

## Cell Division in *Escherichia coli*: Role of FtsL Domains in Septal Localization, Function, and Oligomerization

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**In *Escherichia coli*, nine essential cell division proteins are known to localize to the division septum. FtsL is a 13-kDa bitopic membrane protein with a short cytoplasmic N-terminal domain, a membrane-spanning segment, and a periplasmic domain that has a repeated heptad motif characteristic of leucine zippers. Here, we identify the requirements for FtsL septal localization and function. We used green fluorescent protein fusions to FtsL proteins where domains of FtsL had been exchanged with analogous domains from either its *Haemophilus influenzae* homologue or the unrelated MalF protein to show that both the membrane-spanning segment and the periplasmic domain of FtsL are required for localization to the division site. Mutagenesis of the periplasmic heptad repeat motif severely impaired both localization and function as well as the ability of FtsL to drive the formation of sodium dodecyl sulfate-resistant multimers in vitro. These results are consistent with the predicted propensity of the FtsL periplasmic domain to adopt a coiled-coiled structure. This coiled-coil motif is conserved in all gram-negative and gram-positive FtsL homologues identified so far. Our data suggest that most of the FtsL molecule is a helical coiled coil involved in FtsL multimerization.**

Cell division in bacteria culminates with the formation of the midcell septum followed by cell separation. In *Escherichia coli*, at least nine gene products, FtsZ, FtsA, ZipA, FtsQ, FtsL, FtsI, FtsK, FtsW, and FtsN, have been identified as being essential for septation, and the septal localization of all of them has been reported previously (1–3, 5, 8, 12, 20, 29, 30, 31). The nature of the mechanism leading to the selection of the midcell division site is still unknown and poses one of the most fundamental questions in cell biology. One approach to this issue is to determine the nature of the septal localization signal that targets cell division proteins to the division site.

Recent progress indicates that only portions of certain Fts proteins are necessary for septal localization. In the case of FtsK, the N-terminal-15% portion of this protein (comprising the membrane-spanning segments) is sufficient for localization to the division site but not for complementation of an *ftsK* defect (16). For FtsN, the periplasmic domain promotes targeting of this protein to the septum (29). Studies with replacements of the cytoplasmic and transmembrane domain of the bitopic membrane protein FtsQ suggest that the cytoplasmic domain and the membrane-spanning segment have little or no role in FtsQ targeting and function at the division site (3, 5). In contrast, for FtsI, a bitopic membrane protein with a large periplasmic enzymatic domain involved in peptidoglycan biosynthesis, replacements of the cytoplasmic or transmembrane domain of FtsI failed to complement and localize at the division site. These results, along with the fact that no common targeting motif can be identified in these proteins, suggest that each cell division protein carries a distinct and specific localization signal.

In this paper, we identify the requirements for septal localization and function of the cell division protein FtsL. Although the function of FtsL is still unknown, its sequence is conserved in gram-negative and gram-positive bacteria, and it is an es-

essential cell division protein in *E. coli* and *Bacillus subtilis* (7, 9). FtsL is a bitopic cytoplasmic membrane protein whose structure includes a small amino-terminal cytoplasmic domain (37 residues), a single transmembrane segment (20 residues), and a small periplasmic domain (64 residues). The sequence of the periplasmic domain of FtsL exhibits a stretch of five leucines separated from each other by seven residues which has features of an  $\alpha$ -helical leucine zipper. The presence of this motif raises the possibility that FtsL interacts with itself or other proteins to form homo- or heterodimers. Studies in which the three domains of FtsL were exchanged with those of other bitopic membrane proteins showed that all three domains of FtsL are required for its function (11). Different possible roles can be attributed to FtsL domains, including that of signaling localization to the cell septum. Thus, one possible consequence of exchanging FtsL domains might be to interfere with its localization.

We recently used fluorescence microscopy to demonstrate that *E. coli* FtsL (FtsL<sub>E<sub>coli</sub></sub>) is localized to the division site and that FtsL localization depends on the prior recruitment of FtsZ, FtsA, ZipA, and FtsQ but not FtsI and FtsN. These results, along with similar experiments done with FtsI and FtsQ, led us to propose that FtsL is recruited after FtsZ, ZipA, FtsA, and FtsQ and before FtsI and FtsN (8).

In this paper, we use green fluorescent protein (GFP) fusion technology to analyze the septal localization and function of FtsL derivatives in which the cytoplasmic domain and/or membrane-spanning segment of FtsL is replaced with analogous parts of either MalF, a maltose transport protein with no role in cell division, or FtsL<sub>Hinf</sub>, the *Haemophilus influenzae* FtsL<sub>E<sub>coli</sub></sub> homologue. Our main finding is that both the membrane-spanning segment and the periplasmic domain of FtsL<sub>E<sub>coli</sub></sub>, but not the cytoplasmic domain, are required for localization to the division site. Further, we found that mutagenesis of the leucine repeat heptad motif in the periplasmic domain of FtsL severely impaired both localization and function. Finally, we report that the FtsL periplasmic domain can drive formation of sodium dodecyl sulfate (SDS)-resistant multimers in vitro. These findings are consistent with the likely

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existence of a coiled-coil structure predicted for the periplasmic domain of all FtsL homologues, which may be involved in a homo- or heterodimerization process important in the mechanism of cell division.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phage.** Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Strain construction was done by generalized transduction with P1 (24) or specialized transduction with  $\lambda$ InCh.  $\lambda$ InCh picks up plasmid-borne genes by homologous recombination. In this case, the genes were *gfp-ftsL* or *gfp-ftsL* derivatives, *bla*, and *lacI<sup>q</sup>* from pDSW207-based plasmids (31). Chromosomal insertions made with  $\lambda$ InCh were subsequently stabilized by selecting for a deletion that removed most of the phage, including its killing functions (D. Boyd et al., unpublished data; see also <http://beck1.med.harvard.edu/LambdaInCh.html>).

**Media.** Media were NZY or Luria-Bertani (LB) medium (9, 27). L-Arabinose or D-glucose was added as indicated to modulate expression of genes under control of the P<sub>BAD</sub> promoter (10). For localization studies, isopropyl- $\beta$ -D-thiogalactoside (IPTG) was used to modulate expression of *gfp* fusions under the control of modified *trc* promoters: 2.5  $\mu$ M for P<sub>207</sub>-*gfp-ftsL* derivatives (8, 31). The IPTG-dependent plasmid pJMG64 was maintained with 5 mM IPTG. Antibiotics were used for selection at the following concentrations: ampicillin (AMP) at 200  $\mu$ g/ml for plasmids and 25  $\mu$ g/ml for chromosomal alleles, kanamycin (KAN) at 40  $\mu$ g/ml, chloramphenicol at 30  $\mu$ g/ml, tetracycline (TET) at 15  $\mu$ g/ml, and spectinomycin (SPEC) at 25  $\mu$ g/ml. In complementation experiments, pJMG64 was counterselected with streptomycin used at 1.5 mg/ml.

**Molecular biological procedures.** Standard procedures were used for cloning and analysis of DNA, PCR, electroporation, and transformation (27). Enzymes used to manipulate DNA were from New England Biolabs (Beverly, Mass.). Oligonucleotides were from Gibco BRL (Gaithersburg, Md.). DNA sequencing was performed by the Micro Core Facility in the Department of Microbiology and Molecular Genetics at Harvard Medical School.

**Sequence analysis.** Amino acid sequence comparisons were performed with the Clustal package program (14). Homology searches were performed with Blast 2.0 at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html>). Hydropathy plots and DNA sequence manipulations were done with DNASTrider 1.3 (22). Coiled-coil propensity was determined with Coiled 2.2 (19) on the ISREC website ([http://www.isrec.isb-sib.ch/software/COILS\\_form.html](http://www.isrec.isb-sib.ch/software/COILS_form.html)).

**Construction of *gfp* fusions.** (i) *gfp-ftsL* and *gfp-LLL*. Plasmids for making gene fusions to *gfp* were based on pDSW207 (31). pDSW236 is described in reference 8. To make pDSW207-LLL, LLL was amplified by PCR with pLD45 (11) as template and oligonucleotides L1 (CGAGAATTCACAACAACATGATCAG CAGACTGACAGAA) and L2 (TCGAAGCTTTTTATTTTGGCACTACGAT ATTTTC) as primers. The amplified DNA was digested with *EcoRI* and *HindIII* (sites underlined) and ligated into the same sites of pDSW207 to create pJMG453. The fusion protein encoded by pJMG453 has the linker sequence YKEFNINMI, where YK are the last residues of GFP and MI are the first two residues of FtsL.

(ii) **Fusions to FtsL-MalF swap proteins.** To make *gfp-FLL*, *gfp-LFL*, and *gfp-FFL*, the *FLL*, *LFL*, and *FFL* genes were amplified by PCR with pLD63, pLD94, and pLD90 (11), respectively, as template and oligonucleotides F1 (CGAGAATTCACAACAACATGATGTCATTAATAAAGAAACATTTGGTG GC) (FLL and FFL) or L1 (LFL) and pBADrev (ACCGTTCGCGTTTG ATT) as primers. The amplified DNA was digested with *EcoRI* (site underlined) and *HindIII* (site present downstream of the constructs in pLD63, pLD94, and pLD90) and ligated into the same sites of pDSW207 to create pJMG447, pJMG448, and pJMG449. The fusion protein encoded by pJMG448 has the same linker sequence as that in GFP-LLL. The fusion proteins encoded by pJMG447 and pJMG449 have the linker sequence YKEFNINMMD, where YK are the last two residues of GFP and MD are the first two residues of FLL and FFL, whose amino terminus is derived from MalF.

To make *gfp-FFF*, the *FFF* gene was amplified by PCR with pLD47 (11) as template and oligonucleotides F1 and F2 (TCGAAGCTTTTAATCAAACCTTC ATTCGCGTGGC) as primers. The amplified DNA was digested with *EcoRI* and *HindIII* (site underlined) and ligated into the same sites of pDSW207 to create pJMG450. The fusion protein encoded by pJMG450 has the same linker sequence as that in GFP-FLL.

To make *gfp-LLF*, the *MscI-HindIII* fragment of pLD47, corresponding to the MalF region beyond the first transmembrane segment, was introduced into pJMG453 (pDSW207-LLL) with its *MscI-HindIII* fragment deleted to create pJMG451. The fusion protein encoded by pJMG451 has the same linker sequence as that in GFP-FLL.

(iii) **Fusions to FtsL-FtsI swap proteins.** To make *gfp-LLI*, the *LLI* gene was amplified by PCR with pLD116 (11) as template and oligonucleotides L1 and pBADrev. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pDSW207 to create pJMG474. The fusion protein encoded by pJMG474 has the same linker sequence as that of pDSW236.

(iv) **Fusions to FtsL<sub>Ecol</sub>-FtsL<sub>Hinf</sub> swap proteins.** *ftsL<sub>Hinf</sub>* was amplified by PCR with pGHIA44 (American Type Culture Collection [ATCC], Manassas, Va.) as template and oligonucleotides HiL-5 (CCCCGAATTCAGAGAGGACGAATG

CATGTCGAAAATAATAAGCCTCG and HiL-3 (CCCCAAGCTTTATCCT TATTCTCAATTTCAACTTCTTGC) as primers. In HiL-5, the sequence located downstream of the *EcoRI* site corresponds to the region placed upstream of *E. coli* FtsL (the ribosome binding site is in boldface) followed by the *H. influenzae* coding sequence (italicized). The amplified DNA was digested with *EcoRI* and *HindIII* (sites underlined) and ligated into the same sites of pBAD18 to create pJMG452.

To make *gfp* fusions to FtsL<sub>Ecol</sub>-FtsL<sub>Hinf</sub> swap proteins, the swap constructs were first made in pBAD18 derivatives. To make *HLL*, the DNA fragment encoding the cytoplasmic domain of FtsL<sub>Hinf</sub> was amplified by PCR with pJMG452 as template and oligonucleotides pBADfw (CTGACGCTTTTATCGCAAC) and Hieag-3 (GGGCGGCCGGAAGAAAATAAATCTTCG) as primers. The amplified DNA was digested with *EcoRI* (site present upstream of the constructs in pJMG452) and *EagI* (site underlined) and ligated into the same sites of pLD45 to create pJMG429.

To make *LHL*, the DNA fragment encoding the membrane-spanning domain of FtsL<sub>Hinf</sub> was amplified by PCR with pJMG452 as template and oligonucleotides Hieag-5 (CCCCGCGCGTTAGTGGTGTGGTGAATAGGG) and Hims-3 (GGGTGGCCAATCCAAATCGTCCCCATTGCAGAAACTA) as primers. The amplified DNA was digested with *EagI* (site underlined) and *MscI* (site underlined) and ligated into the same sites of pLD45 to create pJMG430.

To make *LLH*, the DNA fragment encoding the periplasmic domain of FtsL<sub>Hinf</sub> was amplified by PCR with pJMG452 as template and oligonucleotides Hims-5 (CCCTGGCCATAAAACTCGCCAATTAATTTC) and HiL-3 as primers. The amplified DNA was digested with *MscI* (site underlined) and *HindIII* and ligated into the same sites of pLD45 to create pJMG434.

To make *HLL*, the *EagI-HindIII* DNA fragment of pJMG430 was inserted into the same sites of pJMG429 to create pJMG431.

To make *gfp-LHH*, the *MscI-HindIII* DNA fragment of pJMG434 was inserted into the same sites of pJMG430 to create pJMG433.

To make *HLH*, the *EagI-HindIII* DNA fragment of pJMG434 was inserted into the same sites of pJMG432 to create pJMG446.

Plasmids for making gene fusions to *gfp* were all based on pDSW207. *ftsL<sub>Hinf</sub>* and *HLH* were amplified by PCR with, respectively, pJMG452 and pJMG446 as templates and oligonucleotides Haeinf1 (CCCCGAATTCACAACAACATGTCGAAAATAATAAGCCTCG) and pBADfw as primers. The amplified DNA was digested with *EcoRI* (site underlined) and *HindIII* and ligated into the same sites of pDSW207 to create pJMG454 and pJMG457. The fusion proteins encoded by pJMG454 and pJMG457 have the linker sequence YKEFNINMS, where YK are the last residues of GFP and MS are the first two residues of FtsL. *HLL* and *HHL* were amplified by PCR with pJMG429 and pJMG431 as templates and oligonucleotide HiL-1 and pBADrev as primers. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pDSW207 to create pJMG455 and pJMG456, respectively. *LHH* and *LLH* were amplified by PCR with pJMG433 and pJMG434 as templates and oligonucleotides L1 and pBADrev as primers. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pDSW207 to create pJMG458 and pJMG459, respectively.

(v) **Fusions to FtsL<sub>Ecol</sub> periplasmic domain mutants.** LL<sub>HinfL</sub>, LL<sub>GCN4L</sub>, and LL<sub>CEBP<sub>L</sub></sub> were constructed as follows. First, a silent mutation was introduced in the DNA region encoding the periplasmic domain of LLL by the megaprimer methods (28). The megaprimer was generated with pLD45 as template and oligonucleotides Lxho3mp (GAATGGCGCAACTGATCCTCGAGGAGAA TGC) and pBADfw as primers. *LLxL* was then amplified with pLD45 as template and the megaprimer and pBADrev as primers. The mutation modified the 86th and 87th codons of LLL (CTT GAA [Leu Glu]) to (CTCGAG [Leu Glu]) and introduced a unique *XhoI* site in the *LLL* sequence to create pJMG460.

The partial exchange of the periplasmic domain of FtsL<sub>Ecol</sub> with the homologous region of *ftsL<sub>Hinf</sub>* (LL<sub>HinfL</sub>) (see Fig. 3) was achieved as follows: a 104-bp DNA fragment corresponding to residues 45 to 72 of the *ftsL<sub>Hinf</sub>* periplasmic domain, flanked by *MscI* and *XhoI* restriction sites, was amplified by PCR with pJMG452 as template and oligonucleotides LzHinf-5 (CCCTGGCCATAA AACTCGCAATTAATTTC) and LzHinf-3 (GGGGCTCGAGTTGTAAT ACGGTATTC) as primers. The amplified DNA was digested with *MscI* and *XhoI* (sites underlined) and ligated into the same sites of pJMG460 to create pJMG427 (see Fig. 3).

The partial exchange of the periplasmic domain of FtsL<sub>Ecol</sub> with the analogous region of the yeast transcription factor GCN4 was realized as follows: a 104-bp DNA fragment corresponding to residues 248 to 275 of GCN4, flanked by *MscI* and *XhoI* restriction sites, was amplified by PCR with pY88 (25) as template and oligonucleotides LzGCN4-5 (CCCTGGCCAAAGAATGAAACAACCTTGAA GAC) and LzGCN4-3 (GGGGCTCGAGCTTAAATCTGGCAACCTCATT) as primers. The amplified DNA was digested with *MscI* and *XhoI* (sites underlined) and ligated into the same sites of pJMG460 to create pJMG428 (see also Table 5).

The partial exchange of the periplasmic domain of FtsL<sub>Ecol</sub> with the analogous region of the mammalian transcription factor C/EBP was achieved as follows: a 104-bp DNA fragment corresponding to residues 312 to 339 of the rat liver C/EBP, flanked by *MscI* and *XhoI* restriction sites, was amplified by PCR with pGB017 (4) as template and oligonucleotides LzCEBP-5 (CCCTGGCCAGA AGGTGTTGGAGTTGACCAG) and LzCEBP-3 (GGGGCTCGAGCCGAC CGTGTCCAGTTCACGG) as primers. The amplified DNA was digested with

TABLE 1. Bacterial strains used

Strain	Relevant genetic markers or features	Construction <sup>a</sup>	Source or reference
<b>Strains without <i>gfp</i> fusions</b>			
KS272	F <sup>-</sup> <i>ΔlacX74 galE galK thi rpsL ΔphoA</i> (PvuII)		Laboratory collection
MC4100	F <sup>-</sup> <i>araD139 ΔlacU169 relA1 rpsL150 thi mot ffb5301 deoC7 ptsF25 rbsR</i>		Laboratory collection
JP313	MC4100 <i>Δara714</i>		J. Pogliano
LMG145	KS272 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pLMG180		9
JMG47	KS272 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pLD45	P1 on LMG145 × KS272 pLD45, selecting for Km <sup>r</sup>	This study
DHB4	F <sup>-</sup> <i>Δlacpro lacI<sup>q</sup>/Δ(malF) ΔphoA</i> (PvuII) <i>phoR ΔlacX74 Δ(ara leu)7697 araD139 galE</i> (or <i>U</i> ) <i>galK Str<sup>r</sup></i>		D. Boyd
DHB9	DHB4 <i>recA1 srl::Tn10</i>		D. Boyd
JMG48	KS272 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG64	P1 on LMG145 × KS272 pJMG64, selecting for Km <sup>r</sup>	This study
JMG33	KS272 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> ) <i>recA1 srl::Tn10</i> /pJMG64	P1 on DHB9 × JMG48, selecting for Tet <sup>r</sup>	This study
DHB6521	SM551 <i>λInCh</i> (Kan <sup>r</sup> )		D. Boyd et al., unpublished data
<b>Depletion backgrounds</b>			
JMG343	JMG33/pAM238		This study
JMG344	JMG33/pJMG40		This study
JMG100	JMG48/pJMG473		This study
JMG348	JMG33/pJMG38		This study
JMG347	JMG33/pJMG39		This study
JMG359	JMG33/pJMG444		This study
JMG350	JMG33/pJMG157		This study
JMG351	JMG33/pJMG158		This study
JMG354	JMG33/pJMG162		This study
JMG355	JMG33/pJMG163		This study
JMG356	JMG33/pJMG164		This study
JMG353	JMG33/pJMG161		This study
JMG352	JMG33/pJMG159		This study
<b><i>gfp</i> fusion in wild-type background</b>			
EC452	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp</sub></i>		30
EC438	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-ftsL</sub></i>		8
EC531	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-ftsL</sub></i>		8
JMG287	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG453	This study
JMG441	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG453	This study
JMG377	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-FL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG447	This study
JMG469	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-FL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG447	This study
JMG289	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LFL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG448	This study
JMG292	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-FFL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG449	This study
JMG290	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLF</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG441	This study
JMG291	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-FFF</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG450	This study
JMG295	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLI</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG474	This study
JMG299	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLΔL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG465	This study
JMG300	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LL<sub>Hinf</sub>L</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG461	This study
JMG371	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LHL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG455	This study
JMG372	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LHL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG475	This study
JMG374	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-HHL</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG456	This study
JMG373	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LHH</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG458	This study
JMG370	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLH</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG459	This study
JMG375	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-HLH</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG457	This study
JMG376	JP313 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-FtsL<sub>Hinf</sub>L</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG454	This study
JMG303	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLL<sub>(L70H)</sub></sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG466	This study
JMG304	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LLL<sub>(L84D)</sub></sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG467	This study
JMG301	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LL<sub>GCN4</sub>L</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG462	This study
JMG302	MC4100 <i>Δ(λattL-lom)::bla lacI<sup>q</sup> P<sub>207-gfp-LL<sub>CIEBPL</sub>L</sub></i>	<i>λInCh</i> using pDWS207 derivative pJMG463	This study
<b><i>gfp</i> fusions in depletion backgrounds</b>			
JMG366	JMG531 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197		8
JMG367	JMG441 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG441, selecting for Km <sup>r</sup>	This study
JMG420	JMG377 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG377, selecting for Km <sup>r</sup>	This study
JMG416	JMG371 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG371, selecting for Km <sup>r</sup>	This study
JMG418	JMG373 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG373, selecting for Km <sup>r</sup>	This study
JMG415	JMG370 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG370, selecting for Km <sup>r</sup>	This study
JMG419	JMG375 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG375, selecting for Km <sup>r</sup>	This study
JMG422	JMG376 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG376, selecting for Km <sup>r</sup>	This study
JMG417	JMG372 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG372, selecting for Km <sup>r</sup>	This study
JMG442	JMG374 <i>ftsL::TnphoAL81ΔIS50R</i> (Kan <sup>r</sup> )/pJMG197	P1 on LMG145 × JMG374, selecting for Km <sup>r</sup>	This study

<sup>a</sup> P1 indicates P1 transduction. For example, JMG367 was constructed by infecting JMG441 with P1 lysate made from LMG145.

TABLE 2. Plasmids used

Plasmid	Relevant features	Source or reference
pBAD18	P <sub>BAD</sub> arabinose regulation, Amp <sup>r</sup>	10
pBAD39	Arabinose regulation, IPTG dep <sup>a</sup> , <i>lacI</i> <sup>q</sup> Strep <sup>S</sup> Amp <sup>r</sup>	L. M. Guzman, unpublished data
pAM238	P <sub>lac</sub> regulation, Spec <sup>r</sup>	J. P. Bouché, unpublished data
pGHIIA44	<i>H. influenzae</i> ATCC clone, <i>ftsL</i> <sub>Hinf</sub> Amp <sup>r</sup>	ATCC
pGB017	<i>C/EBP</i> Amp <sup>r</sup>	4
pY88	<i>GCN4</i> Amp <sup>r</sup>	24
pDSW207	<i>P</i> <sub>207-<i>gfp</i></sub> Amp <sup>r</sup>	30
pDSW236	pDSW207- <i>ftsL</i> , Amp <sup>r</sup>	8
pJMG453	pDSW207- <i>LLL</i> , Amp <sup>r</sup>	This study
pJMG447	pDSW207- <i>FLL</i> , Amp <sup>r</sup>	This study
pJMG448	pDSW207- <i>LFL</i> , Amp <sup>r</sup>	This study
pJMG449	pDSW207- <i>FFL</i> , Amp <sup>r</sup>	This study
pJMG450	pDSW207- <i>FFF</i> , Amp <sup>r</sup>	This study
pJMG451	pDSW207- <i>LLF</i> , Amp <sup>r</sup>	This study
pJMG474	pDSW207- <i>LLI</i> , Amp <sup>r</sup>	This study
pJMG454	pDSW207- <i>ftsL</i> <sub>Hinf</sub> , Amp <sup>r</sup>	This study
pJMG455	pDSW207- <i>HLL</i> , Amp <sup>r</sup>	This study
pJMG456	pDSW207- <i>HHL</i> , Amp <sup>r</sup>	This study
pJMG475	pDSW207- <i>LHL</i> , Amp <sup>r</sup>	This study
pJMG457	pDSW207- <i>HLH</i> , Amp <sup>r</sup>	This study
pJMG458	pDSW207- <i>LHH</i> , Amp <sup>r</sup>	This study
pJMG459	pDSW207- <i>LLH</i> , Amp <sup>r</sup>	This study
pJMG461	pDSW207- <i>LL</i> <sub>Hinf</sub> , Amp <sup>r</sup>	This study
pJMG462	pDSW207- <i>LL</i> <sub>GCN4</sub> , Amp <sup>r</sup>	This study
pJMG463	pDSW207- <i>LL</i> <sub>C/EBP</sub> , Amp <sup>r</sup>	This study
pJMG465	pDSW207- <i>LL</i> $\Delta$ , Amp <sup>r</sup>	This study
pJMG466	pDSW207- <i>LLL</i> <sub>(L70H)</sub> , Amp <sup>r</sup>	This study
pJMG467	pDSW207- <i>LLL</i> <sub>(L84D)</sub> , Amp <sup>r</sup>	This study
pJMG197	<i>ftsL</i> Cm <sup>r</sup>	8
pJMG64	<i>ftsL</i> , IPTG dep, <i>lacI</i> <sup>q</sup> Strep <sup>S</sup> Amp <sup>r</sup>	This study
pJMG180	pBAD18- <i>ftsL</i> , Amp <sup>r</sup>	10
pJMG460	pBAD18- <i>LLxL</i> , Amp <sup>r</sup>	This study
pLD45	pBAD18- <i>LLL</i> , Amp <sup>r</sup>	10
pLD63	pBAD18- <i>FLL</i> , Amp <sup>r</sup>	10
pLD94	pBAD18- <i>LFL</i> , Amp <sup>r</sup>	10
pLD90	pBAD18- <i>FFL</i> , Amp <sup>r</sup>	10
pLD47	pBAD18- <i>FFF</i> , Amp <sup>r</sup>	10
pLD116	pBAD18- <i>LLI</i> , Amp <sup>r</sup>	10
pJMG427	pBAD18- <i>LL</i> <sub>Hinf</sub> , Amp <sup>r</sup>	This study
pJMG429	pBAD18- <i>HLL</i> , Amp <sup>r</sup>	This study
pJMG446	pBAD18- <i>HLH</i> , Amp <sup>r</sup>	This study
pJMG433	pBAD18- <i>LHH</i> , Amp <sup>r</sup>	This study
pJMG434	pBAD18- <i>LLH</i> , Amp <sup>r</sup>	This study
pJMG430	pBAD18- <i>LHL</i> , Amp <sup>r</sup>	This study
pJMG431	pBAD18- <i>HHL</i> , Amp <sup>r</sup>	This study
pJMG452	pBAD18 <i>ftsL</i> <sub>Hinf</sub> , Amp <sup>r</sup>	This study
pJMG464	pBAD18- <i>LL</i> $\Delta$ , Amp <sup>r</sup>	This study
pJMG438	pBAD18- <i>LLL</i> <sub>(L70H)</sub> , Amp <sup>r</sup>	This study
pJMG439	pBAD18- <i>LLL</i> <sub>(L84D)</sub> , Amp <sup>r</sup>	This study
pJMG428	pBAD18- <i>LL</i> <sub>GCN4</sub> , Amp <sup>r</sup>	This study
pJMG440	pBAD18- <i>LL</i> <sub>C/EBP</sub> , Amp <sup>r</sup>	This study
pJMG40	pAM238- <i>LLL</i> , Spec <sup>r</sup>	This study
pJMG473	pAM238- <i>LLxL</i> , Spec <sup>r</sup>	This study
pJMG38	pAM238- <i>FLL</i> , Spec <sup>r</sup>	This study
pJMG39	pAM238- <i>LFL</i> , Spec <sup>r</sup>	This study
pJMG444	pAM238- <i>FFL</i> , Spec <sup>r</sup>	This study
pJMG158	pAM238- <i>LL</i> <sub>Hinf</sub> , Spec <sup>r</sup>	This study
pJMG162	pAM238- <i>ftsL</i> <sub>Hinf</sub> , Spec <sup>r</sup>	This study
pJMG157	pAM238- <i>LL</i> $\Delta$ , Spec <sup>r</sup>	This study
pJMG155	pAM238- <i>LLL</i> $\Delta$ , Spec <sup>r</sup>	This study
pJMG163	pAM238- <i>LLL</i> <sub>(L70H)</sub> , Spec <sup>r</sup>	This study
pJMG164	pAM238- <i>LLL</i> <sub>(L84D)</sub> , Spec <sup>r</sup>	This study
pJMG161	pAM238- <i>LL</i> <sub>GCN4</sub> , Spec <sup>r</sup>	This study
pJMG159	pAM238- <i>LL</i> <sub>C/EBP</sub> , Spec <sup>r</sup>	This study

<sup>a</sup> IPTG dep, IPTG-dependent replication.

*MscI* and *XhoI* (sites underlined) and ligated into the same sites of pJMG460 to create pJMG440 (see also Table 5).

Plasmids for making gene fusions to *gfp* were based on pDSW207. pDSW207-*LL*<sub>Hinf</sub>, pDSW207-*LL*<sub>GCN4</sub>, and pDSW207-*LL*<sub>C/EBP</sub> were made by PCR

with, respectively, pJMG427, pJMG428, and pJMG440 as template and oligonucleotides L1 and L2 as primers. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pDSW207 to create pJMG61, pJMG462, and pJMG463. The fusion proteins encoded by pJMG461, pJMG462, and pJMG463 have the same linker as pJMG236.

LL $\Delta$  was made as follows: the 86-bp *MscI*-*XhoI* fragment of *LLxL* was deleted from pJMG460. The resulting deleted plasmid was treated with mung bean nuclease, removing four extra hanging bases from the *XhoI* site. The resulting construct was self-ligated, creating a 30-residue in-frame deletion in the periplasmic domain of LLL (LL $\Delta$ ) in pJMG464. pDSW207-LL $\Delta$  (pJMG465) was made as described above with pJMG464 as template and L1 and L2 as primers.

To create LLL<sub>(L70H)</sub>, we mutagenized *LLL* by incorporation of a phosphorylated oligonucleotide during PCR amplification (23). We used pLD45 as template and oligonucleotide pBADf, phosphorylated oligonucleotide MnL2 (GCTCAGCGGAACAACACGTGCTGGAGCGAGATGC), and pBADrev as primers. MnL2 introduced an L-to-H mutation in the 70th codon of FtsL. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pBAD18 to create pJMG438.

To create LLL<sub>(L84D)</sub>, we mutagenized *LLL* by incorporation of a phosphorylated oligonucleotide during PCR amplification (23). We used pLD45 as template and oligonucleotide pBADf, phosphorylated oligonucleotide MnL4 (GACATGGAATGGCGCAACGATATCCITGAGAGAATGC), and pBADrev as primers. MnL4 introduced an L-to-D mutation in the 84th codon of FtsL. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pBAD18 to create pJMG439. pDSW207-LLL<sub>(L70H)</sub> (pJMG466) and pDSW207-LLL<sub>(L84D)</sub> (pJMG467) were made as described above with pJMG438 and pJMG439 as template and L1 and L2 as primers.

**Other constructs.** pAM238 derivative plasmids described in Table 2 were created as follows: pBAD18-based constructs were amplified by PCR with oligonucleotides pBADf and pBADrev as primers. The amplified DNA was digested with *EcoRI* and *HindIII* and ligated into the same sites of pAM238. LLL $\Delta$  was constructed as follows: a stop linker (LzStop1 [TCGACAAAATAATA CGTAA] and LzStop2 [ACGTTTACGTATTATTTTGG]) was inserted at the *XhoI* site of *LLxL* in pJMG473. The resulting plasmid (pJMG155) presents a deletion of residues 87 to 119 in the FtsL coding sequence. For pJMG64, the *SacI*-*HindIII* fragment of pLMG180 was introduced into pBAD39 linearized with the same enzymes to create pJMG64.

**Construct verification.** All constructs were checked by PCR with specific and vector-based primers. Then each construct was sequenced in the pBAD18 or pAM238 context. For GFP fusions, the gene coding for the protein fused to GFP was sequenced from a PCR product obtained by amplification of chromosomal DNA. The sequence was checked up to the GFP-swap junction point. The constructs FFF and LLF were sequenced up to the junction point with the end of the region coding for the first membrane-spanning segment. The integrity of the rest of the *malF* portion was then checked by testing the ability of LLF (pJMG451) to complement a *malF* mutation in DHB4 on a MacConkey maltose plate.

**Growth conditions. (i) GFP-fusion protein expression. (a) Wild-type background.** Cells expressing GFP fusions ("GFP fusion in wild-type background" section of Table 1) were grown overnight at 30°C in NZY medium plus AMP, diluted 1:200 in fresh NZY medium (without AMP) containing 2.5 to 10  $\mu$ M IPTG, and grown on a roller at 30°C until the optical density at 600 nm (OD<sub>600</sub>) reached 0.25.

**(b) Depletion experiments.** To deplete FtsL from strains indicated in the "GFP fusion in depletion background" section of Table 1, strains were grown overnight in NZY with AMP, KAN, chloramphenicol, and 0.2% arabinose (which allowed high-level expression of FtsL from plasmid pJMG197). Cultures were diluted into fresh medium containing chloramphenicol and 0.02% arabinose and grown to an OD<sub>600</sub> of 0.5. Cells were washed and resuspended in NZY medium and then diluted 1:200 into NZY with 0.2% glucose (which shut off synthesis of FtsL from pJMG197) or NZY with 0.2% arabinose plus 2.5 to 5  $\mu$ M IPTG. After about 3 to 5 h, an OD<sub>600</sub> of 0.25 was reached and cells had become filamentous in the glucose culture. Samples were harvested and prepared for microscopy and Western blot analysis.

**(ii) FtsL derivative protein expression from pBAD18-based constructs.** pBAD18-based FtsL derivatives were introduced into KS272, grown overnight at 37°C in LB medium plus AMP, diluted 1:200 in fresh LB medium, and grown on a roller at 37°C until the OD<sub>600</sub> reached 0.25. Then, arabinose or glucose (0.2%) was added and cultures were grown for 1 to 2 more h until the OD<sub>600</sub> reached 0.6 to 1.

**Complementation of an *ftsL* null mutation. (i) Complementation of FtsL derivative as GFP fusions inserted on the *E. coli* chromosome.** GFP-FtsL fusions were tested for complementation as follows. Strains corresponding to the "GFP fusion in depletion background" section in Table 1 were grown on low-AMP (25  $\mu$ g/ml)-KAN-chloramphenicol-arabinose plates and were tested on plates containing low AMP, KAN, and 2.5  $\mu$ M IPTG, supplemented with 0.2% glucose. Under these conditions, where the pJMG197-borne copy of *ftsL* is repressed, only strains expressing a functional GFP-FtsL derivative could grow.

**(ii) Complementation of null mutant by counterselection.** The functionality of FtsL derivatives in the pAM238 context was tested as follows. FtsL variants made in pBAD18 were subcloned in pAM238 (a plasmid with a pSC101 origin of replication) under plac promoter control and introduced in strain JMG33 con-

TABLE 3. Localization frequencies of and complementation by GFP-FtsL and GFP-FtsL<sub>Ecol</sub>-MalF swap proteins

Construct	Structure <sup>a</sup>	Localization frequency <sup>b</sup>		Complementation <sup>c</sup>	
		No. of cells scored	Band at midcell (%)	Swap not fused to GFP <sup>c</sup>	Swap fused to GFP <sup>d</sup>
GFP-FtsL		342	72	+++	+++
GFP-LLL		310	69	+++	+++
GFP-FLL		307	70	-	-
GFP-FFL		295	<1	-	ND
GFP-LFL		301	<1	-	ND
GFP-LLF		293	<1	ND	ND
GFP-LLI		320	<1	-	ND
GFP-FFF		311	<1	-	ND

<sup>a</sup> Open boxes represent domains of FtsL. Shaded boxes denote GFP. Hatched boxes represent domains derived from MalF. The checkered box represents the periplasmic domain of FtsI. E and M indicate borders of the transmembrane domain where restriction sites for *EagI* and *MscI*, respectively, have been introduced. In FtsL, the former changes a Lys to an Arg and the latter changes an Ala to Gly. Diagrams of fusion proteins are not to scale. The cytoplasmic and transmembrane domains of FtsL contain 37 and 20 amino acids, respectively, while those from MalF contain 16 and 25 amino acids, respectively. The periplasmic domain of FtsL contains 64 amino acids. The periplasmic domain of FtsI contains 537 amino acids. The region past the first transmembrane domain of MalF contains 478 amino acids.

<sup>b</sup> Strains used: FtsL, EC438; LLL, JMG287; FLL, JMG469; FFL, JMG292; LFL, JMG289; LLF, JMG290; LLI, JMG295; FFF, JMG291.

<sup>c</sup> Strains used: FtsL, strain from reference 10; LLL, JMG344; FLL, JMG348; FFL, JMG359; LFL, JMG347; LLI, strain from reference 10; FFF, strain from reference 10.

<sup>d</sup> Strains used: GFP-FtsL, JMG366 (8); GFP-LLL, 367; GFP-FLL, JMG420.

<sup>e</sup> For complementation test, plus signs indicate colony size and minus signs indicate no growth. ND, not determined.

taining pJMG64 (Table 1 and Table 2). pJMG64 is a pBAD39-derived plasmid expressing a wild-type allele of *ftsL*, whose induction (with arabinose) is required to complement the chromosomal *ftsL-phoA* null mutation. pBAD39 is a counterselectable pBAD18-derived plasmid, carrying a *lacI* gene, whose replication is IPTG dependent and which expresses *rpsL*, conferring a dominant sensitivity to streptomycin (1.5 mg/ml). The complete features of pBAD39 will be described in detail elsewhere (L. M. Guzman, unpublished data). The rationale of the counterselection-based complementation test is the following: without IPTG and AMP and in the presence of streptomycin, cells carrying pJMG64 are counterselected. The expression of genes carried by the pAM238-based plasmid is then constitutive in JMG33 deprived of pJMG64, as there is no longer a *lacI* gene in the cells. Any functional FtsL derivatives would then support the growth of JMG33 in the absence of pJMG64.

Strains corresponding to the "depletion background" section in Table 1 were grown on plates containing IPTG, AMP, KAN (Tn5 associated with the chromosomal *ftsL* null allele), TET (Tn10 associated with the chromosomal *recA* allele), SPEC, and arabinose (0.2%). Under counterselection conditions without IPTG and AMP but with SPEC, KAN, TET, streptomycin, and glucose (0.2%), where the pJMG64-borne copy of *ftsL* is counterselected, only strains expressing a functional FtsL derivative could grow.

**Microscopy and image analysis.** Cells were grown and processed for protein localization experiments as described in reference 8. Microscopy and image analysis were performed as described in reference 8.

**Western blotting.** Western blotting was performed by standard techniques as described in reference 8. To determine the steady-state levels of the GFP fusion proteins, we harvested cells from the same cultures as those used for fluorescence microscopy. Cells were pelleted by centrifugation and resuspended with 10  $\mu$ l of SDS sample buffer per OD<sub>600</sub> unit of 0.1. Samples were boiled for 3 min, and 10  $\mu$ l of sample was loaded onto an SDS-polyacrylamide gel. To determine the multimerization state of the FtsL derivatives, cells were pelleted by centrifugation and resuspended with 10  $\mu$ l of SDS sample buffer per OD<sub>600</sub> unit of 0.1. In most of the experiments, samples were not boiled and 10  $\mu$ l of sample was loaded onto a 10% (GFP fusion) or 13% (FtsL swaps) SDS-polyacrylamide gel.

## RESULTS

**The membrane-spanning segment and the periplasmic domain of FtsL are both required for septal localization.** In previous complementation tests, we used swap proteins where the three domains of FtsL were exchanged with topologically equivalent domains of MalF, FtsI, and FtsQ. This analysis indicated that all three domains were required for FtsL function (11). One simple interpretation of these data is that all three domains participate in septal localization. To test this hypothesis, we fused a bright variant of GFP (6) at the amino terminus (cytoplasmic domain) of several swap proteins: LLL, FLL, FFL, LFL, LLF, LLI, and FFF (Table 3). The three-letter names indicate the source of each domain in the swap proteins: the first letter indicates that of the cytoplasmic region, the second indicates that of the transmembrane segment, and the third indicates that of the periplasmic domain. For example, LFL refers to a protein in which the cytoplasmic and periplasmic domains are derived from FtsL and the membrane-spanning domain is derived from the first transmembrane segment from MalF. In LLI, I stands for the periplasmic domain of FtsI, another bitopic membrane protein involved in cell division. LLL and FFF constructs differ from wild-type FtsL and MalF in that two and four amino acids at the borders of the membrane-spanning segment have been altered due to introduction of restriction sites in corresponding regions of the *ftsL* and *malF* genes, respectively (11). We integrated the genes coding for GFP fused to the N terminus of LLL, FLL, FFL, LFL, LLF, LLI, and FFF to generate merodiploid strains sim-

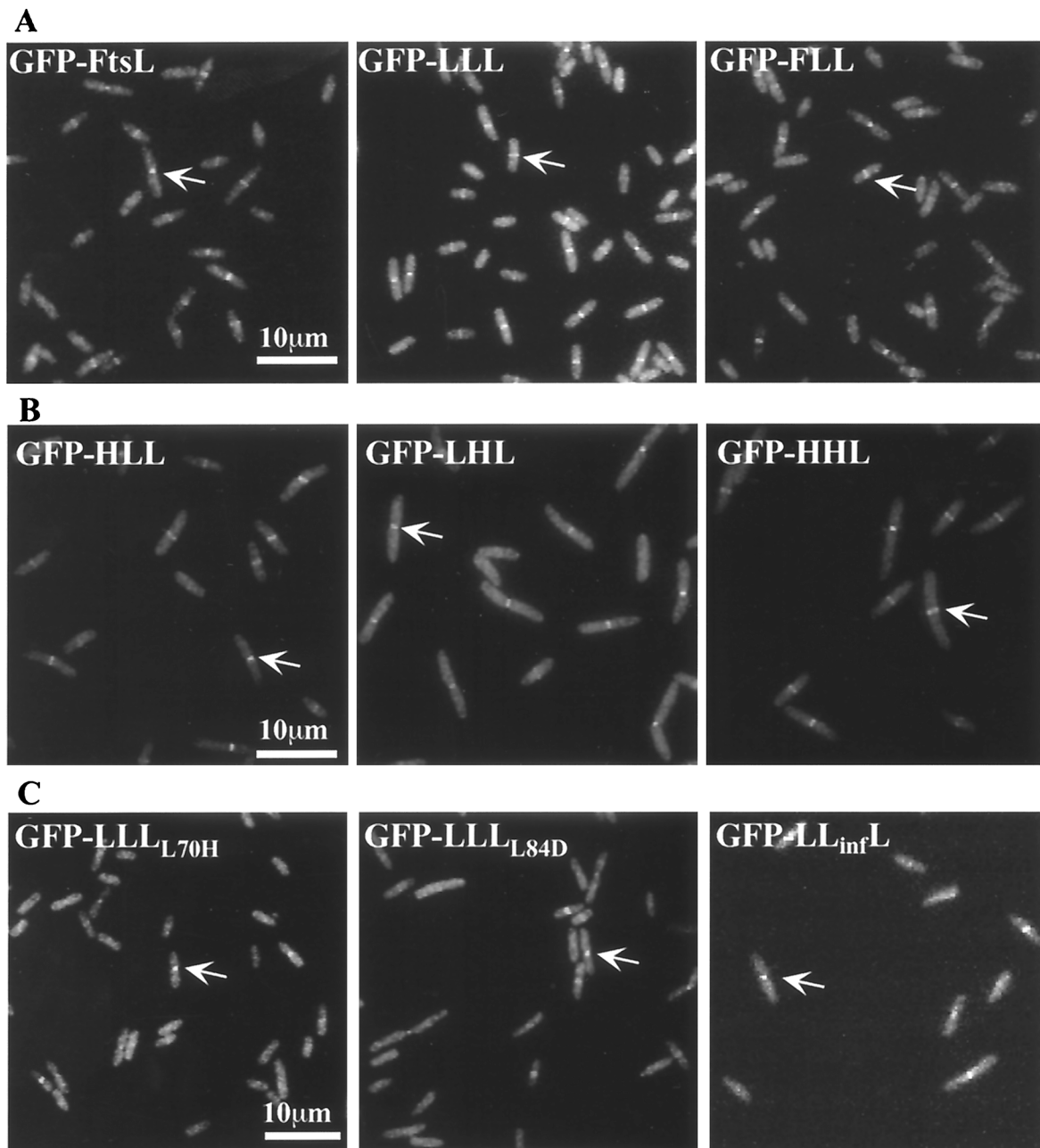


FIG. 1. Subcellular location of GFP-FtsL swap proteins in cells fixed during exponential growth. Arrows indicate rings at division sites. (A) Septal localization of FtsL<sub>Ecol</sub>-MalF swap proteins fused to GFP. Strains used were as follows: GFP-FtsL, EC438; GFP-LLL, JMG287; and GFP-FLL, JMG469. (B) Septal localization of FtsL<sub>Ecol</sub>-FtsL<sub>Hinf</sub> swap proteins fused to GFP. Strains used were as follows: GFP-HLL, JMG371; GFP-LHL, JMG372; and GFP-HHL, JMG374. (C) Septal localization of FtsL periplasmic mutants fused to GFP. Strains used were as follows: GFP-LLL<sub>(L70H)</sub>, JMG303; GFP-LLL<sub>(L84D)</sub>, JMG304; and GFP-LL<sub>Hinf</sub>L, JMG300.

ilar to those used for localization of GFP-FtsL (5, 8, 31). Cells expressing GFP fusions to the FtsL-MalF swap proteins were fixed and examined by fluorescence microscopy. GFP-LLL localized as well as the control protein in which GFP was fused to the wild-type FtsL, while GFP-FFF did not localize to the septum. The GFP-FLL protein was the only swap protein to localize to the septum with a frequency similar to that of GFP-LLL (Table 3 and Fig. 1A).

The lack of localization of GFP-LFL, GFP-FFL, GFP-LLF, and GFP-LLI is consistent with the inability of these constructs, without GFP attached, to complement an *ftsL* null mu-

tant. The efficient septal localization frequency of GFP-FLL contrasted with the lack of functionality of the FLL protein in the cell division process (11) (Table 3). This finding led us to test the complementation of a null mutation in *ftsL* by GFP-FLL. The *gfp-LLL* and *gfp-FLL* fusions were tested for complementation in the FtsL depletion strain. Cells expressing GFP-LLL grew well in the presence of glucose (FtsL depletion) while cells expressing GFP-FLL did not. These complementation results are consistent with those obtained with the LLL and FLL proteins when not fused to GFP (Table 3). Differences in localization did not result from differences in

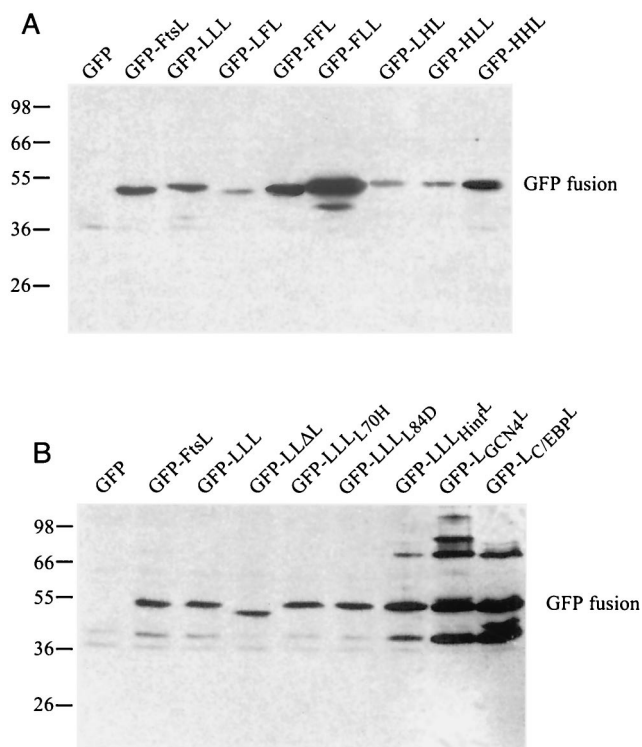


FIG. 2. Steady-state levels of GFP-FtsL-MalF swap proteins as determined by Western blotting with an anti-FtsL antibody. Molecular mass standards (kilodaltons) are indicated to the left of the blot, while the positions of GFP-FtsL fusions are shown to the right. The GFP fusion protein produced is indicated above each lane. Strains used were as follows: (A) GFP, EC452; GFP-FtsL, EC438; GFP-LLL, JMG287; GFP-LFL, JMG289; GFP-FFL, JMG292; GFP-FLL, JMG469; GFP-LHL, JMG372; GFP-HLL, JMG371; and GFP-HHL, JMG374; (B) GFP, EC452; GFP-FtsL, EC438; GFP-LLL, JMG287; GFP-LLAL, JMG299; GFP-LLL<sub>(L70H)</sub>, JMG303; GFP-LLL<sub>(L84D)</sub>, JMG304; GFP-LL<sub>HinfL</sub>, JMG300; GFP-LL<sub>GCN4L</sub>, JMG301; and GFP-LL<sub>C/EBpL</sub>, JMG302.

expression; Western blotting was performed and indicated that, although the stability and abundance of each swap protein vary, they are all expressed at a significant level (Fig. 2). The fact that GFP-FFF and GFP-LLF complement a *malF* mutation (Materials and Methods) suggests that these proteins are also expressed at appropriate levels. The amount of GFP-LLI was determined with anti-FtsI antibodies (data not shown).

The results in Table 3 suggest that the FtsL septal localization signal is carried by a combination of the membrane-spanning segment and the periplasmic domain but that localization of these two domains to the division site is not sufficient to complement for FtsL function.

**The *H. influenzae* FtsL homologue neither complements nor localizes in *E. coli*.** To further investigate the essential features of the FtsL localization signal, we asked whether a homologue of *E. coli* FtsL from another bacterial species could complement for FtsL function. Such an approach has proven to be informative in studies of the function of the cell division proteins FtsA and FtsZ (21). We chose the *H. influenzae* FtsL (FtsL<sub>Hinf</sub>) as a good candidate to test for complementation and localization in *E. coli*. The FtsL<sub>Hinf</sub> amino acid sequence is 40% identical to that of the FtsL of *E. coli*, and the chromosomal position and locus genetic organization of *ftsL*<sub>Hinf</sub> are very similar to those of *ftsL*<sub>Ecol</sub>. The homology between the two proteins is distributed evenly across the sequence except for the N terminus of FtsL<sub>Hinf</sub>, which is shorter by 14 residues. FtsL<sub>Ecol</sub> and FtsL<sub>Hinf</sub> present the same predicted bitopic to-

pology and have a conserved heptad motif in their periplasmic domain (Fig. 3). We cloned the *ftsL*<sub>Hinf</sub> gene from the *H. influenzae* ATCC cosmid library and expressed it under the pBAD arabinose-inducible promoter with the *ftsL*<sub>Ecol</sub> translation signals. Complementation tests showed that *ftsL*<sub>Hinf</sub> did not complement an *ftsL*<sub>Ecol</sub> null mutant. To verify the expression of FtsL<sub>Hinf</sub> under these conditions, we generated fusions of FtsL<sub>Hinf</sub> to alkaline phosphatase, where alkaline phosphatase is fused after the last residue of the FtsL<sub>Hinf</sub> periplasmic domain. In this construct, the promoter and translation signals are the same as in constructs expressing FtsL<sub>Ecol</sub> and FtsL<sub>Hinf</sub>-PhoA fusions have been used to verify expression and proper topology of FtsL<sub>Ecol</sub> (11). The hybrid protein FtsL<sub>Hinf</sub>-PhoA gave high levels of alkaline phosphatase expression, as observed on indicator plates containing 5-bromo-3-chloro-indolylphosphate (data not shown), indicating that FtsL<sub>Hinf</sub>, as FtsL<sub>Hinf</sub>-PhoA, was expressed and assumed the predicted topology and arrangement in the membrane. Therefore, the lack of functionality of FtsL<sub>Hinf</sub> does not appear to be due to lack of expression or proper insertion in the envelope.

Based on sequence homologies and similarity of genetic organization of the *ftsL*<sub>Hinf</sub> chromosomal region, it is likely that FtsL<sub>Hinf</sub> fulfills in *H. influenzae* the role played by FtsL<sub>Ecol</sub> in *E. coli*. Our results suggest that FtsL<sub>Hinf</sub> in *E. coli* is deficient for a key functional feature required to support cell division. Whether this deficiency relates to an inability to localize at the division site is analyzed below.

**Portions of FtsL<sub>Hinf</sub> and FtsL<sub>Ecol</sub> are functionally interchangeable.** To test whether portions of the FtsL<sub>Hinf</sub> protein could functionally substitute for the homologous regions of FtsL<sub>Ecol</sub>, we replaced the first half of the periplasmic domain of the swap protein LLL (residues 58 to 85 [Fig. 3]) with its corresponding region from FtsL<sub>Hinf</sub> (residues 45 to 72). This new protein, named LL<sub>Hinf</sub>L, is able to complement an *ftsL* null mutant, although less efficiently than LLL or wild-type FtsL (Table 4).

To test whether FtsL<sub>Hinf</sub> and LL<sub>Hinf</sub>L localize to the division site, we fused GFP to the N terminus of FtsL<sub>Hinf</sub> and LL<sub>Hinf</sub>L. As expected from complementation studies, the GFP-FtsL<sub>Hinf</sub> protein did not localize to the division site but rather showed a uniform fluorescent background. In contrast, GFP-LL<sub>Hinf</sub>L localized in 40% of the cells, compared to 70% septal localization for GFP-LLL (Fig. 1C and Table 4). The level of localization was consistent with the complementation results: weaker complementation correlates with less frequent localization (Table 4). These results indicated that FtsL<sub>Hinf</sub> is non-functional in *E. coli* but that part of its periplasmic domain can be functionally exchanged with the homologous region of the FtsL<sub>Ecol</sub> domain.

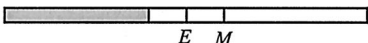
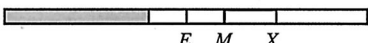
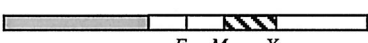
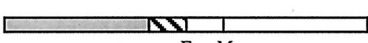

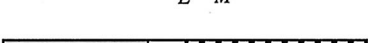
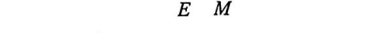
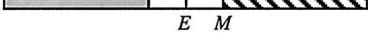
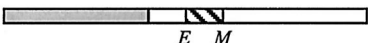
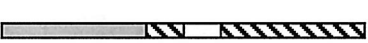
We then constructed several swap proteins where the three domains of FtsL<sub>Ecol</sub> were exchanged with topologically equivalent domains of FtsL<sub>Hinf</sub>: HLL, HHL, LHH, LLH, and LHL, where, for example, LHL refers to a protein in which the cytoplasmic and periplasmic domain are derived from FtsL<sub>Ecol</sub> and the transmembrane domain is from FtsL<sub>Hinf</sub>. To test for localization of the swap proteins, cells expressing GFP fusions to the N terminus of the swap genes were fixed and examined by fluorescence microscopy. As shown in Fig. 1B and Table 4, all fusions containing the periplasmic domain of FtsL<sub>Ecol</sub> showed efficient localization to the division site. In contrast with the localization results obtain with GFP-LL<sub>Hinf</sub>L, the whole periplasmic domain of FtsL<sub>Hinf</sub> is not sufficient to direct the fusion protein to the division site and plausibly accounts for the lack of function of FtsL<sub>Hinf</sub> in *E. coli*.

We then tested the functionality of these GFP fusion proteins in the FtsL depletion strain. Complementation results are





TABLE 4. Localization frequencies of and complementation by GFP-FtsL<sub>Ecol</sub>-FtsL<sub>Hinf</sub> swap proteins

Construct	Structure <sup>a</sup>	Localization frequency <sup>b</sup>		Complementation <sup>c</sup>	
		No. of cells scored	Band at midcell (%)	Swap not fused to GFP <sup>c</sup>	Swap fused to GFP <sup>d</sup>
GFP-LLL		310	75	+++	+++
GFP-LLxL		ND <sup>f</sup>	ND	+++	ND
GFP-LL <sub>Hinf</sub> L		255	40	+	ND
GFP-HLL		278	82	ND	+++
GFP-HHL		310	76	ND	-
GFP-LHH		268	<1	ND	-
GFP-LLH		319	<1	ND	-
GFP-LHL		308	80	ND	-
GFP-HLH		242	<1	ND	-
GFP-FtsL <sub>Hinf</sub>		273	<1	-	-

<sup>a</sup> Open boxes represent domains of *E. coli* FtsL (FtsL<sub>Ecol</sub>). Shaded boxes denote GFP. Hatched boxes represent domains derived from wild-type *H. influenzae* FtsL (FtsL<sub>Hinf</sub>). E and M indicate borders of the transmembrane domain where restriction sites for *EagI* and *MscI*, respectively, have been introduced. X indicates the position of the *XhoI* site introduced to allow partial swapping inside the region encoding the periplasmic domain of FtsL. In FtsL<sub>Ecol</sub>, the former changes a Lys to an Arg and the latter changes an Ala to a Gly. In FtsL<sub>Hinf</sub>, the former changes an Asn and a Lys to a Gly and an Arg, respectively, and the latter changes a Thr to a Gly. Diagrams of fusion proteins are not to scale. The cytoplasmic and transmembrane domains of FtsL<sub>Ecol</sub> contain 37 and 20 amino acids, respectively, while those from FtsL<sub>Hinf</sub> contain 24 and 20 amino acids, respectively. The periplasmic domain of FtsL<sub>Ecol</sub> contains 64 amino acids. The periplasmic domain of FtsL<sub>Hinf</sub> contains 63 amino acids.

<sup>b</sup> Strains used: GFP-LLL, JMG441; GFP-LL<sub>Hinf</sub>L, JMG300; GFP-HLL, JMG371; GFP-HHL, JMG374; GFP-LHH, JMG373; GFP-LLH, JMG370; GFP-LHL, JMG372; GFP-HLH, JMG375; GFP-FtsL<sub>Hinf</sub>, JMG376.

<sup>c</sup> Strains used: LLL, JMG344; LLxL, JMG100; LL<sub>Hinf</sub>L, JMG351; FtsL<sub>Hinf</sub>, JMG354.

<sup>d</sup> Strains used: GFP-LLL, JMG367; GFP-HLL, JMG416; GFP-HHL, JMG442; GFP-LHH, JMG418; GFP-LLH, JMG415; GFP-LHL, JMG417; GFP-HLH, JMG419; GFP-FtsL<sub>Hinf</sub>, JMG422.

<sup>e</sup> For complementation test, plus signs reflect colony size and minus signs indicate no growth.

<sup>f</sup> ND, not determined.

the periplasmic domain of FtsL is required for accurate septal localization. None of the swap proteins in which a portion of this domain was removed or exchanged yielded a fully functional and correctly localized protein. The fact that the two leucine mutants GFP-LLL<sub>(L70H)</sub> and GFP-LLL<sub>(L84D)</sub> showed a deficient localization pattern suggests that the heptad motif could be instrumental in the localization process; however, merely exchanging this motif with a well-characterized leucine zipper does not allow localization in GFP-LL<sub>GCN4</sub>L and GFP-LL<sub>C/EBP</sub>L.

**The heptad motif of the periplasmic domain of *E. coli* FtsL is conserved in all identified FtsL homologues and has the characteristics of a coiled-coil domain.** The results presented above suggested that the heptad repeats of the periplasmic domain of FtsL may be required for FtsL function and that they may be indicative of structural features of its periplasmic domain. Heptad repeat motifs such as those found in FtsL<sub>Ecol</sub> and FtsL<sub>Hinf</sub> have been reported to have a high propensity to form  $\alpha$ -helical multimeric coiled coils. Coiled coils are helical bundles of two to five helices that have a distinctive packing of amino acid side chains at helix-helix interfaces (18). This packing results in every seventh residue occupying an equivalent

position on the helix surface. The seven positions are named **a** to **g**; **a** and **d** are usually occupied by hydrophobic residues and form the helix interface (core). Oppositely, charged residues in positions **e** and **g** flank the core and stabilize the protein through ionic interactions. The outer positions, **b**, **c**, and **f**, are not directly involved in the formation of the helical bundle and are free to interact with surrounding molecules. A wide variety of proteins involved in maintaining cell shape, in organizing the cytoplasm, and in cell movement and membrane fusion are coiled coils of two or more  $\alpha$ -helices (17).

In order to estimate the degree of conservation of the heptad motif among FtsL homologues, we carried out a multiple alignment between several previously identified as well as newly described FtsL homologues. FtsL homologues so identified were found in *B. subtilis*, *Streptococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Yersinia pestis*, and *Buchnera aphidicola*. These homologues were identified by us and others in both gram-negative and gram-positive bacteria in complete and incomplete genome databases based on (i) their homologies with either FtsL<sub>Ecol</sub> or FtsL<sub>Hinf</sub>, (ii) the similarity of their genetic locus position (upstream of *ftsI* in a cell division operon encoding other *fts* gene homologues), and (iii) their

TABLE 5. Localization frequencies of and complementation by GFP-FtsL mutants

Construct	Structure <sup>a</sup>	Localization frequency <sup>b</sup>		Complementation <sup>c</sup>	
		No. of cells scored	Band at midcell (%)	Swap not fused to GFP <sup>c</sup>	Swap fused to GFP <sup>d</sup>
GFP-LLL		342	69	+++	+++
GFP-LLΔL		355	—	—	ND
GFP-LLL <sub>(L70H)</sub>		310	17	+	ND
GFP-LLL <sub>(L84D)</sub>		380	28	+	ND
GFP-LL <sub>GCN4</sub> L		232	<1	—	ND
GFP-LL <sub>C/EBP</sub> L		218	<1	—	ND

<sup>a</sup> Open boxes represent domains of *E. coli* FtsL (FtsL<sub>Ecol</sub>). Shaded boxes denote GFP. Hatched boxes represent GCN4 leucine zipper. The checked box represents C/EBP leucine zipper. E and M indicate borders of the transmembrane domain where restriction sites for *EagI* and *MscI*, respectively, have been introduced. X indicates the position of the *XhoI* site introduced to allow partial swapping inside the region encoding the periplasmic domain of FtsL. In LLL, the former changes a Lys to an Arg, and the latter changes an Ala to a Gly. The cytoplasmic and transmembrane domains of LLL contain 37 and 20 amino acids, respectively. The periplasmic domain of LLL contains 64 amino acids.

<sup>b</sup> Strains used: GFP-LLL, JMG287; GFP-LLΔL, JMG299; GFP-LLL<sub>(L70H)</sub>, JMG303; GFP-LLL<sub>(L84D)</sub>, JMG304; GFP-LL<sub>GCN4</sub>L, JMG301; GFP-LL<sub>C/EBP</sub>L, JMG302.

<sup>c</sup> Strains used: LLL, JMG344; LLΔL, JMG350; LLL<sub>(L70H)</sub>, JMG355; LLL<sub>(L84D)</sub>, JMG356; LL<sub>GCN4</sub>L, JMG353; LL<sub>C/EBP</sub>L, JMG352.

<sup>d</sup> Strain used: GFP-LLL, JMG367. ND, not determined.

<sup>e</sup> For complementation test, plus signs indicate colony size and minus signs indicate no growth.

predicted bitopic membrane protein topology. Multiple alignment of the FtsL homologues so identified is presented in Fig. 5, where the **a** to **g** positions of the residues in the heptads are indicated above the alignment. In gram-negative bacterial FtsL homologues, the **d** position is mostly occupied by hydrophobic (Leu) and, to a lesser extent, by charged residues (E). Interestingly, in a typical coiled-coil core, the **a** position is often occupied by charged residues instead of hydrophobic residues. The **g** position is mainly occupied by charged residues, especially in the gram-negative bacterial homologues. Gram-positive bacterial FtsL proteins conform more to a canonical coiled coil, where **a** and **d** hydrophobic positions define the apolar stripe between helices.

In order to determine the propensity of FtsL<sub>Ecol</sub> to form coiled coils, we submitted the FtsL<sub>Ecol</sub> amino acid sequence to a widely used coiled-coil prediction algorithm, COILS 2.2 (18, 19). This analysis revealed that FtsL<sub>Ecol</sub> has a low propensity (5% depending on the window length [Table 6]) to form a coiled-coil structure in the periplasmic domain. Strikingly, when we performed the same analysis with FtsL from *H. influenzae*, *B. subtilis*, *S. aureus*, *S. pneumoniae*, and *E. faecalis*, the periplasmic domain of these FtsLs showed a maximum propensity score of 100%. As noted by Lupas and coworkers (18), one of the limits of the method is that the absence of a peak is not as conclusive as the presence of a peak; there are many explanations of why the program might fail to predict a coiled-coil structure. On the other hand, a propensity of 100% is a very strong indication of a coiled-coil structure. This predicted coiled-coil propensity is very high in most of the FtsL homologues except for *E. coli* FtsL and its closest homologue, *Y. pestis* FtsL, where a low coiled-coil propensity is predicted (5% for *Y. pestis* and 80% for *B. aphidicola* [Table 6]).

Interestingly, the coiled-coil propensity of *E. coli* and *Y. pestis* FtsL increases up to almost 100% if only one residue is changed in their amino acid sequence (Y81 instead of W81 for *E. coli* FtsL and Y66 instead of W66 for *Y. pestis* FtsL). Apparently, the tryptophan instead of tyrosine residue at position 81 (or 66 for *Y. pestis* FtsL) greatly reduces the coiled-coil propensity probability (Table 6). This result may be due to the

fact that the scoring matrix for the COILS program is derived exclusively from long, parallel, two-stranded coiled coils where W is highly disfavored at the **a** position. Indeed, certain peculiarities of two-stranded coiled coils, such as a restricted amount of space in packing the core layers, will be used to judge coiled-coil sequences that do not have these problems by virtue of the fact that they form three-, four-, or even five-stranded coiled coils, which have increasingly more packing space in their core. This signifies that if the FtsL coiled-coil is not two stranded, then it may well be easy to accommodate the W in position **a** of the core, even though this is very difficult in a two-stranded coiled coil (A. Lupas, personal communication). In *B. aphidicola* FtsL, which is one-third shorter than *E. coli* FtsL, the disruptive effect of the tryptophan W81 located at the end of the putative coiled coil is predicted to be smaller than in the case of *E. coli* or *Y. pestis* FtsL, where it is located in the middle of the coiled coil.

Besides the striking conservation of the heptad repeats in the periplasmic domain, the FtsL multiple alignment also shows that the helical structure may extend through the membrane-spanning segment and the cytoplasmic domain (Fig. 4).

**The periplasmic domain of *E. coli* FtsL is a multimerization domain.** The data presented above suggest that FtsL may adopt a coiled-coil structure that could encompass both the transmembrane and the periplasmic domain. Coiled-coil structures have been involved in the formation of multimers where

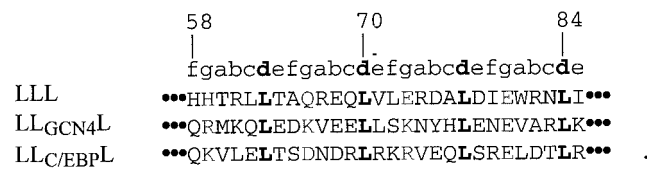


FIG. 4. Sequence alignment of the regions that are not common to LLL, LL<sub>GCN4</sub>L, and LL<sub>C/EBP</sub>L swap proteins. Dotted lines represent flanking FtsL sequence which is the same in the three proteins. Leu70 and Leu84 are mutated in LLL<sub>(L70H)</sub> and LLL<sub>(L84D)</sub>.

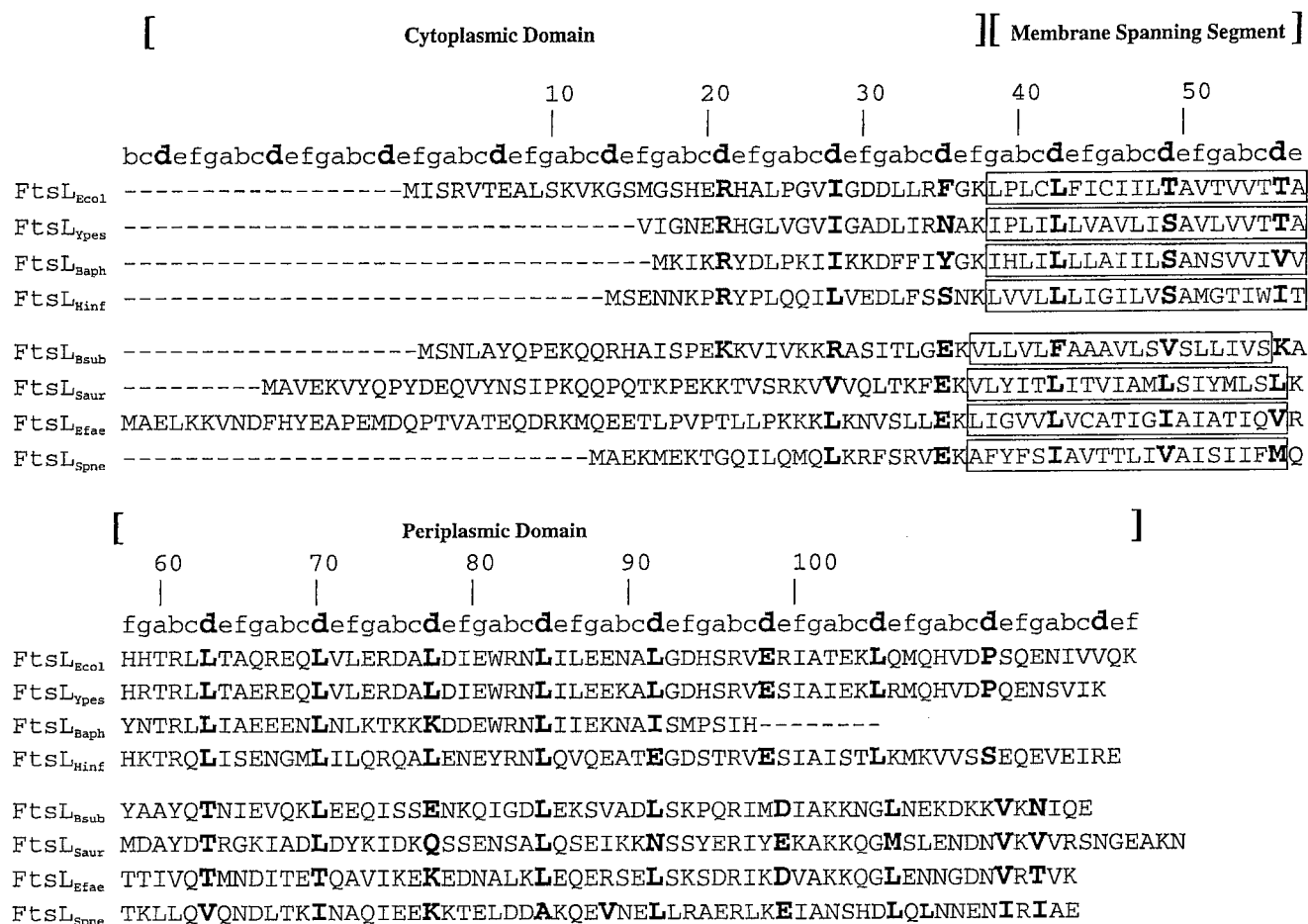


FIG. 5. Multiple alignment of FtsL homologues. Predicted membrane-spanning segments are boxed. The a to f positions of the heptad repeat of the potential coiled-coil domain are indicated above the sequence. The d position is indicated in boldface. The numbers above the alignment refer to the *E. coli* FtsL amino acid position. FtsL<sub>Ecol</sub>, *E. coli* FtsL; FtsL<sub>Ypes</sub>, *Y. pestis* FtsL; FtsL<sub>Baph</sub>, *B. aphidicola* FtsL; FtsL<sub>Hinf</sub>, *H. influenzae* FtsL; FtsL<sub>Bsub</sub>, *B. subtilis* FtsL; FtsL<sub>Saur</sub>, *S. aureus* FtsL; FtsL<sub>Efae</sub>, *E. faecalis* FtsL; FtsL<sub>Spne</sub>, *S. pneumoniae* FtsL.

two to five helices interact to form a superhelical bundle. The stability of the coiled-coil interactions is variable but may be high and in some cases thermostable and SDS resistant (13). To test whether potential FtsL multimers were stable enough to resist denaturation by SDS, we carried out an SDS-polyacrylamide gel electrophoresis analysis of a protein extract from a strain where FtsL was expressed from a multicopy plasmid under an inducible promoter. On SDS-polyacrylamide gels, two forms could be detected with anti-FtsL antibodies: a lower form that ran at the expected monomeric FtsL molecular mass (about 14 kDa) and an upper form running at 30 kDa that may correspond to an FtsL homodimer (Fig. 6). In some experiments, higher-order multimers could also be seen, although less consistently than the apparent dimer (data not shown). The abundance of the dimeric form was considerably reduced in boiled samples (data not shown). In order to determine whether the formation of this putative dimer is dependent on the presence of the periplasmic domain, cell extracts from strains expressing LLL, FLL, LFL, and FFL swap proteins as well as LLL<sub>(L70H)</sub> and LLL<sub>(L84D)</sub> were submitted to the same analysis. Samples from cells expressing LLL and FLL, but not LFL or FFL, displayed the putative dimer bands (Fig. 6A). Samples from strains expressing the two leucine mutants displayed a much-reduced ability to dimerize that could not be observed consistently (not shown in Fig. 6 and noted as +/- in Table 7). These results demonstrate that only constructs car-

rying the membrane-spanning segment and periplasmic domain of FtsL multimerize and that mutagenesis of two d positions [LLL<sub>(L70H)</sub> and LLL<sub>(L84D)</sub>] of the putative coiled-coil motif reduces the dimerization ability. To carry further this

TABLE 6. Coiled-coil-forming potential in FtsL gram-negative and gram-positive homologues

Organism	Predicted coiled coil <sup>a</sup>	Avg score <sup>b</sup> (%)
<i>Escherichia coli</i>	60-90	5
<i>Escherichia coli</i> (W81 to Y)	60-90	90 <sup>c</sup>
<i>Yersinia pestis</i>	40-80	5
<i>Yersinia pestis</i> (W81 to Y)	40-80	90 <sup>c</sup>
<i>Buchnera aphidicola</i>	35-70	80
<i>Haemophilus influenzae</i>	40-80	100
<i>Bacillus subtilis</i>	60-100	100
<i>Streptococcus aureus</i>	60-120	95
<i>Streptococcus pneumoniae</i>	40-110	100
<i>Enterococcus faecalis</i>	75-115	100

<sup>a</sup> Approximate location of the strongest prediction for coiled coil in FtsL amino acid sequence.

<sup>b</sup> Scores were calculated according to the work of Lupas et al. (18) with the COILS program on the ISREC web server ([http://www.isrec.isb-sib.ch/software/COILS\\_form.html](http://www.isrec.isb-sib.ch/software/COILS_form.html)). Window length, 21; matrix, MTIDK weighted. For *E. coli* and *Y. pestis* FtsL, the value reported is the average obtained with all four matrices used.

<sup>c</sup> Coiled-coil propensity prediction: consequence of the replacement of W81 (1) by Y (2) in *E. coli* FtsL and of W66 (3) by Y (4) in *Y. pestis* FtsL.

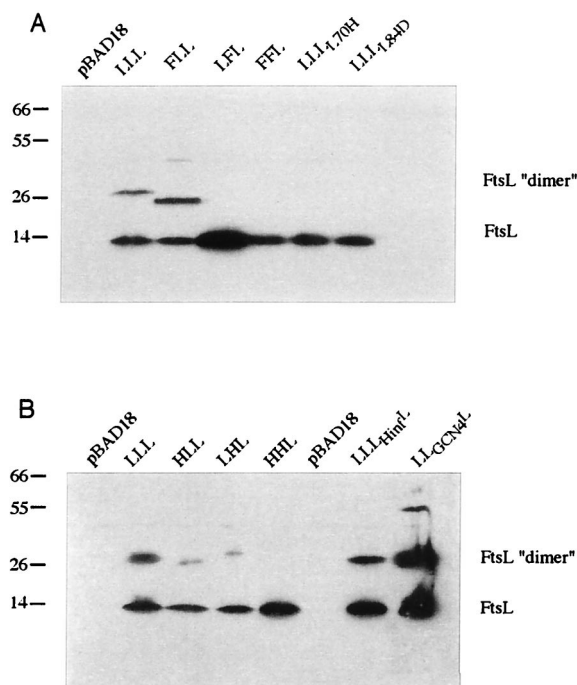


FIG. 6. Western blot of FtsL SDS-resistant multimers. The figure shows the multimerization state of FtsL swap proteins as determined by Western blotting with an anti-FtsL antibody. Molecular mass standards (kilodaltons) are indicated to the left of the blot, while the positions of FtsL monomers and dimers are shown to the right. The FtsL swap protein produced is indicated above each lane. Background strain used was KS272. Plasmids used were as follows: (A) control, pBAD18; LLL, pLD45; FLL, pLD63; LFL, pLD94; FFL, pLD90; LLL<sub>(L70H)</sub>, pJMG438; and LLL<sub>(L84D)</sub>, pJMG439; (B) control, pBAD18; LLL, pLD45; HLL, pJMG432; LHL, pJMG430; HHL, pJMG431; LL<sub>HinfL</sub>, pJMG427; and LL<sub>GCN4L</sub>, pJMG428.

analysis, we analyzed cell extracts from strains expressing LLL, HLL, LHL, and HHL swap proteins as well as LL<sub>HinfL</sub> and LL<sub>GCN4L</sub>. Figure 6B shows that LLL, HLL, LHL, and LL<sub>HinfL</sub> dimerize while HHL dimers could not consistently be observed (Fig. 6 and noted as +/- in Table 7). The levels of dimerization of HLL and LHL were found to be consistently lower than the one observed with FLL, although HLL and LHL were stable enough at the steady-state level. Samples from strains expressing LL<sub>HinfL</sub> displayed about the same ability to dimerize as LLL. The introduction of a well-characterized dimerization motif in LL<sub>GCN4L</sub> led to the efficient formation of dimers and even higher-order multimers (tetramers?) (Fig. 6). These results suggest that the FtsL<sub>Hinf</sub> membrane-spanning segment cannot fully replace its *E. coli* homologue with regard to the multimerization phenotype although it fully complements the localization phenotype. It also shows that multimerization alone is not sufficient to fulfill the FtsL role in cell division.

Taken together, these results are consistent with the hypothesis that both the transmembrane segment and the periplasmic domain of FtsL form a coiled-coil structure that could lead to multimerization. Since poorly dimerizing constructs still localize efficiently, multimerization may be a functionally critical event that could take place after FtsL localization to the division site.

## DISCUSSION

In this paper, we have analyzed the structure-function relationship of the essential cell division protein, FtsL. This analysis is facilitated by the simple domain structure of FtsL, a

bitopic membrane protein composed of an N-terminal cytoplasmic domain, a transmembrane segment, and a periplasmic domain. We have studied the role of these domains by swapping them or portions of them with other cell division proteins or with an unrelated protein. In these studies, we have examined the role of these different domains in three properties of FtsL. These properties are (i) the ability to form dimers or even multimers on SDS gels, (ii) the ability to localize to the site of formation of the cell septum, and (iii) the ability to provide an essential function in the cell division process. The results of the swap studies (summarized in Table 7) show that the three functions of FtsL are separable.

**Oligomerization of FtsL.** We have shown that, on SDS gels, a substantial proportion of FtsL is found in a band corresponding to dimers. We believe that this oligomerization is made possible by the apparent coiled-coil structure that the periplasmic domain, with its heptad repeats of leucines, may assume, perhaps along with the two other domains of the protein (see below). We find that dimerization is correlated with the presence of the intact *E. coli* FtsL periplasmic and transmembrane domains; the presence of the periplasmic domain alone is not sufficient. Furthermore, mutations in either of two leucines of the heptad repeats in the periplasmic domain reduce the efficiency of dimerization. However, dimerization per se is not sufficient for the two other properties of FtsL: localization to the septum and function in cell division. This we showed by providing an alternative dimerization signal in the periplasmic domain, the leucine zipper region of the *Saccharomyces cerevisiae* GCN4 protein. In this case, the protein still forms dimers on gels but is nonfunctional for the other properties of FtsL. Thus, specific sequences in the periplasmic domain are required for the localization process, beyond those required for dimerization.

TABLE 7. Localization frequencies, complementation, and dimerization phenotypes of some of the FtsL swap constructs described in this study

Construct	Localization frequency (%)	Complementation <sup>a</sup>	SDS-resistant dimers <sup>b</sup>
<b>Controls</b>			
FtsL	72	+++	++++
LLL	69	+++	+++
<b>MalF-<i>E. coli</i> FtsL swaps</b>			
FLL	70	-	+++
LFL	<1	-	-
FFL	<1	-	-
<b><i>H. influenzae-E. coli</i> FtsL swaps</b>			
LL <sub>HinfL</sub>	40	+/-	++
HLL	82	+	+
LHL	80	-	+/-
HHL	76	-	+/-
HLH	<1	-	-
LHH	<1	-	-
<b><i>E. coli</i> FtsL mutants</b>			
LLL <sub>(L70H)</sub>	17	+/-	+/-
LLL <sub>(L84D)</sub>	28	+/-	+/-
<b><i>E. coli</i> FtsL leucine zipper swap</b>			
LL <sub>GCN4L</sub>	<1	-	++++

<sup>a</sup> For the complementation test, plus signs reflect colony size, minus signs indicate no growth, and +/- indicates poor growth.

<sup>b</sup> The plus signs reflect the propensity to form SDS-resistant dimers. +/-, dimers were not consistently observed in all the experiments.

**Localization to the septum.** Localization of FtsL to the septal region of the *E. coli* cells also appears to be dependent on the presence of its periplasmic domain and on a transmembrane segment either from *E. coli* FtsL or from the *H. influenzae* homologue. The HLL, HHL, and LHL swap constructs all localize efficiently to the septum. Furthermore, the FLL construct, in which a cytoplasmic domain of the unrelated membrane protein, MalF, replaces the normal FtsL cytoplasmic domain, also localizes to the septum with a frequency comparable to that of the wild type. Thus, the cytoplasmic domain is needed neither for dimerization nor for localization. However, since the localization studies were done in a background that expresses wild-type FtsL from the chromosome, we cannot distinguish between the possibility that FLL localizes on its own to the septum and the possibility that it localizes via an interaction of the dimerization domain with an already localized wild-type FtsL.

The mutations altering the conserved leucines in the periplasmic domain reduce the efficiency of localization, as does the replacement of the heptad repeat region of FtsL with the comparable region from the *H. influenzae* FtsL. While the studies of dimerization suggested that not just any dimerization domain will allow proper localization, the results for localization indicate that not even a close relative of FtsL from *H. influenzae* provides enough of the specific sequence to the periplasmic domain that is required for efficient localization. The degree of specificity required in this case suggests that the periplasmic domain of FtsL may interact with another cell division protein that is not strongly conserved in sequence between bacterial species.

**Function in cell division.** Finally, even certain swap constructs that localize very efficiently to the cell septum do not provide the complete set of functions required to complement an FtsL null mutation. The constructs FLL, HHL, and LHL all localize to the septum with the same efficiency as wild-type FtsL, but none of them complement. The finding with the FLL swap protein indicates that the cytoplasmic domain of FtsL, which is not essential for dimerization or septal localization, is, nevertheless, required for function. Complementation by the HLL construct indicates that the *H. influenzae* cytoplasmic domain of FtsL is sufficiently close in structure to that of the *E. coli* protein, despite a low degree of sequence conservation, that it can function properly. This is somewhat surprising since the FtsL<sub>Hinf</sub> cytoplasmic domain is 14 residues shorter than the one of FtsL<sub>Ecol</sub>. Thus, the essential region of the cytoplasmic domain of FtsL<sub>Ecol</sub> may lie in the sequence that starts after residue 15 (Fig. 3). Two other gram-negative bacterial FtsL homologues, identified in *Y. pestis* and *B. aphidicola*, also display short cytoplasmic domains. Multiple alignments show that each of the three homologues has two conserved charged residues in common (R21 and D31 in FtsL<sub>Ecol</sub> [Fig. 5]) that may be good mutagenesis targets for identifying critical residues in the FtsL cytoplasmic domain.

In sum, all three domains of FtsL are required for its function in cell division, but they play different roles. The cytoplasmic domain appears to be needed for some step late in the functioning of FtsL, after it has arrived at the cell septum. The periplasmic domain is involved in dimerization, a step that may be required after localization can take place or a step that may be required to maintain a stably localized protein. The transmembrane segment of FtsL is required for complementation also; even the related membrane-spanning segment from the *H. influenzae* FtsL will not suffice for function. Again, such a finding suggests that the interactions of *E. coli* FtsL with other components of the cell division machinery require specific sequences of both the transmembrane and periplasmic domains.

Interestingly, *B. subtilis* DivIC protein stability was reported to depend on *B. subtilis* FtsL synthesis (7). The DivIC product is required for formation of the vegetative and sporulation septum (15) and bears a striking structural resemblance to *E. coli* FtsL, including a predicted coiled-coil periplasmic domain. Although there is no obvious DivIC homologue in *E. coli*, an attractive possibility would be that FtsL interacts in a complex containing a DivIC homologue.

**A potentially unbroken transmembrane and periplasmic coiled-coil motif is involved in localization to the division site.** Localization and complementation analysis as well as coiled-coil predictions is compatible with a helical structure prolonged throughout the membrane-spanning segment and the cytoplasmic domain. This raises the possibility that the FtsL coiled coil could run essentially unbroken through the membrane and be involved in the formation of multimers. FtsL SDS-resistant multimers of higher order than dimers can be observed in some conditions, suggesting that, at least in vitro, interaction of more than two molecules may take place. Whereas the presence in vivo of such multimers remains to be demonstrated, preliminary data involving in vivo cross-linking between cysteines introduced in the periplasmic domain (Leu63 to Cys and Leu70 to Cys) suggest that the observed SDS-resistant multimers are likely to be homomultimers (J. M. Ghigo, unpublished results).

What is the function of FtsL? Coiled-coil domains are found in transcription factors and fibrous and structural proteins as well as membrane fusion proteins. As pointed out before (8), based on its small size and the absence of recognizable catalytic domain, it seems unlikely that FtsL fulfills a biosynthetic function of its own. Rather, the results presented in this study are consistent with the hypothesis of a structural role for FtsL. Although there is limited information on the roles of proteins involved in septation in *E. coli*, so far, they often fall into two hypothetical categories: cytokinesis (i.e., FtsZ) and cell envelope biogenesis (i.e., FtsI). Since FtsL homologues are known in gram-positive bacteria, it is unlikely that FtsL functions in outer membrane biosynthesis or interaction. Interestingly, most cell division proteins known to act before FtsL, i.e., FtsZ, ZipA, FtsA, and FtsQ (8), have known homologues in gram-positive bacteria or have been shown to interact with FtsZ. An attractive possibility would then be that FtsL participates in a process that takes place in both gram-positive and gram-negative bacteria and that is related to cytokinetic aspects of septation.

FtsL, through the formation of densely packed multimers, may be involved in linking together the cytokinetic elements or in providing a membrane anchor to the FtsZ contractile ring. Such an anchor may help to resist the shearing forces developed during the contraction of the cytokinetic ring. Alternatively, since coiled-coil domains have been shown elsewhere to be involved in membrane interactions (26), an attractive hypothesis would be that the FtsL coiled coil could be playing a similar role in septation.

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