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Cell Division Studies of Escherichia Coli: Expression and Protein Localization of Cell Septation Gene, FtsA.

Chong Chon Younghae

Louisiana State University and Agricultural & Mechanical College

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Cell division studies of *Escherichia coli*: Expression and protein localization of cell septation gene, *ftsA*

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The Louisiana State University and Agricultural and Mechanical Col., 1988
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Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Microbiology

by

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ABSTRACT

FtsA is a gene essential for cell septation. It has been postulated to have both regulatory and structural functions. To study the structural involvement of the FtsA protein in septum formation, four FtsA-LacZ protein fusions were constructed using a mini-Mu transposon (MudII1734). When maxicells containing either ftsA-lacZ or ftsA specifying plasmids were radioactively labelled and fractionated, FtsA-LacZ fusion protein and FtsA were located in both the membrane and cytoplasmic fractions. The FtsA-LacZ fusion proteins were also located in dividing wild type dividing cells by measuring β-galactosidase activity. Cell envelope fractions from a sucrose equilibrium gradient had β-galactosidase activity in outer membrane-heavy(OM_{H}) and outer membrane-light(OM_{L}) fractions. To verify the cellular location of native FtsA protein in membrane fractions, an expression plasmid was constructed by placing a ftsA-lacZ gene fusion downstream from a λ P_{L} promoter. The FtsA-LacZ fusion protein was overproduced and purified by β-galactosidase affinity column chromatography. Polyclonal antibodies to this protein were isolated and demonstrated to be specific for the FtsA-LacZ fusion, FtsA protein and β-galactosidase. Cross reactivity to FtsA protein was found in outer membrane fractions, OM_{H} and OM_{L}, and inner membrane fraction of wild type cells. These data suggest that the FtsA protein may be functioning as an inner-outer membrane protein in septation.
Electron microscopy of thin sections treated with FtsA-LacZ antibodies and immunogold labelling also suggest that the FtsA protein is located in the cell envelope. Furthermore, direct identification of FtsA protein by immuno-electron-microscopy in normal and filamentous cells indicates an enrichment of FtsA protein near septation sites.
INTRODUCTION

Cell division in *Escherichia coli* is a complex biological process that involves the coordination and regulation of several physiological growth processes including increases in cell length, mass and volume, initiation of DNA replication, and segregation of replicated chromosomes. The final steps in cell division are the coordinated invagination of the three envelope layers (inner membrane, murein, outer membrane) at the cell center (septation) and then physical separation of the newly formed daughter cells.

Progress has been made in understanding cell division at the genetic level. Conditional lethal mutants that cannot form septa have been isolated and referred to as filamentation temperature-sensitive (*fts*) mutants. A number of genes involved in the formation of the septum are clustered at the 2 minute region on the *E. coli* genetic map. Based on phenotypic properties, these genes have been postulated to have essential roles in the initiation of septum formation (*ftsZ* and *ftsQ*) and later invagination stages (*ftsA* and *ftsI*). These genes have been cloned and the DNA sequenced. DNA sequence analysis has confirmed genetic studies that three of these *fts* genes (*ftsQ, ftsA, ftsZ*) are closely linked, that there is no strong transcription terminator between the genes, and that promoters exist upstream of each of the genes. However, information concerning the regulatory and morphogenetic functions of these genes in the septation process at the molecular level is only starting to be accumulated. At present the biochemical roles of the FtsQ, FtsA, and FtsZ proteins in septum formation are not known.
Microscopic studies of cells and physiological analyses of cell division mutants have suggested that the septation process is a morphogenetic pathway. Phenotypic properties of conditional cell division mutants, such as cell shape, the amount of residual division, the percentage of persisting constrictions at the restrictive temperature and the pattern of division recovery after a shift back to the permissive temperature have suggested that the cell division genes are activated in the order FtsZ-FtsQ-FtsA-FtsI. For example, conditional lethal mutants of \textit{ftsA} when shifted to nonpermissive temperature do not divide but filament with partial constrictions at potential cell division sites. Thus, the FtsA protein appears to function after septation initiation. The ordered functions of these cell division genes may be controlled at the level of new transcription or the activation of previously existing proteins. It is probable that both events occur but the current evidence strongly favors the protein activation model as the primary mechanism which commits a cell to septum formation.

In this thesis, I attempt to verify the hypothesis that the synthesis and invagination of surface layers constituting the new septum require the formation of a structure which has been called the "septalsome". The septalsome is a multienzyme-protein complex in which the known septation proteins (FtsZ, FtsQ, FtsA and FtsI) and perhaps others would initiate and control the septation steps. This septalsome would contain both specific peptidoglycan synthesizing and modifying enzymes plus envelope structuring proteins essential for
septation. This organelle would thus ensure the precise coordination of the enzymatic activities and localization of the septation proteins within the cell envelope.

In this study, I have focus primarily upon the FtsA protein as a potential septalsome protein. Since the amount of FtsA within a cell is small, the construction of FtsA fusion proteins were necessary. The location of these fusion proteins in dividing wild type cells was accomplished by assaying β-galactosidase activities of the FtsA-LacZ fusion proteins in soluble and membrane fraction of cells. To investigate location of authentic FtsA protein, a plasmid which overproduced the FtsA-LacZ protein was constructed and fusion protein was purified and injected into rabbits for production of polyclonal antibodies. Western blotting experiments were conducted with these antibodies on cell membrane fractions. Direct visualization of FtsA protein at septation sites was done by immunoelectron microscopy. In addition, various promoter fusion plasmids were constructed in attempts to investigate the regulation of the cell division genes, ftsA and ftsZ.
I. Identification of cell division genes.

A number of genes essential for cell division in *Escherichia coli* have been mapped to the 2 min region of the genetic map (36,3). These genes have been mainly identified as temperature sensitive (ts) lethal mutations, so called *fts* (filamentation temperature sensitive) phenotype, that result in multi-nucleated filamentous growth and eventual cell death at the nonpermissive temperature (11,77,94,120). The *ftsZ* (*sulB*) gene and two neighboring cell division genes, *ftsQ* and *ftsA* in this 2 min region (94,96,109,129,130) are part of a large cell envelope-cell division gene cluster (Fig. 1). That is, in addition to *fts* genes this 2 min region encodes for murein genes (8 genes) which specify enzymes required for the peptidoglycan synthesis of the cell wall, cell permeability-cell separation gene, *envA* (7), and the *secA* gene which is essential in protein export across the cytoplasmic membrane (90). This close clustering of cell envelope genes and cell division genes suggests possible coordinated interactions among the gene and gene products that result in peptidoglycan synthesis and septum formation. The *ftsZ*, *ftsQ*, *ftsA* and *ftsI* gene in this cluster encode proteins that probably are required to initiate, form and complete a cell septum. They have been hypothesized to act in a morphogenic pathway (9, Fig. 2) although little is known about their specific functions or biochemical activities in septum formation.

*FtsZ84* is a temperature sensitive (ts) mutant that at the nonpermissive temperature forms multinucleated nonseptated filaments lacking any persisting or newly initiated septal constriction (77). When double mutants that were both temperature sensitive for
elongation(rodA) and septation were analyzed(9), the results indicated that the formation of any septum-like constriction required expression of the FtsZ gene product. That is, FtsZ acts earlier than FtsQ, FtsA, or FtsI(PBP3). Analyses of new peptidoglycan synthesis(127) revealed that radioactive DAP(Diaminopimellic acid) was found to be incorporated near newly initiated constriction (septum sites) of ftsI mutant filaments, but was not found in nonconstricting ftsZ84 mutant filaments. In addition, a recent morphogenic and physiological study(113), investigating the formation of septal constrictions and the amount of cell division of ftsZ, ftsQ, ftsA, and ftsI mutants after a temperature shift from 28°C to 42°C, has suggested that the functioning of these cell division genes is in the order FtsZ- FtsQ- FtsA, FtsI.

Genetic and biochemical evidences (45,46,56,57,58) have demonstrated that the FtsZ(SulB) gene product is the target of SOS(DNA damaged) induced cell filamentation. SulA(SfiA) synthesis is induced following DNA damage as are DNA repair enzymes that are regulated by the LexA-RecA regulatory circuit(86,71). SulA protein is labile with a half-life of 3 min in wild type cells(64). In a lon mutant(101,180) SulA is more stable with a half-life near 20 minutes, which enhances its inhibitory effects and leads to filamentation and death of the lon mutant after SOS induction such as UV irradiation(56,58). SulB(sfiB) mutations (like sulA(sfiA) mutations) were isolated as second site mutations that confer UV resistance to a lon mutant. The sulB locus was found to be identical to ftsZ gene locus(45,73,64). The hypothesis that FtsZ is the target of SulA protein is supported by the findings that the overproduction of FtsZ
can suppress the SOS-induced lethal filamentation in lon mutants(75), and that biochemical studies suggest direct interactions between the FtsZ protein and SulA proteins because the half-life of radioactively labelled SulA in maxicells was increased when cells contained the wild type ftsZ gene but not sulB mutation(65). The inhibition of cell septation by SulA interacting with FtsZ is reversible in wild type cell since SOS-induced filaments eventually divide even in the absence of de novo protein synthesis(80).

It has been hypothesized that a possible role of FtsZ protein is as an element of the cell division apparatus to which the cell transmits information about growth conditions(67). This role for FtsZ is based on studies with a ftsZ(sfiB114) mutant. This mutant is unable to interact with SulA(64) but it had a longer cell septation delay (50 to 60 min.) compared with wild type (20 min) after a shift-up from glucose minimal medium to complex media, and it had a shortened cell septation delay (10 to 16 min) after a nutritional pulse (a shift up to complex media followed rapidly by a return to glucose minimal medium).

When the level of ftsZ gene expression was increased, minicell formation and increased septation that results in slightly smaller cells have been observed. Ward and Lutkenhaus(123) found that just a two to seven-fold increase of FtsZ synthesis induces polar divisions producing minicells. These minicells were not formed at the expense of normal divisions, as they are in a minB minicell producing mutants, but were the result of increased septation. The small cell size of cells containing multicopy-plasmids specifying ftsZ appears to be because medial septal invaginations are initiated earlier in
the cell division cycle. However, the introduction of a \textit{ftsZ}
specifying multicopy-plasmid into \textit{minB} mutants results in just
increased polar division producing more minicells with normal medial
divisions\cite{123}. This observation suggests possible interactions
between the FtsZ and \textit{minB} locus products. These findings suggest
that FtsZ protein is involved in the maturation stage of the
potential\textit{(medial)} division sites. The FtsZ-induced production of
minicells was suppressed in \textit{cya} mutant\cite{27}. \textit{cya} mutant cell phenotype
is short rods because cAMP-\textit{CRP} (Catabolic Regulatory Protein) is
necessary for longitudinal cell growth. This finding is consistent
with earlier observations\cite{55} that introduction of \textit{cya} or \textit{crp}
mutation into a \textit{minB} minicell strain suppresses minicell production.
The function of cAMP-\textit{CRP} in minicell formation is postulated to be
between the division machinery and PBP2 (penicillin binding protein
\textit{2}). Minicell strains are deficient in PBP2. (see Sect2 for role
PBP2).

The \textit{ftsZ}(\textit{suJ}B) gene has been subcloned and sequenced \cite{95,129}
and identified as a 45 Kdal protein that is membrane associated\cite{53}.
Ward and Lutkenhaus have purified the FtsZ protein and have made
antibodies to it \cite{75}. They found that when cells were screened by
Western blotting a FtsZ analogue was antigenically conserved in all
tested procaryotes both Gram-negative and Gram-positive. Only
closely related enterobacteriaceae, however, hybridized with an \textit{E. coli}
\textit{ftsZ} DNA probe by Southern blotting. Furthermore, the induction
of minicells phenotype was observed in other strains transformed
with multicopy-plasmids containing \textit{ftsZ} gene, demonstrating that the \textit{E. coli} FtsZ protein could function in several other bacterial species\cite{24}.

\textit{FtsQ} mutant, TOE1, was obtained by temperature-oscillation enrichment\cite{11}. Its temperature sensitivity is salt remedial by 1\% sodium chloride (as is several other \textit{fts} mutants\cite{94}). The \textit{ftsQ} gene has been subcloned and sequenced\cite{130}, from which the DNA sequence predicts that FtsQ protein is 31.4 Kdal in size. However, no radioactive polypeptide has been identified as the FtsQ gene product even though several \textit{in vivo} labelling procedures were tried that included maxicell and minicells containing \textit{ftsQ} encoding plasmids and UV irradiated cells infected with \textit{ftsQ} transducing phage. Thus, FtsQ protein is likely to be either synthesized in very small amount or is extremely labile or both. Filamentous cells of \textit{ftsZ} and \textit{ftsQ} mutant at the nonpermissive temperature have similar phenotypes. Both mutant filaments are resistance to \textit{β}-lactam lysis and both quickly have septa after a shift back to the permissive temperature, 30°C \cite{113}. However, filaments of \textit{ftsQ} mutant can have constrictions indicating that septation can continue pass the initiation step. Thus the FtsQ gene functions after FtsZ gene but before FtsA, FtsI genes.

Directly blocking DNA synthesis (e.g. \textit{dnaA}(ts), \textit{dnaC}(ts), \textit{dnaB}(ts) etc.) causes inhibition of septation and produces transient filamentous cells\cite{32}. This SOS independent division inhibition(TER pathway: 18,37,61,62,115,117) can result in short uninucleoid filaments that can produce normal size cells from their end which are DNA-less. This anucleated cell production requires a functional
cAMP-CRP complex and is affected by suIB mutations which suggests an involvement of FtsZ in the TER pathway. The division promoting role of the cAMP-CRP may involves activation of FtsZ (that promotes division) by protein(s) or other metabolic factors (67) by the cAMP-CRP, since FtsZ expression is not directly regulated by cAMP-CAP (27).

The reversal of the SOS independent division inhibition requires a short period of protein synthesis at the end of a round of chromosome replication (66). FtsA has been proposed as a possible termination protein because it controls late events (35, 118) of division and its synthesis appears to be coordinated with DNA replication in the TER pathway (27, 115, 117).

The gene product of FtsA has been postulated to be a membrane protein (53) and a structural component of the septum (116). The ftsA gene has been cloned and DNA sequenced (95); and identified as a 50 Kdal polypeptide on SDS-PAGE of extracts of in vivo labelled cells containing ftsA on plasmid or a λ transducing phage (76). The expression of FtsA is very low, even when amplified by introduction of a multicopy plasmids encoding ftsA (109, 129). Possible protein interaction between FtsA and PBP3 (119) suggests an involvement of FtsA in peptidoglycan synthesis specific for septum formation. The properties of double mutants harboring the Ion mutation in combination with either the ftsQ1, ftsA10, ftsA12, or ftsZ84 mutations suggests that FtsQ, FtsA, and FtsZ proteins also possibly interact with each other and perhaps form a molecular complex (29).

FtsI (pbpB or sep), which is closely linked but separate from ftsQAZ locus, has been cloned and sequenced (87). Its gene product
(60 Kdal) is present in about 50 copies per cell in the cell membrane(103). FtsI codes for a minor penicillin binding protein, PBP3 (see Sect. 2), which possesses both transpeptidase and transglycosylase activities on peptidoglycan substrates in vitro(14,31,60). Mutations affecting FtsI(94) or selective inhibition of PBP3 by β-lactam antibiotics such as cephalexin(58) impair cell septation and produces filaments. PBP3 is the only known protein target for β-lactam antibiotics that involves cell septation. β-lactam induced cellular lysis at potential division sites occurs only after the action of FtsZ,Q,A division genes. The sensitivity of fts mutants to β-lactam lysis plus other morphogenic and physiological studies (113) are also consistent with ordering FtsI function after FtsZ,FtsQ,FtsA function. Furthermore, indirect evidence from autoradiographic analysis of peptidoglycan synthesis(127) suggests the PBP3 is not involved in the initiation of constriction but rather in its proper completion.

Recently, the envA gene located immediately downstream of ftsZ has been sequenced and found to be an essential gene by insertion mutagenesis(7). The phenotype of a single non-temperature sensitive envA mutant(envA1) is filamentous cell chains that have inner membrane separation but not outer membrane-cell wall constriction. The envA defect is probably due to decreased N-acetyl muramyl-L-alanine amidase activity(128).

MinB at 26 min on the genetic map seems to inactivate old septal sites (28,114), since minB mutants produces minicells. Rothfield's group(13) has recently cloned the minB locus and found that minB locus codes for several gene products indicating it is
probably a multi-gene operon. They have suggested that the role of the \textit{minB} locus is localization of the septation sites, since under-expression of \textit{minB} locus results in minicell production.

Another \textit{fts} gene cluster maps at 76 min and contains \textit{ftsY}, \textit{ftsE}, and \textit{ftsX}(48). Gene \textit{ftsS} is closely linked to, but separate from \textit{ftsY}. The location of the heat shock regulatory gene, \textit{rpoH(htpR/hin)}, immediately downstream of this \textit{ftsYEX} cell division operon, suggests a possible function of the \textit{ftsE} cluster is linking the cell division process to some of the major global regulatory networks of the cell (e.g. the SOS or DNA repair regulon, the heat shock regulon, the stringent response regulon). Changes of Ca++ level during division(22,88) and homology of the FtsE protein to bacterial ATP-binding proteins involved in membrane transport(50) also suggests a role of FtsE in cell cycle-dependent ion transport.

Other \textit{fts} mutants that may block division includes \textit{ftsB}(48 min) and \textit{ftsH}(69 min). \textit{FtsB} mutant has been shown to be allelic with \textit{nrdB}(68,111) which is involved in the synthesis of DNA precursors. Cell division inhibition of this mutant is growth rate dependent suggesting indirect involvement of FtsB in the septation process. \textit{FtsH} was previously identified as a gene involved in some initial stage of septum formation due to a slow stop of cell division at 42°C(1). A possible role of FtsH protein is to regulate the synthesis or stability or/both of PBP3(41). This was suggested by a recent finding that there was absence of PBP3 in cell envelopes of an \textit{ftsH} mutant at 42°C even when the mutant strain contained an overproducing PBP3(\textit{ftsI}) plasmid. The function of \textit{ftsM}, which maps near \textit{leu}, appear to be in SOS-associated repair. The \textit{ftsM} mutant are
affected in Weigle reactivation and its expression appears to be regulation by LexA (38,39). A possible interaction of FtsM with FtsZ protein has been suggested because there was amplification of the ftsZ71 mutation phenotype when in the ftsM1 mutation was present in the mutant strain (12,89).

II. Roles of penicillin binding proteins in morphogenetic cycle.

Penicillin binding proteins (PBPs), located in the intermembrane structures (4,97) within the cell envelope of Escherichia coli, represents a set of proteins responsible for peptidoglycan biosynthesis involved in cell shape, elongation and septation (55). These proteins are the actual enzymes that catalyze the insertion of new material into the peptidoglycan sacculus and are engaged in the control of cell elongation (106) and cell division (14,63).

PBP1A and 1B are transpeptidases present at 200 molecules per cell (104) which are responsible for the crosslinking of the peptidoglycan (PG) net. Inhibition of the function of PBP1A and 1B by mutations or cephaloridine (a β-lactam antibiotic) results in an inhibition of PG synthesis causing lethal cell lysis.

PBP2 (pbpA gene product) is involved in the control of elongation of the rod-shaped sacculus and indirectly cell septation. Mecillinam, a β-lactam antibiotic that specifically binds PBP2, inhibits cell elongation and produces spherical cell (106) and that cannot divide (63). It has been proposed that the lethality of mecillinam bound to PBP2 is primarily the result of this septation inhibition (26). Most mecillinam-resistant mutant cells were
mutations in \textit{pbpA} and \textit{rodA} genes. These mutants have a spherical cell phenotype in the absence of mecillinam and they apparently grow by constant septation\textsuperscript{(27)}. Other mecillinam resistant mutants were in \textit{cya}, \textit{crp} genes. These mutants have a rod shaped cell phenotype in the absence of mecillinam. The \textit{cya} and \textit{crp} mutants formed filaments when DNA synthesis was interrupted, since the functional cAMP–CRP complex is required for the production of anucleated cell (see sec 1). Recent \textit{cya} expression studies using a \textit{cya-lac} transcriptional fusion plasmids\textsuperscript{(120)} have revealed that \textit{cya}(adenyl cyclase gene) is transcribed during cell elongation and its transcription is repressed during cell septation. Interestingly, it was found that increased expression of \textit{cya} inhibits cell division resulting in filamentous cell growth. These data support a possible role of the cAMP–CRP complex in cell elongation and suggest cAMP–CRP may have a negative function on cell septation. The fact that \textit{cya} and \textit{crp} mutants are mecillinam resistant suggest that the cAMP–CRP regulates some aspects of the interactions between PBP2 and the septation apparatus. Furthermore, the inhibition of lateral wall elongation by mecillinam stimulates septation \textit{crp(ts),ftsA} mutant\textsuperscript{(19)}. This observation suggests that cAMP–CRP complex may be regulating the transition between peptidoglycan synthesis of lateral wall elongation and septum formation.

PBP3 (the gene product of \textit{ftsI, pbpB, sep}) is a complex protein of 60 Kdal with transglycosylase and transpeptidase enzymatic activities which is involved in murein synthesis and septum formation\textsuperscript{(14,31,60)}. Septal peptidoglycan synthesis is inhibited during the period of actual septation by selective inhibition of PBP3
or FtsI mutations. Biochemical data(31) has revealed that PBP3 is responsible for viability when there is a under limitation of murein pentapeptides, since PBP3 can use the available tripeptide for nonseptal transpeptidation. Other biochemical studies(31) have demonstrated that at least part of the septal peptidoglycan, which contains increased amounts of cross linked peptidoglycan with a high ratio of tripeptide cross linked subunits, is synthesized by PBP3 using UDP-N-acetylmuramyl tripeptide-substrates as acceptors.

PBP4 a 49 Kdal polypeptide that has been shown to have carboxypeptidase and DD-endopeptidase activity in vitro and transpeptidase activity in vivo. PBP4 is believed to be involved in secondary transpeptidation resulting in additional peptidoglycan cross linking, that in a way converts new murein into old murein(55).

PBP5(dacA) is a 42 Kdal polypeptide that has DD-carboxypeptidase activity which is high just before septation(8). PBP5 is responsible for converting peptidoglycan pentapeptide subunits into tri- and tetra-peptide subunits, the preferred substrate for PBP3 and septal peptidoglycan synthesis. Overproduction of PBP5 results in spherical cells, which is the same phenotype exhibited by PBP2 mutants. A recent model(8,82,85) for the morphogenetic cycle in E. coli invokes a shifting balance in the relative activities of PBP2 and PBP3 that is driven by periodic changes in the activities of D-alanine carboxypeptidase(PBP5). This model does explain the spherical cell phenotype when PBP5 is overproduced or PBP2 is mutated. Increased conversion of pentapeptide chains into tetrapeptides suppresses crosslinking required for cell elongation because PBP2 requires nascent
peptidoglycan chains with complete pentapeptides. The location of dacA(PBP5) gene's chromosomal location is near gene locus with rodA, pbpA genes at 15 min on the E. coli genetic map (105,108).

The low numbers of PBP3(50 molecules/cell) and PBP2(20 molecules/cell) molecules which are produced at a constant rate in each cell cycle from a small number of mRNA molecules(104,126) suggest that the balanced activities between PBP2 and PBP3 is obtained by the modulation of their enzymatic activities rather than by their synthesis. Biochemical studies(84) have shown that ftsZ84(ts) mutants exhibit a low activity of D-alanine carboxypeptidase at 42°C when the cells are filamentous, suggesting FtsZ may be a modulator. In addition, other data(31,42) point to FtsA and FtsH as possible modulators of PBP3 either regulating the enzymatic activity and/or protein stability. A possibility for protein-protein interaction between PBP3 and FtsA was suggested by the increase resistance of ftsA mutants to the lytic action of β-lactam antibiotics and by the finding of decreased binding of $^{125}$I-benzypenicillin to PBP3 in ftsA mutant strains at the nonpermissive temperature. Possible interactions of PBP3 with FtsZ have also been implied(31), which was based on decreased binding of PBP3 to $^{125}$I-ampicillin in a strain carrying the su1B mutation. In addition, PBP3 and RodA(10) proteins may also interact because a combination of mutation in both rodA or pbpB gene can results in mutants with normal shape and division. These findings indicate that the activities of the cell division genes and the enzymatic activities of PBPs are highly coordinated and regulated, possibly as part of a multimeric bifunctional complexes for both cell elongation
and septum formation. These multimeric bifunctional complexes could be, in turn, modulated by multiple factors linked to global regulatory metabolic circuits at discrete places (possibly potential division sites) within the cell envelope of *E. coli*.

II. Periseptal anulii and potential division sites.

Donachie et al. (36) observed that in dnaA(ts) mutant at the nonpermissive temperature (42°C) unreplicated nucleoid at potential division sites resulted in a block of cell septation and the production of short anucleoid filamentous cells. Normal sized DNA-less cell were produced from these anucleoid filaments when the transient chromosomal replication blockage was removed by returning these cells to the permissive temperature. This finding indicated that the cell continues to develop potential division sites of unicellular length in the absence of new chromosomal replication (50). Thus DNA replication and cell septation can be uncoupled. This finding also indicated that the initiation of the septation process occurs before completion of chromosome replication. Jones and Donachie (66) and by Holland (52) have proposed that activation of cell septal division sites occur after the termination of chromosome replication. In fact, Donachie and Beggs (34) found that potential division sites could be detected by their sensitivity to penicillin and that these sites appear to be present at regular intervals early in the cell cycle. Teather et al (114) proposed that the synthesis of quantumly produced division factor controlled the location and frequency of the cell septa. This hypothesis was to explain why a
normal potential division site was lost in the minB mutant, in which the synthesis of a minicell results in the parent cell usually being two cell lengths.

Recently, Rothfield's group observed these potential division sites by TEM in serial sections of plasmolized bacterial cells(78) as concentric rings of tightly bound inner-outer membrane [commonly called adhesion zones(5)]. They called this organelle the "periseptal annuli". The periseptal annuli flank the constriction site around the potential septa and they appear to act as an inner-outer membrane gasket compartmentalizing the periplasmic space between the dividing cells(Fig 3). Ishidate et al.(59) have developed a membrane fractionation procedure that enables the isolation of a membrane fraction, called outer membrane light(OM_L), which contains this inner-murein-outer membrane fraction. Biochemical studies(59) demonstrated that this OM_L fraction is involved in the translocation of newly synthesized lipopolysaccharides and peptidoglycan synthesis. Additional evidence, supporting the model that the periseptal annuli is an cellular organelle associated with cell septation, is that the OM_L fraction was increased in cell division mutants, IkyD and cha. Both mutants are defective in outer membrane invagination during septum formation(21). Incomplete, nascent annuli that do not extend completely around the cell were also observed at nonseptal regions along growing cells(25). These nascent annuli appear to be generated from complete annuli already in position at the midpoint of newborn cells, and were displaced laterally during cell elongation to position at 1/4 and 3/4 cell lengths that become the midpoint annuli.
of newborn cells of forthcoming divisions (25). These newly generated annuli have been hypothesized to be the cell structure involved with chromosome partitioning during the cell division.

The finding of periseptal annuli at potential division sites in a divC mutant filament (26), in which cell division was blocked before any septal invagination, indicated that divC gene product acts after biogenesis, maturation and localization of the periseptal annuli. Thus, the formation of the periseptal annuli precedes the initiation of septal invagination. The identification of a second division related structure, septal attachment sites, SAS (79), suggest that it may be a first step in septal invagination.

The minB locus has been proposed (13) to be involved in the localization of the division septum. The single polar periseptal annulus, which remains at the cell pole in each daughter from the preceding division event, could be the foci for the abnormal polar division and minicell formation in minB mutant strains (13, 29).

IV. Preliminary studies on the regulation of ftsQAZ gene expression.

The 2 min region of E. coli genetic map including ftsQAZ genes contains a large cluster of 14 genes, approximately 20 kilobases, involved in cell envelope growth and division (96, Fig. 1). The close clustering of these genes suggest a possible coordinate expression and/or interaction between the genes. The cloning of DNA from this region and the sequencing and subcloning of DNA fragments into promoter assay have enabled the construction of a transcription map of the region (95, 96, 109, 129, 130). Although the contiguous genes
between ddl (D-ala:D-ala ligase) and envA are transcribed in the same direction, this regions forms an atypical operon with overlapping transcriptional units since each gene, when subcloned, can be expressed from its own promoter (110, 130). The promoters (Pz3 and Pz2) of the ftsZ gene are within the coding sequence of the upstream ftsA gene (110), the promoter of ftsA in turn is within the coding sequence of upstream ftsQ gene (95), and the promoter for ftsQ may be within its upstream gene neighbor. S1 mapping (Corton and Lutkenhaus, unpublished) and the used of a terminator-probe vector has recently revealed the location of a typical strong rho-independent transcriptional terminator just downstream of envA (7).

The absence of strong transcriptional terminators between ddl and envA suggests that transcription originating from ddl could continue through to envA. However, it remains to be determined what transcripts are produced form this gene cluster in response to different metabolic signal in vivo. A possibility is that gene expression involves promoter specific transcription initiation and RNA polymerase transcriptional pausing. A computer search of the published DNA sequence of the ftsQ-ftsA region (95) has identified two potential NusA sites with a possible DNA sequence that could form a stem-loop structure (R. Gayda, unpublished data), one at the beginning of the ftsQ gene sequence and the other near the ftsZ promoter Pz3. NusA specific DNA sequence has been demonstrated to be necessary for lambda N gene antitermination (43).

The expression study of ftsZ gene has revealed substantial reduction of ftsZ synthesis when a Tn1000 transposon was inserted within the ftsA gene (64). Similarly, an ftsZ encoding DNA fragment,
containing only the two promoter (P_{Z2} and P_{Z1}) cloned into \( \lambda \) vector, was insufficiently expressed to complement an \textit{ftsZ} mutant. However, a \( \lambda \) \textit{ftsZ} transducing phage was able to complement a \textit{ftsZ} mutant\cite{74} when it contained additional upstream DNA including the promoter(P_{Z3}) within the \textit{ftsA} gene but not the entire \textit{ftsA} gene coding region. Sullivan and Donachie\cite{109} found that the transcription from P_{Z2}P_{Z1} and P_{Z3} when together was more than the sum of their separate promoter activities. Similarly, there is evidence\cite{33} that upstream DNA in addition to the \textit{ftsA} promoter is required for proper expression of the \textit{ftsA} gene. These data suggest that regulation of the \textit{ftsQAZ} genes is not simple but complex.

Transcriptional expression of \textit{ftsQAZ} encoding region also seem to involve different regulatory interactions among the various promoters. The amount of FtsZ per cell was invariable over a 10 fold range of cell mass, thus suggesting that \textit{ftsZ} expression is subject to some type of autoregulation\cite{36,65}. Transcription from P_{Z3} was found to be increased in a \textit{cya} mutant, which suggested \textit{ftsZ} expression may be negatively regulated by cAMP-CRP complex. However, (as previously mentioned) a recent study\cite{27} has demonstrated that cAMP-CRP does not regulate \textit{ftsZ} gene expression but that the increased FtsZ protein in the \textit{cya} mutant was a compensation for the small cell size of the \textit{cya} mutant. Transcription from P_{Z3} was increased as much as 10 fold when \textit{ftsA} had a nonsense mutation and to a lesser extent in mutants of \textit{ftsI}, \textit{ftsQ} and \textit{ftsZ}.

Post-transcriptional processing and/or modification of the Fts gene products have not been reported.
Materials and Methods

I. Bacterial strains, plasmids and phages.

The *E. coli* K-12 strains, plasmids and phages used in this study are listed in Table 1 and 2.

100 μl of λ1098(10 X 10⁸ plaques forming units) that contains a mini-Tn10 transposon were mixed with 100 μl of an overnight culture of strain RGC103-9(*sulB9*,*lon*). After 20 min at room temperature the mixture was plated out on a YET-plate containing tetracycline(12.5 μg/ml). Tetracycline-resistant (Tet<sup>R</sup>) colonies were pooled. Strain YC99 was constructed by P1-mediated transduction of RGC123 to tetracycline resistance and nitrofurantoin (NF) resistance (to select for *sulB9* allele) from such a random Tn10 bacterial pool. *SulB9* allele was found to be 90% cotransduced with Tn10 in YC99. YC100 was constructed by P1 mediated transduction of *ftsA*:Tn10 into strain AMC290 by selecting for tetracycline resistance and temperature sensitivity. Strain YC200 was obtained by P1-mediated transduction of Lac<sup>+</sup> allele from Hfr3000 into AMC290 by isolating blue colonies on minimal lactose plates containing X-gal(40 μg/ml) after 3 days incubation at 30°C.

II. Media and growth conditions.

*E. coli* K-12 strains were routinely grown on complex medium, YET (per liter): 10g Bacto-tryptone(Difco), 5g Bacto-yeast extract(Difco) and 10g NaCl. The final pH was 7.0. YET agar plates included 15 g/l Bacto-agar(Difco). Low salt complex medium, TEY was identical to YET
but without any NaCl. Other media used for *E. coli* growth included:

(i) M9 minimal medium (83) which consisted of minimal salts (per liter): 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.15 g NaCl, and 1 g NH₄Cl, to which after autoclaving and cooling were added 2 mM MgSO₄ and 0.1 mM CaCl₂, the carbon source was 0.2% glucose and amino acids and vitamins added as required. The final pH was 7.4. (ii) KU-medium (100) was M9 minimal salts plus 10 g glucose and 10 g NZamine (Sheffield products), 0.5 g uridine per liter. (iii) Sulfate free Hershey medium (100) that was (per liter) 4 g glucose, 1.0 mg thiamine (B1), 5.4 g NaCl, 3 g KCl, 1.1 g NH₄Cl, 15 mg CaCl₂·2H₂O, 203 mg MgCl₂·6H₂O, 0.2 mg FeCl₃·6H₂O, 87 mg KH₂PO₄, 12.1 g Trizma base (tris[hydroxymethyl]aminomethane). The final pH was 7.0. (iv) Lactose agar plates (83) consisted of (per liter) 10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 0.5 g (NH₄)₂SO₄, 0.5 g sodium citrate, 15 g Bacto-agar, to which after autoclaving, 1.0 ml of 20% MgSO₄·7H₂O, 0.2 ml of 5% thiamine, 10 ml of 20% lactose, 5 ml of 2% amino acids as required were added. Final pH was 7.0 without adjustment. (v) L medium consisted of (per liter) 10 g Bacto-tryptone, 5 g Bacto yeast extract and 5 g NaCl.

Carbenicillin (carb, 25 μg/ml), tetracycline (tet, 12.5 μg/ml) kanamycin (kan, 25 μg/ml), and 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal, 40 μg/ml) were added when needed.

Overnight cultures at 30°C were diluted approximately 100 fold in fresh media and grown to OD₆₀₀ = 0.1 to 0.5 before cells were challenged by experimental conditions such as a temperature shifts. The permissive temperature for the *ftsZ*, *ftsA*, *ftsQ*, *ftsI* mutants was 30°C and the restrictive temperature was 42°C.
ftsA mutants, YC100, was unable to grow on TEY plates at 30°C, and exhibited temperature sensitive phenotype on YET media at 42°C. ftsI mutant, AMC493, was sensitive to growth at 42°C on both YET and TEY media. ftsZ mutant, AMC419, and ftsQ mutant, TOE1, was temperature sensitive only on TEY (no salt) media. Growth of ftsZ, ftsQ mutants on YET plates incubated at 42°C results in colonies.

Strain YC200(lac+) was induced for expression of β-galactosidase with 1mM IPTG(isopropyl β-D-thio-galactopyranoside) at OD₆₀₀ = 0.12 for 2 hrs.

III Enzymes and chemicals.

Restriction endonucleases were obtained from either New England Biolabs, Inc., or Bethesda Research Laboratories (BRL). T4 DNA ligase, bacterial alkaline phosphatase and the large fragment of DNA polI (Klenow) were obtained from BRL.

p-chloromercurophenyl sulfonic acid (pCMS), used as an inhibitor of contaminating nuclease activity, DL-dithiothreitol, and Trizma Base (tris[hydroxymethyl] aminomethane) were purchased from Sigma Chemical Co.

Alkaline-phosphatase linked goat anti-rabbit immunoglobulin G antibody (Western blot AP system, W3930) was obtained from Promega. The goat anti-rabbit-immunoglobulin G antibody labelled with 10nm gold particles was purchased from Jenssen (Pharmaceutical).

β-lactamase substrate, PADAC([1-(thienyl-2-acetamido)]-3-[2-(4-N,N-dimethylaminophenylazo)pyridium methyl]-3-cephem-4-carboxylic acid) was purchased from Calbiochem (Behring Diagnostics).
IV. Methods of DNA preparation

a. Large scale plasmid DNA isolation.

Plasmid DNA was prepared by the method of Robert Kolter with some modifications. Five mls of an overnight culture of cells that were grown in L broth plus 40 µg/ml ampicillin were used to inoculate 1 liter fresh L+amp. The culture was grown at 37°C (or 30°C) with shaking (200 rpm) to a optical density of OD₆₀₀=0.4. Plasmid DNA was amplified in the cells by adding chloramphenicol (150mg/l) and continuing incubation overnight with shaking. The cells were harvested by centrifugation for 5 minutes at 5,000 X g which yields a soft pellet of cells. These cells were then resuspended in 5 ml of 50mM Tris-Cl (pH 8.0) with 25% (w/v) sucrose. 0.5 mls of lysozyme (10mg/ml in a solution of 50mM Tris-Cl, pH 8.0, and 25% sucrose) was added and the mixture was incubated on ice for 5 minutes. Then, 0.5ml of 0.25M EDTA (pH 8.0) was added and further incubated on ice for another 5 minutes. Next, 5 ml of Triton X-100 lytic mixture (50mM Tris-Cl (pH 8.0), 62mM EDTA and 0.4% Triton X-100) at room temperature was added quickly, mixed, and left at room temperature for 5 minutes or until a clear lysate was achieved. Cellular debris was sedimented by ultracentrifugation at 40k r.p.m. for 45 minutes in a Beckman type 75 Ti rotor. The supernatant fluid was decanted into a plastic centrifuge tube. One gram of CsCl and 0.1ml of a solution of 5mg/ml ethidium bromide was added per ml of supernatant. The protein debris was then removed by centrifugation at 10K rpm (Sorvall SS34 rotor) for 5 minutes. The supernatant was transferred to quick-seal polyallomer tubes and CsCl density gradients were established by centrifugation to equilibrium at 49k rpm in a Beckman VTi65 rotor for 20 hrs at 15°C.
Covalently closed circular plasmid DNA band was visualized with long-wave ultraviolet light and extracted from the side of the tube with a number 21 needle and syringe. To remove the ethidium bromide, the plasmid DNA in CsCl solution was extracted 6 times with water-saturated butanol. The solution was then diluted with two volumes of water; the DNA was precipitated by the addition of ethanol at 6 times the original volume, and solution kept at -20°C for 2 hours. The plasmid DNA was pelleted by centrifugation at 10,000 X g, washed with 80% ethanol, dried in vacuo, and resuspended in T.E. (10 mM Tris-HCl, 1mM EDTA, pH 8.0) buffer.

b. Small-scale (rapid) plasmid isolation.

Small quantities of plasmid DNA were prepared for rapid screening by the method of Holmes and Quigley (54) with modifications. 5 mls of bacteria that were grown overnight were pelleted at 3000 X g for 5 min and resuspended in 0.35 ml of STET2 buffer (8% sucrose, 0.05% Triton X-100, 50mM EDTA, 10mM Tris-HCl(pH 8.0)). To the resuspended cells 25 μl of freshly prepared lysozyme (10mg/ml) was added, mixed briefly and then the tube was placed in a boiling water bath for 40 secs. The tubes were chilled in ice bath and immediately centrifuged at 12,000g for 20 min at 4°C. The supernatant was drawn, 1/10 vol of 3M sodium acetate (pH 4.8) was added and then an equal volume of cold isopropanol was added and the solution was placed at -18°C for 10 min. The DNA was precipitate was pelleted by centrifugation in a microcentrifuge for 5 min at 12,000 g and washed 2 times with ether. The resulting plasmid DNA pellet was resuspended in 50 ul of TE buffer and kept at -18°C.
V. General genetic and recombinant DNA techniques.

Pl transduction for strain construction were performed as previously described (80,102). Similarly, λ phage growth and infection were as described (80,102).

DNA recombinant techniques were performed essentially as described by Maniatis et al (81). Restriction enzyme digestions were for 60 min at 37°C in buffers recommended by the manufacturers. When DNA was to be digested with more than one enzyme, the enzyme reaction requiring lower salt concentration was performed first, then reaction mix was inactivated by heating at 65°C for 10 min, cooled and adjusted by the addition of salt to meet the requirement of the next enzyme in the sequence. After completion of enzyme digestion, pCMS was added to 10 mM to inhibit nuclease activity (without interfering with subsequent ligation reaction) before the usual inactivation at 65°C for 10 min. Otherwise, the digestion reactions were terminated by phenol extraction of proteins followed by ethanol precipitation of the DNA.

In some DNA fragment sub-clonings, the vector DNA after restriction enzyme digestion was dephosphorylated with bacterial alkaline phosphatase (0.1 unit/µg of DNA) at 65°C for 1 hr. The DNA was then ethanol precipitated after phenol-chloroform ether extraction, and then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

DNA fragments from agarose gels were recovered by electro-elution onto dialysis membrane as described in section VII, and purified through either Elutip-D columns according to manufacturer's specification or by chromatography on a DEAE-50 column. The DNA
fragments were further cleaned by phenol-chloroform-ether extraction and then ethanol precipitation. DNA pellet was finally resuspended in TE(10:1) buffer.

Ligation of DNA fragments were performed in ligation buffer (66 mM Tris-Cl, pH 8.0, 1 mM EDTA, 5 mM MgCl₂, 5 mM dithiothreitol, 0.1 M ATP). Optimal ratio of vector DNA to insert DNA was determined as described by Goodman et al (40).

Plasmid DNA transformations were performed as previously described (70). Unless otherwise noted, strain AMC290 was used as the recipient in the DNA transformations.

The orientation and in some cases whether or not a vector has an insert DNA fragment was determined by restriction enzyme cleavage mapping of isolated plasmid DNA as previously described (107).

VI. Bacterial transformations.

Transformation with plasmid DNA followed a modification of Lederberg and Cohen (70) as previously described (101). Competent cells of appropriate host were prepared as follows. A 50 ml L broth culture was inoculated with 0.5 ml of an overnight culture and grown to OD₆₀₀ = 0.2. The cells were chilled on ice, centrifuged and resuspended in 50 mls of cold 0.2 M MgCl₂. After a second centrifugation at 4°C, the cell pellet was resuspended in 25 ml of 0.1 M CaCl₂ and chilled on ice for 20 minutes. A third centrifugation in the cold was carried out, and the cell pellet was finally resuspended in 0.7 mls of cold 0.1 M CaCl₂. Transformation involved mixing 0.1 ml DNA(approximately .05µg up to 1µg) with 0.2 ml of competent cells and incubating mixture on ice for 30 minutes. To aid
DNA uptake the mixture was heat pulsed 30 secs before 30 minute at 37°C ice incubation and after for 2.5 minutes at 42°C. The transformation mixture was diluted 2 to 10 fold with L broth containing 10% glycerol and incubated at either 37°C for 1hr or 30°C for 2 hrs before spreading onto selective agar plates. Transformants were verified by antibiotic resistance and/or phenotype (i.e. temperature resistance).

After initial transformation with a newly constructed plasmid, the plasmid DNA was isolated and purified as described in section IV.b and then used in a second transformation to determine the phenotype of the new plasmid in the appropriate strains.

VII Electrophoretic techniques.

Horizontal agarose gels were prepared in TBE electrophoresis buffer (89mM Tris-borate, 2 mM EDTA, pH 8.0). Loading buffer (6X) contained 0.25% bromphenol blue, 0.25% xylene cyanol and 15% Ficoll in dH2O. After electrophoresis, the agarose gels were stained with a solution of ethidium bromide (0.5 mg/ml) for 1 hr. DNA was visualized on a U.V. trans-illuminator. HindIII digested lambda DNA was used as DNA fragment molecular weight markers.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed utilizing the discontinuous system as described by Laemmli(69), that was modified as described previously(44). The main gel was polymerized in a solution containing 0.37M Tris-HCl (pH 7.8), 0.1% SDS, 0.03% (vol/vol) TEMED, 0.33% (wt/vol) ammonium persulfate, with concentration of acrylamide ranging from 10 to 30% maintaining a N,N'-methylene bisacrylamide-acrylamide ration of 1:173.
10% to 30% linear gradient acrylamide gels were prepared with a gradient maker (Hoefer Scientific Instruments). The stacker consisted of 0.14M Tris-HCl (pH 6.8), 5.7% acrylamide, 0.15% N,N'-methylene bisacrylamide, 0.1% SDS, 0.05%(vol/vol) TEMED, 0.05%(wt/vol) ammonium persulfate. The electrode buffer at pH 8.5 contained 0.05 M Tris-HCl, 0.384M glycine and 0.01% SDS. The gels were run at 20 milliamps at constant current until the dye front reached the gel bottom. Protein extracts were solubilized by heating at 100°C for 5 min (at 65°C for 30 min for certain membrane fractions) in the presence of 1% SDS, 0.625 Tris-HCl (pH 7.0), 5% β-mercaptoethanol, 4M urea, and 0.015% bromophenol blue. Protein in SDS-PAGE were stained and fixed with a solution of 0.25% Coomassie Brilliant Blue, 43% methanol and 7% acetic acid and destained in 43% methanol and 7% acetic acid.

VIII. Extraction of DNA fragments from agarose gels.

DNA fragments were extracted from agarose gels by cutting a slot just ahead of the desired band, placing a piece of dialysis membrane to line the distal side of the slot, filling the slot with 1X TBE and then electro-eluting the DNA into the slot and against the dialysis membrane. The DNA was pulled off the membrane by reversing the current for 30 seconds, and the buffer in the slot was collected. The slot was rinsed two times with buffer which was also collected. The DNA fragments was cleaned by passage through an Elutip-D column (Schleicher and Schuell) or by DEAE-52 chromatography. DNA from column was concentrated by the addition of one-tenth volume 3M sodium acetate (pH 4.8) and ethanol precipitation.
IX. Maxicell labelling of plasmid specified proteins.

The procedure used was a modification of Sancar et al. (99,100). CSR603 was transformed with specific plasmid DNA to be analyzed for protein expression. Three ml's KU medium inoculum of a plasmid containing maxicell strain was grown at 37°C to an OD₆₀₀ of 0.2 (2 X 10⁸ cells/ml). These cells were then diluted 1:50 into 10 ml of fresh KU medium and grown again to an OD₆₀₀ of 0.2. The cells were then transferred to a 6 cm petri dish (Falcon). The dish containing the cells and a small magnetic stirring bar was placed onto a magnetic stir plate. A portable 254 nm ultraviolet light (ultraviolet products, Inc.) was clamped to a stand at a distance of 20 cm from the dish. The top of the petri dish was removed and the vigorously stirred cells were irradiated for 30 seconds. The cells were then transferred to a 125 ml flask, diluted 1:2 with K medium and incubated for 2 hours at 37°C. It should be noted that all incubation were carried out in the dark as the bacteria are photo-reactive. Cycloserine (200 µg/ml) was added to each cell suspension and the incubation with shaking was continued for 13 to 14 hours. An addition 200 µg/ml cycloserine was added during the final hour incubation. The cells were sedimented by centrifugation at 6000 rpm (SS34 rotor) at 4°C. The supernatant was discarded and the cells were washed twice with sulfate-free Hershey medium (50 ml flask) and incubated with shaking for 1 hour at 37°C. The plasmid specific protein were labeled for 1 hr at 37°C with [³⁵S]-methionine (New England Nuclear) which was added at a concentration of 5 µci/ml. The labelled maxicells were pelleted by centrifugation at 6000 rpm and washed twice with 0.1M NaCl to remove non-specific radioactivity. All supernatants and washes were treated as
radioactive waste. The cell pellets were then resuspended in 200 μl of sample buffer and boiled for 5 minutes to denature the proteins.

The cpm/μl of samples were determined in a Beckman LS6800 Scintillation counter. Approximately 500,000 to 750,000 cpm/lane was loaded onto 10% to 30% gradient SDS-Polyacrylamide gel and electrophoresed at 20 mA constant current until the dye front ran off the end of the gel (approximately 16 hours). The gel was then dried at 80°C on a BioRad gel dryer. Dried gels were placed directly against Cronex X-ray film (Dupont).

Development and visualization of the resulting autoradiograms were as previously described (44,101).

X. Plasmid constructions.


The procedure developed by M. Casadaban (20) for mini-Mu transposon mutagenesis of plasmids was used to isolate insertion mutation of plasmid pRGC31. The mini-Mu transposon used, MudII1734 (Fig. 1) is a 9.7 Kb derivation of MudII phage (20) that confers kanamycin resistance (kanR). Plasmid pRGC31 was transformed into strain POII1734 (20) which contained a Mucts and mini-Mu, MudII1734. The strain was heat induced and the resulting phage lysate was used to infect strain YC99. Phage transductants that conferred both the original plasmid resistance (ampicillin from pRGC31) and mini-Mu resistance to kanamycin contained plasmids with mini-Mu inserts. Phage transductants that expressed β-galactosidase activity, indicating a gene fusion with LacZ, were identified with X-gal on
selective plates(YET with kan and amp, 101). The location and orientation of the mini-Mu inserts were determined by restriction enzyme mapping of the isolated plasmid DNA(Fig 6).


Plasmid pGW7(30) is derived from the E. coli plasmid pBR322 and contains a 4.0 Kb EcoRI-BamHI fragment derived from the bacteriophage lambda control region (Fig. 4). This enable foreign genes to be placed under the control of the highly active λ PL promoter. The PL promoter is regulated by the thermolabile CI repressor protein (CI<sub>65</sub>) contained on the plasmid. Also present is the λ N gene whose product allows for efficient readthrough of rho-dependent and rho-independent terminator site.

The plasmid pGW7 and pYC16 were isolated and prepared as described in section IV. a. The plasmid vector pGW7 was completely digested with BamHI and followed by treatment with bacterial alkaline phosphatase. The plasmid pYC16 was partially digested with BamHI (1u/4μg, 3 min at 37°C). The 8.5 Kb BamHI DNA fragment containing the FtsA-LacZ hybrid gene was recovered from an agarose gene and purified as described in section VII. The two DNA fragments were ligated together and then transformed into strain AMC290(Lac). Transformants that grew as blue colonies on X-gal,amp, kan plates were selected. The insertion and orientation of the 8.5 Kb DNA fragment was determined by restriction enzyme mapping of one isolated plasmid DNA, which was designated pYC3.
c. Construction of promoter fusion plasmids.

The *ftsZ* regulatory region gene fusions were performed by subcloning the appropriate purified restriction fragment into the promoter probe plasmid, pKK232-8 (16, Fig. 5) that give transcription fusions to the chloramphenicol acetylase gene (CAT). pRGC31 was used as the source of *ftsZ* DNA.

pYC500 consists of an agarose gel purified 1.7Kb *BamH1-HindIII* restriction fragment from pRGC31 that was ligated into *BamH1, HindIII* digested pKK232-8. Carb<sup>r</sup>, Cm<sup>r</sup>(25ug/ml) transformants were verified by restriction enzyme analysis of *BamH1 - HindIII* digestions and *BgIII* digestions.

In pYC530 the *BamH1-BgIII* DNA fragment of pYC500 was deleted by a double digestion and ligation. Again, restriction analysis of carb<sup>r</sup>, cm<sup>r</sup> transformants verified the construction.

pYC580 consists of an agarose gel purified 0.5Kb *HindIII-EcoR1* restriction fragment from pRGC31 in which the overlapping DNA ends were filled in with DNA polymerase I and then blunt end ligated into *SmaI* ends of pKK232-8 that were dephosphorylated. One carb<sup>r</sup>, cm<sup>r</sup> plasmid transformant contained the proper DNA fragment in the desired orientation based on *HindII* and *BssHII* double digestions.

pYC600 consists of an agarose gel purified 2.2 Kb *BamH1-EcoR1* restriction fragment from pRGC31 in which the overlapping DNA ends were filled in with DNA polymerase I and then blunt end ligated into the *SmaI* ends of pKK232-8 that were dephosphorylated. Seven carb<sup>r</sup>, cm<sup>r</sup> transformants contained the proper fragment in the desired orientation based on *HindII-BgIII* double digests and *HindIII* digest of plasmid DNA.
pY620 consists of an agarose gel purified 1.2 Kb BgIII–EcoRI restriction DNA fragment form pRGC31 in which the overlapping DNA ends was filled in with DNA polymerase I and then blunt end ligated into the Smal ends of pKK232-8 that was dephosphorylated. Plasmid transformants were verified to contain the proper DNA fragment based on HindIII digestions.

XI. Purification of FtsA-LacZ hybrid protein.

The purification of FtsA-LacZ fusion protein was by β-galactosidase affinity columns as described by Bastia(47) and Ullmann(121) with some modifications.

E. coli cells AMC290/pY3C were grown in YET broth at 30°C in the presence and carb(25 μg/ml). The λ pL promoter of pY3C was induced by heating to 43°C when the culture reach an OD₆₀₀ of 0.3. After 2.5 hrs at 43°C the cells were harvested by centrifugation at 6000 g for 5 min. Pellet was suspended so that a gram of cells was in 2 mls of buffer I (0.2M Tris-HCl, pH 7.0/ 0.25M NaCl/0.01M magnesium acetate/ 10 mM 2-mercaptoethanol/ 5% glycerol/ 1mM PMSF). This cell suspension was frozen at -80°C and thawed quickly in a 30°C water bath. Pancreatic DNase and RNase was added at 10 μg/ml each. The cell suspension was then disrupted by two passages at 10,000 psi through a pre-chilled French pressure cell. Unbroken cells were removed by centrifugation for 10 min. at 2000 X g. The lysate was clarified by centrifugation at 35,000 rpm for 30 min at 4°C in a type 75 Ti rotor(Beckman). The β-galactosidase activity in the cleared lysate was determined as described(83).
The protein from above cleared lysate was salted out with ammonium sulfate. Ammonium sulfate crystals were added slowly with stirring to 40% saturation. The precipitate was collected by centrifugation and the pellet was suspended in buffer II (10mM Tris, pH 7.0/10mM magnesium acetate/0.2M NaCl/0.1M KCl/0.1mM EDTA/1mM DTE/0.1mM PMSF) and dialysed against 100 vol of the same buffer for 3 hrs with 3 buffer changes.

The above dialysate was mixed with 5M NaCl to obtain a final concentration of 1.5M NaCl. A volume of 2 ml of this solution was applied to 2 ml bed volume of p-aminobenzyl-β-D-thiogalactopyranoside-agarose(NH2BenSGal-Agarose) column which was equilibrated with buffer III (10mM Tris, pH 7.0/1.5 M NaCl/0.1M KCl/0.1M EDTA/1mM DTE/0.1mM PMSF). The flow rate was 1 ml/hr. The column was washed with buffer III (about 100 bed volumes) until no more material absorbing at 280nm appeared in the flow through. The protein retained was eluted from the column with 0.1M sodium borate, 10mM β-mercaptoethanol, pH 10, and the proteins was promptly precipitated with ammonium sulfate. This precipitate was collected by centrifugation and the protein was resuspended in buffer IV (40mM Tris-HCl, pH 7.5/1mM MgCl2/0.1mM EDTA/1mM DTE/50% glycerol) and kept at -80°C. Before injection into rabbits the protein was dialysed against phosphate buffered saline(PBS; 10mM phosphate buffer/150mM NaCl, pH 7.4).

XII. Production of FtsZ-LacZ Antibodies

Antiserum against the FtsA-LacZ fusion protein was produced using a immunization protocol previously described(98). A rabbit was
injected intradermally with antigen (500 μg) emulsified in an equal volume of complete Freund adjuvant (Difco). After 4 week, the rabbit was given a booster injection of another 500 μg of antigen in same volume with incomplete Freund adjuvant. To insure high titer of FtsA specific antibodies, the rabbit was given a second booster injection with 200 μg of FtsA-LacZ hybrid protein isolated from SDS-PAGE gels. Strips of acrylamide excised from gels which had been stained and dried were rehydrated in PBS buffer and macerated in a mortar and emulsified in an equal volume of incomplete adjuvant.

Blood was obtained before the initial injection for control experiments (pre-immune serum). Antibody production against FtsA-LacZ was titered at 1 week intervals after the booster injection. Detection and titer of the blood antiserum were carried out by ring precipitin and dot blotting tests (17) with a constant dilution of antigen. Pure immunoglobulin, IgG antibody fraction, was prepared from the serum by chromatography on protein A-sepharose CL-4B column according to manufactures procedure. Affinity purified rabbit IgG was dialysed against PBS buffer containing 0.02% sodium azide overnight with four changes of buffer. To enhance the specificity of immunodetection, the IgG antibodies were absorbed against a cleared lysate of YC100 (ftsA10 mutant) prepared after a temperature shifted to 42°C for 2 hrs.

XIII. Western blot analysis.

Polypeptides separated on a 10 to 30% gradient SDS-PAGE gel were electrophoretically transferred to a Nytran Sheet (Schleicher &
Schuell) at 250 mA for 3 hrs by the method of Burnette (17) with the following modifications.

The transfer buffer consisted of 20 mM Tris-Cl, 150 mM Glycine, pH 8.0. Transfer was monitored by prestained molecular weight standards (BRL). The immobilized polypeptides were blocked in TBST buffer (0.1M Tris-Cl, pH 7.5, 0.1M NaCl, 0.05% v/v Triton X-100) containing 10% (w/v) bovine serum albumin (BSA) overnight at 48°C. The blocked membrane filter was then incubated with appropriate dilutions of antibodies (1:1000) in TBST buffer containing 0.5% BSA for 2 to 4 hrs. After being washed in six changes of buffer (10 min each), the membrane sheets were incubated for 1 hr with alkaline phosphatase linked-goat anti-rabbit IgG (1:7500 dilution) and then washed as described above. All the above steps were performed with gentle agitation at room temperature throughout unless otherwise noted.

Antibody reactivity was detected with color development by the substrates BCIP and NBT. If labelled proteins were used, the resulting immunoblot was subjected to autoradiography after being dried.

XIV. Cell fractionation procedure.

Membrane, cytosol and Sarkosyl insoluble membrane fractions were prepared as described previously (44,101). Ten mls of cells or labelled maxicells with plasmids were pelleted by centrifugation at 12,000 X g for 10 min, suspended in 5 ml of 10 mM phosphate buffer, pH 7.4, centrifuged again, and finally resuspended in 2 mls of the same buffer. The cells were then lysed by sonic oscillation. The un-lysed cells were removed by centrifugation at 2000 X g for 10 min. This
cleared lysate was fractionated into cytosol and membrane fraction by centrifugation at 20,000 X g for 15 min. The envelope-containing pellet was then suspended in 2 mls of buffer. One half (1 ml) of the envelope pellet preparation was further fractionated into Sarkosyl insoluble outer membrane by being solubilized with 1% Sarkosyl at 37°C for 20 min. The total membrane and Sarkosyl-insoluble membrane preparations were centrifuged again at 20,000 X g for 15 min and resuspended in phosphate buffer. The soluble cytoplasmic proteins were concentrated by precipitation with 10% trichloroacetic acid, washed twice with 80% cold acetone and resuspended in smaller volume of phosphate buffer.

Proteins from the above fractions were separated by PAGE-SDS gels, and in case of labelled proteins from maxicells were detected by autoradiography.

XV. Isolation of membrane fractions OM₅, OM₅, IM.

Cell fractionation of membrane envelope into OM₅, OM₅, IM was performed by the procedure of Ishidate et al (59) as briefly described below.

Cells were grown at 30°C in YET medium (1 l) to OD₆₀₀= 0.4, rapidly cooled and then centrifuged at 2000 X g for 10 min. Pellet was resuspended in 10 mls of 20% sucrose and pancreatic DNase and RNase (10 μg/ml each) were added. The cell suspension was then mechanically disrupted by two passages at 1000 psi through a pre-chilled French pressure cell. (American Instrument Co., Silver Spring, MD.). After removal of unbroken cells by centrifugation for 30 min at 2000 X g, EDTA was added to the supernatant fraction to a final concentration of
5 mM. The supernatant (1.7 ml) was layered onto a two step gradient (SGO) consisting of 0.8 ml of 60% sucrose and 2.5 ml of 25% sucrose. The crude membrane fraction, isolated from SGO by centrifugation in a Beckman SW50.1 rotor at 40K rpm for 3.5 hrs, was diluted with 10 mM Hepes, pH 7.4, 5 mM EDTA to a refractive index of 1.365-1.37 at 20°C. The resulting suspension (2.0 ml) was then applied to the top of the following sucrose gradient (SGI): 60% (0.5 ml), 55% (0.8 ml), 50% (2.2 ml), 45% (2.2 ml), 40% (2.2 ml), 35% (1.4 ml), 30% (1.0 ml). The gradient was centrifuged in a Beckman SW41 rotor at 36K rpm for 16 hrs. Fractions (0.3 to 0.4 ml) were collected from the top using a fraction recovery system (Beckman) and refractive index was determined with a refractometer (Bausch & Lomb). Based on the refractive index, each membrane fraction was pooled and concentrated by centrifugation at 50K rpm for 3.5 hrs after being diluted with 3 volumes of 10 mM HEPES buffer (pH 7.4), and the final pellets were resuspended in 10 mM HEPES buffer.

Total cytosol and membrane fractions were prepared by centrifugation of the supernatant (3 ml) obtained above by centrifugation at 100,000 X g for 1.5 hr after dilution with 2 volumes of 10 mM HEPES, pH 7.4. Pelleted membrane was resuspended in same buffer and soluble proteins were concentrated by precipitation with 10% cold trichloracetic acid (TCA). The TCA precipitate was allowed to stand on ice for 20 min, pelleted by centrifugation, washed twice with 80% acetone, and finally resuspended in same buffer.

Approximately equal amounts of protein from each sample (50 µg) were loaded onto SDS-PAGE gels used for protein separation and Western immunoblotting as described above.
XVI Immunogold labelling and electron microscopy.

Immunogold labelling was performed as described by Bayer et al. (6) and by Reid et al. (93) with following modifications.

Cells (AMC290) were grown in YET broth at 30°C to mid-log phase and a portion of cells were then fixed with an equal volume of L broth with 4% formaldehyde and 0.2 M cacodylate-HCl buffer (CD), pH 7.2 for 30 min. The fixed cells were collected on a filter by drawing about 2 mls of the suspension of fixed cells into a 5 ml syringe and passing through an attachable filter holder containing a 13 mm diameter polycarbonate membrane filter (0.40 um). The filter was washed with 3 ml of 0.1M CD buffer and then dehydrated with graded alcohol series (50% to 100%). Dehydration was started in 50% ethyl alcohol. 10% increases of alcohol concentration were used in the dehydration steps. The filter membrane was removed from the holder, infiltrated in LR write resin at room temperatures for 1 hr (two changes of LR white resis) and embedded in fresh accelerated LR white resin in 30 min. on ice.

For gold-labelling, ultrathin sections were placed on parlodion and carbon coated copper grids. These grids were blocked with TBST/5% BSA buffer (pH 7.5) for 30 min to saturate nonspecific protein binding sites and then reacted for 1.5 hr with affinity purified FtsA specific antibodies (1 μg/ul) in dilutions of 1:50 in TBST/1% BSA buffer. In parallel, sample grids were processed with preimmune IgG antibodies (0.5 μg/ml) in dilutions of 1:25 as controls. After five subsequent washings in TBST/1% BSA buffer (5 min each), the sections were floated on goat anti-rabbit IgG 10 nm gold conjugate (Janssen's) in TBST/1%
BSA buffer. The grids were again washed 5 times in TBST/1% BSA buffer and rinsed in distilled water.

Sections were stained in 1% uranyl acetate before being examined with a JEOL 100CX transmission electron microscope at 80 KV.

Ultra thin sections of IPTG induced cells (YC200) were immunogold-labelled with FtsA-LacZ antibodies as described above.

XVII. Preparation of cell extracts for enzyme assays.

Cell extracts for CAT/β-lactamase assays were prepared by the method of Lupski et al (72). Cells containing plasmid were inoculated in L broth supplemented with 0.2% glucose and ampicillin(50 µg/ml) or carbenicillin(25 µg/ml). An overnight culture was diluted 100 fold in fresh broth and grown to an OD₆₀₀ = 0.2 to 0.5 (Spectronic 20, Baush and Lomb). Six mls of cells were collected in a 15 ml corex tube by centrifugation at 7000 rpm for 10 min in Sorvall SS34 rotor. The cells were washed in 2 ml of 50 mM Tris, 30 µM DTT, pH 7.8, resuspended in 600 µl of same buffer and transferred to 1.5 ml Eppendorf tubes. The cells were frozen by placing at -70°C for 1 hr. Cells were then thawed and sonicated with 2 or 3 pulses of 10 sec with at least 15 sec rest between pulses using a MSE sonicator with a micro-tip. To remove cell debris, the cell extracts were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. The supernatant was placed in another Eppendorf tube and kept on ice for subsequent enzyme assays or at -20°C for storage.
XVIII Enzyme assays.

a. β-galactosidase assay

β-galactosidase activity was determined using o-nitrophenyl-
galactoside (ONPG) as a substrate according to the method of
Miller(83). An aliquot of the sample to be assayed was added to Z
buffer (final volume 1 ml) containing 60 mM Na₂HPO₄-7H₂O, 40 mM
NaH₂PO₄-H₂O, 10 mM KCl, 1mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0.
If the sample consisted of intact cells, 40 ul CHCl₃ and 20 ul 0.1%
SDS were added and vortex for 10 secs. Sample tubes are incubated in
a water bath at 28°C for 5 minutes for equilibration. The reaction
was started by adding 0.2 ml of ONPG(4 mg/ml) and stopped by adding
0.5 ml of 1M Na₂CO₃ solution after sufficient yellow color had
developed. The optical density at both 420nm and 550nm for each tube
was determined and the units of β-galactosidase activity was
calculated from the following formula:

units = 1000 X (OD₄₂₀ - 1.75 X OD₅₅₀)/t X v X OD₆₆₀.

Where t is length of reaction time in minutes and v is the volume of
the enzyme extract used per ml of assay mixture.

b. CAT Assay.

Cell extracts as described in section XVII were used in the CAT
assay. The reaction mixture was freshly prepared by dissolving 8 mg
of DTNB (5,5'-dithiobis-2-nitro benzoic acid) in 2.0ml 1.0 M Tris-HCl,
pH 7.8, adding 0.4 ml acetyl-CoA (5 mM) stock solution, and adjusting
the total volume to 20 mls. 600 µl of the reaction mixture was placed
in a reference cuvette and sample cuvette in a Beckman double beam
recording Model 30 spectrophotometer. 20 µl of enzyme extract was
added to the reaction mixture and the $A_{412}$ was adjusted to zero, then measured for 30 sec to 3 min using the spectrophotometer recorder to obtain the background activity. To start the reaction, 12 μl of 5 mM chloramphenicol was added. The increased absorbance at $A_{412}$ was recorded for about 5 min. The CAT enzyme specific activity (in nmol/min/mg) was determined by dividing nmol/min·ml by the protein concentration(mg/ml) as follows:

$$\text{nmoles/min/mg} = \Delta A_{412} \times \frac{1}{0.0136} \times \frac{1}{\text{mg protein}}$$

C. β-lactamase Assay.

Cell extracts as described in section XVII were used. 600 μl of a reaction mixture consisting of 41.5 μg/ml cephaloridine (10^{-4}M) in 0.1M phosphate buffer pH 7.0 was placed in a sample cuvette. Spectrophotometer was blanked against 0.1M phosphate buffer pH 7.0 and the $A_{255}$ was recorded for 30 sec. to get a base-line level. The absorbance of the cephaloridine mixture ($A_{255}$) was between 1.2 and 1.4. To start the reaction 20 μl of the enzyme extract was added to the reaction mixture and the $A_{255}$ was recorded for approximately 5 min. A fall of the optical density from 1.4 to 0.6 would indicate complete hydrolysis. Specific activity for β-lactamase (in nmole/min/mg protein) was calculated by ($\Delta A_{255}$/min) X 375/mg protein.

An alternate substrate used was PADAC. Again a 600 μl reaction mixture was used consisting of 15.6 μg/ml PADAC (2.8 X 10^{-5}M) in 0.05 M phosphate buffer, pH 7.0. PADAC hydrolysis was measured at $A_{566}$. The Specific activity for β-lactamase in nmole/min/mg protein was determined by ($\Delta A_{566}$/min) X (1/0.0605/mg protein).
XIX. Protein Assay.

Amount of protein was measured by the method of Bradford(15) using the Biorad Protein Assay dye reagent concentrate. Bovine serum albumin (Sigma) was diluted and used for protein standard concentrations.

XX. Plasmolysis of bacterial cells

Plasmolysis of bacterial cells were performed by a modified method of MacAlister and Rothfield(128). A strain AMC410(ftsZ84) grown in YET broth overnight at 30°C were diluted (1:100) in fresh medium. A portion of the growing cells at 30°C (OD600 = 0.2) were shifted to 42°C for 90 min. Culture was harvested by centrifugation in a microfuge for 1 min. The pellet was suspended in 0.1 volume of fresh culture medium at room temperature. A portion of this cell suspension was plasmolyzed by adding equal volume of plasmolyzing solution (26% sucrose(wt/vol), 0.2 M cacodylate-Cl buffer, pH 7.2). These cells were kept at room temperature for 3 min before they were fixed by the addition of equal volume of 4% formaldehyde in plasmolyzing buffer. The cell suspension was allowed to stand for an additional 60 min. at room temperature, before being centrifuged for 30 sec in microfuge and being suspended in fresh plasmolyzing solution. Unplasmolyzed cells were also fixed with formaldehyde in parallel. Micrographs were taken with a phase contrast microscope (BH-2; Olympus, Splan 100pl objective) with a 35 mm camera. Portion of the fixed plasmolyzed and unplasmolyzed cells were thin sectioned and immunogold-labelled as described in section XVI.
RESULTS


pRGC31 plasmids with mini-Mu, MudIII1734, insertions were selected as Mu-ductants that were both amp<sup>r</sup> and kan<sup>r</sup> and grew as light blue colonies on X-gal indicator plates. Approximately 1% of the total kan<sup>r</sup> colonies expressed β-galactosidase indicating an in-frame protein fusion with lacZ. Of these approximately one in fifty (2%) were found to be within the ftsA gene by restriction enzyme mapping. The mini-Mu insertion sites of four isolated ftsA-lacZ plasmids are shown in Fig. 6. In plasmid pYC40, MudIII1734 transposon was inserted approximately 550 bases from the start of the ftsA amino-terminal end. In plasmid pYC39, the transposon insertion was approximately 650 bases, and in plasmids, pYC28 and PYC16, the mini-Mu was inserted 850 bases from amino-terminal end.

Temperature sensitive ftsA mutant strains, TKF10 or YC100, remained temperature sensitive at the nonpermissive temperature (42°C) when the ftsA-lacZ fusion plasmids were introduced. However, the fusions may retain some functional interactions. When the fusion plasmids, pYC39 and pYC40, were transformed into strain YC100 it became filamentous at the permissive temperature (30°C). Some filaments were observed with the wild type strain, AMC290, when transformed with pYC39 and pYC40. Interestingly, plasmids encoding slightly larger protein fusions, pYC16 and pYC28, did not effect septation in AMC290.
II. Expression of FtsA-LacZ fusion polypeptide in maxicells.

The synthesis of FtsA-LacZ fusion proteins was confirmed by labelling the plasmid specified proteins using the maxicell labelling procedure\(^{99,100}\). Autoradiograms of the \(^{35}\)S-methionine labelled polypeptides encoded by several plasmids in strain CSR603 is shown in Fig. 7. On this gel system, the FtsA protein migrates as a 48 kD polypeptide, FtsZ as a 45 kD polypeptide and \(\beta\)-lactamase as a 28 kD polypeptide\(^{(99)}\). All transformants with presumed \(ftsA-lacZ\) fusion containing plasmids synthesized large polypeptides migrating near the top of the SDS-PAGE gel with concomitant loss of the FtsA polypeptide band\(^{(100)}\). Plasmid pYC40 specified a new 142 kD fusion peptide. Plasmids, pYC28 and pYC16 encoded 154 kD fusion peptides. Plasmid pYC39 specified a hybrid peptide of 146 kD (data not shown).


It was imperative to determine whether the amino-terminal end of the FtsA protein could localize the FtsA-LacZ fusion polypeptide similar to the FtsA protein. Native FtsA protein was labelled in maxicells containing pRGC31\(^{(99)}\). CSR603 strains containing pYC28, pYC40 or a control plasmid pRGC43 which encodes \(\beta\)-galactosidase and FtsA were also labelled. The labelled maxicells were fractionated into membrane and cytoplasmic fractions by centrifugation and part of the membrane fraction was further extracted with 1% Sarkosyl detergent to solubilize the inner membrane proteins. Table 3 presents the quantitative data as relative molar yields of labelled proteins from an electrophoresis autoradiogram. The \(\beta\)-galactosidase peptide
specified by pRGC43 was localized in the cytoplasmic fraction with only a small contaminating amount visible in the membrane fractions. The FtsA protein, specified by both pRGC43 and pRGC31, fractionated almost equally between the cytoplasmic fraction and the total membrane fraction. Furthermore, approximately 50% of the membrane fraction of FtsA remained after Sarkosyl extraction. The FtsA-LacZ fusion proteins specified by plasmids pYC28 and pYC40 behaved identically to the FtsA protein. The presence of FtsA and FtsA-LacZ labelled proteins being found in both the soluble and membrane fractions is most likely a consequence of the maxicell protein labelling procedure. Labelled proteins are likely to be synthesized in excess, and maxicells are division arrested due to extreme chromosome DNA damage.

Since the FtsA-LacZ fusion protein has β-galactosidase activity, this activity could be used to localize the FtsA-LacZ protein in wild type cells. We fractionated the membranes of strain AMC290 containing fusion plasmids by the procedure of Ishidate et al.(59). This procedure enables the isolation of unique membrane fraction called "outer membrane-light(OM_L)" which contains the inner-murein-outer membrane fraction. The β-galactosidase activity from the final isopycnic sucrose gradient was determined for the different density fractions collected (Fig. 8). Most (80%) of the β-galactosidase enzyme activity in membrane preparation of the FtsA-LacZ fusion specified by pYC40 was found in the two fractions corresponding to OM_n(density 1.24 to 1.26) and OM_L(density 1.21-1.23). A small amount of LacZ specified β-galactosidase activity was found in the inner
membrane(IM) fraction and slightly lighter fraction (density 1.16 to 1.12). Similar results were obtained with cells containing fusion plasmid pYC16.

IV. **Overproduction and purification of FtsA–LacZ protein.**

In order to produce large quantities of the FtsA–LacZ hybrid protein, the 8.5 Kb *BamH1* fragment of plasmid pYC16(23) encoding the hybrid protein was subcloned into the expression plasmid pGW7(30). Plasmid pYC16 was partially digested with *BamH1* and the 8.5 Kb fragment was subsequently recovered from a preparative agarose gel and ligated into *BamH1* digested and dephosphorylated pGW7 DNA. One kan^R^ carb^R^ colony contained a plasmid, designated pYC3, which had the 8.5 Kb insert in the correct orientation as determined by restriction enzyme digestion with *BgIII* (Fig.9).

When strain AMC290 (Δlac) containing pYC3 are temperature shifted from 30°C to 42°C, transcription of the *ftsA–lacZ* DNA was induced resulting in increased fusion protein production. The level of hybrid protein production was measured by β-galactosidase specific activity. The optimal β-galactosidase activity was found after 2.5 hr at 42°C. These temperature shifted pYC3 containing cells accumulated up to 1% of the total cell protein as FtsA–LacZ protein. β-galactosidase activity was approximately 10 fold higher (7230 U/mg) after induction (687 U/ml) and 20 fold higher than same cells containing the original fusion plasmid pYC16(347 U/mg).

The hybrid protein was purified from the soluble fraction of disrupted cells by two purification step: 40% (NH₄)₂SO₄ precipitation cut and affinity chromatography on p-aminobenzyl-β-D-thio-
galactopyranoside-agarose. In Fig. 10, the composition and degree of purity of the total soluble proteins, the 40% (NH₄)₂SO₄ fraction and the affinity-purified fractions separated by SDS-PAGE are shown. The 40% ammonium sulfate precipitant fraction retained approximately 90% of total β-galactosidase activity and there was an enrichment of the hybrid protein (Fig. 10, lane D). The hybrid protein had the expected size compared to β-galactosidase molecular weight marker (Fig.10, lane E), and was identical to fusion protein specified by plasmid pYC16 (which had been labelled by maxicell labelling). A β-galactosidase polypeptide band, that was probably a degradation product of the fusion protein, was always present in the affinity-purified protein fraction. This protein degradation may be due to enzymatic activity occurring during the purification which was performed at room temperature (to increase the binding of the fusion protein to the affinity matrix). The cleavage of fusion to β-galactosidase polypeptide can be expected because FtsA-LacZ fused protein is likely to have two distinct protein domains.

V. Identification of wild type FtsA protein by Western blots.

Polyclonal antibodies from rabbit against the FtsA-LacZ fusion protein antigen were prepared. The antiserum contained a significant titer of antibodies to both the FtsA-LacZ and LacZ determined by the ring precipitin tests and by dot blot analysis (data not shown). Western immunoblotting experiments were performed with an FtsA-LacZ immunoglobulin fraction (a final protein concentration of 1 mg/ml that was purified from serum using a protein A affinity column).
To test wild type FtsA protein cross reactivity with the FtsA-LacZ antibodies, the FtsA protein was \[^{35}\text{S}]\text{methionine}
labelled using maxicell containing plasmids encoding FtsA before Western immunoblotting was performed. \[^{35}\text{S}]\text{methionine}
labelled FtsA polypeptides, visualized by autoradiography, was also found by Western blotting to be enzymatically labelled by FtsA-LacZ antibodies (Fig. 11B). As expected, both \[^{35}\text{S}]\text{methionine}
labeled fusion polypeptides specified by plasmids pYC16, PYC40 (Fig. 11D, lane 3 & 4) and labeled \(\beta\)-galactosidase
specified by control plasmid pRGC43 also cross reacted with FtsA-LacZ antibodies (Fig. 11D, lane 2). The FtsA-LacZ fusion
proteins synthesized in maxicell appeared to be unstable because a strong antibody cross-reactivity band similar in mobility to
\(\beta\)-galactosidase was observed upon Western blotting. Thus, the results indicated that the polyclonal antibodies from FtsA-LacZ protein
antigen was specific for FtsA, FtsA-LacZ and LacZ polypeptides.

Additional immunoreactive bands which stained less intensely (Fig. 11B, lane 1 & 2) were visible in total cell extracts. One at 37
Kdal and several small peptides at the bottom of the Western blot. These bands, however, were not observed with the soluble protein
fraction probed by Western blotting with FtsA-LacZ antibodies. That is, these extraneous bands appeared in only the membrane fractions.
These bands could be the result of cross reactivity of FtsA antibodies with other membrane proteins, modified FtsA protein or its breakdown
products. Nonspecific binding could not be completely excluded because preabsorption to remove nonspecific antibodies were performed
with a cleared lysate. However, immunoglobulins from pre-immune serum
did not show any reactivity in Western blots of membrane proteins under the same experimental conditions (data not shown).

To determine whether the FtsA protein synthesized in wild type cell could be detected with the FtsA-LacZ antibody preparation, total cellular polypeptides from a Lac deletion strain AMC290 were separated on SDS-PAGE and Western blotted with several concentration of FtsA-LacZ antibodies (data not shown). The amount of FtsA within a wild type cell was apparently too small to be detected in a total cell extract. Wild type FtsA protein was detected in an immunoblot of protein from a 40% ammonium sulfate pelleted fraction of AMC290/pYC3 which was temperature shifted to overproduce the FtsA-LacZ fusion peptide (Fig. 12). Apparently, this plasmid containing strain either synthesized more FtsA or wild type FtsA became more stable because of the FtsA-LacZ fusion protein synthesis. However, this experiment gave us confidence that we could detect wild type FtsA if a cell fraction was enriched for FtsA.

VI Cellular localization of FtsA protein.

Membrane fractionation studies with FtsA-LacZ expressing strains revealed that β-galactosidase activity was associated with membrane fractions corresponding to outer membrane heavy (OMₘ) and outer membrane light (OMₗ). Previous studies above demonstrated that we can detect FtsA protein by Western blotting. Thus, to verify that native FtsA protein indeed was enriched and localized to membranes in wild type E. coli cells two experimental approaches using the FtsA specific polyclonal antibodies were performed: A. Western immunoblots of cell fractions and B. Immunoelectron microscopic localization.
A. Western blots of cell fractions.

AMC290Δlac74 cells were fractionated into cytosol and envelope membrane fractions first by centrifugation. The protein from both fraction were separated by SDS-PAGE, blotted onto Nytran membrane filters and probed with FtsA-LacZ antibodies. A band in the envelope fraction, corresponding in molecular weight to native FtsA, interacted with FtsA-LacZ antibodies but not in the soluble fraction (Fig. 13, lanes 5, 6, 8, 9).

Increased β-mercaptoethanol concentration from 1 to 5% in PAGE buffer in attempts to remove the cross antigenic smearing that was found with SDS-PAGE of the envelope fraction (Fig 13) resulted in the labelling of two bands (60Kdal and 52Kdal) above the 48K FtsA band. In addition, another band (37Kdal) below the FtsA band was labelled in SDS-PAGE of envelope and soluble fractions(Fig. 14, lane E, F). Two bands corresponding to 33Kdal and 23Kdal polypeptides and one immunoreactive band at the bottom of the gel were also labelled in SDS-PAGE of the envelope protein fraction. The later two bands, 33Kdal and 23Kdal, are probably degradation products of FtsA. This is because these polypeptide bands are observed, although less intensively, as [35S]methionine labelled polypeptides of maxicell containing plasmid pRGC31(specifying FtsA).

The cell envelope was further fractionated into specific membrane domains using neither lysozyme or EDTA but French pressure cell lysis followed by sucrose floatation steps and isopycnic sucrose gradients as described previously (section III). OMH, OMl and IM membrane fractions were separated on SDS-PAGE(10-30%) and the proteins
were electro-transferred to Nytran sheet. Fig. 13 and 14, show the result of Western blotting with FtsA-LacZ antibodies. In Fig. 13 (lanes 1,2,3,4) the data revealed that native FtsA cross reactivity to FtsA-LacZ antibodies were found in OM$_H$ and OM$_L$ fraction of AMC290 (OD$_{600}$=1.6). This result is consistent with the FtsA-LacZ fusion studies. Similar results were obtained after Western immunoblotting membrane fractions of YC100(ftsA0) strain that was shifted to 42°C for 2 hrs. This indicated that ftsA0 does make a cross reactive protein which is incorporated into the envelope but probably is not functional.

When B-mercaptoethanol concentration was increased in PAGE buffer, the cross reactivity to FtsA was found not only in OM$_L$ fraction but also in IM fraction (Fig. 14, lane A,B,C,D) of AMC290(OD$_{600}$=0.4). The labelling of the cross reacting peptides were due to antibody specificity and not due to any spurious enzyme-conjugant labelling because similar results were obtained when Western immunoblotting was performed with a different secondary antiserum, $^{125}$I-labelled goat anti-rabbit IgG under same experimental conditions.

Fig 15 presents the SDS-PAGE profile of Coomassie blue stained polypeptides which Western blotted and presented in Fig. 14.

The membrane fractions (Fig.14,lanes A,B,C,D) contained additional immunoreactive labelled bands similar to labelled bands observed in total membrane fraction and the soluble fraction (Fig 14, E,F). To exclude the possibility that the appearance of these additional bands was due to nonspecific binding (for example, cross reactivity to the major outer membrane protein, OmpA(37Kda1)), FtsA-LacZ IgG fraction was preabsorbed twice with a lysate containing cell
membrane from SGO fraction (see Material and Methods) to remove nonspecific antibodies. The result of Western blotting with this IgG fraction is shown in Fig. 16. The labelling intensity of the additional bands and also of the FtsA band decreased. These results, although not conclusive, indicated that the several additional immunoreactive bands are not result cross reactivity of FtsA-LacZ antibodies with any major *E. coli* membrane proteins. It is more likely they are precursors, breakdown products or modified forms of FtsA protein.

The presence of 4M urea in the sample solubilization buffer was required to cleanly detect the FtsA protein by Western blotting. If urea was left out of sample buffer, FtsA band was not visible and cross immunoreactive material migrated near top of gel. The urea effect, probably indicates that FtsA aggregates with other membrane protein or itself even in the presence of 1% SDS. (Increased SDS in solubilization buffer resulted in the inability of FtsA-LacZ antibodies to cross-react with gel proteins.)

B. Immunogold labelling and electron microscopy.

a. Wild type cells.

Direct immuno-electron microscopic observations of FtsA antibody using goat anti-rabbit immunoglobulin conjugated with colloidal gold to mark *E. coli* cell bound FtsA antibody were performed. Basically, ultrathin sections of AMC290 cells were isolated by low temperature embedding of cells in LR white resin with accelerator at 4°C and reacted with FtsA-LacZ antibodies. Binding of
these antibodies was detected with goat anti-rabbit IgG conjugated to gold particles.

In fig. 17(A,B,C,D) the gold particles were found mostly as aggregates in the envelope with few being found in the central cytoplasmic region. No gold particles were found in samples treated with preimmune antibodies (Fig. 17; E,F) instead of FtsA-LacZ antibodies. The gold particles appeared to be aggregated at sites that appear to be septa or one polar end of the cell.

As a control, β-galactosidase was induced with IPTG (YC200, lac+) and the cells were thin section and treated as above. Most of the gold particles were found throughout the cytoplasm (Fig. 17; G,H).

b. Plasmolyzed cells.

Potential septation sites can be identified by phase contrast microscopy and by electron microscopy using plasmolyzed bacterial cells (78). The resulting plasmolysis bays define potential septal sites and a cellular region consisting of inner-outer membrane adhesion zone. To determine whether inner-outer membrane regions within Fts filaments could be visualized, cells were plasmolyzed and electron micrographs of ultrathin sections were analyzed. As described previously, ftsZ84(ts) cells at elevated temperature results in the formation of long filaments without any visible septa. When ftsZ(AMC419) mutant filaments were plasmolyzed, multiple plasmolysis bays were visible along the length of the filament (Fig. 18). The various sized nonseptal plasmolysis bays appeared at a near cell length intervals expected if bays define potential septation sites.
However, the number and distance of the plasmolysis bays along the length of the filaments varied somewhat with sucrose concentration and with growth conditions. Similar distribution patterns of plasmolysis bays were observed by phase contrast microscopy of ftsA10(ts) or ftsI(ts) filaments which also have some visible constrictions identifying septal sites (data not shown).

To study distribution of FtsA protein in ftsZ nonconstricting filamentous cells direct immunoelectron microscopy was performed. The electron micrographs (Fig. 19) show that gold particles representing antibody bound FtsA were found mostly as aggregates at intervals along the envelope of the nonseptal filaments.

Immunoelectron micrography of plasmolyzed ftsZ filaments, to locate inner-outer membrane regions, were also analyzed under same experimental conditions. The micrograph in Fig. 20 reveals that gold particles were distributed as aggregates at inner-outer membrane junction sites defined by the plasmolysis bays. Furthermore, the gold particles were clustered at regular intervals along the length of the filament similar to the non-plasmolyzed cells (Fig. 19). These results confirm that FtsA proteins are associated with potential septation sites.

VII. Investigation of ftsZ gene expression.

A. Promoter gene fusions and quantitation assays.

The vector used in all of the promoter fusion experiments was pKK232-8 (16). It contains a chloramphenicol acetylase (CAT) structural gene whose natural promoter has been deleted (Fig. 21). The CAT gene in this vector can be expressed from a promoter inserted into an upstream restriction enzymes polylinker site. Translation
proceeds only from a start site immediately flanking the gene. The amount of CAT activity in a cell harboring the plasmid is thus an accurate index of the promoter strength of DNA fragments containing promoters which are inserted into the polylinker site. The constitutively expressed β-lactamase gene on the plasmid provides a selectable marker and an internally expressed gene for normalization. That is, the ratio of CAT activity to β-lactamase activity gives a measure of the strength of the inserted promoter that is independent of plasmid copy number and overall metabolic activity of the host cell.

The different ftsZ promoters (P_{z1}P_{z2}P_{z3}) and ftsA (pA) promoter regions were subcloned into pKK232-8 polylinker region and then CAT/β-lactamase specific activity in wild type strain (AMC290) background (Table 4) were determined. The potential proximal promoters (110), P_{z1} and P_{z2}, within the HindIII-EcoRI DNA fragment of the plasmid pYC580 was poorly expressed in vivo being approximately 21.8 X 10^{-3} units. This is consistent with low level of ftsZ expression from a λenvA(77), that only partial complemented a fts284(ts) mutant. In contrast to P_{z1}P_{z2}, the promoter P_{z3} within the coding sequence of ftsA, as in pYC530, showed a high level of promoter activity, approximately 235.2 X 10^{-3} units. If the ftsA gene promoter, P_{A}, was fused to P_{z3} (BamHI-BglII), as in pYC500, then the promoter activity increases slightly indicating a weak ftsA promoter. This indicates that the ftsA promoter is weak and contributes little to the transcription from P_{A}P_{z3}-CAT fusion. The total promoter activity from pYC620 was lower than the sum of the separate activities of P_{z3} and P_{z1}P_{z2}. When P_{A} was included with P_{z3}P_{z2}P_{z1}, as in pYC600, the promoter activity was
increased by 1.8 fold but still lower than the level of transcription from pYC53(Pz3).

Lutkenhaus and his colleagues(75) have shown that the presence of part of ftsQ structural gene, as in pYC600, results in the decreased ftsZ expression and Tn10 insertion into ftsQ gene recovers ftsZ gene expression enough to suppress the SOS induced lethal filamentation of the lon mutant. However, the maximal expression of ftsZ either with a single copy phage vector (130), or with a multicopy plasmid(122) has been shown to require approximately 2 Kb of DNA upstream of the ftsZ gene, including ftsQ promoter.


Expression of ftsZ has been cited to be negatively regulated at the transcriptional level by FtsI, FtsQ, FtsA and FtsZ itself(36). Temperature shift experiments with temperature sensitive mutants of ftsQ, ftsA, and ftsZ were performed to assess whether any of the fts gene products regulate the transcription of another fts cell division gene. Since temperature sensitive fts mutants result in an inactive protein product at the nonpermissive temperature, the effect on transcription is easily determined by measuring the CAT/β-lactamase specific activities, using the promoter fusion plasmids constructed above.

Table 5 shows that FtsZ temperature inactivation increased CAT expression from both P_A P_z3 and P_z3. The level of increase was about 1.5 to 3 fold. Wild type control also increases the promoter activity from P_A P_z3 and P_z3 by 3 to 4 fold. Autoregulation of ftsZ expression can't be completely excluded although the data suggest that
the increased CAT expression by FtsZ inactivation could result from the temperature shift from 30°C to 43°C. The data is comparable to LacZ-transcriptional studies which showed no apparent \textit{ftsZ} autoregulation (R. Gayda, unpublished).

FtsQ inactivation (Table 6) had stimulating effects both on P\textsubscript{APZ3} promoter combination and on P\textsubscript{Z3} promoter alone.

The FtsA inactivation (Table 7) had a slight decreasing effect on P\textsubscript{Z3} promoter with only little effect on P\textsubscript{Z3PZ1} promoter when compared with wild type control. A low level of CAT expression was obtained because of the temperature sensitivity of the \textit{ftsA} mutant strain even in the presence of high salt. These data are opposite to the finding\cite{36} that transcription of \textit{ftsZ} is increased as much as 10 fold when cells have a nonsense mutation in the chromosomal copy of \textit{ftsA} gene. On the other hand, there is a evidence supporting that the expression of \textit{ftsA} may be controlled by FtsZ protein through activation of transcription because the FtsA gene expression of plasmid pRGC43 was no longer sufficient to complement \textit{ftsA} mutants when the \textit{ftsZ} gene was inactivated by the mini-Mu insertion (R. Gayda, unpublished).

Possible \textit{dnA} consensus sequence within \textit{ftsZ} promoters(P\textsubscript{Z3PZ1}) led us to study if \textit{dnA} effected the expression of \textit{ftsZ} gene. Table 8 shows that \textit{dnA} temperature inactivation increased CAT activity nearly 2 fold from both P\textsubscript{APZ3} and P\textsubscript{Z3}, suggesting that \textit{dnA} regulation may link septation with DNA synthesis.
DISCUSSION

Previous genetic studies have postulated that the function of FtsA protein is both regulatory and structural in septum formation (115-118). The purpose of this study was to investigate the structural involvement of FtsA in septum formation. The establishment of the cellular location of FtsA is important because current data (10, 29, 31, 41, 65, 119) strongly proposed interactions among the components of the division machinery. Thus the locating FtsA within a specific membrane domains will make possible the determination of the specific functions or biochemical activities of FtsA in septation and will begin to unravel the ordered morphogenic molecular mechanisms involved in the septation process.

Evidence obtained that FtsA protein is acting as an inner-outer membrane junction protein are as follows. First, FtsA-LacZ fusion proteins demonstrated that the amino terminal end of the FtsA protein can localize FtsA-LacZ hybrid protein to the cell envelope (Fig. 6 and Fig. 7). Furthermore, FtsA-LacZ translational fusions behaved like FtsA protein when radioactively labelled using the maxicell technique and when fractionated into membrane and cytoplasm (Table 3). Second, wild type dividing cells synthesizing the FtsA-LacZ hybrid proteins, when the membranes were fractionated by a modified sucrose equilibrium gradient exhibited, β-galactosidase enzyme activities in fraction corresponding to OMw and OMl (Fig. 9). The OMl fraction contains inner-outer membrane fusions (59). Third, when polyclonal antibodies were made from purified FtsA-LacZ proteins, Western-blotting experiments with these antibodies on cell membrane fractions
demonstrated that native FtsA was found localized to OM$_m$ and OM$_L$ fractions (Fig. 13). (Other experiments conducted with increased concentration of \( \beta \)-mercaptoethanol in SDS-PAGE showed that the FtsA can also be detected in inner membrane(IM) fraction (Fig 15,16)).

Fourth, direct immunoelectron microscopic localization confirmed the specific localization of FtsA protein at septation sites within the wild type cells. The immunogold-labelling of thin sections of filamentous cells indicated an enrichment of FtsA protein at potential septation sites which were identified as inner-outer membrane junction sites by plasmolysis. Thus, our model is that FtsA along with other septation proteins forms part of a "septalsome" core which initiates and modulates the sequence of biochemical changes that lead to formation of the division septum.

Ishidate et al. (59) have reported that the OM$_L$ membrane fraction is involved in murein synthesis and the translocation of newly synthesized lipopolysaccharides. Recently, MacAlister et al. (78) have demonstrated that OM$_L$ fraction also contains the periseptal annuli which flank the constriction sites around the potential septum as an inner-outer membrane gasket to compartmentalize the periplasmic space between the dividing cells.

Similarly, it has been reported (4,97) that the penicillin binding proteins (PBPs) are located at discrete zones within the cell envelope, an inter-membrane fraction probably corresponding to inner-outer membrane adhesion sites or periseptal annuli(5,78). PBPs are a set of actual enzymes that catalyze the insertion of new material into the peptidoglycan sacculus and are engaged in the control of cell elongation(106) and cell division(14,63). One of them, PBP3(ftsI) gene
product, is a known septation proteins which has transglycosylase and transpeptidase enzymatic activities which are involved in murein synthesis and septum formation. Overproduction of inactive PBP3 results in inhibition of cell septation implying that PBP3 is part of a protein complex in vivo(15B). FtsA mutants have increased resistance to β-lactam induced lysis and decreased binding of [125I] ampicillin to PBP3. This supports a possible interaction of PBP3 with FtsA. In fact, it has been suggested that FtsA functions as part of an active multimeric complex by regulating or directing murein biosynthesis for septum formation(23). Furthermore, indirect evidence, which comes from murein synthesis studies by topographic autoradiography, show that increased incorporation of radioactive diaminopimelic acid in the central zone representing the present or future site for septum formation (127).

Observations(data not shown), that fusions specified pYC39 or pYC40 caused some filamentous cell growth even in wild type strains also supports a functional role of FtsA as a component of multimeric complex in septation. FtsA-LacZ fusions may be competing with wild type FtsA protein that may be functioning as an oligomer or mixed oligomer with other septation proteins.

The localization of FtsA10 protein to OMn and OMl membrane fractions like native FtsA protein indicates that FtsA10 probably leads to a nonfunctional complex due to an altered protein structure.

FtsZ probably is also involved in the multienzyme complex. A highly expressed LacZ-FtsZ protein under control of the lac promoter(124) when induced by IPTG(123) result in filamentation and death. Whereas, overproduction of FtsZ results in early cell
septation and minicell formation\(^{(124)}\). This functional similarity between specific alterations of FtsZ and overproduction of FtsZ supports an involvement of some type of stoichiometric regulation of this complex division machinery.

The \textit{ftsA} gene consists of 420 residue amino acids\(^{(95)}\) with a calculated molecular weight of 45.4 Kdal. The FtsA protein has been identified as a 50 Kdal polypeptide \(^{(76)}\) on SDS polyacrylamide gels of extracts of in vivo labelled cells with \textit{ftsA} on plasmid or \textlambda transducing phage. This 10\% discrepancy of this protein on SDS-PAGE gels that has been reported \(^{(95)}\) is probably due to fact that it is a membrane protein.

The FtsA gene product in relationship to FtsZ is synthesized much less (a few molecules\(^{(53)}\) of FtsA to maximally a hundred molecules of FtsA per cell \(^{(52,76)}\): based on intensity of autoradiograms\(^{(76)}\), FtsA is expressed as much as 10 fold less than FtsZ, and based on the amount of \(\beta\)-galactosidase enzyme activities of FtsA transcriptional fusions compared with FtsZ transcriptional fusions FtsA is expressed as much as 40 fold less than FtsZ. The low amount of FtsA protein within cells could be because FtsA protein is synthesized at a low level, or because FtsA is degraded at a high rate. Our results indicate that both are likely occurring. Assuming that the specific activity of native \(\beta\)-galactosidase is very similar to that of fused FtsA-LacZ protein activity (where 0.06 units of \(\beta\)-galactosidase activity corresponds to 0.3 monomers of protein per cell\(^{(92)}\)). Assuming that the cell on average contains 50 copies of fusion plasmid per cell (1 transcript or 1 translation per transcript per plasmid per generation) We estimate that there are 20 to 200
(probably averaging around 50) FtsA protein molecules per cell from the amount of β-galactosidase enzyme activities of FtsA transcriptional and translational fusions.

Promoter strength assay (Table 4) of ftsA promoter and the immunoelectron microscopic localization (Fig. 17) are consistent with this low level expression of ftsA. Immunoblot (Fig. 11D) of extracts containing fusion protein specified by pYC40 and enriched fusion protein specified by pYC3 also strongly suggest that FtsA protein is unstable because only β-galactosidase part of hybrid proteins were intensely cross-reacted with FtsA-LacZ antibodies whereas no immunoreactive band corresponding to the FtsA part of fusion protein was observed.

The difficulty in identifying FtsA protein on immunoblots even when cells carrying ftsA multicopy plasmid could be ascribed to this small amount of FtsA protein. A 10 fold increased antibody concentration did not increase the sensitivity of FtsA antigenicity in the immunoblots under same experimental condition.

Interestingly, the amount of the native FtsA protein was sufficient to be detected in immunoblot (Fig. 12) when 40% (NH₄)₂PO₄ precipitate of FtsA-LacZ hybrid proteins enriched cell extracts were analysed. This observation suggests that overproduced FtsA-LacZ protein could act as a positive regulator of ftsA expression or remove its negative regulator. Alternatively, FtsA-LacZ hybrid protein could increase the stability of the native FtsA protein by a mixed multimeric formation or autoregulation.

Conditional temperature sensitive mutants of ftsA form long multinucleated non-septated filaments that have slight indentations at
the septation sites. This mutant phenotype plus other genetic studies(9,35,76) suggest that FtsA protein acts after the indentation of the new septation sites. Morphogenic and physiological analysis(113) of cell division mutants have proposed that the morphogenetic pathway is in the order of FtsZ-FtsQ-FtsA,FtsI.

Subcloning, promoter fusion assay, and DNA sequencing (95,96,109,129,130) have confirmed that contiguous fts genes (ftsQ-ftsA-ftsZ) form an atypical operon with overlapping transcriptional units that transcribe in the same direction from the promoters (Fig. 1) locating upstream of each of the genes. There are no transcription terminators between the genes. This closely packed organization may imply that the variable levels of coupled expression of these genes are critical to the ordered functions of cell septation gene products. The observation that the presence of the HindII-EcoRl DNA sequences decreased the ftsZ expression from Pz3 by 2 to 4 fold (Table 4) suggests that the normal chromosomal location of ftsZ genes for proper functioning of the gene is critical to their coordinate expression and/or mutual regulation. A report(133) that integration of a λ phage that carries a wild type ftsA sequence into the attλ site of a ftsA mutant, can only partially complements this ftsA mutant, point to an importance of the neighboring DNA sequences.

In vivo quantitative promoter strength assays (Table 4) of the ftsA and ftsZ promoters in BamH1-EcoRl fragment(2.2Kb) subcloned in promoter probe vector pKK232-8 confirms that ftsA promoter is a relatively weak promoter. The potential proximal promoter Pz1 and Pz2 within the HindIII-EcoRl fragment was also poorly expressed in vivo compared to the distal promoter Pz3 in BglII-HindIII region.
comparison of the homology scores of known promoter sequences -35 and -10 region to the *ftsZ* promoter sequences gave the following scores: \( P_{z1} = 53.3\% \), \( P_{z2} = 45.6\% \) and \( P_{z3} = 37.3\% \). A score of 45\% is the lower limit for an effective promoter. These homology scores plus our *in vivo* data together suggest that \( P_{z1} \) and \( P_{z2} \) may be negatively controlled whereas \( P_{z3} \) must be positively controlled to be an effective promoter.

There is some disagreement of our expression data with expression data published by Sullivan and Donachie (109) and by YI and Lutkenhaus (130). However, there is also disagreement between these two published papers. The differences suggest that this DNA region may be highly regulated by multiple factors linked to cellular events and by multiple interactions to control the variable levels of coupled expression at different times in the cell cycle. The differences are also likely to be the result of our plasmid constructions and the interaction of DNA sequences around these promoters.

Expression study (Table 7) with temperature sensitive mutant *ftsA* showed that FtsA appears to increase the *ftsZ* expression from \( P_{z3} \) at transcriptional level. Interestingly, there is an observation that *ftsA* expression may be controlled by FtsZ protein through activation of transcription because the *ftsA* gene of plasmid pRGC43 is no longer able to complement an *ftsA* mutant when the *ftsZ* gene was inactivated by the mini-Mu insertion (R. Gayda, unpublished).

On the other hand, *ftsZ* expression (Table 8) may be regulated negatively by *dnaA* protein essential for initiation of chromosome replication. DnaA protein has been shown to autoregulate its own synthesis and is an important control element of *E. coli* origin.
region(43). Consensus sequences for DnaA binding have been found in the regulatory regions of pyrBl, argFl, polA, uvrA and dam genes (111). The promoter expression data plus the DnaA consensus binding sequence within ftsZ promoters (P_{Z2}P_{Z1}) suggest that dnaA regulation of ftsZ expression may link septation with DNA synthesis.

Protein sequence of FtsA(95) doesn't contain a typical leader signal peptide, that is present in precursors of most periplasmic and outer membrane proteins. This indicates that localization of FtsA into specific inner-outer membrane junction sites may occur through a different mechanism by which an internal protein sequences of FtsA is sufficient for proper insertion at a particular cellular location. The inner-outer membrane fraction is likely to be a labile structure. The membrane fractionation procedure employed neither lysozyme or EDTA treatments in order to preserve envelope structures. Furthermore, this lability probably explains FtsA-LacZ and FtsA being found in the OM_m membrane fraction(Fig. 8 and 13). This is supported by the report(4) that PBPs are localized in the intermembrane fraction under the mild conditions for cell rupture and usually found in both the IM and OM membrane fractions when the inner-outer adhesion sites are destroyed by the cell breakage and fractionation procedures.

Immunoblot analysis with membrane fractions isolated from sucrose equilibrium gradients indicates that the FtsA is localized to the specific domain of the envelope, inner-outer membrane junction sites. However, immunoreactive bands in addition to FtsA antigen at 60Kdal, 52Kdal, 33Kdal, 23Kdal were observed. Several pieces of evidence exclude the possibility that nonspecific binding is
responsible for the appearance of these immunoreactive bands. 1. Preimmune serum did not show binding on Western blots under same experimental conditions. 2. Two of the bands were also detectable as corresponding $^{35}S$ labelled bands, but less intensely, when FtsA specifying plasmid proteins were labelled by maxicell procedure. 3. Instability of FtsA protein suggest that 33Kdal and 23 Kdal polypeptides are probably FtsA breakdown products or modified FtsA present in the membrane. The report that outer membrane protein OmpT is a protease(49) may support this. 4. Two times preabsorption of FtsA-LacZ antibodies fraction with lysate containing total membranes, to remove antibodies reacting with the major membrane proteins of E. coli, decrease FtsA antigenicity but did not eliminate these immunoreactive bands.

The mechanisms used by the cell to identify the proper site and to localize the cell division machinery at this site are unknown. Rothfied's group(25) has proposed a model that nascent annuli are generated from a previous periseptal annuli acting as a boundaries of tseptation site and then laterally displaced till it reaches a midpoint between a cell pole and the generating periseptal annuli. Phase contrast and electron micrographs demonstrate the patterns of localized plasmolysis bays of periseptal annuli at septation sites and annular adhesion zones at nonseptal locations corresponding to future sites of cell division.

Similar patterns of localized plasmolysis bays plus similar phenotype properties in different fts cell division mutants($ftsZ$, $ftsA$, $ftsI$) (data not shown) suggest that FtsZ function, leading to the initiation of septal invagination, appears to be activated after
the maturation and localization of nacent periseptal annuli. The fact that *ftsZ84* filaments after temperature shift back to 30°C, recovers cell septation quicker than *ftsA* or *pbpB* filaments supports this model. However, the possibility of FtsZ protein is involved in localization of the septation sites must also be considered because of minicell formation due to increased FtsZ proteins. FtsA's localization by immunogold electron microscopy near inner-outer membrane junction sites, when visualized by plasmolysis bays, suggests another possible role of nascent annuli. It is probable that the maturation of periseptal annuli mediates the localization of the septation proteins to the proper cell locations.

Thus, our data supports that the septation proteins form a core, part of an hypothesized "septalsome", a multienzyme-protein complex at septation sites. Formation of this multimeric complex is probably modulated by multiple factors linked to several metabolic processes. Multiple interactions appear to be involved in activation of the septation proteins in the septalsome which intern modulates the enzymatic activities of the septation steps at precise times during the cell division cycle.
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Table 2. Plasmids and phages.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant markers</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pRGC31</td>
<td>$\text{amp}^r, ftsZ^+, ftsA^+$</td>
<td>R. Gayda(23)</td>
</tr>
<tr>
<td>pRGC43</td>
<td>$\text{amp}^r, \text{kan}^*, ftsA^+$</td>
<td>R. Gayda(23)</td>
</tr>
<tr>
<td></td>
<td>$ftsZ::\text{mini-MuI1734(lac)}^+$</td>
<td></td>
</tr>
<tr>
<td>pGW7</td>
<td>$\text{amp}^r$</td>
<td>(30)</td>
</tr>
<tr>
<td>pKK232-8</td>
<td>$\text{amp}^r$</td>
<td>J. Brosius(16)</td>
</tr>
<tr>
<td>pYC16</td>
<td>$\text{kan}^*, ftsZ^+$</td>
<td>This work(23)</td>
</tr>
<tr>
<td></td>
<td>$ftsA::\text{mini-MuII1734(lac)}^+$</td>
<td></td>
</tr>
<tr>
<td>pYC28</td>
<td>$\text{kan}^*, ftsZ^+$</td>
<td>This work(23)</td>
</tr>
<tr>
<td></td>
<td>$ftsA::\text{mini-MuII1734(lac)}^+$</td>
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<tr>
<td>pYC39</td>
<td>$\text{kan}^*, ftsZ^+$</td>
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</tr>
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<td>$ftsA::\text{mini-MuII1734(lac)}^+$</td>
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</tr>
<tr>
<td>pYC40</td>
<td>$\text{kan}^*, ftsZ^+$</td>
<td>This work(23)</td>
</tr>
<tr>
<td></td>
<td>$ftsA::\text{mini-MuII1734(lac)}^+$</td>
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</tr>
<tr>
<td>pYC500</td>
<td>$\text{amp}^r, \text{cm}^r$</td>
<td>This work</td>
</tr>
<tr>
<td>pYC530</td>
<td>$\text{amp}^r, \text{cm}^r$</td>
<td>This work</td>
</tr>
<tr>
<td>pYC580</td>
<td>$\text{amp}^r, \text{cm}^r$</td>
<td>This work</td>
</tr>
<tr>
<td>pYC600</td>
<td>$\text{amp}^r, \text{cm}^r$</td>
<td>This work</td>
</tr>
<tr>
<td>pYC620</td>
<td>$\text{amp}^r, \text{cm}^r$</td>
<td>This work</td>
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<table>
<thead>
<tr>
<th>Phages</th>
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<tr>
<td>PlVir</td>
<td></td>
<td>R. Gayda</td>
</tr>
<tr>
<td>$\lambda_{1098}$</td>
<td>$\lambda::\text{mini-Tn10}$</td>
<td>N. Kleckner</td>
</tr>
<tr>
<td>(125)</td>
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Table 3. Cellular Location of FtsA and FtsA–LacZ Fusions

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Cytoplasmic</th>
<th>Total Membranes</th>
<th>Sarkosy Extracted Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FtsA β-gal fusion</td>
<td>FtsA β-gal fusion</td>
<td>FtsA β-gal fusion</td>
</tr>
<tr>
<td>pRG43</td>
<td>.14</td>
<td>.18</td>
<td>.07</td>
</tr>
<tr>
<td>pRG31</td>
<td>.13</td>
<td>.12</td>
<td>.06</td>
</tr>
<tr>
<td>pYC40 (142,000 fusion)</td>
<td>.04</td>
<td>.05</td>
<td>.04</td>
</tr>
<tr>
<td>pYC28 (154,000 fusion)</td>
<td>.03</td>
<td>.04</td>
<td>.03</td>
</tr>
</tbody>
</table>

1 - Relative molar yield of plasmid encoded proteins.
Relative molar yield = % of polypeptide/molecular weight × 10³
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction frag. fused to Cm&lt;sup&gt;+&lt;/sup&gt; gene</th>
<th>Relevant Promoters</th>
<th>CAT/β-lactamase activity 10&lt;sup&gt;3&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>pKK232-8</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pYC600</td>
<td>BamH1 - EcoR1</td>
<td>P&lt;sub&gt;A&lt;/sub&gt;P&lt;sub&gt;z3&lt;/sub&gt;P&lt;sub&gt;z2&lt;/sub&gt;P&lt;sub&gt;z1&lt;/sub&gt;</td>
<td>108</td>
</tr>
<tr>
<td>pYC620</td>
<td>BgIII - EcoR1</td>
<td>P&lt;sub&gt;z3&lt;/sub&gt;P&lt;sub&gt;z2&lt;/sub&gt;P&lt;sub&gt;z1&lt;/sub&gt;</td>
<td>59</td>
</tr>
<tr>
<td>pYC580</td>
<td>HindIII-EcoR1</td>
<td>P&lt;sub&gt;z2&lt;/sub&gt;P&lt;sub&gt;z1&lt;/sub&gt;</td>
<td>22</td>
</tr>
<tr>
<td>pYC530</td>
<td>BgIII-HindIII</td>
<td>P&lt;sub&gt;z3&lt;/sub&gt;</td>
<td>235</td>
</tr>
<tr>
<td>pYC500</td>
<td>BamH1-HindIII</td>
<td>P&lt;sub&gt;A&lt;/sub&gt;P&lt;sub&gt;z3&lt;/sub&gt;</td>
<td>249</td>
</tr>
</tbody>
</table>

Cell extracts of AMC290 containing above plasmids were prepared and CAT specific activity/β-lactamase activity was determined as described in Material and Methods. The background CAT activity in this assay was zero in AMC290 (OD<sub>600</sub> = 0.5). All measurements were performed in duplicate and the mean is presented.
Table 5. FtsZ effects on promoters.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction frag. fused to Cm(^r) gene</th>
<th>Relevant Promoters</th>
<th>CAT/β-lactamase activity X 10(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMC419(new) AMC421(wt)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>time (min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>PYC500</td>
<td><em>BamH1-HindIII</em></td>
<td>P(<em>A)P(</em>{z3})</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>pYC530</td>
<td><em>BglII-HindIII</em></td>
<td>P(_{z3})</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

nd = not done. A portion of actively growing cells (OD\(_{600}\)=0.1) were temperature shifted to 43°C and the cells were harvested after 30 and 60 minutes as described in Material and Methods.
Table 6. FtsQ effects on promoters.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction frag. fused to Cm&lt;sup&gt;r&lt;/sup&gt; gene</th>
<th>Relevant Promoters</th>
<th>CAT/β-lactamase activity X 10&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOE1(ftsQ)</td>
<td>time 30°C 43°C (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYC500</td>
<td>BanHI-HindIII</td>
<td>PA, P&lt;sub&gt;Z3&lt;/sub&gt;</td>
<td>0 334</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>30 369 957</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 325 1184</td>
</tr>
<tr>
<td>pYC530</td>
<td>BgIII-HindIII</td>
<td>P&lt;sub&gt;Z3&lt;/sub&gt;</td>
<td>0 331</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 319 709</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 219 1158</td>
</tr>
</tbody>
</table>

Cell extracts were prepared and the activity was assayed as described in Table 5.
Table 7. FtsA effects on promoters.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction frag. fused to Cmr gene</th>
<th>Relevant Promoters</th>
<th>CAT/β-lactamase activity ( \times 10^5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>YC100(ftsA10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>time 30°C 4°C 30°C 43°C</td>
</tr>
<tr>
<td>PYC530</td>
<td>BglII-HindIII</td>
<td>( P_z3 )</td>
<td>30 234 208 54 94</td>
</tr>
<tr>
<td>pYC580</td>
<td>HindII-EcoRI</td>
<td>( P_z2P_z1 )</td>
<td>30 26 18 17 12</td>
</tr>
</tbody>
</table>

Cell were grown in L plus 0.2% glucose medium. Cell extracts were prepared and the activity was assayed as Table 5.
Table 8. DnaA46 mutants effects on promoters.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Restriction frag. fused to Cm^r gene</th>
<th>Relevant Promoters</th>
<th>CAT/β-lactamase activity X 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>E177(dnaAts)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>time 30°C 43°C (min)</td>
</tr>
<tr>
<td>PYC500</td>
<td>BamH1–HindIII</td>
<td>P_aP_z3</td>
<td>0 114</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 131 201</td>
</tr>
<tr>
<td>pYC530</td>
<td>BglII–HindIII</td>
<td>P_z3</td>
<td>0 84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 88 186</td>
</tr>
<tr>
<td>pYC580</td>
<td>HindIII–EcoRI</td>
<td>P_z2P_z1</td>
<td>0 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 9 11</td>
</tr>
</tbody>
</table>

Strains culture was grown overnight in LB-0.2% glucose at 30°C and then diluted 100 fold and placed at 30°C. A portion of exponentially growing cells (OD_{600} = 0.5) was temperature shifted to 43°C for 60 min. The cells were collected and extracts prepared for assays as described in Materials and methods.
Fig. 1. Locations of coding sequences and promoters within the *ddl-envA* region of the *E. coli* chromosome. (a) The 14 loci concerned with cell envelope growth, organization and division have been identified within the 2-min region of the *E. coli* chromosome. (b) Restriction enzyme map of the *ddl-envA* region and, beneath, location of the structural genes. P, promoter locations; arrows, directions of transcription. Abbreviations for restriction sites: B, *BamH*I;Bg, *BglII*; C, *ClaI*; E, *EcoR*I; H, *HindIII*; K, *KpnI*; P, *PstI*; Pv, *PvuII*; S, *Sall*; X, *XhoI*. 
Fig. 2. Proposed steps in the morphogenesis of E. coli cells. Three morphogenic systems are represented. System i: gene specifying a set of enzymes involved in the net synthesis of peptidoglycan (P.G.) including mraA, mraB, mrbA, mrbB, mrhA, mrbB, mrcA, mrcB, murE, murF, murG, murC, ddl and possibly dacA and dacB. System ii: shape modifying system for the cylindrical P.G. sacculus. System iii: At least six gene products (those of ftsZ, ftsI, ftsQ, ftsA, envA, and minB) assigned for successive steps in cell division; the initiation, formation, completion, separation and inactivation (as potential division sites) of new cell poles. The phenotype of cells which have been blocked at different stages in system iii division process are shown at the top of the diagram.
Fig. 3. Diagram of periseptal annuli in LkyD− cells (a). Surface view of the chain of cells (b). Abbreviations: IM, inner membrane; M, murein; OM, outer membrane; P, plasmolysis bays; D, zones of membrane-murein adhesion that form the periseptal annuli; D', zones of adhesion that form incomplete annuli at the mid points of the cells; S, septal cleft. (Modified from MacAlister et al (78)).
Fig. 4. Schematic representation of the expression vector pGW7. Thick line, DNA derived from phage λ; thin line, DNA derived from pBR322(30). Arrows indicate the direction of transcription initiating from the λ P_L and λ P_R promoters.
Fig. 5. The vector pKK232-8. A partial restriction map shows the site in the polylinker used in promoter cloning. T; terminators, Amp; β-lactamase gene, CAT; chloramphenicol gene. Restriction enzyme codes: R₁, EcoR₁; H₃, HindIII.
Fig. 6. Restriction maps of pRGC31, MudII 1734 and mini-Mu insertion locations. Encoding sequence for ftsA showed as hatch line. Mini-Mu insertion sites are shown by lollipop structure with associated plasmid designations. Restriction enzyme codes: R1, EcoR1; pst, Pst1; B1, BamH1; H3, HindIII. Kan\textsuperscript{r} is abbreviation for kanamycin resistance (25 μg/ml). Amp\textsuperscript{r} is abbreviation for ampicillin resistance (50 μg/ml).
Fig. 7. Autoradiograph of plasmid-specified polypeptides labelled in UV-irradiated maxicells. Polypeptides were separated on a 10% to 30% gradient SDS-PAGE. Lane A contained approximately 50% of the total radioactivity that was added to lanes B,C,D (500,000 c.p.m.). Molecular weight standards used were: myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), albumin, bovine (66 kD), albumin, egg (45 kD), carbonic anhydrase (29 kD). Lanes: 1, CSR603(pRG31); 2, CSR603(pYC40); 3, CSR603(pYC28); 4, CSR603(pYC16). Label point to FtsA-LacZ fusions, FtsA, FtsZ, Bla(β-lactamase, Amp<sup>r</sup>) and Kan(Kan<sup>r</sup> protein specified by mini-Mu).
Fig. 8. Separation of membrane fraction by sucrose equilibrium gradient. Strain AMC290(pYC40) and AMC290(pRGC43) were disrupted and fractionated as described in materials and methods (Section XV). Areas corresponding to OM₉, OM₈, F/B and IM as described by Ishidate et al. (59) are indicated at top of Figure.
Fig. 9. Schematic diagram for construction of expression plasmid, pYC3. DNA segments of pYC16 (FtsA-LacZ plasmid) and vector pGW7 (λ P_L promoter plasmid regulated by thermolabile CI repressor, C_{587C}, were combined to generate pYC3. [ ], MudII1734 DNA (9.7 Kb); ftsA, ftsA gene; P_L, K P_L promoter; kan^r, kanamycin resistance; amp^r, ampicillin resistance; β', β-galactosidase(truncated); N, λ N gene. Restriction enzyme codes: R1, EcoR1; P, Pst1; B1, BamH1; H3, HindIII; Bg, BglIII.
BH1 digestion and Bacterial alkaline phosphatase treatment

Partial digestion with BH1 and purification of 8.5Kb DNA fragment.

Ligation
Selection (Lac\textsuperscript{+}, Amp\textsuperscript{r}, Kan\textsuperscript{s})
Fig. 10. SDS-PAGE profile of proteins for purification of FtsA-LacZ protein from temperature shifted AMC290(Δlac) strain containing plasmid pYC3. The cells were shifted to 42°C for 2.5 hrs. The hybrid protein was purified from the soluble fraction of French pressure lysed cells by ammonium sulfate precipitation and affinity chromatography on p-aminobenzyl-β-D-thio-galactopyranoside agarose (Sigma) as described in the Material and Methods. Polypeptides were separated on a 10 to 30% gradient gel and stained with Coomassie blue (0.2% w/v). Lanes: A. molecular weight standards (same as described in Fig. 8); B. total protein in broken cells (140 μg); C. total protein in cleared lysate (130 μg); D. 40% (NH₄)₂SO₄ pellet (130 μg); E. purified FtsA-LacZ fusion protein eluted from p-aminobenzyl-β-D-thio-galactoside column (10 μg).
Fig. 11. Identification of FtsA by Western blot analysis. FtsA protein was \[^{35}\text{S}\]methionine labelled in maxicells with plasmids specifying FtsA and/or \(\beta\)-galactosidase. Each sample was heated for 5 min at 100°C in sample buffer containing 4M urea and 1% \(\beta\)-mercaptoethanol. Proteins were separated by SDS-PAGE(10-30%) and electro-transferred onto Nytran sheets. Blocked membranes were incubated with purified IgG antibodies (1:1000 dilution) against FtsA-LacZ for 2 hrs and washed. Immunogenicity was detected with alkaline phosphatase linked goat-anti-rabbit-IgG with color development by BCIP and NBT. Maxicell labelled polypeptides were detected in the same blot by autoradiography. A). Autoradiogram of blot B. Lane 1 - CSR603/pRGC43; lane 2 - CSR603/pRGC31. B). Immunoblotting detection of FtsA in total cells (blot B). Lane 1 - CSR603/pRGC43; lane 2 - CSR603/pRGC31. C). Autoradiogram of blot D. Lane 1 - CSR603/pRGC31; lane 2 - CSR603/pRGC43 (1/5 cpm); lane3 - CSR603/pYC40 (FtsA-LacZ fusion); lane 4 - CSR603/pYC16 (FtsA-LacZ fusion). D). Immunoblotting detection of FtsA or FtsA-LacZ fusion (blotD). lanes 1-4 same as C. pRGC31 specifies FtsA, FtsZ, Amp. pRGC43 specifies \(\beta\)-galactosidase, FtsA, FtsZ, Amp, Kan.
Fig. 12. Immunodetection of FtsA in AMC290 strain harboring pYC40 or overproducing plasmid pYC3. Western blotting experiments were performed under same conditions as described in the legend of Fig. 12. Lanes: A. 40% (NH₄)₂SO₄ precipitate of AMC290/pYC40 extract (150 µg); B. 40% (NH₄)₂SO₄ precipitate of temperature induce AMC290/pYC3 extract (65 µg).
Fig. 13. Cellular localization of the FtsA protein by Western blot with FtsA antibodies. Membrane fractions of AMC290 were isolated by the procedure of Ishidate et al. (59) as described in Fig. 9. Each sample (~50µg) was heated for 20 min at 90°C in sample buffer containing 4M urea and 1% β-mercaptoethanol prior to SDS-PAGE and Western blot analysis. The Western blot was done under the same experimental conditions described in Fig. 12. Lanes: 1. IM - inner membrane; 2. F/B - Flagellar fraction with membrane fragments; 3. OM_L - outer membrane light fraction; 4. OM_H - outer membrane heavy fraction; 5. Total membrane; 6. Total soluble proteins; 7. Control- 40% (NH₄)₂SO₄ of temperature induced AMC290/pYC3 extract; 8. Total membrane; 9. Total soluble proteins.
Fig. 14. Western blot with FtsA-LacZ antibodies of cell fractions of AMC290. Approximately equal amount (~50μg) protein from cellular fractions of AMC290 were applied to each lane. Experimental conditions were as described in Fig. 14. Lanes: A. IM - inner membrane; B. F/B - flagellar fraction with membrane fragments; C. OM_L - outer membrane light fraction; D. OM_H - outer membrane heavy fraction; E. total membrane pellet; F. total soluble proteins; G. control - CSR603/pRGC31 total cell proteins; H. control - 40% (NH₄)₂SO₄ of temperature induced AMC290/pYC3 extract.
Fig. 15. SDS-PAGE Coomassie blue stain gel profile of AMC290 cellular fractions analysed in immunoblot of Fig. 15. Each sample was prepared and separated as described in Fig. 15. Lanes: A. IM - inner membrane; B. F/B - flagellar fraction with membrane fragments; C. OM$_L$ - outer membrane light fraction; D. OM$_H$ - outer membrane heavy fraction; E. total membrane pellet; F. total soluble proteins; G. control - CSR603/pRGC31 total cell proteins; H. control - 40% $(NH_4)_2SO_4$ of temperature induced AMC290/pYC3 extract.
Fig. 16. Western blot of FtsA antibodies preabsorbed with total membrane of cell fractions of AMC290. Approximately equal volume of each sample was prepared and analyzed as described in Fig. 15. FtsA-LacZ specific antibody fraction was preabsorbed twice with lysate of ftsA10 mutant containing the total membrane fractions isolated from a sucrose step gradient. Lanes: 1. IM - inner membrane(60 μg); 2. F/B - flagellar fraction with membrane fragments(10 μg); 3. OMₐ - outer membrane light fraction(60 μg); 4. OMₐ - outer membrane heavy fraction(40 μg); 5. total membrane pellet(50 μg); 6. total soluble proteins(50 μg); 7. control - CSR603/pRG31 total cell proteins; 8. control - 40% (NH₄)₂SO₄ of temperature induced AMC290/pYC3 extract(50 μg).
Fig. 17. Electron microscopic localization of FtsA in *E. coli* cells by immunogold labelling. Cells were embedded, thin sectioned, treated with FtsA-LacZ polyclonal antibodies and labelled with goat anti-rabbit IgG colloidal gold conjugates (10nm) as described in the Material and Methods. Lanes: A,B,C,D - AMC290(Δlac) treated with FtsA-LacZ polyclonal antibodies; E,F - control, AMC290 treated with pre-immune serum antibodies; G,H - control, YC200(Δlac−) treated with FtsA-LacZ polyclonal antibodies.
Fig. 18. Phase contrast light and electron micrographs of plasmolyzed ftsZ84(ts) filaments. Strain AMC419 growing cells at 30°C (OD₆₀₀ = 0.2) in YET broth were shifted to 42°C for 90 min. A portion of the culture was plasmolyzed in 13% sucrose, fixed with paraformaldehyde and prepared for phase contrast (A) and electron microscopy(B). Plasmolysis bays are visible along the length of the filaments. Bar, μm - light micrograph; Bar, 1 μm - electron micrograph.
Fig. 19. Immunoelectron micrographic localization of FtsA in fts284 filament. A portion of culture in Fig. 18 was fixed with paraformaldehyde and prepared for immuno-electromicroscopy as described in the legend to Fig. 18. Bar, 1 μm.
Fig. 20. Localization of FtsA in plasmolyzed ftsZ84 filament by immunoelectron micrograph. This section of plasmolyzed ftsZ filamentous cells in Fig. 18 was subject of immunogold labelling as described in Fig. 18. Bar 1 μm.
Fig. 21. Promoter fusions of the *ftsA-ftsZ* regulatory region to the chloramphenicol acetylase gene (*Cm*<sup>r</sup>). A partial restriction map of the 2.2 Kb fragment from pRGC31 (Fig. 6) is shown together with the extent of cloned fragments and their orientation relative to *Cm*<sup>r</sup> structural gene in pKK232-8. Also depicted are the promoter (P<sub>z1</sub>, P<sub>z2</sub>, P<sub>z3</sub>, P<sub>A</sub>) and the structural regions of *ftsQ*, *ftsA*, *ftsZ*. (" " indicated incomplete coding region). Abbreviation used for restriction enzymes are E<sub>1</sub>, EcoRI; B<sub>1</sub>, BamHI; Bg, BgIII; Bs, BssHI; H<sub>3</sub>, HindIII.
VITA

Younghae Chong Chon was born August 4, 1953 in Seoul, Korea. She graduated from Jeongshin Girls High School in Seoul, Korea in 1972. She attended Ewha Women's University in Seoul, Korea and received a Bachelor of Pharmacy degree in 1976. After graduation she worked for more than 3 years at National Institute of Scientific Investigation in Seoul, Korea in a laboratory as a pharmacist. Younghae entered the Graduate School of Louisiana State University in August 1982 and received the Master of Science degree in Microbiology in December 1984. She is currently a candidate for the degree of Doctor of Philosophy in Microbiology at Louisiana State University.
Candidate: Younghae Chong Chon

Major Field: Microbiology

Title of Dissertation: Cell Division Studies of Escherichia coli: Expression and Protein Localization of a Cell Septation Gene, ftsA.

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

Tuesday, July 12, 1988