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# Interaction Network among *Escherichia coli* Membrane Proteins Involved in Cell Division as Revealed by Bacterial Two-Hybrid Analysis

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**Formation of the *Escherichia coli* division septum is catalyzed by a number of essential proteins (named Fts) that assemble into a ring-like structure at the future division site. Several of these Fts proteins are intrinsic transmembrane proteins whose functions are largely unknown. Although these proteins appear to be recruited to the division site in a hierarchical order, the molecular interactions underlying the assembly of the cell division machinery remain mostly unspecified. In the present study, we used a bacterial two-hybrid system based on interaction-mediated reconstitution of a cyclic AMP (cAMP) signaling cascade to unravel the molecular basis of septum assembly by analyzing the protein interaction network among *E. coli* cell division proteins. Our results indicate that the Fts proteins are connected to one another through multiple interactions. A deletion mapping analysis carried out with two of these proteins, FtsQ and FtsI, revealed that different regions of the polypeptides are involved in their associations with their partners. Furthermore, we showed that the association between two Fts hybrid proteins could be modulated by the coexpression of a third Fts partner. Altogether, these data suggest that the cell division machinery assembly is driven by the cooperative association among the different Fts proteins to form a dynamic multiprotein structure at the septum site. In addition, our study shows that the cAMP-based two-hybrid system is particularly appropriate for analyzing molecular interactions between membrane proteins.**

In *Escherichia coli*, the cell division process, also referred to as cytokinesis, constriction, or septation, is one of the most central yet poorly understood aspects of the bacterial physiology (for reviews, see references 5, 33, and 45). The event takes place at the midcell and starts after the bacterial chromosomal DNA has been duplicated and segregated into two daughter nucleoids. Cell division genes, named *fts*, have been identified mainly through conditional mutants that form long filamentous cells at nonpermissive temperatures (4, 5). At present, at least fourteen proteins are known to be specifically required for the *E. coli* cell septation (for reviews, see references 1, 5, 33, 42, and 45). The majority of the Fts proteins are anchored to the cell membrane, and most of them appear to localize to the bacterial septum in a sequential order (for reviews, see references 5, 33, 35, and 40). Fluorescence microscopy studies using immunofluorescence or the green fluorescent protein (GFP) fused to the Fts proteins have revealed that assembly of the septum starts with the positioning of an FtsZ ring in the cell center. The FtsZ ring is stabilized by FtsA and ZipA, which localize to the septum independently of each other but only in the presence of the FtsZ protein. FtsQ follows FtsK, whose localization requires both FtsA and ZipA proteins, in this hierarchical assembly. Then FtsL, FtsB, FtsW, FtsI, FtsN, and AmiC are successively recruited to the FtsZ ring (for reviews, see references 1 and 5). Recently, Schmidt et al. (42) showed that two proteins, FtsE and FtsX, could localize to the septum site in an FtsZ-, FtsA-, and ZipA-dependent manner. The position of EnvC in the sequential pathway is not yet established (2).

The hierarchical appearance of the Fts proteins at the septum site suggests potential protein-protein interactions between the *E. coli* division proteins. Deciphering these interactions is an essential step in understanding the role(s) of these different proteins in the cytokinesis process. Direct associations between *E. coli* cell division proteins have been demonstrated for FtsZ, FtsA, and ZipA. FtsZ is able to polymerize to form a ring at the cell center (3, 38), and the C-terminal cytosolic domain of FtsZ has been shown to associate with ZipA and FtsA (24, 26, 32, 34, 44). Moreover, FtsA is able to dimerize (9, 46). Characterization of the interactions involving the other Fts proteins has been limited, probably because these proteins are membrane bound and some of them are expressed at very low levels. Yet, recently, using an immunoprecipitation technique, Buddelmeijer and Beckwith have succeeded in demonstrating that FtsQ, FtsL, and FtsB can form a multimeric protein complex (6). In addition, Di Lallo and coauthors have used an in vivo approach to detect potential protein-protein interactions between different *E. coli* Fts proteins (18). By using a two-hybrid assay based on the formation of chimeric transcriptional repressors, they were able to confirm well-documented interactions such as the FtsZ dimerization-oligomerization, the association of FtsZ with FtsA, and the FtsA dimerization. They also suggested numerous additional associations between various cell division proteins (18).

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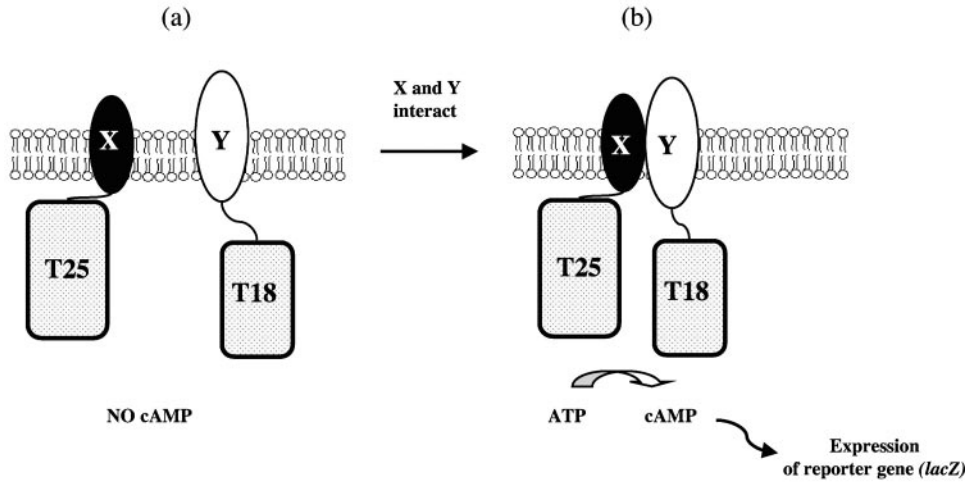


FIG. 1. Detection of membrane protein associations with the BACTH system. (a) Proteins of interest X and Y are genetically fused to the two complementary fragments, T25 and T18, from the catalytic domain of *B. pertussis* AC and coexpressed in *E. coli cya* cells. (b) Interaction between the two hybrid proteins results in functional complementation between the T25 and T18 fragments, leading to cAMP synthesis. cAMP, upon binding to the catabolite activator protein (a transcriptional regulator) triggers the expression of *E. coli* catabolic operons, allowing the bacteria to utilize sugars such as lactose and maltose. Efficiencies of complementation can be determined by measuring β-galactosidase activities in the transformed cells.

In this work, we attempted to characterize the interactions between the *E. coli* proteins involved in the cell division machinery by using a different bacterial two-hybrid system, the bacterial adenylate cyclase two-hybrid (BACTH) system, which is based on the interaction-mediated reconstruction of a cyclic AMP (cAMP) signaling cascade (29). In this assay, the proteins of interest are genetically fused to two fragments (T25 and T18) of the catalytic domain of *Bordetella pertussis* adenylate cyclase (AC) and coexpressed in an *E. coli cya* strain (i.e., a strain deficient in endogenous AC). Interaction of the two hybrid proteins results in a functional complementation between the T25 and T18 fragments, leading to cAMP synthesis and in turn to transcriptional activation of catabolic operons (such as the lactose operon and the maltose regulon). Importantly, as the BACTH assay involves a cAMP signaling cascade, the interaction between the hybrid proteins does not need to take place near the transcription machinery as is the case with yeast or other bacterial two-hybrid systems (17, 19, 21, 27, 31). For this reason, the BACTH system seems to be particularly appropriate for studying interactions among membrane proteins (Fig. 1).

Our present data, besides confirming most of the previously established interactions between various *E. coli* Fts proteins, reveal several novel associations between these proteins, mediated by distinct polypeptide regions of each partner. Furthermore, we show that the interaction between two Fts hybrid proteins could be modulated by the coexpression of a third Fts partner. Altogether, these results suggest that the assembly of the cell division machinery is driven by the cooperative association among the different Fts proteins to form a dynamic multiprotein structure at the septum site.

## MATERIALS AND METHODS

**Bacterial strains and growth media.** The *E. coli* K-12 strain XL1-Blue (Stratagene) was used in all of the cloning steps. BACTH complementation assays were carried out with the *E. coli cya* strain DHM1 [$F^-$ *glnV44*(AS) *recA1 endA gyrA96 thi-1 hsdR17 spoT1 rfbD1 cya-854*], which carries a 200-bp deletion within the *cya* gene. DHM1 was constructed by P1 transduction of the wild-type *ilv* locus from strain FB8 into strain DHT1 (16) by selection on minimal medium M63 supplemented with glucose and vitamin $B_1$ (37). To enable P1 transduction, DHT1 was transiently made *recA*$^+$ as described previously (28). To ensure the presence of the mutated *cya* locus, $Ilv^+$, transposon-free ($Tet^s$) clones were then reisolated on MacConkey plates containing 1% maltose.

Bacteria were grown at 30°C in Luria-Bertani (LB) broth supplemented with ampicillin at 100 μg/ml and kanamycin at 50 μg/ml as needed. For cloning steps, glucose (0.4%) was added to the growth medium to decrease the expression of Fts hybrid proteins. Screening for the ability to utilize sugar was performed on LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml), isopropyl-β-D-galactopyranoside (IPTG; 0.5 mM), and appropriate antibiotics.

**Plasmid constructions.** Standard protocols for molecular cloning, PCR, DNA analysis, and transformation were used (41). PCR was performed with DyNAzyme EXT polymerase from Finnzymes (Espoo, Finland). Oligonucleotides were from MWG Biotech (Ebersberg, Germany). DNA fragments were sequenced by the GENOME Express company (Meylan, France).

To construct the recombinant plasmids used in the BACTH complementation assays, the genes coding for the different Fts proteins (or their subdomains) were PCR amplified by using appropriate primers (DNA sequences available upon request) and the genomic DNA from *E. coli* K-12 MG1665 as a template. Briefly, amplified DNA fragments were digested with BamHI and KpnI (for *ftsB*, *ftsL*, *ftsN*, *ftsW*, and *ftsX*), with BamHI and EcoRI (for *ftsA* and *ftsI*), or with XbaI and EcoRI (for *ftsQ*) and subcloned into the corresponding sites of the pKT25 and pUT18C vectors (30). The resulting recombinant plasmids expressed hybrid proteins in which the polypeptides of interest were fused to the C termini of the T25 and T18 fragments of AC, respectively. The PCR-amplified full-length *ftsZ* gene was digested with XbaI and BamHI and subcloned into the corresponding sites of a new BACTH vector, pKNT25 (G. Karimova, unpublished data), that, in contrast to pKT25, has a multicloning site located upstream of the T25 open reading frame. Therefore, in the resulting plasmid, pKNT25-*ftsZ*, FtsZ is fused to the N-terminal end of the T25 fragment of AC. Attempts to clone the full-length *ftsZ* gene into the high-copy-number vector pUT18 were unsuccessful.

To construct the pUT18C-*ftsB*/*ftsL* plasmid, which coexpresses the T18-FtsB hybrid protein and the wild-type FtsL polypeptide, a DNA fragment encompassing the Shine-Dalgarno sequence and the structural part of *ftsL* was PCR amplified using appropriate primers, cleaved with KpnI and EcoRI (these restriction sites were included in the PCR primers), and subcloned into the corresponding sites of the plasmid pUT18C-*ftsB*. In the resulting plasmid, *ftsL* is cotranscribed with the T18 gene-*ftsB* fusion from an IPTG-induced *lac* promoter (see Fig. 5a).

To make the pUT18C-*ftsI*/*ftsL* plasmid, which coexpresses the T18-FtsI hybrid protein and the wild-type FtsL polypeptide, the above-described *ftsL* fragment was amplified by appropriate primers and subcloned into the EcoRI site of pUT18C-*ftsI*. The proper orientation of the *ftsL* insertion (see Fig. 5b) was selected by endonuclease restriction analysis.

DNA sequences of the cloned *fts* DNA fragments in all recombinant plasmids were verified by sequencing.

**BACTH complementation assays.** For BACTH complementation assays, recombinant pKT25 and pUT18C carrying the *fts* genes were used in various combinations to cotransform DHM1 cells. The transformants were plated onto LB–X-Gal–IPTG medium and incubated at 30°C for 24 to 36 h. Efficiencies of interactions between different hybrid proteins were quantified by measurement of β-galactosidase activity in liquid cultures. For this measurement, bacteria were grown in LB broth in the presence of 0.5 mM IPTG and appropriate antibiotics at 30°C for 14 to 16 h. Before the assays, the cultures were diluted 1:5 into M63 medium and the optical density at 600 nm ($OD_{600}$) was recorded. To permeabilize cells, 30 to 35 μl of toluene and 30 to 35 μl of a 0.1% sodium dodecyl sulfate solution were added to 2.5 ml of bacterial suspension. The tubes were subjected to a vortex for 10 s and incubated at 37°C for 30 to 40 min for evaporation of toluene. For the enzymatic reaction, aliquots (0.1 to 0.5 ml) of permeabilized cells were added to buffer PM2 (70 mM $Na_2HPO_4 \cdot 12H_2O$, 30 mM $NaHPO_4 \cdot H_2O$, 1 mM $MgSO_4$, and 0.2 mM $MnSO_4$, pH 7.0), containing 100 mM β-mercaptoethanol, to a final volume of 1 ml. The tubes were incubated at 28°C in a water bath for 5 min. The reaction was started by adding 0.25 ml of 0.4% *o*-nitrophenol-β-galactoside (ONPG) in PM2 buffer (without β-mercaptoethanol). The reaction was stopped by adding 0.5 ml of a 1 M $Na_2CO_3$ solution. $OD_{420}$ was then recorded. The enzymatic activity, $A$ (in units per milliliter), was calculated according to the following equation: $A = 200 \times (OD_{420}$ of the culture $- OD_{420}$ in the control tube)/minutes of incubation) $\times$ dilution factor.

One unit of β-galactosidase activity corresponds to the hydrolization of 1 nmol of ONPG per min at 28°C (39). The specific activity of β-galactosidase is defined in units per milligram (dry weight) of bacteria. It is determined from the $OD_{600}$ of the bacterial culture by considering that 1 ml of culture at an $OD_{600}$ of 1 corresponds to 300 μg (dry weight) of bacteria.

A level of β-galactosidase activity at least four- to fivefold higher than that measured for DHM1(pKT25/pUT18C) cells (100 to 150 U per mg [dry weight]) was considered to indicate an interaction.

## RESULTS

**BACTH analysis of *E. coli* cell division proteins.** To characterize the physical associations between components of the *E. coli* cell division machinery, the following Fts proteins (in their full-length forms), FtsA, FtsB, FtsI, FtsL, FtsN, FtsQ, FtsW, FtsX, and FtsZ, were tested systematically for pairwise interactions by using the BACTH assay. For this testing, DNA fragments encoding the selected proteins (except FtsZ) were cloned into pKT25 and pUT18C vectors to generate recombinant plasmids expressing hybrid proteins in which the examined polypeptides were fused at the C terminus of either the T25 or the T18 fragment of the *B. pertussis* AC. The full-length *ftsZ* gene was cloned into a different vector, pKNT25, to express a hybrid FtsZ protein fused to the N terminus of T25 (FtsZ-T25).

To probe putative interactions between the Fts hybrid proteins, an *E. coli cya* strain, DHM1, was cotransformed with pairs of recombinant plasmids expressing the T25 and T18 hybrids. The efficiencies of functional complementation between the different hybrids were determined by β-galactosidase assays as described in Materials and Methods.

Despite the fact that the Fts hybrid proteins were overexpressed in DHM1 cells, only 3 out of 19 appeared to be, to some extent, deleterious to the host cells. We did not succeed in constructing a pUT18 derivative expressing the FtsZ protein fused to the N terminus of the T18 fragment (FtsZ-T18), probably because the high-copy-number recombinant plasmid was toxic for *E. coli*. The T18-FtsA protein, when coexpressed with either T25-FtsA or FtsZ-T25, appeared to slow down cell growth, whereas overproduction of the T18-FtsQ protein resulted in a certain degree of instability of the corresponding plasmid, pUT18C-*ftsQ*. In addition, several Fts hybrid proteins, such as FtsN, FtsQ, and FtsX, induced a moderate cellular filamentation phenotype (data not shown). This suggests that the Fts moieties in the corresponding hybrids were able to interfere somehow with the cell division process. It is noteworthy that previous studies showed that all the above-mentioned Fts proteins can be fused at their N termini to GFP without impairing their ability to localize to the septum site and, in several instances, to be functional in cell division (for reviews, see references 5 and 35).

As shown in Fig. 2, most Fts proteins were able to associate with multiple partners. The high number of unexpected interactions among the tested Fts proteins, as deduced from the results of the BACTH complementation assays, appeared striking at first sight, especially in view of the sequential recruitment of Fts proteins to the septum site. However, several observations suggest that these BACTH data reveal authentic and direct protein-protein interactions.

(i) None of the Fts hybrid proteins gave any complementation signal when tested either with control T25 and T18 polypeptides or with unrelated membrane protein hybrids, like MalF and MalG, two polytopic membrane proteins of the *E. coli* maltose ABC transporter (20) (Fig. 2).

(ii) In most cases, complementation between the T25 and the T18 hybrids could be detected in both configurations, that is, when the given Fts proteins were fused to either the T25 or the T18 AC fragment. The most noticeable exception was FtsW, for which only the T25-FtsW hybrid appeared to be functional, probably because overexpression of the T18-FtsW hybrid protein may prevent correct insertion of the 10 membrane-spanning segments of FtsW into the inner membrane.

(iii) Under our experimental conditions, the Fts hybrid proteins were expressed from multicopy plasmids under the control of the IPTG-regulated *lac* promoters, in the presence of IPTG and, therefore, their expression levels exceeded the physiological concentrations of most wild-type Fts proteins (e.g., FtsI, FtsL, FtsN, FtsQ, and FtsW). Under these circumstances, it seems likely that the T25- and T18-fused Fts hybrid proteins were distributed over the entire cell membrane and not restricted only to the cell septum. Similarly, several GFP-Fts chimeric proteins have been shown to localize to the cell septum at low expression levels but not when overexpressed (11, 23, 25, 35, 45). Therefore, BACTH complementation between two particular hybrids likely resulted from the association between the polypeptides occurring outside the septum site rather than from colocalization within the cell division machinery.

(iv) The fact that, in the BACTH assay, the interacting Fts hybrid proteins were overexpressed makes it unlikely that the complementation data (Fig. 2) resulted from indirect interactions mediated by endogenous Fts proteins acting as bridging molecules. Indeed, to act as a bridging molecule, an endogenous Fts protein should be present at a level at least similar to that of the hybrid Fts protein tested. This is not the case for most Fts polypeptides, except FtsZ, whose level of expression is naturally high (40). Nevertheless, as shown in Fig. 2, the FtsZ

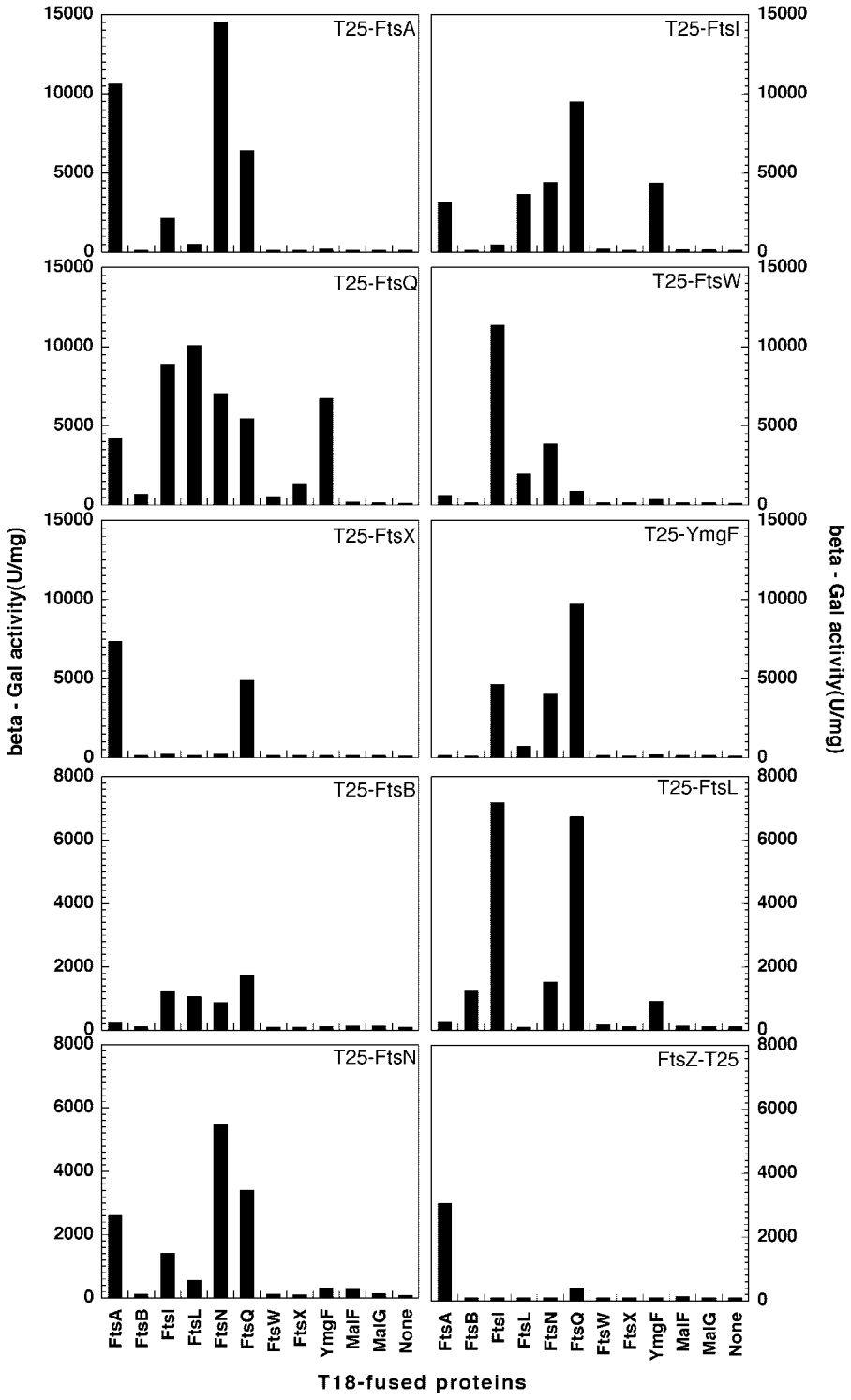


FIG. 2. BACTH analysis of interactions between Fts proteins. The efficiencies of functional complementation between the indicated hybrid proteins were quantified by measuring β-galactosidase (beta-Gal) activities in suspensions of toluene-treated *E. coli* DHM1 cells harboring the corresponding plasmids, as described in Materials and Methods. Each bar represents the mean value from results for at least three independent cultures. In all cases, standard deviations were within 20% of the mean except in the assays involving the T18-FtsQ hybrid, for which higher standard deviations (up to 40%) were observed due to the instability of the pUT18C-*ftsQ* plasmid. The two membrane proteins MalF and MalG of the *E. coli* maltose ABC transporter (20) were used as controls. Under the same assay conditions, functional complementation between T25-MalF and T18-MalG and between T25-MalG and T18-MalF yielded 10,000 ± 1,200 and 1,000 ± 150 U of β-galactosidase/mg (dry weight) of bacteria, respectively.

hybrid protein did not interact with any tested Fts protein except FtsA, whose association with FtsZ is well known (34, 44). We conclude that even FtsZ, the most abundant Fts protein, is unlikely to serve as a linking molecule between two Fts hybrids under the assay conditions used in the present study.

(v) Finally, as shown below, for two of the Fts proteins, FtsQ and FtsI, we were able to map the polypeptide regions that are critical for interactions with their partners. The fact that particular deletions selectively abolished complementation with specific partners suggests that the BACTH complementation results from intrinsic associations between cognate molecules rather than from coassembly of the hybrid Fts proteins into the septal ring.

**In vivo interaction network among Fts proteins.** Among all the tested proteins, the dimerization of only FtsA and FtsA-FtsZ association has been experimentally documented by several independent approaches (protein overlay and yeast and bacterial two-hybrid systems). These interactions were clearly confirmed by the BACTH system (Fig. 2). Importantly, FtsA was also found to interact with several of the Fts membrane-associated proteins, in particular FtsI, FtsN, and FtsQ. Potential interaction between FtsA and FtsI was suggested by the earlier work of Tormo et al. (43) and supported by recently published data (13, 18). FtsN, the latest recruit to the septum, has been originally identified as a multicopy suppressor of *ftsA12*(Ts), and our data provide experimental evidence for direct interaction between these two components of the cell division apparatus. Furthermore, FtsN, when expressed from a multicopy plasmid, can also suppress *ftsQ*(Ts), *ftsI*(Ts), and *ftsK*(Ts) mutations, suggesting that FtsN may interact with the corresponding Fts proteins (15). Our present BACTH data indicate that FtsN can indeed associate with FtsQ and FtsI, which is also in agreement with results from Di Lallo et al. (18). In another study, Dai and Lutkenhaus (14) have observed that overproduction of the FtsQ protein in *ftsA*, *ftsI*, and *ftsZ* thermosensitive mutants induces cell filamentation at permissive temperatures, also suggesting that FtsQ may interact with the corresponding gene products. Interactions of FtsQ with FtsA and FtsI were clearly confirmed here by BACTH assays. Interestingly, our results indicate that FtsQ could associate with nearly all the tested Fts proteins, although with quite different efficiencies. These data suggest that FtsQ may have a central role in the assembly of all the components of the septosome.

FtsI contains a large periplasmic domain that expresses a transpeptidase activity involved in the synthesis of septal peptidoglycan (for reviews, see references 4 and 5). Indirect data have suggested that FtsI may associate with FtsW (36), and indeed, a strong interaction between FtsW and FtsI was detected by the BACTH assay (Fig. 2).

The BACTH analysis also showed that, in addition to FtsA, two other Fts proteins, FtsN and FtsQ, were able to dimerize or multimerize, as also reported by Di Lallo et al. (18); however, at variance with their results, we detected only a weak signal for FtsI dimerization. In addition, no evidence for homodimerization could be obtained for FtsL and FtsB, although both proteins have a leucine zipper-like motif in their periplasmic parts. Nevertheless, FtsB and FtsL formed heterodimers, supporting the suggestion of Buddelmeijer et al. (6, 7) that these two proteins may interact through a coiled-coil structure.

The integral membrane protein FtsX was found to associate specifically with FtsA and FtsQ, thus suggesting that FtsX may contribute to the cytokinetic process. Recently, Schmidt et al. (42) reported that FtsX is recruited to the septum in an FtsZ, FtsA-, and ZipA-dependent manner.

Finally, we tested YmgF, a 72-residue-long polypeptide with two potential transmembrane segments, which was isolated as an interacting partner of FtsL during the screening of an *E. coli* library with the BACTH system (G. Karimova, unpublished data). As shown in Fig. 2, YmgF was able to associate with three other Fts proteins: FtsI, FtsN, and FtsQ. Further studies using a YmgF-GFP fusion revealed that YmgF is a membrane-bound protein, although it did not localize to the *E. coli* septum site, at least when cells were grown in rich medium (N. Buddelmeijer, personal communication). Interestingly, *ymgF* is located near the *minCDE* region within the genome of *E. coli* K-12, yet *ymgF* seems to be nonessential, as the corresponding chromosomal DNA region can be deleted without affecting cell viability (22). At present, the role of YmgF in cell division remains elusive, but as this polypeptide interacted specifically with several Fts proteins, one may speculate that it may be involved in the fine tuning of *E. coli* cell division.

**Mapping of interacting domains of FtsQ and FtsI.** The striking degree of connectivity among the Fts proteins as revealed by BACTH analysis prompted us to further examine the specificity of the observed interactions by mapping the domains critical for protein-protein interaction. For this mapping, we chose two of the Fts proteins, FtsQ and FtsI, that appeared to provide strong complementation signals with a variety of partners.

**FtsQ interacting domains.** To delineate the region(s) of FtsQ involved in the interactions with its different partners, four truncated FtsQ variants were constructed and genetically fused to the C terminus of T18. The resulting hybrid proteins were then tested in BACTH complementation assays with the FtsA, FtsI, FtsL, FtsN, FtsQ, FtsX, or YmgF polypeptide fused to the T25 fragment. As shown in Fig. 3, deletion of a major part of the N-terminal cytosolic segment of FtsQ ($FtsQ_{19-276}$) significantly reduced its association with FtsA or FtsX but it did not affect either FtsQ dimerization or its association with the FtsI, FtsL, FtsN, or YmgF protein. Deletion of the last 10 amino acids of FtsQ ($FtsQ_{1-266}$) diminished its ability to dimerize and to interact with FtsA and FtsX and to some extent with FtsI but did not affect its association with FtsL, FtsN, and YmgF. Deletion of the last 30 amino acids of the FtsQ protein ($FtsQ_{1-246}$) abolished the capacity of FtsQ to dimerize as well as to associate with all tested Fts proteins. Yet this truncated FtsQ hybrid efficiently complemented T25-YmgF, indicating that T18-$FtsQ_{1-246}$ was properly assembled in the inner membrane in a stable form. This was not the case for a fourth truncated form, containing only the first 60 amino acids, encompassing the cytosolic and transmembrane segments of FtsQ ($FtsQ_{1-60}$). In this case, no complementation with any of the tested partners could be detected. In the absence of a positive control signal, we could not conclude whether this lack of complementation resulted from a lack of interaction among hybrid proteins or alternatively from the instability of the T18-$FtsQ_{1-60}$ fusion or its inability to assemble in a proper manner in the membrane.

Taken together, our data indicate that the C-terminal region of FtsQ is essential for interaction with other Fts partners, a

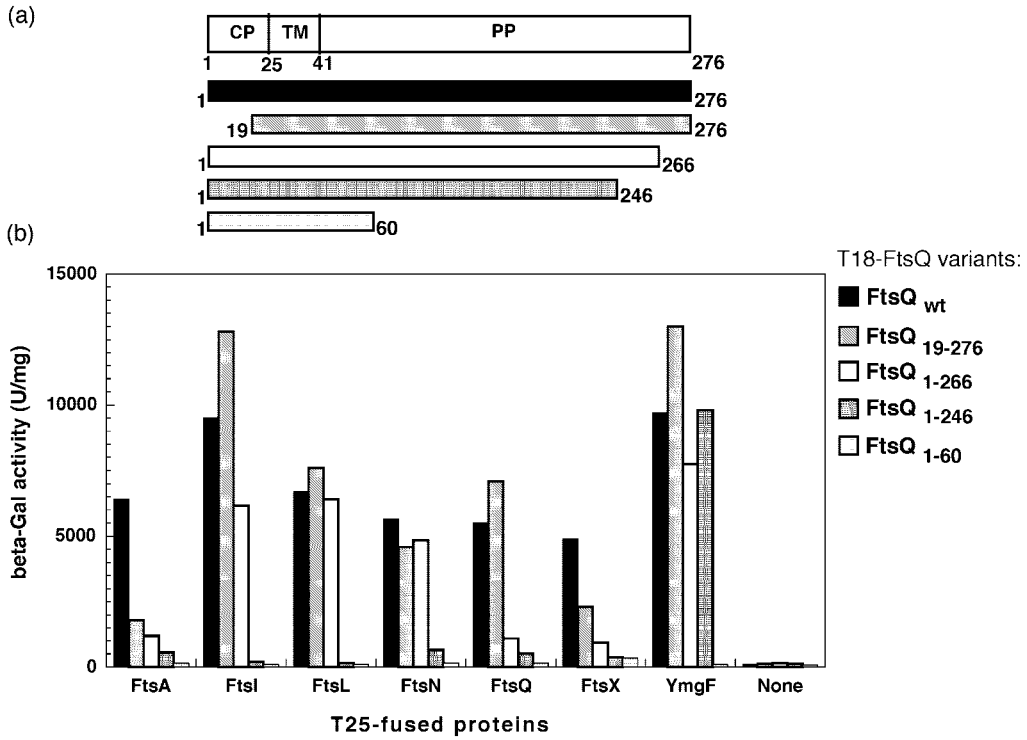


FIG. 3. Mapping of the FtsQ interacting domains. (a) Schematic representation of FtsQ and its truncated variants. The cytosolic part (CP), transmembrane domain (TM), and periplasmic part (PP) of FtsQ are indicated. (b) Functional complementation between the indicated hybrid proteins was quantified by measuring β-galactosidase (beta-Gal) activities in suspensions of toluene-treated *E. coli* DHM1 cells harboring the corresponding plasmids, as described in Materials and Methods. Each bar represents the mean value from results for at least three independent cultures. Standard deviations were within 40% of the mean for the wild-type T18-FtsQ assays (due to pUT18C-*ftsQ* instability) and below 20% of the mean for all other assays (plasmids carrying the T18-truncated FtsQ derivatives were stable). $FtsQ_{wt}$, wild-type FtsQ.

finding that is in good agreement with the work of Chen et al., who showed that an FtsQ variant missing the last 29 amino acids can localize to the cell septum (although less efficiently than wild-type FtsQ) but fails to recruit FtsL and other downstream proteins (10). Whether the C-terminal segment is directly implicated in the association with different partners or whether it is involved mainly in FtsQ dimerization, a prerequisite for permitting the interaction of FtsQ with other Fts proteins, remains to be clarified.

**FtsI interacting domains.** Several truncated variants of FtsI (fused to T18) were constructed and tested in the BACTH complementation assays with FtsL, FtsQ, FtsW, and YmgF, all fused to T25. As shown in Fig. 4, the deletion of most of the C-terminal transpeptidase domain (i.e., amino acids [aa] 251 to 576) of FtsI ($FtsI_{1-250}$) did not significantly alter its ability to associate with each tested partner. A shorter FtsI variant ($FtsI_{1-70}$), missing most of the periplasmic part of the protein, interacted with FtsQ and FtsW proteins as efficiently as the full-length FtsI. However, association of $FtsI_{1-70}$ with FtsL and YmgF was significantly reduced (Fig. 4). Another variant, $FtsI_{1-42}$, containing the N-terminal cytosolic part (aa 1 to 18) and the transmembrane segment (aa 19 to 42) of FtsI, failed to interact with any of the tested Fts partners. The $FtsI_{19-51}$ variant, encompassing the transmembrane domain and a short extension of 10 amino acids (aa 42 to 51) from the periplasmic part of FtsI, did not interact with FtsL, FtsW, and YmgF but still associated with FtsQ, albeit with a reduced efficiency compared to that of $FtsI_{1-70}$.

Taken together, these data indicate that the multiple FtsI interactions are mediated by different segments of the protein: the FtsQ interacting site is located primarily between amino acids 19 and 51; the FtsW binding site encompasses an extended transmembrane region of FtsI (aa 1 to 70), whereas interactions with FtsL and YmgF require a longer fragment of the FtsI polypeptide extending up to residue 250. The C-terminal transpeptidase domain (aa 251 to 576) of FtsI was not required for association with FtsL, FtsQ, FtsW, and YmgF.

Importantly, the observations that various regions of the FtsQ or FtsI polypeptide are required for association with different partners support the view that BACTH complementation results from direct interactions between the two coexpressed hybrid proteins rather than from colocalization of the proteins within the septum machinery.

**Three-hybrid experiments.** All the above-described results suggest that the tested Fts proteins are able to associate with various components of the cell division apparatus. Whether they can contact these multiple partners simultaneously within a division complex or whether they associate sequentially in a mutually exclusive manner remains unknown. To clarify this point, we attempted to study the effect of coexpression of a third Fts component on the association between two Fts hybrid proteins. We chose to examine the FtsB component as this

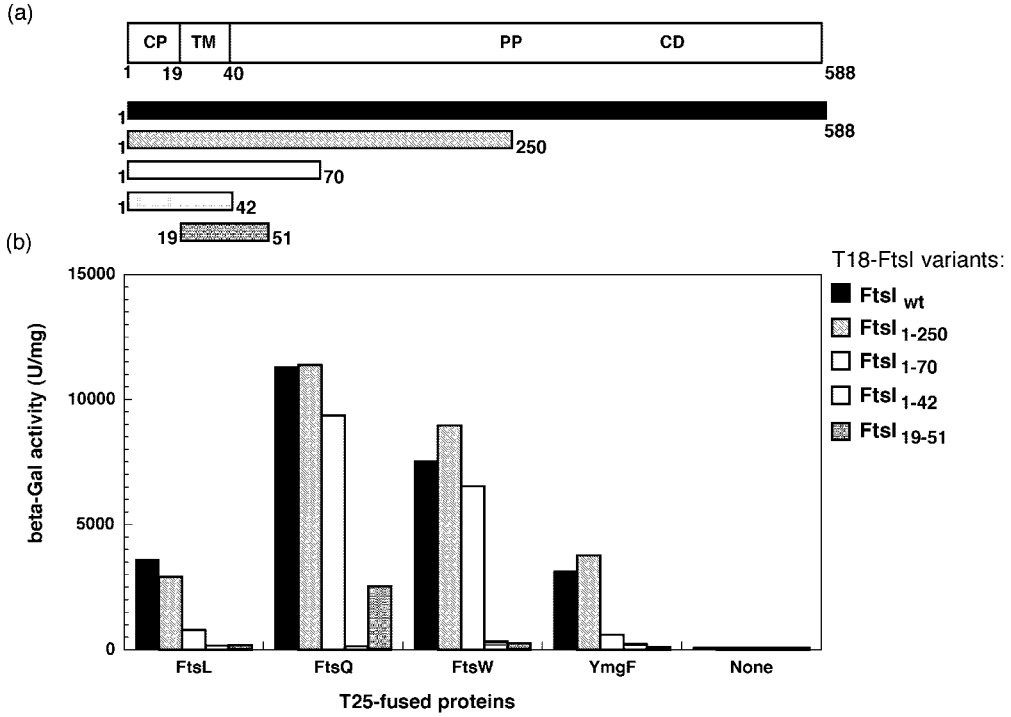


FIG. 4. Mapping of the FtsI interacting domains. (a) Schematic representation of FtsI and its truncated variants. The cytosolic part (CP), transmembrane domain (TM), and periplasmic part (PP), including the catalytic domain (CD), of FtsI are indicated. (b) Functional complementation between the indicated hybrid proteins was quantified as described in the legend to Fig. 3. Each bar represents the mean value from results for at least three independent cultures, with standard deviations below 20% of the mean. beta-Gal, β-galactosidase; $FtsI_{wt}$, wild-type FtsI.

protein appeared to provide relatively low levels of complementation with various partners and, therefore, we expected to detect more easily a potential modulation of complementation by a third protein. We constructed a derivative of plasmid pUT18C-*ftsB* that, in addition to the T18-FtsB fusion, also expresses the FtsL polypeptide. Various T25-Fts hybrids were then tested in complementation assays with either T18-FtsB alone or T18-FtsB coexpressed with FtsL (Fig. 5a). As shown in Fig. 5a, coexpression of FtsL modulated the association of T18-FtsB with its T25-Fts partners in essentially three different ways.

Firstly, and not surprisingly, coexpression of FtsL reduced complementation between T18-FtsB and T25-FtsL, suggesting that the free FtsL polypeptides competed with T25-FtsL for binding to T18-FtsB.

Secondly, we found that overexpression of FtsL strongly enhanced association between T18-FtsB and T25-FtsQ, as revealed by a seven- to eightfold increase in β-galactosidase activity. These data suggest that the presence of large amounts of FtsL molecules (compared to the normal cellular level) either stabilized the T18-FtsB hybrids and, therefore, favored their association with T25-FtsQ or stabilized the FtsB-FtsQ complex by interacting simultaneously with both components. Recently, Buddelmeijer and Beckwith reported immunoprecipitation experiments indicating that FtsB and FtsL are associated in a heterodimer that partially coimmunoprecipitates with FtsQ (6).

Thirdly, upon overexpression of FtsL, T18-FtsB was shown to complement efficiently T25-FtsW whereas no complementation was detected between these two hybrid proteins in cells expressing normal levels of FtsL. Similarly, in DHM1 cells overexpressing FtsL, a weak complementation between T18-FtsB and T25-FtsI could be detected. Indeed, as FtsL could interact with both FtsB and FtsW (or FtsI), these results suggest that, in vivo, FtsL may bridge the two hybrid proteins T18-FtsB and T25-FtsW (or T25-FtsI) to form a ternary complex. Interestingly, DHM1 cells coexpressing T18-FtsB, T25-FtsW, and FtsL exhibited higher β-galactosidase activity (5,600 U/mg) (Fig. 5a) than cells expressing either T18-FtsL and T25-FtsW (2,200 U/mg) (Fig. 2) or T18-FtsL and T25-FtsB (1,200 U/mg) (Fig. 2). This suggests that the ternary complex is more stable than each of the heterodimeric complexes separately. In contrast, the β-galactosidase activity in cells coexpressing T18-FtsB, T25-FtsI, and FtsL (580 U/mg) (Fig. 5a) was lower than that measured in cells expressing T18-FtsL and T25-FtsI (3,800 U/mg) (Fig. 2) or in cells expressing T18-FtsL and T25-FtsB (1,200 U/mg) (Fig. 2). In the latter case, the heterotrimeric complex may be less stable than individual heterodimers.

Taken together, these data indicate that the association of two Fts proteins can be stabilized or even triggered by a third Fts partner and support the idea that cooperative associations of the various Fts proteins may promote the assembly of the cell division machinery at the cell septum site.

In a second set of experiments, we also examined the effects of overexpression of FtsL on the association of T18-FtsI with its various partners. As shown in Fig. 5b, overproduction of FtsL selectively abolished the complementation signal between

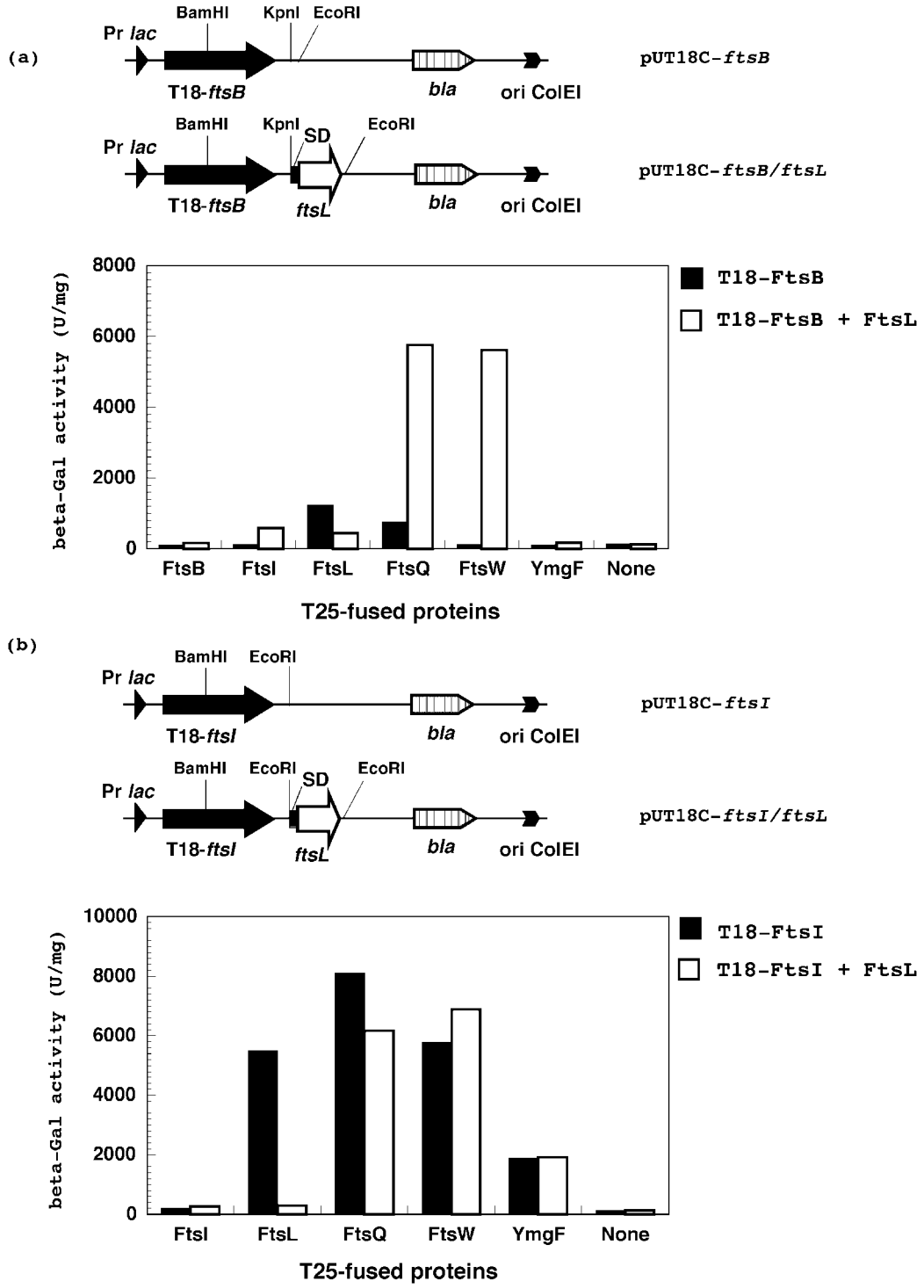


FIG. 5. Three-hybrid experiments. (a) Schematic maps of pUT18C-*ftsB* and pUT18C-*ftsB*/*ftsL* plasmids show the locations of the T18 gene-*ftsB* fusion and the *ftsL* and *bla* (ampicillin resistance) genes, the ColE1 origin of replication (ori ColE1), the Shine-Dalgarno sequence (SD), and the *lac* promoter (Pr *lac*). DHM1 cells were cotransformed with the pKT25 derivative expressing the indicated T25-Fts protein fusion, either plasmid pUT18C-*ftsB*, expressing the T18-FtsB fusion, or plasmid pUT18C-*ftsB*/*ftsL*, coexpressing the T18-FtsB fusion and the wild-type FtsL polypeptide. beta-Gal, β-galactosidase. (b) Schematic maps of pUT18C-*ftsI* and pUT18C-*ftsI*/*ftsL* plasmids show locations of the T18 gene-*ftsI* fusion and the *ftsL* and *bla* (ampicillin resistance) genes, the ColE1 origin of replication, the Shine-Dalgarno sequence, and the *lac* promoter. DHM1 cells were cotransformed with the indicated pKT25 plasmid, either pUT18C-*ftsI*, expressing the T18-FtsI fusion, or pUT18C-*ftsB*/*ftsL*, coexpressing the T18-FtsI fusion and the wild-type FtsL polypeptide. Efficiencies of the interactions were monitored as described in the legend to Fig. 3. Each bar represents the mean value from results for at least three independent cultures, with standard deviations below 20% of the mean.

T18-FtsI and T25-FtsL without affecting complementation with T25-FtsQ, T25-FtsW, or T25-YmgF. These results indicate that the wild-type FtsL efficiently competes with T25-FtsL for binding to T18-FtsI but does not modulate the association of T18-FtsI with the other T25-Fts hybrids.

## DISCUSSION

We have used a bacterial two-hybrid system based on the interaction-mediated reconstitution of a cAMP signaling cascade (29) to unravel the molecular basis of septum assembly by analyzing the protein interaction network among several *E. coli* cell division proteins. Strikingly, our results indicate that most Fts proteins are able to interact with multiple partners. We confirm well-characterized interactions (e.g., FtsA dimerization and FtsZ-FtsA interaction) and provide experimental evidence for several other postulated associations (associations of FtsN with FtsA, FtsI, and FtsQ and of FtsQ with FtsA and FtsI). The BACTH genetic assay also revealed many unexpected interactions among the tested Fts components. Several of the observed interactions were also recently detected by Di Lallo et al. with a different bacterial two-hybrid system (18). The fact that the same interactions between specific Fts proteins were observed, although the corresponding proteins were fused to different reporter moieties (chimeric repressors versus AC fragments), strongly supports the physiological relevance of these associations. Furthermore, a deletion mapping analysis was carried out with two of these Fts proteins, FtsQ and FtsI, and established that different regions of the polypeptides were involved in the associations with their partners. Finally, we also showed that the interactions between two Fts hybrid proteins could be modulated (strengthened or diminished) by coexpression of a third Fts partner.

Two important points should be highlighted regarding the significance of the interaction data deduced from this bacterial two-hybrid analysis.

(i) In the BACTH assay, the hybrid proteins are overexpressed compared to the expression level of native molecules, raising questions about the physiological relevance of the detected interactions. Whereas under native conditions, most Fts proteins are expressed at rather low levels (50 to 100 copies per cell), in our two-hybrid screen the Fts hybrid polypeptides were expressed at levels of up to a few thousand copies per cell. In these conditions, the BACTH assay probably revealed many weak associations among the hybrid proteins. Even if such interactions would not occur at low protein concentrations, they may take place when the Fts polypeptides are assembled within the septosome, where their local concentrations should be much higher than those expected for the same amounts of proteins distributed all over the cell membrane.

(ii) As the BACTH assays are carried out with *E. coli*, it is possible that some of the detected complementation results from an indirect association of the two hybrid proteins triggered by an endogenous protein acting as a tethering molecule. In fact, such indirect interactions, mediated by a third partner, cannot be easily ruled out. Nevertheless, we think that most of the reported interactions (Fig. 2) correspond to direct physical associations, mainly because the endogenous Fts proteins are expressed at rather low levels to serve as potential bridging molecules between the overproduced hybrid Fts proteins. For example, we showed that the endogenous FtsL level was insufficient to trigger association between T18-FtsB and T25-FtsW (Fig. 2) and only in three-hybrid experiments could overproduction of a wild-type FtsL trigger this association (Fig. 5a).

The network of Fts protein interactions deduced from the present two-hybrid data, as well as those of Di Lallo et al. (18), indicates that the sequential assembly of the Fts proteins within the septosome cannot be explained by simple binary interactions between the components that are successively recruited to the cell division sites. The two-hybrid results show that Fts proteins that are recruited early into the septum (e.g., FtsA and FtsQ) can interact with late recruits, such as FtsI and FtsN, although in vivo the latter require the intermediate proteins FtsB, FtsL, FtsI, and FtsW to assemble into the septal ring. The two-hybrid data may yet be reconciled with prior immunocytological results if one assumes that most of the interactions detected by the BACTH assay are characterized by low affinity: weak interactions between two overexpressed hybrid proteins (e.g., FtsA and FtsN) would not be strong enough to maintain a stable complex between these proteins present at low levels in the cells, and this, therefore, may explain why FtsA alone is unable to recruit FtsN to the septum.

However, the fact that most Fts proteins could establish multiple interactions of low affinity with other Fts partners would suggest a model for the assembly of the septosome that may be driven by the cooperative association of the different Fts polypeptides to form a dynamic multiprotein structure at the septum site. This assembly would be stabilized by the multiple weak interactions among Fts proteins in a manner similar to that observed for the organization of neuronal synapses (for a review, see reference 12). Recent studies have shown that the neurotransmitter receptors are concentrated at synaptic sites through the interactions with specific scaffolding proteins but can also be found dispersed in the membrane. Receptor movements in the plane of the membrane alternate between periods of high mobility outside synapses and periods of relative immobility within synapses, the latter state originating, in part, from the transient association with the scaffolding proteins (12). A similar dynamic equilibrium may be involved in the formation of *E. coli* septosome supramolecular machinery. Stable assembly of the different Fts proteins at the septal site may also be achieved through a network of multiple interactions of low affinity, each component behaving as a scaffolding molecule for the other partners. Indeed, our three-hybrid experiments (Fig. 5a) demonstrated that association between two Fts hybrid proteins could be enhanced when a third Fts polypeptide was coexpressed.

The septosome assembly may be triggered by the central positioning of the Z ring that would recruit the FtsA and ZipA components and then the downstream components. The observed sequential recruitment may reflect the progressive build up of the septosome machinery by incremental stabilization provided by the association of the successive partners. Interestingly, in *Bacillus subtilis* the corresponding cell division proteins appear to be recruited in a much more concerted manner (for a review, see reference 8). The *B. subtilis* cell division proteins DivIB, DivIC, FtsL, PBP-2B, and probably FtsW are all completely interdependent for assembly at the division site: mutation or depletion of any of these proteins prevents all the others from assembling. These observations may support a

model of mutual stabilization of the whole septosome machinery through multiple interactions between individual components. The difference between concerted (*B. subtilis*) and sequential (*E. coli*) assembly may ultimately reside within the range of affinities of the interactions between the different polypeptides. A precise analysis of equilibrium constants of these different associations will be required to clarify this critical issue. In the proposed model of dynamic assembly of septosome machinery, like in the neuronal synapses, the Fts integral membrane polypeptides, through lateral diffusion, may constantly enter and exit the septal zone, diffusing rapidly outside it and being trapped in a slow mobility state within the septosome. The observed septal localization of each component would result from the statistical distribution of the polypeptides between the midcell and the whole bacterial inner membrane.

In addition, the dynamic assembly of Fts proteins may confer a significant robustness to the septosome machinery in that it may buffer large perturbations in the amounts of the components. In contrast, a scheme in which the successive Fts proteins are connected to upstream and downstream partners by selective interactions should be more sensitive to imbalances in the amounts of the different components as any overexpressed Fts protein would efficiently titrate out the downstream polypeptides. We observed, indeed, that overexpression of hybrid Fts proteins was in most cases well tolerated by the bacteria.

Finally, our present data indicate that the BACTH system is a potentially attractive tool for delineating specific interactions between the various Fts proteins and should be useful for further characterization of the molecular basis of the septosome assembly as well as other transmembrane protein complexes.

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# ERRATUM

## Interaction Network among *Escherichia coli* Membrane Proteins Involved in Cell Division as Revealed by Bacterial Two-Hybrid Analysis

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Volume 187, no. 7, p. 2233–2248, 2005. Page 2238, column 1, line 23: "$FtsI_{19–51}$" should read "$FtsI_{19–61}$."
Page 2238, column 2, line 6: "51" should read "61."
Page 2239, Fig. 4. "$FtsI_{19–51}$" should read "$FtsI_{19–61}$."