1998

Localization and Biochemical Characterization of the Cell Division Protein FtsA in Escherichia Coli.

Anand Immaneni

Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation


https://digitalcommons.lsu.edu/gradschool_disstheses/6680
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
LOCALIZATION AND BIOCHEMICAL CHARACTERIZATION OF
THE CELL DIVISION PROTEIN FtsA IN *Escherichia coli*

A Dissertation

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
Anand Immaneni
B.Sc.(Hons), Osmania University, 1987
May, 1998
DEDICATION

I dedicate this work to my beloved late sister Swathi L. Immaneni.
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my major professor, Dr. Randall C. Gayda, for accepting me as a graduate student and for his advice, support and providing me the opportunity to learn research.

My sincere gratitude to all my committee members Dr. Eric Achberger, Dr. Sue Bartlett, Dr. Alan Biel, Dr. Ronald Siebeling and Dr. John Harbo for their advice, support and patience. Special thanks are extended to Dr. Siebeling for his encouragement and help with the purification of the FtsA polyclonal antibodies; Dr. Achberger for the unrestricted access to his lab equipment and computers; Mrs. Cindy Henk for her support, technical assistance with the electron microscopy, photography etc; Dr. Srinivasan for his counsel and advice; and Dr. Socolofsky for his generosity and critical reading of my dissertation. I am grateful to Dr. John Larkin who was instrumental in my admission to the department.

I specially thank my parents and all other family members for their love, support and encouragement in all my endeavors over the years. I am greatly indebted to my aunt and uncle Mrs. and Dr. Madireddi for their generosity and help. I would like to pay my respects to my late grandmothers and my uncle Ranga who passed away during the course of my study.

I thank all the graduate and undergraduate students, names of whom are too many to mention, for their friendship, fun and support over the years. I am grateful to the Department of Microbiology and Louisiana State University for giving me the opportunity and the financial support to pursue my graduate study.
# TABLE OF CONTENTS

**DEDICATION** ................................................................. ii

**ACKNOWLEDGEMENTS** .................................................... iii

**LIST OF TABLES** ............................................................ v

**LIST OF FIGURES** .......................................................... vi

**LIST OF ABBREVIATIONS** ................................................ viii

**ABSTRACT** ................................................................. ix

**INTRODUCTION: AN OVERVIEW OF CELL DIVISION IN E.coli.** .... 1

**CHAPTER I**
Cellular localization studies with FtsA polyclonal antibodies .......... 15
Introduction ............................................................................. 16
Materials and methods ......................................................... 21
Results ..................................................................................... 28
Discussion .............................................................................. 51

**CHAPTER II**
Purification and biochemical characterization of cell division protein FtsA from *Escherichia coli*. .......................................................... 61
Introduction ............................................................................. 62
Materials and methods ......................................................... 65
Results ..................................................................................... 72
Discussion .............................................................................. 92

**CHAPTER III**
Immunological screening for FtsA-related proteins in other microorganisms ................................................... 98
Introduction ............................................................................. 99
Materials and methods ......................................................... 101
Results ..................................................................................... 104
Discussion .............................................................................. 114

**CONCLUSIONS** ........................................................... 116

**REFERENCES** ............................................................. 118

**VITA** ............................................................................. 133
LIST OF TABLES

1. List of microorganisms used in this survey and their sources.........................102

2. Calculated molecular weights of FtsA homologues in Gram-negative bacteria....107

3. Calculated molecular weights of FtsA homologues in Gram-positive bacteria.....110

4. Calculated molecular weights of FtsA-like proteins in other organisms..........113
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Immunoblot demonstrating the specificity of the affinity purified FtsA polyclonal antibodies</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>Immunogold localization of FtsA protein</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Immunogold localization of FtsA in cross sections</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>Phase contrast light microscope photographs of D1210/pLYH1 carrying the ( ftsA^c )</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>Immunogold localization of FtsA in cells overexpressing FtsA(^c) protein</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>Immunogold labelled sections of curved cells treated as in Fig. 5</td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>Immunogold localization of FtsA in cells in which FtsA(^c) protein was induced for an hour</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>([^{35}S])methionine labelling and pulse chase of FtsA</td>
<td>47</td>
</tr>
<tr>
<td>9</td>
<td>Evidence for cleavage of FtsA during cell lysis and extraction</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>Western blot analysis of the cellular fractionation of native FtsA</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>Primary and secondary sequence evaluations of FtsA protein profile</td>
<td>56</td>
</tr>
<tr>
<td>12</td>
<td>Secondary structure prediction for FtsA protein</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>Optimization of the Sarkosyl concentration</td>
<td>73</td>
</tr>
<tr>
<td>14</td>
<td>UV cross-linking of Sarkosyl solubilized FtsA to ([\alpha-^{32}P])ATP</td>
<td>75</td>
</tr>
<tr>
<td>15</td>
<td>Evidence for aggregation of FtsA on columns</td>
<td>78</td>
</tr>
<tr>
<td>16</td>
<td>Coomassie stained SDS-PAGE showing FtsA protein isolation from membranes by Sarkosyl(0.2%) and octylglucoside exchange</td>
<td>80</td>
</tr>
<tr>
<td>17</td>
<td>Chromatographic purifications of FtsA in octylglucoside</td>
<td>81</td>
</tr>
<tr>
<td>18</td>
<td>Coexpression of FtsA and GroESL chaperones</td>
<td>84</td>
</tr>
<tr>
<td>19</td>
<td>SDS-PAGE profile of FtsA at each stage of purification</td>
<td>85</td>
</tr>
</tbody>
</table>
20. UV cross linking of $[\alpha^{32}\text{P}]$ATP to partially purified FtsA ........................................ 87

21. $[\alpha^{32}\text{P}]$ATP photo cross-linking with purified FtsA ........................................... 90

22. Immunoblot identification of FtsA like proteins from Gram-negative rod shaped bacteria ................................................................. 106

23. Immunoblot detection of FtsA-related proteins in Gram-positive bacteria ................................. 109

24. Screening for FtsA-related proteins in other unique microorganisms and eukaryotic specimens ........................................................................ 112
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>IEX</td>
<td>Ion exchange chromatography</td>
</tr>
<tr>
<td>OG</td>
<td>Octylglucoside</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
</tbody>
</table>
ABSTRACT

Cell division protein, FtsA, is an inner membrane protein with a putative ATP binding motif. Although FtsA has been studied genetically, it has not been characterized biochemically. Immunoelectron microscopy with FtsA polyclonal antibodies permitted FtsA to be detected within the cell. Since native FtsA is present in low amounts, the cellular level of this protein was increased by induction of $ftsA$ from an overexpression plasmid before processing the cells for immunogold labeling. The immunogold particles localized FtsA to the inner cytoplasmic membrane. Immunogold labeling of cells overproducing C-terminal deleted FtsA displayed the gold particles throughout the cytoplasm. This suggested that the C-terminal domain of FtsA may be the membrane anchor. In Western blots of proteins from membrane and soluble fractions obtained from wild-type cells, FtsA polyclonal antibody cross-reacting bands were found in both fractions. FtsA solubilized from membranes using sarkosyl bound [$\alpha-^{32}$P]ATP. Urea-solubilized FtsA did not bind ATP under similar conditions. Sarkosyl-solubilized FtsA irreversibly aggregated during column purification attempts, and hence, sarkosyl was exchanged with octylglucoside. FtsA was purified successfully in the presence of octylglucoside. This FtsA did not bind ATP. To obtain FtsA in a soluble and functional conformation, FtsA was co-expressed with chaperones, GroES and GroEL. FtsA was purified from the supernatant by successive anion exchange and nucleotide affinity columns. The FtsA fraction from an Affigel Blue column demonstrated [$\alpha-^{32}$P]ATP binding activity. But on further purification, only a dimer species of the purified FtsA bound [$\alpha-^{32}$P]ATP. This ATP binding was intrinsic to FtsA, and was abolished by
EDTA, unlabeled ATP, heat or FtsA antibodies. The dimer form was barely detectable on SDS-PAGE where the monomeric FtsA band was distinctly seen. However, immunoblotting with anti-FtsA verified the band in the dimer region as FtsA. Western blot screening of the proteins from cell lysates of various eubacteria and unicellular eukaryotes for the presence of FtsA homologs revealed that all contained a major immunoreactive protein band. No FtsA band was found in cell wall-less *Mycoplasma*, consistent with FtsA being required for peptidoglycan biosynthesis.

x
INTRODUCTION: AN OVERVIEW OF CELL DIVISION IN *E. coli*

The bacterial cell cycle is a complex process that can be delineated into two segments, 1) chromosome replication and segregation stage, 2) cytokinesis and cell separation stage. These processes are effected by a set of complex cascade of signals. Three regions of the *Escherichia coli* genetic map encompass genes particularly involved in cell division and murein biosynthesis. A 12 Kb region at 2 minutes is designated the *mra* region, the region at 15 minutes is designated as *mrd* region and the one located at 71 minutes, is the *mre* region. The "dcw" (division and cell wall) cluster is present in the *mra* region. This cluster includes the *mraA, mraB, ftsI, murE, murF, murG, murC, ddl, ftsQ, ftsA, ftsZ, envA, secA* and *azi* genes. These are essential morphogenes for cell division and the biosynthesis of peptidoglycan precursors (Donachie and Robinson, 1987; Donachie, 1993). The last visible stage of cell division that results in the physical separation of two daughter cells is termed "septation". It involves a topological shift in the direction of cell wall synthesis from longitudinal to a transverse mode with the circumferential invagination of the cytoplasmic membrane, the rigid murein and the outer membrane. This process is well coordinated with chromosome replication.

A number of temperature sensitive conditional filament forming mutants have been isolated and they all map in the 'dcw' cluster. They are classified as *fts* (filamentous temperature sensitive) because these mutants grow but are unable to divide at a restrictive temperature. The genes *ftsI, ftsQ, ftsA, ftsZ* are the best studied. Temperature sensitive mutants of these individual genes produce aseptate multinucleate
filaments with distinct morphological characteristics at the non-permissive temperature that relate to the order in which these genes function during cell division (Taschner et al., 1988). The *ftsZ* mutants at the non-permissive temperature exhibit filaments with no constrictions, whereas mutations in *ftsA* and *ftsI* result in filaments with periodic indentations along the length of the filaments indicating abortive division events (Begg and Donachie, 1985). The *ftsQ* defect results in long filaments only on a certain growth medium (Carson et al., 1991). This genetic evidence suggested that FtsZ protein functions the earliest followed by FtsA, FtsI and FtsQ during septation. All these gene products are required in stoichiometric amounts for productive division. Under or overproduction of FtsZ or FtsA protein results in septation inhibition and filament formation (Dewar et al., 1992).

The essential division genes *ftsQ*, *ftsA*, *ftsZ* and *envA*, all encoded in the 'dcw' (division cell wall) cluster of the *mra* region form an atypical operon. They are contiguous genes and are transcribed in the same direction from the same strand (Descoteaux and Drapeau, 1987). Although each of these genes has individual promoters, DNA sequence analysis showed the presence of additional promoters within the coding sequences of the genes, two for *ftsA* within *ftsQ* coding region and three for *ftsZ* within the coding sequence of *ftsA*. The proteins are encoded by successive open reading frames which either overlap or are separated by only a few bases. These internal promoters transcribe an overlapping set of mRNAs. There are no transcriptional terminators between the individual genes, but a strong transcription terminator for the whole operon was found at the end of *envA* gene (Beall and
Such a combination of overlapping expression ensures the progressively higher rates of expression of the downstream genes than their immediately preceding ones inversely proportional to the growth rate of the cells. An additional promoter preceding \textit{ftsQ} within \textit{ddl}, termed "gearbox promoter," has been identified. It is believed to be necessary for gene expression during stationary phase growth (Aldea et al., 1990).

The close proximity of the ribosome binding sequences (RBS) and translational terminators allows the coordinated expression of the genes of the "dcw" cluster (Robinson et al., 1984). Translational studies showed that FtsA and FtsQ proteins are inefficiently produced. Their relatively low levels of synthesis are due to the presence of rare codons (Grosjean et al., 1982). FtsA is translated independently of FtsQ from a ribosome binding site within \textit{ftsQ}. But FtsA translation is stimulated when upstream FtsQ sequences are more actively translated which implies that FtsA and FtsQ are translationally coupled. There is no auto-regulation on translation of FtsA or FtsQ (Mukherjee and Donachie, 1990). The differential expression of these three contiguous genes \textit{ftsQ, ftsA, ftsZ} results from differential translation of the mRNA (Mukherjee and Donachie, 1990). While FtsQ is produced at 25 molecules per cell, FtsA is synthesized at 200 molecules per cell and FtsZ at 20,000 molecules per cell. Thus the amounts of these proteins found in normal cells correspond to their relative translation efficiencies from a single mRNA transcript (Dewar et al., 1989).

There seem to exist inverted repeats (IR) of two fold symmetry proximal to this dcw gene cluster which reflect probable binding sites for regulatory proteins or mRNA
structuring for post transcriptional regulation (Robinson et al., 1984). A newly discovered trans-acting factor called SdiA, seems to be involved in transcriptional regulation of \textit{ftsQAZ}, at one of the two promoters immediately upstream of this cluster (Wang et al., 1991). When expressed from a plasmid, this factor not only caused the increase in cellular concentration of FtsZ protein but also complemented the \textit{ftsZ(Ts)} mutant.

FtsZ is a 41 kDa essential division protein in \textit{Escherichia coli} (Dai et al., 1991; Pla et al., 1991). It acts earliest during the initiation of septum formation. At non-permissive temperature, temperature sensitive \textit{ftsZ} mutant forms smooth morphology filaments with no septal indentations (Lutkenhaus et al., 1980). Increasing the wild type cellular concentrations of FtsZ 2-10 fold leads to the formation of chromosome less mini-cells due to hyperdivision activity (Ward and Lutkenhaus, 1985). Gross higher expression of FtsZ inhibits septation completely resulting in filaments similar to those exhibited by FtsZ mutants at the restrictive temperature. This division block can be overcome simply by increasing the levels of FtsA (Dewar et al., 1992; Dai et al., 1993). This block is apparently due to an abnormality in the ratio of these two proteins. FtsZ is relatively abundant and present at about 5000-20,000 molecules per cell. Immunolabeling studies showed FtsZ localizes to the midcell region in the form of a circular ring (Bi and Lutkenhaus, 1991). FtsZ, which is predominantly a cytoplasmic protein at the onset of division, accumulates at the septal primordium as a polymeric ring. This Z-ring appears to contract as the septation progresses. Upon completion of the septum formation, the FtsZ ring is disassembled and diffuses away. Hence it
shuttles between two states, a cytoplasmic state and a membrane associated polymeric state. A tubulin signature sequence motif is present within the FtsZ amino acid sequence. This predicted that FtsZ is a GTP binding protein and a GTPase. This was confirmed by experimental studies from three different investigators (de Boer et al., 1992; Raychaudhari and Park, 1992; Mukherjee and Lutkenhaus, 1994). FtsZ shows a concentration dependent lag in its GTPase activity suggesting that the GTPase activity is dependent upon polymerization of the FtsZ monomers. The two mutant FtsZ proteins, FtsZ3 and FtsZ84, showed a deficiency in the GTP binding ability and were incapable of forming the Z-ring during cell division in the respective mutants.

In vitro polymerization studies with purified FtsZ protein (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994) demonstrated that it can form filamentous micro-tubular structures similar to tubulin filaments. The protein assembly was dependent on the presence of guanine nucleotides, GDP or GTP. FtsZ could be cycled through rounds of polymerization and depolymerization. The FtsZ84 mutant protein is defective in GTP hydrolysis and as such could not polymerize.

FtsZ protein is required for septation not only in all eubacteria (Corton et al., 1987), but also in the cell wall-less Mycoplasma and archaebacteria which lack peptidoglycan. This raises the possibility that FtsZ primarily helps in the invagination of the cytoplasmic membrane (Wang and Lutkenhaus, 1996). Recently immunoelectron microscopy studies confirmed the presence of Z-rings in wild type dividing cells and its absence in the ftsZ mutants (Addinall and Lutkenhaus, 1996). Furthermore, they found that the Z-rings formed in the absence of active ftsA, ftsQ, ftsI gene products thus
demonstrating that these are not required for Z-ring formation. So the smooth filament morphology discussed earlier, is due to the absence of Z-ring formation. Similarly, situations under which FtsZ is inhibited, i.e. excess of MinCD or SulA production, Z-ring formation was blocked resulting in multinucleate filaments (Bi and Lutkenhaus, 1993). It can be proposed that FtsZ functions as the prokaryotic cytoskeletal equivalent of eukaryotic tubulin.

FtsA is a 45 kDa protein (Lutkenhaus and Donachie, 1979; Yi and Lutkenhaus, 1985) associated with the inner side of the cytoplasmic membrane (Pla et al., 1990). Many temperature shift experiments with \textit{ftsA(Ts)} conditional mutants indicated that FtsA forms part of the septum structure and functions throughout the septation process (Tormo et al., 1980; Tormo and Vicente, 1984). At the restrictive temperature of 42°C, \textit{ftsA} mutants form filaments with periodic constrictions along their length unlike the \textit{ftsZ} mutant filaments which have no indentations. However chromosome replication and segregation occurs normally (Lutkenhaus and Donachie, 1979; Tormo and Vicente, 1984). Overexpression of FtsA also inhibits cell septation, probably by inhibiting the formation of septalsome multiprotein complex (Wang and Gayda, 1990). This inhibition may be due to insufficient FtsZ protein because filament formation due to overexpressed FtsA protein can be relieved by the concomitant overexpression of FtsZ protein (Dewar et al., 1992; Dai et al., 1992).

Little is known about the native FtsA cellular function or its biochemical activities. From the gene sequence data it has been predicted that FtsA belongs to the actin-hexokinase-DnaK like proteins with a putative ATP binding motif (Bork et al.,
Sequences in the FtsA protein also have similarity with yeast CDC2 and CDC28 kinase domains (Robinson, 1987). Genetic evidence suggests that FtsA may be interacting with the FtsZ and FtsI (PBP3) at the membrane (Dai and Lutkenhaus, 1992; Dewar et al., 1992). A point mutation in the FtsA protein apparently diminished the radiolabeled ampicillin binding ability of FtsI (PBP3) derived from these mutant membrane fractions (Tormo et al., 1986, Sanchez et al., 1994). FtsA has also been implicated in coupling DNA replication and septation (Tormo et al., 1985).

FtsQ is a 31 kDa integral membrane protein with a bitopic membrane topology (Yi and Lutkenhaus, 1985; Storts et al., 1989). It is estimated to be present at 25 molecules per cell and is required throughout septation (Carson et al., 1991). Temperature sensitive mutants of \textit{ftsQ} form long multinucleate filaments (Begg et al., 1980). Overproduction of FtsQ protein had no effect on morphology of cells when grown in LB medium but causes constricted filaments in minimal medium (Dai et al., 1992). Overexpression of \textit{ftsQ} in a \textit{ftsI} (Ts) mutant is lethal even at the permissive temperature (Mukherjee et al., 1993) suggesting a possible interaction between FtsQ and FtsI. Increasing the levels of FtsQ in other \textit{fts} mutants results in filaments in LB medium at the permissive 30°C temperature. This can be interpreted that the mutated division proteins are more sensitive to disruptions in the septal apparatus due to excess FtsQ.

Other Fts proteins implicated in cell septation such as FtsL, FtsN, FtsW, ZipA are all transmembrane proteins. Their respective mutants exhibit aseptate multinucleate filaments at their non-permissive temperature thus implicating them in the septation
process. Each of these gene products is present in very low amounts (~50 molecules) per cell. FtsL has a potential leucine zipper motif in its periplasmic domain for dimerization (Ishino et al., 1989; Ueki et al., 1992; Guzman et al., 1992). FtsN was identified as a multicopy suppressor of \textit{ftsA12(Ts)} mutant (Dai et al., 1993). When on a plasmid it also complements mutations in \textit{ftsI} and \textit{ftsQ} but not \textit{ftsZ84}. The mechanism of FtsN protein function is yet unknown. FtsW protein is believed to function in maintaining cell shape along with PBP2 (penicillin binding protein 2) by affecting peptidoglycan biosynthesis (Ikeda et al., 1989; Ishino et al., 1986). ZipA is a recently discovered protein. It interacts with FtsZ at the midcell site. The \textit{zipA} null mutants form \textit{ftsZ(Ts)} like multinucleate filaments and hence it is deemed essential for cell division. ZipA is the membrane anchor for the FtsZ ring (Hale and deBoer., 1997).

\textit{Escherichia coli} cell division cycle is a repeating series of events punctuated by chromosome replication and septum formation (Helmstetter, 1987). Division occurs once per cell cycle only after the DNA replication and segregation are completed and by which time a critical cell length is attained (Donachie and Begg, 1989). Initiation of chromosome replication occurs once per copy of \textit{oriC} at each doubling of cell mass (Donachie, 1968). This precise coupling is due to the critical concentration of an initiator substance which depletes with each initiation and has to be resynthesized for the next cycle. DnaA protein is required for initiation of chromosome replication and not for later steps in replication. DnaA is present at the same concentration in cells growing at different rates and hence it is hypothesised to be the initiator substance. The reinitiation is also controlled by the methylation of GATC sites present in the \textit{oriC}
region of the DNA. Following replication, these sequences remain hemimethylated due to sequestration of oriC to the cell membrane. Reinitiation cannot occur until oriC is released from the membrane and methylated (Russel et al., 1987; Campbell et al., 1990). Mutants defective in post replication DNA uncoupling are identified as defective in various DNA topoisomerases function (Hiroto et al., 1968). These resulted in cells with large masses of unresolved DNA (Hussain et al., 1987). Another class of mutants, defective in partition muk form DNA free cells with twice the DNA content that is due to the irregular assortment of the sister chromosomes (Hiraga et al., 1989).

Jones and Donachie (1973) showed that a 5-10 minute period of RNA and protein synthesis was needed to occur 35 minutes after the initiation of DNA synthesis, in order for cell septation to progress. This led to the proposal that "termination proteins" formed shortly after termination of replication are required for initiation of septation. FtsA protein, a structural component, is synthesized during the late stages of cell cycle and requires active DNA replication for its synthesis (Tormo et al., 1985). DNA, RNA or protein synthesis inhibition with antibiotics in fisA(Ts)/recA double mutants using temperature shift experiments supported this further (Tormo et al., 1985). FtsA can fit the profile of the mediator protein.

The mechanism of the symmetric placement of the septum between the two nucleoids equidistant from the poles in dividing cells is explained by different models. Although, how the cell identifies the midcell site is still an enigma, the preferential selection of the midcell in deference to other potential division sites along the cell has been elucidated. The evidence for the presence of potential division sites at the poles.
came from the studies on minicell mutants of *Escherichia coli* (Adler et al., 1967). These mutants differentiate small chromosome less minicells towards the poles, indicating that these polar septation sites are derived from the previous division sites that are being reused (Teather et al., 1974). But in normal cells, these residual division sites are suppressed allowing only the midcell site to be active. This is accomplished by the cooperative action of the gene products of *minC, minD, minE* of the minB locus (de Boer et al., 1989). The MinC and MinD act in concert to form a non-specific septation inhibitor that blocks septation at all probable sites, while MinE imparts the topological specificity that enables division at the midcell site and vetoes the effect of MinCD. Overexpression of MinC and D or loss of MinE leads to division inhibition and filament formation. Conversely, elevated levels of MinE protein or loss of either MinC or MinD synthesis leads to the formation of minicells (Zhao et al., 1995). Wild type MinE is normally present in a limited amounts just enough to saturate the midcell topological target site and reverses the inhibition exerted by MinCD. But when overexpressed MinE overcomes the MinCD inhibition effect even at the poles and leads to the formation of minicells. The N-terminal domain of MinE contracts MinCD protein whereas the C-terminal domain acts directly or indirectly as the topological sensor (Zhao et al., 1995).

The periseptal annuli model demonstrates that when aseptate filaments of *ftsA* are exposed to hypertonic solution, plasmolysis bays occur along the length of the filament (Rothfield et al., 1987). These regions of plasmolysis indicated the presence of circumferential zones of adhesion which have been termed the periseptal annular apparatus (PSA) flanking the putative septal site (MacAlister et al., 1983; Anba et al.,
These annular regions are formed due to the adhesion of outer membrane, the murein and the inner membrane. As the cell grows the PSA duplicate and move laterally to the 1/4 and 3/4 cell length marking the midpoint of the daughter cells (Cook et al., 1986; de Boer et al., 1990). As a result, the periseptal annuli of the mother cell become the polar annuli of the daughter cells retaining an inherent potential for division (Teather et al., 1974). Recently it has been shown that plasmolysis bays are formed at the putative septal sites in both ftsA and ftsZ filaments (Cook et al., 1986). The periseptal annuli divide the periplasmic space into periseptal domain and act as physical barriers to prevent the unrestricted movement of molecules between periseptal and polar domains. This compartmentalization of the periplasm allows the cell to maintain proteins and other precursors required for septum formation at the proper location by preventing them from diffusing away (Foley et al., 1989).

The nucleoid occlusion model was proposed by Mulder and Woldringh, 1989, 1990; Woldringh et al., 1991) based on the fact that the cell divides at nucleoid free regions. They hypothesized that a diffusible positive factor produced during the termination of replication influences the cell to divide in between the segregating sister chromosomes. Thus the physical presence of the nucleoid itself prevents septation while the actively replicating nucleoid stimulates it to occur in the vicinity. However this model does not explain many questions.

The central stress model was proposed (Koch and Holtje, 1995) on the principle that the cytoplasmic membrane experiences a higher stress at the center of the rod shaped cell than at the poles as it elongates. It is based on the assumption that murein
is incorporated diffusely over lateral walls and intensely at the invaginating septum; cytoplasmic membrane (CM) is synthesized uniformly over the entire wall; CM and murein interact non-covalently; and that differential stresses are created throughout the surface due to differential syntheses of cytoplasmic membrane and murein. The elongation of the cell wall causes a tension in the cytoplasmic membrane which it relieves by the incorporation of phospholipid quickly. The localized murein insertion causes localized stresses in the cell wall which gradually decrease towards the center of the rod. But since the incorporation is diffusely uniform the net stress peaks exactly at the physical center. This causes the cell wall to invaginate to form the septum.

Peptidoglycan is composed of alternately arranged hexosamine derivatives, NAG (N-Acetyl glucosamine) and NAM (N-acetyl muramic acid) residues linked together by peptide cross bridges. These glycan chains are organized in a parallel fashion perpendicular to the long axis of the cell (Holtje and Schwarz, 1990). Prefabricated precursors of peptidoglycan material are enzymatically inserted and covalently bonded to the preexisting murein layer. The directionality of this insertion is from the inside to outside of the existing cell wall. Arthur Koch's "make-before-break" model (1983) states that new peptidoglycan material is attached to the existing strands before breakage of the old strands occurs. Once inserted, the new fragment is stretched effecting elongation.

The synthesis of the peptidoglycan precursors per se is brought about by the _mur_ set of genes encoding for various enzymes involved. Peptidoglycan or murein biosynthesis is accomplished by a set of enzymes called "penicillin binding proteins"
(PBP) which act specifically at different stages during cell cycle (Matsuhashi, 1982; Spratt, 1975; Tanaki et al., 1977; Suzuki et al., 1978). These proteins are bifunctional peptidoglycan synthetases with activities of peptidoglycan transglycosylase and β-lactam sensitive transpeptidase. They are named PBP1A, PBP1B, PBP2, PBP3, PBP4, PBP5, PBP6. PBPs 1–4 are high molecular weight PBPs. The N-terminal domains encode for the transglycosylase function which extends the glycan chains by the use of isoprenolpyrophosphate bound repeating units as precursors. The C-terminal catalyses the transpeptidase role that forms the peptide cross linkages between the two adjacent glycan strands (Suzuki et al., 1978).

The cell elongation process occurs when PBP2 synthesizes an initiation piece of peptidoglycan around the growing zone of sacculus. Then PBP1A and PBP1B start synthesis of the cell wall in the correct direction (Tanaki et al., 1977; Suzuki et al., 1978). Peptidoglycan synthesis is preceded by a reaction of a specific nicking enzyme which splits the existing network of murein to allow the incorporation of the newly formed peptidoglycan through cross bridges (Matsuhashi, 1981b). PBP3 is the septum forming peptidoglycan synthetase (Ishino et al., 1989) while PBP4, PBP5, PBP6 are involved in the formation and maturation of the peptidoglycan network.

FtsA interaction with PBP3 was deduced by the fact that binding of β-lactam antibiotics to PBP3 is affected by mutations in ftsA (Tormo et al., 1986). This was seconded by the observation ³H labeled DAP (diaminopimelic acid) in a temperature sensitive ftsA mutant was inhibited by PBP3 specific furazlocillin at permissive temperature (Nanninga et al., 1989). Prior experiments in this laboratory (Wang et al.,
1993) demonstrated that overexpression of FtsA protein increases the hexosamine content of the cells. This inferred FtsA involvement in peptidoglycan synthesis. Insertion of labeled DAP occured diffusely over the entire cell wall surface during elongation. But there was a topological shift of incorporation from lateral wall towards the cell center upon cell division especially at the leading edge of constriction (Woldringh et al., 1985; Cooper., 1988; Weintjes and Nanninga, 1989). Autoradiographic evidence using labeled DAP incorporation showed that peptidoglycan strand insertion is single stranded during elongation and multistranded during septation (DeJonge et al., 1989). This topographic shift is due to the change in murein specific enzymatic activities from PBP2 to PBP3.

The work presented in this dissertation attempts to further the understanding of the structural and biochemical aspects of FtsA protein. These studies sought to 1) visually immunolocalize FtsA protein and to see the effects of carboxy terminal truncation on its cellular localization; b) investigate the presence of different forms of FtsA protein in the cell; c) quantitate the partitioning of the protein between the soluble and membrane states; d) purify the membrane bound and soluble FtsA from the overproducing cells and to demonstrate its putative ATP binding activity; e) screen for the presence and estimate the size of FtsA protein homologues in cellular extracts of various Gram positive and Gram negative bacteria, archaebacteria, Mycoplasma, eukaryotes and eukaryotic organelles.
CHAPTER I

CELLULAR LOCALIZATION STUDIES OF FtsA WITH POLYCLONAL ANTIBODIES
Introduction

Bacterial cell septation in Escherichia coli involves the coordinated invagination of the three layers of the cell envelope between the segregated chromosomes and the precise assembly of a new cross wall at a site called the division septum. This complicated process is carried out with remarkable fidelity such that only one septum is formed per division event. Formation of this septum requires the combined action of a number of specific gene products. Among the many cell division genes identified, \textit{ftsQ}, \textit{ftsA}, \textit{ftsZ} and \textit{ftsI} are the best studied (Bi and Lutkenhaus, 1990; Donachie et al., 1984). These genes are all located in a morphogene cluster involved in cell wall biosynthesis and cell division at the 2 minute region on the \textit{Escherichia coli} genetic map. FtsQ and FtsI are transmembrane proteins; FtsZ is a highly abundant cytoplasmic protein while FtsA is a cytoplasmic membrane protein. All these proteins supposedly interact with each other based on the genetic evidence (Descoteaux et al., 1987).

Each of these genes are essential for cell viability and hence are indispensable to the cell. As such, they are amenable to study only as conditional temperature sensitive lethal mutants. The \textit{fts} (filamentous temperature sensitive) phenotype is characterised by multinucleate filamentous cell growth and eventual cell death when the mutants are grown at a non-permissive temperature of 42°C (Allen et al., 1974; Begg et al., 1985). This effect is due to the inactivation of the gene products at that temperature. The filamentous phenotype resulting from each of these mutant genes is morphologically distinct. The mutations in the \textit{ftsA}, \textit{ftsQ} or \textit{ftsI} genes result in filaments at the restrictive temperature with constrictions along their length which appear to be
aborted septa. Mutations in $ftsZ$ result in the formation of filamentous cells with no such constrictions (Begg et al., 1980; Lutkenhaus et al., 1980). These findings suggested that these gene products have a structural function and that FtsZ protein acts earliest during cell septation followed by FtsA and FtsI, while FtsQ is required throughout the process (Begg and Lutkenhaus, 1985; Donachie et al., 1980). Kinetics of cell division after the protein synthesis which was inhibited by the addition of chloramphenicol, compared to that of specific inhibition of FtsA using $ftsA(Ts)$ mutants, further demonstrated that FtsA protein is needed just before cell division occurs. Temperature shift experiments performed using some $ftsA$ mutant alleles showed that inactivation of FtsA at $42^\circ$C can be reversed upon temperature shift down to $30^\circ$C. Hence the potential septal primordia that resumed normal cell division were not inactivated. In contrast, in an irreversible $ftsA3(Ts)$ mutant, septa formed at $42^\circ$C were inactivated completely and could not resume septum formation when returned to the permissive temperature. This suggested that a physical block was established on the nascent septal structure by the inactive FtsA gene product (Tormo et al., 1984). These findings implied that FtsA is directly incorporated into the septum complex. Furthermore, the pattern of septation recovery in $ftsA(Ts)$ filaments after a temperature shift down in the presence of chloramphenicol revealed that de novo synthesis (Donachie et al., 1979) of FtsA protein is required during a short period just before the final stages of cell division and that DNA replication is also necessary for this synthesis. Hence it was deduced that FtsA could couple DNA replication and cell division (Tormo et al., 1980). In the presence of mutant $ftsA2(Ts)$ allele, PBP3 (penicillin binding protein 3) had diminished ability to
bind radiolabeled ampicillin, implying an interaction between PBP3 and FtsA proteins (Tormo et al., 1986).

FtsA is a 45 kDa protein located in the inner cytoplasmic membrane and it exerts its visible action after the FtsZ protein functions. Earlier fractionation experiments carried out in this laboratory (Chon and Gayda, 1988) found that FtsA partitioned with the inner-outer membrane fraction. Protease accessibility studies with spheroplasts and inverted membrane vesicles, confirmed that FtsA localized only on the inner side of the cytoplasmic membrane, and that it was probably anchored in such a way that the protein protruded into the cytoplasm (Pla et al., 1990). A computer generated 3D model of FtsA protein was proposed based on the predicted amino acid sequence. A putative ATP binding sequence motif was found similar to that present in diverse proteins like actin, Hsc70 and hexokinase (Bork et al., 1992). Thus, FtsA protein’s tertiary structure is hypothesized to be similar to that of actin.

The \textit{ftsZ} gene product, encodes a 41 kDa cytoplasmic protein (Pla et al., 1991) that plays a key role in cell division (Dai and Lutkenhaus, 1991). The level of FtsZ protein in the cell determines the frequency of cell division (Bi and Lutkenhaus, 1990b); and as such, it is the target for the endogenous cell division inhibitors, SulA and MinCD (Bi and Lutkenhaus, 1990a,b,c). Purified FtsZ protein undergoes GTP dependent assembly into filaments (Bramhill et al., 1994; Mukherjee and Lutkenhaus, 1994; Erickson et al., 1996). Immunogold labeling experiments using FtsZ antibodies demonstrated that FtsZ forms a dynamic ring at the predivisional site, termed the Z ring (Bi and Lutkenhaus, 1991). This ring maintains its position at the leading edge of the
invaginating cytoplasmic membrane and contracts as septum formation ensues. Once the septum is completed FtsZ protein depolymerizes and disperses into the cytoplasm. In similar, FtsZ localization experiments with the *ftsZ84(Ts)* mutant, which forms smooth filaments at non-permissive temperature, Z rings were not detected. In *ftsA, ftsI, ftsQ* mutants grown at the non-permissive temperature, FtsZ rings are formed at the putative septal sites, a finding that suggests that the functionality of these gene products is not necessary for Z ring formation or its localization (Addinall et al., 1996). In *ftsZ26(Ts)* mutant cells, internal non-ring FtsZ spirals were observed. These FtsZ spirals seemed to direct the invagination of the advancing septa resulting in morphological deformities of the elongated cells. Thus a single mutation that skews the Z-ring geometry can effect the septal morphology. In *rodA* elongation mutant cells, which grow as spheres, FtsZ localizes as Z-arcs leading to abnormal septa. These Z-arcs later develop into full Z-rings. In immunofluorescence labeled cells with no sign of separation, a single dot of fluorescence appeared at predivisional site. Taken together, these results demonstrate that FtsZ polymerizes bidirectionally from a single nucleation site on the inner membrane around the circumference and perpendicular to the long axis via an intermediate Z-arc stage (Addinall et al., 1996). However the question whether the invagination occurs by the contractile properties of FtsZ protein itself or in conjunction with other cell septation proteins such as FtsA and FtsI is yet to be elucidated.

Expression of morphogenes seems to be restricted to the times when their products are required by the cells. This is supported by the fact that both under
expression and overexpression of these morphogenes is lethal or detrimental to the cell. Overproduced levels of FtsA or FtsZ causes filamentation without septation (Wang and Gayda, 1990; Ward and Lutkenhaus, 1985) and ultimately cell lysis. Increasing the levels of FtsA can suppress the filamentation caused by the overexpression of FtsZ. Conversely, increasing the levels of FtsZ can relieve the division inhibition caused by overexpression of FtsA (Dai and Lutkenhaus, 1992). This study indicated that a proper ratio between the proteins FtsA and FtsZ is needed for septation to occur normally and the apparent possibility that these two proteins interact in vivo.

Overexpression of \textit{jisA} gene synthesizing a truncated FtsA protein, causes C-shaped cells and gyroidal cells (Gayda et al., 1992). Transmission electron micrographs of these cells revealed the presence of aggregates of striated structures. These aggregates were arrayed diagonally to the long axis of the cell. The composition of these aggregates was not determined but it is thought to be a composite of proteins involved in cell division (Gayda et al., 1992).

In this chapter, the cellular localization of both FtsA and that of the C-terminal truncated protein (FtsA\textsuperscript{c}) were investigated using TEM and immunogold labeling with FtsA specific polyclonal antibodies. Firstly, the localization of FtsA in FtsA expressed cells was studied. Secondly, similar experiments were performed to define the composition of the aggregated structures in the C-shaped cells and study the importance of the C-terminal domain of FtsA in cell septation. Thirdly, the presence of cytoplasmic and membrane bound forms of FtsA protein was investigated in subcellular fractions from wild-type cells using western blotting method. In addition, the question whether
FtsA protein is post-transcriptionally processed from a precursor form into a second form was investigated using $^{35}$S methionine pulse chase labeling experiments. For this purpose a new $ftsA$ overexpression plasmid (pQA5) under the control of T$_7$ promoter was specifically constructed.

**Materials and Methods**

**Bacterial strains and media:** Cells of *E.coli* strain D1210 (*recA13, lacF*) (Wang and Gayda, 1991) containing the FtsA overexpressing plasmid, pLHW3, or the FtsA$^c$ (truncated FtsA) overexpression plasmid, pLYH1, were grown in minimal medium (M9 medium, Miller, 1972) enriched with 1% vitamin free casaminoacids (DIFCO) and supplemented with 75 $\mu$g/ml of carbenecillin. The wild type control strain, X7102 was grown in YET complex medium (0.5% yeast extract, 1% NaCl, 1% Tryptone). The temperature sensitive cell division mutants YC100 ($ftsA10::Tn10$) or AMC419 ($fts84::Tn10$) (Chon and Gayda, 1988) were grown in YET medium and TEY medium (YET-NaCl) respectively supplemented with 15 $\mu$g/ml tetracyclin.

**FtsA protein purification for polyclonal antibodies:** D1210 cells with plasmid pLHW3 were grown overnight in enriched minimal medium at 37°C. This overnight culture was used to seed 1 l of fresh minimal medium at a 1:50 dilution which was then incubated in a 37°C shaker. The culture was monitored constantly until O.D.$_{600}$ was 0.3 at which time FtsA overexpression was induced by the addition of isopropyl $\beta$ D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 mM. The cells were incubated for a period of 8 hours, harvested by centrifugation at 6,500 rpm, 30 min.
4°C, in a GS-3 rotor (Sorvall-Dupont). The cells were washed in 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 50 mM NaCl and repelleted.

The FtsA isolation and extraction procedure was a modification of the method described by Frankel et al., (1991) for the purification of actin. The cell pellet was resuspended in 20 ml of lysis buffer, 10 mM triethanolamine pH 7.5, 1 mM PMSF (phenylmethylsulfonylfluoride), 1 mM Benzamidine, 1 mM DTT (dithiothreitol), 10 μg/ml each of Leupeptin and Pepstatin (Frankel et al.,1991). The cells were mechanically disrupted by two consecutive passages through a precooled French pressure cell (American Instrument Co., MD) at 10,000 psi to achieve maximal lysis. The unbroken cells were pelleted by a low speed centrifugation for 15 minutes at 1000 X g. This cell free lysate was further centrifuged at 20,000 X g for 15 minutes at 4°C to obtain the membrane fractions which contained the FtsA protein along with other membrane proteins. This pellet was gently suspended in solubilization buffer (25 mM triethanolamine, 1 mM EDTA (ethylenediaminetetracetate) and 0.2% (w/v) Sarkosyl) and allowed to stand for 10 minutes. This suspension was then ultracentrifuged at 100,000 X g for about 6 hours. This supernatant was enriched in FtsA protein based on SDS-PAGE analysis (Fig.9, lane 5). This protein extract was then chromatographed on an DEAE-Sephadex anion exchange column. The FtsA protein aggregated on the column and could only be eluted from the column with a 0-8 M urea gradient.

The FtsA eluted from the above column was further gel purified as follows. Equivalent volumes of this protein extract were mixed with sample disruption buffer (20% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 8 M urea, 0.03% bromophenol
blue, 1.25 M Tris-HCl pH 7.0) and electrophoresed on a gradient (10-20%) polyacrylamide gel (Laemmli, 1970) in the presence of SDS. Appropriate uninduced and induced cellular extract controls were also run in parallel lanes. To avoid the laborious Coomassie staining and fixing steps, the gel was treated for 15 minutes with cold 0.25 M potassium chloride (KCl) solution. KCl causes dodecyl sulfate to form an opaque precipitate. Since the concentration of the SDS is greater in the region of the proteins, they appear as white bands against a clear background of the gel (Sheer, 1994). The FtsA protein was distinguished using the prestained molecular weight markers and the uninduced-IPTG induced controls. The areas of the gel pertaining to FtsA were sliced out and soaked in phosphate buffered saline (PBS) to allow the elution of FtsA. A sample of the gel eluted FtsA protein was verified against appropriate controls on SDS-PAGE. This eluted FtsA was once again separated on a 4-15% SDS-PAGE, excised and repurified from the gel as before. The protein concentration was determined using BCA protein assay kit (Pierce Chemical Co.). Approximately 0.5 mg of FtsA protein was mixed with an equivalent volume of Freund’s incomplete adjuvant and this mixture was injected intraperitoneally into a rabbit. Before injecting the protein, a sample of pre-immune serum was bled and tested for any reactivity with Escherichia coli protein extracts by Western blotting. After three weeks, the rabbit was test bled and the serum was analysed for the production of FtsA polyclonal antibodies by means of Western blotting. A second booster dose of the FtsA antigen (0.5 mg) was injected at this time to enhance the antibody production. After a month, the antiserum
was tested again for the FtsA specific antibodies and the polyserum was harvested from
the rabbit.

The FtsA antibodies were purified from the serum as follows. The serum was
heated at 56°C for 30 minutes to inactivate the complement and then it was clarified by
passing through a 0.45 μm filter (Nalgene). In order to remove the non-specific cross
reacting antibodies, a 1:5 dilution (25 ml) of the polyserum in PBS (phosphate buffered
saline) was adsorbed against *E.coli* cell lysate (Chon and Gayda, 1988). The lysate was
obtained from YC100 (*ftsA*Δ0) mutant grown in 100 ml YET broth with tetracycline (15
μg/ml) at 30°C till log phase and shifted to 42°C for 3 hours. The cells were
resuspended in PBS and lysed by French pressure cell and this crude extract was then
added to the diluted antiserum. The mixture was incubated by shaking on ice for about
8 hours. The precipitate was pelleted by centrifugation and the supernatant was clarified
once again by passing through 0.45 μm filter (Nalgene). This purified serum was then
passed on a 2 ml Protein-A affinity column (Pierce) equilibrated with PBS buffer (0.067
M, pH 8.0) (Dr. Siebeling’s protocol). The column was washed thoroughly with PBS
buffer (30 ml) until the serum albumin was eluted and the absorbance OD_{280} returned
to baseline. The bound antibodies (IgG) were then eluted with 10 ml of 0.1 M citric
acid/citrate, pH 2.9 buffer into 10 ml of 2X PBS with 0.01 M inositol. This antibody
containing eluate was dialysed twice against 1 l of PBS, and it was used as the
polyclonal antibody preparation for all future experiments. The effectiveness of the
polyclonal antibodies against FtsA was verified by Western blots. The concentration of
the antibody preparation was determined by BCA protein assay kit (Pierce) against an
albumin protein standard as 2.5 mg/ml. Aliquots (0.5 ml) of the antibodies were frozen at -20°C.

**Immunogold labeling technique for TEM:** Immunogold labelling was performed as described by Bayer et al.,(1997) and Reid et al.,(1985) with modifications. Bacterial cells were immobilized on a filter support and fixed in two successive changes of a mixture of freshly prepared 1% formaldehyde, 0.5% glutaraldehyde, and 1% osmium tetroxide in sodium cacodylate buffer (50 mM, pH 7.1), for a total of 30 minutes. After a brief water rinse, the cells were stained with 0.5% uranyl acetate in the dark for 30 minutes followed by dehydration in an ethanol gradient and embedded in LR white resin. Thin sections were collected on nickel grids coated with collodion and carbon.

Immunolabeling was preceded by treating the grids with 2.5% sodium periodate. After a water rinse, samples were blocked with 2% BSA in PBS. The Protein-A affinity purified FtsA antibodies, were diluted 1:10 with the blocking solution, and the grids were exposed to it for 60 minutes. Appropriate control grids were exposed to normal pre-immune serum diluted 1:10 or to 2% BSA for similar time periods. Samples were washed with PBS then blocked again with 2% BSA before labeling with Protein-A conjugated to 10, 15 or 20 nm colloidal gold (Sigma) for 30-60 minutes. Grids were washed in PBS and water, then stained with Reynolds lead citrate. The sections were viewed in a JEOL 100CX transmission electron microscope at 80 kv.

**Light Microscopy:** Cells to be photographed were either directly placed on a microscope slide with a cover slip or fixed with 10% formaldehyde and then spread on
microscope slides. An Olympus BH2 phase contrast microscope with a 100X phase objective was used to observe the cells and Kodak Technical Pan Film was used for photography.

**Western Blotting Method:** The protein samples were solubilized by mixing with sample disruption buffer as described previously in the ratio of 1:1 and boiling for for about 5 minutes. The proteins were separated on a 4-15% gradient SDS-polyacrylamide mini gel cast according to Bio-Rad labs specifications. The proteins were then electrophoretically transferred onto Nytran membrane (S&S) at a constant voltage for 1-3 hours. Prestained Rainbow markers (Amersham) served as a control to monitor the efficiency of the transfer. The Western blotting procedure (Burnette, 1981) was performed with Immunoselect Assay kit (Gibco-BRL) according to the manufacturer's instructions. The Nytran or PVDF membrane was blocked non-specifically with 5% non-fat milk in TBS (Tris buffered saline; 50 mM Tris-Cl, 200 mM NaCl, pH 7.5). The membrane was then incubated with FtsA polyclonal antibody dilution 1:1000 in TBS and incubated for 1 hour to overnight at room temperature. After washing the membrane 3 times with TTBS (TBS+0.05% Tween) for 15 min each to remove the unbound antibody, it was then incubated with goat anti-rabbit biotinylated secondary antibody (1:3000) for an hour. Following this incubation period, the membrane was again washed 3 times with TTBS and incubated with streptavidin-alkaline phosphatase conjugate (1:20,000) for 30 min. The membrane was washed successively for 15 min each with TBS buffer and then with substrate buffer (100 mM Tris-Cl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). The membrane was then treated with the alkaline phosphatase.
substrate BCIP 116 µl, NBT 154 µl (50 mg/ml) in substrate buffer. The membrane was allowed to incubate until color bands developed and the reaction was stopped by thorough wash with distilled water. Band intensities were quantitated by Scanalytics program (Bio-Rad Inc).

Cloning of *ftsA* gene into pET5 vector and ^35^S methionine labeling experiments: Cell division gene *ftsA* was subcloned from the plasmid pZAQ (Ward and Lutkenhaus, 1985) encoding *ftsQ*, *ftsA* and *ftsZ*. A 2.3 kb DNA fragment consisting of the entire *ftsA* gene was generated by *EcoRI* digestion of *pZAQ*. This 2.3 kb DNA fragment was cloned into pET5 vector (Novagen Inc). Equal amounts of vector pET5(Amp') and pZAQ plasmid DNA (1 µg) were digested with *EcoRI* endonuclease for an hour at 37°C. The restriction enzyme was inactivated at 65°C and the digests were mixed in a 3:1 insert to vector ratio. DNA ligase was added to the mixture along with the ligation buffer according to manufacturer's recommendation and the ligation of the 2.3 kb fragment into pET5 was allowed by overnight incubation in a 16°C water bath. Appropriate positive and negative controls were also included. The ligated mixture was then transformed into YC100 (*ftsA* mutant) and the colonies that survived at 42°C on YET-ampicillin agar plates were selected. The plasmid DNA was purified from bacterial cultures of positive colonies by using the Qiagen plasmid purification kit (Qiagen Inc.) according to the manufacturer's recommended procedure. The size and the orientation of the 2.3 kb fragment was verified by restriction enzyme analysis and agarose gel electrophoresis. The plasmid, designated (pQA5), was then transformed into the host BL21(DE3) that encodes the gene for T7 RNA polymerase under the control
of an IPTG inducible lac promoter. The pQA5 transformants of BL21(DE3) were used for the labeling experiment. BL21(DE3) cells transformed with the vector plasmid pET5 were used as negative control. $^{35}$S Methionine labeling was performed as previously described (Gayda et al., 1992).

Results

Specificity of the polyclonal antibodies for FtsA: The visualization of FtsA by immunoelectron microscopy depends on the specificity of the polyclonal antibodies. Antiserum raised against gel purified FtsA protein was purified on Protein-A column matrix as stated in the Materials and Methods section. The antibodies were tested for reactivity by Western blotting various protein extracts resolved on SDS-PAGE. The purified antibodies reacted with a singular band of about 45 kDa, demonstrating good specificity. Only a faint band of FtsA was detected in whole cell lysate of uninduced control, D1210/pLHW3 (Fig. 1, lane 2), inspite of loading twice as much total protein. The intensity of the FtsA band increased in the whole cell extract of D1210/pLHW3 with induction of the encoded $ftsA$ gene with IPTG (Fig. 1, lane 3). An intense band with altered mobility (43 kDa) was evident with the whole cell extract obtained from D1210/pLYH1 upon IPTG induction of the carboxy truncated $ftsA$ gene (Fig. 1, lane 4). Partially purified proteins of FtsA and Fts$A^c$ were obtained from the respective amplified cell extracts. The membrane fractions from the two cellular lysates were subjected to Sarkosyl (0.2% w/v) solubilization as detailed in Materials and Methods (Chapter II). Both the FtsA and Fts$A^c$ enriched protein extracts (Fig. 1, lane 5 & 6) showed intense bands of the similar sizes as seen in lanes 3 & 4, as well as intense
Figure 1. Immunoblot demonstrating the specificity of the affinity purified FtsA polyclonal antibodies. Lane 1, Rainbow molecular standards; lane 2, Uninduced control D1210/plHW3 whole cell pellet; lane 3, whole cells after IPTG induction of FtsA for 6 hours; lane 4, D1210/pLYH1 whole cells overexpressing FtsA<sup>c</sup> after IPTG induction for 6 hours; lane 5 & 6, FtsA and FtsA<sup>c</sup> proteins Sarkosyl solubilized from the respective membranes (Chapter II, Materials & Methods); lane 7, gel purified FtsA protein used to raise the polyclonals. The proteins were run on a 4-15% SDS-PAGE, transferred onto nitrocellulose membrane and probed with FtsA polyclonal antibodies.
smaller bands of approximately 32 kDa & 30 kDa representing the breakdown products. The subtle difference in the molecular sizes of FtsA and FtsA\textsuperscript{c} breakdown products was similar to that of the full length products. Similar size breakdown bands were also observed in the respective whole cell extracts (lanes 3 & 4). Lane 7, depicts the gel purified FtsA protein antigen used to inject the rabbit. The high molecular weight bands of 90 kDa and 180 kDa seen in this lane represent FtsA protein aggregates that do not dissociate even after boiling in the presence of 8M urea in the sample disruption buffer. Thus the polyclonal antibodies purified are highly specific for the FtsA protein and they detect the breakdown products and oligomeric complexes of FtsA.

**Localization of cellular FtsA by immunoelectron microscopy:** The spatial organization of FtsA within *E.coli* cells was investigated by Protein-A conjugated immunogold labeling technique as described in the Materials and Methods. The presence of a cross-reacting FtsA antibody on cellular thin sections was visualized with Protein-A conjugated to gold particles.

The normal cellular concentrations of FtsA in wild type *E.coli* cells is very low. During the cell cycle FtsA is synthesized de novo, immediately after chromosomal replication and just prior to cell division (Sullivan et al., 1987). In a population of exponentially growing cells, only 10-20\% of them are in an active state of cell division (Bi and Lutkenhaus, 1992). This made it improbable to localize FtsA with normal levels of expression and hence, our lack of success. In order to increase the intensity of immunogold labeling and to mimic wild type conditions, the numbers of FtsA molecules were mildly enhanced by overexpressing it from a plasmid copy. This was achieved by
using D1210/plHW3 cells with the FtsA overexpression plasmid. The overproduction of FtsA was effected by inducing the tac promoter with 0.5 mM IPTG. After an hour, samples of cells were removed and processed for transmission electron microscope thin sections followed by immunogold labeling with FtsA antibodies as described in Materials and Methods. Figures 2 and 3 show that most of the immunogold label decorated along the inner side of the cell membrane. All of the FtsA antibody labelled cross sections which were screened, showed this pattern of gold labeling. No labeling was seen in control cells that were uninduced or cells labeled with pre-immune serum (Fig.2A).

Localization of C-terminal truncated FtsA: Earlier published data from our laboratory demonstrated that removal of the C-terminal end of the ftsA gene and expressing it from a plasmid resulted in a novel curved cell phenotype. Eventually these cells developed into spiral or gyroidal filaments (Gayda et al., 1992). Thin sections of these cells when observed under electron microscope, showed long axial structures traversing the cytoplasm. The ends of these aggregates appeared to be associated with the cytoplasmic membrane. It was speculated that the curved cell phenotype was a result of 1) an internal structural formation probably involving FtsA and FtsAc directly and 2) concomitant asymmetric alteration in peptidoglycan synthesis. Hence it was imperative to investigate these structures using immunogold labeling to determine protein composition of these aggregates, the cellular location of these structures and deduce the functional importance of the C-terminus of the FtsA protein.
Figure 2. Immunogold localization of FtsA protein. Immunoelectron micrographs of longitudinal sections of D1210/pLHW3 cells overexpressing FtsA, A) cells labeled with preimmune serum; B) cells treated with FtsA antibodies. D1210/pLHW3 cells were induced with IPTG for 1 hour to increase cellular FtsA. 10 nm gold particles conjugated to Protein-A were used for this experiment.
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 3. Immunogold localization of FtsA in cross sections. D1210/pLHW3 cells were induced as in the Figure 2 and panels A and B labeled with 20 nm gold particles; C and D labeled with 10 nm gold particles.
The \textit{ftsA}\textsuperscript{c} was sub-cloned from the initial construct pDLL8 (Gayda et al., 1992), into a more efficient expression plasmid pTTQ18 (Stark, 1987). The cells hosting this plasmid pLYH1, were induced with IPTG for a period of 2-6 hours and aliquots were taken out to be fixed for immunogold labeling of the FtsA as described earlier. The phenotypic morphology of the cells was observed periodically under a phase contrast microscope as shown in Figure 4. The immunogold labeled thin sections of these cells showed only a few gold particles outlining the inner membrane which was unlike the labeling seen with overproduced full length FtsA. However the axial structures were specifically labeled with the immunogold particles (Fig.5 & 6). Thus these protein aggregates contain FtsA\textsuperscript{c} and FtsA protein. Since wild type FtsA is present in limited numbers, it can be deduced that the immunolabeled protein is predominantly FtsA\textsuperscript{c}. As previously speculated, there could be other proteins interacting with FtsA that compose these aggregates. A most probable one is FtsZ, an abundant cytoplasmic protein. The location of these aggregates in the cells was random. In Figure 5, the aggregates appear to be crossing the cytoplasmic bridge between the constricting cell or conversely, originating from the septum. Some of these structures appeared to be running tangential to the inner membrane. This displacement could be the result of the physical obstruction by the nucleoid. However, cross-sections through the cells showed that most aggregates were generally suspended in the cytoplasm. Each aggregate seemed to be packed as individual filaments in bundled arrays. Some cells showed the aggregates emanating from the polar region (Fig.6 A,C).
**Figure 4.** Phase contrast light microscope photographs of D1210/pLYH1 carrying the \( ftsA^c \) gene. Panel A, shows cells before IPTG induction of FtsA\(^c\), panels B,C,D shows cells after induction for 2,4,6 hours respectively.
Figure 5. Immunogold localization of FtsA in cells overexpressing FtsA\(^6\) protein. *E.coli* D1210/pLYH1 cells were induced with IPTG for 6 hours, processed for TEM, treated with FtsA antibodies and immunolabeled as in Materials and Methods. A and B thin sections through partially septating cells. 15nm gold particles were used.
Figure 6. Immunogold labeled sections of curved cells treated as in Figure 5. Panels A,B show the FtsA\(^{-}\) aggregates labeled with the gold particles. Cross sections show that these aggregates are essentially cytoplasmic. Panels C,D are similar sections labeled with preimmune serum as control.
In prior studies, the diagonal location of most of the aggregates in many C-cells gave the impression that 1) these aggregates may be responsible for the physical distortion of these growing cells causing them to become curved 2) and that they may be emanating from a membrane site at the putative septum. With FtsA antibodies we were able to investigate the cellular origin or source of these structures. Thin sections of cells after FtsA c was induced only for an hour were examined. Representative cells are shown in Fig7B & C. Figure 7B shows a curved cell in which the immunogold labeling pattern displays random gold particles with no localization sites. Other cells (Fig.7C) were 2 or 3 cell lengths long due to FtsA c expression also show immunogold labeling that was non-specific. In all of the thin sections of cells in which FtsA c was induced only for an hour, no axial aggregates, as seen previously, were observed.

The distribution of FtsA in the wild type *E.coli* strain, X7102, was also investigated by immunoelectron microscopy. The general model, based on the genetic evidence was that FtsA acts at the invaginating septal complex in a membrane dependant manner immediately after FtsZ forms the Z ring. It was our goal to localize FtsA in wild-type cells when they are in an actively dividing state. However, immunogold particles identifying FtsA were rarely seen in approximately 200 thin sections that were screened. Only one cellular thin section (Fig.7D) showed a pattern of immunogold label perpendicular to the long axis of the cell, albeit poorly and it was discounted as statistically insignificant.

**In vivo ³⁵S Methionine labeling and pulse-chase experiments of FtsA:** The possibility of processing of the FtsA protein after synthesis was investigated with ³⁵S
Figure 7. Immunogold localization of FtsA in cells in which FtsA⁶ protein was induced for an hour. D1210/pLYH1 cells were induced for an hour and fixed for TEM thin sections and probed with FtsA antibodies as described in Fig.6. Panel A, cells labeled with preimmune serum; panels B, C, D cells exhibiting gold particles non-specifically scattered across the cells.
methionine labeled FtsA. FtsA was specifically labeled for 30 and 60 minutes and then chased with excess unlabeled methionine for 30, 60 or 90 minutes. Aliquots of labeled or pulse chased whole cell pellets were directly lysed in sample disruption buffer followed by SDS-PAGE (10-30%) analysis and autoradiography. The relevant autoradiograph (Fig. 8) showed a band at approximately 45 kDa (FtsA) which appears only after IPTG induction. It was conspicuously absent in the control sample with ftsA inserted in incorrect orientation (Fig. 8, lane 2). This labeled band increased in intensity with labeling time. When labeled cells were subjected to a chase with excess unlabeled methionine for similar periods of time, the 45 kDa band did not decrease in intensity or a lower molecular weight labeled band appear simultaneously. If such a band appeared, it would have been suggestive of a precursor of FtsA being processed into a mature protein. Thus the possibility that FtsA protein is processed was ruled out.

Evidence for cleavage of FtsA during cell lysis: In cell lysates of FtsA overproduced cells, most of the FtsA was recovered in the 20,000 X g membrane fractions under two different lysis conditions tested. The SDS-PAGE (Fig. 9) analysis of whole cell pellets when lysed directly with sample buffer, before (Fig. 9, lane 1) and after induction (Fig. 9, lane 2), showed only one FtsA protein band, identified by the increase in its intensity after induction. But when the cells were lysed in a French pressure cell or with lysozyme, the FtsA enriched protein extracts showed two distinct bands pertaining to FtsA (Fig. 9, lanes 3,4,5,6). The lower band in this doublet pertaining to FtsA appeared intensely only after the cell lysis. A strong inference is that FtsA is being cleaved at a site causing it to be released from the membrane during cell
Figure 8. ($^{35}$S) methionine labeling and pulse chase of FtsA. BL-21(DE3)/pQA5 cells labeled for times 30, 60 mins. (lanes 3, 4) with ($^{35}$S) methionine and then chased with excess cold methionine for the times 30, 60, 90 mins. (lanes 6, 7, 8) processed and subjected to SDS-PAGE and exposed to autoradiograph. Lane 2 shows cells with control plasmid carrying $ftsA$ in incorrect orientation labelled for 90 min; lanes 1, 5, 9 show molecular weight standards. Arrow shows the FtsA band.
Figure 9. Evidence for cleavage of FtsA during cell lysis: One ml whole cell pellets (D1210/pLHW3) before (lane 1) and after (lane 2) IPTG induction for 6 hrs, lysed directly in SDS/urea sample disruption buffer; lane 3, FtsA induced cells lysed directly with Lysozyme/Sarkosyl; lane 4, Sarkosyl solubilized FtsA from the membranes of overproduced cells; lane 5, 20,000 X g pellet after solubilization as in lane 4; lane 6, supernatant after removal of FtsA enriched membranes; lane 7, Octylglucoside (2%) soluble proteins after sedimentation of the ribosomes and membranes at 100,000 X g; lane 8, 100,000 X g pellet. Samples were analysed on 10-30% SDS-PAGE and Coomassie Blue stained. Five fold more sample was loaded in lane 8.
disruption procedure. This is believed because this lower molecular weight FtsA band was retained in the post-membrane supernatant after 20,000 X g centrifugation (Fig. 9, lane 6). Whereas the higher molecular weight FtsA that was in the supernatant, pelleted upon centrifugation at 100,000 X g (Fig. 9, lane 7).

**Western Blot analysis of two forms of FtsA in wild type cells:** Wild-type D1210 cells were grown to log phase and cellular fractions after French pressure cell lysis were obtained by ultra-centrifugation. The total protein in the lysate, soluble and membrane fractions was quantitated. Equal volumes of each fraction were separated on an SDS-PAGE and then Western blotted with FtsA antibodies. The anti-FtsA polyclonal antibodies identified two closely migrating bands in the cellular lysate, one of which segregated with the membrane fraction (Fig. 10, lanes 4, 7) and the other with the soluble fraction (Fig. 10, lanes 3, 6). Some of the membrane form was also present in the soluble fraction probably due to the smaller size of the bound membrane fragments. The band in the membrane fraction appeared to run with a retarded mobility compared to the lower band in the cytoplasmic fraction. These results support the earlier conclusion that the slightly smaller cytoplasmic form of FtsA could be the result of cleavage of the membrane bound form during cell breakage. To find out, if phosphorylation could be responsible for this increase in molecular size of the membrane FtsA form, membrane extract was treated with alkaline phosphatase and analysed by SDS-PAGE followed by Western blotting with FtsA antibodies (data not shown). There was no molecular weight decrease of this band. However, alkaline
Figure 10. Western blot analysis of cellular fractions of wild type FtsA. *E. coli* X7102 cells were grown to OD$_{600}$ of 0.3 (log) or 1.4 (stationary), resuspended in buffer-A to obtain the same cell density, lysed by French press followed by ultra centrifugation at 100,000 X g for 1 hr to sediment the membranes. Each membrane fraction was resuspended in original volume of buffer-A. Equivalent volumes of each of the lysate, supernatant and membrane fractions of log (lanes 2,3,4) or stationary (lanes 5,6,7) phases were run on 4-15% SDS-PAGE, blotted onto Nytran membrane and probed with FtsA polyclonal antibodies. Lanes 2,5 lysates; lanes 3,6 supernatants; lanes 4,7 membranes of respective samples; lane 8 shows the gel purified FtsA protein control.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
phosphatase treatment is not a reliable and sensitive method as it does not necessarily dephosphorylate all proteins, hence this result was inconclusive.

Discussion

Genetic and biochemical studies revealed that many proteins (FtsZ, FtsA, FtsI, FtsQ, ZipA, FtsN) should interact together sequentially as a multimeric complex at the putative division site. The formation and placement of the septum is generally believed to be temporally and spatially regulated (Mukherjee and Lutkenhaus, 1996). Previous studies indicate that FtsA is anchored to the membrane and plays a structural role within the septation (Chon and Gayda, 1989; Pla et al., 1990).

In this study, we attempted to localize FtsA within the cell using immunoelectron microscopy. By enhancing the physiological levels of the cellular FtsA protein by overexpression and immunogold labeling of thin sections, we showed that FtsA was almost exclusively associated with the inner membrane throughout the cell. This strongly confirmed that it is inner membrane localized. These results only establish that, when FtsA is overexpressed it is a cytoplasmic membrane protein and does not answer the question whether it is preferentially located at the septal region under normal conditions. It could also be argued, based on the above evidence, that it is normally distributed throughout the inner membrane but is activated only at the division site. The explanation for finding random localization of newly synthesized membrane proteins is not well understood. Two possibilities proposed are that the membrane proteins are synthesized and inserted at multiple random sites or they are synthesized at a few discrete sites and rapidly diffused over the membrane surface (Funnel, 1996). The
membrane specific immunogold labeling pattern we observed represents individual molecules of FtsA and we did not see inclusion bodies since the electron micrographs of these sections did not reveal any classical dense masses of protein (Schoemaker et al., 1985; Blum et al., 1992; Bowden et al., 1991) which are normally observed upon gross overexpression of some proteins. Furthermore, some of the cells used for above immunogold labeling had FtsA overexpressed for only an hour. In fact, FtsA when extracted, partitioned with the membrane pellet even at very shorter induction times, low temperature of induction (25°C) and low IPTG concentration (0.1 mM), conditions which generally preclude the formation of protein inclusion bodies.

Function of the C-terminus of FtsA: Lack of any immunogold labeling of FtsA on the inner membrane of thin sections prepared from D1210/pLYH1, cells overexpressing the FtsA\(^c\) (truncated) protein, suggested that the deletion of the C-terminus of FtsA prevents its apparent normal association with the membrane. Thus, the C-terminal domain of FtsA may be required for membrane anchoring. The aggregate formation in the curved cells was probably due to the inherent nature of the protein to self-aggregate. The wild-type FtsA that is synthesized in these curved cells is probably sequestered within these aggregates and is unavailable for normal septation. The protein-protein interaction domains in the FtsA\(^c\) protein are probably towards the N-terminus of the protein and hence still accessible for interactions with itself and with FtsZ protein.

The absence of any aggregates in the thin sections of D1210/pLYH1 curved cells, induced for only an hour, indicated that these aggregates are not physically
responsible for the cell curvature and that the nucleation for this aggregation occurs only after a critical concentration of FtsA\(^c\) is attained. When the cells with overproduced FtsA\(^c\) (6 hrs) were fractionated, the protein aggregates were found to sediment with the membranes although significant amount of FtsA\(^c\) protein was present in the supernatant (data not shown). Probably the sheer size of the aggregates enabled them to be pelleted.

The D1210/pLYH1 curved cells eventually grow into aseptate gyroidal filaments upon prolonged expression of FtsA\(^c\). This morphology could be due to impairment of the Z ring formation by sequestering FtsZ and/or normal FtsA by the high levels of FtsA\(^c\). This sequestering perhaps causes asymmetric nucleation of FtsZ. Since wild-type FtsA and FtsZ are expressed normally in these cells, one would not expect the cell division to be abnormal. If only the wild-type FtsA protein but not FtsZ, was sequestered in these aggregates, the cells should show aseptate filamentation with periodic constrictions as seen with \(ftsA\) temperature sensitive mutants. But since the spiral cells do not show any constrictions, the conclusion is that both FtsA and FtsZ must both be sequestered in these protein aggregates, and it explains the loss of normal cell division in the spiral cells. Another hypothesis for the curved cell morphology is that the dislocation of the wild type FtsA from the putative septum site misdirects the PBP3, required for septal peptidoglycan synthesis. Because the C-terminal domain, which is lacking in FtsA\(^c\) must interact with PBP3 and direct the insertion of the peptidoglycan material at the membrane/septum site. On the other hand, since the polymerizing domains and FtsZ interacting domains of FtsA\(^c\) are still intact, FtsA\(^c\)
perhaps sequesters the wild type FtsA and FtsZ present. Although FtsAc has a deficiency of only C-terminal 28 amino acids, a plasmid expressing it cannot rescue the YC100(Ts) transformants at the non-permissive temperature. PBP3 is the best candidate for this FtsA interaction because of the fact that it is the only cell division protein whose cytoplasmic N-terminal domain is indispensable for its peptidoglycan synthesis function during cell septation (Addinall and Lutkenhaus, 1996). Replacement of this cytoplasmic domain resulted in the loss of ability to support cell division (Guzman et al., 1997).

FtsA, along with other division proteins like FtsI and FtsQ, is a relatively scarce protein (200 molecules) and hence immunoelectron microscopy (IEM) method is not sensitive enough to detect its timed localization in a septating cell. Moreover, since the polyclonals were raised against a denatured antigen and in vivo the protein functions in a native conformational state, only a few surface epitopes were accessible for the polyclonal antibodies to bind in that particular dimension (Beveridge, 1993).

**Complementation tests:** Neither the plasmid construct pLYH1 nor its parent plasmid pDLL8, carrying the truncated FtsA gene, could complement the YC100(T*) FtsA mutant at the non-permissive temperature of 42°C. This result implied that the FtsAc protein is non-functional and that the C-terminus plays a essential role in FtsA's activity. The importance of FtsA's C-terminal was further strengthened by a HisTag fusion to the C-terminal of FtsA, that was recently constructed in this laboratory, which did not restore normal cell division activity to the YC100(Ts) FtsA mutant at the non-permissive temperature (unpublished data).
As shown in Figure 9, a second lower molecular weight form of FtsA with slightly altered mobility appears upon lysis of the overexpressed cells. Most of the upper form pellets with the membrane fractions and was only solubilized by detergents, while the lower form is retained in the high speed supernatant. By definition, any protein that is present in the supernatant after centrifugation at 100,000 X g for an hour is considered soluble (Frankel et al., 1991). Is the appearance of two forms of FtsA an artifact resulting from cell lysis. We believe it is not. This is based on Western blot analysis of wild-type cellular fractions (Fig. 10), wherein FtsA partitioned into membrane fraction and the cytoplasmic form. Western blot analysis of protein fractions eluted from chromatographic columns constantly identified at least two major bands of FtsA. Since FtsA is believed to be a phosphoprotein (Freestone et al., 1995), one possibility is that FtsA is dephosphorylated during purification and resolves into multiple isoforms with slightly altered mobilities on SDS-PAGE.

A model for FtsA membrane anchoring: The primary sequence of FtsA does not contain any signature sequence that would target it to the membrane, although it is not required for the type of anchoring proposed for FtsA. The bacterial secretory protein, SecA, does not have a membrane anchoring signature sequence either but it is found both in the membrane and cytoplasm. The hydropathy profiles of the FtsA primary sequence was derived using the "Protlyze" algorithm. A Kyte-Doolittle plot (Fig.11B) shows that FtsA protein sequence has hydrophobic amino-acid patches distributed over the entire sequence. From the primary sequence of FtsA (Fig.12A), it was noted that most of the protein would be involved within the putative ATP binding
Figure 11. Primary and secondary sequence evaluations of FtsA protein profile. Panel A, shows the aminoacid charge distribution of FtsA primary sequence; Panel B, hydrophilicity plot for FtsA protein as predicted by Kyte-Doolittle method. Negative scale depicts hydrophobicity peaks. Panel C, Chou-Fasman helix prediction for *E.coli* FtsA. Boxes represent the extent of each helix.
A

Scale Used: [ ] >= 70%  [ ] >= 60%  [ ] >= 50%

Polar AAs

Nonpolar AAs

B

C

Chou-Fasman Prediction for ecolifise
Figure 12. Secondary structure prediction for FtsA protein. A) The FtsA amino-acid sequence shows an ATP binding motif with amino-acid residues which are conserved among many other ATP binding proteins suggesting a functional ATPase activity for FtsA. B) shows the Chou-Fasman secondary structure cartoon based on predicted helix, turn, sheet motifs. Note the terminal 30-40 aminoacids are not included in the ATP binding fold.
domains except the C-terminal 30-40 amino acids. This stretch of amino acid residues are predominantly positively charged groups (>70%) (Fig. 12A) with interspersed hydrophobic residues and could be accessible to interact with the membrane when required. The Chou-Fasman secondary structure prediction plot (Fig. 12B) did not show any helix pattern that would allow it to wholly span the membrane. It could also be that FtsA can shuttle between membrane bound and free cytosolic states from the time it is synthesized to being associated with the membrane at the time of septation.

Possible mechanisms for this type of reversible insertion and release of cytoskeletal proteins into membranes include 1) reversible phosphorylation on serine, threonine or tyrosine of proteins 2) local changes in lipid composition 3) covalent lipid modification of these proteins (Funnel., 1997). The additional requirement for another transmembrane protein in such cytoskeletal protein and membrane associations cannot be excluded. In eukaryotes, specific linker proteins (cytoplasmic/peripheral membrane) like actinin and vinculin mediate the direct or indirect interaction of actin filaments (cytoplasmic) with transmembrane receptors (integral membrane proteins). This can very well be applied to the bacterial system i.e. FtsA, FtsZ, FtsI interactions with FtsA acting as a linker between FtsI and FtsZ at the constricting septum. It was shown recently that a new protein, ZipA forms a ring at the midcell region as an anchor for FtsZ ring (deBoer et al., 1996) even before the cell begins to divide. Recently, it was demonstrated by immunofluorescence microscopy, that FtsI apparently also forms a ring at the predivisional site even prior to the FtsZ ring localization. Hence, concerted action of many proteins is required to accomplish the task of septation. FtsN, seems to
organize as a ring at the nascent septum but the time at which it acts is still unclear (Dai et al., 1996).

The inhibition of cell division in the presence of excess FtsA is probably due to concentration dependent titration of other essential cell division proteins such as FtsZ from the putative septum complex (Chon and Gayda., 1988; Wang and Gayda., 1990; Tormo et al., 1984, 1986). In fact, recently Ma et al., (1996) using GFP fusions to FtsA and FtsZ demonstrated that FtsA localizes as a ring at the putative septum in an FtsZ dependant manner. The FtsA ring was not found in cells in situations when functional FtsZ was absent. They also showed that GFP-FtsA or GFP-FtsZ proteins in the presence of excess wild type FtsZ forms non-productive spirals. Furthermore, Addinall et al. (1996) reported that Z ring formation is prevented when FtsA is overproduced. Recently immunoflorescence microscopy studies have also revealed that FtsA is also localized to a ring in a FtsZ dependent manner at the septum (Addinall et al., 1996). These results, along with the prior cell morphology studies (Dai et al., 1992), confirms that stoichiometric amounts of each of these essential proteins are required for productive cell septation. With the panoply of cell division proteins, all localized as rings at the midcell, septum formation is becoming a much more intricate process than it is believed.
CHAPTER II

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF CELL DIVISION PROTEIN FtsA FROM \textit{Escherichia coli}
Introduction

The cell division protein, FtsA, is a 45 kDa membrane associated protein. FtsA normally localizes to the inner cytoplasmic membrane (Chon and Gayda, 1988; Pla et al., 1990). The levels of the native protein in *Escherichia coli* cells can be characterized as very low, ranging from 50-200 molecules per cell (Wang et al., 1992). Another predominant cell division protein FtsZ is highly abundant and is present at about 5000-20,000 molecules per cell (Dai and Lutkenhaus, 1992). FtsA interacts with FtsZ after it localizes at the cell mid-point in an annular fashion (Tormo et al., 1980; Addinall et al., 1996). Overproduction of FtsZ, at least 10 fold, causes inhibition of cell division (Ward and Lutkenhaus, 1985) which can be overcome by simultaneous overexpression of FtsA. This suggests an interaction between both these proteins and that FtsA is required to be in a stoichiometric ratio to FtsZ (Dewar et al., 1992).

Overexpression of FtsA also inhibits cell septation resulting in filamentous cells (Wang and Gayda, 1990). These aseptate multinucleate filaments produce periodic bulges at the putative septal sites. Such bulged cells were found to contain enhanced hexosamine content due to enhanced peptidoglycan precursors, suggesting the direct or indirect involvement of FtsA in peptidoglycan synthesis (Wang et al., 1993). Morphogenetic observations revealed that FtsA is a component of the septalsome complex, since an altered form of FtsA produced by the temperature sensitive *ftsA3* mutant at 42°C blocks cell septation (Tormo and Vicente, 1984) and removal of that block requires Lon protease function (Dopazo et al., 1987; Pla et al., 1990). Biochemical studies with the aforementioned *ftsA3* mutant suggested that FtsA may
interact with penicillin binding protein 3 (PBP3) which is specifically required for septal peptidoglycan synthesis. Membrane fractions of this mutant at the restrictive temperature showed diminished ability to bind $[^{3}H]$-ampicillin (Tormo et al., 1986) and this was $ftsA3$ allele specific (Sanchez et al., 1994).

An amino acid sequence comparison study of FtsA (420 aa) revealed it is similar to proteins such as actin, hexokinases, hsp70 and other ATPases. A set of common conserved amino acid residues, distributed in five sequence motifs which are involved in ATP binding were found (Bork et al., 1992). Based on the predicted 3D structural analysis of actin, it was predicted that FtsA will have five loops, one for adenosine binding, two for phosphate interactions, and two representing interdomain hinges. This ATP binding motif is distinctly different from the single phosphate binding loop motif as exhibited by adenylate kinase, RecA, EF-Tu or Ras oncprotein-p21 (Schulz, 1992). Doolittle (1981) has pointed out that any alignment of two proteins will show some accidental homology which makes it to difficult to interpret the significance of weakly homologous sequences. But the alignment of several like protein sequences with each other avoids this problem, because it is much less likely that several proteins will have the same conserved sequence.

It was also reported that FtsA has a region of amino acids homologous to eukaryotic cell cycle kinases CDC2 (Robinson et al., 1987). Analysis of the primary sequence of FtsA revealed a highly significant region of 60 amino acids, suggesting that FtsA may be a a regulatory kinase. Protein phosphorylation has been shown to occur in bacteria (Manai et al., 1979). In *E.coli*, at least 130 phosphorylated proteins have
been observed in vivo (Cortay et al., 1986). Novel phosphoproteins have been identified using chromatographic fractionation of labeled proteins (Freestone et al., 1995). One of these proteins was a 45 kDa intensely phosphorylated protein which is present in tiny amounts barely detectable by Coomassie Blue protein staining. This protein was speculated to be FtsA.

The aforementioned homologies and the predicted structure of FtsA, prompted the investigation of the biochemical properties of FtsA, i.e. putative ATP binding function. In order to investigate the predicted enzymatic activities, FtsA had to be obtained in a soluble, pure and functionally active state. Hence the goal of this research was to purify FtsA and then to characterize its predicted biochemical functions.

Since FtsA is a membrane protein (Pla et al., 1990) and is synthesized in minute amounts in the cell, it was overexpressed in order to obtain it in ample amounts. Previous overexpression studies in the lab showed that FtsA fractionated with the insoluble membrane pellet probably due to its inherent hydrophobicity. Hence it had to be solubilized from the membranes with either detergents or as a urea denatured protein (Chon and Gayda, 1988; Wang and Gayda, 1992).

This chapter focusses on characterizing the nature of FtsA during its purification. Initial studies using urea solubilized refolded FtsA protein did not show ATP binding activity. Thus it became a necessity to develop methods to successfully extract and purify FtsA from membrane fractions utilizing mild, non-denaturing conditions. Two methods of membrane solubilization and purification of FtsA that prevented aggregation, are presented here along with a brief mention of other
procedures attempted. A novel in vivo protein folding achieved by co-expressing bacterial chaperones GroES and GroEL along with FtsA was also attempted. This method prevented the apparent aggregation of FtsA protein with the outer membrane proteins due to misfolding and allowed the isolation of FtsA from the soluble fraction. FtsA was purified from this GroESL enriched soluble fraction and used for biochemical studies.

Many problems were resolved to obtain purified soluble FtsA. These problems included: FtsA expression was poor, FtsA’s irreversibly aggregated during purification even in the presence of detergents, and lack of a biochemical assay to track the activity of the protein during purification.

Materials and Methods

Bacterial strains and plasmids: *E. coli D1210 (recA13, lacI*) strain containing either the vector plasmid, pKK223-3, or an FtsA overexpressing plasmid, pLHW3 were used in this study. The plasmid pLHW3, was constructed by sub-cloning an EcoRI-BamHI DNA fragment obtained by partial digestion of another plasmid construct, pLHW1 into pKK223-3 vector (Wang and Gayda., 1992). For chaperone co-expression experiments, the plasmid pGroESL containing genes for GroES, GroEL and Lac repressor (Amrein et al., 1995) was transformed into the above D1210/pLHW3 strain. Both plasmids can be stably maintained in the same cells in the presence of antibiotic ampicillin and both GroES and GroEL along with FtsA can be overproduced simultaneously from their promoters.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Growth medium and expression conditions: The D1210 cells containing any of the above mentioned plasmids were grown at 30°C in M9 minimal medium (Miller, 1972) that was supplemented with or without 1% vitamin amino acids and 100 μg/ml carbenicillin. Overnight seed cultures were diluted (50X) into fresh medium and incubated until the OD_{600} of cells was between 0.3-0.4. Overexpression of the proteins was achieved by the addition of isopropyl-β,D-thiogalactopyranoside (IPTG-Sigma Chemical Co.) to the culture at a final concentration of 0.5 mM. These cells were incubated from 2-6 hours depending on the experiment, harvested by centrifugation (5000 X g, 20 min., 4°C) and the cell pellets were stored at -80°C until use.

Extraction of FtsA from membranes by Sarkosyl solubilization (Method I):
Cell pellet (wet weight 6 g) was resuspended in 20 ml of low salt lysis buffer [10 mM triethanolamine, pH 8.0; 1 mM dithiothreitol (DTT); 0.5 mM ATP; 1 mM PMSF; 10 μg/ml each of the protease inhibitors Leupeptin and Pepstatin] (Frankel et al., 1990) at 4°C and the cells were broken by two passages through a French pressure cell at 1000 lb/in². The lysate was centrifuged at 1000 X g for 15 min. (Sorvall SS34 rotor) to remove the unlysed cells. Cell free lysate was then spun at 20,000 X g for 15 min. to pellet the large membrane fragments. This pellet is the FtsA enriched fraction and was used for direct Sarkosyl solubilization (Frankel et al., 1990). The pellet was gently resuspended in Sarkosyl solubilization buffer [25 mM triethanolamine (TEA), pH 7.5; 1 mM EDTA; 50 mM NaCl; protease inhibitor cocktail (Frankel et al., 1990)]. sarkosyl was added to a final concentration of 0.2% (w/v) and the mixture was incubated on ice for 30 minutes. The supernatant obtained by centrifugation at 100,000 X g (Beckman,
L5-65B, Ty75 rotor) for 4 hours, was carefully removed into a fresh tube and the pellet was saved for PAGE analysis. This solubilized FtsA enriched extract (I) was concentrated in a centicon (Amicon Inc.) and tested for ATP binding. Membrane fraction obtained from D1210 cells with the vector pKK223-3, was subjected to similar Sarkosyl solubilization and used as a control in the ATP assay.

In the second phase of purification, Sarkosyl was exchanged with octylglucoside (Frankel et al., 1990). Exchange buffer (25 mM TEA pH 7.5; 50 mM NaCl; 1 mM EDTA with 2% octyl glucoside) was added to the sarkosyl solubilized extract and stirred for 30 min. Divalent cations MgCl₂ and CaCl₂ were added (final 1.25 mM and 1.06 mM respectively) and clarified by centrifugation at 100,000 x g for 2 hours to remove sarkosyl insoluble proteins, membranes and ribosomes. The soluble fraction was then subjected to column chromatographic separation on a 5 ml DEAE-Sephadex column (A-50, Pharmacia) equilibrated with octylglucoside exchange buffer. The column was washed by 4 column volumes of exchange buffer with 0.5% detergent to remove the unbound proteins. Bound proteins from the column were eluted by a salt gradient of 50 mM NaCl to 0.5 M NaCl in TEA pH 7.5. The proteins were collected in 0.5ml fractions and were analysed on 4-15% SDS-PAGE (Laemmli, 1970). Bio-Rad Econo System was used for chromatographic separations. FtsA enriched fractions were pooled and chromatographed on the same column with a gradient of 0.2 M-0.6 M NaCl in the same buffer. FtsA enriched fractions that eluted at a salt concentration around 0.4 M were pooled and stored at 4°C for ATP binding studies.
Extraction of FtsA by lysozyme/Sarkosyl lysis of cells (Method II): Cell pellet from 1 l culture (6 g) was resuspended in 7.5 ml STE buffer (10% sucrose; 100 mM Tris pH 8.0; 1 mM EDTA) and 100 μg/ml lysozyme (Frankel et al., 1990). The cells were incubated on ice for 15 min and then about 65 ml of lysis buffer (15mM TEA; pH 8; 50 mM NaCl; 1 mM ATP; 1 mM DTT; and protease inhibitor cocktail as mentioned above) was added. While stirring on ice, 10% Sarkosyl was added to final concentration of 0.2% w/v. The viscous lysate was sonicated (5 X with 10 sec bursts, Heat systems Ultrasonic). This lysate was then centrifuged for 15 minutes at 30,000 x g (Sorvall SS34). The supernatant was collected and octylglucoside (25% w/v) was added to a final concentration of 2% (w/v). After shaking for about 15 min. MgCl₂ and CaCl₂ was added to a final concentrations of 1.25 mM and 1.05 mM respectively. The solution was shaken for another 30 minutes and centrifuged at 100,000 X g for 4 hrs to pellet the ribosomes and insoluble membrane debris. This supernatant was designated as solubilized FtsA (II).

Purification of soluble FtsA by co-overexpression of bacterial chaperones: D1210 cells containing both the FtsA expressing plasmid, pLHW3 and chaperone expressing, pGroESL (Drs.J.Mous & D.Gillessen) and were induced with 0.5 mM IPTG at 30°C for 6 hrs. Samples were taken out every hour, pelleted and used for SDS-PAGE analysis. A cell pellet of 10 g wet weight was resuspended in 25 ml of lysis buffer (25 mM HEPES, pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM MgSO₄, 1 mM EDTA, 1 mM DTT, 1 mM PMSF (phenylmethylsulfonylflouride), leupeptin, pepstatin, benzamidine (10 μg/ml) at 4°C. The cells were broken in a precooled French
pressure cell (SLM Aminco) at 10,000 psi. Unlysed cells were removed by successive centrifugations at 1000 X g (SS34) for 30 min. The suspension was clarified by centrifugation at 20,000 X g for 30 min followed by a high speed ultracentrifugation at 100,000 X g (Ty75 rotor; Beckman,L5 65B) for 4 hours. The supernatant was dialysed against 4 M ammonium sulfate. The precipitated proteins were pelleted by centrifugation at 12,000 X g for 15 min. dissolved in and dialysed against 1 l start Buffer-S (20 mM Tris pH 7.2, 100 mM KCl, 10% glycerol, 1 mM DTT).

The dialysate was clarified by centrifugation and applied onto a 10 ml Q-sepharose column (Pharmacia) equilibrated with the start buffer-S. The column was washed with 5 volumes of column buffer and the bound proteins were eluted with 10 column volumes of a linear salt gradient of 100 mM-0.5 M KCl in buffer-S. 1 ml fractions were collected and 100 µl of peak fractions were analyzed by SDS-PAGE (4-15%) and FtsA protein was confirmed by immunoblot with FtsA antibodies. FtsA enriched fractions were pooled and concentrated by a diafiltration device (Amicon). The floculants that formed were pelleted by centrifugation. The supernatant was applied on a 10 ml Affigel-Blue F3GA (Bio-Rad Inc.), a dinucleotide affinity column, equilibrated with the same start buffer-S as above. The proteins were eluted with a linear gradient of 100 mM-0.5 M KCl in start buffer-S. FtsA protein was found to elute over a range of 0.3-0.5 M KCl and it was the major band observed on SDS-PAGE and western blot analysis. The eluted fractions were pooled (Fraction-I) and concentrated in a diafiltrator (Amicon). Significant amount of protein floculated out of solution during the concentration step. The SDS-PAGE and Western blot analysis of this floculant pellet
showed that FtsA was 98% of the proteins. Addition of excess salt (KCl) or glycerol (20%) prior to concentration did not prevent the proteins from precipitation. The unbound fractions from the Affigel-Blue column were found to contain considerable amounts of FtsA based on immunoblotting analysis. These unbound fractions were pooled and rechromatographed on the Affigel-Blue column. Most of the FtsA eluted off the column during the column wash stage itself. Some of the homogeneously clean FtsA fractions (5 ml) were carefully pooled and concentrated in a centricon (Amicon) once again. But this time, only minute amount of proteins precipitated. The protein were further separated on a Biogel P-60 size exclusion column (1 cm X 60 cm) and developed with the buffer S. 0.5 ml fractions were collected and analysed by SDS-PAGE (4-15% minigel) and FtsA was followed by immunoblot. Fractions essentially isolated as homogeneous FtsA were stored at 4°C.

**ATP binding assay by photo cross-linking:** UV induced cross-linking of [α-^{32}P]ATP (10 mCi/mmol, Amersham) to FtsA protein was carried out as described by deBoer et al., (1991). About 3 µg of FtsA protein was added to 50 µl reaction mixture containing Buffer-P (25 mM Tris-Cl, pH 7.2, 50 mM potassium glutamate, 1 mM DTT, 10 mM MgCl₂) and 0.5 µM [α-^{32}P]ATP. Samples were mixed at 4°C in eppendorf tubes and then transferred to the wells of a siliconized microtiter plate which was placed on top of a chilled metal block placed on an ice bath. The samples were then irradiated at 254 nm for 30 min. by placing a UV lamp (SL-2537; UVP Inc.,) directly over the microtiter dish. The irradiated samples were transferred to tubes containing 50 µl of 3 mM unlabeled ATP in deionized water. The wells were washed
with 100 μl of Buffer-P and this wash was added to the corresponding tubes. The proteins were then precipitated by the addition of 0.75 ml of 10% trichloroacetic acid (TCA). After 45 min on ice, the precipitants were centrifuged, washed once with 10% TCA and once with acetone. 25 μl of SDS sample buffer was added and the proteins were analysed by SDS-PAGE followed by autoradiography. Where necessary additional components were added to the reaction mixture according to the variations tested. Actin (Sigma Co.) served as the positive control for ATP binding and as a molecular size marker.

**ATP-Agarose binding experiments:** Sarkosyl solubilized and octylglucoside exchanged FtsA, were tested for ATP binding activity on a 2 ml ATP-Agarose affinity matrix (Sigma,Co). 5 ml of extract (6 mg/ml) was incubated along with the column matrix for 2-8 hrs by constant shaking. The unbound fractions were removed by washing the matrix five times with column buffer (25 mM TEA, pH 7.5; 50 mM NaCl; 0.5% octylglucoside) and bound proteins were eluted with 1 M NaCl or 5 mM ATP in column buffer. Actin (Sigma Co.) was used as a positive binding control in the same buffer conditions, since it was known to bind ATP and it has similar molecular size as FtsA.

**Western Blotting Method:** The bands pertaining to FtsA were verified by immunoblotting of the proteins onto PVDF (Millipore) or Nytran (S&S) membranes and screening with anti-FtsA polyclonals raised in rabbit. The immunoblot kit (Immunoselect, Gibco-BRL) using alkaline phosphatase colorimetric detection was used for this purpose. The protocols and conditions for immunoblotting were essentially the
same as those mentioned in Chapter I. Rainbow markers (Amersham Inc.) served as molecular weight standards and to judge the efficiency of protein transfer. Biotinylated broad range markers (Broad range; Bio-Rad) were also used.

Results

Sarkosyl solubilization of overexpressed FtsA: Based on aminoacid sequence comparison, FtsA was hypothesized to be similar to actin protein structurally and enzymatically (Bork et al., 1992). FtsA has been predicted to have an ATP binding pocket similar to actin. Native FtsA is a membrane protein present in low amounts in the cells whereas actin is a ubiquitous cytoplasmic protein in eukaryotic cells. Yeast actin when overexpressed in \textit{E.coli} was found to associate with the membranes upon cell lysis and caused it to aggregate (Frankel et al., 1991). They successfully purified actin using two different procedures that utilize Sarkosyl detergent without denaturation. Initial studies by Wang and Gayda (1992) found that FtsA when overexpressed from a plasmid also associates with the inner membrane fractions which could be only dissociated with 6-8 M urea buffer. In pilot experiments, during the extraction of FtsA from overexpressed membrane fractions FtsA was found to aggregate even in the presence of detergent or urea and thus behaved very similar to actin. This suggested that the actin protocols could be successfully adopted to purify native FtsA in a non-denaturing manner. Two different published methods were tried. Method-I involved the solubilization of the protein with the detergent directly from the membranes and the method-II involved the prevention of coaggregates by directly lysing the cells with lysozyme in the presence of the Sarkosyl detergent (See Materials and Methods).
Figure 13. Solubilization of FtsA from membranes with various concentrations of Sarkosyl: Solubilized proteins were separated on SDS-PAGE. Lanes 1 & 15, Molecular size Rainbow markers; lane 2, French pressure cell lysate; lanes 3,6,9,12 membranes before Sarkosyl solubilization; lanes 4,7,10,13 unsolubilized pellet fractions; lanes 5,8,11,14: 0.1%, 0.2%, 0.5%, 1% Sarkosyl (w/v) solubilized proteins respectively. In each case, the FtsA enriched membrane fractions were solubilized and unsolubilized material is pelleted at 100,000 X g for 1 hr.
Initial solubilization studies with FtsA enriched membrane extracts, demonstrated that Sarkosyl (0.2%) selectively solubilized FtsA protein from these membrane extracts. Figure 13 is a Coomassie blue stained SDS-PAGE that shows that enriched membrane extracts treated with various concentrations of Sarkosyl detergent. Comparison of soluble protein (lanes 5,8,11,14) to the respective insoluble fractions (lanes 4,7,10,13) shows that a concentration of 0.2% Sarkosyl effectively and selectively solubilized FtsA. Hence this concentration was chosen for further studies.

To verify whether the Sarkosyl solubilized FtsA was functionally active, its ATP binding ability was tested. A sample of Sarkosyl solubilized FtsA was mixed with $[^{32}\text{P}]$ATP and ATP binding was performed by UV induced photo cross linking. A membrane extract, which was prepared from the cells carrying plasmid vector only, served as a wild type control. The proteins cross-linked to $[^{32}\text{P}]$ATP were separated on SDS-PAGE, Coomassie blue stained, dried and exposed to X-ray film. A representative autoradiograph is shown in Figure 14. In lane 3, a band pertaining to the molecular weight of FtsA (45 kDa) was capable of binding labeled $[^{32}\text{P}]$ATP very strongly compared to the relevant faint band in the vector control extract (Fig. 14, lane 2). This suggested that the amplified 45 kDa labeled band was indeed FtsA. The actin positive control also bound labeled ATP effectively (lane 5), while the urea purified FtsA did not (lane 4). A 60 kDa protein band, hitherto absent in control extract also bound ATP very intensely. This 60 kDa protein was confirmed as a heat shock protein induced in response to the stress due to overexpression of FtsA. Other weakly labeled bands were visible at lower molecular range.
Figure 14. UV cross-linking of Sarkosyl solubilized FtsA to [α-32P]ATP. Cross linked samples were TCA precipitated and analysed on 4-15% SDS-PAGE (B) Coomassie stained gel (A) autoradiograph of the dried gel. Lane 1, 14C MW markers; lane 2, wild type Sarkosyl soluble membrane extract (D1210/pKK223-3); lane 3, FtsA solubilized from overproduced membrane D1210/pLHW3, lane 4, Urea purified FtsA, lane 5, pure actin protein.
Column chromatography purification of Sarkosyl solubilized FtsA: Sarkosyl solubilized FtsA protein extracts were subjected to further purification on anion exchange columns of DEAE-cellulose or DEAE sephadex (A-50) columns. These columns were tried initially in the absence of any Sarkosyl detergent in the column running buffers. A linear NaCl gradient (50 mM-1 M) could not elute FtsA from the columns apparently due to aggregation of the FtsA protein. Washing the column with Sarkosyl buffer would not elute the bound proteins. This indicated that FtsA once solubilized requires detergent to retain its solubility. Ironically, when chromatographed in Sarkosyl buffer, FtsA still aggregated on the column. Similar results ensued when size exclusion chromatography (Sephacryl S-200) was attempted in the presence of Sarkosyl.

To verify if in fact FtsA aggregated irreversibly on the top of the column, samples of the column bed matrix were removed and treated with various eluants and then analysed on SDS-PAGE (Fig. 15A). The aggregated proteins could not be dislodged with 2% Triton X-100 (lane 5) or 1 M NaCl (lane 7) although 2% Sarkosyl (lane 3) seemed to elute some protein. The proteins could only be released upon disruption with 8 M urea/SDS sample buffer (lane 2,4,6).

The aggregated proteins on the column, could not be eluted by extensive washing (Fig. 15B) with high salt (2 M NaCl) buffer (lanes 1-3) or 2% Sarkosyl buffer (lanes 4-6) but only with 8 M urea in the buffer (lanes 7-9). Thus we had evidence for irreversible aggregation of Sarkosyl solubilized FtsA and concluded that sarkosyl solubilized FtsA could not be purified further. An alternate method is to
Figure 15. Evidence for aggregation of FtsA on columns. A) SDS-PAGE of spin test. 50µl of protein bound column matrix was treated with either 2% Triton X-100 (lane 7), 1M NaCl (lane 5), 2% Sarkosyl (lane 3); lanes 2, 4, 6 matrix pellets after disrupted in Urea/SDS sample buffer. B) SDS-PAGE representing fractions eluted off the IEX column with 2M NaCl (lanes 1-3), 2% sarkosyl (lanes 4-6), 8M urea (lanes 7-9). FtsA could be dislodged completely only with 8M Urea.
exchange Sarkosyl for a non-ionic detergent like octylglucoside as described with actin protocol (Frankel et al., 1991). Although in pilot studies octylglucoside by itself very poorly solubilized FtsA from membranes, upon exchange with Sarkosyl FtsA still remained in a soluble state.

**Octylglucoside exchange of Sarkosyl solubilized FtsA:** FtsA extracted with Sarkosyl from the membranes was exchanged with 2% octylglucoside buffer as explained in Materials and Methods section. Samples of protein obtained from various stages of Sarkosyl extraction, octylglucoside exchange and clarification were analysed on 10-30% SDS-PAGE. The gel was Coomassie blue stained to visualize the protein bands. Figure 16 shows the representative SDS-PAGE. No perceptable FtsA band could be seen in uninduced sample even though twice as much total protein was loaded (lane 2). But upon induction with IPTG, the FtsA band was distinguishable (lanes 3,4,6). However, a second band below the FtsA appeared only after cell lysis and extraction with Sarkosyl (lanes 8) and remained in the supernatant (lane 10). The upper FtsA band was associated with the membrane fractions and was solubilized effectively with Sarkosyl.

**Column purification of FtsA in octylglucoside (OG):** Sarkosyl solubilized FtsA protein was exchanged with octylglucoside buffer and applied to an ion exchange column DEAE-sephadex (A-50). The optimal concentration of the OG detergent in column buffers required to prevent any spontaneous aggregation of FtsA was found to be 0.5% (w/v). The bound proteins were eluted with a 50 mM-0.5 M NaCl gradient in 0.5% octylglucoside. The SDS-gel (Fig.17A) shows that most of the FtsA eluted...
Figure 16. Coomassie stained SDS-PAGE showing FtsA protein isolation from the membranes with Sarkosyl (0.2%) and Octylglucoside exchange. Lanes 1, MW markers; 2,5 whole cells before induction; lanes 3,4,6 after induction for 2,4,8 hrs. respectively; lanes 7, 8 FtsA solubilized by lysozyme/sarkosyl lysis and French press lysis; 9, 10, supernatant after sedimentation of large membranes; lane 11, Octyl glucoside (2%) exchanged soluble fraction after removal of ribosomes and membrane debris at 100,000 X g; lane 12, insoluble fraction of lane 11 (5X more membrane samples were loaded on PAGE for judging the efficiency of FtsA solubilization. Filled arrow points to FtsA band(s) & shaded arrow shows major outer membrane protein.
Figure 17. Chromatographic purifications of FtsA in octylglucoside. SDS-PAGEs show the elution profiles of peak fractions with two separate column matrices A) Experiment 1, (DEAE-sephadex) peak FtsA fractions eluted around 0.4M NaCl B) Experiment 2, (DEAE-sephacel) separated fractions of FtsA eluted about 0.5M NaCl. 50μl of each sample was analysed on SDS-PAGE after TCA precipitation. lane 1, in each gel indicates the MW markers (Sigma, Co), lane10, shows the sample load control.
with the low molecular weight proteins around 0.4 M NaCl. FtsA enriched fraction was attempted to concentrate in Centricon (Amicon) for size exclusion chromatography (Bio-Gel P-60). But the proteins completely aggregated, thus preventing it from being concentrated. Different conditions like the addition of excess 2% OG buffer, or 2M NaCl or dilution with buffer failed to prevent this aggregation.

The eluted fractions from the above DEAE-Sephadex were then subjected to separation directly on an ATP-agarose affinity column to devise a single step purification scheme (Material and Methods). But the FtsA proteins did not show any binding to the matrix, even when it was batch adsorbed for different times. Similar results were obtained when AffiGel Blue (nucleotide analog) column. A conclusion was that FtsA protein lost its ATP binding potential probably due a collapse of the nucleotide binding pocket or masking of the ATP binding sites by OG detergent.

**Purification of soluble FtsA from GroESL co-expressed cells:** The plasmid pGroESL encoding the genes for bacterial chaperones GroES and GroEL, along with the FtsA encoded plasmid pLHW3 can be stably maintained in the same cells. Co overproduction of FtsA along with the bacterial chaperones resulted in considerable increase in the solubility of overexpressed FtsA. However the yield of FtsA protein dropped dramatically due to the runaway expression of the two chaperone proteins. This effect had been seen in similar instances when chaperones were co-expressed with other insoluble proteins (Makrides, 1996). The SDS-PAGE (Fig.18) shows various stages of FtsA-GroESL chaperone co-expression and isolation of FtsA from the supernatant. Considerable amount of FtsA protein was obtained in the supernatant for further
Figure 18. Coexpression of FtsA and GroESL chaperones. Lane 1, whole cell sample before induction with IPTG; lanes 2-6, samples taken after 2, 4, 6, 8 hours; lanes 6 & 7, lysate before & after removal of unlysed cells at 1000 X g; lane 8, Unlysed cell pellet; lane 9, 10,000Xg pellet; lane 10, pellet after > 100,000 X g; lane 1, soluble protein fraction containing FtsA. Arrow points to FtsA band, shaded arrow points to GroESL.
Figure 19. SDS-PAGE profile of FtsA at each stage of purification. Fractions of eluted proteins were analysed by SDS-PAGE after staining with Coomassie Brilliant Blue. Lane 1, sample applied to the Q-sepharose column (60 μg); lane 2, Q-sepharose eluted proteins applied to Affigel-Blue column (38 μg); lane 3, unbound proteins from the Affigel Blue-I; lane 4, Affigel Blue-I eluted FtsA fraction (23 μg); lane 5, Affigel Blue-II eluted fraction upon rechromatography (9 μg); lane 6, molecular weight standards from top, Myosin (205 kDa), Galactosidase (116 kDa), Phosphorylase (97.4 kDa), Fructose 6-phosphate kinase (84 kDa), Bovine serum albumin (66 kDa), Glutamic dehydrogenase (55 kDa), Ovalbumin (45 kDa), Glyceraldehyde 3-phosphate dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), Trypsin inhibitor (20 kDa); lanes 7-10, FtsA purified fractions from the Affigel Blue-II column.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
column purification. Significant amount of the FtsA also remained in the membrane pellet. As demonstrated in the earlier purification method (Fig. 16), a second band of FtsA appeared only after the French press cell lysis.

The soluble FtsA was fractionated by successive use of Q-sepharose anion exchange chromatography and Affigel Blue affinity chromatography. The FtsA protein eluted from the column over a wide range of salt concentrations from the Q-sepharose ion-exchange column. Eluted protein was tracked with a UV detector by referring the absorbance at 280 nm. After each purification step, TCA (Trichloroaceticacid) precipitated samples of various fractions were analyzed by SDS-PAGE and FtsA was identified by immunoblotting with anti-FtsA. Since there some abundant proteins like FtsZ, EF-Tu in the molecular weight range of FtsA, it was necessary to verify the FtsA at each stage of purification by Western blotting before FtsA enriched fractions were pooled. Significant amount of FtsA precipitated when the samples were concentrated whether by ammonium sulfate or by diafiltration in an Amicon device. The final fractionation of the proteins yielded pure FtsA with no visible contaminant bands on SDS-PAGE analysis. This protein was used for UV induced ATP binding assays.

ATP binding activity of soluble FtsA (GroESL): Fractions of FtsA eluted from an early stage of purification were tested for their ATP binding ability again by using UV induced cross linking to radiolabeled [$\alpha$-32P]ATP (Fig. 20). Two fractions that eluted off the Affigel (nucleotide affinity) column and recognized as FtsA by western blotting were used for the initial ATP binding experiment. The labeling was done according to the Materials and Methods and proteins separated on a SDS-PAGE. To
Figure 20. UV cross linking of $[\alpha^{32}\text{P}]\text{ATP}$ to partially purified FtsA. About 3 $\mu$g protein sample was used for each reaction. Lane 1, $^{14}\text{C}$ MW markers; lane 2, actin control; lane 3, HisTag purified FtsA; lane 4, octylglucoside purified FtsA; lane 5, cHA column purified FtsA (GroESL); lanes 6 & 7, FtsA fractions(I) eluted from Affigel Blue column; lanes 8 & 9, unbound FtsA fractions from Affigel Blue; lanes 7 & 9, with 0.1 $\mu$M EDTA in the reaction mixture; lane 10, Biotinylated molecular weight markers. Panel A, autoradiograph; Panel B, Westernblot of the same samples as shown in Panel A; Panel C, Coomassie blue staining showing the total proteins.
ascertain the positive identity of the labeled band, the gel was also blotted onto a PVDF membrane directly for immunoblot screening by FtsA polyclonal antibody. After the Western analysis, the blot was dried and exposed to X-ray film (Fig.20A). In lanes 6,7,8,9 of Figure 20A, the bands pertaining to FtsA monomer showed a weak labeled fuzzy band on the autoradiograph. This band migrated close to the 46 kDa $^{14}$C marker and actin positive control band (45 kDa) in lane 2 of Figure 20A. These bands were slightly smaller in size than the FtsA in the previous lane because this FtsA was purified from the supernatant and probably represents FtsA cleaved during cell lysis as shown earlier (Fig.20B, lanes 5,6,7). This could be the reason for the poor ATP binding. The other labeled bands were extraneous ATP binding proteins in the crude sample. Presence of 0.1μM EDTA, slightly decreased this binding (lanes 7,9). Western blot of these samples (Fig.20 B) demonstrated a single species identified as FtsA, that aligned with the labeled FtsA bands on the autoradiograph when overlaid. This data confirms that the FtsA binds [$\alpha$-$^{32}$P]ATP.

Based on the above results, the ATP binding with a homogeneously purified FtsA protein was tested. The unbound FtsA rich fractions from Affigel Blue column, when rechromatographed on the same column, yielded fairly clean preparation of FtsA when verified on SDS-PAGE. This FtsA sample was used, for further confirmatory ATP cross linking experiments (Fig.21). More variables were included in the experiment to prove the authenticity of the ATP binding ability of FtsA. The representative autoradiograph (Fig.21B) showed that a dimer species of about 90 kDa bound labeled ATP (lane 3) and this binding enhanced with increased UV crosslinking.
Figure 21. [α-32P]ATP photo cross-linking with purified FtsA. Pure FtsA fractions eluted from extended Affigel Blue chromatography were tested for ATP binding ability under various conditions. A) Coomassie stained SDS-PAGE of cross linked samples; B) Autoradiograph after exposure of the dried gel, lane 1, 14C MW markers; lane 2, actin control; lane 3, protein cross-linking for 5 min; lane 4, sample cross-linked for 30 min; lane 5, sample preincubated with anti-FtsA; lane 6, +1% Sarkosyl; lane 7, 10 mM cold ATP chase.
time to 30 minutes (lane 4) with the same amount of FtsA protein and labeled ATP. This demonstrated that there was a cooperative effect in the ATP binding phenomenon of FtsA i.e. as FtsA formed more dimers, it bound more ATP. The pre-incubation of the protein with FtsA antibody abolished this ATP binding (Fig. 21B, lane 5). Inclusion of 10 mM EDTA in the cross-linking buffer (not shown) addition of 0.1 mM unlabeled ATP (lane 7) or heating the sample for 1 min at 90° C (not shown) all abrogated the ATP binding ability. But when 1% Sarkosyl detergent was included in the reaction mixture, a very high molecular weight labeled band was evident at the top of the gel (Fig. 21B, lane 6) which was probably an oligomer. This was an interesting observation because of the fact that FtsA localizes at the membrane during septation it probably binds ATP effectively only in a membrane bound conformation. This agrees with the ATP binding result (Fig. 14) obtained with the Sarkosyl solubilized FtsA from the membranes. Coomassie blue staining of the representative PAGE gel, showed a consistent band pertaining to the labeled 90 kDa dimer observed on the autoradiograph. However, this dimer band was weakly recognized by FtsA polyclonal antibodies on a Westernblot.

Discussion

The goal of this project was to obtain pure FtsA protein, in a soluble, properly folded, active form and then to study its predicted biochemical properties, namely the ATP binding and/or ATPase activity. The function of FtsA was based on its primary amino acid sequence comparison with other diverse protein sequences (Bork et al., 1992). It was predicted that FtsA is similar to eukaryotic structural protein actin, DnaK.
and other kinases in containing the ATP binding pocket and could be an ATPase. Robinson et al., (1987) previously also suggested that FtsA could be a kinase, akin to CDC2 and CDC28 in yeasts.

Studies in this lab and others (Chon and Gayda, 1988; Pla et al., 1990), demonstrated that FtsA is a membrane protein and is present in very low amounts in wild type cells. Hence to obtain it in significant amounts, FtsA had to be overexpressed. Ensuing over expression studies in this lab revealed that FtsA, when overproduced from an expression plasmid, was membrane bound and formed inclusions that could only be solubilized with 8 M urea (Wang and Gayda, 1992). In this study, it was found that urea purified FtsA was ineffective in binding [α-32P]ATP either by UV induced cross linking or in blot overlay assays.

The essence of this study was to extract FtsA from either the insoluble membrane fractions and soluble fraction and to purify it to homogeneity so as to assay its ATP binding activity. In order to achieve this, many problems, some of which were stated earlier, had to be overcome. Preliminary investigations involved the assessment of the protein expression conditions followed by solubilization conditions. These studies found that best yields of soluble FtsA was obtained when the temperature of induction was 30°C compared to earlier 37°C, and the growth of cells was in minimal enriched medium. The lower growth rate conditions slowed the growth rate down and allowed time for the overexpressed protein to fold properly. Inspite of these changes, FtsA still tended to associate with the membrane fractions. At low IPTG concentrations (0.1 mM), the induction was poor apparently due to the lacI genotype of the strain which provides
constitutive Lac repressor. Sarkosyl (0.2%) detergent was found to selectively solubilize
FtsA from membranes very efficiently, affirning the fact that it is indeed a cytoplasmic
inner membrane protein. Significantly, the FtsA protein extracted with Sarkosyl from
the membranes showed effective ATP binding. To further purify this Sarkosyl
solubilized FtsA from membranes various chromatographic columns were attempted.
FtsA protein aggregated within the columns even in the continued presence of the
Sarkosyl detergent. Actin is much like FtsA protein, in that it is a structural protein of
45 kDa and aggregates when overproduced in E.coli. Frankel et al., (1991) established
a procedure to purify yeast actin from E.coli in a soluble and active form. So this
protocol was applied to effect further purification of FtsA. This method exchanges
octylglucoside, a non-ionic detergent for sarkosyl (mildly anionic) detergent. FtsA was
found to be amenable on a single step chromatographic column in the presence of this
detergent. But further purification of eluted FtsA fractions was precluded due to FtsA
aggregating again. Dialysing out the detergent slowly, or protein concentration in a
Centricon-30 (Amicon) concentrator caused irreversible aggregation of the protein.
Hence the protein was not amenable to further fractionation by size exclusion even in
the presence of detergent. Attempts were also made to purify FtsA remaining in the
supernatant after the membranes were pelleted by high speed centrifugation, using
isoelectric focussing method (Rotofer). The FtsA protein once again flocculated in an
irreversible manner. Why should FtsA aggregate if it is predominantly exposed into the
cytoplasm. One possibility is that the protein may be unfolding and exposing the
hydrophobic surfaces. This was supported by the observation that this aggregation was
enhanced by increasing the salt concentration. Salt sequesters the water molecules exposing the hydrophobic surfaces of the protein which enable it to stay soluble (Roepe et al., 1989).

Alternate methods to purify FtsA were also attempted. The co-expression of GroES and GroEL was used because it was found to be productive for many recalcitrant proteins (Amrein et al., 1995). The chaperones co-expression mildly enhanced the in vivo levels of soluble FtsA in the supernatant. This method maintained considerable quantities of FtsA in a soluble form which enabled it to be further purified by column chromatography. However, significant amounts of FtsA protein still precipitated out of solution after each chromatography step. This aggregation occurs when FtsA was pooled and concentrated either by ammonium sulfate or diafiltration in a concentrator. Addition of salt (0.5 M KCl) or 20% glycerol or 5 mM DTT did not prevent this aggregation. It is worth noting that following pilot experiments, EDTA was totally eliminated from the buffers, suspecting that the FtsA aggregation could be enhanced by the dislodging of the ATP from its binding pocket and exposing the core hydrophobic residues. The protein aggregates when checked on SDS-PAGE were found to contain predominantly FtsA. However, after repeated trials, some FtsA enriched soluble protein fractions were obtained and shown to be homogeneous for FtsA and these fractions were used for further biochemical ATP binding assays.

The ATP binding studies show that a small fraction of the monomeric FtsA in a partially purified extract was found to bind ATP, although weakly. Extended ATP cross-linking times showed that a dimer species of FtsA apparently bound ATP better,
which showed a diffused band on the autoradiograph. Preincubation of the sample with FtsA polyclonal antibodies, abrogated this ATP labeling affirming that this ATP binding was intrinsic to the FtsA in the sample. Thus our finding suggests that FtsA may bind ATP as a dimer. This ATP binding was also abolished by EDTA, unlabeled ATP, heat. However, added Sarkosyl enabled an oligomer to bind ATP which was unable to move through the SDS-PAGE. FtsA forms dimers and oligomers even in presence of urea as often seen in control samples of the Western blots which were identified with the polyclonal antibody. FtsA being a membrane associated protein, the requirement of phospholipid for its ATP binding and hence ATPase activity cannot be ruled out. The poor labeling by ATP may be due to weak binding or binding followed by instant ATP hydrolysis which could be tested by using non-hydrolysable ATP analogues.

The presence of soluble FtsA in the supernatant following the overproduction could be attributed to a "spill over effect" which translates that when membrane proteins are overexpressed the protein primarily seeks the membrane and after the membrane is saturated with FtsA, it is forced to stay in the cytoplasm (Roepe et al., 1989). The cytoplasmic fraction of the protein could be in the form of aggregates or inclusions depending upon the internal concentration of the protein. This explains the presence of FtsA in both membrane and supernatant upon gross overexpression. But, in the wild-type cellular fractions (Fig. 10), a subtle molecular weight difference was found between the membrane and soluble forms of FtsA. The higher molecular weight of the membrane FtsA could be due to the membrane phospholipid, which the membrane protein carries upon cell lysis and keeps it soluble (Roepe et al., 1989).
However, SDS-PAGE profile of FtsA protein from overexpressed extracts (Fig. 9) clearly showed the appearance of a second band just below the FtsA band this altered size protein only appears upon lysis of the cells, which strongly indicated that it was a result of cleavage occurring during cell lysis.

The present study demonstrated that FtsA is capable of binding ATP, as predicted from the presence of a putative nucleotide binding domain within the protein (Bork et al., 1992). In summary, this method of chaperone co-expression for obtaining ample amounts of FtsA in a soluble and active form will make it easy to test the other biochemical activities of the protein. However conditions must be refined to prevent precipitation due to irreversible aggregation.
CHAPTER III

IMMUNOLOGICAL SCREENING FOR FtsA-RELATED PROTEINS IN OTHER MICROORGANISMS
Introduction

Cell division in most eubacteria involves the coordinated circumferential invagination of the cytoplasmic membrane and rigid peptidoglycan cell wall, resulting in the partitioning of the cytoplasm into two compartments. This is characterized by the formation of a septal cross wall precisely between the two nucleoids. For this to occur, peptidoglycan synthesis must be switched from elongation mode to septal mode of synthesis in the case of rod-shaped bacteria (Nanninga, 1991). Genetic studies implicated many essential genes \( ftsA, ftsZ, ftsQ, ftsI, ftsN, ftsW, ftsL \) that are involved in this process, directly or indirectly (Donachie, 1993). The cell cycle is under the spatial and temporal control of these gene products in that the cell always divides at mid-cell only after it attains twice its original length (Lutkenhaus, 1993).

Among those proteins involved, FtsZ has the predominant and the most visible role in the process. It forms a Z-ring at the putative division site and contracts as septation progresses at the leading edge (Bi and Lutkenhaus, 1991). The ring disassembles once septation is complete thus dissociating as the polar cap is formed. This Z-ring is hypothesized to function as a cytoskeletal structure. In vitro studies have demonstrated a GTP dependent self-assembly of FtsZ into filaments (Mukherjee et al., 1994; Bramhill et al., 1994). It has also demonstrated that FtsZ is an GTP binding protein and a GTPase (Raychaudhari et al., 1992; deBoer et al., 1992; Mukherjee et al., 1993). Z rings with abnormal morphologies have been found in a \( ftsZ(Ts) \) mutant (Bi et al., 1992), suggesting that the pattern of septum growth depends on the shape of
the Z-ring. The fact that FtsZ has a tubulin like signature sequence and assembles into filaments suggest it is the progenitor of eukaryotic tubulin (Ericksin, 1995).

Genetic evidence suggested that FtsA may also be a component of the septum, interacting directly both with FtsZ and FtsI (Tormo et al., 1984, 1986). Morphological studies have shown that \( ftsZ \) acts earlier than the other division genes (Begg et al, 1985; Taschner et al., 1988). Addinall et al., (1996) demonstrated that the Z rings formed in \( ftsA, ftsQ \) and \( ftsI \) mutants but their contraction was blocked, suggesting the involvement of these gene products at later stages of septation. FtsA has an ATP binding domain that is homologous to other known ATPases such as actin, DnaK and sugar kinases (Bork et al., 1992). FtsI (PBP3) is required for septal peptidoglycan synthesis (Spratt et al., 1977; Botta and Park, 1981).

Phylogenetic studies showed that FtsZ is well conserved in almost all eubacteria both Gram-positive and Gram-negative (Corton et al., 1987; Bi and Lutkenhaus, 1992). Genetic homologues of \( ftsZ \) were recently been found in archaebacteria (Margolin et al., 1996; Wang et al., 1996) and also in \textit{Arabidopsis thaliana} chloroplasts (Osteryoung and Vierling, 1995) suggesting the retention of the primary cytokinetic machinery in eukaryotic organelles. Genetic evidence suggested that FtsA and FtsZ are required in proper ratios for septation to occur normally (Dewar et al., 1992; Dai and Lutkenhaus, 1992). It was likely, that FtsA would be conserved like FtsZ, at least in all eubacteria. Protein extracts from various Gram-positive and Gram-negative bacteria, other unusual organisms including \textit{Mycoplasma}, some eukaryotes like yeasts, and organelles like chloroplasts and mitochondria were examined for the presence or absence of the FtsA
protein homologs by Western immunoblotting with anti-FtsA antibody. The
immunologically cross-reacting bands were identified and approximate molecular weight
determined.

Materials and Methods

Organisms and growth conditions: Microorganisms used in this survey and
their sources are listed in Table 1. The media and growth conditions were as described

Preparation of cell extracts: The protein extracts of different organisms were
obtained for the Western blot survey were provided by Shao Xiaoping (1992). The
extracts were frozen at -80°C. The chloroplast and mitochondrial samples from spinach
were provided by Ms. Kathy Mason.

Protein quantitation: Protein concentration of the various extracts used was
determined by BCA protein assay (Pierce Co.) according to the manufacturers
instructions. BSA (bovine serum albumin) 2 mg/ml stock was used to prepare a set of
five dilutions to generate a known standard curve from which the concentrations of the
unknowns were calculated. The diluent used was 10 mM Tris-Cl buffer (pH 7.25).
Protein extracts of different organisms were serially diluted in the ratios 1:2, 1:4; 1:8;
1:16; 1:32. A tenth ml of each set of dilution of protein standard or unknown were
pipetted into labeled tubes. 0.1 ml of the diluent served as a blank. 2 ml of working
reagent made according to the protocol was added to each tube and incubated at 37°C
for 30 min. After cooling down to room temperature, the absorbance was measured by
using Beckman Du-65 spectrophotometer. A standard curve was plotted with
Table 1: List of microorganisms used in this survey and their sources

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Salmonella choleraesuis</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Serratia marscecens</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Rhizobium fredii</em></td>
<td>Dr. R. Gayda</td>
</tr>
<tr>
<td><em>Caulobacter vibrioides</em></td>
<td>Dr. J. Larkin</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>Dr. R. Siebeling</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Dr. E. Achberger</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Sarcina luea</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Sporosarcina ureae</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Arthrobacter crystalopoetus</em></td>
<td>Dr. E. Achberger</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisae</em></td>
<td>Dr. M. Orlowski</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>Dr. M. Orlowski</td>
</tr>
<tr>
<td><em>Deinococcus radiodurans</em></td>
<td>Dr. J. Battista</td>
</tr>
<tr>
<td><em>Mycoplasma smegmatis</em></td>
<td>Dr. E. Achberger</td>
</tr>
<tr>
<td><em>Rhodobacter capsulatus</em></td>
<td>Dr. A. Biel</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>Dr. M. Potter</td>
</tr>
<tr>
<td><em>Mycoplasma hyorhinis</em></td>
<td>Dr. G. Stemke</td>
</tr>
<tr>
<td><em>Halobacterium halobium</em></td>
<td>ATCC</td>
</tr>
<tr>
<td>Chloroplast(spinach)</td>
<td>K. Mason</td>
</tr>
<tr>
<td>Mitochondria(spinach)</td>
<td>K. Mason</td>
</tr>
</tbody>
</table>
absorbance at 562 nm vs concentrations. The protein concentrations were determined by using this standard curve.

**Immunoblotting procedure:** Amount of sample from the extract of each organism used for the SDS-PAGE was based on pilot experimental data. The amount of protein from each organism that showed a detectable band on a Western blot was loaded on the gel. Western blots were performed using the Immunoselect kit (Gibco BRL) by Streptavidin-alkaline phosphatase reaction. Although chemiluminiscent immunoblot method, was highly sensitive in detecting minute amounts of FtsA protein, there was significant back ground. However, the chemiluminiscent blots were used as secondary evidence to verify the actual FtsA band from each organism. The protein extracts from each sample were individually solubilized by mixing 1:1 vol/vol Urea-SDS solubilization buffer (1.25 M Tris-Cl buffer pH 7.0; 20% SDS; 10% β-mercaptoethanol; 8 M urea; 0.03% Bromophenol Blue). The samples were boiled for 10 minutes and separated on 4-15% SDS-PAGE mini gradient gels. Rainbow™ protein molecular weight markers (Amersham Co.) were loaded in the first lane to serve as size standards and to monitor the efficiency of protein transfer. An internal standard of gel purified *E.coli* FtsA was also included in each gel. The proteins were separated at a constant voltage of 150V. The proteins on the gel were then electrophoretically transferred onto PVDF (Millipore) membranes at constant voltage for 2-3 hours.

The membranes were blocked by incubating with 5% non-fat dry milk in TBS (Tris buffered saline: 50 mM Tris-Cl, pH 7.5; 200 mM NaCl) with gentle agitation for 2-3 hours. The membrane blots were then incubated with the primary FtsA antibody.
(1:1000 dilution of the polyclonals) in 1% non-fat dry milk in TBST (TBS with 0.05% Tween-20) over night at R.T. The membranes were then washed in TBST 3X to remove the unbound antibody and subsequently incubated with the biotinylated secondary antibody (goat anti-rabbit) at a dilution of 1:5000 for about 30 min. Unbound secondary antibody was removed by rinsing the blots again in TBST 3 times. The final incubation was performed with Streptavidin-Alkaline Phosphatase conjugate in TBST for 30 min followed by successive washing steps with TBST, TBS and substrate buffer (100 mM Tris-Cl pH 9.5; 100 mM NaCl; 50 mM MgCl₂). Then the blots were developed with NBT/BCIP mixture in substrate buffer according to the manufacturer’s recommendation. As soon as the initial bands appeared, the development was stopped by rinsing in running water.

**Calculation and estimation of the molecular weight of FtsA bands:** The molecular weights were calculated by using the molecular weight mode on the Eagle Eye (Stratagene Inc.). A standard curve was generated by plotting the known protein standards in the marker lane (y-axis) against the relative distances moved by those bands (x-axis). The molecular weights were calculated by measuring the relative distance moved by each band from top of the blot to the base of each band and plotting against the standard.

**Results**

This particular survey for FtsA protein homologues included several prokaryotic species and some eukaryotic organisms. Protein extracts from a total of 20 diverse eubacterial species were examined as well as the extracts from *Mycoplasma*, a cell wall-
less microorganism and two yeasts, *Saccharomyces* and *Schizosaccharomyces*. In addition protein extracts from two eukaryotic organelles, mitochondria and chloroplast from spinach were also screened for FtsA like protein. The size of the homologs were compared to that of *Escherichia coli* FtsA (45 kDa) which served as a reference along with the molecular weight rainbow markers.

**FtsA antigenic homologues in Gram-negative rod shaped bacteria:** The immunoblot of various Gram-negative bacteria using polyclonal antibody is shown in Figure 22. The Gram-negative bacteria *Salmonella cholereaosis*, *Serratia marscecens*, *Rhizobium meliloti*, *Rhizobium fredii* and *Caulobacter* all showed a representative band at 45 kDa similar to that of the FtsA control. *Escherichia, Salmonella and Serratia* also showed a faint second band at about 44 kDa MW. *Vibrio cholera* had two doublet bands, the first one at 47 kDa with a faint lower band running very close, and a second doublet at 32 kDa. The two doublets reacted with equal intensity. Alternatively, comparing the second doublet with the *E.coli* standard FtsA in the last lane, suggested that the second doublet could be a breakdown product. In *Caulobacter* in addition to the 45 kDa protein, there was a lower band of 42 kDa, running parallel and was similar in intensity to the upper band. This second band may be a cleaved product much like the one observed in *E.coli* (Fig.9). In *Citrobacter freundii* the immunoreactive band at 45 kDa was very faint but there was a significant diffused band at about 92 kDa. Addition of excess β-mercaptoethanol in sample buffer or extensive boiling did not resolve this diffused band. This band runs parallel to the aggregate in control lane suggesting that this may be a dimer. All of the above organisms were
Figure 22. Immunoblot identification of FtsA like proteins from Gram-negative rod shaped bacteria. Lane 1, molecular weight markers; lane 10, pure FtsA protein; lanes 2-9, contained the following bacterial lysates: 2, Escherichia coli; 3, Salmonella choleraesuis; 4, Citrobacter freundii; 5, Serratia marcescens; 6, Vibrio cholerae (14035) 7, Rhizobium meliloti; 8, Rhizobium fredii; 9, Caulobacter vibrioides. Approximately equal amount of protein was loaded.
Table 2: Calculated molecular weights of FtsA homologues in Gram-negative bacteria

<table>
<thead>
<tr>
<th>GRAM-NEGATIVE BACTERIA</th>
<th>CALCULATED MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Salmonella choleraosis</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Serratia marscecens</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Rhizobium fredii</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Caulobacter vibiodes</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>47 kDa, 32 kDa</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>45 kDa</td>
</tr>
</tbody>
</table>
Gram-negative rods, and contained FtsA relatively similar in size to the FtsA in *E.coli* predicting the conservation in the mechanism of cell division. The relative molecular weight of FtsA protein homologues from the above Gram-negative bacteria are listed in Table 2.

**Identification of FtsA-related proteins in Gram-positive bacteria:** Immunoblot of various Gram-positive bacteria is shown in Figure 23. *Bacillus subtilis* showed a strong cross-reacting band of 48 kDa with a minor band at about 35 kDa. *Bacillus cereus* showed five intense bands, a sharp band at 90 kDa, a broad band of 73 kDa, a smaller broad band at 36 kDa and two relatively faint bands at 46 kDa and 45 kDa. While the smaller 36 kDa band could be attributed to differential modification products or break down products, the higher migrating bands could be construed as dimers of the 36 kDa and 45 kDa monomers respectively. *Staphylococcus aureus* and *Streptococcus faecalis* displayed a band of 54 kDa, and a band at about 100 kDa. This band is faint in *Staphylococcus aureus* compared to *Streptococcus faecalis* in the colorimetric method of immunoblotting, but was clearly present in chemiluminiscent blot (Not shown). This demonstrates the relatedness between these two gram positive cocci. In many trial immunoblots done, *Clostridium sporogenes* shows a dense smudge at the top of the lane, obviously due to aggregates. *Sarcina lutea* and *Sporpsarcina ureae* exhibit a single strong band at 45 kDa. *Arthrobacter* showed an intense FtsA antigenic homologue of 53 kDa.
Figure 23. Immunoblot detection of FtsA-related proteins in Gram-positive bacteria. Lane 1 depicts molecular weight markers; lane 10 shows pure FtsA protein control; lanes 2-9 were loaded with the following bacterial lysates: 2, *Bacillus subtilis*; 3, *Bacillus cereus*; 4, *Staphylococcus aureus*; 5, *Streptococcus faecalis*; 6, *Clostridium sporogenes*; 7, *Sarcina lutea*; 8, *Sporosarcina ureae*; 9, *Arthrobacter crystallopoietes*. Approximately equal amount of protein extract was loaded.
Table 3: Calculated molecular weights of FtsA homologues in Gram-positive bacteria

<table>
<thead>
<tr>
<th>GRAM-POSITIVE BACTERIA</th>
<th>CALCULATED MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>48.35 kDa</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>36.46, 45.73, 90 kDa</td>
</tr>
</tbody>
</table>
| *Staphylococcus aureus*
| 54.100 kDa              |
| *Staphylococcus faecalis*  
| 54.100 kDa               |
| *Clostridium sporogenes*  
| smear at ~ 100kDa       |
| *Sarcina lutea*         | 45 kDa                     |
| *Sporosarcina ureae*    | 45 kDa                     |
| *Arthrobacter crystalopoetus*  
| 53 kDa                   |
Anti-FtsA cross reactive proteins in other eubacteria and eukaryotes: In addition to the eubacterial samples screened for FtsA-like proteins, two types of yeast, Saccharomyces (budding yeast) and Schizosaccharomyces (fission yeast); Mycoplasma hyorhinis (cell wall-less microorganism); eukaryotic organelles, mitochondria and chloroplast were also included in the study. A representative Western blot is shown in Figure 24. Three weak immunoreactive bands were observed in Saccharomyces cerevisiae. The calculated molecular weights (Table 4) were 98 kDa protein band, a faint 46 kDa band and a lower 40 kDa band. Schizosaccharomyces pombe lane had a single 45 kDa cross reacting band. These cross-reacting bands were relatively weak in yeast strains in comparison to Deinococcus radiodurans contained in addition to a 45 kDa band, a band at 60 kDa. In the Mycobacterium extract there was a large smear with a possible band at about 43 kDa. The smear could be due to lipids attached to the FtsA protein. Rhodobacter capsulatus and Azotobacter vinelandii exhibit positive bands at 46 kDa and 45 kDa respectively. Two faint anti-FtsA cross-reacting bands were observed in the lanes that carried chloroplast and mitochondrial extracts (Fig.24, lanes 7,8). The molecular size of these bands approximate with those of Rubisco enzyme subunits which is abundantly found in chloroplasts and mitochondria. The extract of Mycoplasma hyorhinis (not shown) did not display a band pertaining to FtsA while Halobacterium salinarium, an archaebacterium exhibited a extremely faint band of about 90 kDa.
Figure 24. Screening for FtsA-related protein in other unique microorganisms and eukaryotic specimens. Lane 1 shows molecular weight markers; lane 10, FtsA control; lanes 2-9 carried the following cell extracts: 2, *Saccharomyces cerevisae*; 3, *Schizosaccharomyces pombe*; 4, *Deinococcus radiodurans*; 5, *Mycobacterium phlei*; 6, *Rhodobacter capsulatus* SB1003; 7, *Azotobacter vinelandii*; 8, Mitochondria (spinach); 9, Chloroplast (spinach). Variable amount of protein was loaded in each lane.
Table 4: Calculated molecular weights of FtsA-like proteins in other organisms

<table>
<thead>
<tr>
<th>OTHER EUBACTERIA, EUKARYOTES &amp; ORGANELLES</th>
<th>CALCULATED MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>98, 46, 40 kDa</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td><em>Deinococcus radiodurans</em></td>
<td>45 kDa, 60 kDa</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>43 kDa</td>
</tr>
<tr>
<td><em>Rhodobacter capsulatus</em></td>
<td>46 kDa</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>45 kDa</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>absent</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>absent</td>
</tr>
</tbody>
</table>
Discussion

The proteins FtsZ and FtsA are essential for cell division in *Escherichia coli* and *Bacillus subtilis* (Beall and Lutkenhaus, 1992). FtsZ is very well conserved throughout eubacteria including *B. subtilis* (Wang et al., 1993), *Staphylococcus aureus* (Lutkenhaus, 1993); *Streptomyces coelicolor* (Beall et al., 1991), *Mycoplasma* (Fraser et al., 1995), *Acholeplasma* (Wang et al., 1996) and Archaea and even chloroplast of *Arabidopsis* (Osteryoung and Vierling, 1995). FtsA interacts with FtsZ protein and hence could be theoretically be present in all organisms in which FtsZ is present. FtsA directed polyclonal antibodies were used to detect for FtsA protein homologues in prokaryotes and some eukaryotes as well.

The results clearly demonstrate the presence of FtsA immunoreactive band among all the eubacteria screened. All the Gram negative rod shaped bacteria contain an FtsA like homologue generally around 45 kDa molecular weight. Among the Gram positive bacteria, *Bacillus subtilis* displayed a band at 48 kDa which matches the size determined from the gene sequence. *Staphylococcus* and *Streptococcus* showed similar bands but also displayed a second 90 kDa band which could be a dimer. This larger protein may be involved in controlling the cocci shape of the bacteria. But this higher molecular size band was not detected in *Sarcina* and *Sporosarcina* species both of which are cocci shaped and divide in two different planes giving rise to the ordered cubic arrangement. However, not much is known about cell division in these genera. *Arthrobacter* has a conspicuous band at 53 kDa. The absence of FtsA related band in *Mycoplasma* is strengthened by the published genome sequence of *Mycoplasma*.
genitalium genome (Fraser et al., 1995) in which no gene similar to \textit{ftsA} was identified. This strongly suggests the role of FtsA protein is in peptidoglycan cell wall synthesis.

FtsA polyclonal antibodies cross-reacted with two faint bands of identical molecular weight in both the chloroplast and mitochondrial extracts. These bands are probably Rubisco enzyme subunits based on the molecular weight. Chloroplast is placed in the same phylogenetic group as that of cyanobacteria, the evolutionary precursors of plastids. Chloroplasts divide by binary fission like bacteria. During chloroplast division a CD ring forms at the putative division site much like the FtsZ ring in bacteria (Suzuki et. al., 1994). Recently, a gene similar to FtsZ, has been isolated from the chloroplast DNA of \textit{Arabidopsis thaliana} (Osteryoung and Vierling, 1995). Membrane import experiment demonstrated its functionality in the chloroplast. Although it is suggestive that there could also be a FtsA-like protein in chloroplast which could play a role in chloroplast division cycle, the absence of peptidoglycan in chloroplast, rules out this possibility.

Mitochondria on the other hand are presumed to have evolved from a different lineage through \textit{Archaebacteria}. They do not have a peptidoglycan cell wall either. Hence, the possibility of the presence of FtsA-like protein in these organelle could be ruled out.
CONCLUSIONS

Immunogold labeling of thin sections of cells overexpressing FtsA established that FtsA is localized in the inner membrane as previously suggested (Chon and Gayda, 1988; Pla et al., 1990). When cells overexpressing the C-terminal deleted FtsA, FtsAc, were immunogold labeled, the truncated protein did not localize to the inner membrane. Instead, large cytoplasmic FtsAc aggregates were observed. This suggested that the C-terminal domain was involved in the membrane anchoring function of FtsA. Protease accessibility studies with inverted membranes and spheroplasts (Pla et al., 1990) demonstrated that only part of the FtsA is located in the membrane such that most of the FtsA is exposed towards the cytoplasm. Analysis of the amino acid sequence of FtsA shows that the C-terminal residues could form an α-helix to enable it to be inserted into the membrane. When FtsA was overexpressed a single polypeptide was amplified, but after cellular fractionation two bands of FtsA with slightly altered mobilities were observed. The slower mobility form partitioned with the membrane pellet and was effectively solubilized with Sarkosyl, a detergent that solubilizes inner membrane proteins. The faster mobility form of FtsA retained in the soluble fraction. These results suggested that the full length FtsA is cleaved during cell lysis resulting in the lower molecular weight form. Fractionation of wild-type cells and Western blot analysis confirmed the presence of two forms of FtsA, a cytoplasmic form and a membrane form. The above evidence confirmed that FtsA is an inner membrane protein and that its C-terminus may be involved in membrane anchoring.
FtsA was successfully solubilized from the membranes of FtsA overexpressed cells with Sarkosyl. This detergent solubilized FtsA bound \([\alpha^{32}\text{P}]\text{ATP}\). Urea extracted FtsA was inactive since it did not bind ATP under similar conditions. FtsA was further purified by column chromatography after the substitution of octylglucoside detergent for Sarkosyl in buffers. However, the octylglucoside purified FtsA could not bind ATP. FtsA partitioned with the membrane fraction even under many conditions that prevent the formation of inclusion bodies. FtsA was co-expressed with GroESL chaperones in order to prevent in vivo aggregation and to achieve solubility of FtsA without the aid of detergents. FtsA was purified from the soluble fraction by successive anion (Q-sepharose) and affinity (Affigel-Blue) column chromatography. The FtsA purified by this method bound labeled ATP weakly as a monomer and strongly as a dimer.

Immunoblot screening of various Gram-positive and Gram-negative organisms, using FtsA directed polyclonal antibodies revealed that FtsA protein homologues are present in all eubacteria surveyed. While all the Gram-negative organisms contained an FtsA band of about 45 kDa, the Gram-positive organisms displayed FtsA homologue of variable sizes. FtsA is conspicuously absent in extract of cell wall-less \textit{Mycoplasma}. FtsA-like proteins were also found in the extracts of unicellular eukaryotes, \textit{Saccharomyces} and \textit{Schizosaccharomyces}. FtsA was also absent in the eukaryotic organelles mitochondria and chloroplast. The general conclusion is that FtsA is highly conserved among all eubacteria. But its absence in \textit{Mycoplasma} implicates its involvement in peptidoglycan synthesis during septation which is in agreement with other experimental evidence.
REFERENCES


Aldea, M., T. Garrido, J. Pla, and M. Vicente. 1990. Division genes in Escherichia coli are expressed coordinately to cell septum requirements by gearbox promoters. EMBO J. 9:3787-3794.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
VITA

Anand Immaneni was born on June 30, 1966 in the city of Madras, Tamil-Nadu, India. He was awarded the degree of Bachelor of Science (Honors) majoring in Microbiology and Botany, minoring in Chemistry and Biochemistry from Osmania University, Hyderabad, India, in 1987. He entered the Graduate school at Louisiana State University, Baton Rouge, in June, 1990. He is currently a candidate for the Doctor of Philosophy degree in Microbiology with a minor in Biochemistry, which will be awarded in May of 1998.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Anand Immaneni

Major Field: Microbiology

Title of Dissertation: Localization and Biochemical Characterization of the Cell Division Protein FtsA in *Escherichia coli*

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

12, January, 1998