A down expressing PBP4 DR361A, p15A origin, Cam ^R	This work

TABLE 2 Primers used in this study.

Primer	Sequence 5'-3'	Purpose
priXL51	ACCATGGCTAATTCCCATGTCAG	GA_pkd3_F
priXL54	GTGTAGGCTGGAGCTGCTTC	PCR_pkd3_R
priXL187	CCGGCCGAATTCAACAACAACCTCGATGATTCGCTTTGAACATGTCAGC	FtsE-EcoRI fw
priXL188	GGCCAAGCTTTTATTCATGGCCACGCCTCC	FtsE-EcoRI rv
priXL221	CATGAAGCCGTTGGCTTAACC	ΔFtsE-FA-F
priXL222	TAGGAACTTCGAAGCAGCTCCAGCCTACACTGTTAATCCTCTC GGGCAAAAAG	ΔFtsE -FA-R
priXL223	GGATATTCATATGGACCATGGCTAATCCCATGTCAGGCGGT	ΔFtsE- 1-150
priXL224	GGTGAACAAGCCCGC	ΔFtsE-1:150
priXL225	GGTGAGCATGCGATAGGAACG	ΔFtsE-clean
priXL226	TGAGCGGATAACAATTTACACAGGAAACAGACCATGAATAA GCGCGATGCAATC	ΔFtsE-clean
priXL230	CTTTTCAGATCCTGCAATGCG	FtsE-K41Q-F
priXL231	ATTCGGGCGCAGGGCAAAGTACTCTCCTGAAGCTG GATCAGCTTCAGGAGAGTACTTTGCCCTGCGCCGGA	FtsE-K41Q-R

priXL232	CGGTACTGCTAGCGGCTGAACCGACTGGTAACCTG	FtsE-D162A-F
priXL233	AGGTTACCAGTCGGTTCAGCCGCTAGCAGTACCGC	FtsE-D162A-R
priXL234	GTAAGTCTAGCGGACGCTCCGACTGGTAACCT	FtsE-E163A-F
priXL235	AGGTTACCAGTCGGAGCGTCCGCTAGCAGTACCGC	FtsE-E163A-R
priXL236	AGATGGCGCTGCCTGCCGGCACCCAGAAAG	PBP4-S62G-F
priXL244	GCGCAATTcatgCGATTTTCCAGATTATCA	EcoRI-PBP4-F
priXL245	GCGCAAGCTTctaATTGTTCTGATAAATATC	HindIII-PBP4-R
	GTTGACCGCAACGGTGGCGGAAGCGGGTCTGTGCAGGATGG	
priXL250	AGCCAGCTATG	PBP4 Δ D3-F
	TCCATCCTGCACAGACCCGCTTCCGCCACCGTTGCGGTCAACT	
priXL251	ATGGCGGC	PBP Δ D3-R
priXL266	CCGGCTGGCCATGGAATGCCATGACACAATGCTTTAGCGCTC	PBP4-D155A-F
	GGAGCGCTAAAGCATTGTGTCATGGCATTCCATGGCCAGCCG	
priXL267	G	PBP4-D155A-R
	GCCGATGGTTCAGGGCTTTCCGGCGCATAACCTGATTGCCCCC	
priXL268	GCC	PBP4-R361A-F
	GGCGGGGGCAATCAGGTTATGCGCCGAAAGCCCTGAACCAT	
priXL269	CGGC	PBP4-R361A-R

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986

Midcell localization of PBP4 of *Escherichia coli* is essential for the timing of divisome assembly

Jolanda Verheul^{1#}, Adam Lodge^{2,7#}, Hamish C.L. Yau^{2,8#}, Xiaolong Liu^{1,9#}, Alexandra S. Solovyova³, Athanasios Typas^{4,5}, Manuel Banzhaf^{6*}, Waldemar Vollmer^{2**}, and Tanneke den Blaauwen^{1***}

¹Bacterial Cell Biology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam; Science Park 904, 1098 XH Amsterdam, The Netherlands.

²Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, NE2 4AX, United Kingdom.

³NUPPA, Leech Building, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

⁴European Molecular Biology Laboratory, Genome Biology Unit, Heidelberg, Germany

⁵European Molecular Biology Laboratory, Structural & Computational Unit, Heidelberg, Germany

⁶Institute of Microbiology & Infection and School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK. B15 2TT

⁷Present address: Iksuda Therapeutics, The Biosphere, Draymans Way, Newcastle Helix, Newcastle upon Tyne, NE4 5BX, UK

⁸Present address: Procter & Gamble, Newcastle Innovation Centre, Whitley Road, Newcastle-Upon Tyne, NE12 9TS, UK.

⁹Department of Biochemistry, University of Oxford, OX1 3QU, Oxford, United Kingdom.

#contributed equally.

*Corresponding author. Tel: +44 121 4145586; Email: m.banzhaf@bham.ac.uk

**Corresponding author. Tel: +44 191 208 3216; Email: w.vollmer@ncl.ac.uk

***Corresponding author. Tel: +31 20 525 3852; Email: t.denblaauwen@uva.nl

FIGURES AND LEGENDS

FIG 1 Structure of PBP4 and its localization at midcell and in the lateral wall during fast growth. A. Crystal structure of PBP4 in which the three domains (numbers 1-3) and some of the residues that are essential for its endopeptidase activity are indicated. B. MC4100 cells were grown exponentially in TY medium at 37°C to an OD600 of 0.3 and fixed and immunolabeled with BW25113 Δ *dacB* pre-adsorbed anti-PBP4. Upper panel phase contrast image corresponding to the SIM fluorescence image in the lower panel. Scale bar equals 2 μ m C. BW25113 wild-type cells were grown in TY at 37°C and harvested in the exponential phase

at an OD600 of 0.3 and fixed. Samples were divided into three aliquots and cells were immunolabeled with antibodies against PBP3, FtsN and PBP4. The first aliquot was immunolabeled without any treatment, the second after permeabilizing the cells with TritonX-100 (right: phase contrast and fluorescence images) and the third after permeabilizing cells with Triton-X100 and lysozyme (left: phase contrast and fluorescence images). The scale bar equals 5 μ m. PBP4, but not PBP3 or FtsN, is accessible without degrading the peptidoglycan layer.

FIG 2 Activity of PBP4 is not required for localization. Cells were grown in minimal glucose medium to steady state at 28°C, fixed and immunolabeled with antibodies against PBP4. (A) The extra fluorescence at midcell (FCPlus) in the Δ *dacB* strain transformed with the empty plasmid (EP, yellow), or plasmids expressing wild-type PBP4 (black), PBP4S63G (blue), PBP4D155A, (purple) PBP4R361A (red), PBP4 Δ D3 (green) was determined and plotted as function of the cell division cycle age as in bins of 5% age classes with the error bar indicating the 95% confidence interval. (B) Demographs of the localization fluorescence pattern of the PBP4 variants shown in (A) with the cells sorted according to their cell length. The white line indicates the length of the cells. Intensity scaling is identical for all maps. Number of cells analyzed for each immunolabeling was at least 2000 cells.

FIG 3 PBP1A and LpoA contribute to PBP4 midcell localization. Cells were grown exponentially to an OD of 0.3 in TY at 37°C, then fixed and immunolabeled with antibodies against PBP4. Because fluorescence imaging by microscopes is usually not directly comparable between different experiments, all results in each experiment were normalized to the parental strain BW25113. (A) PBP4 fluorescence at midcell per μm circumference of the cell. (B) Concentration of PBP4 in the cells. (C) length of the cells. (D) Diameter of the cells. $\Delta lpoA$ (n=5), $\Delta lpoB$ (n=5), $\Delta mrca$ (PBP1A, n=2), $\Delta mrcaB$ (PBP1B, n=2). Each point is the average of 1000-2000 cells. Based on the one way Anova the difference in midcell localization is

significant ($P=0.009$) while the difference in concentration of PBP4 is not significant ($P=0.0484$).

FIG 4 PBP4 interacts with PBP1A, LpoA and Nlpl. (A) Summary of apparent K_D values of the interactions of PBP4 or a version lacking domain 3 (PBP4 Δ D3) with LpoA, the C-terminal domain of LpoA (LpoA^C), the N-terminal domain of LpoA (LpoA^N), Nlpl and PBP1A determined by microscale thermophoresis (MST). Corresponding binding curves are shown in Suppl. Figure S7. (B) Pulldown experiment showing that domain 3 of PBP4 is required for interaction with Nlpl. Oligo-histidine tagged Nlpl (His-Nlpl) pulled down PBP4 to Ni-NTA beads. His-tagged PBP4 Δ D3 did not pull down untagged Nlpl. A, applied sample; E, eluted sample. (C) *In vivo* cross-linking/co-immunoprecipitation showing interaction between PBP1A and PBP4. Growing cells of BW25113 (wt) or BW25113 Δ *dacB* (Δ PBP4) were chemically cross-linked with

93 DTSSP and cell extract was immunoprecipitated with purified anti-PBP4 antibody. Control
 94 samples did not receive anti-PBP4 antibody. The cross-linker was cleaved by reducing agent
 95 and proteins were separated by SDS-PAGE and transferred to a membrane, followed by
 96 detection of PBP1A with specific antibodies. PBP1A was detected in the elution of the sample
 97 from wild-type and not from Δ PBP4. (D) *In vivo* cross-linking/co-immunoprecipitation (as in
 98 panel C) showing that PBP4 and be cross-linked with LpoA in cells.
 99

FIG 5 PBP4 or NlpI do not affect the activity of PBP1A whereas LpoA reduces the activity of PBP4. (A) Summary of the results from an *in vitro* PG synthesis assay with PBP1A (in the presence or absence of LpoA) and radio-labelled lipid II substrate. The presence of NlpI, catalytically inactive PBP4(S62A) or both together, does not affect the cross-linkage of the PG product of a PBP1A or PBP1A/LpoA reaction (left side), and NlpI, PBP4(S62A) or both together,

don't induce enhanced carboxypeptidase activity of PBP1A, which would be seen as a higher content of tetrapeptides (right side). Values are mean $\pm$ variation of two independent repeats. Example chromatograms are shown in Figure S8. (B) The presence of LpoA modestly reduces the activity of PBP4 in a DD-carboxypeptidase assay using the substrate *N*-acetyl-L-Lys-D-Ala-D-Ala. (C) LpoA also reduces slightly the activity of PBP4 against PG sacculi from BW25113, as seen by the reduced digestion of TetraTetra upon a 30 min incubation period. (D) The $\Delta dacB$ and $\Delta dacB\Delta lpoA$ mutant strains are equally sensitive to overexpression of PBP4. The average growth over time as measured by OD650 nm is plotted against the added IPTG concentration. The culture grown in TY with glucose to suppress the expression is represented by -5 on the X-axis. All other data points are from cell grown in TY without glucose (n=4).

FIG 6 Cell division cycle timing of the localization of PBP4. (A) MC4100 cells were grown to steady state in minimal glucose medium at 28°C and immunolabeled with antibodies specific for PBP4. Phase contrast (top) and fluorescence is shown (bottom). Scale bar equals 2 μ m. (B)

The extra fluorescence at midcell compared to the rest of the cell (FCPlus) is plotted as function of the normalized cell division cycle age. The dots (grey for MC4100 and light pink for the BW25113 $\Delta dacB$ strain) are the values measured for the individual cells and the markers with bars (black for the wild-type MC4100, red for the BW25113 $\Delta dacB$) are 5% age bins with 95% confidence. (C) Comparison of the timing of the localization at midcell of FtsZ (grey), PBP4 (blue), PBP3 (green) and FtsN (black) and the general membrane stain bodipy-12 (yellow) during the cell division cycle age. The FCPlus values are min-max normalized to enable timescale comparison, despite differences in molecule number and antibody affinities.

FIG 7 PBP4 localization is dependent on the presence of the proto-ring. Isogenic strains producing different temperature sensitive (ts) versions of cell division proteins where grown to steady state in Gb1 medium at 28°C and split in two parts. These were 1:4 diluted in prewarmed medium and grown for two mass doublings (MD) at either 28°C or 42°C. The cells

135 were fixed, labeled with antibodies specific for PBP4 (a4) and, in the case of FtsA (ts), with
 136 antibodies against FtsZ (aZ). From left to right: the phase contrast, corresponding
 137 fluorescence image of the PBP4 labeling and the merged images at the permissive
 138 temperature, demograph of PBP4 fluorescence localization where cells are sorted according
 139 to their cell length (contours in white). This is followed by the same series from the non-
 140 permissive temperature samples. The number of cells analyzed were 3641 and 1642 for
 141 LMC511 FtsA (ts), 2809 and 1576 for LMC515 FtsE(ts), 3833 and 1203 for LMC531 FtsQ (ts)
 142 and 3876 and 926 for LMC510 PBP3(ts), for the cells grown at 28°C and 42°C, respectively.
 143 The scale bar equals 5 μ m.

FIG 8 PBP4 is recruited by the septal cleavage complex FtsEX. (A) Cells of BW25113 expressing the proteins indicated were grown in LB at 37°C to OD₆₀₀ = 0.3, fixed and labeled with specific antibodies against PBP4. From left to right, the phase contrast, corresponding fluorescence image of the PBP4 labeling, and the merged former two images, map of fluorescence with the cell lengths outlined (with the same contrast and brightness for all maps) of PBP4 localization where cells are sorted according to their cell length are shown. The number of cells analyzed were 687 for $\Delta envC$, 434 for $\Delta ftsE$, and 346 for $\Delta ftsEX$. The scale bar equals 5 μm . (B) Cells of LMC500 or XL36 (LMC500 $\Delta ftsE::pTrc99A$ down $ftsX$) expressing wild-

type or active site versions of FtsE were immunolabeled with anti-PBP4 antibodies. Cells were grown in TY at 30°C and FtsE expression from plasmid was induced for 2 mass doubling with 30 μ M IPTG, and cell were fixed and harvested at an OD₆₀₀ of 0.3. The maps of PBP4 fluorescence with the cell lengths outlined is shown for the indicated strains. Number of cells analyzed: LMC500 (2462), (wt) (2588), (ts) (2540), K41Q (728), D162A (554), and E163E (1052).

FIG 9 Localization of PBP4 depends on the presence of domain 3 but not on activity. Cells

were grown in minimal glucose medium to steady state at 28°C, fixed and immunolabeled with the indicated antibodies. (A and B) Graphs with the percentage of constricting cells and the average cell length in μm for the various mutants expressed from plasmid in the ΔdacB strain and also of the parental strain BW24113 ($n = 4$). (C) The concentration of PBP4 in fluorescent units per μm^3 in these cells for a representative experiment (out of the four repeats).

(D) Normalized FCplus of FtsN, FtsZ, and PBP4 plotted against normalized cell division cycle age for the parental strain BW25113, the ΔdacB strain carrying plasmids for the expression of PBP4WT, PBP4S63G, PBP4D155A, PBP4R361A or no protein (EP) showing that despite having a relative normal length and constriction percentage, FtsZ and FtsN localize rather late at midcell in the ΔdacB strain. Plasmids were not induced.

FIG 10 Model of the organization of PBP4 in the periplasm of *E. coli*. In a minimal medium grown cell of average size of 3 by 0.86 μm 230000 Lpp trimeric proteins are present of which 1/3 is bound to PG. (A) During elongation PBP4 (or a fraction of the PBP4 molecules) is kept away from its substrate through its interaction with NlpI and possibly LpoA. (B) PBP1A and LpoA associate with ZipA and the Z-ring to assist in preseptal PG synthesis. Because of their presence, the absence of NlpI and the newly synthesized PG, PBP4 is attracted by PBP1A and LpoA. More PBP4 is accumulating at midcell and becoming clearly visible by immunolabeling upon maturation of the divisome and synthesis of septal PG. All proteins apart from the FtsZ polymer have been drawn according to their crystal structure shape and hydrated crystal structure sizes (scale bar equals 4 nm). The membranes are assumed to be 2 nm and the distance between the outer and the inner membrane 21 nm.