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The Transmembrane Helix of the *Escherichia coli* Division Protein FtsI Localizes to the Septal Ring

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FtsI (also called PBP3) of *Escherichia coli* is a transpeptidase required for synthesis of peptidoglycan in the division septum and is one of about a dozen division proteins that localize to the septal ring. FtsI comprises a short amino-terminal cytoplasmic domain, a single transmembrane helix (TMH), and a large periplasmic domain that encodes the catalytic (transpeptidase) activity. We show here that a 26-amino-acid fragment of FtsI is sufficient to direct green fluorescent protein to the septal ring in cells depleted of wild-type FtsI. This fragment extends from W22 to V47 and corresponds to the TMH. This is a remarkable finding because it is usual for a TMH to target a protein to a site more specific than the membrane. Alanine-scanning mutagenesis of the TMH identified several residues important for septal localization. These residues cluster on one side of an alpha-helix, which we propose interacts directly with another division protein to recruit FtsI to the septal ring.

The use of fluorescence microscopy to visualize proteins in bacteria has revealed that many proteins are not distributed randomly but instead localize to specific subcellular sites, such as the midcell or pole(s) (22, 37). Moreover, proteins that are targeted to specific sites often fail to function properly if they are mislocalized. Despite the importance of proper localization, little is known about how targeting information is encoded in the amino acid sequences of bacterial proteins. In this report, we describe a small peptide from a bacterial cell division protein, FtsI, that is sufficient to target green fluorescent protein (GFP) to the division site in *Escherichia coli*. Interestingly, this peptide is a transmembrane helix (TMH). These findings help to clarify how targeting information is encoded in FtsI's primary sequence and demonstrate that a bacterial TMH can serve as a targeting signal.

FtsI, also known as penicillin-binding protein 3 (PBP3), is a transpeptidase needed for cross-linking septal peptidoglycan (1, 3, 38). Previous studies from a number of laboratories have shown that FtsI is one of over a dozen proteins that localize to the division site, where they form a structure called the septal ring (for recent reviews, see references 12 and 43). As division proceeds, the ring constricts so as to remain at the leading edge of the developing septum. The septal ring is thought to be a multiprotein complex that mediates cell division. Studies of septal ring assembly in various mutant backgrounds have revealed that, at least in *E. coli*, the division proteins are recruited to the ring in a sequential fashion. In this hierarchy, FtsI is one of the last proteins to join the ring; localization of FtsI appears to depend upon the prior localization of FtsZ, FtsA, ZipA, FtsEX (though this is a leaky requirement), FtsK, FtsQ, FtsBL, and FtsW. This scheme suggests that FtsI is recruited to the septal ring by a cascade of protein-protein interactions involved in the assembly of a multiprotein complex. Moreover, FtsI might localize by binding to FtsW, since FtsW appears to be directly responsible for recruiting FtsI to the septal ring (28). A recent study employing a bacterial two-hybrid system reported that FtsI interacts with itself, FtsW, FtsQ, and FtsA (10), but it is not clear whether any of these interactions are direct.

In terms of structure, FtsI is a bitopic membrane protein with a short N-terminal cytoplasmic domain, a single TMH, and a large periplasmic region that contains two domains—one of unknown function, the other a transpeptidase catalytic domain (Fig. 1) (4, 33). The question of what part(s) of FtsI serves to direct the protein to the division site has been addressed by random mutagenesis of the entire gene followed by a screen for localization-defective mutant proteins (45). Mutant proteins specifically defective in septal localization all had lesions in one of three amino acids—R23, L39, or Q46—that are in or near the TMH. These lesions did not prevent insertion of the mutant proteins into the membrane, although subtle effects on how the mutant proteins sit in the lipid bilayer could not be ruled out. Nevertheless, the simplest interpretation was that the most important localization signals in FtsI reside in the TMH, not in the domain of unknown function, as had been suggested previously (27, 33).

Here we show that the TMH of FtsI can direct GFP to the septal ring, albeit not as efficiently as the full-length FtsI protein. This finding allays concerns that the TMH lesions identified previously (45) impaired septal localization indirectly by altering the domain of unknown function. As further evidence that the TMH is the primary localization determinant in FtsI, we show that suppressors of a TMH lesion that prevents septal localization map back to the TMH, not the domain of unknown function. Finally, alanine-scanning mutagenesis indicates that one face of the TMH is particularly important for septal localization. We propose that this surface is the site of a protein-protein interaction involved in recruiting FtsI to the septal ring.

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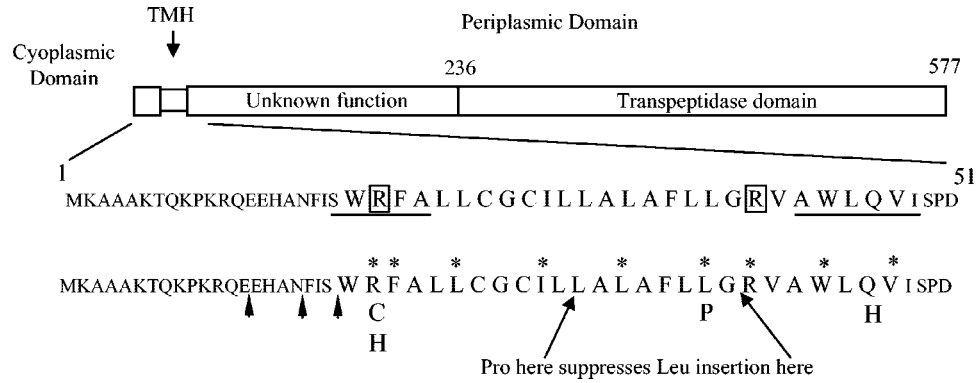


FIG. 1. Domain structure of FtsI. On top is a ribbon diagram showing that mature FtsI is 577 residues in length. It contains a short cytoplasmic domain, a single TMH, and a large periplasmic region that includes both a domain of unknown function and a transpeptidase catalytic domain. The sequence below the ribbon diagram shows the 51 N-terminal amino acids. The minimal fragment found here to direct GFP to the septal ring is indicated in large type and extends from W22 to V47. The borders of the TMH as determined by five different computer programs are indicated by underlining; underlined residues were predicted by at least one program to be in the TMH, while residues between the underlined regions were predicted by all five programs to be in the TMH. Arginines at positions 23 and 41 that were presumed in earlier studies to be the borders of the TMH are boxed. The bottom sequence summarizes results of our mutagenesis studies. Arrowheads indicate the positions of cytoplasmic domain deletions. An asterisk marks each residue found to be important for septal localization by alanine scanning. Arrows show the positions of the leucine insertion that abolishes septal localization and of the L33P substitution that corrects this defect. Letters beneath the sequence indicate lesions found in a previous study (45) to cause localization defects (R23C, R23H, L39P, and Q46H).

MATERIALS AND METHODS

Strains. Strains used include EC295 [MG1655 *ftsI23*(Ts) *leu*::Tn*10*], EC812 [MG1655 *ftsI*::*cat* Δ(λ*att-lom*)::*bla araC* P_{BAD}-*ftsI*], MC4100 [F^- λ^- *araD139* Δ*lacU169* Δ*relA1 rpsL150 thi mot flb-5301 deoC7 ptsF25 rbsR*], LMG64 [*ftsI23*(Ts) *leu*::Tn*10 recA*::*cat*], and DHB4 [F′ *lacI*^{q} pro/λ^- Δ*lacX74 galE galK thi rpsL phoR* Δ*phoA*(PvuII) Δ*malF3*]. These strains have been described previously (5, 16, 45).

Media. Strains were grown in Luria broth (LB) (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter with 15 g of agar per liter for plates) containing the following antibiotics as appropriate: chloramphenicol at 10 μg/ml, kanamycin at 40 μg/ml, and ampicillin at either 25 μg/ml for chromosomal alleles or 200 μg/ml for plasmids. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce expression from P_{lac}, while L-arabinose and D-glucose were used for induction and repression, respectively, of P_{BAD} (15).

Molecular biological procedures and oligonucleotides. Standard procedures for cloning and analysis of DNA, PCR, electroporation, and transformation were used (2). Enzymes used to manipulate DNA were from New England BioLabs (Beverly, Mass.). Oligonucleotides were from Integrated DNA Technologies (Coralville, Iowa). DNA sequencing was performed by the DNA Core Facility of the University of Iowa by using dye terminator cycle-sequencing chemistry. All constructs made by PCR were sequenced to verify their integrity.

Plasmids. Plasmids used in this study were based on pDSW521 (Kn^r P_{lac}::*gfp-ftsI*$_{2\text{-}577}$, where *ftsI*$_{2\text{-}577}$ indicates the *ftsI* gene in which the bases that code for residues 2 to 577 have been retained). pDSW521 is a derivative of pTH18-kr, which has a copy number of ~5 (19, 45).

(i) Deletions of the cytoplasmic domain. To delete the first 14 codons, *ftsI* was amplified from pLMG173 (16) with primers MW1 (CGGAATTCAACAACAA CCGTAAGGAACATGCCAACTTTATCAGT) and pBAD-rev (ACCGCTTC TGCGTTCTGATT). The first primer encodes an EcoRI site (underlined) that is in frame with an EcoRI site at the junction between *gfp* and *ftsI* in pDSW521. It also encodes a linker sequence (NNNRK) followed by codons 15 to 21 of *ftsI*. The second primer anneals to vector sequences downstream of *ftsI* in pLMG173. The PCR product was digested with EcoRI and SacII (site in *ftsI*), and the ~200-bp product was ligated into the same sites of a *gfp-ftsI* fusion vector named pDSW254 (44) to make pDSW372. A ~1.8-kb EcoRI-HindIII fragment that carries *ftsI* was subsequently excised from pDSW372 and used to replace the corresponding fragment in pDSW521 to create pDSW723 (Kn^r P_{lac}::*gfp-ftsI*$_{15\text{-}577}$). Plasmids pDSW724 (Kn^r P_{lac}::*gfp-ftsI*$_{19\text{-}577}$) and pDSW725 (Kn^r P_{lac}::*gfp-ftsI*$_{22\text{-}577}$) were constructed similarly, except that the upstream primers were MW2 (CGGAATTCAACAACAACCGTAAGTTTATCAGTTGGCGTT TTGCG) and MW3 (CGGAATTCAACAACAACCGTAAG TGGCGTTTTG CGTTGTTATGC), respectively. The pDSW254 derivatives constructed as intermediates were pDSW373 and pDSW374.

(ii) Deletions of the periplasmic domain. The transpeptidase domain was deleted by digesting pDSW521 with PstI, treating the remaining sequence with T4 DNA polymerase to remove 3′ overhangs, and religating the ends. This procedure introduces a frameshift mutation, so Arg 239 of FtsI is followed by 7 nonnative residues (RRWFIAQ) and a stop codon. The resulting plasmid is pDSW669 (Kn^r P_{lac}::*gfp-ftsI*$_{2\text{-}239}$). A deletion that removed all *ftsI* codons downstream of Arg 80 was constructed by PCR. The upstream primer was *gfp*-461 (5′ ATACATCATGGCAGACAAACA), which binds in the middle of *gfp*. The downstream primer was Arg80 (CTAAGCTTTTAcccggg CGACCAGAACGG TCAGTAAT), which carries 7 codons of *ftsI* (the last being codon 80) followed by a SmaI site (lowercase letters), a stop codon, and a HindIII site (underlined). The SmaI site was included to permit making fusions to the C terminus of this construct in the future. The ~500-bp PCR product was digested with HpaI and HindIII and ligated into pDSW521 that had been cut with the same enzymes to produce pDSW714. Other deletion derivatives were made similarly using the following downstream primers: Ser70 (CTAAGCTTTTAcccgggGGAGGTGGAAAC TTGCTGAAC), Arg60 (CTAAGCTTTTAcccgggACGCATGTCGCCCTCTTTC AC), Lys55 (CTAAGCTTTTAcccgggTTTCACCAGCATATCCGGGGA), Asp51 (CTAAGCTTTTAcccgggATCCGGGGAGATAACTTGTAA), Pro50 (CTAAGC TTTTAcccgggCGGGGAGATGACTTGTAACCA), Val47 (CTAAGCTTTTAccc gggAACTTGTAACCACGCTACGCG), Leu45 (CTAAGCTTTTAcccgggTAACC ACGCTACGCGTCCGAG), and Ala43 (CTAAGCTTTTAcccgggCGCTACGCG TCCGAGCAGAAA). Finally, a control construct that expressed only *gfp* was made with the following downstream primer: CTAAGCTTTTAcccgggGTTGTTGTTGA ATTCTTTGTA. The corresponding plasmids and the proteins that they produce are pDSW715 (GFP-FtsI$_{2\text{-}70}$), pDSW716 (GFP-FtsI$_{2\text{-}60}$), pDSW717 (GFP-FtsI$_{2\text{-}55}$), pDSW718 (GFP-FtsI$_{2\text{-}51}$), pDSW719 (GFP-FtsI$_{2\text{-}60}$), pDSW720 (GFP-FtsI$_{2\text{-}47}$), pDSW721 (GFP-FtsI$_{2\text{-}45}$), pDSW722 (GFP-FtsI$_{2\text{-}43}$), and pDSW729 (GFP).

(iii) Combining cytoplasmic and periplasmic domain deletions. Plasmid pDSW726 (GFP-FtsI$_{2\text{-}60}$) was made exactly like pDSW716, except that the template for PCR was pDSW374 rather than pDSW521. Plasmid pDSW727 (GFP-FtsI$_{22\text{-}47}$) was made exactly the same as pDSW726 by using the downstream primer that truncates *ftsI* at codon 47.

Alanine scanning of *ftsI*. Alanine substitutions in the TMH of *ftsI* were made by using either the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) or the Gene Tailor kit (Invitrogen, Carlsbad, Calif.). The template was pDSW521 (Kn^r P_{lac}::*gfp-ftsI*). Primer sequences are available upon request. Constructs were confirmed by sequencing.

Isolation of suppressor alleles. Random mutagenesis was done by PCR with *Taq* DNA polymerase as described previously (45). The template was pLD75, which carries an *ftsI* allele with an extra leucine codon in the TMH (16). The insertion becomes codon 41, so the allele will be referred to here as *ftsI*$^{+L41}$; in a previous publication (16), it was referred to as I/I_{Stu}/I to indicate that the

insertion creates a StuI restriction site near the border of the TMH with the periplasmic domain. The primers used were pBAD-SacI (GAAGAGCTCGAG GAGGAAGAACCGATGCCG) and I3′-Xba (GTCTCTAGATTAATTACTA CCAAACATATCC), which anneal upstream and downstream of *ftsI*$^{+L41}$ in pLD75. The resulting ~1.8-kb product was digested with SacI and XbaI (sites underlined) and cloned into the same sites of pDSW204, which confers Amp^r and has a modified Trc promoter (44). The resulting plasmid pools were introduced into the *ftsI23*(Ts) mutant LMG64 by electroporation. Cells were plated on LB-ampicillin at either 30°C to assess transformation efficiency or 42°C to select for suppressor mutations. Suppressor mutations were mapped to the *ftsI* gene on the plasmid by recovering the *ftsI* allele on an ~1.8-kb SacI-XbaI fragment, ligating the fragment back into pDSW204, and retesting the phenotype. Three independent isolates were ultimately sequenced and found to have the same suppressor mutation, T98 to C, which changes Leu 33 to Pro. One plasmid was saved and designated pDSW744. To fuse *gfp* to *ftsI*$^{+L41}$ and *ftsI*$^{L33P,+L41}$, the genes were amplified by PCR from pLD75 and pDSW744, respectively, by using primers P496 CCAGAATTCAACAACAACAAAGCAG CGGCGAAAACGCAG and P497 CCTAAGCTTACGATCTGCCACCTGT CCC. The resulting ~1.8-kb product was digested with EcoRI and HindIII (sites underlined) and ligated into the same sites of pDSW521 to create pDSW658 (Kn^r P$_{lac}$::*gfp-ftsI*$^{+L41}$) and pDSW659 (Kn^r P$_{lac}$::*gfp-ftsI*$^{L33P,+L41}$).

Complementation assays. pDSW521 or derivatives were transformed into EC812 with selection for kanamycin resistance on LB containing kanamycin, chloramphenicol, ampicillin, and arabinose. The arabinose was present to induce chromosomal P$_{BAD}$-*ftsI* in EC812. Isolates were tested for complementation by streaking onto LB plates containing the same antibiotics except glucose (to repress P$_{BAD}$-*ftsI*) and IPTG (to induce the *ftsI* allele on the plasmid). Growth was scored after 18 h at 37°C on a scale from poor (−), meaning that no isolated colonies formed, to good (+++), meaning that colonies were as large as those obtained with wild-type *gfp-ftsI*$_{2-577}$ expressed from pDSW521. To test for dominance (which was not observed), similar plates that contained arabinose rather than glucose were used. Complementation was also tested in the *ftsI23*(Ts) strains LMG64 and EC295. Transformants were recovered at 30°C by selecting for kanamycin resistance, and then isolates were tested for complementation by streaking onto LB-kanamycin with IPTG. Growth was scored after 18 h at 42°C. Identical control plates were incubated at 30°C to test for dominance (which was not observed).

Microscopy. GFP-FtsI proteins were visualized in fixed cells as described previously (45). All cultures were grown at 30°C. (i) Localization of GFP-FtsI proteins in merodiploids was tested in transformants of DHB4 and MC4100. An overnight culture grown in LB-kanamycin was diluted 1:1,000 into the same medium plus IPTG, grown to an optical density at 600 nm of ~0.3, and then fixed for microscopy. (ii) Localization of GFP-FtsI proteins in filamentous cells depleted of FtsI was done with transformants of EC812 as described previously (45).

Western blotting. At the time of harvesting cells for fluorescence, a sample of culture was taken and examined by Western blotting. FtsI and GFP-FtsI were detected with anti-FtsI antibody as described previously (45).

Prediction of the TMH by using computer programs. Residues K11 to G57 of FtsI were analyzed with five programs that predict the location of TMHs: TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (no documentation published), PHDhtm (http://cubic.bioc.columbia.edu/predictprotein/submit_def.html) (36), HMMTOP (http://www.enzim.hu/hmmtop/) (39), TopPred II (http://www.sbc.su.se/~erikw/toppred2/) (41), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (21). Default settings were used in all cases. The predictions returned were as follows: with TMpred, A25 to W44; with HMMTOP, F24 to I48; with PHDhtm, L26 to W44; with TMHMM, S21 to A43; and with TopPred II, W22 to V42. We also analyzed the full-length FtsI sequences with all five programs and got the following predictions: with TMpred, F24 to L45; with HMMTOP, F19 to L38; with PHDhtm, L26 to A43; with TMHMM, S21 to A43; and with TopPred II, W22 to V42. Note that only HMMTOP gave a significantly different prediction, shifting the TMH by 6 residues depending on how much of FtsI was submitted for analysis. The HMMTOP prediction for the full-length protein is unlikely to be correct, because it is clearly an outlier among the 10 predictions made and, unlike the other programs, is sensitive to how much of the N terminus is included in the analysis. Moreover, if the TMH were to start at F19, then the GFP-FtsI$_{22-577}$ protein would have two additional charged residues in the TMH (derived from the linker between GFP and FtsI). Unless these residues can "snorkel" to the surface, such a drastic change in the TMH might be expected to compromise the function of the protein; nevertheless, it supports cell division. For these reasons, predictions obtained with the K11-G57 peptide were used to obtain the results shown in Fig. 1.

Molecular modeling of the TMH. Residues W22 to W44 of FtsI were assigned an alpha-helical structure by using Sybyl 6.8 (1999–2001; Tripos, Inc.). Hydrogens were added to the structure, the molecular dictionary was set to "protein," and then a steric minimization was computed by using the following settings: the method was Powell, the initial optimization was simplex, the termination was a gradient of 0.05 kcal/mol · Å, there were 8,000 maximum iterations, and the energy force field was "not in use." Hydrogens were then removed, as were lone-pair electrons from the two cysteines. Files were saved as Brookhaven database files and processed in Protein Explorer (Eric Martz, 2002).

RESULTS AND DISCUSSION

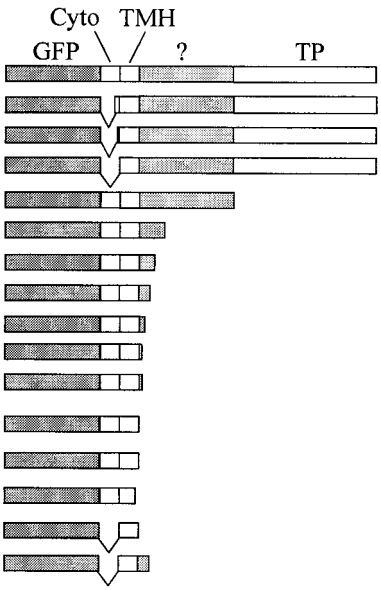
Defining the TMH. Bowler and Spratt (4) used a combination of β-lactamase fusions and protease sensitivity to determine the membrane topology of FtsI, which they called PBP3. They found that FtsI is a bitopic membrane protein with a short N-terminal membrane anchor of ~40 amino acids and a large periplasmic domain of ~500 amino acids. Hydropathy plots revealed a span of 17 consecutive nonpolar amino acids from F24 to G40 that could serve as a TMH (Fig. 1). This hydrophobic span is flanked by Arg 23 and Arg 41, which were considered to define the borders of the TMH in two reports that analyzed the functions of the cytoplasmic domain and TMH by constructing hybrid proteins (8, 16). It is important to note, however, that the borders of the TMH are not known so precisely, and one caution in the interpretation of results obtained with hybrid FtsI proteins is that the domains might not have been replaced as cleanly as intended.

To better define the TMH and to get an estimate of the uncertainty involved in that definition, we used five different computer programs that identify transmembrane segments in proteins. Residues K11 to G57 of FtsI were submitted for analysis because one of the programs used gave an unlikely prediction when the full-length protein was analyzed (see Materials and Methods). No two programs predicted exactly the same TMH, but all included the hydrophobic region from L26 to V42 (Fig. 1). Interestingly, all of the programs placed Arg 41 inside the TMH, and two of them included Arg 23 as well. While this phenomenon may seem counterintuitive, studies of membrane proteins and model peptides have revealed that arginines can be accommodated near the end of a TMH because the long side chain allows the positively charged guanidinium group to interact with the phospholipid headgroups, an arrangement called snorkeling (reviewed in reference 20). Several of the algorithms included Trp 22 and/or Trp 44 in the TMH. Aromatic amino acids, especially Trp and Tyr, are often found near the ends of TMHs, where they serve as anchors because the aromatic rings have a preference for the interface between the hydrophobic and hydrophilic phases (reviewed in references 20 and 35).

We conclude from this analysis that the TMH is probably longer than the hydrophobic core defined operationally as the TMH in previous studies (8, 16). The TMH is not likely to be longer than from S21 to I48. Given the evidence that Trp residues are often found at the borders of TMHs, it is tempting to speculate that the TMH extends from Trp 22 to Trp 44, but biochemical approaches will be needed to determine the boundaries of the TMH precisely.

FtsI proteins with truncations of the cytoplasmic domain. We made a series of deletion constructs that removed progressively increasing amounts of the cytoplasmic domain of FtsI

TABLE 1. Characterization of truncated FtsI proteins (fused to GFP)

Map of GFP-FtsI constructs[a]	FtsI fragment	Complementation[b]	Septal localization[c]	
			Merodiploid	Depletion strain
	K2-V577	+++	35	45 (745)
	E14-V577	+++	41	50 (264)
	F19-V577	+++	34	49 (215)
	W22-V577	++	28	51 (259)
	K2-R239	−	9	93 (57)
	K2-R80	−	0	100 (97)
	K2-S70	−	0	100 (32)
	K2-R60	−	0	96 (93)
	K2-K55	−	ND	100 (34)
	K2-D51	−	ND	94 (52)
	K2-P50	−	ND	94 (139)
	K2-V47	−	1	92 (124)
	K2-L45	−	0	0 (125)
	K2-A43	−	ND	0 (150)
	W22-V47	−	1	58 (197)
	W22-R60	−	ND	98 (88)

[a] For clarity, domains are not drawn to scale. Cyto, cytoplasmic domain; ?, domain of unknown function; TP, transpeptidase catalytic domain.

[b] Complementation was scored in three *ftsI* mutant backgrounds and ranged from +++ to indicate complementation as good as that of the wild type to − to indicate the level of complementation with the vector alone. The GFP-FtsI$_{W22\text{-}V577}$ protein was given an intermediate score because it supported division in only two of the three backgrounds (see the text).

[c] Values are percentages of cells or filaments having at least one fluorescent band, indicative of septal localization. At least 200 cells were scored for each merodiploid configuration. The number of cells or filaments of the depletion strain that were scored is given in parentheses. ND, not determined.

(Fig. 1). The largest deletion, M1 to S21, removed essentially the entire domain, replacing it with GFP and a linker sequence (NNNRK) followed by the presumed first residue of the transmembrane segment (W22) of FtsI. These constructs were tested for their ability to complement two *ftsI*(Ts) strains and one FtsI depletion strain. The two temperature-sensitive (TS) strains carry the same *ftsI23*(Ts) allele (the lesion is Y380→D [M. C. Wissel and D. S. Weiss, unpublished data]). Curiously, one of the TS strains, LMG64, is tighter than the other, EC295. We do not know the reason for this, but EC295 is an MG1655 derivative, and we have observed that several other widely used *fts* alleles are not as tight when they are moved to an MG1655 background (D. S. Weiss, unpublished data). The LMG64 TS strain is also tighter than the FtsI depletion strain used in these studies, EC812.

All but one of these constructs complemented both *ftsI*(Ts) strains and the depletion strain when they were expressed at roughly wild-type levels from a *lac* promoter on a low-copy-number plasmid (Table 1). The exception was the largest deletion, M1 to S21, which, according to one of the computer prediction programs, lacks the first residue of the TMH. This deletion derivative complemented the depletion strain and the EC295 TS mutant but not the more stringent LMG64 TS mutant. Thus, even the largest deletion retained substantial function in cell division.

We conclude that little if any of the cytoplasmic domain of FtsI is required for function in cell division. At most, only the last few residues of the cytoplasmic domain (F19 to S21) might be important, but even this is unclear given the strain dependence of the complementation studies and the concern that deleting so close to the TMH might perturb the TMH. A previous study employing domain swapping concluded that the cytoplasmic domain of FtsI was essential for its function (16). All of the constructs in that study changed M1 through W22, and complementation was tested in LMG64. Thus, the results of that study and the present one are not in conflict, as we observe that replacing M1 to S21 leads to loss of complementation in LMG64. Rather, it is the use of shorter deletions and different strain backgrounds for complementation, coupled with our heightened awareness of the uncertainty of the domain boundaries, that leads us to infer that the cytoplasmic domain does not have an essential function, such as interacting with another division protein in the cytoplasm to target FtsI to the septal ring, or send signals across the cytoplasmic membrane.

All of the GFP-FtsI derivatives with cytoplasmic domain deletions were tested for septal localization in two genetic configurations. One, referred to here as merodiploid, involves expressing *gfp-ftsI* fusions at approximately wild-type levels from a low-copy-number plasmid in a strain that also produces FtsI from the native chromosomal gene. This is a stringent test for localization, in that the GFP-FtsI protein has to compete with wild-type FtsI for assembly into the septal ring. The other configuration, called the depletion strain, involves expressing GFP-FtsI derivatives from the same low-copy-number plasmid but in a strain that has been engineered to express a chromosomal *ftsI* gene under the control of the arabinose-dependent P_{BAD} promoter. Growth on glucose depletes the cells of FtsI

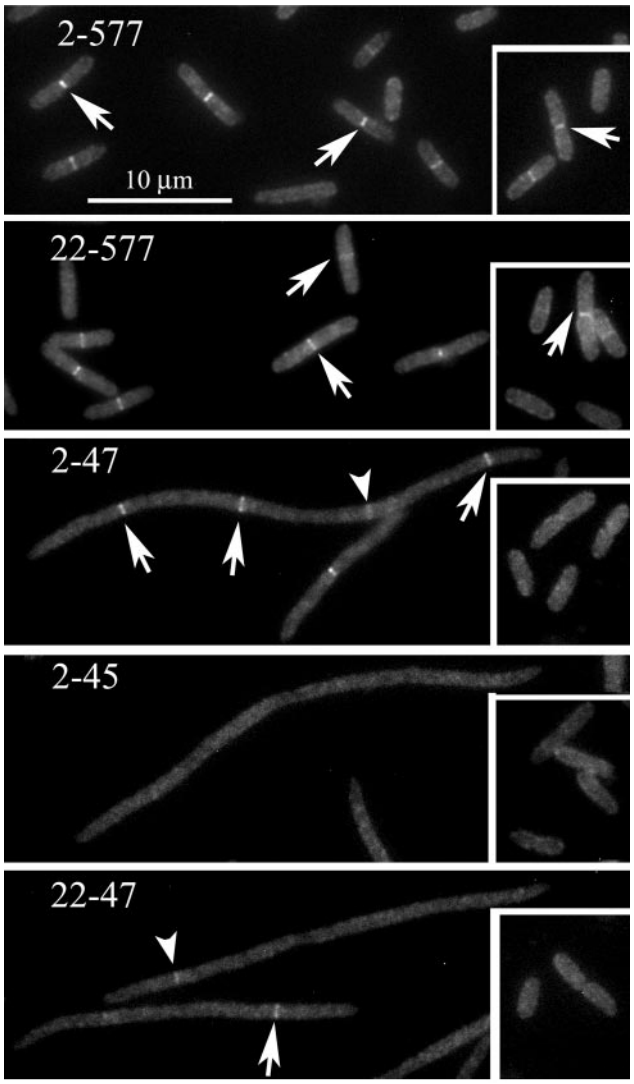


FIG. 2. Localization of GFP-FtsI deletion derivatives. (Large images) Localization of GFP-FtsI in cells depleted of wild-type FtsI; (inset) localization of GFP-FtsI in cells that also contain wild-type FtsI (i.e., an *ftsI*/*gfp-ftsI* merodiploid). Numbers refer to the fragment of FtsI fused to GFP. Arrows indicate examples of septal localization. For this experiment, EC812 (FtsI depletion strain) or MC4100 (merodiploid) were transformed with a low-copy-number plasmid that expresses a *gfp-ftsI* derivative, as indicated. EC812 transformants were grown in LB with 0.2% glucose (to deplete FtsI) and 100 μM IPTG (to induce *gfp-ftsI* from the plasmid) for 5 h, at which time the cells were fixed and examined by fluorescence microscopy. MC4100 transformants were grown in LB with IPTG (even though MC4100 is *lacI* negative) and processed similarly. Localization of these mutant proteins was also assayed in DHB4 transformants, with similar results. Like MC4100, DHB4 is wild type for *ftsI* but carries *lacI*^{q}.

and enables us to observe localization of GFP-FtsI derivatives that retain some ability to localize but compete poorly with the wild type. (In principle, the use of these two strains might allow us to detect mutant GFP-FtsI proteins that cannot localize by themselves but can be recruited into the septal ring by the wild-type protein via, for example, formation of an FtsI–GFP-FtsI heterodimer. In practice, however, we have yet to observe better localization in a merodiploid than in a depletion strain.)

Consistent with the complementation results, GFP-FtsI derivatives with deletions of the cytoplasmic domain localized to the septal ring in both tests (Fig. 2; Table 1). Most remarkably, even the GFP-FtsI$_{22\text{-}577}$ protein, which probably lacks the entire cytoplasmic domain, localized in the merodiploid configuration, indicating that it retains enough targeting information to compete effectively with wild-type FtsI for assembly into the septal ring.

FtsI proteins with truncations in the periplasmic domain. We also made a series of deletions that removed portions of the periplasmic domain. These proteins did not support division (Table 1), which was to be expected since all lacked the transpeptidase domain critical for FtsI function in peptidoglycan synthesis. Removal of only the transpeptidase domain reduced localization about fourfold in a merodiploid, but deletion extending into the domain of unknown function prevented localization in a merodiploid (Fig. 2; Table 1). Interestingly, many of the constructs localized well in filamentous cells that had been depleted of FtsI. The shortest fragment that localized lacked all residues beyond V47. We were unable to detect these deletion derivatives consistently by Western blotting with anti-GFP antibody, so we have no direct information on their expression level, stability, or efficiency at inserting into the cytoplasmic membrane. Because they were expressed from the same plasmid as full-length *gfp-ftsI*, and because similar exposure times (~10 s) were needed to photograph both full-length and truncated *gfp-ftsI* proteins, we suspect that weak localization of, for example, the GFP-FtsI$_{2\text{-}47}$ protein is not simply due to low abundance or inefficient membrane insertion.

Why derivates that lacked most of the periplasmic domain localized poorly compared to full-length FtsI is a matter of conjecture. Periplasmic sequences might affect the conformation of the TMH, or they might engage other division proteins and contribute to septal localization. The domain of unknown function has been suggested to be a site of interaction with other division proteins (27, 33). These interactions may help recruit FtsI to the septal ring, enable FtsI to recruit downstream proteins such as FtsN, and/or regulate the catalytic (transpeptidase) activity of FtsI. Previously, we described several single-amino-acid substitutions in the domain of unknown function that cause subtle localization defects in a merodiploid (≤2-fold) (45). Together with the larger defect seen when the entire domain is deleted, it seems likely that the domain of unknown function contributes to septal localization, but we doubt that it is the primary targeting signal in FtsI. In this context, it is worth noting that our lab has described amino acid substitutions in the domain of unknown function that impair localization of the next protein in the recruitment hierarchy, FtsN (45).

While this work was in progress, Piette et al. also reported the localization of GFP-FtsI proteins with progressive deletions of the periplasmic domain (34). Their findings and ours are in general agreement. One notable difference is that they observed localization when residues up to Val 42 were deleted from FtsI (i.e., GFP-FtsI$_{2\text{-}42}$), whereas the protein with the largest deletion that localized in our studies was truncated at Val 47 (i.e., GFP-FtsI$_{2\text{-}47}$). Another difference was that Piette et al. were able to verify expression of their truncated proteins by Western blotting with anti-GFP the antibody. We think

TABLE 2. Alanine-scanning mutagenesis of GFP-FtsI

Amino acid substitution[a]	Complementation[c]	Localization in merodiploid[d]	No. of cells scored[e]
None (wild type)	+++	31	2,035
W22A	+++	33	432
R23A	−	4	400
F24A	+++	1	430
L26A	+++	26	177
L27A	+++	12	311
C28A	+++	18	380
G29A	+++	29	510
C30A	+++	25	489
I31A	+++	2	1,235
L32A	+++	29	542
L33A	+++	23	142
L35A	+++	13	208
F37A	+++	46	177
L38A	+++	21	399
L39A	+++	12	199
G40A	+++	33	153
R41A	+++	12	321
V42A	+++	18	283
W44A	+++	10	196
L45A[b]	−	0	100
Q46A	+++	18	331
V47A	+++	5	271
Vector	−	0	129

[a] Residues that are alanine in the wild type are omitted.
[b] Western blotting revealed that the L45A protein is subject to proteolysis, presumably by the leader peptidase.
[c] Complementation was scored in three *ftsI* mutant backgrounds and ranged from +++ to indicate complementation as good as that of the wild type to − to indicate the level of complementation with the vector alone. Results were consistent in all three backgrounds.
[d] Values are the percentages of cells exhibiting a fluorescent band at midcell.
[e] All constructs were tested on at least two occasions. Results were consistent, and data were pooled.

both differences can be attributed to higher levels of expression of *gfp-ftsI* alleles in the study of Piette et al., as they employed a higher-copy-number plasmid with a stronger promoter and a stronger ribosome binding site.

Localization of the TMH. We combined cytosolic and periplasmic domain deletions and found that a 26-amino-acid fragment extending from W22 to V47 localized in cells depleted of wild-type FtsI (Fig. 2). This fragment localized poorly—the fluorescent bands tended to be faint, and only some of the septal rings in the depletion filaments were decorated as indicated by the low number of rings per unit of cell length (Table 1). Because the filaments were smooth (i.e., the fluorescent GFP-FtsI$_{22\text{-}47}$ bands were not associated with indentations), we do not think that residual native FtsI recruited the GFP-FtsI$_{22\text{-}47}$ protein to these sites. On the contrary, our data imply that GFP-FtsI$_{22\text{-}47}$ competes with native FtsI for septal localization and that the short fragment localizes by the same mechanism as that used by authentic FtsI. The fact that there is competition helps rule out the possibility that localization of the fragment is some sort of artifact.

The W22 to V47 fragment corresponds very well with the TMH (Fig. 1). The fact that the isolated TMH can localize at all underscores its importance as a targeting signal. Our lab has previously identified amino acid substitutions that cause large (≥8-fold) defects in septal localization of full-length FtsI (45). Strikingly, all of these lesions fall within the fragment that we have now shown is sufficient to target GFP to the septal ring (Fig. 1).

Alanine-scanning mutagenesis. To identify TMH residues important in septal localization, we performed alanine-scanning mutagenesis on the region from W22 to V47 (in the context of a GFP fusion to full-length FtsI). When expressed at wild-type levels from a low-copy-number plasmid, all but two of the alanine substitution alleles complemented two *ftsI*(Ts) strains and an FtsI depletion strain (Table 2). The exceptions were R23A and L45A, which failed to complement in any background. Western blotting with anti-FtsI antibody revealed that the L45A protein was truncated (Fig. 3), presumably by leader peptidase. The L45A substitution creates an Ala-Trp-Ala sequence near the periplasmic end of the TMH, which

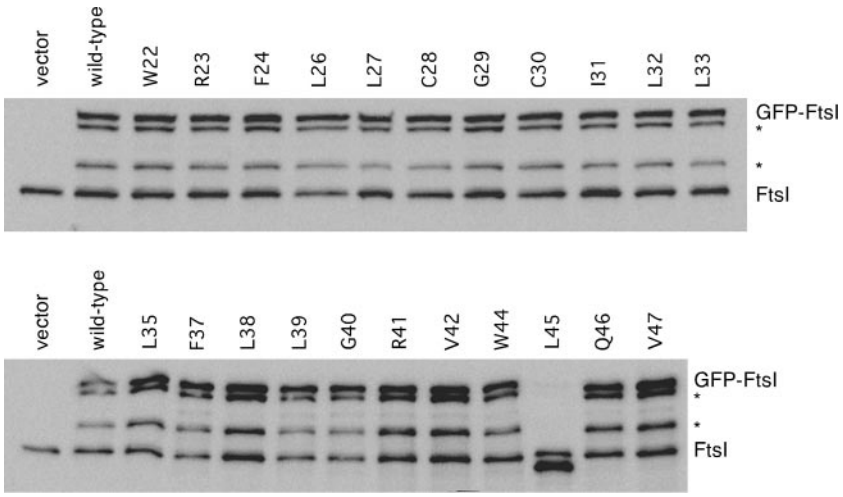


FIG. 3. Expression of alanine-scanning derivatives of GFP-FtsI. DHB4 transformants were grown in LB with 100 μM IPTG to an optical density at 600 nm of 0.3 and then harvested for analysis by Western blotting with anti-FtsI antibody. The first lane on each blot is the vector, pDSW729, which expresses *gfp* alone. The second lane is pDSW521, which expresses the *gfp-ftsI* wild type. The remaining lanes show alanine-scanning derivatives of pDSW521, as indicated by the residue changed to alanine. Two prominent breakdown products of GFP-FtsI are indicated on the right with an asterisk.

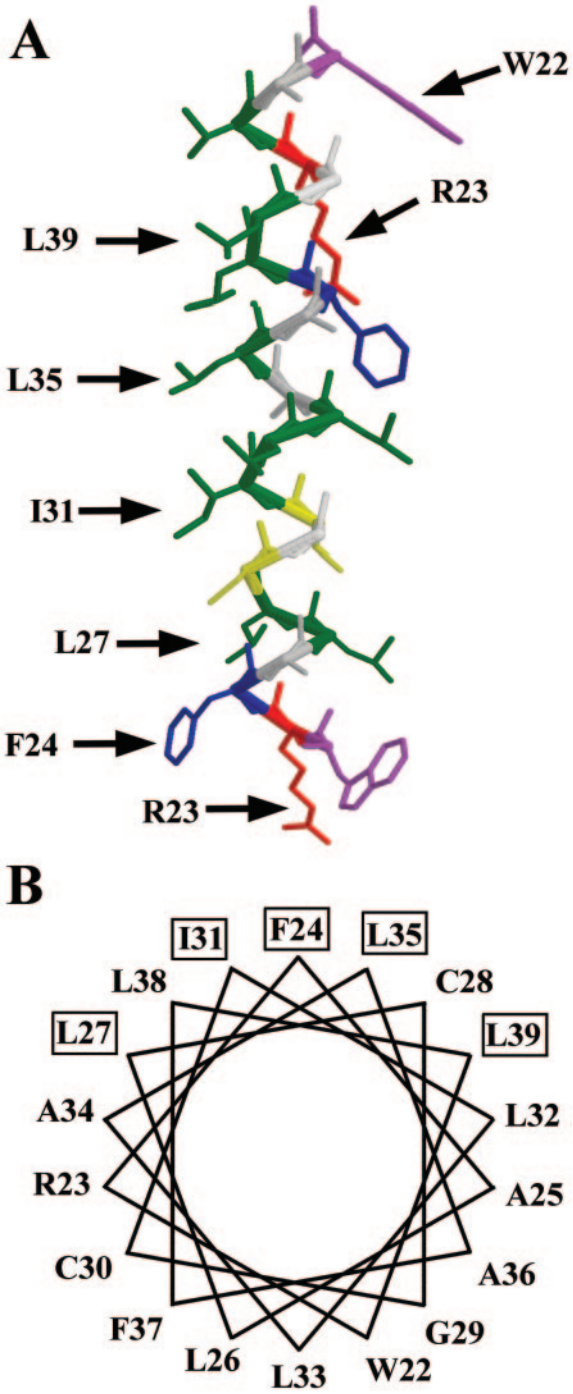


FIG. 4. Proposed structure of the localization helix. (A) Model of TMH residues W22 to W44 as an alpha-helix. Residues identified by alanine scanning as important for septal localization are indicated with arrows. Amino acids are color coded as follows: red indicates Arg; blue indicates Phe; light gray indicates Gly; dark gray indicates Ala; yellow indicates Cys; green indicates Leu, Val, and Ile; and purple indicates Trp. Because the modeling software assumes an aqueous environment, the side chain of Arg 41 points down. This alignment places the positively charged guanidinium moiety inside the lipid bilayer. More likely, the side chain points up so that the guanidinium can interact with the lipid phosphate groups. (B) Helical wheel diagram of W22 to L39. Boxes highlight hydrophobic residues from F24 to L39 that were identified by alanine scanning as important for septal localization. Note that these residues fall on one face of the helix.

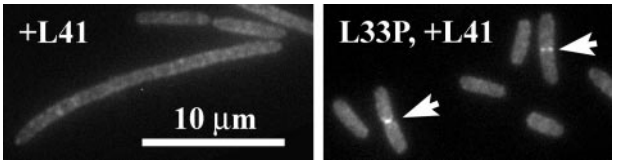


FIG. 5. Localization of GFP-FtsI^{+L41} and GFP-FtsI$^{L33P,+L41}$. EC812 transformants carrying pDSW658 or pDSW659 were grown for 5 h in LB with 0.2% glucose (to deplete FtsI) and 100 μM IPTG (to induce the *gfp-ftsI* derivative) and then fixed and examined by fluorescence microscopy. Arrows indicate fluorescent bands indicative of septal localization.

matches the Ala-X-Ala cleavage site recognized by leader peptidase (42). Why the R23A protein failed to support division is not obvious. While this substitution caused a localization defect, the R23A protein localized better than the I31A mutant protein that nevertheless rescued division. R23 was previously identified as a residue important for FtsI function and septal localization (Fig. 1) (45).

We assayed localization of the alanine substitution derivatives in merodiploids, where competition with wild-type FtsI produced from the chromosome provides a stringent test for localization. In such an assay, nine of the mutant proteins showed a decrease of more than twofold, and a couple had severe defects (Table 2). In a depletion background, the mutant proteins localized efficiently (not shown), which probably explains why a protein such as the I31A derivative supported division despite localizing poorly (in a merodiploid).

Some of the critical residues are near the borders of the TMH (R23, R41, W43, and Q46). These residues are likely to be important for positioning the helix properly in the membrane, so lesions here might impair localization indirectly. The remaining critical residues probably lie in the hydrophobic core (F24, L27, I31, L35, and L39). Models of the TMH indicate that these residues are on the same face of the alpha-helix (Fig. 4). We propose that this surface of the TMH interacts with a helix from another membrane protein. A likely candidate is FtsW, which has 10 TMHs (13, 23) and is, according to the recruitment hierarchy, the protein that recruits FtsI to the septal ring (28).

Other potential mechanisms by which the TMH may target FtsI to the septal ring involve the idea that FtsI dimerizes or forms some other oligomer. In one scenario, the TMH might mediate dimerization of FtsI, and the TMH has to be a dimer to localize. Alternatively, FtsI may localize as a monomer via its periplasmic domain but then recruit additional molecules of FtsI via TMH-TMH interactions. For the latter scenario to hold, one has to further postulate that the mutant forms of FtsI compete poorly with native FtsI for oligomer formation—otherwise, the mutant proteins would have localized better in merodiploids than in a depletion background. These ideas cannot be ruled out in part because the oligomeric state of FtsI is not known and in part because the filaments used for localization studies contain a small amount of residual wild-type FtsI even after extended depletion (data not shown). The strongest argument against such schemes is that they imply the existence of a primary targeting domain distinct from the TMH. In principle, such a domain should have been found in

our previously published screen for localization-defective mutants (45), but it was not.

Intragenic suppression of an insertion in the TMH of FtsI. Insertion of a single Leu residue between G40 and R41 lengthens the hydrophobic core of the TMH by 1 residue and results in a loss of function in cell division (16). The mutant protein, referred to herein as FtsI^{+L41}, is stable as determined by Western blotting. Using a fusion to GFP, we found that the Leu insertion prevents septal localization either in a merodiploid (not shown) or in filamentous cells depleted of wild-type FtsI (Fig. 5).

We considered two explanations for this observation. Lengthening the TMH might change the angle at which it crosses the cytoplasmic membrane and thus disrupt interactions between the TMH of FtsI and the TMH of another protein. Alternatively, the Leu insertion might alter the position of the periplasmic domain so that it no longer interacts appropriately with other proteins. If the latter explanation is correct, then one might expect to find intragenic suppressor mutations that map to the domain of unknown function.

We mutagenized a plasmid-borne *ftsI*$^{+L41}$ allele by PCR. Plasmids were transformed into an *ftsI*(Ts) strain, which was plated at either 30°C to determine transformation efficiency or 42°C to select for suppressors. Out of an estimated ~70,000 transformants plated at 42°C, 26 colonies were obtained. All appeared phenotypically similar upon restreaking. Three isolates that arose from independent PCRs were characterized further. All had the same mutation, T98→C, which changes Leu 33 to Pro. As expected, all three isolates retained the extra Leu codon in the parental allele. Changing Leu 33 to Pro is expected to bend the alpha-helix (35), which might restore the helix more closely to its proper length despite the presence of the extra leucine. A GFP fusion was then constructed and used to test for septal localization. GFP-FtsI$^{L33P,+L41}$ did not localize in a merodiploid (data not shown) but did localize when cells were depleted of wild-type FtsI (Fig. 5). Note that the depletion strain did not become filamentous in this experiment because the mutant GFP-FtsI protein supports division.

The location of the suppressor mutation suggests that the primary effect of the original leucine insertion was manifested locally (i.e., in the TMH itself) rather than at a remote site (i.e., the domain of unknown function). Given that there are several leucines in the TMH that could be changed to proline by a single nucleotide substitution, one wonders why only the L33P lesion was recovered. Perhaps bends introduced at other sites do not restore function; even if these bends also shorten the helix, they might not permit the TMH to make a precise fit with the presumed target TMH to which it binds.

Requirements for TMHs in localization of other proteins. The results of this and other studies (34, 45) establish that the TMH of FtsI is directly involved in targeting FtsI to the septal ring. Alignments of FtsI proteins from several bacterial species indicate that the length of the TMH is well conserved, as indicated by the spacing of the two arginines that flank the hydrophobic core, but the sequence of the hydrophobic core is not conserved (data not shown). Thus, we see no evidence of a targeting motif that might be shared among different Fts proteins. Moreover, studies of the bitopic membrane proteins that localize to the septal ring in *E. coli* have shown that targeting information can reside in a variety of domains—cytoplasmic, TMH, or periplasmic (Fig. 6). The lack of a conserved targeting motif is consistent with the notion that division proteins localize by a cascade of protein-protein interactions rather than by binding to a common target in the septal ring. Nevertheless, it is interesting that FtsA and ZipA do not appear to share a targeting motif even though each protein binds the C-terminal tail of FtsZ (11, 17, 18, 24, 26, 29, 30).

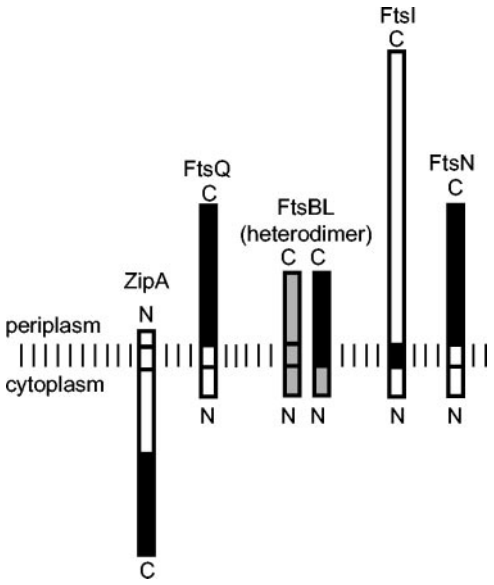


FIG. 6. Targeting domains in bitopic membrane proteins needed for cell division in *E. coli*. For clarity, proteins are not drawn to scale. Regions sufficient for septal localization are shown in black, while regions known to be dispensable are in white. FtsB has not been studied with respect to this function and is shown in gray. Even where targeting information has been studied, only a few constructs have been evaluated in some cases, so further work may narrow the localization determinant to a smaller protein fragment than indicated here. Previous reports and the present study were the sources of information for localization determinants ZipA (17), FtsQ (7), FtsL (14), FtsI (this study), and FtsN (8).

We expect the targeting sequence to be a conserved feature of FtsI proteins from different species, an expectation that is hard to reconcile with the lack of sequence conservation in the TMH. One potential explanation is that the relative contributions of the TMH and domain of unknown function to septal targeting might differ from species to species. Consistent with this theory, studies of *Bacillus subtilis* indicate that assembly of FtsI (called PBP2b in that organism) into the septal ring involves a complex network of interactions among several division proteins (9). An alternative explanation for the lack of conservation in the TMH is that it has coevolved with its target TMH (in FtsW?) to such an extent that sequence conservation is no longer readily recognizable. Our alanine-scanning mutagenesis results indicate that the TMH of FtsI and its target may plausibly drift, without intermediate states being lethal.

We think that it is unusual for a TMH to target a protein to a site more specific than the membrane. Besides FtsI, the only example of which we are aware involves several bitopic membrane proteins that localize to the Golgi apparatus of eukaryotes (reviewed in reference 31). These proteins have short transmembrane helices (~15 hydrophobic residues) compared to those of proteins found in the plasma membrane (~20

hydrophobic residues), and it is the length rather than the sequence of these short TMHs that appears to be important for proper localization. Golgi apparatus membranes contain little cholesterol and are therefore thinner than the plasma membrane, which is ~50% cholesterol (40). It has been proposed that short TMHs target certain proteins to the Golgi apparatus by matching the thickness of the bilayer to the length of the TMH (reference 6, but see also reference 25). In contrast to the situation with Golgi body proteins, at least one of which can localize properly if it is given a polyleucine TMH of the appropriate length (32), our studies of FtsI indicate that the sequence of the TMH is important for septal localization. This finding suggests that the TMH targets FtsI to the septal ring by binding to another protein rather than partitioning passively into a region where the lipid bilayer has unique properties.

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