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Topological Characterization of the Essential *Escherichia coli* Cell Division Protein FtsN

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Genetic and biochemical approaches were used to analyze a topological model for FtsN, a 36-kDa protein with a putative transmembrane segment near the N terminus, and to ascertain the requirements of the putative cytoplasmic and membrane-spanning domains for the function of this protein. Analysis of FtsN-PhoA fusions revealed that the putative transmembrane segment of FtsN could act as a translocation signal. Protease accessibility studies of FtsN in spheroblasts and inverted membrane vesicles confirmed that FtsN had a simple bitopic topology with a short cytoplasmic amino terminus, a single membrane-spanning domain, and a large periplasmic carboxy terminus. To ascertain the functional requirements of the N-terminal segments of FtsN, various constructs were made. Deletion of the N-terminal cytoplasmic and membrane-spanning domains led to intracellular localization of the carboxy domain, instability, and loss of function. Replacement of the N-terminal cytoplasmic and membrane-spanning domains with a membrane-spanning domain from MalG restored subcellular localization and function. These N-terminal domains of FtsN could also be replaced by the cleavable MalE signal sequence with restoration of subcellular localization and function. It is concluded that the N-terminal, cytoplasmic, and transmembrane domains of FtsN are not required for function of the carboxy domain other than to transport it to the periplasm. FtsQ and FtsI were also analyzed.

The process of cell division in *Escherichia coli* involves the formation of a septum at the midpoint of the cell in coordination with DNA replication and segregation (14). The septum is formed by circumferential invagination of the cytoplasmic membrane accompanied by the synthesis of septal peptidoglycan and invagination of the outer membrane. At least six essential genes, *ftsA*, *ftsI*, *ftsL* (*mraR*), *ftsN*, *ftsQ*, and *ftsZ*, are required (10, 13, 17). Two of these, *ftsA* and *ftsZ*, encode products that are located in the cytoplasm but function at the cytoplasmic membrane. During cell division, FtsZ is located at the leading edge of the invaginating septum at the cytoplasmic surface of the inner membrane (2). FtsZ is a GTPase (11, 22, 25) that may function as a cytoskeletal element. It has been suggested that FtsZ self-assembles at the division site to orchestrate invagination of the septum (2, 20). Consistent with this possibility, FtsZ has been shown to assemble into filaments in vitro in the presence of GTP (6, 23). In cell fractionation studies, FtsA partitions between the cytoplasm and inner membrane and is thought to be a component of the septum (26, 27). Morphological evidence suggests that FtsA functions after FtsZ in cell division, although the ratio of FtsZ to FtsA is important for cell division to occur (9, 12). Although the biochemical activity of FtsA is unknown, sequence comparisons suggest that FtsA has an ATP binding site similar to that found in actin and DnaK (26).

The *ftsI* (*pbpB*), *ftsQ*, *ftsL*, and *ftsN* genes encode distinct gene products although they share several features. Each is a cytoplasmic membrane protein present in relatively low numbers (~20 to 50) per cell (4, 8, 10, 17). Of these proteins, only the biochemical activity of the *ftsI* gene product, PBP3, is known (18). It is a high-molecular-weight penicillin-binding protein with transglycosylase and transpeptidase activities required for septum-specific peptidoglycan biosynthesis. The biochemical activities of the other three Fts proteins are unknown. The *ftsN* gene was isolated as a multicopy suppressor of a temperature-sensitive *ftsA* mutation and was found to also suppress a temperature-sensitive *ftsI* mutation (10). Little is known about the activity of FtsQ or FtsL.

The products of the *ftsL*, *ftsQ*, and *ftsI* genes have been shown to have a simple bitopic membrane topology consisting of a short N-terminal cytoplasmic domain fused through a single transmembrane domain to a large C-terminal periplasmic domain (4, 8, 17). Although FtsN has not been as well characterized, cell fractionation studies revealed that FtsN is a membrane protein, and inspection of the amino acid sequence suggested that it also had a bitopic membrane topology (10). Proteins such as these Fts proteins may interact with FtsZ or FtsA through their N-terminal cytoplasmic tails. In this study, we examined the membrane topology of FtsN and examined the requirement and specificity of the N-terminal cytoplasmic and transmembrane domains for FtsN function.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains utilized in this study are all *E. coli* K-12 derivatives and were as follows: JKD41 [*ftsN*::*kan*(pKD123)] (10), DHB4 [F′ *lac pro lacI*^{q}/Δ(*ara leu*)*7697 araD139* Δ(*lac*)*X74 galE galK rpsL phoR* Δ(*phoA*) *Pvu*II Δ(*malF*)*3 thi*] (5), PR700 (Δ*malB*), and MC4100. The plasmids are listed in Table 1. pKD123 carries *ftsN* on a temperature-sensitive replicon and was described previously (10). The cloning in this study utilized two plasmids. pJF118HE is an expression vector containing the *lacI* gene, the *tac* promoter, and a multicloning region (16). pDH5730 contains a truncated *phoA* gene with restriction sites for introducing DNA fragments to generate fusions to *phoA* (5). pMal-c is a vector for construction and expression of *malE* fusions obtained from New England Biolabs. Bacteria were routinely grown in L broth or on L agar plates supplemented with 50 μg of thymine per μl and appropriate antibiotics as described previously (10).

Construction of *ftsN-phoA* fusions. Two *ftsN-phoA* fusions, A and B, were made by the method recommended by Dana Boyd (5). In this approach, PCR is used to make gene fusions with the junctions at predetermined positions. In our case, two DNA fragments with different end points in the 5′ end of *ftsN* were synthesized by PCR with pKD123 as the template and cloned into a vector containing a 5′-end-truncated *phoA*. Both fragments started 470 bp upstream of

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TABLE 1. Plasmids

Plasmid	Description	Source or reference
pKD123	pEL3 (temperature-sensitive replicon) containing *ftsN*	10
pKD2-5K	Contains *ftsN* with a *kan* cassette at codon 70	10
pJF118HE	Expression vector	16
pDHB5059	Intact *phoA* gene	5
pDHB5730	Vector for making fusions to *phoA*	5
pKD140	*ftsN*	10
pKD135	*ftsQ ftsA*	9
pKD146	*malE-ftsN* fusion made by cloning an *Eco*RI-*Sal*I fragment from pKD2-5K into pMal-c	This study
pKD180	*ftsI* cloned into pUC19	This study
pKD151	$ftsN^{1-28}$-*phoA* fusion A in pDHB5730	This study
pKD152	$ftsN^{1-55}$-*phoA* fusion B in pDHB5730	This study
pKD159-2	$malG^{1-3}$-$ftsN^{46-319}$ fusion in pJF118HE	This study
pKD159	$ftsN^{\Delta 4-45}$ in pJF118HE	This study
pKD157	$malG^{1-33}$-$ftsN^{46-319}$ fusion in pJF118HE	This study
pKD160	$malE^{1-26}$-$ftsN^{56-319}$ fusion in pJF118HE	This study
pKD161	$malG^{1-33}$-$ftsQ^{41-276}$ fusion in pJF118HE	This study
pKD162	$malG^{1-33}$-$ftsI^{37-588}$ fusion in pJF118HE	This study

ftsN, and one (fragment A) ended at codon 28 whereas the second (fragment B) ended at codon 55. Primer N/PhoA5′ was utilized for the 5′ ends of both fragment A and fragment B, and primers N/PhoA3′A and N/Pho3′B were used for the 3′ ends of fragments A and B, respectively. To facilitate cloning, an *Mlu*I site was incorporated in the 5′-end primer and a *Bsp*EI site was incorporated in the 3′-end primers. The two PCR products, A and B, were digested with *Mlu*I and *Bsp*EI, gel purified, and cloned into the *Mlu*I and *Bsp*EI sites of plasmid pDHB5730, resulting in pKD151 and pKD152, respectively. In these plasmids, the 5′ end of *ftsN* is fused in frame with the 3′ end of *phoA*. pKD151 should express a fusion protein with the cytoplasmic domain of FtsN fused to PhoA, whereas pKD152 should express a fusion protein with the cytoplasmic domain plus the putative transmembrane domain of FtsN fused to PhoA.

Construction of fusions of *fts* genes to *mal* genes. Fusions between various *mal* genes and *fts* genes were constructed by using a PCR strategy described by Zhao et al. (28). All fusion genes were then cloned in the expression vector pJF118HE. Three primers were used to synthesize each fusion in two steps as outlined below. One primer (primer A) was complementary to a sequence upstream of the 5′ end of gene A, another primer (primer B) was complementary to a sequence downstream of the 3′ end of gene B, and a third primer (primer A/B) was complementary to the junction of the desired fusion. Primers A and A/B were first used for the synthesis of the 5′ portion of a fusion by using a plasmid containing gene A as the template. For the second PCR step, primers A and B and the product of the first PCR were combined in a PCR mixture which contained a plasmid containing gene B as the template to produce the desired fusion. A *Hin*dIII site and an *Eco*RI site were incorporated in primer A and primer B, respectively, to facilitate cloning into the expression vector. The PCR products were cloned into the *Hin*dIII and *Eco*RI sites of pJF118HE. The templates used for PCR were pKD135 (*ftsQ*), pKD123 (*ftsN*), and pKD180 (*ftsI*).

Generation of antisera to FtsN. To obtain antisera to FtsN, a MalE-FtsN fusion protein was made and purified by using a kit obtained from New England Biolabs. The procedures described by the manufacturer were followed. To construct the plasmid for overexpression of the fusion protein, the *Eco*RI-*Sal*I fragment from pKD2-5K (10), which includes *ftsN* lacking its first 70 codons, was cloned into the *Eco*RI-Sal1 sites of pMAL-c. The fusion protein was overexpressed, purified, and sent to Cocalico Biologicals Inc. to raise an antiserum to FtsN in rabbits. Western blots (immunoblots) of cell extracts revealed two bands of similar molecular weights. One of these bands was determined to be the FtsN band, as described in Results. The second band was determined to be MalE, since it was not present in a strain (PR700) with the *malB* region deleted (data not shown).

Cell fractionation, proteinase K accessibility assay, AP assay, and Western blot. Cell fractionation was done according to the procedure of Ito et al. (19). The alkaline phosphatase (AP) assay was as described elsewhere (5). Preparation of spheroblasts and inverted vesicles and the proteinase K accessibility assay were carried out as described previously (7). Quantitative Western blotting was carried out as described previously (1).

RESULTS

Analysis of AP fusions to FtsN. FtsN has a stretch of 27 hydrophobic amino acid residues located near its N terminus flanked by several positively charged amino acids. On the basis of this sequence characteristic and the observation that FtsN expressed in minicells is located in the membrane fraction, we suggested that FtsN was a cytoplasmic membrane protein with a bitopic membrane topology (Fig. 1) similar to that of FtsQ, FtsL, and FtsI (10). To test this model, AP (PhoA) fusions, which have been widely employed to study membrane protein topology (21), were constructed. Two FtsN-PhoA fusions, designated A and B (Fig. 1), were constructed as described in Materials and Methods by using a strategy described by Boyd et al. (5). Fusion A, contained on pKD151, has PhoA without its N-terminal signal sequence fused to FtsN after the 28th amino acid residue immediately before the hydrophobic segment. Fusion B, contained on pKD152, has the truncated PhoA joined to FtsN after the 55th amino acid residue just after the hydrophobic segment. pKD151, pKD152, and control plasmids were introduced into the *phoA* deletion strain DHB4 containing p184*lacI*^{q} (to supply sufficient *lac* repressor) and the AP activity was measured (Fig. 2). To determine the relative specific activities of the fusion proteins, they were quantitated by immunoblotting with anti-PhoA antiserum (Fig. 2). DHB4 containing pKD151 carrying fusion A gave a relative specific AP activity of 2 U, while DHB4 carrying pKD152 expressing

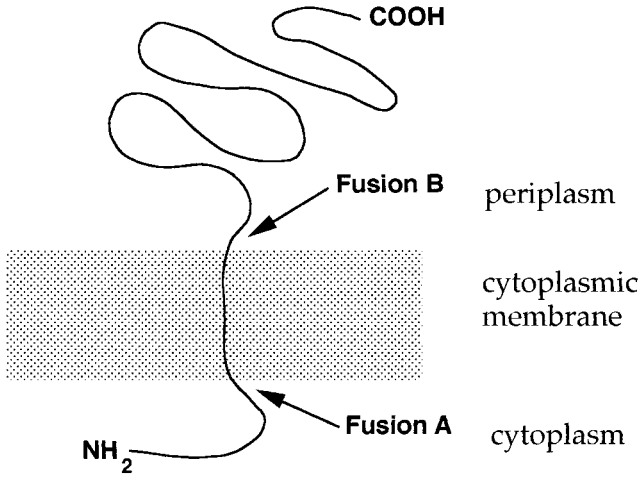


FIG. 1. Topological model of FtsN and junctions of PhoA fusions. This diagram illustrates the topological model proposed previously for FtsN, which was confirmed by the data obtained in this study. Two PhoA fusions (A and B) were constructed with the N-terminally truncated PhoA added at the indicated positions.

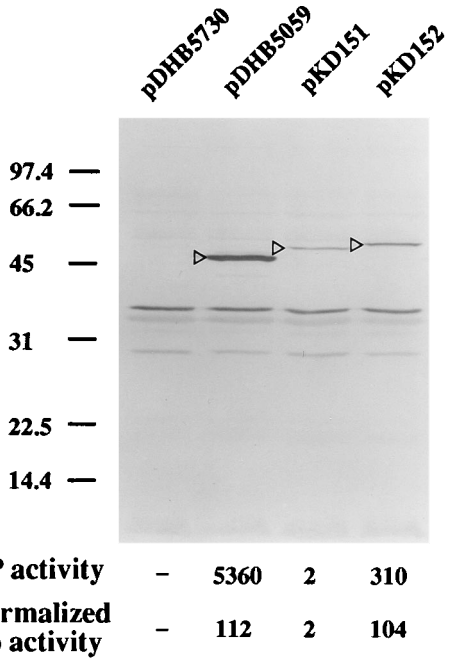


FIG. 2. Characterization of FtsN-PhoA fusions. Equivalent quantities of cells of DHB4 containing the plasmids indicated were isolated by centrifugation, lysed in sodium dodecyl sulfate (SDS) sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and immunoblotted with antibody to PhoA. The positions of PhoA (encoded by pDHB5059), fusion A (encoded by pKD151), and fusion B (encoded by pKD152) are indicated by the open arrowheads. AP activity (shown in units) was determined for each culture and normalized by determining the relative amount of PhoA or PhoA fusion protein in each sample following quantitative immunoblotting as described in Materials and Methods.

fusion B gave a much higher specific AP activity of 104 U. This specific activity is comparable to that of the wild-type PhoA protein, encoded by the control plasmid pDHB5059, indicating that the PhoA portion of the fusion was efficiently translocated to the periplasm. These results indicated that the N-terminal portion of FtsN present in fusion B is sufficient to mediate the translocation of PhoA into the periplasmic space, and the failure of fusion A to do so argues that the hydrophobic segment is essential for this process.

Protease accessibility of FtsN in spheroblasts and inverted membrane vesicles. In order to directly examine the topology of the FtsN protein, an antiserum to a MalE-FtsN fusion protein was generated as described in Materials and Methods. Thus, this antiserum should recognize both FtsN and MalE. Immunoblot analysis of a total cell lysate from JKD41(pKD123) revealed two bands with molecular weight of 38,000 and 36,000, as expected for MalE and FtsN, respectively (Fig. 3, first lane). To confirm that one of these proteins was FtsN, a culture of JKD41(pKD123) was shifted to 42°C and subsequent samples were analyzed by immunoblotting. JKD41(pKD123) has the chromosomal *ftsN* gene disrupted by a *kan* insertion and wild-type *ftsN* supplied by a temperature-sensitive replicon, pKD123. Following a shift to 42°C, the intensity of the band corresponding to FtsN should decrease since replication of the plasmid is blocked and the FtsN protein will be diluted by continued cell growth. As expected, the intensity of the lower-molecular-weight band decreased with incubation time at 42°C whereas the intensity of the higher-molecular-weight band was constant (Fig. 3). This result confirmed that the lower-molecular-weight band was FtsN. The absence of the higher-molecular-weight

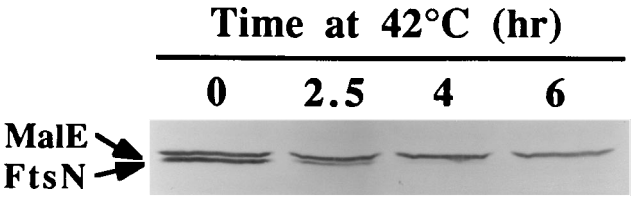


FIG. 3. Antisera recognize FtsN and MalE. A culture of JKD41(pKD123) growing exponentially in L broth at 30°C was shifted to 42°C at zero time. Samples were taken at the indicated times and adjusted so that equivalent amounts of optical density units were loaded in all lanes. The samples were analyzed for FtsN and MalE content by immunoblot analysis with antisera raised against a MalE-FtsN fusion protein.

band in a Δ*malB* strain confirmed that it was MalE (data not shown).

Previously (10), we had demonstrated that FtsN expressed in minicells was membrane bound. The localization of FtsN in cells of the wild-type strain MC4100 was examined by cell fractionation and immunoblotting. FtsN was detected in the inner membrane fraction but not the outer membrane fraction, confirming that FtsN is a cytoplasmic membrane protein (data not shown). To determine if FtsN had the membrane topology indicated by the AP fusions, proteinase K accessibility analysis was performed with spheroblasts and inverted membrane vesicles prepared from MC4100. According to the topological model (Fig. 1), treatment of spheroblasts with protease should result in almost complete degradation of FtsN whereas treatment of inverted vesicles should remove only about a 3-kDa, N-terminal fragment; the remainder of FtsN should be inside the vesicle and be protected. When spheroblasts were treated with proteinase K and analyzed by immunoblotting (Fig. 4A), the band corresponding to FtsN disappeared, indicating that the majority of FtsN was accessible to protease. As a control, the blot was simultaneously probed with antibody to the cyto-

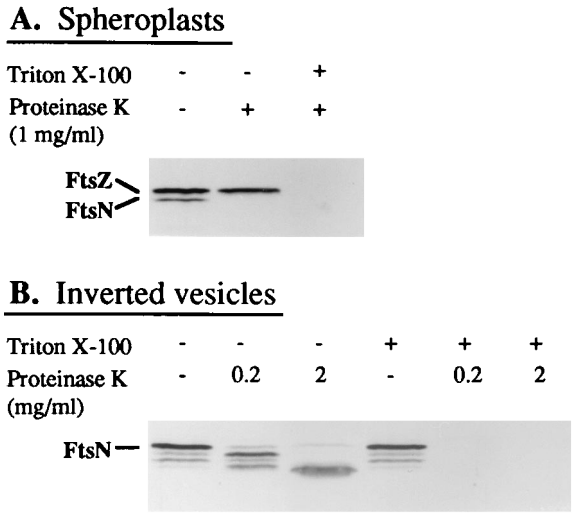


FIG. 4. Protease accessibility of FtsN in spheroblasts and inverted vesicles. (A) Spheroblasts of MC4100 grown in L broth were treated with proteinase K and Triton X-100 as indicated. After treatment, samples were analyzed by immunoblotting with antisera raised against the MalE-FtsN fusion protein and antisera to FtsZ. The positions of FtsZ and FtsN are indicated. (B) Inverted vesicles of MC4100 grown in L broth were treated with proteinase K and Triton X-100 as indicated. Following treatment, the samples were analyzed by immunoblotting with antisera raised against the MalE-FtsN fusion protein and the position of full-length FtsN is indicated. +, present; −, absent.

plasmic protein FtsZ. In contrast to FtsN, FtsZ was protected by the cytoplasmic membrane and remained intact. Upon inclusion of Triton X-100 to solubilize the membrane, FtsZ was degraded. When the inverted membrane vesicles were treated with proteinase K, the size of the FtsN band decreased by about 3 kDa, as expected (Fig. 4B). Inclusion of Triton X-100 led to complete degradation of FtsN, indicating that the membrane was responsible for protecting most of FtsN from proteolysis. On the basis of these results and the PhoA fusion studies, it is concluded that the topological model for FtsN presented in Fig. 1 is correct.

Translocation of FtsN is required for its function. To determine if the localization of FtsN to the cytoplasmic membrane is required for its function, a deletion of the hydrophobic segment was constructed. This construct, *ftsNΔ1*, whose product had most of the N terminus, including the hydrophobic signal sequence, removed, was cloned into pJF118HE downstream of the *tac* promoter to give pKD159. This plasmid could not complement the *ftsN* conditional null mutant, JKD41 (pKD123), either in the presence or in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). However, the mutant protein could not be detected even in the presence of IPTG, indicating that the deletion protein was quite unstable (data not shown). To increase expression of FtsN lacking the hydrophobic segment, the region encoding the periplasmic domain was fused in frame to the third codon of MalG. This fusion protein, FtsNΔ (Fig. 5), should be translated much more efficiently by employing the start codon and ribosome binding site provided by the *malG* gene. pKD159-2, expressing this fusion protein, still failed to complement JKD41(pKD123), although the level of the mutant protein, even in the absence of IPTG, was higher than that of the wild-type FtsN (Fig. 6). Cell fractionation confirmed that the mutant protein was in the cytoplasm (Fig. 6). These results strongly indicate that localization of FtsN in the membrane was required for its function.

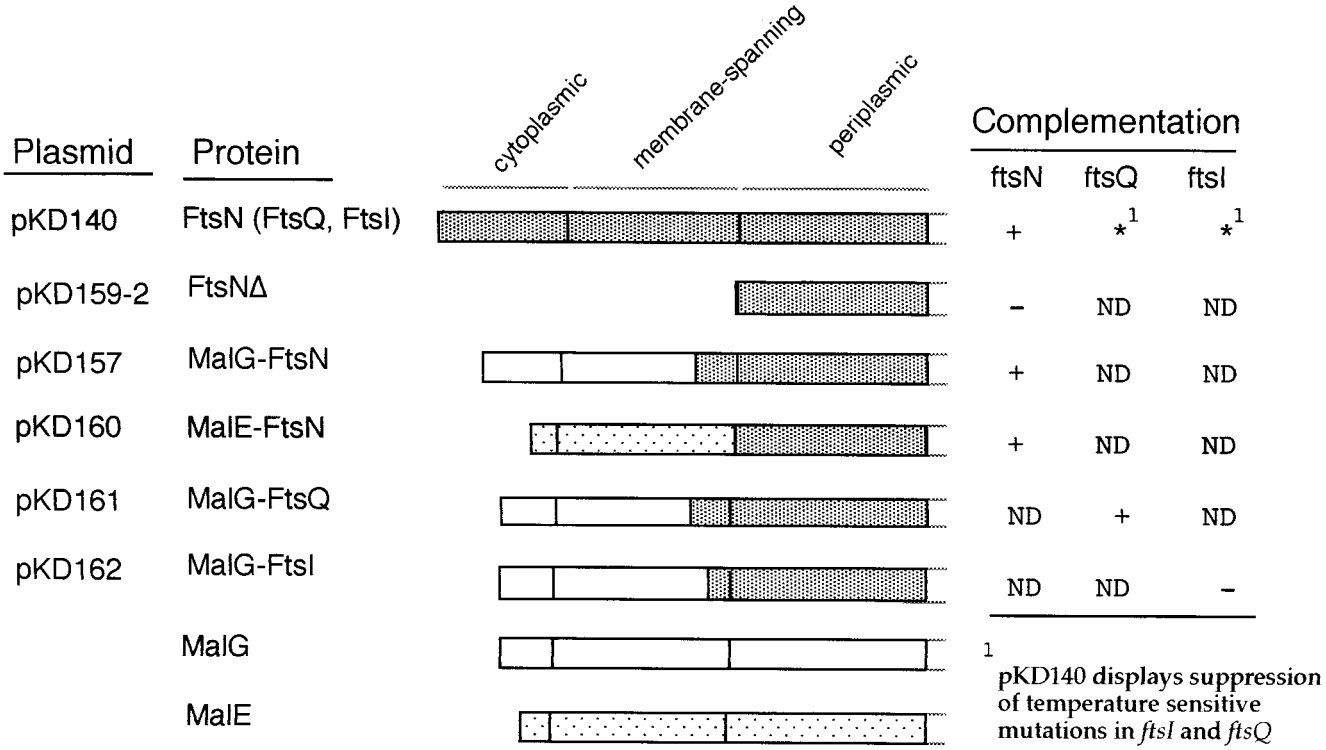
The N-terminal cytoplasmic and membrane-spanning domains of FtsN and FtsQ can be replaced by the analogous segments from an integral membrane protein. Like FtsN, several other cell division proteins, FtsQ, FtsL, and FtsI, have simple bitopic membrane topologies. It has been speculated that the N-terminal cytoplasmic domains of these proteins might play critical roles by sensing signals in the cytoplasm to coordinate the circumferential invagination of the septum (8, 17, 20). To test this possibility, we replaced the N-terminal portions of FtsN, FtsQ, and FtsI that contained the small cytoplasmic domain and most of the membrane-spanning domain with the N-terminal sequence of MalG. MalG is a component of the maltose transport system and is an integral inner membrane protein with multiple membrane-spanning segments (5). An N-terminal segment of MalG, including the first membrane-spanning domain, was used to replace the N-terminal sequences of FtsN, FtsQ, and FtsI (Fig. 5). It was expected that the membrane-spanning segment of MalG would allow these fusion proteins to attain a bitopic membrane topology similar to those of the wild-type proteins. If the N-terminal domains of FtsN, FtsQ, or FtsI play a critical role(s) other than mediating translocation, these fusion proteins would lose their function despite assuming the proper topology. On the other hand, if these N-terminal domains specify only topological information, they should be interchangeable and the MalG fusions should retain function. Functionality was assessed by complementation of the corresponding mutants (Fig. 5). The MalG-FtsN (pKD157) and the MalG-FtsQ (pKD161) fusion proteins were functional since plasmids expressing these proteins could complement the *ftsN* null mutation and *ftsQ1*(Ts), respectively. In contrast, the MalG-FtsI (pKD162) fusion protein appeared to have lost function as it no longer complemented *ftsI23*(Ts).

The bitopic membrane topology of the MalG-FtsN fusion protein was confirmed by the demonstration that the fusion protein was present in the membrane fraction (Fig. 6, first three lanes) and was accessible to proteinase K in spheroblasts (Fig. 7, leftmost gel). Although the levels of the fusion proteins were higher than the normal physiological level of FtsN (Fig. 6, leftmost lane) and a possible lack of full activity of these fusion proteins might be compensated for by the higher levels, the results indicate that the cytoplasmic and transmembrane domains of FtsN do not play an additional essential role in the function of this protein. From our results, it appears that the same is true for FtsQ, since *ftsQ1*(Ts) is complemented by the MalG-FtsN fusion. Although it would appear that the cytoplasmic and transmembrane domains of FtsI are not replaceable, we could not draw a firm conclusion because we did not have FtsI antibody to confirm stability and transmembrane topology of the MalG-FtsI fusion protein.

The FtsN membrane-spanning segment can be replaced by the cleavable MalE signal sequence. From the above results, it appeared that as long as FtsN and FtsQ were inserted into the cytoplasmic membrane and had the proper topology, they remained active. We next tested if the periplasmic domain of FtsN must be anchored to the membrane in order for it to be functional. To do this, the periplasmic domain of FtsN was fused to the signal sequence of MalE. MalE is a periplasmic protein whose signal sequence is cleaved during translocation to the periplasm. If the MalE signal sequence functioned properly, the periplasmic domain of the MalE-FtsN (pKD160) fusion should be translocated to the periplasm and the MalE signal sequence should be cleaved during translocation. Immunoblot analysis detected both the fusion protein and the processed form of the fusion protein in the total cell lysate (Fig. 6, seventh lane). The identification of the lower-molecular-weight band as the processed form is consistent with its location primarily in the soluble fraction (Fig. 6, eighth and ninth lanes) and its accessibility to proteinase K in spheroblasts (Fig. 7, rightmost gel). pKD160 carrying the *malE-ftsN* fusion was then tested for its ability to complement JKD41(pKD123) containing the null allele of *ftsN* on the chromosome and wild-type *ftsN* on a temperature-sensitive replicon. pKD160 allowed JKD41 to grow at a high temperature and was able to replace pKD123. This strongly suggested that the cytoplasmic and the transmembrane domains of FtsN are not essential for cell survival and that the periplasmic domain of FtsN need not be anchored to the membrane to be functional. However, it should be noted that this form of FtsN is expressed at a higher level than is wild-type FtsN, and is possible that this may compensate for loss of membrane attachment. Also, not all of the fusion protein is processed (more-complete processing was obtained in other experiments), and it is possible that this form is the active form. Nonetheless, the ability of the MalE-FtsN fusion to complement the *ftsN* null allele confirms that the N-terminal cytoplasmic and membrane-spanning domains of FtsN are only needed for proper cellular localization of FtsN's periplasmic domain.

DISCUSSION

The *ftsN* gene was isolated as a multicopy suppressor of the *ftsA12* temperature-sensitive mutation (10). Multicopy *ftsN* also suppressed the *ftsI23* temperature-sensitive mutation, although it did not suppress *ftsZ84*(Ts) or a variety of *dna*(Ts) mutations. Since depletion of FtsN led to filamentation without a noticeable effect on DNA segregation, it was concluded



FtsN **MAQRDYVRRSQPAPSRRKKSTSKKQRNLPAVSPAMVAIAAAVLVTFIGGLYITHHKKEE**

FtsN MAM**HHKKEE**

MalG-FtsN MAMVQPKSQLARLFITHLLLLLFIAAIMFPLLM**TFIGGLYFITHHKKEE**

MalE-FtsN MLILTGARILALSALTTMMFSASALA**HHKKIEE**

FtsQ **MSQAALNTRNSEEEVSSRRQQGTRLAGILFLLTVLTTVLVSGWVVLGWMEDAQRLP**

MalG-FtsQ MAMVQPKSQLARLFITHLLLLLFIAAIMFPLLM**SGWVVLGWMEDAQRLP**

FtsI **MKAAAKTQDPKRQGGHANFISWRFALLCGCILLALAFLLGRVAWLQ**

MalG-FtsI MAMVQPKSQLARLFITHLLLLLFIAAIMFPLLM**FLLGRVAWLQ**

FIG. 5. Diagram and N-terminal sequence of the fusions used in this study. FtsN, FtsQ, and FtsI all have the same organization, with an N-terminal cytoplasmic domain fused to a larger periplasmic domain by a hydrophobic membrane-spanning segment. Although MalG is an integral membrane protein, the N-terminal region has an organization similar to that of the Fts proteins, with the extreme N terminus in the cytoplasm followed by a membrane-spanning domain. MalE also has similar organization; however, its membrane-spanning segment is cleaved during transport. The following fusions were made: in FtsNΔ, the ribosome binding site and first three codons of *malG* were fused to the periplasmic domain of FtsN; in MalG-FtsN, the 5′ end of *malG* was fused to *ftsN* with the junction occurring within the membrane-spanning segments; in MalE-FtsN, the 5′ end of *malE* was fused to *ftsN* with the junction occurring within the membrane-spanning segments; in MalG-FtsQ, the 5′ end of *malG* was fused to *ftsQ* with the junction occurring in the membrane-spanning segments; and in MalG-FtsI, the 5′ end of *malG* was fused to *ftsI* with the junction occurring in the membrane-spanning segments. The amino acid sequences of these fusions are shown, with amino acids from the Fts proteins in larger, boldface type and amino acids from the Mal proteins in regular type. The membrane-spanning segments are underlined.

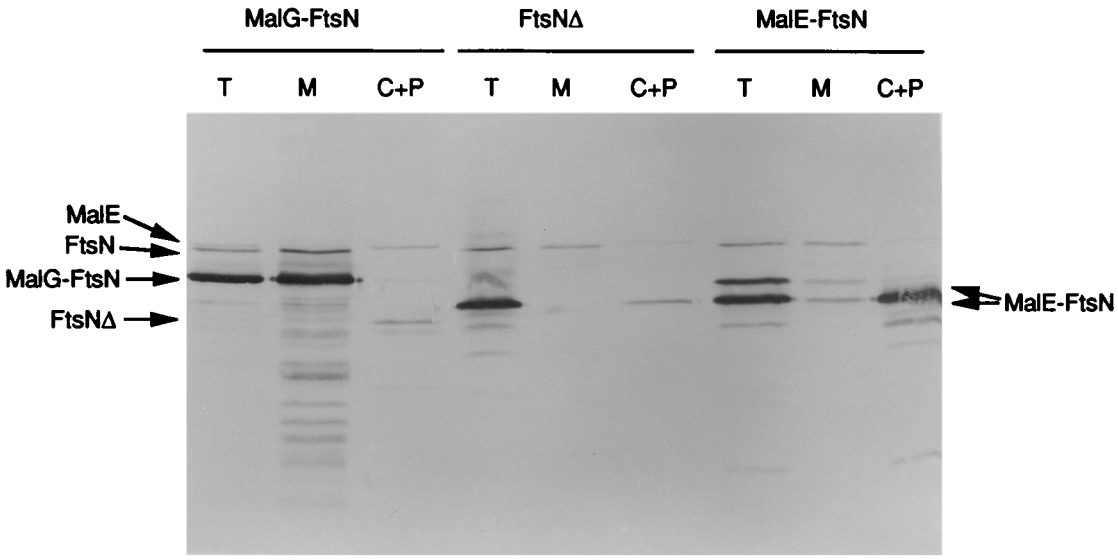


FIG. 6. Expression and cellular location of the FtsN fusions used in this study. MC4100 containing the plasmids indicated was grown in L broth supplemented with ampicillin. Cells from exponentially growing cultures were collected, and a sample was taken (T [total cell]). The remaining cells were lysed and fractionated into a high-speed pellet (M [membrane fraction]) and supernatant (C+P [cytoplasmic plus periplasmic fractions]). The samples were then analyzed by immunoblotting.

that *ftsN* was an essential cell division gene. Furthermore, FtsN was localized to the membrane fraction, and the hydropathy profile of FtsN suggested that it had a simple bitopic membrane topology. This topology was confirmed in this study.

Fusions constructed between FtsN and an N-terminally truncated AP confirmed that FtsN's single, long hydrophobic segment could act as an export signal. In addition, protease accessibility studies confirmed the postulated topological model for FtsN. Protease treatment of spheroblasts resulted in complete degradation of FtsN, whereas treatment of inverted vesicles reduced the size of FtsN by approximately 3,000 Da. This reduction in size is expected if the N-terminal segment that precedes the hydrophobic segment was removed and the remainder of the protein was protected by the cytoplasmic membrane. This interpretation, as opposed to the hypothesis of a protease-resistant fragment, is supported by the complete degradation of FtsN in the presence of Triton X-100. Thus, FtsN is anchored to the membrane by a noncleavable, hydrophobic segment that functions to transport the large carboxy segment to the periplasm.

Having confirmed the topological model for FtsN, we wished to determine if the N-terminal segments of FtsN were required for function. The short N-terminal, cytoplasmic domain of FtsN consists of 28 amino acids of which 10 are positively charged at neutral pH. This high charge density, along with the length of this domain (it is relatively long in comparison with the majority of exported proteins), led us to suspect that it might be required for FtsN function. However, replacement of this region and the transmembrane domain of FtsN with the corresponding segments of MalG resulted in a MalG-FtsN fusion that was properly localized and retained FtsN function. This fusion contains 17 amino acids preceding the hydrophobic domain, only 3 of which are positively charged at neutral pH. In addition, replacement of this region of FtsN with the MalE cleavable signal sequence resulted in a fusion protein with FtsN function, despite the majority of the fusion protein being processed. These results indicate that the N-terminal cytoplasmic and transmembrane domains of FtsN do not have unique functions but are only required to transport the carboxy domain to the periplasm. One caveat is that the fusion proteins are expressed at higher levels than is FtsN, and it is possible that this may overcome a lack of efficiency conferred by the missing N-terminal fragment.

In addition to FtsN fusions, we also analyzed fusions between MalG and both FtsQ and FtsI. A MalG-FtsQ fusion was able to complement an *ftsQ1*(Ts) mutant, whereas a MalG-FtsI fusion was unable to complement *ftsI23*(Ts). These results indicate that the N-terminal cytoplasmic and hydrophobic domains of FtsQ are dispensable whereas the corresponding domains of FtsI are not.

During cell division, the cytoplasmic membrane has to invaginate and peptidoglycan synthesis must be switched from an elongation to a septal mode (24). Failure of either of these events would result in division inhibition. Several cell division

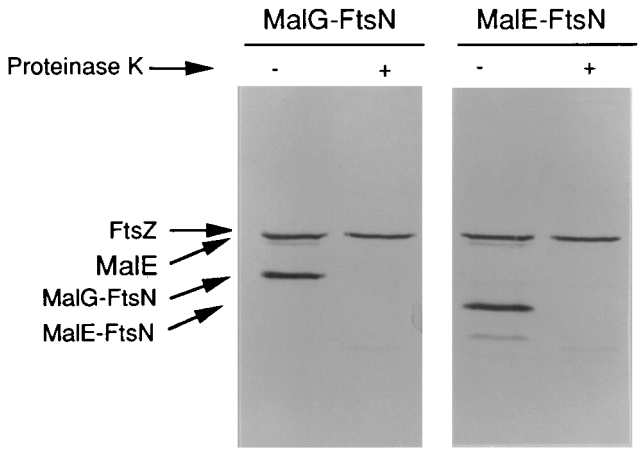


FIG. 7. Protease accessibility of FtsN derivatives in spheroblasts. Spheroblasts containing the MalG-FtsN fusion and the MalE-FtsN fusion were treated with proteinase K and analyzed by immunoblotting with antisera raised against the MalE-FtsN fusion. As a control, the samples were simultaneously immunoblotted with antibodies to the cytoplasmic FtsZ protein. −, absent; +, present.

genes identified to date are directly or indirectly implicated in septal peptidoglycan synthesis (3, 13). It has been proposed that at least some of these cell division gene products form a division complex at the leading edge of the invaginating septum which is responsible for directing and carrying out septum-specific peptidoglycan synthesis (24). One of the components of such a putative complex is likely to be FtsZ, a key cell division protein that is organized into a ring pattern at the leading edge of the septum. The FtsZ ring could function as a cytoskeletal element to mediate septal invagination. In this role it could act as an anchor for other possible components of the division machinery, including the products of *ftsA*, *ftsI*, *ftsQ*, *ftsL*, and *ftsN*. However, our results demonstrating that the cytoplasmic and transmembrane segments of FtsN can be replaced by analogous segments of other membrane or secreted proteins would rule out a model in which these segments bound directly to FtsZ. An alternative possibility is that FtsZ plays an indirect role by initiating septation and providing a topologically restricted substrate. This type of model for topologically restricting FtsN function would be consistent with the observation that a 50-fold excess of FtsN has little effect on cellular morphology (10). The same might be true for FtsQ.

The recent sequence analysis of the *Haemophilus influenzae* Rd genome revealed a homolog of the *ftsN* gene designated *msgA* (15). MsgA consists of 204 amino acids and is homologous (21% identity) to the carboxy-terminal domain of FtsN. Interestingly, the MsgA homolog lacks a signal sequence and an N-terminal cytoplasmic domain. However, inspection of the sequence upstream of the selected initiation codon for *msgA* revealed an in-frame stretch of codons that would encode a signal sequence consisting of 20 hydrophobic residues. This region is preceded by another stretch of codons that would encode a sequence rich in positively charged amino acids similar to that found at the N terminus of FtsN. Two potential intiation codons are present. One (TTG) is in frame and is at the start of the putative signal sequence, and a second (GTG) is out of frame just upstream of the region encoding the positively charged region. On the basis of our analysis of FtsN localization and functionality in *E. coli*, we would suggest that for *msgA* to be a functional homolog of *ftsN* one of the above initiation codons is probably correct and that MsgA from *H. influenzae* also contains a signal sequence.

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