The Cell Division Protein FtsN: Identification of SPOR Domain Amino Acids Important for Septal Localization, Peptidoglycan-binding, and a Disulfide Bond

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Abstract

SPOR domains are about 75 amino acids long and probably bind septal peptidoglycan during cell division. We mutagenized 33 amino acids with surface-exposed side chains in the SPOR domain from an *Escherichia coli* cell division protein named FtsN. The mutant SPOR domains were fused to Tat-targeted green fluorescent protein (TTGFP) and tested for septal localization in live *E. coli* cells. Lesions at 5 residues reduced septal localization by a factor of three or more: Q251, S254, W283, R285 and I313. All of these residues map to a β-sheet in the published solution structure of FtsN\(^{SPOR}\). Three of the mutant proteins (Q251E, S254E, and R285A) were purified and found to be defective in binding to peptidoglycan sacculi in a co-sedimentation assay. These results match closely with results from a previous study of the SPOR domain from DamX, even though these two SPOR domains share <20% amino acid identity. Taken together, these findings support the proposal that SPOR domains localize by binding to septal peptidoglycan and imply the binding site is associated with the β-sheet. We also show that FtsN\(^{SPOR}\) contains a disulfide bond between β-sheet residues C252 and C312. The disulfide bond contributes to protein stability, cell division, and peptidoglycan binding.
Introduction

Cell division in *Escherichia coli* is mediated by structure called the “septal ring” or “divisome” that assembles at the midcell (1-4). The septal ring contains at least 30 different types of proteins, most of which localize by binding to other septal ring proteins (e.g., (5-13)). But proteins that contain a SPOR domain (Pfam 05036) may localize by a completely different mechanism because SPOR domains are thought to bind to septal peptidoglycan (PG) (14-16). The details of the SPOR-PG interaction have yet to be elucidated. This is of interest because it is not known how septal PG differs from PG elsewhere in the sacculus and understanding the SPOR-PG interaction might lead to new insights into PG biogenesis during cell division.

SPOR domains are ~75 amino acids long and have been identified using bioinformatics approaches in over 7000 proteins from over 2000 bacterial species (17). At least seven of these SPOR domains have been shown to localize to the midcell *in vivo* and bind PG *in vitro* (14-16, 18), and we are not aware of any documented counter examples, so PG-binding is probably a general property of these domains. In contrast, there is good evidence for diversity in the biochemical and physiological functions of the numerous SPOR domain proteins. For starters, most SPOR domain proteins contain additional domains, which are different in different proteins (17). Moreover, although most of the SPOR domain proteins that have been studied are components of the septal ring that mediates cell division (14-16, 19), there are exceptions. Indeed, the name “SPOR” domain arose because the founding member of the family, CwlC of *Bacillus subtilis*, is a cell wall amidase that helps degrade PG to release the spore from the mother cell (20-22). Another exception is a small *Vibrio parahaemolyticus* protein designated VPA1294 that has been implicated in swarmer cell differentiation (23).
The present manuscript is more concerned with the structure-function relationships of SPOR domains than with their physiological roles, so we now summarize what is known about this topic. The solution structures of three SPOR domains have been solved by nuclear magnetic resonance spectroscopy (NMR). These domains come from two *E. coli* cell division proteins, FtsN and DamX, and the *Bacillus subtilis* sporulation protein, CwlC (24-26). Hereafter we will refer to these domains as FtsN$^{\text{SPOR}}$, DamX$^{\text{SPOR}}$ and CwlC$^{\text{SPOR}}$, respectively. All three domains exhibit a similar core architecture comprising a $\beta\alpha\beta\alpha\beta$ secondary structure that folds into a 4-stranded anti-parallel $\beta$-sheet buttressed on one side by 2 $\alpha$-helices (Figure 1). We recently identified three surface-exposed amino acids in the $\beta$-sheet of DamX$^{\text{SPOR}}$ that are important for septal localization and binding to PG sacculi from whole cells (25) (Figure 1). These residues are probably part of a binding site for septal PG. Here we address two important questions that were not addressed in our structure-function study of DamX$^{\text{SPOR}}$. First, can the results obtained with DamX be generalized to SPOR domains from other proteins? This is an issue because SPOR domains display less than 20% identity in pairwise comparisons, and it is not known whether all SPOR domains bind the same PG structure. Second, are additional residues, especially residues outside of the $\beta$-sheet, also important for septal localization? We only mutagenized nine amino acids in DamX$^{\text{SPOR}}$, so important residues might have been overlooked.

To address these questions, we undertook a comprehensive mutagenesis of the SPOR domain from FtsN, an essential cell division protein found in a variety of proteobacteria (16, 19).

By way of background, *E. coli* FtsN comprises a short N-terminal cytoplasmic domain (∼30 a.a.), a single transmembrane helix (∼20 a.a.), and a comparatively large periplasmic domain (∼265 a.a.), which is mostly unstructured except for the SPOR domain (residues 243-319) at the very C-terminus of the protein (26, 27). *In vitro*, FtsN$^{\text{SPOR}}$ binds to intact PG sacculi and to the
glycan strands released by digestion of PG with an amidase (18). In vivo, FtsN\textsuperscript{SPOR} localizes to
the septal ring in wild-type \textit{E. coli} but not in a triple amidase mutant (15). Although FtsN\textsuperscript{SPOR} is
required for obvious localization of GFP-FtsN fusion proteins to the septal ring (15, 16), truncated FtsN proteins that lack the SPOR domain support cell division almost as well as wild-type (15, 16, 18). The essential function of FtsN has been mapped to a 35 a.a. region at the
beginning of the periplasmic domain (15). This region of FtsN is thought to interact with some
other division protein(s), perhaps a PG synthase, to trigger constriction (13, 15, 26, 28-30). In
summary, current thinking is that a portion of FtsN near the start of the periplasmic domain
mediates the critical function of FtsN, while the SPOR domain improves the efficiency of this
process by targeting FtsN to the septal ring.

The only other well-characterized SPOR domain is from DamX of \textit{E. coli}. Although
mutants lacking DamX have only subtle phenotypic changes (14, 15, 31), the SPOR domain of
DamX is very important for these functions (25). This makes DamX a more tractable model
protein than FtsN for exploring the physiological importance of the SPOR domain.
Nevertheless, as explained below, FtsN\textsuperscript{SPOR} is a good model system for exploring the generality
of the structure-function relationships reported for DamX\textsuperscript{SPOR}.

First, the solution structure of FtsN\textsuperscript{SPOR} is available and assays for studying septal
localization and PG binding have been established (15, 18, 26). Second, there are some striking
structural differences between FtsN\textsuperscript{SPOR} and DamX\textsuperscript{SPOR}, suggesting that functional differences
might exist as well. In FtsN\textsuperscript{SPOR} the \(\beta\)-sheet is relatively flat, whereas in DamX\textsuperscript{SPOR} it is
strikingly curved (Figure 1). The \(\beta\)-sheet of CwlC\textsuperscript{SPOR} is also rather curved (Figure 1), making
FtsN\textsuperscript{SPOR} the odd one out (24-26). This is notable because residues important for septal
localization and PG-binding in DamX\textsuperscript{SPOR} map to the \(\beta\)-sheet (25). Thus, unless there are
conformational changes, it is doubtful that FtsN\textsuperscript{SPOR} and DamX\textsuperscript{SPOR} could interact with PG using homologous residues. A third difference is that DamX\textsuperscript{SPOR} contains a short C-terminal α-helix that interacts with the β-sheet and is needed for domain stability (25). No such α-helix exists in FtsN\textsuperscript{SPOR} or CwlC\textsuperscript{SPOR}, as these two domains are slightly shorter than DamX\textsuperscript{SPOR}, but FtsN\textsuperscript{SPOR} possesses a rather flexible C-terminus (AAGG), which may destabilize the domain. A final indication that FtsN\textsuperscript{SPOR} and DamX\textsuperscript{SPOR} might not use homologous residues to bind PG is that these domains share <20% amino acid identity. These considerations prompted us to conduct a thorough mutagenesis of FtsN\textsuperscript{SPOR}, the results of which are presented in this report.

**Materials and Methods**

**Strains, plasmids, primers and media.** All strains used in this study are listed in Table S1. Unless noted otherwise, *E. coli* was grown at 30°C in Luria-Bertani (LB) medium containing 1% NaCl or in M9 minimal medium containing 0.2% glucose and thiamine at 1 μg/ml (32). Plates contained 15 g agar per liter. Ampicillin and kanamycin were used at 200 μg/ml and 40 μg/ml, respectively.

**Plasmid construction.** Plasmids for localization studies of mutant forms of FtsN\textsuperscript{SPOR} were variants of pDSW992 [ColE1 ori, bla, lacI\textsuperscript{q}, a weakend trc promoter designated P\textsubscript{204,TT}, gfp-ftsN residues 240-319]. Most amino acid substitutions in FtsN\textsuperscript{SPOR} were introduced using degenerate primers and a multistep PCR procedure involving megaprimering (33). Plasmids for overproducing hexahistidine (His\textsubscript{6}-) tagged FtsN\textsuperscript{SPOR} variants were based on pQE80L (Qiagen). See supplemental information for details of all plasmid constructions, including primer sequences in Table S2.
Protein localization and microscopy. Cells for localization of $^{\text{T}}$T-GFP-FtsN$^{\text{SPOR}}$ proteins were grown and visualized essentially as described (25) except that overnight cultures were diluted 1:200 and examined when the OD$_{600}$ reached ~0.5. To assay localization experiments done in the CH34/pMG20 background, the media included 0.2% L-arabinose and chloramphenicol at 30 $\mu$g/ml. Our microscope, camera, and software have been described (34).

Purified proteins. Wild-type and mutant His$_6$-tagged FtsN$^{\text{SPOR}}$ proteins were overproduced in *E.coli* SHuffle T7 (35) (New England Biolabs) and purified at 4°C by cobalt affinity chromatography. Procedures were similar to as described (14), except cells grown in LB at 37°C to OD$_{600}$ ~0.5 were induced overnight at 20°C with 1 mM isopropyl-$\beta$-D-thiogalactoside (IPTG). The purified proteins were dialyzed against binding buffer (25 mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.5) at 4°C. Typical yields were ~1 mg from a 500 ml culture and purity was judged to be ~95% by SDS-PAGE. Aliquots were stored at 4°C and PG binding assays were conducted within three days.

PG binding assay. Whole PG sacculi were isolated and quantified based on amino sugar content as described previously (14). Binding assays were performed as described, in 100 µl reaction mixtures containing 1 nmol of protein and 75 µg of PG sacculi, which corresponds to about 75 nmol of disaccharide units. When testing the effect of the disulfide bond on PG-binding, 1 mM dithiothreitol (DTT) was included in the buffers during dialysis and the binding assay.

Western blotting. Western blotting was performed as described (14). Typically cells from 1 mL of culture grown to an OD$_{600}$ ~0.5 were pelleted in a microfuge, taken up in 0.2 mL of sample buffer and boiled for 10 min. Then 10 µl of sample were loaded onto an SDS-
polyacrylamide gel. Purified rabbit anti-FtsN and anti-FtsQ were diluted 1:2,000 or 1:4,000, respectively (36). Rabbit anti-GFP serum was obtained from C. Ellermeier and used at a dilution of 1:8,000. The secondary antibody was goat anti-rabbit conjugated to horse-radish peroxidase (Thermo Scientific, Rockford, IL) and detection was with SuperSignal West Pico chemiluminescent substrate (also from Thermo Scientific). Blots were photographed using a Fujifilm LAS-1000 imager.

Results

Localization-defective mutants of FtsN<sup>SPOR</sup> map to the β-sheet. The SPOR domain from FtsN contains 76 residues, 33 of which we judged by visual inspection of the structure in PyMOL to have surface-exposed R-groups (37). These 33 residues are distributed throughout the domain (Figure 2), including many in the two α-helices that were not explored in our previous mutagenesis of DamX<sup>SPOR</sup>. An alignment of FstN SPOR domains from several species revealed that the residues targeted for mutagenesis are not simply the most highly conserved ones (38). Many of the highly conserved residues are internal to the domain and presumably make important contributions to protein folding. The 33 targeted residues were changed to alanine using a strategy that involved degenerate primers, allowing other substitutions to be obtained as well, for a total of 92 mutants. The mutant SPOR domains were fused to Tat-targeted GFP (T<sub>T</sub>GFP) to direct export of the properly folded T<sub>T</sub>GFP-FtsN<sup>SPOR</sup> fusion proteins into the periplasm (39).

Wild-type E. coli cells harboring the T<sub>T</sub>GFP-FtsN<sup>SPOR</sup> plasmids were grown to mid-log phase in LB and then immobilized on an agarose pad for fluorescence microscopy. Septal localization was quantified by scoring cells for the presence or absence of a fluorescent band at 8
the midcell, with results reported as the fraction of cells in the population that exhibited septal localization. We used an arbitrary statistical cut-off to identify localization-defective proteins; this cut-off was a 3-fold reduction in localization, which corresponds to ≤ 23% of the cells in the population being scored positive for a fluorescent band at the midcell. Samples were also taken for Western blotting with anti-GFP antiserum to ascertain whether non-localizing TTGFP-\textsuperscript{SPOR} proteins were produced in normal amounts. The results for our most interesting mutants are presented in Figure 3. Quantitative localization data for all mutants are in Table S3.

A wild-type TTGFP-FtsN\textsuperscript{SPOR} construct localized to the septal ring in ~70% of the cells, similar to previous reports (14, 15). Lesions at Q251, S254, W283, R285, and I313 reduced septal localization by a factor of at least 3 without destabilizing the protein as judged by Western blotting (Figure 3). All of these residues map to the β-sheet (Figure 2). Proteins with substitutions at P290 also localized very poorly, but in this case Western blots revealed folding or stability defects (Figure S1), so we did not classify P290 as being important for septal localization per se.

If the cut-off used to classify residues as important for septal localization were relaxed to include mutants with a 2-fold defect (i.e., septal localization in ≤ 35% of the population), some additional amino acids would come into play: R256, E262, T263, R265, and F270. These amino acids are in the loop connecting β1 to α1 (R256) or in α1 proper (E262, T263, R265 and F270). Western blotting indicated these residues are not important for the overall stability of the domain (Figure S1). We suspect substitutions at these sites of secondary importance perturb septal localization indirectly by altering the conformation of the β-sheet (see Discussion).

As noted above, these results pertain to a wild-type E. coli host. If the SPOR domain from the native FtsN protein interacts with the SPOR domains produced from the plasmid, either
directly by dimerization or indirectly by competition for binding sites in the PG, then use of a
wild-type host might lead to misinterpretations. We therefore assayed localization of wild-type
and 14 mutant $^{14}$GFP-FtsN$_{SPOR}$ constructs in an $E. coli$ strain that produces a truncated FtsN with
no SPOR domain (15). The mutants chosen included two with severe localization defects
(Q251E, W283D), two with intermediate defects (T263D, F250A) and 10 that localized well
when assayed in a wild-type background. Although $^{14}$GFP-FtsN$_{SPOR}$ constructs did not localize
quite as well in the FtsN SPOR null strain, the trends were very similar to what we observed
when the native FtsN protein was present (Table S4). For example, localization of the wild-type
construct fell from 70% to 60%, while localization of the W283D mutant fell from 9% to 4%.
We conclude that the presence or absence of authentic FtsN produced from the chromosome
does not affect the interpretation of our experiments to identify FtsN$_{SPOR}$ residues important for
septal localization.

Finally, it should be noted that we did not introduce any SPOR domain amino acid
substitutions into full-length FtsN to determine whether these impair cell division. It is unlikely
that such mutations would have much effect on cell division because even a complete deletion of
the SPOR domain is surprisingly well tolerated (15, 16, 18). For DamX, whose SPOR domain is
critical for the overall function of the protein, we found that SPOR domain point mutations are
not as deleterious as SPOR domain deletions (14).

Localization-defective mutants bind PG poorly. FtsN$_{SPOR}$ probably localizes to the
septal ring by binding to septal PG (14, 15, 18, 25). This hypothesis predicts that localization
defective FtsN$_{SPOR}$ mutant proteins will also be defective in PG binding. To test this idea, we
subcloned coding sequences for wild-type and three localization-defective SPOR domains
(Q251E, S254E and R285A) into a vector that provides an N-terminal histidine tag to facilitate
purification. These constructs also included two C-terminal aspartate residues (DD) to mask a potential recognition signal for the ClpXP protease (40). Addition of DD to the C-terminus did not impair septal localization of a TTGFP-FtsN^{SPOR} construct (Figure 3B, Table S3). [The problems with overproduction that led us to modify the C-terminus are described in Supplemental Information and Figure S2.] All proteins were overproduced at 20 °C in an *E. coli* strain engineered to promote disulfide bond formation in the cytoplasm (35).

Approximately 25 ± 5 % of wild-type FtsN^{SPOR} protein co-sedimented with purified *E. coli* PG sacculi upon ultracentrifugation, but for the mutant proteins this was reduced to 7 ± 3% (Q251E, S254E) or 19 ± 1% (R285A) (Figure 3C). These values correlate with the relative proficiencies of septal localization: WT > R285A > Q251E ≈ S254E (Table S1). It should be noted that because these experiments employed whole sacculi, it is not known whether the binding observed reflects a general affinity for PG or the specific binding to septal PG inferred from localization studies *in vivo*.

The β-sheet of FtsN^{SPOR} contains a disulfide bond. FtsN from *E. coli* contains two cysteines, C252 and C312, both of which are conserved and located in the β-sheet (Figure 2). Paired cysteines in *E. coli* periplasmic proteins usually form disulfide bonds (41, 42).

Examination of the published structure of FtsN^{SPOR} revealed C252 and C312 are adjacent (Figure 4A) (26), but the two sulfur atoms are separated by about 5.3 ± 0.9 Å (Table S5), which is longer than the ~2 Å distance expected for a disulfide bond. However, the domain used to determine the structure was overproduced in the cytoplasm and purified in the presence of DTT, so any disulfides normally present in FtsN^{SPOR} might have been reduced. To test for a disulfide in FtsN, we prepared whole cell extracts in Laemmli sample buffer containing or lacking 5% β-mercaptoethanol (~700 mM). Western blotting revealed that reducing the thiols in FtsN resulted...
in slightly lower mobility during polyacrylamide gel electrophoresis, whereas the mobility of FtsQ, which lacks cysteines, was unchanged (Figure 4B; Because the mobility difference is subtle, a second blot is shown in Figure S3 to document reproducibility). The location of C252 and C312 suggests they would form an intramolecular disulfide; consistent with this, a larger species indicative of an FtsN dimer was not detected in the unreduced samples (Figure 4B).

The major catalyst of disulfide bond formation in the periplasm is DsbA (43). We obtained several dsbA null mutants from Jim Bardwell, along with the corresponding parental wild-type strains. It has been reported that an ftsN SPOR domain deletion mutant exhibits normal cell length in LB but is slightly elongated in M9 minimal medium (15). Similarly, we observed that dsbA strains are the same length as wild-type when grown in LB, but when grown in M9 the dsbA mutant averaged 30% longer (Figure 4D). There was also a higher fraction of constricting cells, 30 ± 7% for vs. 15 ± 9%, suggesting loss of DsbA retards the rate of cytokinesis. The dsbA mutant strains also averaged about 10% thinner than wild-type, a difference observed in both LB and M9 (Figure 4D). Western blotting revealed the absence of DsbA reduced steady-state levels of FtsN by about 4-fold in M9 (Figure 4C). The difference was closer to 2-fold in LB (Figure S4). In contrast, the absence of DsbA had no effect on the abundance of DamX, a SPOR domain protein that does not contain any cysteines (Figure 4C, Figure S4).

Cells producing TTGFP-FtsNSPOR were dim and septal localization was reduced in a dsbA background (Figure 5A), but a TTGFP-DamXSPOR construct localized well even in cells lacking DsbA (Figure S5). Consistent with the localization results, Western blotting revealed about 4-fold less TTGFP-FtsNSPOR in dsbA mutants, but no change in the abundance of TTGFP-DamXSPOR (Figure 5B). Changing the cysteines to alanine, either alone or simultaneously, reduced TTGFP-
FtsN<sup>SPOR</sup> stability so severely that the proteins were essentially undetectable in Western blots (Figure 5C). Finally, inclusion of DTT in a co-sedimentation assay caused a 25% reduction in PG binding by wild-type FtsN<sup>SPOR</sup>, from 25 ± 5% (N = 4) of the input protein to 19 ± 1% (N = 2) (Figure 3C).

**Discussion**

The binding site for septal PG in FtsN<sup>SPOR</sup> is probably the β-sheet. A comprehensive mutagenesis of FtsN<sup>SPOR</sup> residues with surface-exposed side-chains revealed only five amino acids that, when mutagenized, reduced localization by a factor of three or more: Q251, S254, W283, R285, and I313. All five of these residues are in the β-sheet. Three of the localization-defective mutant proteins (Q251E, S254E and R285A) were purified and found to bind PG poorly as compared to wild-type. Taken together, these findings argue FtsN<sup>SPOR</sup> localizes by binding to septal PG and suggest the binding site is associated with the β-sheet.

Mutations at S254 returned noteworthy results because changes to E and K reduced localization to 13% and 5%, respectively, but the S254A mutant localized as well as wild-type (Figure 3B, Table S3). Similar results emerged from our analysis of DamX<sup>SPOR</sup>, where changes of S354 to T or K greatly impaired septal localization but an S354A mutant localized like wild-type (25). Alignments show S and A occur with roughly equal frequency at this position (17, 25). We infer that the R-group at this position has to be small but does not make a direct contribution to PG-binding.

One of the motivations for this study was to determine whether any residues outside of the β-sheet are important for septal localization, as this would implicate additional surfaces of
FtsN\textsuperscript{SPOR} as potential PG-binding sites. Although no critically important residues outside of the \( \beta \)-sheet were found, we did identify a few that make modest contributions to septal localization: R256, E262, T263, R265, and F270. We suspect these residues affect localization indirectly. R256 is in the loop connection \( \beta 1 \) to \( \alpha 1 \), and interacts with one of the residues critical for septal localization, W283. Residues E262, T263, R265 and F270 are all in \( \alpha 1 \), which has extensive interactions with the \( \beta \)-sheet, especially strand \( \beta 2 \). In particular, parts of the side-chain of R265 contact \( \beta 2 \) residue I277. Residue E262 also comes close to I277, though whether these two amino acids make direct contact is not clear.

All SPOR domains probably engage PG via the \( \beta \)-sheet. Residues Q251 and S254 of FtsN\textsuperscript{SPOR} align with the most highly conserved surface-exposed SPOR domain residues and the corresponding amino acids of DamX\textsuperscript{SPOR}, Q351 and S354, were found to be important for septal localization and PG binding in that domain (25). The SPOR domains from FtsN and DamX have <20\% identity and noteworthy differences at the level of tertiary structure, particularly the curvature of the \( \beta \)-sheet and the presence of an extra \( \alpha \)-helix at the C-terminus of DamX\textsuperscript{SPOR} (Figure 1). Thus, the fact that we obtained similar results for two such divergent SPOR domains suggests most, perhaps all, SPOR domains bind PG in a similar fashion.

SPOR domains exhibit a ribonucleoprotein fold (RNP-fold) (25, 26). Despite the name, RNP-folds are found in a variety of proteins and mediate interactions with a variety of ligands, not just RNAs. There is much precedent for the \( \beta \)-sheet being the primary ligand-binding site in RNP-fold domains. For example, the human splicosomal protein U1A has two RNP-fold domains, both of which bind RNA via residues in the \( \beta \)-sheet. In particular, U1A residues in \( \beta 1 \) and \( \beta 3 \) play a critical role in binding to the cognate RNA hairpin (44, 45), reminiscent of our finding that the most important residues in FtsN\textsuperscript{SPOR} are in \( \beta 1 \) (Q251, S254) or \( \beta 3 \) (W283, R285).
Although the features of PG recognized by SPOR domains remain to be elucidated, current thinking is that they bind to glycan strands lacking oligopeptide side-chains. “Naked” glycan strands are likely enriched at the division site by action of periplasmic amidases that process septal PG to facilitate separation of daughter cells (46-49). The strongest support for this hypothesis is that FtsN$^{\text{SPOR}}$ binds the glycan backbone of PG in vitro and fails to localize in a triple amidase mutant in vivo (15, 18). Stacking of aromatic amino acids, especially tryptophan, with the sugar rings of oligosaccharides is known to be important for many protein-carbohydrate binding interactions (50-53). Both FtsN$^{\text{SPOR}}$ and DamX$^{\text{SPOR}}$ have a β-sheet tryptophan that plays a key role in septal localization, W283 in FtsN and W416 in DamX. Curiously, however, these residues are not homologous—W283 of FtsN is in β3 whereas W416 of DamX in β4.

**The β-sheet has a disulfide bond.** FtsN$^{\text{SPOR}}$ has a disulfide bond that links residues C252 in β1 to C312 in β4. The disulfide is not present in the protein used for structure determination, which was obtained under reducing conditions. An interesting question is whether the absence of the disulfide explains why the β-sheet in FtsN$^{\text{SPOR}}$ is relatively flat as compared to DamX$^{\text{SPOR}}$ and CwlC$^{\text{SPOR}}$ (Figure 1). Although we cannot be certain, we doubt that this is the explanation because modeling the effect of the disulfide bond on the structure of the domain does not change the conformation of the β-sheet very dramatically (Figure S6). This interesting anomaly deserves further exploration because it stands to reason that the β-sheet of all three SPOR domains has to adopt the same conformation for homologous residues to bind PG.

Assessing the importance of the disulfide bond for FtsN$^{\text{SPOR}}$ function is complicated by the potential pleiotropy of a *dsbA* null mutation and the fact that FtsN is less stable and gets degraded when the disulfide bond is absent. Despite these caveats, multiple observations argue the disulfide bond is important. First, the cysteines that form this bond are highly conserved in
sequences from different organisms. Second, FtsN levels were reduced in \textit{dsbA} mutants, especially when cells were grown in minimal media, where the mutants also exhibited a small division defect. Third, the wild-type \textit{TT}GFP-FtsN\textsuperscript{SPOR} construct was not well produced and localized poorly in a \textit{dsbA} background. Fourth, changing the cysteines to alanine, either alone or in combination, destabilized the \textit{TT}GFP-FtsN\textsuperscript{SPOR} construct so severely that it was difficult to detect in Western blots using anti-GFP antisera. Fifth, overproduction of a histidine-tagged FtsN\textsuperscript{SPOR} construct in the cytoplasm was greatly improved by using an \textit{E. coli} host engineered to produce disulfide bonds in the cytoplasm (Figure S2). Finally, inclusion of DTT in the PG binding assay reduced binding by about 25%, similar to the R285A substitution that caused a 3-fold reduction in septal localization of a \textit{TT}GFP-FtsN\textsuperscript{SPOR} construct.

To our knowledge, FtsN is the first example of a bacterial cell division protein with a disulfide bond. The Pfam database contains a seed alignment of 136 diverse SPOR domains (http://pfam.janelia.org/family/PF05036#tabview=tab3) (17). Of these, 22 have two cysteines (Table S6), suggesting about 15% of SPOR domain proteins contain a disulfide bond. We therefore infer that disulfide bonds will prove to be important for the stability or function of many SPOR domains.

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cellulose binding by a type-A cellulose-binding module (CBM) and bacterial expansin.
Figure Legends

Figure 1. **SPOR domains have similar structures.** The 4-stranded β-sheet and 2 α-helices common to all SPOR domains are shown in blue and green, respectively. For clarity, turns and coils are also illustrated in green, while the C-terminal helix unique to DamX^{SPOR} is orange. Residues important for septal localization are highlighted in red. Coordinates for these domains were obtained from the Protein Data Bank (PDB 1x60, PDB 1UTA and PDB 2LFV) and rendered using PyMOL.

Figure 2. **Location of FtsN^{SPOR} amino acids targeted for mutagenesis.** Above: Ribbon diagram illustrating the location of all residues (red) at which amino acid substitutions were introduced to test for localization defects. Below: An alignment of the SPOR domains from several FtsN proteins. Residues targeted for mutagenesis are red, with arrows pointing to residues where substitutions impaired localization at least 3-fold. Black background highlights the two conserved cysteines proposed to form a disulfide bond. The alignment was prepared in Clustal W (38) with the following FtsN SPOR domain sequences: *Escherichia coli* MG1655 (locus tag: b3833) residues 244-319, *Citrobacter rodentium* ICC168 (ROD_38131) residues 236-310, *Klebsiella pneumoniae* 342 (KPK_5451) residues 245-320, *Yersinia pestis* biovar *Antiqua B42003004* (YpAngola_A0114) residues 206-281, *Proteus mirabilis* ATCC 29906 B42003004 (YpAngola_A0114) residues 206-281, *Proteus mirabilis* ATCC 29906, *Haemophilus aegyptius* ATCC 11116 (HMPREF0693_0033) residues 188-264, and *Haemophilus aegyptius* ATCC 11116 (HMPREF9095_0699) residues 185-256.

Figure 3. **Localization-defective mutants of FtsN^{SPOR}**. (A) Western blot with anti-GFP antibody demonstrating that WT and mutant TT{sup}GFP-FtsN^{SPOR} proteins were produced at similar...
levels. The (-) designation refers to a control strain producing $^{\text{TT}}$GFP not fused to anything. (B) Fluorescence micrographs of cells producing the indicated $^{\text{TT}}$GFP-$\text{FtsN}^{\text{SPOR}}$ fusion protein. Numbers in the corners refer to the percentage of cells scored as exhibiting septal localization. See Table S3 for more detailed quantitative data on localization frequencies. (C) PG binding assay. “Reduced” refers to the presence of 1 mM DTT. Bars represent the mean and standard deviation from at least three independent experiments.

**Figure 4.** $\text{FtsN}^{\text{SPOR}}$ contains a disulfide bond. (A) Close-up view of C252 and C312 (yellow). $\beta$-sheet residues important for septal localization are in red stick figures. (B) FtsN contains a disulfide bond. Whole cell extracts in Laemmli sample buffer containing (lanes 2, 4) or lacking (lanes 3, 5) 5% $\beta$-mercaptoethanol and analyzed by Western blot with anti-FtsN (top panel) or anti-FtsQ (bottom panel). Samples were loaded twice to facilitate visualization of any mobility differences. (C) Western blot showing that FtsN levels are reduced but DamX levels are normal in two $\text{dsbA}$ mutants as compared to their isogenic wild-type controls. Cells were grown on M9-glucose to midlog phase, then total cell extracts were prepared and proteins separated by SDS-PAGE (10% polyacrylamide). After transfer to nitrocellulose, proteins were detected with anti-FtsN or anti-DamX antibodies. (D) Length and width of wild-type and $\text{dsbA}$ mutants grown in LB or M9-glucose. Cells grown to midlog phase were fixed with paraformaldehyde, photographed under phase contrast, and measured using tools in Image-Pro Plus version 4.1 from Media Cybernetics (Silver Spring, Md.). Bars represent the mean and standard deviation from at least two (LB) or three (M9) experiments in which 170 or more cells were measured.
Figure 5. The disulfide bond is important for stability of a TTGFP-FtsNSPOR fusion protein.

(A) Reduced septal localization of TTGFP-FtsNSPOR in the absence of DsbA. The indicated strains carrying pDSW992 were grown to midlog phase, then immobilized on an agarose pad and photographed under phase (above) and fluorescence (below). (B) Reduced abundance of TTGFP-FtsNSPOR in the absence of DsbA. The indicated strains carrying pDSW992 or pDSW997 were grown to midlog phase and levels of TTGFP-FtsNSPOR or TTGFP-DamXSPO were determined by Western blotting with anti-GFP antibody. (C) Effect of Cys to Ala substitutions on the stability of TTGFP-FtsNSPOR. Wild-type strain EC251 carrying plasmids that direct expression of wild-type TTGFP-FtsNSPOR or the indicated mutant derivatives were analyzed as in (B).
A. Proposed disulfide bond

B. (+) (-) (+) (-) βME

C. FtsN

D. Cell length (μm)

E. Cell width (μm)