The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in Escherichia coli

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During the cell cycle of rod-shaped bacteria, two morphogenetic processes can be discriminated: length growth of the cylindrical part of the cell and cell division by formation of two new cell poles. The morphogenetic protein complex responsible for the septation during cell division (the divisome) includes class A and class B penicillin-binding proteins (PBPs). In Escherichia coli, the class B PBP3 is specific for septal peptidoglycan synthesis. It requires the putative lipid II flippase FtsW for its localization at the division site and is necessary for the midcell localization of the class A PBP1B. In this work we show direct interactions between FtsW and PBP3 in vivo and in vitro by FRET (Förster resonance energy transfer) and co-immunoprecipitation experiments. These proteins are able to form a discrete complex independently of the other cell-division proteins. The K2–V42 peptide of PBP3 containing the membrane-spanning sequence is a structural determinant sufficient for interaction with FtsW and for PBP3 dimerization. By using a two-hybrid assay, the class A PBP1B was shown to interact with FtsW. However, it could not be detected in the immunoprecipitated FtsW–PBP3 complex. The periplasmic loop 9/10 of FtsW appeared to be involved in the interaction with both PBP1B and PBP3. It might play an important role in the positioning of these proteins within the divisome.

INTRODUCTION

In every generation, bacteria require morphogenetic protein machineries to produce two identical sacculi by the enlargement and division of one sacculus (Begg et al., 1990; Nanninga, 1998; Errington, 2003). The wall peptidoglycan is a key element in preserving the cell integrity and is related to the bacterial morphogenesis. Peptidoglycan synthesis is catalysed by penicillin-binding protein (PBP) classes A and B (the targets of β-lactams) (Goffin & Ghysen, 1998). Escherichia coli contains three transglycosylase/transpeptidase class A PBPs, PBP1A, PBP1B and PBP1C, which catalyse the formation of polymeric peptidoglycan, and two class B PBPs, the monofunctional transpeptidases PBP2 and PBP3 (also called FtsI), which catalyse peptidoglycan synthesis in coordination with a number of other proteins in two morphogenetic networks, the elongasome during elongation and the divisome during division (den Blaauwen et al., 2008).

The components of the divisome include at least 12 essential cell-division proteins, FtsZ, FtsA, ZipA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, FtsW, PBP3, FtsN, and a number of other proteins including the main peptidoglycan polymerase PBP1B, and the AmiC and EnvC hydrolases (den Blaauwen et al., 2008). To initiate cell division, the GTP-binding tubulin-like FtsZ protein forms an intracellular ring at the division site (Addinall & Lutkenhaus, 1996; Löwe & Amos, 1998). It is stabilized by FtsA, ZapA and ZipA, and this Z-ring serves as a cytoskeletal scaffold for the assembly of the other proteins of the cell-division machinery (den Blaauwen et al., 2008), which assembles in two steps (Aarsman et al., 2005). Some proteins can form a subcomplex, such as FtsQ, FtsL and FtsB (Buddelmeijer & Beckwith, 2004).
The cytoplasmic transmembrane protein FtsW includes 10 transmembrane segments (TMSs) (Lara & Ayala, 2002) and belongs to the SEDS family (for shape, elongation, division and sporulation) (Henriques et al., 1998) (Fig. 1). It interacts with PBP3, FtsQ, FtsL and FtsN in a bacterial two-hybrid assay (Di Lallo et al., 2003; Karimova et al., 2005) and is required to recruit PBP3 to the division site (Mercer & Weiss, 2002). It has been proposed to integrate signals between the cytoplasmic (FtsZ, FtsA, ZipA and FtsK) and the periplasmic (FtsK, FtsQ, FtsL, FtsB, PBP3 and FtsN) components of the divisome (Margolín, 2000), but also to function as a flippase to translocate the lipid II from the cytoplasm to the periplasmic machinery of peptidoglycan assembly (Matsuhashi, 1994). The observation that a ZapA–FtsW fusion and a ZapA–PBP3 fusion can recruit PBP3 and FtsW, respectively, in cells depleted of FtsA or FtsQ to the divisome in cells without a visible constriction suggests the formation of a pre-formed complex between these proteins (Goehringer et al., 2006). The interaction could take place through one of the TMSs of FtsW, as the first 56 residues of PBP3 (containing the TMS) are sufficient to localize it to the division site (Piette et al., 2004). The 9/10 loop of FtsW (between TMSs 9 and 10) could also be involved, since the double FtsW(P368A;P375A) mutant (Fig. 1), while localizing itself at midcell, prevents the interaction of PBP3 with FtsN, which interacts with PBP1B. FtsN stimulates PBP1B activity (Müller et al., 2007). Thus FtsN might coordinate or modulate the activities of a PBP1B–PBP3 complex in the cell.

In this work, we report a direct interaction between FtsW and PBP3 by in vivo FRET ( Förster resonance energy transfer) experiments and in vitro isolation of the FtsW–PBP3 complex by co-immunoprecipitation. FtsW was also shown to interact with PBP1B using a two-hybrid assay, and the periplasmic loop 9/10 of FtsW seemed to be involved in this interaction. A three-protein complex FtsW–PBP3–PBP1B could not be isolated.

**METHODS**

**Bacterial strains, oligonucleotides and media.** Bacterial strains are described in Table 1. Oligonucleotides were from Eurogentec. The rich medium used was Luria–Bertani (LB) medium supplemented with ampicillin (50 or 100 µg ml⁻¹), chloramphenicol (20 or 30 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹) and/or IPTG (0.01, 0.1 or 1 mM) when appropriate. For the FRET experiment, the minimal medium GB1 was used (Bertsche et al., 2006).

**Plasmid construction**

**pDML2454, pDML2455 and pDML2456.** Plasmid pDML2487 carrying the fragment encoding GFP–PBP3(K2–V42) (Piette et al., 2004) was digested by Smal and Scal and cloned into the Smal and Scal sites of pBT/fsls–linker (Bertsche et al., 2006). The NotI–HindIII DNA fragment encoding PBP3(M1–V42) was excised from the resulting plasmid and inserted into the corresponding sites of pDML2453 to create pDML2454, which allows the production of T18–(G4S)₃–PBP3(M1–V42).

The modified fsl gene encoding PBP3(R23C) or PBP3(L39P) was amplified by PCR using plasmids pDSW562 or pDSW566 (gift of D. Weiss) as template and oligonucleotides 5′-TAGGCGGCCGCAGTTAAGGACGTCATAGAAACCGCGGGGAAACCCGGCAGCA and 5′-GTTCTAGGGCTGGAATACCAAACTGGAAC as primers (NotI, SacI and NrdI sites are underlined). The PCR fragment was cloned into pGEM-T Easy (Promega), sequenced, digested with NotI and SacI and inserted into the same sites of pDML2451 (Bertsche et al., 2006). The resulting plasmids pDML2455 and pDML2456 code for T18–(G4S)₃–PBP3(R23C) and T18–(G4S)₃–PBP3(L39P), respectively.

**pDML2445.** The fragment resulting from the Ndel and HindIII digestion of plasmid pDML2413 encoding FtsW(P368A;P375A) (Pastoret et al., 2004) was inserted into the same sites of pDML2424 encoding HA tag–FtsW (Pastoret et al., 2004). The Ndel–HindIII fragment encoding the HA tag–FtsW mutant was exchanged with the corresponding fragment of pDML2443a encoding T25–(G4S)₃–FtsW (Derouaux et al., 2008). The resulting plasmid pDML2445 allows the production of T25–(G4S)₃–FtsW(P368A;P375A)–HA tag.

**pSA060, pSA062, pSA063, pSA066, pSA068 and pSA069.** Fragments resulting from the EcoRI and HindIII digestion of the pDSW234 (Weiss et al., 1999) and pDML2414 (Pastoret et al., 2004) plasmids, carrying the coding sequences for PBP3 and FtsW respectively, were inserted between the same sites of pSAV047 (mCherry) and pSAV058 (mKO) to give pSA060, pSA063, pSA066 and pSA069, coding for the fusion proteins mCh–PBP3, mCh–FtsW, mKO–PBP3 and mKO–FtsW, respectively. The fusion GFP–PBP3(K2–V42) (Piette et al., 2004) was digested by EcoRI and HindIII and the fragment cloned into the corresponding sites of pDML2454, pDML2455 and pDML2456.

*Fig. 1.* Topology of the transmembrane protein FtsW and the transpeptidase PBP3. The positions of the modified amino acids discussed are shown. The HA tag is inserted between R411 and G412.

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pSAV047 (mCherry) and pSAV058 (mKO), yielding pSA062 and pSA068, coding for mCherry–PBP3(K2–V42) and mKO–PBP3(K2–V42), respectively. The mKO sequence was obtained from pmKO1-S1 (MBL, Nagoya, Japan). Tandem (mCherry–mKO, pSAV050), mCherry (pSAV047), mKO (pSAV058), and ‘empty’ plasmids were used for transfection as a positive control, mKO and mCherry expressed as two separate proteins in the cell, mCherry–mKO tandem expressed with unfused mKO, and mKO–mCherry tandem expressed with unfused mCherry as negative controls.

Measurement of β-galactosidase activity in liquid cultures. β-Galactosidase activity was measured in cell extracts from liquid cultures as described by Karimova et al. (2005). A level of β-galactosidase activity at least fourfold higher than that measured for E. coli BTH101 cells producing T18/T25 (16 or 72 U per mg dry weight of bacteria without or with IPTG respectively) was considered to indicate an interaction.

FRET experiment. For the FRET experiment we used a red-shifted fluorescent protein pair with mKO (Karasawa et al., 2004) as a donor and mCherry (Shaner et al., 2004) as an acceptor. E. coli LMC500 co-transformed with two appropriate vectors. When a single protein was to be expressed, a non-coding second plasmid was co-transformed. Each experiment included the mKO–mCherry tandem fusion as a positive control, mKO and mCherry expressed as two separate proteins in the cell, mCherry–mKO tandem expressed with unfused mKO, and mKO–mCherry tandem expressed with unfused mCherry as negative controls. Transformants were grown in TY, 0.4 % glucose (w/v) supplemented with 0.4 % IPTG inducer to induce expression of the HA tag–FtsW(R411HA), or pDSW234 (Weiss et al., 1999), encoding GFP–PBP3, were grown at 37 °C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics to an OD600 of 0.4. Then protein expression was induced for 4 h by addition of 1 mM IPTG. Cells were suspended in 8 ml 30 mM Tris/HCl pH 8, 5 mM EDTA, 100 μg lysozyme ml−1, 5 mM MgCl2, 0.01 U benzonase, 100 μM PMSF. After 20 min incubation at 4 °C, 32 ml cold water was added and the mixture was centrifuged for 1 h at 18 000 r.p.m. (38 830 g). The membrane fraction was then resuspended in 600 μl 10 mM Tris pH 8.0, 10 % (w/v) glycerol, 10 % (v/v) ethylene glycol, 0.5 M NaCl (TGE buffer) and centrifuged for 15 min at 13 200 r.p.m. (13 600 g). The membrane proteins were solubilized in 300 μl 50 mM Tris/HCl pH 7.9, 150 mM NaCl, 35 % (v/v) glycerol, 1.25 % (v/w) octyl glycoside (solubilization buffer). After 15 min incubation on ice, samples were centrifuged for 15 min at 13 200 r.p.m. (13 600 g). The supernatant contained solubilized membrane proteins.

Mouse antibodies (IgG1) directed against GFP (20 μl) were incubated at room temperature for 60 min in the presence of 200 μl magnetic matrix Dynabeads Protein G (Dynal Biotech) in 25 mM HEPES buffer pH 7.5, 150 mM NaCl, 0.1 % (w/v) Triton X-100, 10 % (v/v) glycerol. The matrix/antibodies mixture was washed twice with the same buffer to remove excess antibodies.

Co-immunoprecipitation. This was carried out as follows. A 125 μl sample of each solubilized protein was incubated under agitation at 4 °C with 50 μl matrix (Dynabeads Protein G coupled to GFP antibodies) for 90 min; the matrix was then washed five times with solubilization buffer. Elution was carried out by incubation for 30 min at room temperature with 7.5 μl 66.6 mM Tris/HCl pH 6.8, 11 % (v/v) glycerol, 0.001 % bromophenol blue, 1 % (w/v) SDS and 3.3 % (v/v) β-mercaptoethanol. The eluted proteins were analysed by 10 % SDS-PAGE, followed by Western blotting using antibodies directed against the HA tag.

Fluorescence detection and Western blotting. After SDS-PAGE, the fluorescence of the GFP–protein fusions was detected directly on the gel using an FX Molecular Imager (Bio-Rad) with the same parameter set as designed for fluorescein isothiocyanate detection (excitation by 488 nm wavelength Ar ion laser with a 515–545 nm band emission filter). Western blotting was then carried out as follows: the separated proteins were electro-transferred to a PVDF membrane, blocked with ECL blocking agent and washed and probed by

Table 1. Bacterial strains with relevant properties and references

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genetic marker(s) or features</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>LMC500</td>
<td>F- araD139 Δ(argF-lac)U169 deoC1 flb5301 ptsF25 rbsR relA1 rpsL150 lysA1</td>
<td>Taschner et al. (1988)</td>
</tr>
<tr>
<td>LMC510</td>
<td>LMC500 fsd1258(ts)</td>
<td>Taschner et al. (1988)</td>
</tr>
<tr>
<td>BTH101</td>
<td>F- cry-99 araD139 galE15 galK16 rpsL1 (StrR) hasD2 mcrA1 mcrB1</td>
<td>Karimova et al. (2005)</td>
</tr>
<tr>
<td>Top10F’</td>
<td>F’ [lacPTn10 (TekR)] mcrA Δ(mrr–hsrRMS–mcrBC) 80lacZAM15 ΔlacX74 deoR recA1 araD139 Δ(ara–leu)7697 galU galK rpsL (StrR) endA1 supG</td>
<td>Invitrogen</td>
</tr>
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incubation for 1 h with monoclonal anti-HA-peroxidase (HighAffinity 3F10 Roche). The proteins were visualized by enhanced chemiluminescence (ECL kit, GE Healthcare).

RESULTS

In vivo FtsW–PBP3 interaction revealed by FRET

To study the interaction between FtsW and PBP3, we used the Förster resonance energy transfer (FRET) technique. FRET occurs between two appropriately chosen fluorophores only when the distance separating them is less than 10 nm (Förster, 1948). FtsW and PBP3 were fused to the C-terminal end of mKusabira Orange (mKO) fluorescent protein (Karasawa et al., 2004), the donor, and mCherry red fluorescent protein (Shaner et al., 2004), the acceptor. Previously it was shown that GFP fusions of PBP3 and FtsW are functional (Piette et al., 2004; Pastoret et al., 2004) using the same linker between the fluorescent protein and the cell division proteins as in the mCherry and mKO fusions. E. coli LMC500 was co-transformed with appropriate vectors. Transformants were grown to steady state as described in Methods. Fusion protein expression was initiated by addition of 10 μM IPTG to 40 ml cultures for the next 6 h until the cultures reached an OD_{450} of 0.2. The spectra were recorded with a fluorescence spectrophotometer. Sensitized emission and apparent efficiency of energy transfer \( E_{\text{fA}} \) were derived essentially as described previously (Alexeeva et al., 2010).

The results showed a positive FRET signal between FP–FtsW and FP–PBP3 (FP, fluorescent protein) that was two to three times higher than that of the negative controls (Fig. 2). Significance of the data was assessed using the unpaired double-sided t-test versus the negative control. All samples returning a P-value of the t-test below 0.05 were considered statistically significant for the particular sample. For the mKO–PBP3 and mCherry–FtsW sample a P value of 0.006 (99.4% confidence interval) versus the negative control mKO and mCherry–FtsW was found. In general we found that an average \( E_{\text{fA}} \) of 1.2% represents the percentage of bystander FRET in the membrane in our expression system (Alexeeva et al., 2010). A FRET signal between two FP–PBP3 molecules was also detected (Fig. 2, sample O3 + Ch3) but no significant FRET signal between two FtsW molecules could be detected (Fig. 2, sample OW + ChW). These results indicate that FtsW directly interacts with PBP3 and that PBP3 forms dimers.

In vitro FtsW–PBP3 interaction by co-immunoprecipitation

In order to show that FtsW and PBP3 form a complex, co-immunoprecipitation experiments were carried out. Proteins of membrane fractions isolated from E. coli LMC500 expressing GFP–PBP3 (Piette et al., 2004) or HA tag–FtsW (containing an antigenic YPYDVPDYA peptide epitope inserted between R411 and G412) (Pastoret et al., 2004) were solubilized in the presence of 1.25% (w/v) octyl glucoside as described in Methods. The protein extracts were mixed and incubated with Dynabeads Protein G coupled to GFP antibodies. After washing, the retained proteins were eluted and analysed by SDS-PAGE followed by fluorescence detection and Western blotting (see Methods). Fig. 3 shows that the GFP–PBP3 and HA tag–FtsW were co-eluted from Dynabeads Protein G coupled to GFP antibodies (lanes 9 in Fig. 3a and b). The HA tag–FtsW specifically bound to the GFP–PBP3 since it was not

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**Fig. 2.** FRET efficiency \( (E_{\text{fA}}) \) for various combinations of mKO and mCherry fusions. For each experiment reference spectra were recorded from cells expressing only mKO or only mCherry proteins, and reference background spectra were recorded from cells bearing two non-coding plasmids. The reference spectra were used to calculate the contributions of donor, acceptor and background to the total spectrum of the experimental samples measured at \( \lambda_0 \) using least-square fitting (Alexeeva et al., 2010). O–Ch, tandem expression of mKO and mCh (as positive control, \( n=24 \)); O + Ch, separately expressed mKO and mCh (as negative control, \( n=24 \)); O + ChW, expression of mKO and mCh–FtsW (as negative control, \( n=4 \)); O + Ch3, expression of mKO and mCh–PBP3 (as negative control, \( n=4 \)); O3 + ChW, expression of mKO–PBP3 and mCh–FtsW (\( n=4 \)); OW + Ch3, expression of mKO–FtsW and mCh–PBP3 (\( n=5 \)); O3 + Ch3, expression of mKO–PBP3 and mCh–PBP3 (\( n=4 \)); OW + ChW, expression of mKO–FtsW and mCh–FtsW (\( n=2 \)).
retained and eluted from the Dynabeads Protein G coupled to GFP antibodies in the absence of GFP–PBP3 (Fig. 3a, b, lane 8). This experiment was repeated more than three times with the same results. These results indicate that FtsW and PBP3 are able to form a discrete complex. PBP1B could not be detected reproducibly within the subcomplex when the GFP–PBP3, HA tag–FtsW and His tag–PBP1B proteins expressed from E. coli LMC500 were solubilized with detergent, mixed and incubated with the Dynabeads Protein G coupled to GFP antibodies as described above. Interestingly, the HA tag–FtsW(P368A;P375A) mutant, which reduces the localization of PBP3 at the division site (Pastoret et al., 2004), still bound to GFP–PBP3 (Fig. 3c).

The PBP3 transmembrane segment (TMS) is involved in the interaction with FtsW

It was shown previously that the periplasmic loop 9/10 of FtsW (Fig. 1) was involved in PBP3 recruitment at the division site (Pastoret et al., 2004). It was also shown that the structural determinants required to target PBP3 to the division site are present in the first 56 residues of PBP3 and that PBP3(K2–V42) is weakly recruited to the division site (Piette et al., 2004). Lpp–PBP3, which is obtained by replacement of the first 41 residues of PBP3 by the Lpp signal peptide, does not interact with FtsW (Derouaux et al., 2008). Changing R23 to C or L39 to P, located at both ends of the TMS of PBP3, severely impairs the localization of the protein at the division site but not its membrane insertion (see Supplementary Fig. S1, available with the online version of this paper) or its ability to bind penicillin (Wissel & Weiss, 2004), suggesting that the TMS of the protein is involved in the recruitment of the protein to the division site. In order to define which portions of the proteins were involved in the interactions, we analysed the ability of FtsW mutants to interact with PBP3 and the effect of PBP3 mutants on the interaction with FtsW by using the Cya two-hybrid assay (Karimova et al., 2005; Bertsche et al., 2006). The Cya T18 and Cya T25 fragments of the adenylate cyclase (Cya) were fused to the N-terminus of FtsW mutant and PBP3 mutants, respectively, through a (G4S)3 linker to test their interaction with the respective T25–(G4S)3–FtsW and T18–(G4S)3–PBP3. These two wild-type fusion proteins were functional in vivo as described previously (Derouaux et al., 2008; Bertsche et al., 2006). The production of β-galactosidase was quantitatively measured in cell extracts from cultures grown in LB medium in the absence of IPTG. As shown in Fig. 4, the β-galactosidase activity of the cya-deficient E. coli BTH101 co-producing T18–(G4S)3–PBP3 and T25–(G4S)3–FtsW(P368A;P375A) under non-induced conditions was 64 ± 18 % of that of the wild-type fusion pairs. This result indicates that the interaction was slightly affected by the mutations and is in

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![Fig. 3. Interaction between FtsW and PBP3.](image)

**Fig. 3.** Interaction between FtsW and PBP3. (a) Fluorescent detection of GFP–PBP3 and (b) immunodetection of HA tag–FtsW after 10 % SDS-PAGE. Lanes: 1, solubilized proteins from E. coli strain overexpressing HA tag–FtsW alone (negative control); 2, mixture of solubilized proteins from E. coli strains overexpressing HA tag–FtsW or GFP–PBP3; 3, molecular mass markers; 4, negative control: flowthrough; 5, HA tag–FtsW and GFP–PBP3: flowthrough; 6, negative control: wash; 7, HA tag–FtsW and PBP3: wash; 8, negative control: eluate; 9, HA tag–FtsW and GFP–PBP3: eluate. (c) Left, HA tag–FtsW and GFP–PBP3 eluate; right, HA tag–FtsW(P368A;P375A) and PBP3 eluate (immunodetection of HA tag–FtsW).

![Fig. 4. Interaction between FtsW and PBP3 by the Cya bacterial two-hybrid approach, using the following: 1, E. coli BTH101 (cya) transformants producing T25 and T18 (negative control); 2, T25–(G4S)3–FtsW and T18–(G4S)3–PBP3; 3, T25–(G4S)3–FtsW and T18–(G4S)3–PBP3(M1–V42); 4, T25–(G4S)3–FtsW and T18–(G4S)3–LppPBP3 (negative control); 5, T25–(G4S)3–FtsW and T18–(G4S)3–PBP3(R23C); 6, T25–(G4S)3–FtsW and T18–(G4S)3–PBP3(L39P); 7, T25–(G4S)3–FtsW(P368A;P375A) and T18–(G4S)3–PBP3. The β-galactosidase activity of transformants grown in LB medium in the absence of IPTG for 17 h at 30 °C was determined as described in Methods. Data are means ± SD of six independent experiments. E. coli BTH101 transformants producing T25 with T18–(G4S)3–PBP3, T18 with T25–(G4S)3–FtsW or T18 with T25 gave the same value as found for the β-galactosidase basal activity. A percentage β-galactosidase activity above the horizontal line indicates a significant interaction.
agreement with the data obtained by immunoprecipitation (see above).

The alteration of the TMS of PBP3 strongly affects the interaction between PBP3 and FtsW (Fig. 4). The level of β-galactosidase activity of the cya-deficient E. coli BTH101 co-producing T25–(G4S)3–FtsW and T18–(G4S)3–PBP3(M1–V42), T18–(G4S)3–PBP3(R23C) or T18–(G4S)3–PBP3(L39P) under non-induced conditions was 12, 8 and 7 %, respectively, of the activity of wild-type fusion proteins, whereas in the negative controls it was 2.4 % of that of the wild-type. Under the conditions used, the mutant fusion proteins PBP3(R23C) and PBP3(L39P) were not immunodetected in cell extracts (data not shown). The presence of T18–(G4S)3–PBP3(M1–V42) could not be analysed (the antibodies that recognize PBP3 are not directed against this part of the protein). By contrast, in the presence of IPTG, T18–(G4S)3–PBP3(R23C) and T18–(G4S)3–PBP3(L39P) were immunodetected like the wild-type protein (Supplementary Fig. S1). The level of β-galactosidase activity of E. coli BTH101 co-producing T25–(G4S)3–FtsW and T18–(G4S)3–PBP3(R23C) or T18–(G4S)3–PBP3(L39P) in the presence of IPTG was 28 and 13.6 %, respectively, of the activity of wild-type fusion proteins, whereas in the negative controls it was 6.7 % of that of the wild-type (Supplementary Fig. S2). These results indicate a very weak interaction of FtsW with the PBP3(R23C) mutant and no significant interaction with the PBP3(L39P) mutant.

To verify the interaction between the TMS of PBP3 and the FtsW protein, the interaction between a truncated PBP3 mutant expressing amino acid residues K2 to V42 fused to the C-terminus of mKO and mCherry was studied by FRET. FRET efficiency (E\textsubscript{f}) is shown for various combinations of mKO and mCherry fusions (for details see the legend of Fig. 2). O + ChW, expression of mKO and mCherry–FtsW (negative control); O + Ch3, expression of mKO and mCherry–PBP3 (negative control); OW + Ch3, expression of mKO–FtsW and mCh–PBP3; OW + Ch42, expression of mKO–FtsW and mCh–PBP3(K2–V42); O42 + Ch42, expression of mKO–PBP3(K2–V42) and mCh–PBP3(K2–V42). The values are means ± SD of three independent experiments. Black bars, 28 °C; grey bars, 42 °C.

due to the absence of competing endogenous PBP3. Dimerization was found for the PBP3(K2–V42) mutants at both temperatures, which indicates that the TMS of PBP3 is sufficient for dimerization.

**FtsW interacts with PBP1B**

Previous investigations have shown that PBP3, PBP1B, FtsN and FtsW interact with each other in a bacterial two-hybrid assay (Di Lallo et al., 2003; Karimova et al., 2005; Bertsche et al., 2006; Müller et al., 2007), but prior to this study the interaction between PBP1B and FtsW had not been investigated. We used the Cya two-hybrid system to analyse it. Table 2 shows that cells of E. coli BTH101 co-expressing T18–(G4S)3–PBP1B (Bertsche et al., 2006) and T25–(G4S)3–FtsW (Derouaux et al., 2008) produced β-galactosidase, indicating an interaction between these two proteins. To characterize the interaction, we analysed the effect of the FtsW(P368A;P375A) double mutant on the interaction with PBP1B using the two-hybrid system

**Table 2. Interaction of FtsW(P368A;P375A) double mutant with PBP3, PBP1B and FtsN by the Cya two-hybrid system**

The β-galactosidase activity from E. coli BTH101 (cya) transformants grown in LB medium for 17 h at 30 °C was determined as described in Methods. Data are means ±sd of six independent experiments.

<table>
<thead>
<tr>
<th>β-Galactosidase activity (%)</th>
<th>T18–(G4S)3–PBP3</th>
<th>T18–(G4S)3–PBP1B</th>
<th>T18–FtsN</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25–(G4S)3–FtsW (wild-type)</td>
<td>100 ± 20</td>
<td>100 ± 36</td>
<td>100 ± 22</td>
</tr>
<tr>
<td>T25–(G4S)3–FtsW(P368A;P375A)–HA tag</td>
<td>64 ± 18</td>
<td>46 ± 7</td>
<td>118 ± 15</td>
</tr>
<tr>
<td>T25/T18 (negative control)</td>
<td>2.4 ± 0.3</td>
<td>9 ± 2</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

Fig. 5. Interaction between FtsW and the TMS of PBP3 confirmed by FRET. FRET efficiency (E\textsubscript{f}) is shown for various combinations of mKO and mCherry fusions (for details see the legend of Fig. 2). O + ChW, expression of mKO and mCherry–FtsW (negative control); O + Ch3, expression of mKO and mCherry–PBP3 (negative control); OW + Ch3, expression of mKO–FtsW and mCh–PBP3; OW + Ch42, expression of mKO–FtsW and mCh–PBP3(K2–V42); O42 + Ch42, expression of mKO–PBP3(K2–V42) and mCh–PBP3(K2–V42). The values are means ± SD of three independent experiments. Black bars, 28 °C; grey bars, 42 °C.
Based on bacterial two-hybrid experiments, it is known that FtsW interacts with FtsN (Di Lallo et al., 2003). To determine if the effect of the P368A;P375A modification of FtsW is specific for the interaction with PBP1B and PBP3, we analysed the effects of the FtsW(P368A;P375A) double mutant on the interaction with FtsN. Plasmids allowing the co-production of T25–(G 4S)3–FtsW(P368A;P375A) with T18–FtsN (Müller et al., 2007) were introduced into E. coli BTH101. The FtsW(P368A;P375A) mutant appeared not to affect the interaction, since the β-galactosidase activity of the Cya fusion pair FtsW(P368A;P375A) and FtsN was 118 ± 15 % of that of the wild-type (Table 2). Thus the 9/10 loop of FtsW seems to play a role in the interactions with PBP1B and PBP3 but not in the interaction with FtsN.

**DISCUSSION**

**FtsW and PBP3 form a subcomplex**

The divisome is a dynamic hyperstructure (Norris et al., 2007). Its assembly is mediated by multiple protein interactions. Most of the protein interactions within the divisome have been detected with a two-hybrid system or by genetic methods, which cannot distinguish direct from indirect interactions. Based on these experiments, during the maturation of the divisome, the late proteins appear to be capable of associating into pre-assembled complexes FtsQ–FtsL–FtsB and FtsW–PBP3 (Goehring et al., 2006). The trimeric complex FtsQ–FtsL–FtsB has been identified by biochemical approaches in E. coli and Streptococcus pneumoniae (Buddelmeijer & Beckwith, 2004; Noirclerc-Savoye et al., 2005). The results of in vivo FRET experiments presented in this work show for the first time a direct interaction between FtsW and class B PBP3 in E. coli. In addition, these proteins can be co-immunoprecipitated, demonstrating that they form a discrete complex.

The fact that the FtsW–PBP3 complex was also displayed in Mycobacterium tuberculosis indicates that this complex is a broadly conserved unit of the division machinery like the FtsQ–FtsL–FtsB complex (Goehring et al., 2006). In M. tuberculosis, FtsW and PBP3 formed a ternary complex with FtsZ via the FtsW C-terminal extension, which is not present in E. coli (Datta et al., 2006). In E. coli, PBP3 was shown to interact directly with ZapA, which itself binds FtsZ (Mohammadi et al., 2009; Alexeeva et al., 2010), indicating that there is also an interaction between the Z-ring (via ZapA) and the FtsW–PBP3 complex. During the review of this article, a direct interaction between SpoVE, a non-essential, sporulation-specific homologue of FtsW, and SpoVD, a non-essential PBP homologue to class B PBP2B of Bacillus subtilis, was also demonstrated by co-immunoprecipitations, FRET experiments and co-affinity purification in E. coli (Fay et al., 2010). All these data suggest that this interaction is conserved with SEDS/ PBP pairs (Fay et al., 2010).

**Interaction between FtsW and PBP3**

The interaction between FtsW and PBP3 could take place through one of the TMSs of FtsW, as the first 56 residues of PBP3 (containing the TMS) are sufficient to localize it to the division site (Piette et al., 2004). It has been shown that the peptides K2–V42 and W22–V47 of PBP3 localize, but poorly, to the division site of cells depleted of wild-type PBP3 (Piette et al., 2004; Wissel et al., 2005). Our two-hybrid and FRET results show that the first 42 amino acid residues of PBP3 containing the TMS are still able to interact with FtsW, although not as efficiently as the intact PBP3 protein, when the proteins were overexpressed. It was observed that PBP3(R23C) and PBP3(L39P) mutants localize poorly at the division site (Wissel & Weiss, 2004). Our results show that there is a weak or no significant interaction between FtsW and these PBP3 mutants when the proteins are overproduced and immunodetected, indicating that these residues and/or the conformation of the TMS are essential for the interaction with FtsW. Taken together, these data demonstrate that the PBP3 TMS is involved in a direct interaction with a TMS of FtsW. Therefore the first 42 amino acid residues of PBP3 appear to contain the structural determinants required for the FtsW–PBP3 interaction.

In M. tuberculosis, it was shown that the periplasmic loop 9/10 (residues 376–386) of FtsW (the counterpart of P368–P375 of E. coli FtsW) harbours determinants crucial to the FtsW–PBP3 binding interface (Datta et al., 2006). This loop shows a strong sequence homology with that of E. coli FtsW. The E. coli FtsW(P368A;P375A) mutant, which was altered in this 9/10 loop, was co-immunoprecipitated with PBP3, although it interacts less well with PBP3. All these data suggest that the loop 9/10 of E. coli FtsW is also involved in the interaction with PBP3 in addition to a TMS.

**PBP3 dimers**

From our FRET experiments it can be assumed that PBP3 forms dimers in vivo and that amino acids K2–V42 are sufficient for dimerization, confirming the previous two-hybrid results (Di Lallo et al., 2003; Karimova et al., 2005). However, on the basis of the 3D structure of the soluble form of PBP3(G57–V577), the protein appears as a monomer (E. Sauvage, personal communication). Yet the first 56 residues of PBP3 are targeted just like the wild-type PBP3 to the division site in PBP3-depleted cells (Piette et al., 2004) and are sufficient for its interaction with FtsW. In addition, it has been shown that the first 70 amino acid residues interact with FtsW as efficiently as the wild-type PBP3 (Karimova et al., 2005). All these data suggest that the region involved in the dimerization of PBP3 might consist of
the M1–E56 peptide containing a short intracellular M1–R23 peptide fused to an F24–L39 membrane anchor fused to a short periplasmic G40–E56 peptide. The dimerization region could nevertheless extend further to S70.

**Interaction between FtsW and PBP1B**

Although PBP1B interacts *in vivo* and *in vitro* with PBP3 and its septal localization is PBP3 dependent (Bertsche *et al.*, 2006), the protein is not detected in the FtsW–PBP3 complex after co-immunoprecipitation. One explanation could be that the interaction between these proteins is very weak or is disrupted by the presence of detergent in the membrane extract. Indeed, the PBP1B–PBP3 complex can only be co-immunoprecipitated if PBP1B is covalently attached to PBP3 by *in vivo* cross-linking (Bertsche *et al.*, 2006). It is known that PBP3, FtsN and PBP1B interact with each other and that FtsW interacts with PBP3 and FtsN. To our knowledge there is no previous report suggesting an interaction between FtsW and PBP1B. By using the Cya two-hybrid assay we have shown that FtsW interacts *in vivo* with the main peptidoglycan synthase PBP1B. This interaction might be indirect. Alteration of the F368–P375 periplasmic loop 9/10 of FtsW which is involved in the recruitment of PBP3 at the division site (Pastoret *et al.*, 2004) reduces the interaction with PBP1B 2-fold, and the interaction with PBP3 1.5-fold, but has no effect on the interaction with FtsN. This loop thus appears to play a role in the interaction with both PBP1B and PBP3 and might be involved in the correct positioning of the proteins in the divisome.

Our results demonstrate that FtsW and PBP3 form a conserved subcomplex within the divisome. Although FtsW interacts with PBP1B, an FtsW–PBP3–PBP1B trimeric complex could not be detected, probably because the interactions are not strong enough or are transient.

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**REFERENCES**


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**Fig. S1.** Production of T18-(G₄S)₃-PBP3 mutants from membrane extract labelled with fluorescent ampicillin (0.02 mM) after treatment with 0.5 mM β-iodopenicillanate (*), and then immunolabelled with purified polyclonal antibodies (on the right). Proteins were separated by SDS-PAGE. Lanes: 1, soluble PBP3; 2–5, membrane extract from: BTH101 host strain (2); BTH101 producing T18-(G₄S)₃-PBP3 wt (3); T18-(G₄S)₃-PBP3(R23C) (4); T18-(G₄S)₃-PBP3(L39P) (5). Each loading corresponds to 1.5ml of an induced (0.8 mM IPTG) culture with an OD₆₀₀ of 1.0.

**Fig. S2.** Interaction between FtsW and PBP3 by the Cya bacterial two-hybrid approach. *E. coli* BTH101 (cyα) transformants producing T25 and T18 (negative control) (1); T25-(G₄S)₃-FtsW and T18-(G₄S)₃-PBP3 (2); T25-(G₄S)₃-FtsW and T18-(G₄S)₃-PBP3(R23C) (3); or T25-(G₄S)₃-FtsW and T18-(G₄S)₃-PBP3(L39P) (4) were grown in LB medium in the presence of IPTG (0.8 mM) and appropriate antibiotics for 16 h at 30°C. β-Galactosidase activity was determined as described in Methods. Data are means of six independent experiments. The values of β-galactosidase activity above the horizontal line indicate a significant interaction.