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The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in *Escherichia coli*[*]

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Bacterial cell division requires the coordinated action of cell division proteins and murein (peptidoglycan) synthases. Interactions involving the essential cell division protein FtsN and murein synthases were studied by affinity chromatography with membrane fraction. The murein synthases PBP1A, PBP1B, and PBP3 had an affinity to immobilized FtsN. FtsN and PBP3, but not PBP1A, showed an affinity to immobilized PBP1B. The direct interaction between FtsN and PBP1B was confirmed by pulldown experiments and surface plasmon resonance. The interaction was also detected by bacterial two-hybrid analysis. FtsN and PBP1B could be cross-linked in intact cells of the wild type and in cells depleted of PBP3 or FtsW. FtsN stimulated the *in vitro* murein synthesis activities of PBP1B. Thus, FtsN could have a role in controlling or modulating the activity of PBP1B during cell division in *Escherichia coli*.

Division of a bacterial cell is accomplished by the divisome, which is a transient assembly of a ring-like multiprotein structure at mid-cell. The components of the *Escherichia coli* divisome known so far include 12 essential proteins, FtsZ, FtsA, ZipA, FtsE, FtsX, FtsK, FtsQ, FtsB, FtsL, FtsW, PBP3 (FtsI), and FtsN, and the non-essential PBP1B, AmiC, EnvC, Tol, and Pal, which all have been shown to localize at mid-cell. Although the molecular mechanisms of the assembly and functioning of this dynamic cell division machine are largely unknown, a number of studies have revealed the existence of a network of protein-protein interactions between cell division proteins (1–3).

Cell division involves the synthesis of the murein of two new polar caps of the daughter cells. *E. coli* has a set of six murein synthases, of which only one, the class B penicillin-binding protein 3 (PBP 3)[5] (also named FtsI), is essential for cell division (4). PBP3 interacts with PBP1B, a bifunctional transglycosylase-transpeptidase murein synthase (5) that is produced in the cell in two different isoforms (α and γ) and that is essential only in the absence of the homologous PBP1A (6). PBP1B localizes both at the side wall and at the septum (5), indicating that it participates in murein synthesis at the side wall during elongation and at the septum during cell division. Septal localization of PBP1B depends on the presence, but not on the activity, of PBP3 (5). Because both synthases interact with other proteins, the PBP1B-PBP3 complex could be part of a larger murein-synthesizing multienzyme complex (7) that is controlled by (or an integral part of) the divisome. PBP1B is present in an oligomeric complex in inner membrane vesicles (8). It forms dimers *in vitro* and *in vivo* (9–13) and interacts with the murein hydrolase MltA via MipA (14). PBP3 was found to interact with many other cell division proteins in a bacterial two-hybrid approach (2), and different genetic methods have pointed to interactions of PBP3 with FtsA (15), FtsQ (16), FtsW (16, 17), and FtsN (18). The interaction of PBP3 with FtsN has been confirmed by chemical cross-linking of both proteins in intact cells (5).

ftsN is an essential cell division gene (19) and a multicopy suppressor of *ftsA*(ts), *ftsQ*(ts), *ftsI*(ts), and *ftsK*(ts) mutant strains under certain conditions (20–23). FtsN localizes at mid-cell (19, 24) and is required for septal recruitment of two non-essential septal components: (i) The murein hydrolase AmiC (25) that, among other hydrolases, cleaves the septum for separation of the daughter cells (26), and (ii) the Tol-Pal complex that is required for proper invagination during constriction (27). FtsN has a single transmembrane helix close to the N terminus, followed by the periplasmic part (Fig. 1). NMR analysis of the soluble, periplasmic part revealed the existence of three short α-helices in the region between amino acids 62 and 123 (numbers of the full-length protein), followed by a long, glutamine-rich, unstructured region (amino acids 129 to 225) and a small, globular C-terminal domain (amino acids 243 to 319) (28). The C-terminal domain binds to murein but its function is unknown as it is not essential for cell division (23). Instead, the region of the membrane anchor plus the first ~80 periplasmic

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[5] The abbreviations used are: PBP, penicillin-binding protein; LB, Luria Bertani; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TG, transglycosylation; TP, transpeptidation.

amino acids appears to be the part of FtsN that is essential for cell division (23).

FtsN is only poorly conserved outside the enteric bacteria. Moreover, FtsN can be deleted in *E. coli ftsA* suppressor mutants, indicating that FtsN might not be a core divisome component (29). It has been proposed that FtsN might have a stabilizing function on the divisome, but the precise role of FtsN in cell division remains unknown. We report here that FtsN interacts with the murein synthase PBP1B *in vitro* and *in vivo*. Moreover, FtsN stimulates the murein synthesis activities of PBP1B *in vitro*. Thus, FtsN might have a role in coordinating the murein synthases during cell division in *E. coli*.

MATERIALS AND METHODS

Strains, Growth Medium, and Growth Conditions—Table 1 lists the *E. coli* strains used in this study. Strains were grown aerobically at 30 or 37 °C in Luria Bertani (LB) medium with the supplements indicated in Table 1.

Plasmids Used for the Bacterial Two-hybrid System—pDML2003 producing the T18-$(G_4S)_3$-PBP1B and pDML2014 producing the T25-PBP1B were described previously (5). pDML2437b was derived from pDML2451b (5) in which the *ftsI* gene was replaced by the *ftsW* gene flanked by the SacI and HindIII restriction sites.[6] Plasmids pDML2015 and pDML2016 were constructed for the bacterial two-hybrid system. For this, the *ftsN* gene was amplified by PCR using plasmid pDML2000 as template (5) and the oligonucleotides 5′-CCGGAGCTCGTGGCACAACGAGATTATGTACGC-3′ and 5′-GCCAAGCTTCAACCCCCGGCGGCGAGCCG-3′ as primers (SacI and HindIII sites are underlined). The resulting fragment was digested by SacI and HindIII and cloned into the same sites of pDML2437b (derived from pDML2451b) and pDML2014 (5), respectively, to create pDML2015 coding for T18-FtsN and pDML2016 coding for T25-FtsN. The constructs were sequenced to verify the absence of errors.

A complementation assay was performed as described previously (5) by using the *ftsN* depletion strain JOE565 (MC4100*ftsN*::km *araD*$^+$) harboring the plasmid pBAD33-*ftsN*, in which the *ftsN* gene is under the control of an arabinose-inducible promoter that is repressed with glucose (24). Production of the T18-FtsN protein rescued the division process in the depleted strain grown in LB medium containing 0.2% glucose. The complementation of T25-FtsN was not tested because pBAD33-*ftsN* and pDML2016 have the same origin of replication and bear the same resistance gene.

Growth Medium and Measurement of β-Galactosidase Activity (Two-hybrid System)—Sugar fermentation of *E. coli* DHM1 transformants was detected on LB-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar plates as described previously (5). The β-galactosidase activity was measured in cell extract from liquid culture as described (2, 5). It has been suggested that a level of β-galactosidase activity at least 4-fold higher than that of DHM1 control strain (60–100 units/mg) is indicative of an interaction (2).

Purification of Proteins and Antisera—The His-tagged form of PBP1Bγ (His-PBP1B) was purified from BL21(DE3) pDML924 as described (5) except that 10 mM sodium acetate, pH 5.0, was replaced by 20 mM sodium phosphate, pH 6.0, in the buffers for cation exchange chromatography. For surface plasmon resonance experiments, His-PBP1B was purified as described (30) and concentrated in buffer A (25 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.7% CHAPS) on Microcon YM-30 Millipore to a concentration of 1.9 mg/ml. FtsN-ht was purified from BL21(DE3) pFE42 after growth in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. All buffers used for purification contained a mixture of protease inhibitors (1 μg/ml each of antipain, pepstatin, leupeptin, and aprotinin). The membrane fraction was prepared (5) and incubated with nickel-nitrilotriacetic acid Superflow beads for 2 h. Unbound proteins were washed out with 50 mM Tris/HCl, 500 mM NaCl, 1% Triton X-100, pH 6.0, and with the same buffer containing 20 mM imidazole. Bound protein was eluted with buffer containing 300 mM imidazole. The fractions containing FtsN-ht were pooled and dialyzed against 50 mM Tris/HCl, 500 mM NaCl, pH 6.0. The soluble FtsN variants were purified from BL21 AI cells harboring the corresponding overproduction plasmids upon 3–4 h of growth in the presence of 0.2% arabinose (28). The proteins were purified from the soluble fraction by metal affinity chromatography as described for FtsN-ht but without Triton X-100. Antisera against PBP1A, PBP1B, PBP3, and FtsN were purified as described (5).

FtsN Pulldown Experiment—Murein (0.4 mg) isolated from MC1061 (31) was digested for 18 h at 37 °C with a soluble form of the membrane-bound lytic transglycosylase A (32) in 500 μl of 10 mM HEPES/maleate, pH 5.2, 10 mM $MgCl_2$, 50 mM NaCl to release the 1,6-anhydromuropeptides. After boiling for 10 min, insoluble material was removed by centrifugation. A 50% suspension of Affi-Gel 10 (Bio-Rad) (200 μl) washed three times with ice-cold water and two times with coupling buffer (10 mM HEPES/maleate, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) was incubated with the 1,6-anhydromuropeptide solution. A control Affi-Gel sample received coupling buffer without murein fragments but with 0.1 M ethanolamine. The samples were incubated for 12 h at 6 °C, and the excess coupling sites were blocked by incubation with buffer containing 0.1 M ethanolamine for 3 h. The beads were then washed three times with 1 ml of buffer (see above).

For the pulldown experiments, an FtsN variant (4 μg) and PBP1B (2 μg) were incubated with the beads carrying 1,6-anhydromuropeptides in a total volume of 220 μl for 1 h at 4 °C. Control samples contained either beads without 1,6-anhydromuropeptides or no FtsN. The beads were washed with 1 ml of buffer (25 mM Tris/maleate, pH 6.8, 10 mM $MgCl_2$, 100 mM NaCl, 0.02% NaN_3, 0.25% Triton X-100) and eluted with 120 μl of elution buffer (10 mM Tris/HCl, pH 6.8, 10 mM $MgCl_2$, 500 mM NaCl, 0.2% Sarkosyl, 0.02% NaN_3, 0.25% Triton X-100). The proteins in the eluate were analyzed semi-quantitatively by dot blot analysis. For this, the eluates were dried on a nitrocellulose membrane followed by immunodetection and visualization by staining with chloronaphthol. The amount of FtsN and PBP1B in the eluates was estimated by comparison to signals of standard protein preparations with known concentration. Values from three independent pulldown experiments were averaged.

[6] B. Wolf, Université de Liège, Belgium, unpublished data.

TABLE 1
Strains used in this study
SPEC, spectinomycin; CAM, chloramphenicol; KAN, kanamycin; AMP, ampicillin.

Strain	Relevant property	Supplement to growth medium	References
MC1061	Laboratory strain		(44)
TG4	Inactivation of *ponB*	40 μg/ml SPEC	(45)
EC549	Depletion of PBP3	30 μg/ml CAM 12.5 μg/ml KAN 0.02% arabinose (pre-culture) 0.2% glucose (main culture)	(46, 47)
EC850	Depletion of FtsW	30 μg/ml CAM 12.5 μg/ml KAN 0.02% arabinose (pre-culture) 0.2% glucose (main culture)	(46)
JOE565	Depletion of FtsN	10 μg/ml CAM 40 μg/ml KAN 0.2% arabinose (pre-culture) 0.2% glucose (main culture)	(24)
DHM1	*cya*-deficient, host for two-hybrid system		(2)
BL21(DE3) pDML924	Overproduction of PBP1B	50 μg/ml KAN	(30)
BL21(DE3) pFE42	Overproduction of FtsN-ht	100 μg/ml AMP	(28)
BL21-AI pHis17-ECN2	Overproduction of $FtsN^{58-319}$-ht	100 μg/ml AMP	(28)
BL21-AI pHis17-ECN8	Overproduction of $FtsN^{166-319}$-ht	100 μg/ml AMP	(28)
BL21-AI pHis17-ECN16	Overproduction of $FtsN^{58-125}$-ht	100 μg/ml AMP	(28)

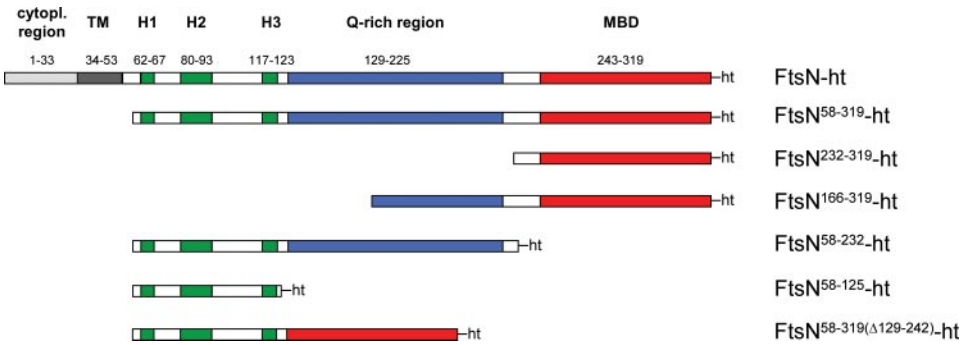

FIGURE 1. **Regions in FtsN and different variants used in this study.** *TM*, transmembrane region; *H1*, *H2*, *H3*, short helical regions; *MBD*, C-terminal murein-binding domain; *ht*, oligohistidine tag. The Q-rich region contains many glutamine residues and is unstructured (28). Numbers indicate the amino acid positions in the native FtsN.

Surface Plasmon Resonance Studies—A Biacore X® (BIAcore AB, Uppsala, Sweden) apparatus was used to analyze the interaction between PBP1B and FtsN. Protein dilutions were made in the HBS running buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 0.05% Triton X-100). FtsN-ht or $FtsN^{58-319}$-ht was immobilized on a CM5 sensor chip with an amine coupling kit as recommended by BIAcore. The amount of immobilized protein was between 1000 and 2000 resonance units (1000 resonance units corresponds to ~1 ng of FtsN/mm^2). A control surface was treated in the same way but did not contain immobilized protein. Differences between the association-dissociation curves observed on the two surfaces indicated a specific interaction. Interaction studies were performed at 25 °C in HBS running buffer at a flow rate of 7 μl/min. His-PBP1B was immobilized on a CM5 sensor chip as described previously (14). Briefly, ampicillin was coupled to the surface via its primary amino group, followed by the binding of PBP1B to the immobilized ampicillin via its active site for transpeptidation. After protein coupling the excess of ampicillin was hydrolyzed by an injection of 100 μl of 0.78 μM TEM β-lactamase.

In Vivo Cross-linking and Co-immunoprecipitation Procedure—Strains were grown in LB medium at 37 °C. For depletion of FtsN, strain JOE565 was grown for ~3.5 mass doublings in LB containing antibiotics and 0.2% glucose to an A_{578} of 0.5, followed by a 23-fold dilution into the same medium and grown to an A_{578} of 0.530 (4.5 mass doublings). The other strains (MC1061, TG4, EC549, and EC850, see Table 1) were grown for ~4–6 mass doublings to an A_{578} of ~0.6. All strains depleted of an Fts protein showed filamentous growth.

Cross-linking of the cells and solubilization of the membrane proteins were done as described (5). Co-immunoprecipitation was performed as described (5) but with protein G-coupled-agarose slurry (ImmunoPure Immobilized Protein G; Pierce) and improved detection of co-immunoprecipitated proteins. A special secondary antibody (Rabbit IgG TrueBlot; eBiosciences) was used for detection of PBP3. For the detection of PBP1B and FtsN, we used goat anti-rabbit IgG (Calbiochem, Merck) as secondary antibody. Both secondary antibodies were coupled to horseradish peroxidase, and the proteins were visualized by enhanced chemoluminescence (ECL) using a detection kit from GE Healthcare.

In Vitro Murein Synthesis Assay—*In vitro* murein synthesis experiments were performed as described (9, 33) (Fig. 10*A*). Lipid II (11 μM, 22000 dpm) was vacuum-dried and dissolved on ice in 10 μl of 2% Triton X-100 for 10 min. Samples contained 11 μM (100 dpm/μl) lipid II and 38 or 730 nM PBP1B (low or high concentration). Some of the samples contained 690 nM FtsN variants. Samples were incubated at 30 °C for 1 h. Prepa-

ration of muropeptides and high pressure liquid chromatography analysis were done as described (9, 33).

Other Methods—SDS-polyacrylamide gel electrophoresis, Western blot, and detection of proteins on the blot membrane with specific antisera were done as described before (5). Coupling of proteins to CNBr-activated Sepharose, preparation of control columns for affinity chromatography, preparation of membrane fraction from *E. coli* cells, and the affinity chromatography experiments were done essentially as described (5, 14). Briefly, a membrane fraction from *E. coli* cells was applied on a Sepharose column containing immobilized protein (FtsN variants or PBP1B). After washing, the bound proteins were eluted successively with buffers containing 150 mM and 1 M NaCl, respectively. Control samples were prepared from the control column (lacking bound protein). Proteins present in the eluates were separated by SDS-PAGE, blotted on nitrocellulose, and immunodetected. Because the sensitivity of detection of proteins by ECL is subject to day-to-day variation, the control samples were always applied on the same polyacrylamide gel as the samples, ensuring that gel electrophoresis, blotting, immunodetection, and ECL reaction (see above) occurred at similar conditions.

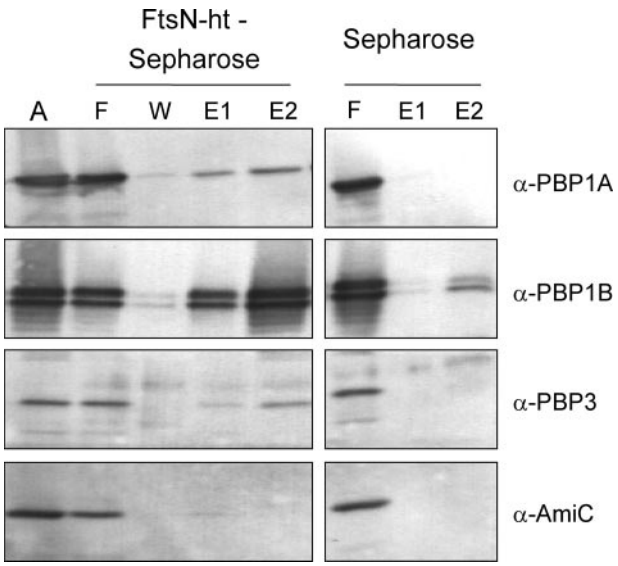

FIGURE 2. **Affinity chromatography with immobilized FtsN-ht and membrane fraction from JOE565.** A Sepharose column served as a control. Murein synthases and AmiC were detected with antisera after SDS-PAGE and Western blot in samples of the membrane fraction (*A*), flow-through (*F*), wash (*W*), low salt elution fraction (*E1*), and high salt elution fraction (*E2*). PBP1A, PBP1B (α and γ), and PBP3, but not AmiC, bound specifically to FtsN-ht-Sepharose.

RESULTS

The different FtsN variants used in this study are indicated in Fig. 1. To detect interactions between FtsN and other membrane proteins we performed affinity chromatography experiments with FtsN-ht (FtsN with a C-terminal hexahistidine tag) immobilized to CNBr-activated Sepharose beads. Sepharose without coupled protein served as a control. A membrane fraction from strain JOE565 grown in the presence of glucose (thus depleted of FtsN) was applied to both columns. After washing, bound proteins were eluted in two steps with buffers containing 150 mM and 1 M salt, respectively. The murein synthases PBP1A, PBP1B, and PBP3 bound in significantly higher amounts to the FtsN-ht-Sepharose than to the control Sepharose (Fig. 2). Both isoforms of PBP1B (α and γ) bound to immobilized FtsN. The murein hydrolase AmiC did not bind to either column.

Affinity chromatography was then performed with different soluble FtsN variants (see Fig. 1) linked to CNBr-activated Sepharose. PBP1B (α and γ) and PBP3 showed an affinity to immobilized $FtsN^{58-319}$-ht, $FtsN^{166-319}$-ht, and $FtsN^{58-125}$-ht (Fig. 3). PBP1A and AmiC were not detected in the eluates (not shown). However, both proteins could be detected in small quantities in the eluates from the $FtsN^{58-319}$-ht and $FtsN^{166-319}$-ht columns after a 10-fold concentration of the sample (Fig. 3).

The next affinity chromatography experiment was performed with PBP1B immobilized to CNBr-activated Sepharose. FtsN was specifically retained by PBP1B-Sepharose (Fig. 4). Interestingly, in these experiments, we frequently detected a double (or triple) band with the α-FtsN antiserum indicating a possible cleavage of FtsN by proteases present in the membrane extract. We confirmed the previously reported dimerization of PBP1B (9) and the interaction of PBP1B and PBP3 (5), as both, PBP1B (α and γ) and PBP3, showed an affinity to the PBP1B-Sepharose but not to the control Sepharose. AmiC also showed weak affinity to immobilized PBP1B, as the majority of bound AmiC eluted with low salt-containing buffer. PBP1A did not bind to immobilized PBP1B.

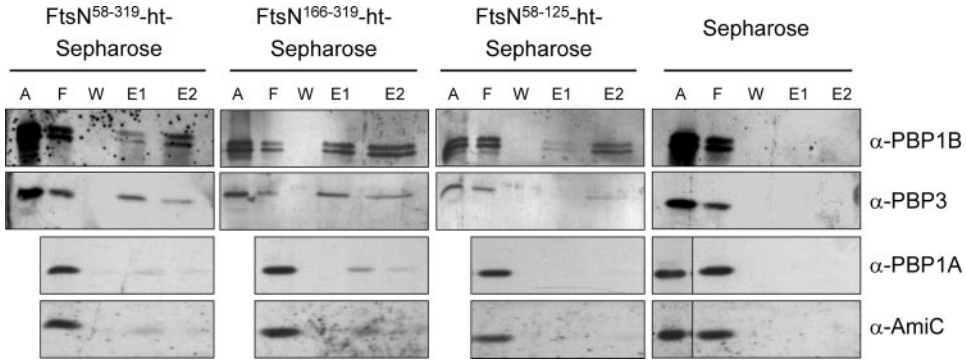

FIGURE 3. **Affinity chromatography with different immobilized FtsN variants and membrane fraction.** A Sepharose column served as a control. After SDS-PAGE and Western blot, murein synthases and AmiC were detected with antisera. Samples: applied membrane fraction (*A*), flow-through (*F*), wash (*W*), low salt elution fraction (*E1*), high salt elution fraction (*E2*). The E1 and E2 samples were concentrated 10-fold for the detection of PBP1A and AmiC. PBP1B (α and γ) and PBP3 bound to FtsN variants lacking the N-terminal part.

To test whether FtsN interacts directly with PBP1B we established a pulldown assay making use of the murein binding property of FtsN (23). We realized that FtsN variants containing the C-terminal murein-binding domain bound to beads with immobilized 1,6-anhydromuropeptides that are the products of digestion of murein with a lytic

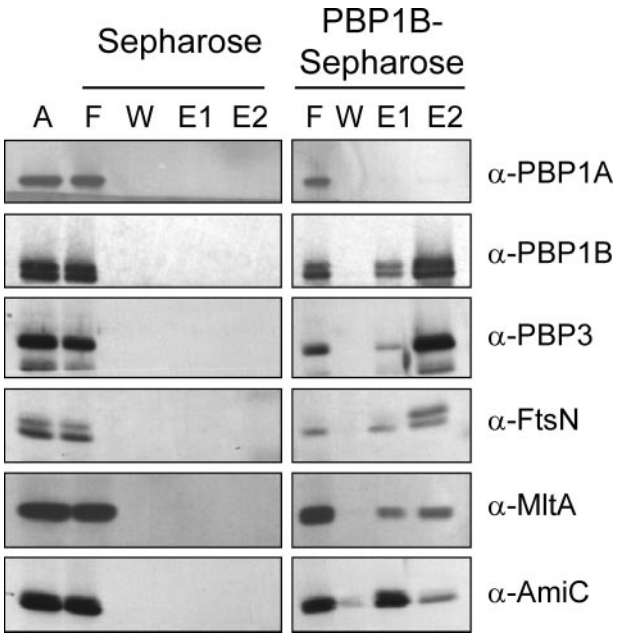

FIGURE 4. **Affinity chromatography with immobilized PBP1B and membrane fraction.** A Sepharose column served as a control. Murein synthases and hydrolases were detected with antisera after SDS-PAGE and Western blot in samples of the membrane fraction (*A*), flow-through (*F*), wash (*W*), low salt elution fraction (*E1*), and high salt elution fraction (*E2*). PBP1B (α and γ), PBP3, FtsN, membrane-bound lytic transglycosylase A (*MltA*), and AmiC, but not PBP1A, bound specifically to PBP1B-Sepharose.

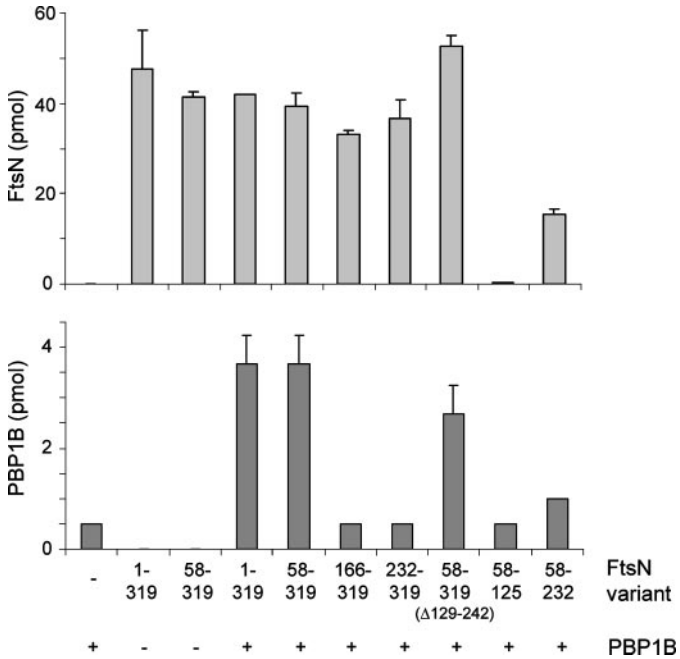

FIGURE 5. **Pulldown experiment with PBP1B and FtsN variants.** PBP1B was incubated with different FtsN variants (with C-terminal oligohistidine tag) and Affi-Gel with immobilized 1,6-anhydro-muropeptides. The numbers indicate the amino acid region of FtsN present in the variants. After washing, the bound proteins were released by SDS, and the amounts of PBP1B and FtsN were estimated semi-quantitatively by dot blotting and detection with antisera. Data from three independent experiments were averaged. *Error bars* indicate S.D. All FtsN variants except $FtsN^{58-125}$-ht bound to immobilized 1,6-anhydro-muropeptides. PBP1B alone did not bind to the beads. PBP1B was pulled down with FtsN-ht, $FtsN^{58-319}$-ht, and $FtsN^{58-319(\Delta 129-242)}$-ht, and not with the other FtsN variants.

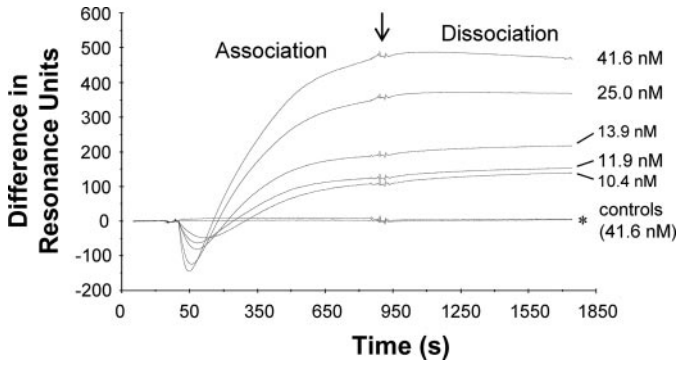

FIGURE 6. **Interaction between FtsN and His-PBP1B by surface plasmon resonance.** FtsN-ht (1035 resonance units) was immobilized, and His-PBP1B was injected at 25 °C at concentrations of 10.4, 11.9, 13.9, 25.0, and 41.6 nM. The control curves (*) show the absence of signal after injection of the soluble form of *E. coli* PBP3 (41.6 nM) or the DD-carboxypeptidase/PBP of *Streptomyces* R61 (41.6 nM) used as control. The *arrow* indicates the beginning of wash with HBS running buffer after the association phase. The values represent the resonance units after substraction of the signals from the control surfaces. There is a concentration-dependent binding of His-PBP1B to immobilized FtsN-ht.

transglycosylase (Fig. 5). PBP1B alone did not bind to beads with these immobilized murein fragments. On the other hand, PBP1B bound to these beads in the presence of FtsN-ht, $FtsN^{58-319}$-ht, and $FtsN^{58-319(\Delta 129-242)}$-ht, indicating that these FtsN variants mediated the binding of PBP1B to the beads.

The direct interaction between PBP1B and FtsN was also detected by surface plasmon resonance (Fig. 6). FtsN-ht was immobilized on the chip surface. After injection of His-PBP1B, there was a specific signal (after subtraction of the signal from the control surface) indicating an interaction with FtsN. There was no binding of the soluble form of *E. coli* PBP3 or of the DD-carboxypeptidase/PBP of *Streptomyces* R61 to the immobilized FtsN, validating the specificity of the observed interaction between FtsN and PBP1B. When various concentrations of PBP1B were injected, the intensity of the signal increased with the concentration of PBP1B. However, the binding constant could not be determined as we failed to completely regenerate the FtsN surface. We also tried to analyze the interaction of FtsN-ht to His-PBP1B immobilized via its active site for transpeptidation to an ampicillin-coated CM5 chip. However, FtsN bound strongly to the control surface, preventing the study of its interaction with the immobilized PBP1B. The soluble $FtsN^{58-319}$-ht (0.34–3.4 μM) did not interact with the immobilized His-PBP1B, and conversely, His-PBP1B (109 nM) did not interact with the immobilized $FtsN^{58-319}$-ht (data not shown).

Having shown a direct interaction between the purified proteins, we next tested whether PBP1B and FtsN interact *in vivo*, applying a bacterial two-hybrid system. The system is based on the reconstitution of the cAMP signal transduction pathway in a *cya*-deficient *E. coli* strain (2, 34). A positive β-galactosidase signal is generated in cells producing T18-X and T25-Y fusion proteins only if X and Y interact with each other. The β-galactosidase activities were significantly higher (48- to 85-fold) in extracts from *E. coli* DHM1 expressing T18-FtsN and T25-PBP1B or T25-FtsN and T18-$(G_4S)_3$-PBP1B compared with control cells expressing T18-FtsN and T25 or T18 and

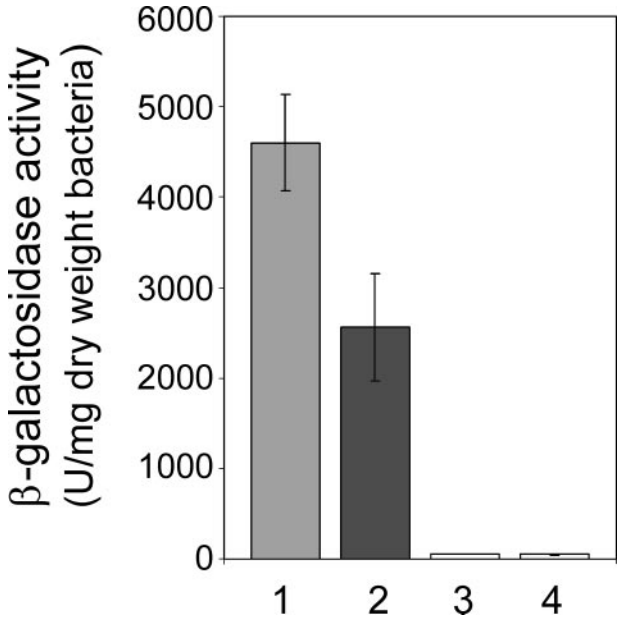

FIGURE 7. **Interaction between PBP1B and FtsN by a bacterial two-hybrid system approach.** DHM1 transformants producing T18-$(G_4S)_3$-PBP1B and T25-FtsN (*lane 1*), T18-FtsN and T25-PBP1B (*lane 2*), T18-FtsN and T25 (*lane 3*), and T18 and T25-FtsN (*lane 4*)were grown in LB medium in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 30 °C. β-galactosidase activity in cell extracts was measured as published (2). The values are a mean of six clones. These results indicate that PBP1B and FtsN interact *in vivo*. *Error bars* indicate S.D.

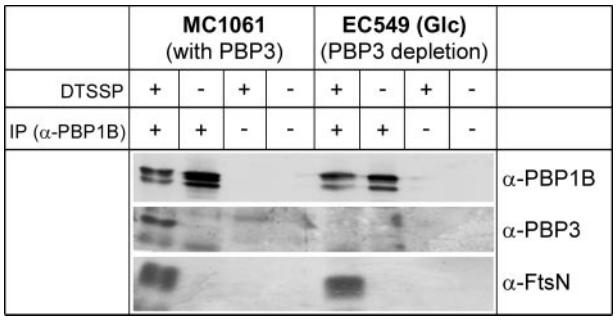

FIGURE 8. ***In vivo* cross-linking of PBP1B and FtsN.** Cells of MC1061 and glucose-grown EC549 were treated with or without dithiobis(sulfosuccinimidylpropionate (*DTSSP*) cross-linker. Membrane fraction was prepared and PBP1B was immunoprecipitated with purified antiserum. Control samples received no antiserum. PBP1B, PBP3, and FtsN were immunodetected after SDS-PAGE and Western blot. PBP1B and FtsN could be cross-linked in the presence and absence of PBP3.

T25-FtsN (Fig. 7). These results show that FtsN interacts with PBP1B *in vivo*.

Therefore, we tested whether PBP1B and FtsN can be cross-linked in intact cells of wild type and EC549 (PBP3 depletion strain) (Fig. 8). We used the water-soluble (3,3′-dithiobis[sulfosuccinimidylpropionate]) (DTSSP) cross-linker, which is capable of diffusing into the periplasm but not into the cytoplasm and can be cleaved by reducing agents (5). The membrane fraction was subjected to immunoprecipitation with an α-PBP1B antiserum, followed by Western blotting and immunodetection of PBP1B, PBP3, and FtsN in the immunoprecipitate. As we had observed before (5), PBP1B and PBP3 could be cross-linked *in vivo* in cells of MC1061. FtsN could be cross-linked with PBP1B in wild-type MC1061 cells

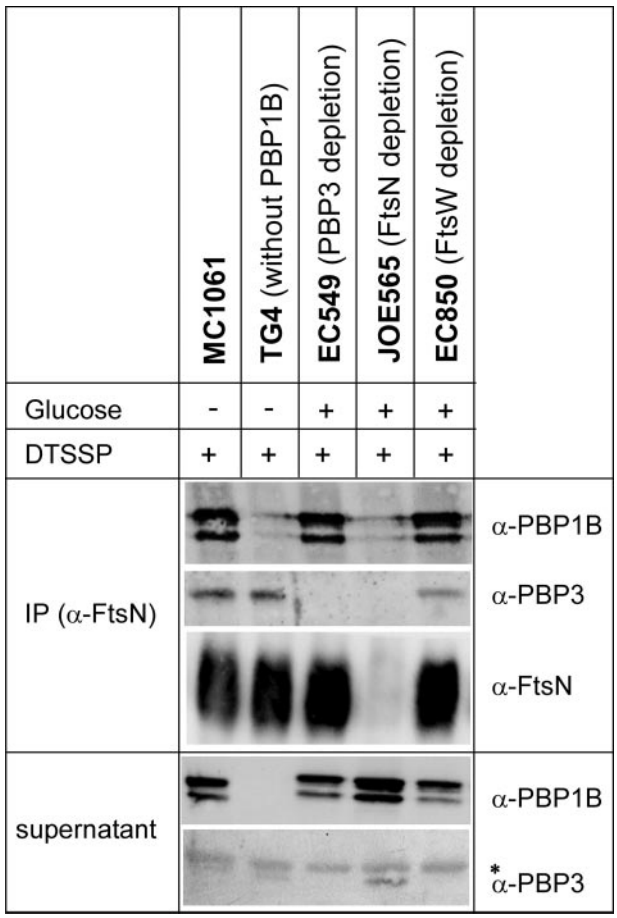

FIGURE 9. ***In vivo* cross-linking of PBP1B, PBP3, and FtsN.** Different strains were treated with dithiobis(sulfosuccinimidylpropionate (*DTSSP*) cross-linker. Membrane extract was prepared and FtsN was immunoprecipitated with purified antiserum. PBP1B, PBP3, and FtsN were immunodetected after SDS-PAGE and Western blot. PBP1B and FtsN were cross-linked *in vivo* in the presence and absence of PBP3 or FtsW. PBP3 and FtsN were cross-linked in the presence and absence of PBP1B and FtsW. In the control sample without FtsN, PBP1B and PBP3 were detected not in the immunoprecipitate but in the supernatant. *Star* indicates an unspecific signal of the α-PBP3 antiserum in samples of the supernatant.

and also in EC549 cells depleted of PBP3, indicating that FtsN and PBP1B interact with each other *in vivo* in the presence and absence of PBP3. Control samples prepared in the same way but omitting the α-PBP1B antiserum did not contain PBP1B, PBP3, or FtsN.

In another experiment the immunoprecipitation of cross-linked proteins was performed with α-FtsN antiserum followed by the detection of PBP1B, PBP3, and FtsN (Fig. 9). PBP1B (α and γ) was cross-linked *in vivo* with FtsN in wild-type cells and in cells lacking PBP3 (EC549) or FtsW (EC850). PBP3 was cross-linked with FtsN in wild type and in cells lacking PBP1B (TG4) or FtsW (EC850). As expected, FtsN, PBP1B, and PBP3 were not present in the immunoprecipitate of a strain depleted of FtsN (JOE565). To summarize these results, PBP1B could be cross-linked *in vivo* to FtsN in the presence and absence of PBP3 and PBP3 could be cross-linked to FtsN in the presence and absence of PBP1B. Both interactions are independent of the presence of the essential cell division protein FtsW and, hence, of ongoing cell division.

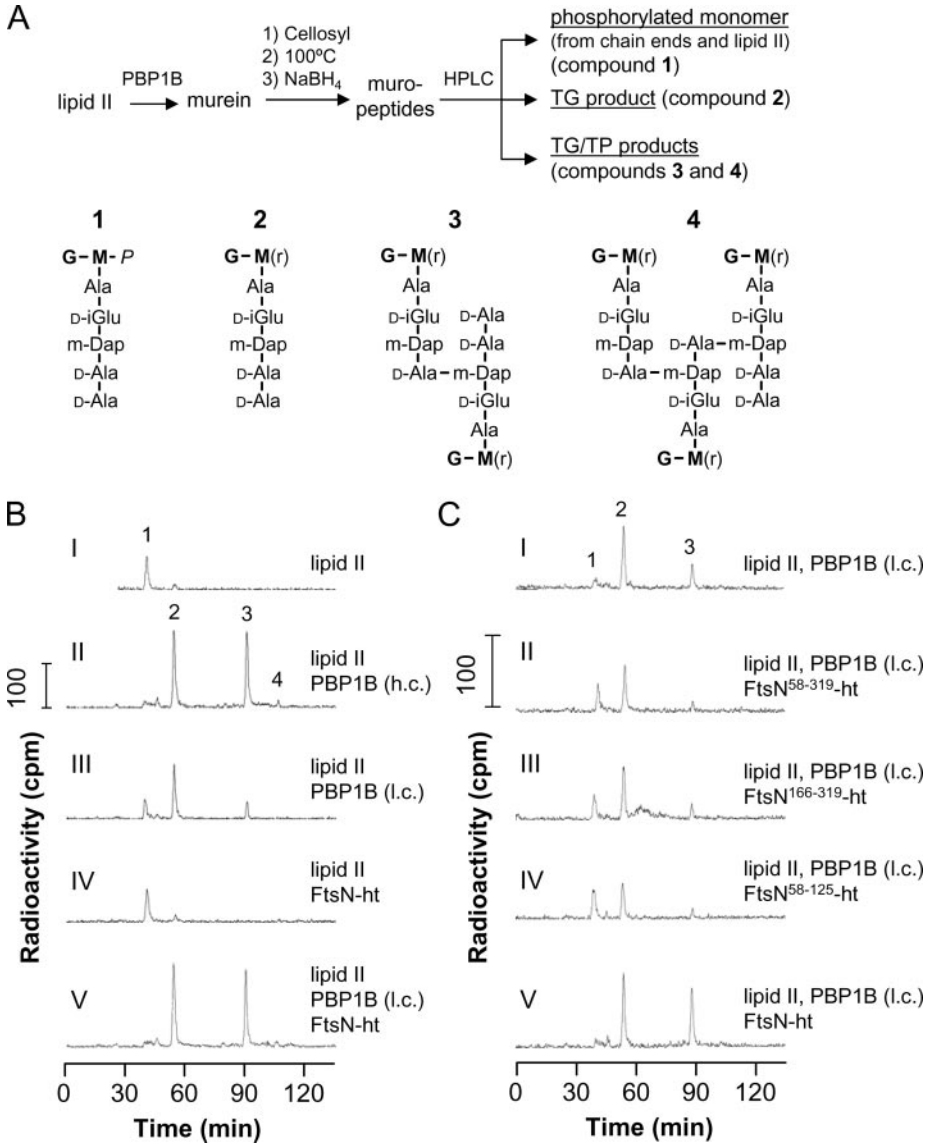

FIGURE 10. **Effect of FtsN on *in vitro* murein synthesis activities of PBP1B.** *A*, scheme of the *in vitro* murein synthesis assay and structures of the products formed. *G*, *N*-acetylglucosamine; *M*, *N*-acetylmuramic acid; *M(r)*, *N*-acetylmuramitol; *P*, phosphate; *TG*, transglycosylation; *TP*, transpeptidation. *B*, the following samples were incubated for 1 h at 30 °C, digested with cellosyl, reduced with sodium borohydride, and analyzed by high pressure liquid chromatography: *Row I*, lipid II alone; *row II*, lipid II and PBP1B (high concentration, *h.c.*); *row III*, lipid II and PBP1B (low concentration, *l.c.*); *row IV*, lipid II and FtsN-ht; *row V*, lipid II, PBP1B (*l.c.*), and FtsN-ht. FtsN-ht enhanced the activity of PBP1B at low concentration. FtsN-ht had no effect on lipid II alone. *C*, PBP1B was incubated at low concentration with lipid II alone (*row I*) or in the presence of $FtsN^{58-319}$-ht (*row II*), $FtsN^{166-319}$-ht (*row III*), $FtsN^{58-125}$-ht (*row IV*), or FtsN-ht (*row V*). Samples were processed as described above and analyzed by high pressure liquid chromatography. Only FtsN-ht containing the N-terminal cytoplasmic and transmembrane regions increased the activity of PBP1B.

Having identified an interaction between PBP1B and FtsN both *in vitro* and *in vivo*, we further tested whether the presence of FtsN-ht has an effect on the enzymatic activities of PBP1B, applying an *in vitro* murein synthesis assay with radioactively labeled lipid II substrate (9). The scheme of the assay and the products formed are shown in Fig. 10*A*. Briefly, the reaction product of PBP1B with lipid II was digested with the muramidase cellosyl, followed by boiling (to remove the lipid anchor present at lipid II and glycan strand ends) and reduction of MurNAc residues present in the transglycosylation products. High pressure liquid chromatography was used to separate the phosphorylated monomer (Fig. 10*A*, *compound 1*), the monomeric transglycosylation (TG) product (*compound 2*), and the cross-linked transglycosylation/transpeptidation (TG/TP) products (*compounds 3* and *4*). We confirmed our previous observation that PBP1B had high TG and TP activities when tested at high protein concentration (favoring dimerization) (9) but was poorly active at low concentration (Fig. 10*B*, *panels II* and *III*) (9). FtsN-ht did not show detectable activity with lipid II (*panel IV*). The presence of FtsN-ht did not alter the pattern of the reaction products when PBP1B was tested at high concentration (not shown). In contrast, FtsN-ht significantly enhanced the TP and TG activities at low PBP1B concentration. In different experiments, the yield of TG products (compounds 2, 3, and 4) increased 2.4- to 5.0-fold and the yield of TP products (compounds 3 and 4) increased 5.1- to 17.3-fold if FtsN were present (Fig. 10*B*, compare *panels III* and *V*). FtsN-dependent stimulation of PBP1B activity (at low enzyme concentration) was independently confirmed by using a different type of *in vitro* murein synthesis assay that allows quantification of the high molecular weight product formed from lipid II substrate (30). In this assay, the presence of FtsN increased the yield of product formed by PBP1B by a factor of 4.7. Only full-length FtsN-ht (containing the N-terminal region) showed a stimulatory effect on PBP1B activities (Fig. 10*C*, compare *panel V* with *panel I*). In contrast, the soluble FtsN variants $FtsN^{58-319}$-ht, $FtsN^{166-319}$-ht, and $FtsN^{58-125}$-ht had no effect on PBP1B activities (compare *panels II*, *III*, and *IV* with *panel I*). In summary, these results show that the presence of FtsN-ht stimulates the TG and TP activities of PBP1B at low enzyme concentrations.

DISCUSSION

Bacterial propagation requires the safe and shape-maintaining enlargement of the murein sacculus. In recent years, a model has emerged in which murein enzymes (synthases and hydrolases) form multienzyme complexes that are spatially and temporally controlled by components of the bacterial cytoskeleton (7, 35). Indeed, many protein-protein interactions between murein synthases, hydrolases, and cell

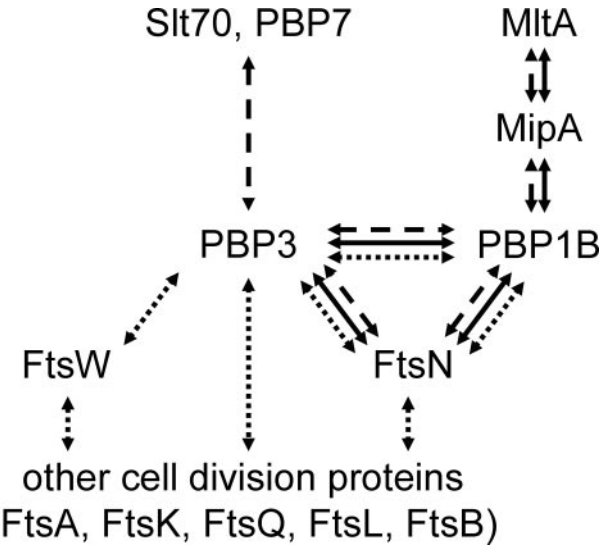

FIGURE 11. **Interaction network between murein synthases (*PBP1B, PBP3*), murein hydrolases (*MltA, Slt70, PBP7*), and cell division proteins (*FtsN, FtsW, FtsA, FtsQ, FtsL, FtsB*).** *Solid arrows*, direct interactions detected with the purified proteins using different methods (Refs. 5, 14 and this work); *dashed arrows*, interactions detected by affinity chromatography (Refs. 14, 40, 41 and this work); *dotted arrows*, interactions detected by genetic methods (Refs. 2, 5, 17, 22, 24, 39, 42, 43 and this work).

division or elongation proteins have been detected (summarized in a recent review, Ref. 36). However, little is known about how the activities of murein synthases are regulated by other proteins. In this study we have discovered, for the first time, *in vitro* stimulation of a murein synthase (PBP1B) by another protein, the cell division protein FtsN. Both proteins interact *in vitro* and *in vivo* as has been shown here by different methods.

The results of our interaction studies with different methods are not completely consistent. Surface plasmon resonance detected an interaction of FtsN-ht (containing the N terminus with the transmembrane anchor), but not of soluble forms of FtsN-ht with His-PBP1B, which is consistent with the finding that only the FtsN-ht, and not the soluble variants, stimulated the activities of PBP1B. These results suggest the importance of the N terminus (with the transmembrane segment) of FtsN for strong interaction with PBP1B and for stimulation of its activities. On the other hand, all FtsN variants (including soluble ones) containing the region between amino acids 58 and 128 pulled down PBP1B to immobilized muropeptides. Affinity chromatography showed also an interaction of $FtsN^{166-319}$-ht with PBP1B. Therefore, there are likely regions of FtsN outside the N terminus (with transmembrane region) involved in weaker and/or indirect interaction with PBP1B (as detected by affinity chromatography and pulldown experiments).

Interestingly, FtsN interacts with two murein synthases, PBP1B and PBP3, which also interact with each other. Studies with the purified proteins and with mutant strains lacking one of the proteins also proved that each of the binary interactions (FtsN-PBP1B, FtsN-PBP3, and PBP1B-PBP3) occurs *in vivo* in the presence and absence of the third partner (this work and Ref. 5). Because all three proteins co-localize at the site of cell division, an obvious interpretation could be that there is a $PBP1B_x$-$PBP3_y$-$FtsN_z$ complex in the cell. However, we cannot exclude the alternative possibility that there are three binary complexes.

The following considerations may argue against the presence of a defined, stoichiometric complex of $PBP1B_x$-$PBP3_y$-$FtsN_z$ inside the cell. (i) Each protein (PBP1B, PBP3, and FtsN) participates in interactions with other proteins (Fig. 11 and the Introduction). They are probably part of a larger multiprotein assembly ultimately linked to the ring of FtsZ molecules. (ii) The copy number of FtsN of 3,000–6,000 copies/cell (23) is >20-fold higher than the copy numbers of PBP1B (127 ± 13 copies/cell) and PBP3 (132 ± 17 copies/cell) (37). Finally, Blue native gel electrophoresis showed that FtsN is capable of self-interacting to form oligomers.[7] Therefore, it is possible that in the cell a murein synthesis complex containing PBP1B and PBP3 (and probably other components such as MipA and membrane-bound lytic transglycosylase A) interacts via the murein synthases with oligomeric FtsN. Interestingly, we have detected interactions between FtsN and PBP1B and between FtsN and PBP3 in non-dividing cells (depleted of FtsW) in which FtsN, PBP3, and PBP1B do not localize at septal positions. This is reminiscent of interactions between PBP1B and PBP3 (5) and between FtsQ, FtsL, and FtsB (38) that have been detected in non-dividing cells. These cell division proteins appear to be recruited in pre-formed complexes to the division site.

The essentiality of FtsN can be by-passed by certain point mutations in *ftsA* that increase the integrity of the Z-ring. This shows that septal murein synthesis can occur in the absence of FtsN in cells with an altered FtsA (29). In this condition, either the PBP1B-PBP3 complex can function in the absence of FtsN or septal murein synthesis involves other murein synthases such as PBP1A or MtgA.

Several functions have been proposed for the N-terminal part of FtsN, including stabilizing the septal ring (39). In this work, we found a significant effect of FtsN on the *in vitro* murein synthesis activity of PBP1B. In particular, FtsN stimulated PBP1B activity at low concentration unfavorable for dimerization. PBP1B is known to be most active at conditions favoring dimerization (9). It is possible that FtsN induces dimerization of PBP1B, thereby activating it. Alternatively, or in addition, interaction with FtsN could increase the processivity of PBP1B. This work provides the first evidence for a possible *in vivo* regulation of murein synthesis complexes by a protein of the divisome, FtsN, that might coordinate or modulate the activities of a PBP1B-PBP3 complex in the cell.

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