Microbiology (2011), **157**, 251–259 **DOI** 10.1099/mic.0.040071-0

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

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Received 2 April 2010
Revised 9 September 2010
Accepted 9 September 2010

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 & Ghuysen, 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 peptide cross-linking (Höltje, 1998; Sauvage *et al.*, 2008). These peptidoglycan synthases work 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).

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Abbreviations: FRET, Förster resonance energy transfer; PBP, penicillin-binding protein; TMS, transmembrane segment.

Two supplementary figures are available with the online version of this paper.

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 (Margolin, 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 (Goehring *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 localization of PBP3 at the division site (Pastoret *et al.*, 2004).

PBP3 interacts directly with PBP1B (Bertsche *et al.*, 2006), which is essential in the absence of its paralogue PBP1A (Suzuki *et al.*, 1978). PBP1B localizes at the lateral wall during elongation and at the division site during septation, and its localization at the division site depends on the presence of PBP3 but not on its activity (Bertsche *et al.*, 2006). These proteins might thus act together to form the peptidoglycan of the two new cell poles. PBP3 also interacts 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.

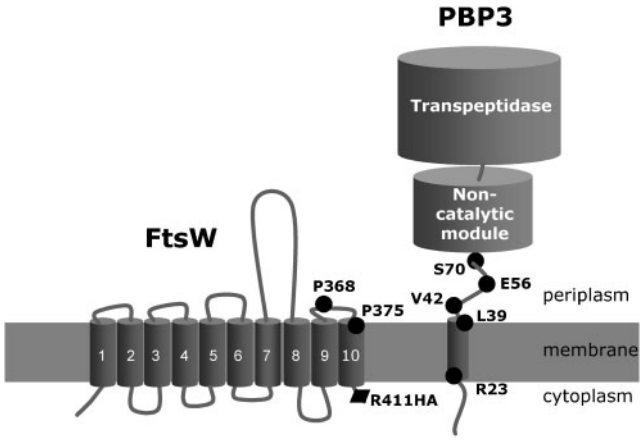

Fig. 1. Topology of the transmembrane protein FtsW and the transpeptidase PBP3. The positions of the modified amino acids discussed are shown. The HA tag is inserted between R411 and G412.

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 *Mlu*I and *Sca*I and cloned into the *Mlu*I and *Sma*I sites of pBT/*ftsI*-linker (Bertsche *et al.*, 2006). The *Not*I–*Hin*dIII 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–$(G_4S)_3$–PBP3(M1–V42).

The modified *ftsI* 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′-TAGGCGGCCGCACTTAAGGAGCTCATGAAAGCAGCGGCGAAAACGCAG-3′ and 5′-GTTCAGTTCGCGATAAACC-3′ as primers (*Not*I, *Sac*I and *Nru*I sites are underlined). The PCR fragment was cloned into pGEM-T Easy (Promega), sequenced, digested with *Mlu*I and *Sac*I and inserted into the same sites of pDML2451a (Bertsche *et al.*, 2006). The resulting plasmids pDML2455 and pDML2456 code for T18–$(G_4S)_3$–PBP3(R23C) and T18–$(G_4S)_3$–PBP3(L39P), respectively.

pDML2445. The fragment resulting from the *Nde*I and *Mlu*I 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 *Sna*BI–*Hin*dIII fragment encoding the HA tag–FtsW mutant was exchanged with the corresponding fragment of pDML2443a encoding T25–$(G_4S)_3$–FtsW (Derouaux *et al.*, 2008). The resulting plasmid pDML2445 allows the production of T25–$(G_4S)_3$–FtsW(P368A;P375A)–HA tag.

pSA060, pSA062, pSA063, pSA066, pSA068 and pSA069. Fragments resulting from the *Eco*RI and *Hin*dIII 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 *Eco*RI and *Hin*dIII and the fragment cloned into the corresponding sites of

Table 1. Bacterial strains with relevant properties and references

Strain	Relevant genetic marker(s) or features	Source
LMC500	F$^-$ *araD139* Δ(*argF-lac*)*U169 deoC1 flB5301 ptsF25 rbsR relA1 rpsL150 lysA1*	Taschner *et al.* (1988)
LMC510	LMC500 *ftsI2158*(ts)	Taschner *et al.* (1988)
BTH101	F$^-$ *cya-99 araD139 galE15 galK16 rpsL1* (Str^R) *hsdR2 mcrA1 mcrB1*	Karimova *et al.* (2005)
Top10F′	F′ {*lacI*^{q}Tn*10* (Tet^R)} *mcrA* Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara–leu*)*7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen

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 pTHV037 (Amp) and pSAV057 (Cam) were used as controls (Alexeeva *et al.*, 2010). The correctness of all constructs was confirmed by sequencing.

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 use 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 was 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–(fusion to protein of interest) expressed with unfused mKO, and mKO–(fusion to protein of interest) expressed with unfused mCherry as negative controls. Transformants were grown in TY, 0.4 % glucose (w/v) supplemented with 100 μg ampicillin ml^{-1} and 25 μg chloramphenicol ml^{-1}. After 8 h growth, the cultures were diluted 1 : 12 500 in 25 ml minimal medium GB1 and grown overnight at 28 °C. Subsequently, the cultures were diluted and cells were grown in minimal medium at 28 °C for ~40 h, keeping the OD_{450} below 0.2 by regular dilution of the cultures in pre-warmed medium. Fusion protein expression was initiated by addition of 10 μM IPTG to 40 ml cultures. Based on the determined growth rates, the cultures were diluted appropriately and cell growth in the presence of IPTG inducer was allowed for the next 6 h until the cultures reached an OD_{450} of 0.2. In the case of LMC510 [PBP3(ts)], after growth at 28 °C as described above, the cultures were split in two and one part was grown at 28 °C, whereas the other part was grown at 42 °C for two mass doublings. The cultures were fixed by addition of 2.8 % (v/v) formaldehyde and 0.04 % (v/v) glutaraldehyde while shaking in a water bath. After 15 min incubation, cells were collected by centrifugation (4 °C, 10 min, 8000 ***g***), washed once with PBS (140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and resuspended in PBS. The samples were incubated overnight at 37 °C to allow maturation of mKO. After adjustment of the OD_{450} of the cell suspension to exactly 1.00, spectra were recorded with a fluorescence spectrophotometer (QuantaMaster 2000-4, PTI) with a red-optimized setup: R928 PMT tube (185–900 nm), 500 nm blaze (1200 lines mm^{-1}) in both excitation and emission gratings. The spectra at donor (mKO, λ_D 538/6 nm) and acceptor (mCherry, λ_A 590/6 nm) excitation wavelengths were recorded for each sample. 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 contributions of donor, acceptor and background to the total spectrum of the experimental samples measured at λ_D using least-square fitting. Sensitized emission and apparent efficiency of energy transfer Ef_A were derived essentially as described previously (Clegg, 1992; Clegg *et al.*, 1992; Gadella, 2009; Alexeeva *et al.*, 2010).

Preparation of fusion proteins and affinity chromatography. *E. coli* LMC500 cells harbouring pDML2424 (Pastoret *et al.*, 2004), encoding 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 OD_{600} 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 $MgCl_2$, 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 % (w/v) octyl glucoside (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 washed and probed by

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 Ef_A 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 Ef_A 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

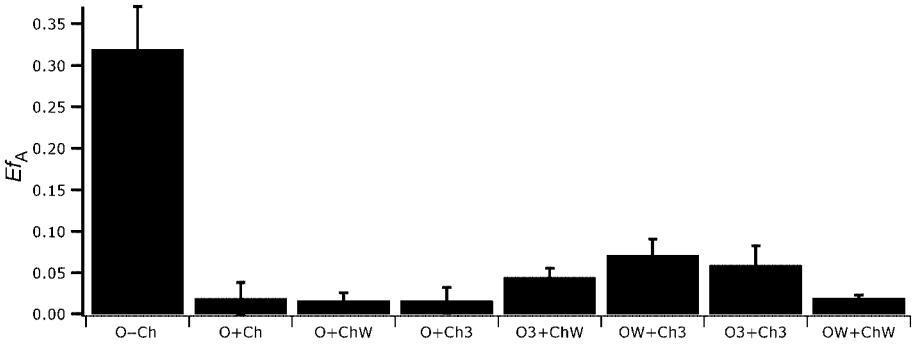

Fig. 2. FRET efficiency (Ef_A) for various combinations of mKO and mCherry fusions. For each experiment reference spectra were recorded from cells expressing only mKO or only mCherry proteins, and reference background spectra were recorded from cells bearing two non-coding plasmids. The reference spectra were used to calculate the contributions of donor, acceptor and background to the total spectrum of the experimental samples measured at λ_D 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 ($n=2$).

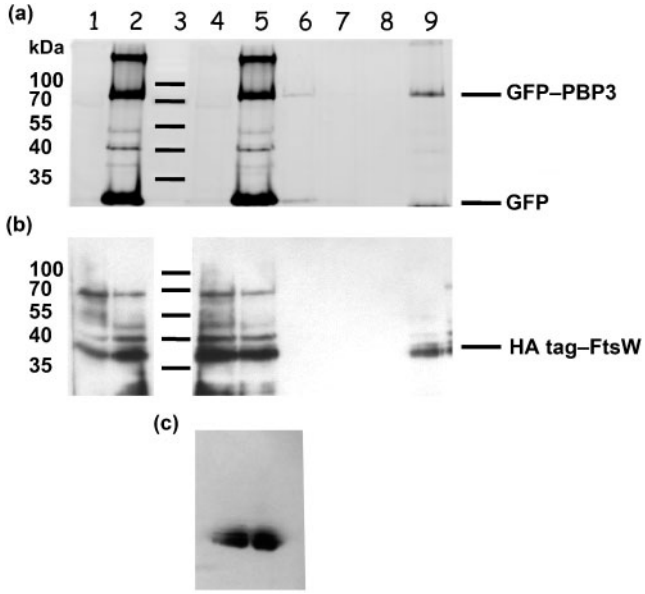

Fig. 3. Interaction between FtsW and PBP3. (a) Fluorescent detection of GFP–PBP3 and (b) immunodetection of HA tag–FtsW after 10 % SDS-PAGE. Lanes: 1, solubilized proteins from *E. coli* strain overexpressing HA tag–FtsW alone (negative control); 2, mixture of solubilized proteins from *E. coli* strains 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).

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 $(G_4S)_3$ linker to test their interaction with the respective T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–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–$(G_4S)_3$–PBP3 and T25–$(G_4S)_3$–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

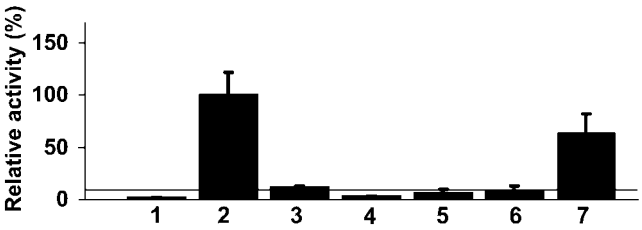

Fig. 4. Interaction between FtsW and PBP3 by the Cya bacterial two-hybrid approach, using the following: 1, *E. coli* BTH101 (*cya*) transformants producing T25 and T18 (negative control); 2, T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3; 3, T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3(M1–V42); 4, T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–LppPBP3 (negative control); 5, T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3(L39P); 6, T25–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3(R23C); 7, T25–$(G_4S)_3$–FtsW(P368A;P375A) and T18–$(G_4S)_3$–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–$(G_4S)_3$–PBP3, T18 with T25–$(G_4S)_3$–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–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3(M1–V42), T18–$(G_4S)_3$–PBP3(R23C) or T18–$(G_4S)_3$–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–$(G_4S)_3$–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–$(G_4S)_3$–PBP3(R23C) and T18–$(G_4S)_3$–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–$(G_4S)_3$–FtsW and T18–$(G_4S)_3$–PBP3(R23C) or T18–$(G_4S)_3$–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 in the presence and the absence of the endogenous PBP3 protein. Strain LMC510 (Taschner *et al.*, 1988) was transformed with mKO and mCherry FRET plasmids. This strain encodes a temperature-sensitive PBP3(G191D;D266N) mutant (*ftsI2158*) that does not localize and gives rise to a very unstable protein, undetectable by immunoblotting, at the restrictive temperature of 42 °C. The wild-type mCherry–PBP3 protein complemented the temperature-sensitive phenotype as expected (not shown) and interacted with FtsW at both temperatures (Fig. 5). The PBP3(K2–V42) mutant also interacted with FtsW, although not as efficiently as the intact PBP3 protein. The FRET efficiency between these proteins was slightly elevated at 42 °C (Fig. 5), possibly 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.

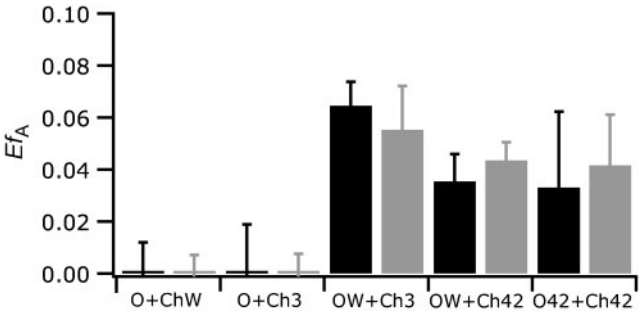

Fig. 5. Interaction between FtsW and the TMS of PBP3 confirmed by FRET. FRET efficiency (Ef_A) is shown for various combinations of mKO and mCherry fusions (for details see the legend of Fig. 2). O + ChW, expression of mKO and mCherry–FtsW (negative control); O + Ch3, expression of mKO and mCherry–PBP3 (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.

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–$(G_4S)_3$–PBP1B (Bertsche *et al.*, 2006) and T25–$(G_4S)_3$–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.

	β-Galactosidase activity (%)		
	T18–$(G_4S)_3$–PBP3	**T18–$(G_4S)_3$-PBP1B**	**T18–FtsN**
T25–$(G_4S)_3$–FtsW (wild-type)	100 ± 20	100 ± 36	100 ± 22
T25–$(G_4S)_3$–FtsW(P368A;P375A)–HA tag	64 ± 18	46 ± 7	118 ± 15
T25/T18 (negative control)	2.4 ± 0.3	9 ± 2	5 ± 1

(Table 2). The level of β-galactosidase activity due to the complementation by the Cya fusion pairs PBP1B and FtsW(P368A;P375A) was 46 ± 7 % of that of the wild-type fusion pair (Table 2). This result suggests that the 9/10 loop of FtsW plays a role in the interaction with PBP1B.

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 P368–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.

ACKNOWLEDGEMENTS

We thank David Weiss, University of Iowa, USA, for plasmids allowing production of R23C and L39P PBP3 mutants, Daniel Ladant for providing the *cya E. coli* strain and Roger Y. Tsien for providing the pRSETBmCherry plasmid. This project was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming (IAP 6/19), by the Fonds de la Recherche Fondamentale Collective (Contract 2.4543.05), by a Dutch NWO 'Van Molecuul tot Cel' programme grant ALW 805.47.200 (S. A. and T. d. B.) by a Vernieuwingsimpuls grant 016.001-024 (T. d. B.) and by the European Commission within the EUR-INTAFAR network (SHM-CT-2004-512138). This work was funded in part by European Framework Programs DIVINOCELL HEALTH F3-2009-223431 (T. d. B. and R. v. P).

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Edited by: J. M. van Dijl

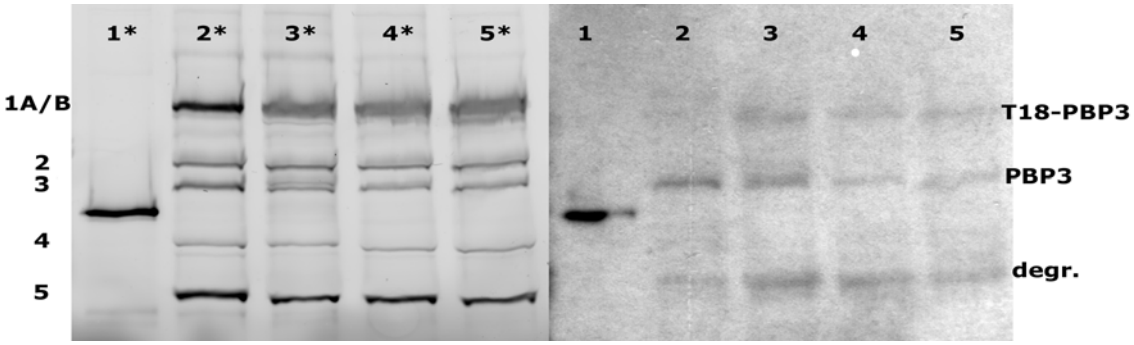

Fig. S1. Production of T18-$(G_4S)_3$-PBP3 mutants from membrane extract labelled with fluorescent ampicillin (0.02 mM) after treatment with 0.5 mM β-iodopenicillanate (*), and then immunolabelled with purified polyclonal antibodies (on the right). Proteins were separated by SDS-PAGE. Lanes: 1, soluble PBP3; 2–5, membrane extract from: BTH101 host strain (2); BTH101 producing T18-$(G_4S)_3$-PBP3 wt (3); T18-$(G_4S)_3$-PBP3(R23C) (4); T18-$(G_4S)_3$-PBP3(L39P) (5). Each loading corresponds to 1.5ml of an induced (0.8 mM IPTG) culture with an OD_{600} of 1.0.

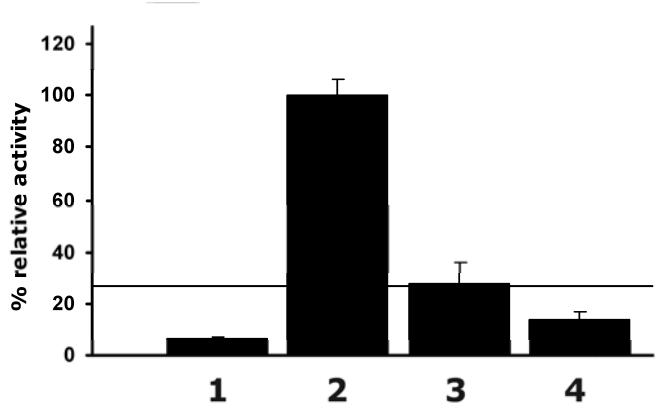

Fig. S2. Interaction between FtsW and PBP3 by the Cya bacterial two-hybrid approach. *E. coli* BTH101 (*cya*) transformants producing T25 and T18 (negative control) (1); T25-$(G_4S)_3$-FtsW and T18-$(G_4S)_3$-PBP3 (2); T25-$(G_4S)_3$-FtsW and T18-$(G_4S)_3$-PBP3(R23C) (3); or T25-$(G_4S)_3$-FtsW and T18-$(G_4S)_3$-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.