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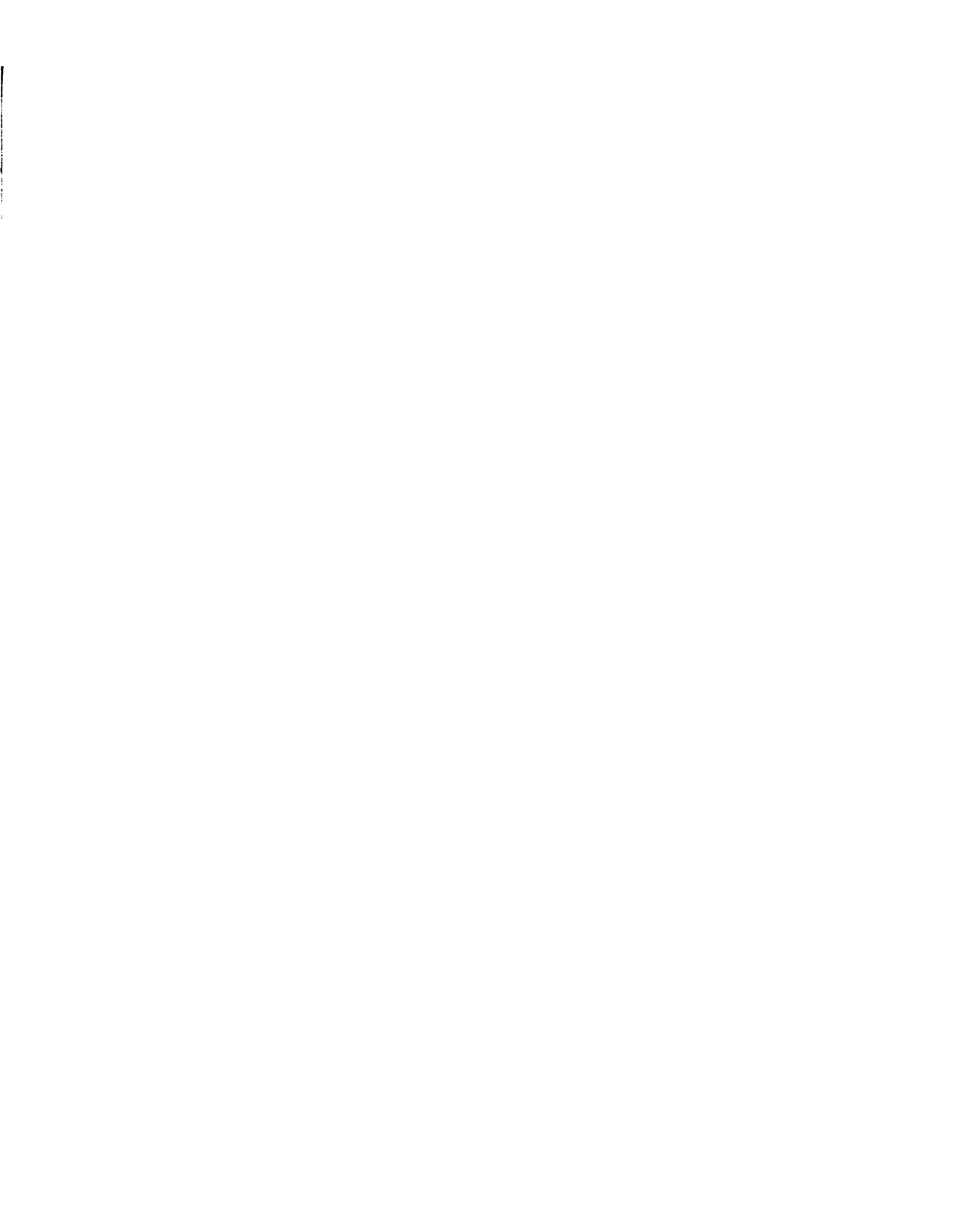
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Université d'Ottawa • University of Ottawa



**MOLECULAR CHARACTERIZATION OF THE  
CELL DIVISION GENE *ftsZ* IN *NEISSERIA GONORRHOEAE***

**AVNI RADIA**

A thesis submitted to the  
School of Graduate Studies and Research  
University of Ottawa  
in partial fulfilment of the requirements for the  
Degree of Master of Science  
Department of Microbiology and Immunology  
Faculty of Medicine

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## ABSTRACT

Cell division is an essential process in all bacteria, although in some organisms like *Neisseria gonorrhoeae* relatively little is known about cell division. The process has been well characterized in *Escherichia coli*, with the identification and characterization of many cell division genes. The *ftsZ* gene is essential in *E. coli* as the gene product plays a critical role in the initiation and formation of the septum at the division site. In efforts to understand cell division at the molecular level in *N. gonorrhoeae*, the *ftsZ* gene has been identified and preliminary characterization is reported. The gonococcal *ftsZ* consists of a 1179 bp open reading frame, encoding a 41.3 kDa protein. Sequence analysis has shown the gonococcal FtsZ to be highly similar to other known FtsZ proteins, with conservation of the GTP binding motif. *In vitro* transcription/translation shows the gene encodes a protein migrating at 43 kDa. Western blot analysis of gonococcal lysates and commensal *Neisseria* lysates detected an immunoreactive band using an anti-*E. coli* FtsZ antisera.

Attempts to clone the gonococcal *ftsZ* gene in an *E. coli* host indicate that expression of the gene from a putative native promoter is lethal to the cell. When expression is controlled by an inducible lactose promoter, the *E. coli* cells show a filamentous phenotype similar to that seen with expression of other heterologous *ftsZ* genes. Attempts to knock out the *ftsZ* gene on the gonococcal chromosome by transformation and recombination indicate that the gene is essential in *N. gonorrhoeae*. The initial characterization of the *ftsZ* gene in *N. gonorrhoeae* provides a basis for further study of cell division in this organism.

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## LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pairs
Da	daltons
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanoine 5'-triphosphate
dig-dUTP	digoxigenin-deoxyuridine-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine 5'-triphosphate
EDTA	ethylenediamineteraacetate
GCMB	GC medium base
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphate dephosphorylase
IPTG	isopropylthio- $\beta$ -D-galactoside
Kb	kilobase pair
kDa	kilodaltons
LB	Luria Bertani
M	molar
mAmp	milliamperes
min	map minutes
min.	minutes
mM	millimolar
NBT	nitroblue tetrazolium salt
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
sec.	seconds
SSC	sodium chloride, sodium citrate buffer
STD	sexually transmitted disease
TAE	Tris-base, sodium acetate, EDTA buffer
TBS	Tris buffered saline
TE	Tris EDTA buffer
Tris	Tris(hydroxymethyl)methylamine
Ts	temperature sensitive
TSA	Tryptic soy agar
TTBS	Tris buffered saline with Tween-20
UDP	uridine diphosphate
X-gal	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside

# 1. INTRODUCTION

## 1.1 Bacterial Cell Division: A General Overview

The process of cell division is an essential biological function for the survival of prokaryotic organisms. Cell division constitutes the third stage in the bacterial cell cycle which starts with initiation of chromosome replication, followed by partition of the sister chromosomes, and ending with division of the cell (Donachie, 1993). Although cell division occurs in all bacteria, it has been most extensively studied in the Gram-negative rod *Escherichia coli*. For cell division to occur in Gram-negative organisms, the ingrowth of the cell membrane, the peptidoglycan sacculus, and the outer membrane is required, leading to the partition of the cytoplasm into two compartments with the replicated chromosomes being segregated (Donachie, 1993). The partition formed in the dividing cell is the septum, and the process of ingrowth leading to partition is septation. Septation requires peptidoglycan synthesis and modification enzymes to form the septum, as well as specific cell division proteins to initiate and direct the process (Lutkenhaus and Mukherjee, 1996).

Many of the genes involved in cell division in *E. coli* have been identified by screening for temperature sensitive mutants. These mutants produce a filamentous phenotype designated *fts* (filamentation temperature sensitive), indicating continuing cell growth in the absence of septation (Lutkenhaus and Mukherjee, 1996). It must be noted that not all cell filamentation is an indication of a cell division gene mutation (Bi and Lutkenhaus, 1991a). Other factors leading to filamentation include DNA synthesis mutants, some protein secretion mutants, and many heat shock response mutants (Bi and Lutkenhaus, 1991a). Filamentation may also be a result of cells undergoing the SOS response, which comprises the physiological

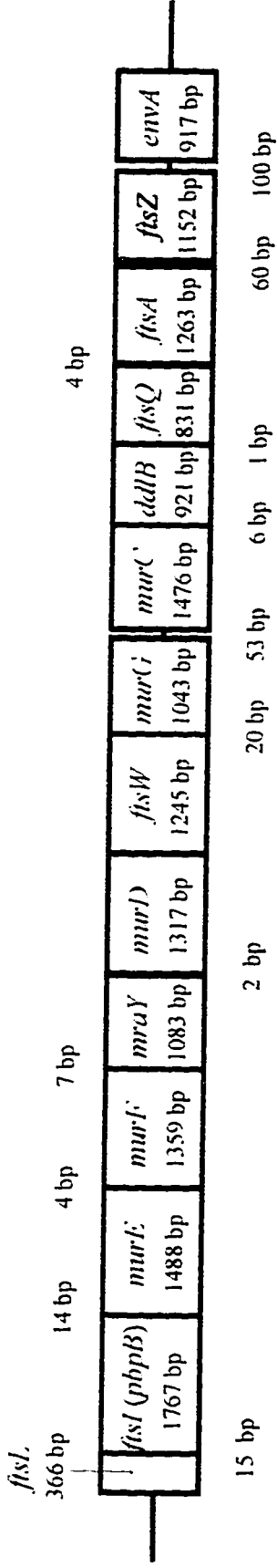
responses of a cell to DNA damage or interference in DNA replication (Walker, 1995).

A group of primary cell growth and cell division genes have been identified in *E. coli* and they form a large operon at 2.2 min on the *E. coli* chromosome. The genes in this operon (Figure 1A) include *ftsL*, *ftsI*, *murE*, *murF*, *mraY*, *murD*, *ftsW*, *murG*, *murC*, *ddl*, *ftsQ*, *ftsA*, *ftsZ*, and *emvA* (Lutkenhaus and Mukherjee, 1996). The genes whose products are involved in peptidoglycan synthesis include *murC* (L-alanine adding enzyme), *murD* (UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase), *murE* (*meso*-diaminopimelate-adding enzyme), *murF* (D-alanyl:D-alanine-adding enzyme), *murG* (UDP-*N*-acetylglucosamine:*N*-acetylmuramyl-pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase), *mraY* (UDP-*N*-acetylmuramoyl-pentapeptide:undecaprenyl-PO<sub>4</sub> phosphatase), *ddl* (D-alanine:D-alanine ligase), and *ftsI* (penicillin binding protein 3(PBP3), a septum peptidoglycan transglycosylase and transpeptidase) (Berlyn *et al.*, 1996; Heijenoort, 1996). The other *fts* gene products (FtsL, FtsW, FtsQ, FtsA, and FtsZ) play a role in forming the actual division apparatus (Lutkenhaus and Mukherjee, 1996). Another operon comprising the *ftsY*, *ftsE*, and *ftsX* genes has been identified at 77.6 min (Gill *et al.*, 1986), but its specific role in cell division has not been defined.

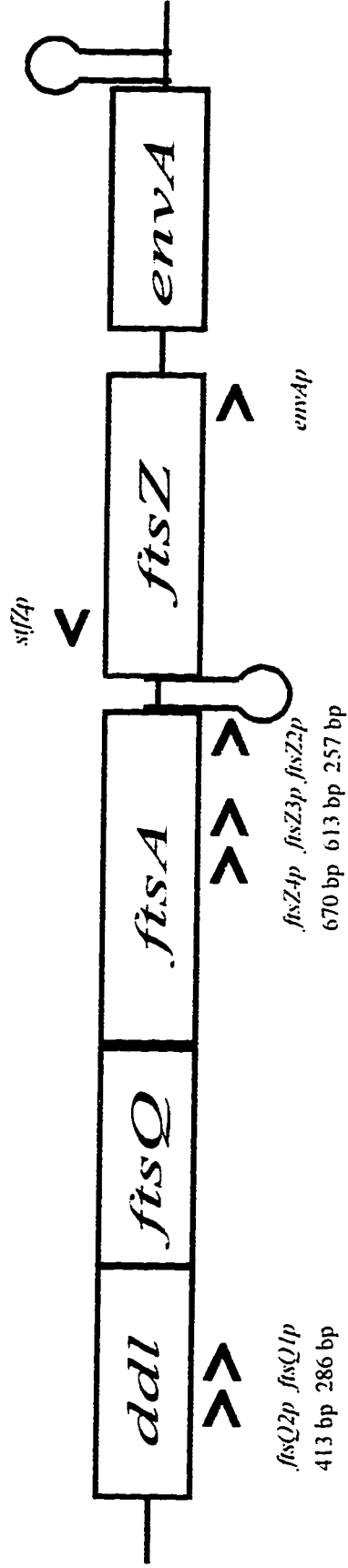
Filamentous mutants were studied to begin defining the functions of the various *fts* gene products (Begg and Donachie, 1985; Taschner *et al.*, 1988). The order of action of the gene products in septation was initially proposed by the presence or lack of constrictions along the filaments at the potential division sites (Begg and Donachie, 1985). The absence of constrictions, resulting in smooth filaments, indicated that the mutant gene product was

FIGURE 1. The cell growth and division gene cluster of *E. coli* located at 2.2 min on the chromosome (modified from Lutkenhaus and Mukherjee, 1996). Figure 1A. The cell growth and division genes in the 2.2 min. region, with *ftsZ* indicated in red. The gene designations and sizes are indicated, and the numbers above the cluster indicate overlaps while the numbers below indicate intergenic spaces. Figure 1B. The locations of the promoters identified for expression of *ftsZ* are indicated (>), with their distance away from the ORFs indicated. The single transcription terminator for the cluster is shown at the end of the *envA* gene, and the reverse promoter (<) and reverse terminator for the antisense *stfZ* RNA are indicated.

A



B



a very early acting protein. The presence of constrictions would indicate a block in the septation after it had already been initiated, thus the gene product would play a role later in the division process. Temperature shift experiments on *fts* mutants were also used to determine the roles of the gene products (Taschner *et al.*, 1988). After growing mutants at the permissive temperature they were shifted to the restrictive temperature and their division was observed (Taschner *et al.*, 1988). Observation of immediate inhibition of cell division might indicate that the mutant gene product was involved in the process of division, whereas the presence of some residual division activity could indicate that the mutant gene product is involved in initiation of division with the remaining gene products functioning to complete the previously initiated division events (Taschner *et al.*, 1988). This type of observation with several mutants led to a proposed order of action of some of the *fts* gene products, with FtsZ acting very early in the division process as an initiator, followed by FtsQ-FtsA-FtsI which continue the process of division (Taschner *et al.*, 1988). Of all the cell division proteins, FtsZ has been shown to play a very central role in the division process. It has been extensively studied and characterized, and this proteins interaction with itself and with various other proteins allows cell division to occur (Lutkenhaus, 1993; Lutkenhaus and Mukherjee, 1996).

## 1.2 Properties of the *ftsZ* Gene and Gene Product

The *ftsZ* locus was first identified in a temperature sensitive filament-forming mutant of *E. coli* (*ftsZ84*(Ts); Lutkenhaus *et al.*, 1980). The *ftsZ* gene maps within a cluster of cell division and cell growth genes at the 2.2 min region of the *E. coli* chromosome (Figure 1A). By complementation, the *ftsZ* locus was found to be distinct from the *ftsA* gene to which

many filament-forming mutations have also been mapped (Lutkenhaus *et al.*, 1980). Observation of *ftsA* mutant cells showed multinucleated filaments with constrictions along the filaments, whereas the *ftsZ* mutant filaments showed no signs of constrictions (Lutkenhaus *et al.*, 1980; Begg and Donachie, 1985). This was the first indication that the product of the *ftsZ* gene is required for cell division before the *ftsA* gene product, perhaps at septum initiation. The temperature shift experiments by Taschner *et al.* (1988) also supported the role of FtsZ in initiation, as the mutant cells were able to complete division at the restrictive temperature, but no new divisions were initiated.

Cells undergoing the SOS response to DNA damage also exhibit a filamentation phenotype (Lutkenhaus, 1983). The *sulB* gene, along with the *sulA* gene, were initially identified in mutants which were not exhibiting the filamentation normally observed when cells are undergoing the SOS response to DNA damage (Lutkenhaus, 1983). During the SOS response, the binding of RecA to single-stranded DNA converts it to an activated form which is able to interact with the product of the *lexA* gene product (Walker, 1995). LexA normally acts as a repressor of the genes in the SOS regulatory network, but the activated RecA cleaves the LexA repressor, and this leads to the induction of those genes (Walker, 1995). The *sulA* gene is one of the genes of the SOS response that is induced, and SulA has been shown to be an inhibitor of cell division during SOS response (Huisman *et al.*, 1984) and SulA was shown to interact with SulB (Jones and Holland, 1985). It was found that mutations of the *sulB* gene mapped to *ftsZ* gene, indicating that they were in fact same gene (Lutkenhaus, 1983). This established the role of *ftsZ* in cell division because the gene product FtsZ (also know as SulB) is the target of the inhibitor SulA.

The nucleotide sequence of the *E. coli ftsZ* gene was determined by Yi and Lutkenhaus (1985). The *ftsZ* open reading frame (ORF) consists of 1149 base pairs (bp), and the FtsZ protein has a predicted molecular weight of 40,294 Daltons (Da) and an isoelectric point of 4.9 (Yi and Lutkenhaus, 1985). It was determined that the amount of FtsZ was in the range of 5,000 to 20,000 molecules per cell (Bi and Lutkenhaus, 1991b; Pla *et al.*, 1991).

The conditionally lethal *ftsZ84*(Ts) mutation provided an indication that the *ftsZ* gene was essential to division, and this was conclusively shown by Dai and Lutkenhaus (1991) and Pla *et al.* (1991). Initial attempts to disrupt the *ftsZ* on the chromosome were unsuccessful (Dai and Lutkenhaus, 1991). It was the construction of a null allele of *ftsZ* on the chromosome in the presence of a cloned *ftsZ* on a temperature sensitive replicon that proved that the *ftsZ* gene was essential for cell division and viability of *E. coli* (Dai and Lutkenhaus, 1991; Pla *et al.*, 1991). The plasmid copy of the gene provided enough FtsZ required for cell division, but a shift of the cells to the non-permissive temperature resulted in loss of the plasmid, decreasing levels of FtsZ, and loss of cell division and cell viability (Dai and Lutkenhaus, 1991; Pla *et al.*, 1991).

To further show the role of *ftsZ* in cell division the level of FtsZ in *E. coli* cells was increased using a multicopy plasmid carrying *ftsZ* (Ward and Lutkenhaus, 1985). An increase of 2 to 7 fold of normal levels resulted in the formation of minicells as a result of additional division events occurring. As the level was increased further 12-fold or more of normal levels, the cells grew as filaments, with inhibition of division. This work indicated that having more FtsZ allowed more than one division event per cycle, thus the amount of FtsZ controls the frequency of division.

The maintenance of the level of FtsZ in the cell appears critical, and the regulation of *ftsZ* expression has been studied. As shown in Figure 1A, the *ftsZ* gene lies near the end of a large cluster of genes which are all in the same orientation, with a single transcriptional terminator downstream of the *envA* gene (Lutkenhaus and Mukherjee, 1996). The promoters controlling the expression of *ftsZ* have been mapped using S1 nuclease protection experiments by Aldea *et al.* (1990). Six promoters were originally identified (Figure 1B): four in the *ftsA* gene just upstream of *ftsZ* (*ftsZ1p-ftsZ4p*) and two upstream of the *ftsQ* gene in the *ddl* gene (*ftsQ1p* and *ftsQ2p*) (Aldea *et al.*, 1990). The promoter *ftsZ1p* mRNA product detected by the mapping was subsequently found to be as a result of an RNase E cleavage site (Cam *et al.*, 1996; not shown). The *ftsQ1p* promoter has been characterized as a “gearbox” promoter (Aldea *et al.*, 1990). Gearbox promoters control the transcription rate such that it is inversely dependent upon growth rate which results in the amount of transcript per cell being constant (Vincente *et al.*, 1991). The expression and regulation of *ftsZ* in the cell cycle has been under investigation, and *ftsZ* transcription was found to fluctuate during the cell cycle (Garrido *et al.*, 1993; Zhou and Helmstetter, 1994). It has been also found that high levels of an antisense RNA transcript from the *ftsA/ftsZ* intergenic region inhibits cell division in *E. coli* grown at 42°C (Dewar and Donachie, 1993). As shown in Figure 1B, a strong promoter (*stfZp*) near the start of the *ftsZ* gene, and a reverse transcriptional terminator in the intergenic sequence of *ftsZ* and *ftsZ* genes have been identified (Dewar and Donachie, 1993). Dewar and Donachie (1993) propose that the transcript may bind to *ftsZ* mRNA to inhibit translation or the transcript may encode a inhibitor of cell division.

The biochemical function of the FtsZ protein was indicated upon analysis of the

deduced amino acid sequence (RayChoudhuri and Park, 1992). Initially, comparison to protein databases found no homology to any known proteins (Yi and Lutkenhaus, 1985; RayChoudhuri and Park, 1992). A closer analysis of the amino acid sequence did reveal that residues 105 to 111 of *E. coli* FtsZ (GGGTGTG) are highly conserved with a glycine rich region of eukaryotic tubulins (G/AGGTGSG), and this tubulin signature sequence is thought to be involved in the binding of GTP by tubulins (RayChoudhuri and Park, 1992). An alignment of FtsZ to eukaryotic tubulins has shown some conserved residues in the N-terminal two thirds of the protein in addition to the tubulin signature sequence (Mukherjee and Lutkenhaus, 1994). It was shown by three groups that *E. coli* FtsZ binds and hydrolyses GTP *in vitro* (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993; RayChoudhuri and Park, 1992). The GTPase activity was found to be dependent on the protein concentration (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993). With one *ftsZ* mutant, FtsZ84, which does not initiate septum formation, a change of one amino acid within the tubulin signature sequence produces significantly reduced binding of GTP and GTPase activity (de Boer *et al.*, 1992).

The similarity to tubulin in terms of GTP binding and hydrolysis lead to the idea that FtsZ may be self-associating as is seen with tubulin which form microtubules (Mukherjee and Lutkenhaus, 1994). FtsZ oligomerization was subsequently indicated by the presence of multiple species upon native gel electrophoresis of FtsZ purified from an overproducing strain (Mukherjee and Lutkenhaus, 1994). Using freeze-etch electron microscopy it was shown that purified FtsZ incubated with GTP formed protofilaments *in vitro* (Mukherjee and Lutkenhaus, 1994), where a protofilament consists of molecules of FtsZ aligned in a row. Using a polycation, DEAE dextran, as is used for tubulin polymerization, FtsZ protofilaments

were formed and observed by negative staining (Mukherjee and Lutkenhaus, 1994; Erickson *et al.*, 1996). Using a 2D crystal formation technique, Erickson *et al.* (1996) showed single protofilaments that were long and straight, large 2D sheets of parallel protofilaments, and curved, ring-shaped (minirings) protofilaments. It was proposed that FtsZ forms protofilaments or sheets *in vivo* to encircle the bacteria at the division site, and perhaps depolymerization or miniring formation could generate the force for contraction to cause ingrowth of the cytoplasmic membrane (Erickson *et al.*, 1996).

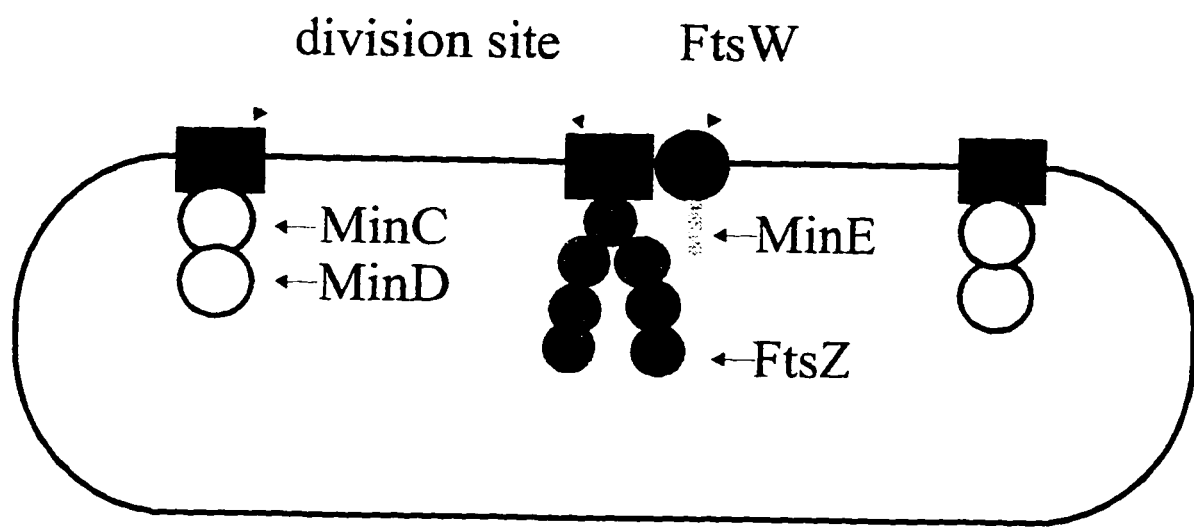
FtsZ of *E. coli* has been localized to the septal region using immunoelectron microscopy (Bi and Lutkenhaus, 1991b). FtsZ was detected at the leading edge of the invaginating cytoplasmic membrane of dividing cells, and the pattern of labelling suggests a ring-like structure of FtsZ at the division site (Bi and Lutkenhaus, 1991b; Addinall *et al.*, 1996). In *ftsZ* mutants grown at the non-permissive temperature, a random distribution of gold particles throughout the cytoplasm was observed in the filamentous cells, indicating that the FtsZ protein was unable to localize at a single site (Addinall *et al.*, 1996). Addinall *et al.* (1996) also used the very sensitive method of immunofluorescence to localize FtsZ; in wild-type dividing cells, a single bright band was observed at the cell center, the diameter of the immunofluorescent band decreased as septation progressed, and when septation was almost complete only a spot of fluorescence was visible between daughter cells. By studying *ftsZ* mutants Addinall and Lutkenhaus (1996a) provide evidence that FtsZ polymerizes from a single point, bidirectionally around the cell at the division site in *E. coli*. Very recently, Ma *et al.* (1996) have localized FtsZ to a division site ring structure in live *E. coli* cells using FtsZ tagged with green fluorescent protein (GFP).

### 1.3 A Model for Cell Division

Although some of the properties of FtsZ and its role in cell division have been well defined, FtsZ alone does not lead to cell division (Lutkenhaus and Mukherjee, 1996). The interactions of FtsZ with other cell proteins and cell division gene products is essential. In *E. coli*, division generally occurs at the center of the long axis of the cell, and it has been found that the placement of the division site is affected by the *min* locus (located at 26.35 min on the chromosome) which encodes the three proteins MinC, MinD, and MinE (deBoer *et al.*, 1989). A mutation in the *min* locus leads to a minicell phenotype where cell division occurs at the cell poles resulting in minicells with no nuclear material. A recent study by Huang *et al.* (1996), using a yeast two-hybrid system to study interactions between FtsZ and the Min proteins, proposed the following model for localization of FtsZ by the *min* system (Figure 2). There exists potential division sites at midcell and at the poles (old division sites) to which FtsZ can localize, but the topological marker for targeting FtsZ has not been identified. MinC interacts with MinD to block the polar sites, and MinE perhaps interacts with the midcell site topological marker to prevent MinCD from masking the division site. FtsZ is thus able to localize to the midcell, but not at the cell poles. One candidate for the targeting of FtsZ is the *ftsW* gene product which appears to act at an early stage in cell division, and this membrane protein may link FtsZ to the membrane, perhaps at the site for polymerization (Khattar *et al.*, 1994).

Once localized to the division site, FtsZ is proposed to undergo polymerization into

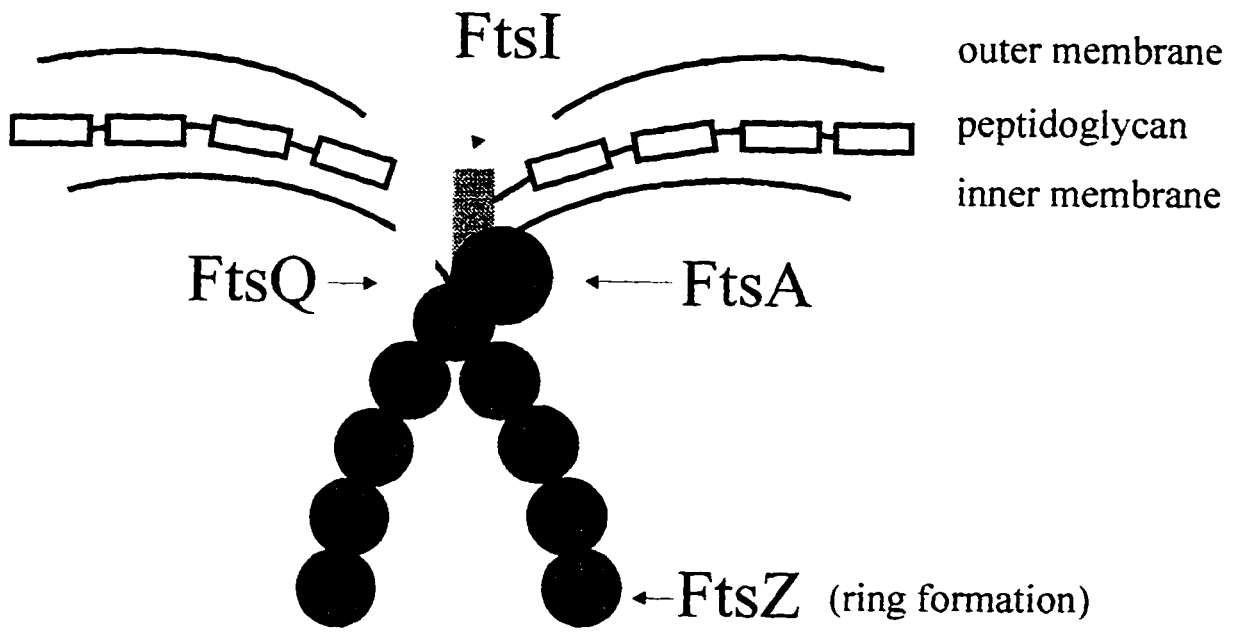
FIGURE 2. Proposed model for the *min* localization system for placement of FtsZ at the midcell division site (refer to text; modified from Huang *et al.*, 1996).



a form of protofilaments around the cell diameter, and then this “FtsZ ring” may be responsible for generating a contractile force to cause ingrowth of the cytoplasmic membrane (Erickson *et al.*, 1996). In addition to this structural role, FtsZ may also be involved in signalling the peptidoglycan synthetic machinery through one or many other cell membrane associated cell division proteins to allow formation of the septum following the membrane ingrowth.

In *E. coli*, some other cell division proteins (FtsL, FtsI (PBP3), FtsW, FtsQ, and FtsA) are those encoded by the genes in the same cluster as *ftsZ* (Figure 1A), and their characterization has become essential in developing a model for cell division (Figure 3). The product of the *ftsA* gene, which lies just upstream of *ftsZ*, is thought to provide a link between the FtsZ ring and the peptidoglycan synthetic machinery. FtsA is a cytoplasmic membrane protein with ATPase activity (Sánchez *et al.*, 1994), and FtsA mutants are blocked in a stage of cell division after FtsZ has initiated septation (Begg and Donachie, 1985; Taschner *et al.*, 1988). The ratio of FtsA to FtsZ is critical for cell division to occur, and FtsA is thought to interact directly with FtsZ (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992). Immunofluorescence microscopy in *E. coli* has localized FtsA to the cell center in a similar pattern as was seen for FtsZ, indicating that FtsA is also localized at the leading edge of the forming septum in dividing cells (Addinal and Lutkenhaus, 1996). It was also shown using *E. coli* FtsZ mutants, that without a functional FtsZ, FtsA could not localize to the division site (Addinal and Lutkenhaus, 1996). Studies using a FtsA-GFP fusion also localized FtsA at the midcell division site, and FtsA appears associated with the FtsZ ring structure (Ma *et al.*, 1996). The interaction of FtsA with PBP3 has been indicated (Tormo

FIGURE 3. Proposed model for cell division in *E. coli*: model for the interactions of FtsZ with other cell division proteins as a link to the peptidoglycan synthetic machinery (modified from Bi and Lutkenhaus, 1991a).



*et al.*, 1986). PBP3 is the *ftsI* gene product, and is one of four high-molecular-weight penicillin binding proteins found in *E. coli* (Lutkenhaus and Mukherjee, 1996). PBP3 has been shown to be specifically required for peptidoglycan synthesis at the septum, and it has transpeptidase and transglycosylase activities (Spratt, 1975). The interaction of FtsA with both FtsZ and FtsI suggests that it serves to link FtsZ to the septal peptidoglycan synthesis (Lutkenhaus and Mukherjee, 1996; Addinall and Lutkenhaus, 1996b). The FtsQ, FtsL, and FtsN proteins are a group of cytoplasmic membrane proteins essential for cell division after the formation of the FtsZ ring, and they are proposed to form a septum-specific peptidoglycan synthetic system interacting with FtsA, FtsZ, and PBP3 (Lutkenhaus and Mukherjee, 1996). Another late stage cell division gene, *ftsK*, has been identified and FtsK is proposed to be involved in peptidoglycan modification or synthesis during septation (Begg *et al.*, 1995). Many questions still remain unanswered, but the cytoskeletal role of FtsZ in the initiation and co-ordination of cell division is strongly supported.

#### **1.4 Homologs of *ftsZ*: Cell Division in other Bacteria**

Most of the properties of FtsZ and the cell division model have been based on studies in the organism *E. coli*, but there has also been limited investigations of cell division in other organisms. Corton *et al.* (1987) used an *E. coli ftsZ* gene probe as well as an anti-*E. coli* FtsZ antibody to examine the presence of a homolog in various bacterial species. The gene probe hybridized to chromosomal DNA of the various Gram negative rods they tested (*Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Serratia marcescens*, *Citrobacter diversus*, and

*Shigella sonnei*) but not to a Gram negative coccus (*Branhamella catarrhalis*) or Gram positive cocci (*Staphylococcus aureus* and *Micrococcus luteus*) tested. However, immunological detection by Western blotting with antibody to the FtsZ protein from *E. coli* identified immunoreactive proteins in the Gram negative rods, as well as for the Gram negative and Gram positive coccal bacteria tested (*Branhamella catarrhalis*, *Neisseria lactamica*, *Neisseria perflava*, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Micrococcus luteus*). The results of this study indicated that the *ftsZ* gene was present in all bacteria tested, although they may not all possess enough sequence homology to be detected at the gene level, and that the process of cell division is somewhat conserved throughout the bacteria.

There have been other reports published on *ftsZ* homologs in some other organisms. Interestingly, homologs have been reported in eubacteria, archaeobacteria, and in one plant chloroplast. In the Gram negative rod *Rhizobium meliloti*, two homologs of *ftsZ* have been identified, one which is about 200 amino acids larger than FtsZ of *E. coli*, and both show GTP binding and a role in cell division (Margolin *et al.*, 1991; and Margolin and Long, 1994). A homolog of *ftsZ* has been identified in the Gram positive rod *Bacillus subtilis* (Beall *et al.*, 1988). The *B. subtilis ftsZ* is an essential gene required for both vegetative growth and sporulation (Beall and Lutkenhaus, 1991), and it was shown to have GTPase activity *in vitro* (Wang and Lutkenhaus, 1993). Immunofluorescence studies have also shown that *B. subtilis* FtsZ localization at the midcell site during vegetative growth switches to polar sites for sporulation (Levin and Losick, 1996).

The *Streptomyces coelicolor ftsZ* has been shown to be required for septation and

spore formation, but not for cell viability (McCormick *et al.*, 1994). However, another filamentous bacterium *Anabaena sp.* PCC 7120 does require *ftsZ* for cell viability (Zhong *et al.*, 1995). In the dimorphic bacteria *Caulobacter crescentus*, *ftsZ* was shown to be essential, and was found to be differentially regulated during the cell cycle (Quardokus *et al.*, 1996).

The *ftsZ* gene has been found in *Mycoplasma pulmonis*, an organism lacking cell wall peptidoglycan (Wang and Lutkenhaus, 1996a). The genome project of *Mycoplasma genitalium* also revealed an *ftsZ* sequence, and interestingly no other *fts* sequences were present (Fraser *et al.*, 1995). This observation further supports the premise that *ftsZ* has the primary role of causing invagination of the cytoplasmic membrane, and that the other *fts* gene products act to link this to septal peptidoglycan synthesis (Wang and Lutkenhaus, 1996a).

Of particular interest is the identification of an *ftsZ* homolog in the archaeobacterium *Halobacterium salinarium* (Margolin *et al.*, 1996). This archaeal FtsZ is similar to the eubacterial FtsZs, and has prompted interesting phylogenetic analysis of FtsZs and tubulins (Margolin *et al.*, 1996). The phylogenetic analysis showed that the FtsZ of *H. salinarium* is more closely related to tubulins than are the eubacterial FtsZ, so the archaeal FtsZ may be a link between tubulins and eubacterial FtsZ (Margolin *et al.*, 1996). Wang and Lutkenhaus (1996b) have also reported the presence of *ftsZ* in the archaeobacteria *Haloferax volcanii* and *Methanobacterium formicum*. The *H. volcanii* FtsZ was found to have GTPase activity and was localized to the division site by immunofluorescence (Wang and Lutkenhaus, 1996b). A homolog of *ftsZ* has been identified in the plant *Arabidopsis thaliana*, and the FtsZ homolog is imported into the chloroplasts (Osteryoung and Vierling, 1995). It may play a role in the division of chloroplasts, which are thought to have evolved from prokaryotes

(Osteryoung and Vierling, 1995).

### **1.5 *Neisseria gonorrhoeae*: The Organism**

*Neisseria gonorrhoeae* is a member of the family *Neisseriaceae*, and is a Gram-negative bacterial pathogen that is diplococcal in morphology (Vedros, 1984). Other members of the family include the pathogenic microorganism *Neisseria meningitidis*, and various non-pathogenic *Neisseria* species referred to as commensal *Neisseria* (for example *Neisseria cinerea* and *Neisseria lactamica*). The only natural host of *N. gonorrhoeae* is the human, and the gonococcus is the causative agent of the sexually transmitted disease (STD) gonorrhea. The gonococcus causes a spectrum of disease of varying severity from asymptomatic infection in men and women to rare cases of bacteremia and meningitis. Infection is usually limited to mucosal surfaces of the urogenital tract, but occasional disseminating infection leads to systemic complications (Kerle *et al.*, 1992). Complications of gonococcal disease are disproportionately borne by women; 15-30% of those with uncomplicated urogenital infections may develop pelvic inflammatory disease (PID) which can cause serious sequelae such as infertility and ectopic pregnancy (Padian and Washington, 1994). The World Health Organization (WHO) estimates that the global burden of new cases of gonorrhea was 62 million for 1995 (World Health Organization, 1995). Also of concern is the “epidemiological synergy” of the relationship between STDs, including gonococcal infections and the human immunodeficiency virus (HIV), with STDs increasing the risk of HIV transmission approximately 3 to 5 fold (Wasserheit, 1992).

The efforts to understand the pathogenesis of gonococcal infection has led to the

identification of some of the genes important to the process. The pili, outer membrane opacity proteins (Opa), and lipopolysaccharide (LPS) are important components of gonococcal pathogenicity and their role in the survival of gonococci in the host has been reviewed by Meyer (1990) and Sparling *et al.* (1990). Many studies have been focused on the molecular genetics of these and other surface antigens, and there has been almost no investigation of gonococcal genetics in other areas such as cell division.

The gonococcal genome consists of a 2.2-2.3 megabasepair circular chromosome (Dempsey *et al.*, 1991; Bihlmaier *et al.*, 1991). Physical and genetic maps of the chromosome have been published, and the majority of the mapped genes include virulence genes, cell surface proteins, and some housekeeping genes (Dempsey *et al.*, 1991; Bihlmaier *et al.*, 1991).

DNA homology between *N. gonorrhoeae* and *N. meningitidis* is relatively high and comparison of the maps shows some conserved gene order, but also some rearrangements (Dempsey *et al.*, 1995). Horizontal genetic exchange occurs between gonococci and other members of the *Neisseriaceae* family (Biswas *et al.*, 1989). Gonococci are naturally competent for DNA uptake and transformation (Sparling, 1966), and a 10 bp gonococcal transformation uptake sequence (GCCGTCTGAA) has been identified (Goodman and Scocca, 1988). These properties have allowed for genetic manipulations in the gonococcal host background.

The genetic mechanisms that *N. gonorrhoeae* has developed to evade the human immune system contribute to the difficulties in the development of an effective vaccine for gonorrhea. Despite extensive research on gonococcal outer-membrane components as

vaccine candidates, no effective vaccine has yet been developed (Blake and Wetzler, 1995). Although infections with *N. gonorrhoeae* can be cured with antimicrobial agents, a concern is the resistance of the organism to antibiotics such as penicillin and tetracycline (Dillon and Yeung, 1989), as well as the emergence of reduced susceptibility to current drugs used such as ceftriaxone and ciprofloxacin. These developments underscore the need for the identification of new antimicrobial agents. We think promising targets for such new drugs could include cell division proteins. Since cell division is an essential process for the viability of the organism, a block in the cell division machinery would essentially eliminate the organism.

#### **1.6 Cell Division and Growth of *N. gonorrhoeae***

Very little is known about the growth and cell division of *N. gonorrhoeae*. Early work by Fitz-James (1964) showed that in thin sections of dividing gonococci, the cell wall and membrane structure was similar to *E. coli*, and it appeared that division occurs by partial constriction and septation. The growth pattern of *N. gonorrhoeae* was shown by Westling-Häggström *et al.* (1977) to be bidimensional using phase-contrast microscopy. It was shown that gonococci expand perpendicular to the division plane, and then daughter cells initiate growth in the second dimension, resulting in perpendicular division planes (Westling-Häggström *et al.*, 1977). They also observed by transmission electron microscopy the ingrowth of cytoplasmic membranes which showed that *N. gonorrhoeae* divides by septation like most other gram-negative organisms, although complete septa were rarely observed (Westling-Häggström *et al.*, 1977).

At the genetic level, there are only a few reports of genes that may have a role in the cell division process of the gonococcus. The *penA* gene of *N. gonorrhoeae* encodes a penicillin binding protein (PBP2) and the gene was cloned and sequenced by Spratt (1988), whose interest was in penicillin resistance, not cell division. Amino acid sequence analysis revealed that the gonococcal PBP2 was a homologue of the *E. coli* PBP3 (Spratt, 1988). This high molecular weight penicillin binding protein is encoded by the *ftsI* gene in *E. coli* and has been shown to be involved in peptidoglycan synthesis at the division site. The role of the gonococcal PBP2 in cell division has not been characterized.

Another report by Fussengger *et al.* (1996) presents a gonococcal gene which may be involved in cell separation. Upon screening for genes involved in natural transformation competence, they identified a novel mutant phenotype called tetrapac (*tpc*). In *tpc* mutants the gonococcal colony morphology was cratered and crinkled, and the gonococcal cells grew in clusters of four (Fussengger *et al.*, 1996). They showed, using electron microscopy, that two diplococci were interconnected at their interface by a double murein layer, and the outer membrane remained continuous around the whole tetrapac. The mutant is unable to complete cell separation, thus it is blocked in cell division at a step subsequent to septum formation. It is possible, but not confirmed, that the *tpc* phenotype is a result of a mutant murein hydrolase which is unable to cleave the murein bonds to separate the daughter cells (Fussengger *et al.*, 1996).

In our laboratory, two other genes that may have a role in cell division have been identified; homologs of the *E. coli* *ftsE* and *ftsX* genes have been cloned and characterized (Bernatchez *et al.*, 1997). FtsE and FtsX are inner membrane-associated proteins in *E. coli*

(Gill and Salmond, 1987) and have been proposed to be constituents of the septalsome that may translocate cell division proteins to the bacterial periplasm (Gill *et al.*, 1986). The gonococcal FtsE shares similarity with FtsE of *E. coli* and the well-conserved regions include the motifs involved in ATP-binding which makes it a member of the ABC (ATP-Binding Cassette) protein family. FtsX from *N. gonorrhoeae* and *E. coli* are predicted to be integral membrane proteins containing 4 transmembrane helices (Bernatchez *et al.*, 1997). It is proposed that FtsE and FtsX are the two domains of an ABC transporter with FtsE serving as an ATP-binding domain and FtsX as its membrane anchor, and that this ABC transporter could participate in the translocation of cell division proteins (Bernatchez *et al.*, 1997).

Apart from these reports there have been no studies on the molecular biology of cell division in *N. gonorrhoeae*. Homologs of the various *E. coli* cell division genes have yet to be identified and characterized as to understand the division process in this coccus.

## 1.7 Hypothesis and Objectives

The focus of our research is cell division in *N. gonorrhoeae*. Cell division in the gonococcus has been investigated at the cellular level, but nothing is known of the molecular biology of cell division in the gonococcus. Identification of cell division gene homologs will help define the cell division process in the gonococcus based on the model developed in *E. coli*. This knowledge will be essential for the development of new therapeutic approaches to control *N. gonorrhoeae*. Targeting cell division proteins to block the division of the gonococcus could be an effective way to control infection.

The overall hypothesis of my thesis is that a homolog of the *E. coli* cell division gene

*ftsZ* exists in *N. gonorrhoeae*, and that the gonococcal FtsZ is structurally and functionally similar to other FtsZ proteins. Specifically, the objectives of my research project were:

- 1) to overview the known *ftsZ* sequences by reviewing the Genbank nucleotide sequence database submissions
- 2) to identify, sequence, and clone the homolog of the cell division gene *ftsZ* in *N. gonorrhoeae* in order to confirm the existence of this gene
- 3) to compare the gonococcal FtsZ to other known FtsZ by multiple sequence alignments to determine overall similarity and specific conserved regions
- 4) to identify a homolog of FtsZ in various *Neisseria* species using an anti-*E. coli* FtsZ antisera
- 5) to determine the effects of expressing the gonococcal FtsZ in *E. coli* by cloning the gonococcal *ftsZ* under the control of an inducible promoter
- 6) to construct a *ftsZ* knock-out mutant in *N. gonorrhoeae* by transformation and recombination
- 7) to determine if a homolog of *ftsZ* exists in *Chlamydia trachomatis*, another important STD pathogen.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Strains and Growth Conditions

*N. gonorrhoeae* strain CH811 (auxotype/serotype/plasmid content (A/S/P) class: non-requiring/IB-2/plasmid free; Moreno *et al.*, 1987), and the commensal strains *N. lactamica* and *N. cinerea* were selected from our culture collection. *N. gonorrhoeae* MS11 was obtained from Dr. J.G. Cannon (University of North Carolina, USA). *Neisseria* species were grown on GC Medium Base (GCMB; Difco, Detroit, MI) supplemented with Kellogg's (1963) defined supplement with the modifications of Dillon (1983) and incubation was at 35°C in a 5% CO<sub>2</sub>, humid environment. *E. coli* strain HB101 (F<sup>-</sup> *thi-1 hsdS20* (r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>-</sup>) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20* (str<sup>r</sup>) *xyl-5 mtl-1*); *E. coli* LigATor (*endA1 hsd17* (rk<sub>12</sub><sup>-</sup>m<sub>k12</sub><sup>+</sup>) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F'*proA<sup>+</sup>B<sup>+</sup>lacI<sup>q</sup>Z ΔM15::Tn10(Tc<sup>R</sup>)*]) (R&D Systems, Minneapolis, MN); and *E. coli* INV $\alpha$ F' (F' *endA1 recA1 hsdR17* (r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>+</sup>) *supE44 thi-1 gyrA96 relA1 φ80dlacZΔM15 Δ(lacZYA-argF)U169 λ<sup>-</sup>*) (Invitrogen Corp., San Diego, CA) were grown on tryptic soy agar (TSA; Difco) or in Luria Bertani (LB) broth (Sambrook *et al.*, 1989) at 37°C. When required, the following antibiotics were used: ampicillin (50 or 100 μg/mL), chloramphenicol (5 μg/mL), and tetracycline (15 μg/mL). *Neisseria* species and *E. coli* strains were stored frozen at -70°C in Brain-Heart Infusion broth (BHI; Difco) containing 20% glycerol (Dillon, 1993).

### 2.2 DNA Isolation and Manipulations

Chromosomal DNA from *N. gonorrhoeae* strains was extracted as described by Dillon *et al.* (1985) and the DNA was purified by caesium chloride density gradient

ultracentrifugation (Sambrook *et al.*, 1989). Chromosomal DNA from *Neisseria meningitidis* strains 1500 and 1501 was previously obtained (Lawson *et al.*, 1996). All plasmid purifications were performed using the Wizard™ Minipreps DNA Purification System from Promega (Fisher Scientific, Nepean, Ontario) following the procedure of the manufacturer for plasmid purification without a vacuum manifold.

DNA concentrations were determined spectrophotometrically using the GeneQuant DNA/RNA Calculator (Pharmacia Biotech; Baie D'Urfé, Quebec), where DNA concentration is equal to optical density at  $\lambda_{260} \times 50 \text{ ng}/\mu\text{L} \times \text{dilution factor}$ .

Restriction enzymes were purchased from Boehringer Mannheim (Laval, Quebec) and from Pharmacia Biotech and used according to the manufacturer's instructions.

DNA was separated on 1% agarose gels using Tris-acetate EDTA buffer (TAE: 40 mM Tris-acetate, 1 mM EDTA, pH 7.8) and a constant voltage of 50 Volts for 2 to 4 hours (h) (Sambrook *et al.*, 1989). The gels were stained in 1 mg/L ethidium bromide solution and the DNA visualized under UV illumination (Fotodyne Inc., New Berlin, WI). Images were captured using a BioPhotonics GelPrint 2000i digital imaging system (BIO/CAN Scientific, Mississauga, Ontario).

### **2.3 Southern Blotting**

DNA separated on agarose gels was transferred to positively charged nylon membranes (Boehringer Mannheim) by the capillary transfer method (Southern, 1975) or by vacuum transfer using BIORAD Model 785 Vacuum Blotter (BIORAD, Mississauga, Ontario) following the instructions of the manufacturer. DNA was fixed to the membranes

by UV-crosslinking for 3 min (Sambrook *et al.*, 1989). DNA amplicons for probes were labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate (Dig-dUTP) using the Genius™ Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim). The DNA amplicons were denatured by heating for 10 min. in a boiling water bath and chilled quickly on ice/ethanol. Hexanucleotide mixture and dNTP labelling mixture (1 mM dATP, 1 mM dCTP, 1mM dGTP, 0.65 mM dTTP, 0.35 mM Dig-dUTP) were added, followed by addition of Klenow enzyme and incubation at 37°C overnight. The reaction was stopped by addition of EDTA (0.2 M, pH 8.0), and precipitated by addition of LiCl (4 M) and ice cold 70% ethanol. After 30 min. at -70°C, the labelled DNA was pelleted by centrifugation, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The membranes were pre-hybridized for at least 1 h in a hybridization incubator (Robbins Scientific Corporation, Sunnyvale, CA) with hybridization solution consisting of 5×SSC (0.75 M NaCl, 0.075 M Na-citrate, pH 7.0), 0.5% (w/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) N-lauroylsarcosine Na-salt, and 0.02% (w/v) SDS. The labelled DNA probe was resuspended in 10 mL of hybridization solution and denatured before use. The pre-hybridization solution was replaced with the probe DNA solution, and the membranes were incubated with the probe overnight. Membranes were first washed twice for 5 min. at room temperature with 2×SSC containing 0.1% SDS, and then twice for 15 min. at the hybridization temperature with 0.1×SSC containing 0.1% SDS. Membranes were used directly for detection, or air dried for storage.

Detection of the hybridized probe was done using the Genius™ kit (Boehringer

Mannheim). Membranes were washed briefly in buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5), and blocked for 30 min. in buffer 2 (1% blocking reagent in buffer 1). The membranes were incubated for 30 min. with polyclonal sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1/5000 dilution in buffer 2). After 2 washes of 15 min. each with buffer 1, the membranes were equilibrated for 2 min. in buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5). The colour reagent was prepared by adding nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to buffer 3, and was applied to the membranes. Blots were incubated in the dark until the colour precipitate formed.

#### 2.4 Oligonucleotide Primers

All oligonucleotide primers were purchased from the University of Ottawa Biotechnology Research Institute (UOBRI, Ottawa, Ontario) unless otherwise indicated. Primers to amplify a partial *ftsZ* gene based on *E. coli* sequence were initially obtained from Dr. R. Levesque (Université Laval, Laval, Quebec). Degenerate oligonucleotide primers for amplification of *ftsZ* were designed manually based on conserved amino acid sequences as determined by multiple sequence alignments of selected FtsZ (McCormick *et al.*, 1994) and based on gonococcal codon usage (West and Clark, 1989). The amino acid sequences of the conserved regions and the sequence of the degenerate primers designed are shown in Table 1.

Primers ar7 and ar10 (Table 2) for amplification of a partial *ftsZ* from *N. gonorrhoeae* were based on the nucleotide sequence of the *N. meningitidis ftsZ* homolog (Genbank

Table 1. Sequences of the degenerate oligonucleotide primers based on the conserved amino acid sequences of FtsZ.

Primer	Degenerate primer sequence (5' to 3')	Amino acid sequence	position on <i>E. coli</i>
ar 1	CTGGAAATTCAACACCCGA(T/C)GC(A/T/G/C)CA(A/G)G	NTDAQA	aa <sup>a)</sup> 43 to aa 48
ar 2	CTTGAAATTCGTGCCCGGT(G/A)CC(G/A)CC(G/A)CCCCA	MGGGTG	aa 104 to aa 109
ar 5	AA(C/T)AC(C/T)GA(C/T)G(G/C)(A/C/T)CA(A/G)GC	NTDAQA	aa 43 to aa 48
ar 6	CG(A/G)AC(A/G)CC(G/A)(C/A)(T/A)(G/A)CCCCAT	MGSGVA	aa 237 to aa 242

a) amino acid

TABLE 2. The sequences of the oligonucleotide primers used for sequencing and amplification of the *ftsZ* gene region of *N. gonorrhoeae* CH811. The positions of the primers is based on the final sequence assembled (Figure 5C).

Primer	Sequence 5' to 3'	Base pair position
ar7	TGCCAATAACGGATGCGCAG	357 to 376
ar10	CTCCGTCCAAGGTTACATCG	1004 to 985 (C) <sup>a)</sup>
ar11	CGCGAATGGCTTCTTCAATG	515 to 496 (C)
ar12	AAGTGGTAACTTGCCCGAGC	825 to 844
ar13	GGGTGAAGACGTAACTATGC	748 to 767
ar14	GTA ACTACGGCAACGGTCAG	630 to 611 (C)
ar15	TCGTACCCA ACTGGATTCTC	423 to 404 (C)
ar16	GTATCGCTATGATGGGTTTCG	897 to 916
ar17	GTCAAACGGCAATCCA ACTG	126 to 107 (C)
ar18	CCGGAAAGGGAAGTAGAAGC	1214 to 1233
ar19	GCCGTCTGAA <sup>b)</sup> -TGGTTGCCAACAATGTG	321 to 337
ar20	GCCGTCTGAA <sup>b)</sup> -CATCTTCGCTCATGGTC	1154 to 1138 (C)
ar23	GATTCTGCCACGTCGTAAAC	252 to 233 (C)
ar24	GAAAGGGAAGTAGAAGCGGT	1217 to 1236
ar25	GGCAAGCGCAATCGGATAGT	1785 to 1767 (C)
ar26	GCCGTAAACTTATTGCCTCC	1413 to 1432
ar27	ACGGTACAGTGCAAGAG	29 to 45
ar28	ATTCTGAACCCGCACAC	1512 to 1496 (C)
ar29	AAACCGTCCTCTGAACG	187 to 203

a) C indicates primer position on complementary strand

b) 5' extension to include the gonococcal uptake sequence

accession U43329). All other primers for PCR and sequencing were designed using the program Primer Designer (Scientific and Education Software). Table 2 shows the primers sequences and their positions on the gonococcal *ftsZ* region sequenced (Figure 5C in Results).

## 2.5 PCR and Inverse PCR Procedures

Each 100  $\mu\text{L}$  PCR reaction contains 1x PCR buffer containing 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs and 2.5 U of *Taq* DNA polymerase (PCR Core Kit, Boehringer Mannheim). All amplifications were done using the Perkin Elmer GeneAmp PCR System 9600 thermocycler (Perkin Elmer Corp. Norwalk, CT).

For PCR amplifications using the *E. coli* based primers and degenerate primers, primers were used at a concentration of 0.2 and 1  $\mu\text{M}$  and genomic DNA from *N. gonorrhoeae* CH811 and MS11 was used at 0.1  $\mu\text{g}$  per reaction. The thermal cycling was as follows: an initial 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec. at various annealing temperatures, 2 min. at 72°C; and a final 5 min. at 72°C.

For amplification of the partial *ftsZ*, primers ar7 and ar10 were designed from the *N. meningitidis ftsZ* homolog sequence (Genbank accession U43329) and were used at 0.2 ng per reaction. Genomic DNA from *N. gonorrhoeae* CH811 and MS11, and *N. meningitidis* 1500 and 1501 was used at 0.1  $\mu\text{g}$  per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles of 15 sec. at 95°C, 15 sec. at 58°C, 1 min. at 72°C; 5 min. at 72°C.

For amplification of *ftsZ* by inverse PCR, *N. gonorrhoeae* CH811 chromosomal DNA was digested to completion with *AluI* and then self-ligated into circles using T4 DNA ligase using DNA concentrations of 0.2, 1, and 5 ng/ $\mu\text{L}$  as recommended in inverse PCR protocols

(Hartl and Ochman, 1994). The religated DNA was cleaned by organic extractions with buffer saturated phenol (Gibco BRL, Gaithersburg, MD), chloroform, precipitated with ice cold 70% ethanol, resuspended in water, and used as the template for inverse PCR. For each PCR reaction, 0.1 ng of DNA from the ligations was used. The reaction conditions described above were used with the divergent primers ar11 and ar12 at 0.2  $\mu$ g per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec. at 58°C, 2 min. at 72°C; 5 min. at 72°C.

For amplification of the entire *ftsZ*, the primer pairs ar27 and ar28, or ar29 and ar28 were used at 0.2  $\mu$ g per reaction, and *N. gonorrhoeae* CH811 chromosomal DNA was used at 0.1  $\mu$ g per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec at 50°C, 1.5 min. at 72°C; 5 min. at 72°C. For amplification of a region of *ftsZ*, primers ar19 and ar20 were used at 0.2  $\mu$ g per reaction, and *N. gonorrhoeae* CH811 chromosomal DNA was used at 0.1  $\mu$ g per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec. at 58°C, 2 min. at 72°C; 5 min. at 72°C.

PCR to confirm orientation of *ftsZ* clones was performed using the T7 primer (5' AATACGACTCACTATAG 3') and either ar28 or ar29 at 0.2  $\mu$ g per reaction. Plasmid DNA from the clones was diluted 20 fold and 10  $\mu$ L was used per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec. at 45°C, 2 min. at 72°C; 5 min. at 72°C.

## **2.6 DNA Sequencing**

The nucleotide sequences of the 650 bp amplicon and of the inverse PCR 1.6 kb amplicons were determined in both directions using automated sequencing services at the University of Ottawa Biotechnology Research Institute (UOBRI). Amplicons were directly sequenced after cleaning with QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA). The Taq Dye-deoxy™ Terminator Cycle Sequencing kit (ABI, Mississauga, Ontario) was used as per the manufacturer's instructions for automated sequencing and Centri-Sep Spin Columns (Princeton Separation, Adelphia, NJ) were used with a 373 Automated DNA Sequencer System (ABI).

## **2.7 DNA and Amino Acid Sequence Analysis**

The partial nucleotide sequences were assembled and analysed using the program PCGene (IntelliGenetics Inc., Geneva, Switzerland). Nucleotide and amino acid sequences were retrieved by the Internet from the Genbank nucleotide sequence database at the National Center for Biotechnology Information, National Institute of Health (NCBI, NIH, Bethesda, MD). Nucleotide and amino acid sequence similarities were investigated using Blast (Alschul *et al.*, 1990) and Clustal V (Higgins *et al.*, 1991). The Genetics Computer Group (GCG) software package (version 7, Madison, WI) was used for Bestfit protein sequence comparisons.

## **2.8 Cloning Strategies and Transformation of *E. coli***

The PCR amplicon cloning vector pTAg (R&D Systems) was chosen for cloning of

various PCR amplicons of *ftsZ* from *N. gonorrhoeae* CH811. This linear cloning vector has deoxythymidine (T) overhangs to allow cloning of PCR amplicons generated with *Taq* polymerase which leaves deoxyadenosine (A) overhangs. The entire *ftsZ* gene amplified using primer pairs ar27/ar28 (1.5 kb amplicon) and ar29/ar28 (1.3 kb amplicon) was ligated into pTAg following the instructions of the manufacturer.

For transformations, commercially available competent *E. coli* LigATor cells (R&D Systems) were used, or competent *E. coli* INV $\alpha$ F' were prepared by the calcium chloride method (Sambrook *et al.*, 1989). Briefly, overnight cultures of *E. coli* were used to inoculate 50 mL of LB and cultures were incubated at 37°C on a rotary shaker until growth reached mid-log phase (0.3-0.6 OD units at  $\lambda_{600}$ ). Cells were placed on ice for at least 30 min. Cells were centrifuged at 4000 rpm for 10 min. at 4°C (Sorvall RC5C centrifuge, Dupont, Newtown, CT), the supernatant removed, and the pellet gently resuspended in 5 mL of ice cold 0.1 M CaCl<sub>2</sub>. Cells were centrifuged again as described, resuspended in 2 mL of 0.1 M CaCl<sub>2</sub>, and kept on ice until use for transformation.

For transformation, 200  $\mu$ L of competent cells were aliquoted to eppendorf tubes for each transformation reaction. 2  $\mu$ L of ligation or control plasmid DNA was added. The competent cells were incubated for 30 min. on ice followed by a 2 min. heat shock at 42°C in a water bath. 800  $\mu$ L of LB broth was added, and the cultures incubated for 1 h at 37°C on a rotary shaker (250 rpm). 75  $\mu$ L of cells were plated on TSA plates with ampicillin for selection of transformants, tetracycline for maintenance of the F' in the LigATor cells, and 80  $\mu$ g/mL 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal) for blue/white screening.

Very few clones were obtained for the 1.5 kb amplicon (primers ar27/ar28), whereas

numerous clones were obtained for the 1.3 kb amplicon (primers ar29/ar28). The clones were initially screened for inserts by restriction endonuclease digestion with *Bam*HI and *Hind*III. No positive clones were obtained for the 1.5 kb *ftsZ* amplicon. Positive clones containing the 1.3 kb *ftsZ* amplicon were further screened by PCR with the T7 primer and either ar28 or ar29 as described above to select clones containing the insert in both orientations with respect to the lactose promoter on the vector. Two clones were maintained: pAR2 contains *ftsZ* downstream and in the same orientation as the *lac* promoter, and pAR3 contains *ftsZ* downstream and in the opposite orientation of the *lac* promoter.

## 2.9 Western Blot Analysis

Overnight growth from agar cultures of *E. coli*, *N. gonorrhoeae* CH811, *N. gonorrhoeae* MS11, *N. cinerea*, and *N. lactamica* cells were harvested in 2 mL of 10 mM Tris, 1 mM EDTA (TE), pH 8.0. Cells were kept on ice and disrupted using a Sonifier<sup>®</sup> cell disruptor W-350 (Branson Sonic Power Co., SmithKline Corporation, Danbury, CT). The sonication was carried out five times, for 6 seconds each, with 6 second intervals using the microtip with an output setting of 7. The disrupted cells were centrifuged at 4°C, 10 min. at 10000 rpm in a Sorvall RC5C centrifuge (Dupont) using the SS35 rotor to obtain the cleared extracts. Approximately equal amounts of protein from the *Neisseria* sp. and *E. coli* extracts were mixed in a 1:1 ratio with SDS sample buffer (2 mL glycerol, 2 mL 10% SDS, 0.25 mg bromophenol blue, 2.5 mL 4× stacking gel buffer, 0.5 mL β-mercaptoethanol) and then boiled for 10 minutes. The proteins of the extracts were separated on sodium dodecyl sulfate polyacrylamide separating gels (10%), with 15% polyacrylamide stacking gels using

the Mini-PROTEAN® II Electrophoresis Cell (BIORAD), run at 100 mAmp for 45 min. The proteins were transferred from the gel to nitrocellulose membrane (BIORAD) using a Mini Trans-Blot® Electrophoretic Transfer Cell (BIORAD) with an overnight transfer at 30 Volts in a 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3 transfer buffer. The immunodetection was performed using the BIORAD Immun Blot® Assay Kit. The membrane was first blocked for 2 h using 3% gelatin in Tris buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5). The membrane was then washed with TTBS (TBS plus 0.05% Tween). The polyclonal rabbit anti-*E. coli* FtsZ antisera obtained from Dr. J. Lutkenhaus (Kansas Medical Center, Kansas City, KA) was used at a 1/2000 dilution in antibody dilution buffer (TTBS plus 1% gelatin) and incubated with the membrane for 1.5 h. Two 5 min. washes were done with TTBS. An alkaline phosphatase conjugated goat anti-rabbit antibody (BIORAD)(1/3000 dilution in antibody buffer, 1 h) was used for detection, and the nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate solution was used for colour development.

### **2.10 *In vitro* Transcription/translation of *ftsZ***

The amplicon of *ftsZ* generated using ar27 and ar28 was cleaned using the QIAquick PCR Purification Kit (Qiagen). The *E. coli* S30 Extract Systems for Linear DNA (Promega) was used for *in vitro* synthesis of the gene product, and was used according to the manufacturer's instructions, with [<sup>35</sup>S]-methionine (Amersham Canada, Oakville, Ontario) and 1 µl of RNAGuard© (Pharmacia) added to each reaction. The reaction products were prepared for electrophoresis as indicated by the manufacturer and were separated on

denaturing 10% SDS-PAGE gels. The gels were dried using a BIORAD model 583 Gel Dryer and exposed to Kodak X-OMAT™ AR films (InterScience, Brampton, Ontario), and films were developed using a Cordell MXR developer (Cordell Engineering, Peabody, MA). The size of the synthesized polypeptides was estimated by comparison with SDS-PAGE broad range molecular weight standards (BIO-RAD).

### **2.11 Expression of the Gonococcal *ftsZ* in *E. coli***

The methods for expression of *ftsZ* in *E. coli* were based on work by Beall *et al.* (1988) and Margolin and Long (1994). Cultures of *E. coli* LigATor containing respectively pTAG, pAR2, or pAR3 were grown in LB containing ampicillin (50 µg/mL) and tetracycline (15 µg/mL) at 37°C from fresh overnight cultures. After 4 h of growth, each culture was divided into two portions and IPTG was added to a final concentration of 1mM to one portion, leaving the other portion as the uninduced control. Samples for microscopy were taken from overnight cultures, immediately before induction, and every hour after induction. Cells were pelleted by centrifugation at low speed using the microfuge, resuspended in 1 mL of 2% glutaraldehyde and fixed for 30 min. Fixed cells were washed with 0.9% NaCl (saline), and resuspended in saline. Wet mounts were observed for cell morphology at 1000X magnification using a Leitz Laborlux K light microscope (Wild Leitz, Willowdale, Ontario) adapted with a Hoffman modulation contrast system (Modulation Optics Inc., Greendale, NY) and photographed using a Wild Photoautomat MP545 camera system (Wild Leitz) using Kodak ASA 400 film.

## 2.12 Insertional Mutagenesis of *ftsZ* in *N. gonorrhoeae*

In order to construct a *ftsZ* knock-out mutant in *N. gonorrhoeae*, the natural transformation and recombination ability of the gonococcus was used. First, a plasmid containing the *ftsZ* gene with an insertion was constructed and this was subsequently used to transform gonococci. A region of *ftsZ* (854 bp) was amplified with primers ar19 and ar20 (primers containing the gonococcal uptake sequence GCCGTCTGAA) and was cloned into pTAg (as described above) to generate pAR1. The clone was confirmed by restriction endonuclease digestion with *Bam*HI and *Hind*III to release the insert. The two primers ar21 (5' ATTACCGCGG-ACATGAAGCACTTCACTGAC 3') and ar22 (5' ATTAGCGCGC-CTGTTACAACAGTCCGCA 3') were designed to amplify the chloramphenicol acetyl transferase gene (*cat*) from pACYC184 (New England Biolabs, Mississauga, Ontario). The plasmid pAR1 was digested with the restriction endonucleases *Mam*I and *Sac*II which releases a 179 bp fragment. The *cat* amplicon was also digested with the restriction endonucleases *Mam*I and *Sac*II, and the marker gene (1452 bp) was inserted into the *ftsZ* of pAR1 by ligation. Transformed INV $\phi$ F' cells containing the new plasmid construct were selected by ampicillin and chloramphenicol resistance. The new plasmid pAR4 was confirmed by restriction endonuclease digestion with *Mam*I, *Sac*II, and *Eco*RI. This plasmid could now be used for transformation of gonococci.

*N. gonorrhoeae* CH811 was grown and type T2 colonies were selected as described by Kellogg *et al.* (1968). Cells from type T2 colonies are piliated and are competent for DNA uptake (Sparling, 1966). Type T2 colonies were streaked onto quadrants of a GCMB agar plate using a swab, and then the DNA for uptake was applied to the cells and spread using

a loop. DNA from a streptomycin resistant derivative of *N. gonorrhoeae* CH811 was used as a positive control for transformation. Plasmid DNA of the pAR4 construct of the *cat* insertion into *ftsZ* was applied to the cells. No DNA was added to a quadrant as a negative control. After 6 h of incubation, cells were streaked onto selective GCMB media and incubated overnight. Selection of the transformation control was done using streptomycin (1 mg/mL), and selection of the *cat* insertion *ftsZ* mutant was with chloramphenicol (50 µg/mL). The negative control cell were streaked onto both selective media, and on non-selective media as a growth control.

### **2.13 Amplification of a Partial *ftsZ* from *Chlamydia trachomatis***

The degenerate primers designed for amplification of *ftsZ* were used on *C. trachomatis* serotype L2 chromosomal DNA obtained from Dr. Mahoney (McMaster University, Hamilton, Ontario). Degenerate primers ar1 and ar2 (Table 1) were used at 10 pmol per reaction, and 0.1 µg of template DNA was used per reaction. The thermal cycling was as follows: 3 min. at 94°C; 30 cycles consisting of 15 sec. at 95°C, 15 sec. at 60°C, 30 sec. at 72°C; 5 min. at 72°C. The amplicon obtained was reamplified with non-degenerate primers ar3 (5' CTGGAATTCAACACCGA 3') and ar4 (5' CTTGAATTCGTGCCGGT 3') designed to the 5' ends of the degenerate primers, and the amplicon was sequenced directly using primers ar3 and ar4.

### 3. RESULTS

#### 3.1 Overview of *ftsZ* Gene Sequences

A search of the Genbank nucleotide sequence database identified 24 reported homologs of the *E. coli ftsZ* cell division gene, many of which are not published sequences. Table 3 summarizes the organisms from which *ftsZ* sequence has been reported, and their description has been indicated. It can be seen that *ftsZ* has been identified in a variety of organisms, including 19 different eubacteria, 4 archaeobacteria, and one plant. Further research (where references are indicated) has been initiated in relatively few organisms (reviewed in the introduction). The identification and study of *ftsZ* has not been undertaken in *N. gonorrhoeae*, nor has a homolog been investigated in *C. trachomatis*.

#### 3.2 Identification of the *ftsZ* Gene of *N. gonorrhoeae* CH811

For the identification of the *ftsZ* gene of *N. gonorrhoeae* CH811, a combination of PCR strategies was attempted. These included PCR amplification using primers based on the *E. coli ftsZ* sequence, using degenerate primers with gonococcal codon bias, and using primers (ar7 and ar10) based on a released *N. meningitidis ftsZ* partial sequence (Genbank accession U43329). PCR amplification using primers ar7 and ar10 was successful in generating a 650 bp PCR product from the chromosomal DNA of *N. gonorrhoeae* strains CH811 and MS11 (Figure 4, lanes 1 and 2) and the expected 650 bp amplicon from the chromosomal DNA of *N. meningitidis* strains 1500 and 1501 (Figure 4, lanes 3 and 4). The 650 bp amplicon from *N. gonorrhoeae* CH811 was sequenced (Figure 5A), and the DNA sequence obtained was analyzed for similarity to other sequences in Genbank using BlastN

TABLE 3. The organisms in which a sequence has been determined for the *ftsZ* gene. The classification of the organisms and the Genbank accession number for the sequence are indicated. Those in which FtsZ has been further characterized are indicated by references.

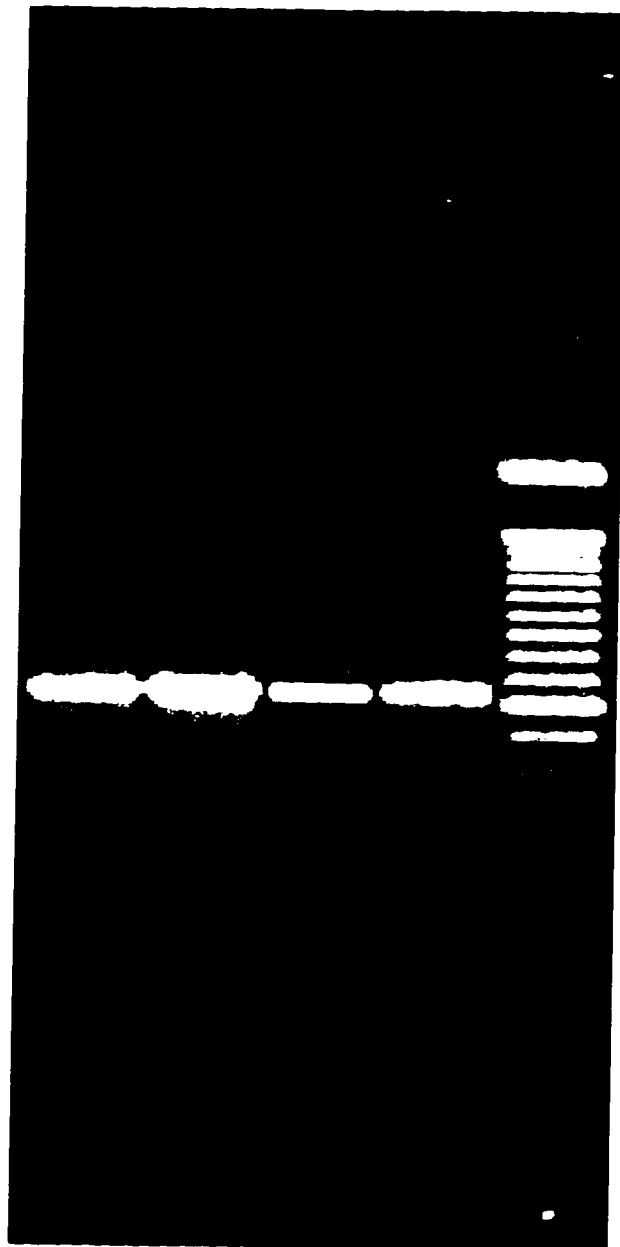
Organism	Classification	Genbank accession	References
<i>Escherichia coli</i>	Gram -ve rod	EC2MIN	many
<i>Rhizobium meliloti</i>	Gram -ve rod	RHMFTSZA RHMFTSZ	Margolin <i>et al.</i> , 1991, Margolin and Long, 1994
<i>Pseudomonas aeruginosa</i>	Gram -ve rod	PAU19797	
<i>Pseudomonas putida</i>	Gram -ve rod	PPU29400	
<i>Azotobacter vinelandii</i>	Gram -ve rod	AVU65939	
<i>Haemophilus influenzae</i>	Gram -ve rod	HIU32794	
<i>Bacillus subtilis</i>	Gram +ve rod	BACFTSZA	Beall <i>et al.</i> , 1988, Wang and Lutkenhaus, 1993, Beall and Lutkenhaus, 1991, Levin and Losick, 1996
<i>Neisseria meningitidis</i>	Gram -ve coccus	NMU43329	
<i>Micrococcus luteus</i>	Gram +ve coccus	MLU10878	
<i>Staphylococcus aureus</i>	Gram +ve coccus	SAU06462	
<i>Streptomyces coelicolor</i>	Gram +ve filamentous	SCU10879	McCormick <i>et al.</i> , 1994
<i>Streptomyces griseus</i>	streptomyces	SGU07344	
<i>Caulobacter crescentus</i>	appendaged	CCU40273	Quardokus <i>et al.</i> , 1996

TABLE 3. continued

Organism	Classification	Genbank accession	References
<i>Borrelia burgdorferi</i>	spirochetes	BBFTSZG	
<i>Anabaena</i> sp.	cyanobacterium	ASU114408	Zhang <i>et al.</i> , 1995
<i>Wolbachia</i> sp.	rickettsias	DMFTSZ	
<i>Mycoplasma pulmonis</i>	mycoplasmas	MPU34931	Wang and Lutkenhaus, 1996
<i>Mycoplasma capricolum</i>	mycoplasmas	MC431	
<i>Mycoplasma genitalium</i>	mycoplasmas	U39700	
<i>Haloferax volcanii</i>	archaeobacterium	HVU37584	
<i>Methanococcus jannaschii</i>	archaeobacterium	MJU67490	
<i>Halobacterium salinarium</i>	archaeobacterium	HSU32860	Margolin <i>et al.</i> , 1996
<i>Pyrococcus woesei</i>	archaeobacterium	PWU56247	
<i>Arabidopsis thaliana</i>	plant	ATU39877	Osteryoung and Vierling, 1995

FIGURE 4. Amplification of a 650 bp amplicon containing a partial *ftsZ* gene from *N. gonorrhoeae* and *N. meningitidis* chromosomal DNA using primers ar7 and ar10 based on *N. meningitidis ftsZ* partial sequence. Lane 1, *N. gonorrhoeae* CH811; lane 2, *N. gonorrhoeae* MS11; lane 3, *N. meningitidis* 1500; lane 4, *N. meningitidis* 1501; M, 100 bp DNA ladder (Gibco BRL).

1 2 3 4 M



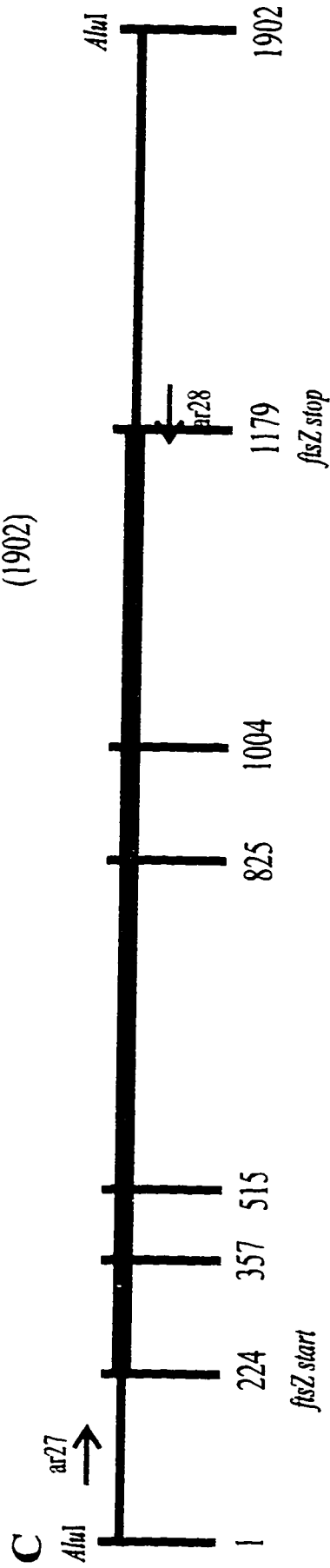
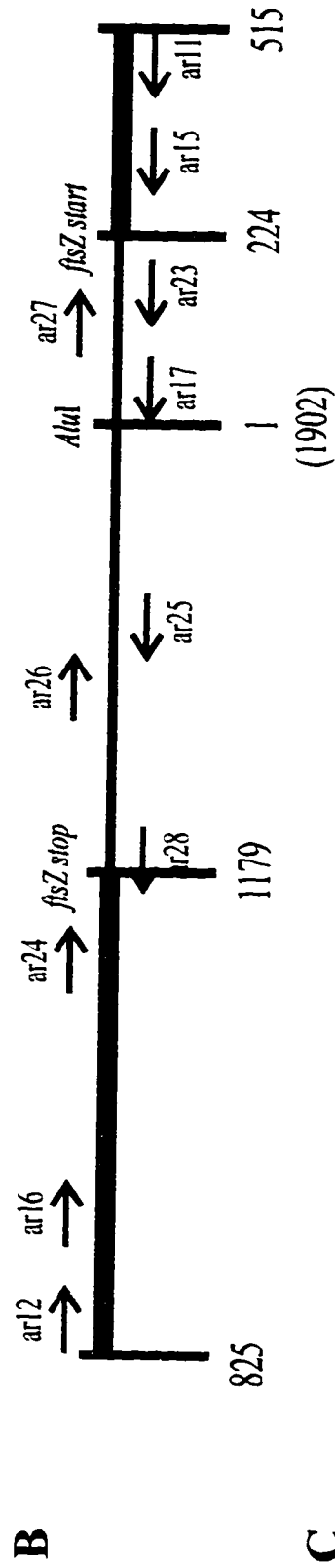
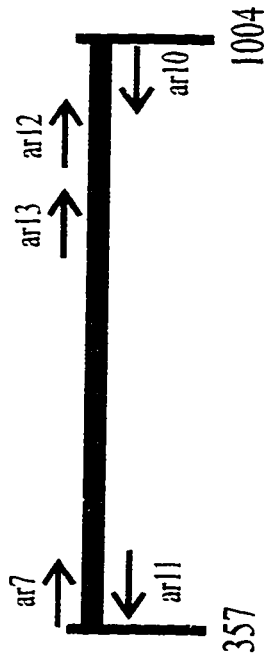
bp

— 1500

— 600

— 100

FIGURE 5. The sequencing strategy of the 650 bp amplicon and the 1.5 kb inverse PCR amplicon containing gonococcal *ftsZ* sequences. Figure 5A. Sequencing of the 650 bp amplicon obtained using primers ar7 and ar10. Figure 5B. Sequencing of the 1.5 kb inverse PCR amplicon obtained using primers ar11 and ar12. Figure 5C. Region obtained by combining the sequences of the 650 bp and 1.5 kb amplicons. Base pair numbering is based on final combined sequence, with the *AluI* site upstream of *ftsZ* being nucleotide 1. Primers used for PCR and sequencing are indicated in blue, primers for sequencing only are indicated in black, and primers for PCR only are indicated in yellow. Primer arrows are not to scale. Regions coding for the gonococcal *ftsZ* gene are indicated in red.



for nucleotide sequence similarity and BlastX for protein translation similarity. The nucleotide sequence showed similarity with other prokaryotic *ftsZ* genes, and the translation showed similarity to FtsZ proteins (data not shown). Therefore, strategies to obtain the entire gene by inverse PCR were initiated.

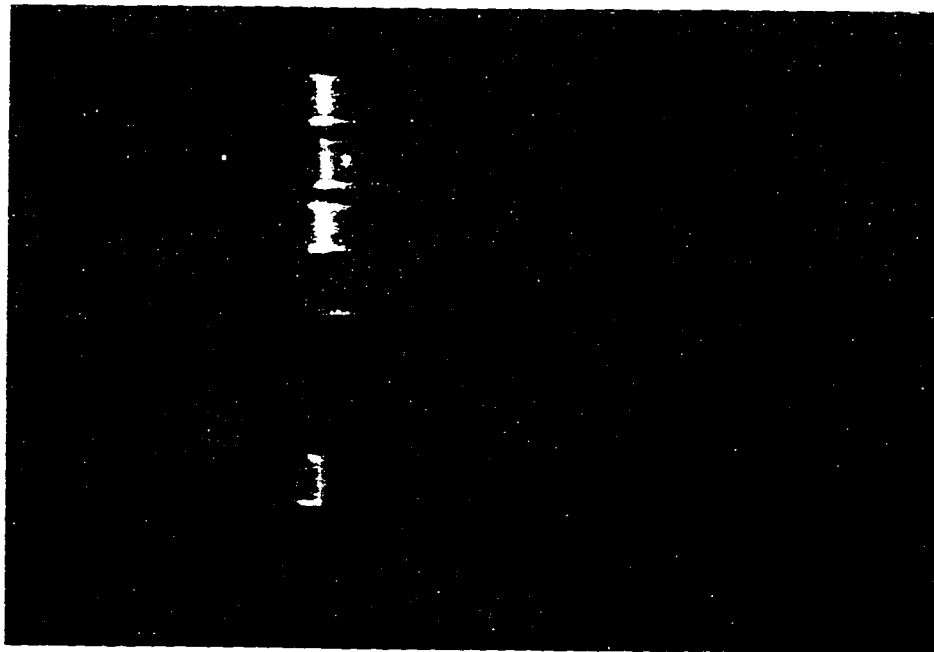
First, a gonococcal chromosomal DNA restriction fragment containing the partial gene sequence was identified. *N. gonorrhoeae* CH811 chromosomal DNA was cut with various restriction enzymes (Figure 6A, lane 1, *AluI*; lane 2, *ApaI*; lane 3, *BglI*; lane 4, *EcoRI*; lane 5, *HindIII*; lane 6, *NheI*; lane 7, *NruI*; lane 8, *XbaI*) and transferred to nylon membrane for hybridization with a labelled partial *ftsZ* amplicon (Figure 6B).

Hybridization at high stringency located the partial *ftsZ* amplicon on a 1.9 kb *AluI* fragment (Figure 6B, lane 1). Another weaker hybridizing *AluI* fragment of around 2.3 kb is also seen (Figure 6B, lane 1) and is probably an incomplete digestion fragment also containing *ftsZ*. In the lanes 2 to 8 of Figure 6B, the gene probe hybridized to very large fragments of the various digests which would be unsuitable for the inverse PCR procedure. In the *ApaI* and *HindIII* digests (lanes 2 and 5) the gene probe hybridized to uncut chromosomal DNA. For the *NheI*, *NruI* and *XbaI* digests (lanes 6, 7, and 8), the hybridizing bands are large because these enzymes are rare cutters generating large fragments of DNA which are not resolved well on this gel. The *BglI* digest generated a fragment around 6.1 kb on which the gene lies (lane 3), which is too large for inverse PCR. The presence of many hybridizing bands in the *EcoRI* digest (lane 4) may be due to star activity of the enzyme. The positive control of the probe hybridizing to itself is in Figure 6B, lane 9. This hybridization to the gonococcal chromosomal DNA digests also confirms the gonococcal

FIGURE 6. Identification of the partial *ftsZ* gene on chromosomal digests of *N. gonorrhoeae* CH811 by Southern hybridization. Figure 6A. Electrophoresis of restriction enzyme digests of *N. gonorrhoeae* CH811 chromosomal DNA. Lane 1, *AluI*; lane 2, *ApaI*; lane 3, *BglI*; lane 4, *EcoRI*; lane 5, *HindIII*; lane 6, *NheI*; lane 7, *NruI*; lane 8, *XbaI*; lane 9, *N. gonorrhoeae* CH811 650 bp amplicon; M, 1 kb DNA ladder (Gibco BRL). Figure 6B. Southern blot of 3A with a 650 bp amplicon probe of the partial *ftsZ* of *N. gonorrhoeae* CH811.

A

M I 2 3 4 5 6 7 8 9

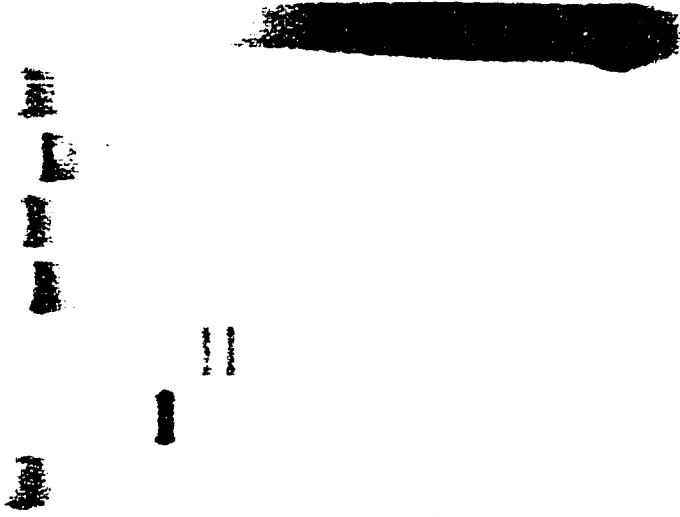


bp

6108 —  
 5090 —  
 4072 —  
 3054 —  
 2036 —  
 1636 —  
 1018 —

B

M I 2 3 4 5 6 7 8 9



bp

6108 —  
 5090 —  
 4072 —  
 3054 —  
 2036 —  
 1636 —  
 1018 —

origin of the partial gene amplicon.

*A*luI digested gonococcal DNA was religated and used at various concentrations as a template for inverse PCR to obtain the remaining *ftsZ* sequence. The divergent primers ar11 and ar12 were designed near the ends of the partial *ftsZ* gene sequence (Figure 5A) and resulted in amplification of a 1.5 kb inverse PCR product (Figure 7, lanes 2, 3, and 4), which is expected considering the size of the hybridizing fragment. Another weaker amplicon of about 2 kb (Figure 7, lane 2) is also obtained as a result of the incomplete digestion fragment which is also present as a template. The 1.5 kb amplicon was sequenced from both ends (Figure 5B), and sequence analysis showed it contained sequence upstream and downstream of the partial *ftsZ* sequence. Sequencing was continued until the *ftsZ* gene start site and the termination codon were identified. The complete coding region of the *ftsZ* gene was obtained by combining the sequences of the 1.5 kb and the 650 bp amplicons (Figure 5C). Although a region of about 1.9 kb was obtained by combining the two sequences, only 1550 bp of sequence containing the *ftsZ* coding region (1179 bp), 223 bp upstream and 148 downstream is being reported (Figure 8) because the remainder of the downstream sequence was not obtained from both strands. This nucleotide sequence has been submitted to the Genbank sequence database with the accession number U76537.

### **3.3 Features of the *ftsZ* Gene and Gene Product**

Analysis of the obtained sequence, shown in Figure 8, revealed that the *ftsZ* ORF comprises 1179 bp encoding a putative protein of 392 amino acids (aa) with a predicted molecular weight of 41542 Da. Other sequence features include a putative ribosome-binding

FIGURE 7. Amplification of the 1.5 kb inverse PCR amplicon from self-ligated *AluI* digests of *N. gonorrhoeae* CH811 chromosomal DNA using primers ar11 and ar12. DNA concentration in ligations was as follows: lane 1, no DNA; lane 2, 0.2 ng/ $\mu$ L; lane 3, 1 ng/ $\mu$ L; lane 4, 5 ng/ $\mu$ L. M, 1 kb ladder (Gibco BRL).

M 1 2 3 4

bp

3054 —

2036 —

1636 —

1018 —

506 —

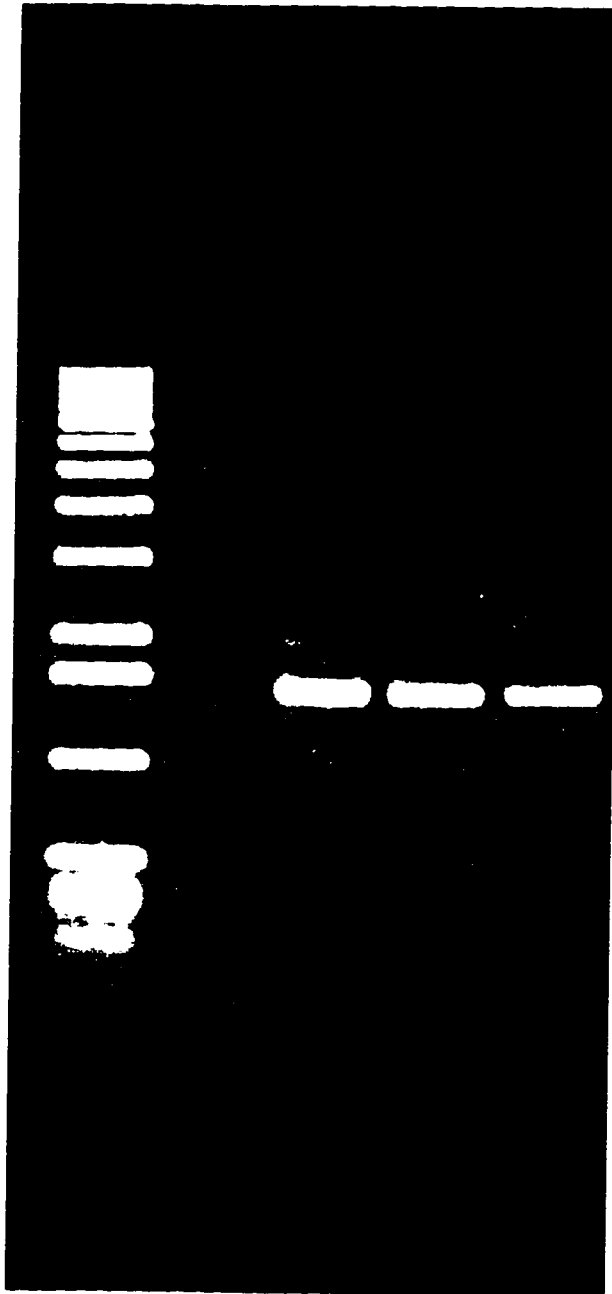


FIGURE 8. A 1550 bp region containing the complete nucleotide sequence and translation of the *ftsZ* gene of *N. gonorrhoeae* CH811. The sequence was obtained by combining the sequence from the 650 bp amplicon and parts of the 1.5 kb inverse PCR amplicon as shown in Figure 5. The *ftsZ* gene coding sequence is shown in red, with the amino acid translation beneath. The putative ribosome binding site is indicated in purple, and putative transcriptional terminator is underlined in black.

1 CTGGAAGGAACTTGCCGCAACCGGAAAACGGTACAGTGCAAGAGAGAGAAGGGGGCGGC  
61 GGTTTGTTGGCAAGATTGAAGCGGTGGATTGAAAACAACCTCTGAACAGTTGGATTGCCG  
121 TTTGACAGGTGAGAAGTATTTTGCCAGCAGTAAGATACTTCTTATATAATAAATAATAAT  
181 TTATTTAAACCGTCCTCTGAACGGGGCGATCAGGAGTTTTTGAATGGAATTTGTTTACGA  
M E F V Y D  
241 CGTGGCAGAATCGGCAGTCAGCCCTGCGGTAATTAAGTAATCGGCTTGGCGGGCGCGG  
V A E S A V S P A V I K V I G L G G G G  
301 TTGCAATGCAATCAATAACATGGTTGCCAACAATGTGCGCAGTGTGGAGTTTATCAGTGC  
C N A I N N M V A N N V R S V E F I S A  
361 CAATACAGATGCGCAGTCTTTGGCAAAAACCATGCGGCGAAGAGAATCCAGTTGGGTAC  
N T D A Q S L A K N H A A K R I Q L G T  
421 GAATCTGACACGCGGTTTGGCGCGGGTGC GAATCCGATATCGGCGGTGCGGCAGCACA  
N L T R G L G A G A N P D I G R A A A Q  
491 GGAAGATCGGGAGGCCATTGAAGAAGCCATTGCGGCGTCCCAATATGCTGTTTATCAGAC  
E D R E A I E E A I R G A N M L F I T T  
541 CGGTATGGCGGGCGGTACCGGTACCGGTTCGCGCGCGGTTGTTGCTGAGATTGCCAAGTC  
G M G G G T G T G S A P V V A E I A K S  
601 TTTGGCATTCTGACCGTTGCCGTAGTTACCCGTCGCTTTTCTTATGAAGGCAAACCGGT  
L G I L T V A V V T R P F S Y E G K R V  
661 CCATGTGGCAGCAGGCGGTTGGAACAGTTGAAAGAACACGTCGATTGCGTGTATTATCAT  
H V A Q A G L E Q L K E H V D S L I I I  
721 CCGGAACGACAAACTGATGACCGCATTGGGTGAAGACGTAACATGCGCGAAGCCTTCCG  
F N D K L M T A L G E D V T M R E A F R  
791 CCGCGCGGACAAATGATTGCGCGATGCGGTGCGAGGCATTTCGGAAGTGGTAACTTGCCC  
A A D N V L R D A V A G I S E V V T C P  
841 GAGCGAAATCATCAACCTCGACTTTGCCGACGTGAAAACCGTGATGAGCAACCGCGGTAT  
S E I I N L D F A D V K T V M S N R G I  
901 CGCTATGATGGGTTCCGGTTATGCCCAAGGTATCGACCGTCCGCGTATGGCGACCGACCA  
A M M G S G Y A Q G I D R A R M A T D Q  
961 GGCCATTTCCAGTCCGCTGCTGGACGATGTAACCTTGGATGGTGCACCGCGGTGTCTGGT  
A I S S P L L D D V T L D G A R G V L V  
1021 CAATATTACGACTGCTCCGGTTGCCGTGAAAATGTCGAGTTGTCTGAAGTCATGAAAAT  
N I T T A P G C L K M S E L S E V M K I  
1081 CGTCAACCAAAGCGCGCATCCCGATTTGGAATGCAAATTCGGTCCCGCTGAAGACGAGAC  
V N Q S A H P D L E C K F G A A E D E T  
1141 CATGAGCGAAGATGCCATCCGGATTACCATATCGCTACCGGTCTGAAAGAAAAGGAGC  
M S E D A I R I T I I A T G L K E K G A  
1201 GGTGATCCTACTCCGAAAGGGAAGTAGAAGCGGTTGCCCCGTCCAAACAGGAGCAAAG  
V D P T P E R E V E A V A P S K Q E Q S  
1261 CCACATTGTGGAAGGTATGATCCGCACCAATCGCGGTATCCGCACCATGAATCTGACAGC  
H I V E G M I R T N R G I R T M N L T A  
1321 AGCGGATTTGCGATAACCACTGCTGCTGACGATTTTGAAATTCCTGCAATTTTGGCTCG  
A D F D N Q S V L D D F E I P A I L R R  
1381 TCAACACAATTGAGACAAATAATGTGCCGTCTGCCGTAAACTTATTGCTCCCGAACCGG  
Q H N S D K End  
1441 TTTTCCGATTTGGGAGGTATATTTTCAATATGCCGAATCCTGTGCGGTTTGGGTTGTG  
1501 CCGGTTGAGATTTTACTTGAACCGACTGCTAGATATGGATATTTCAA

site (RBS) and a putative transcriptional terminator which does not have the gonococcal uptake sequence (Figure 8). Sequence analysis revealed no gonococcal uptake sequences in the entire region sequenced.

Comparison at the nucleotide level of the gonococcal *ftsZ* gene to that of *E. coli* using the program Nalign reveals 60% identity. The amino acid translation of the gonococcal *ftsZ* gene was compared to other FtsZ proteins reported in Genbank using the program Bestfit of the GCG software package. The gonococcal FtsZ shares 72%, 71%, and 64% identity with FtsZ of *E. coli*, *P. aeruginosa*, and *H. influenzae* respectively. The similarity with FtsZ of *B. subtilis* and *S. aureus* is 66% and 63% respectively. A multiple protein sequence alignment comparing FtsZ of *N. gonorrhoeae* to FtsZ of 8 other selected organisms was done using the program Clustal V with manual adjustments (Figure 9). Overall it is conserved in size with the other FtsZ proteins in the alignment. The amino two thirds of the protein is very well conserved having the highly conserved GTP-binding signature sequence GGGTGTG. Some other highly conserved domains were also identified (indicated by \* beneath the amino acids) and these were also indicated by the published alignment of McCormick *et al.* (1994). The carboxyl end of the gonococcal FtsZ does not share significant similarity with any of the other FtsZ in this region, and this lack of conserved sequence is observed between all the other FtsZ proteins.

### 3.4 Amplification and Cloning of the *ftsZ* Gene

In order to obtain the *ftsZ* gene on a single fragment of DNA, the primers ar27 and ar28 were designed to amplify the entire *ftsZ* gene (Figure 5C) from gonococcal

FIGURE 9. Alignment of *N. gonorrhoeae* CH811 FtsZ with other reported FtsZ. Organisms in alignment include Ngo, *N. gonorrhoeae*; Eco, *E. coli*; Hin, *H. influenzae*; Bsu, *B. subtilis*; Sau, *S. aureus*; Mpu, *M. pulmonis*; Sco, *S. colicolor*; and Hsa, *H. salinarium*. Identical residues are indicated by a star (\*) and conservative substitutions are indicated by a period (.). The gonococcal FtsZ is in red, and the conserved GTP binding signature sequence is boxed.

Ngc MEFVYDVAESAV--SPAVIKVIGLGGGGCNAINNMVANNVRS-----  
Eco MFPEMELTND-----AVIKVIGVGGGGGNAVEHVMVRERIEG-----  
Hin MLYPEYPEYDNFNESGALIKVVGVGGGGGNAVNHMVMVMVKQEMGGTFVG  
Pae MFELVDNIAQT-----AVIKVIGVGGGGGNAVNHMMAKNNVEG-----  
Bsu MLEFETNIDGL-----ASIKVIGVGGGGGNAVNRMIENEVQG-----  
Sau MLEFEQGFNHL-----ATLKVIGVGGGGGNAVNRMIDHGMNN-----  
Mpu MSDLEN-FIPT-----ANIKVIGVGGGGGNSVETMIQAGIQG-----  
Sco M---AAPQNYL-----AVIKVIGVGGGGVNAINRMIEVGLKG-----  
Hsa M-----DGDGDGDEFDPRIVIVGCGGAGNNTVNRLYNIGVEGA-----  
\* . . . \* \* \* \* \* . . .

-----VEFISANTDAQSLAKNHAAKRIQLGTNLTRGLGAGANPD  
-----VEFFAVNTDAQALRKTAVGQTIQIGSGITKGLGAGANPE  
ESSLTSEEHGRIVFYAVNTDAQALRKSQVQQTQVQIGGETTKGLGAGANPN  
-----VEFICANTDAQALKNIAARTVLQLGPGVTKGLGAGANPE  
-----VEYIAVNTDAQALNLSKAEVKMQIGAKLTRGLGAGANPE  
-----VEFIAINTDGOALNLSKAESKIQIGEKLTRGLGAGANPE  
-----VEFIVANTDIQALQRSSAPNFIHLGEN-KRGLGAGANPE  
-----VEFIAINTDAQALLMSDADVCLDVGRELTRGLGAGANPA  
-----DTVAINTDKQHLKMIKADTKILVGLSLTNGLGAGGDPS  
\* \* \* \* \* . . . \* \* \* \* \* . . .

IGRAAAQEDREAIEEAIRGANMLFITTGMGGGTGTGSAAPVVAEIAKSLGI  
VGRNAADEDRLRALRAALEGADMVFIAGMGGGTGTGAAPVVAEVAKDLGI  
IGRKAEDDQDEIRKMLEGADMVFIAGMGGGTGTGAAPVVAEIAKELGI  
VGRQALEDREIRISEVLEGADMVFITTGMGGGTGTGAAPVIAQIAKDLGA  
VGKKAEEESKEQIEEALKGADMVFVTAGMGGGTGTGAAPVIAQIAKEMGA  
IGKKAEEESREQIEDAIQGADMVFVTSGMGGGTGTGAAPVVAEIAKEMGA  
VGKKAEEESIVEIKEKLGADMVITSGMGGGTGTGASPIIAKIAARELGA  
VGRKAEDHREEIEEVLKGADMVFVTAGMGGGTGTGAPVVANIARSLGA  
MGERATEMAQGTIKEVLGDADLVFVTAGMGGGTGTGAAPVVSIAKEQGA  
\* \* \* \* \* . . . \* \* \* \* \* . . .

LTVAVVTRPFSYEKG-RVHVAQAGLEQLKEHVDSLIIIPNDKLM TALGED  
LTVAVVTKPFNFEGKKRMAFAEQGITELSKHVNSLITIPNDKLLKVLGRG  
LTVAVVTKPFTFEGKKRMQFAELGIKDLQYVDSMIIIPNQIQKVL PKN  
LTVAVVTRPFPFEGRKRQIADEGIRALAESVDSLITIPNEKLLTILGKD  
LTVGVVTRPFTFEGRKRQLQAAGGISAMKEAVDTLIVIPNDRILEIVDKS  
LTVGVVTRPFSFEGRKRQTQAAGVEAMKAAVDTLIVIPNDRLLDIVDKS  
LTIISIVTTPFTEFENLRNKNAQEGIKNLRVAVSDSIIITISNNKLEQYG-D  
LTIGVVTRPFTFEGRRRANQAEDGIAELREEVDTLIVIPNDRLLSISDRQ  
IVVGMVSTPFNVE-RARTVKAEEGLEKLEKREKADSIIVLDNRRLLDYVP-N  
. . . \* \* \* \* \* . . . \* \* \* \* \* . . .

VTMREAFRAADNVL RDAVAGISEVVTCPSEIINLDFADVKTVM SNRGIAM  
ISLLDAFGAANDVLKGA VQGI AELITRPG-LMNVD FADV RTVM SEMGHAM  
AKLIDAF A AANDVLRNSVMGISDMITSPG-LINVD FADV RTVM SVQGOAM  
ASLLAFAKADVDLAGAVRGISDI IKRPG-MINVD FADV KTV-SEMG MAM  
TPMLEAFREADNVL RQGVQGISDLIATPG-LINLDFADVKT IMSNKGSAL  
TPMMEAFKEADNVL RQGVQGISDLIAVSG-EVNLDFADVKT IMSNQGSAL  
APMKDSFLFADTILKHTVKTITDIIAIPA-HINLDFADVKTVMKDKGDAL  
VSVLDAFKSADQVLLSGVQGITDLITTPG-LINLDFADVKSVMSEAGSAL  
LPIGKA FSVMDQIIAETVKG ISETITQPS-LINLDYADMTA IMNQGGVAV  
. . . \* \* \* \* \* . . . \* \* \* \* \* . . .

Ngc MGSGYAQGI---DRARMATDQAISSPLDDVTLDGARGVLVNIITAPGCL  
Eco MGSGVASGE---DRAEEAAEMAISSPLLEDIDLSGARGVLVNIITAGFD-L  
Hin IGFGSAVGEPGAGRAEEAARLAVRNDLLEKIDLSNAQGILVNIITAGMD-L  
Pae MGTGCASGP---NRAREATEAAIRNPLLEDVNLQGARGILVNIITAGPD-L  
Bsu MGIGIATGE---NRAAEAAKKAISSPLLEA-AIDGAQGVLMNITGGTN-L  
Sau MGIGVSSGE---NRAVEAAKKAISSPLLET-SIVGAQGVLMNITGGES-L  
Mpu IGIGRASGK---DRAVKAAIHAISSPIIET-SIQGASHTIINITGSAN-L  
Sco MGIGSARGD---DRAVAAAEMAISSPLLEA-SIDGARGVLLSISGGSD-L  
Hsa MLVGETQDK---NKTNEVVKDAMNHPLLD-VDYRGASGGLVHITGGPD-L  
. \* . . . \* . . . \*

KMSELSEVMKIVNQSAHPDLECKFGAAEDETMSEDAIRITIIATGLKEKG  
RLDEFETVGNTIRAFASDNATVVIGTSLDPDMNDE-LRVTVVATGIGMDK  
VFEEFNIIGETIGSFASEEATVVVGTSLVPEMSDE-IRVTIVATGLGEIA  
SLGEYSDVGNIEQLASEHATVKVGTVIDADMRDE-LHVTVVATGLGARL  
SLYEYVQAAA-IVASASDQDVNMIFGSVINENLKDE-IVVTVIATGFIEQE  
SLFEAQEAADIVQDAADEDVNMIFGTVINPELQDE-IVVTVIATGFDDKP  
TLTEVHSAVNVIKNAVGPENNTIFGATINESIGDE-IYVSVIATGLSSSK  
GLFEINEAAQLVSEAAHPEANIIFGAVIDDALGDE-VRVTVIAAGFDGGQ  
TLKEAEGIADNITERLDASANVIWGARIQESYKKG-VRVMAIMTGVSQAQ  
\* . . . \*

AVDPTPEREVEAVAPSKQEQSHIVEGMIRTNRGIRTMNLTAADFNDQSVL  
RPEITLVTNKQVQPPVMDRYQQHGMAPLTQEQQPKVAKVVNDNAPQTAKEP  
GNEPIQVVRQGLSTQNIIEGEGRVNIVPELHRRESVEVSRTASEEYQRPLD  
EKPVKVVNDNTVQGSAAQAAAPAQREQQSVNYRDLDRPTVKPITDRLEAFK  
KDVTKQRPVSLNQS IKTHNQSVPKRDAKREEPQQQNTVSRHTSQPADDTL  
TSHGRKSGSTFGTSVNTSSNATSKDESFTSNSSNAQATDSVSERTHTTKE  
KFENSEQEI KDEVSSMLKTMEIDLQASETKTILINDPLPKDEKMLVLSLLD  
PPSKRDNLVGLSSSAKREPTPARPSES RPSFGSLGSKPKKEEPEPAPVPE  
VLGPSTQKQADKSRRELQDVDSKQRAADDAGAGGFGGAHSDGGQDEVEQE

DDFEI PAFLRRQHNSK-----  
DYLDI PAFLRKQAD-----  
NNFFNPAQREEN-----  
KMRNQSHGSAATAAKLNPQDDL DYLDI PAFLRRQAD  
-DIPTFLRNRNKR-----  
DDIPSFIRNREERRSRTR-----  
RDSKILEKDDSQDDTL PFFLKRNV-----  
PVADLPVSPPPVPSRTYSDSAAEELDVPDFLK---  
NGLDVIR-----

chromosomal DNA. A 1.5 kb amplicon of *ftsZ* containing 200 bp upstream of the start was obtained and was cloned into the pTAG vector. Ligations were performed and *E. coli* LigATor cells were transformed. Very few transformants were obtained, and screening of the few clones revealed plasmids with no complete inserts (data not shown). Another primer ar29 was designed to amplify the gene starting just before the ATG start codon (Figure 5C), and was used with ar28 to obtain an 1.3 kb amplicon of *ftsZ* without the upstream region (Figure 10, lane 1). When the 1.3 kb amplicon was used for cloning into the pTAG vector, many clones were obtained. The clones were confirmed by restriction digests with *Bam*HI and *Hind*III which released the 1.3 kb inserts and 3.8 kb vector (Figure 10, lane 2, pAR2 digested; lane 3, pAR3 digested). Since cloning into pTAG is not directional, the orientation of the inserts with respect to the *lac* promoter was determined using PCR. The T7 primer on the vector was used with primer ar28 or ar29 on the insert, and the presence or absence of an amplicon with each primer pair was determined (Figure 11). For pAR2, no PCR product is obtained with T7 and ar28 (Figure 11, lane 1), but with T7 and ar29 an amplicon is obtained (Figure 11, lane 3). The opposite occurred for pAR3, with an amplicon generated with primers T7 and ar28 (Figure 11, lane 2), but no amplicon for T7 and ar29 (Figure 11, lane 4). It was therefore determined that pAR2 contains the *ftsZ* gene downstream and in the same orientation as the *lac* promoter on the vector, and pAR3 contains the *ftsZ* gene downstream and in the opposite orientation of the *lac* promoter (Figure 12).

### 3.5 Western Blot Analysis

An anti-*E. coli* FtsZ antiserum obtained from Dr. Lutkenhaus (Kansas Medical

FIGURE 10. The restriction endonuclease digestion of pAR2 and pAR3 with *Bam*HI and *Hind*III to confirm presence of 1.3 kb *ftsZ* insert. Lane 1, 1.3 kb amplicon; lane 2, pAR2 digested; lane 3, pAR3 digested; M, 1 kb DNA ladder (Gibco BRL).

M 1 2 3

bp

4072 —  
3054 —  
2036 —  
1636 —  
1018 —  
500 —

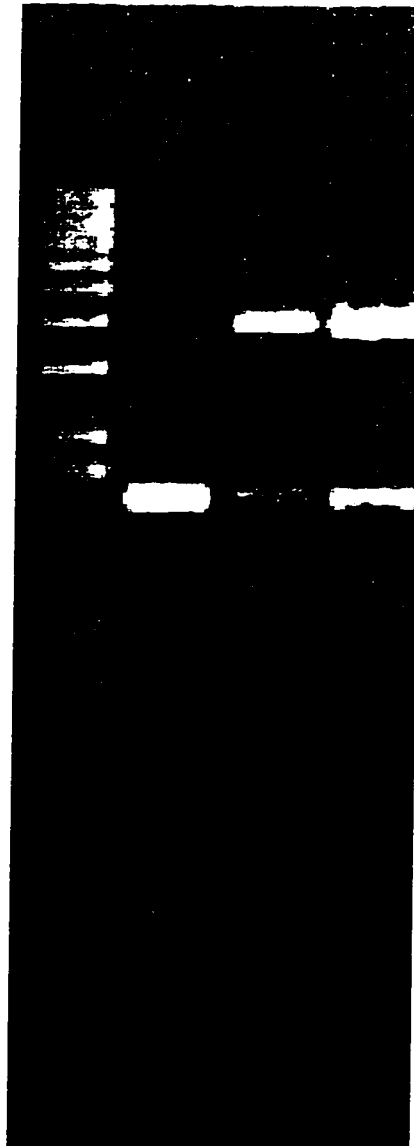
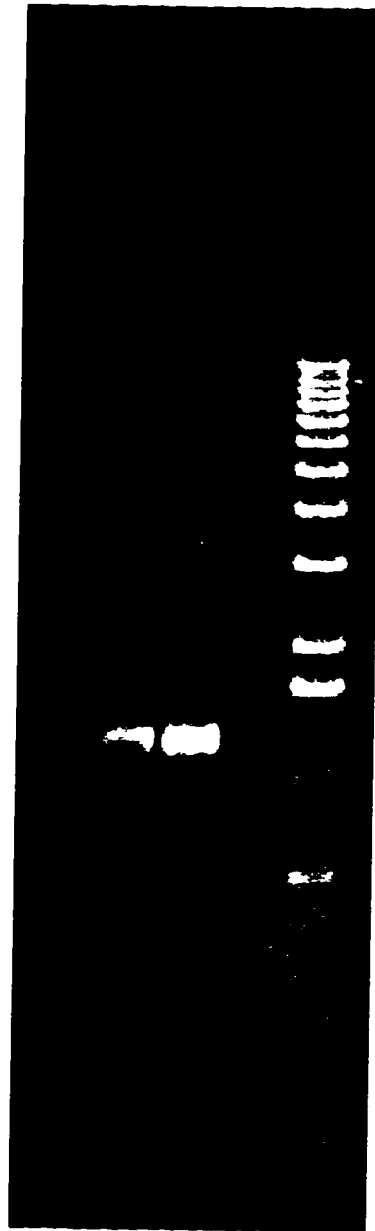


FIGURE 11. Confirmation of the orientation of the *ftsZ* gene cloned in pAR2 and pAR3. The T7 primer on the vector was used with ar28 (lanes 1 and 2) and ar 29 (lanes 3 and 4) for PCR on pAR2 (lanes 1 and 3) and pAR3 (lanes 2 and 4) to establish orientation of the inserts.

1 2 3 4 M



bp

— 3054

— 2036

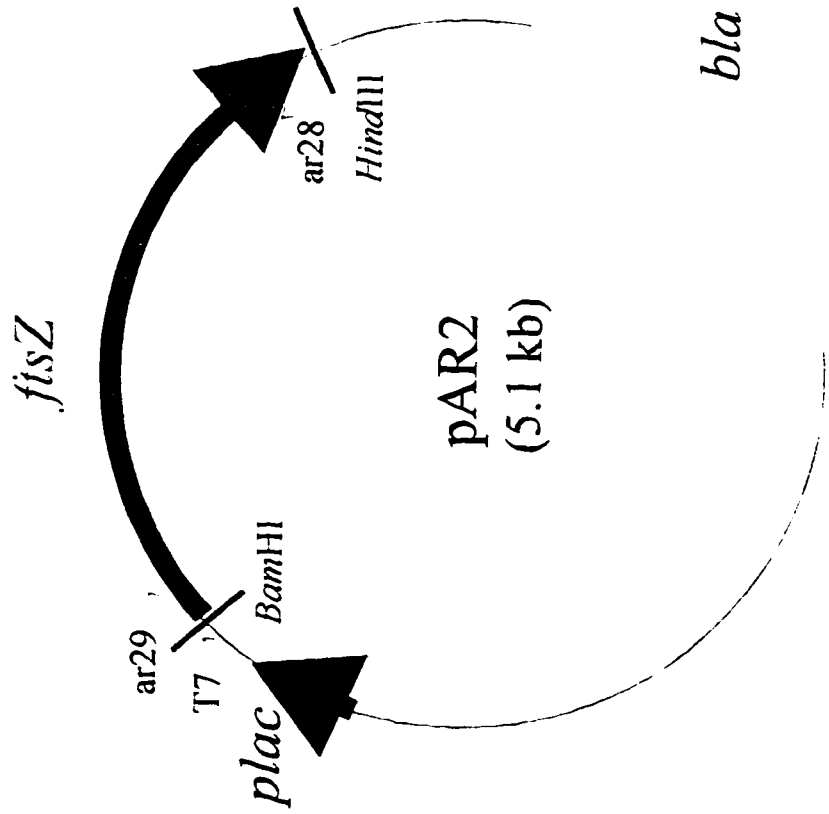
— 1636

— 1018

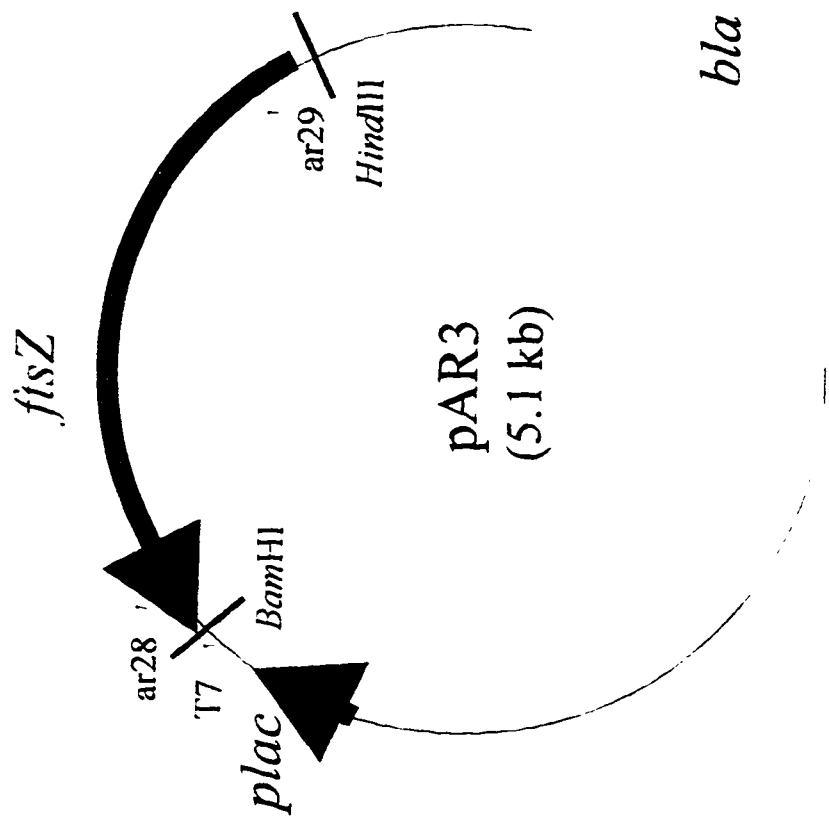
— 506

FIGURE 12. The plasmids pAR2 and pAR3 containing the gonococcal *ftsZ* gene cloned in pTAg. Figure 12A. pAR2 contains *ftsZ* downstream and in the same orientation as the *lac* promoter. Figure 12B. pAR3 contains *ftsZ* downstream and in the opposite orientation as the *lac* promoter. The *ftsZ* gene is shown in red, the *lac* promoter in purple, and the  $\beta$ -lactamase gene in grey. The location of the primers ar28, ar29, and T7 are indicated by black arrows and are not to scale. Relevant restrictions sites are also indicated.

A



B



Center) was used to detect FtsZ in cell extracts of various *Neisseria* species. Cell extracts of *E. coli*, *N. gonorrhoeae* strains CH811 and MS11, *N. cinerea*, and *N. lactamica* were prepared as described in the methods, and proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane for immunodetection. The antibody detected the 40 kDa *E. coli* FtsZ (Figure 13, lane 5) and a major immunoreactive protein migrating at ~43 kDa was detected in the Neisserial extracts (Figure 13, lanes 1 to 4). Since the total protein was standardized, the intensity differences of the immunoreactive bands may be due to differences in the amount of FtsZ present in the cells at the time of harvesting. The immunoreactive band is migrating somewhat slower than expected based on the predicted size of 41.5 kDa. The Neisserial homologue of FtsZ is conserved in size between the two gonococcal strains (CH811, lane 4; MS11, lane 3) and commensal species (*N. cinerea*, lane 1; *N. lactamica*, lane 2-band visible on original immunoblot) and is larger than the *E. coli* FtsZ as is predicted from the translation of the sequence.

### **3.6 *In vitro* Transcription/translation of *ftsZ***

The 1.5 kb amplicon of *ftsZ* containing the upstream region was provided as a linear DNA template for *in vitro* transcription/translation using an *E. coli* S30 Extract System (Promega). A newly synthesized radioactively labelled protein was observed (Figure 14, lane 1). The protein band was migrating at about 43 kDa when compared to molecular weight standards (Figure 14, lane M). This protein corresponds in size to the immunoreactive protein detected in the Western blot analysis (Figure 13).

FIGURE 13. Identification of the Neisserial FtsZ in cell extracts. The bacterial cell extracts were analysed by Western blotting using an anti-*E. coli* FtsZ antiserum. Lane 1, *N. cinerea*; lane 2, *N. lactamica*; lane 3, *N. gonorrhoeae* MS11; lane 4, *N. gonorrhoeae* CH811; lane 5, *E. coli* HB101. Molecular weight marker (BIORAD) positions are indicated on the right.

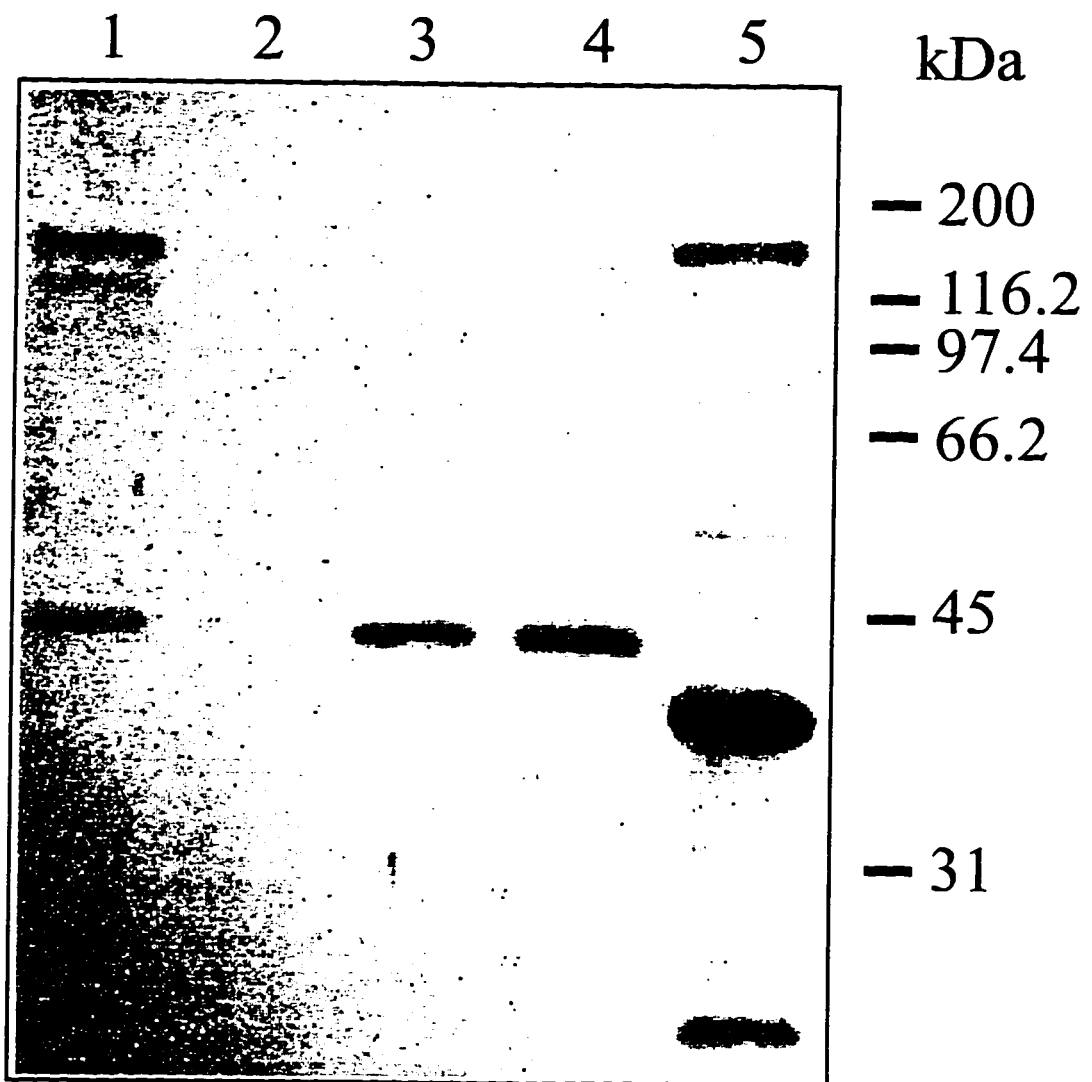


FIGURE 14. *In vitro* transcription/translation of the gonococcal *ftsZ*. Lane 1, 43 kDa transcription/translation radiolabelled product from the 1.5 kb *ftsZ* amplicon as linear DNA template; M, molecular weight size markers (BIORAD).

1

M

kDa



- 200  
- 116.2  
- 97.5  
- 66.2  
- 45  
- 31

### **3.7 Expression of the Gonococcal *ftsZ* in *E. coli***

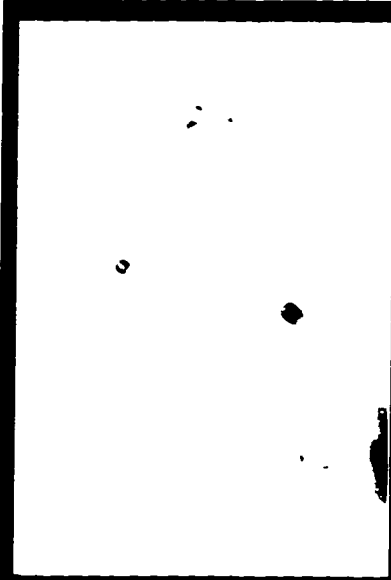
The morphological effect of expressing the gonococcal FtsZ in *E. coli* was followed by microscopy. The plasmid constructs used included pTAg as a vector control, pAR2 containing the *ftsZ* gene cloned downstream and under control of the *lac* promoter (Figure 12A), and pAR3 containing *ftsZ* in the opposite orientation (Figure 12B). Cultures of *E. coli* LigATor containing pTAg, pAR2, and pAR3 respectively were grown to mid- log phase, the cultures were then split in two, and 1mM IPTG was added. The effects of induction of the *lac* promoter expression of the gonococcal *ftsZ* on the *E. coli* cells was observed by glutaraldehyde fixation of cells. All cells were observed under the same magnification of 1000X. Uninduced *E. coli*(pTAg), *E. coli*(pAR2), and *E. coli*(pAR3) had a normal rod-like morphology (Figure 15A, 15C, and 15E) after six hours of growth. Two hours after the addition of IPTG to the other half of the cultures, the induced *E. coli*(pAR2) cells were found to be very elongated (Figure 15D) compared to the induced *E. coli*(pTAg) and *E. coli*(pAR3) (Figure 15B and 15F). Cells were observed 4h after induction, and the cells containing pAR2 with the gonococcal *ftsZ* were extremely filamentous, forming large masses of elongated cells (not shown). The observed filaments appeared to be smooth in morphology, with no signs of constrictions along the length of the cells.

### **3.8 Construction of a *N. gonorrhoeae ftsZ* Knock-out Mutant**

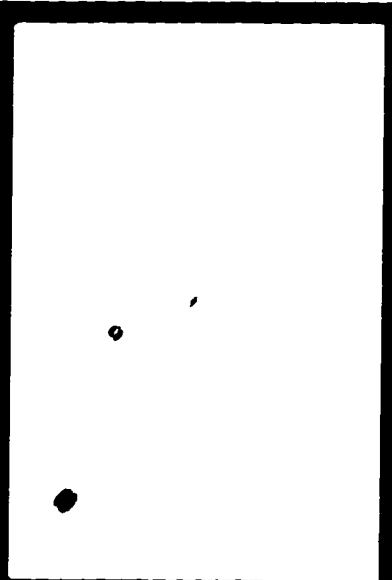
The plasmid construct to deliver the insertionally inactivated *ftsZ* gene to the gonococcal chromosome was constructed as described in the Methods section. First, pAR1 was constructed containing a partial *ftsZ* gene amplicon with gonococcal uptake sequences

FIGURE 15. Expression of *N. gonorrhoeae ftsZ* in *E. coli*. *E. coli* (pTAg) uninduced (A) and 2h after induction (B), *E. coli*(pAR2) uninduced (C) and 2h after induction (D), and *E. coli*(pAR3) uninduced (E) and 2h after induction (F). Cells were fixed in gluteraldehyde and observed using the Leitz Laborlux K light microscope adapted with a Hoffman modulation contrast system, all at a magnification of 1000X.

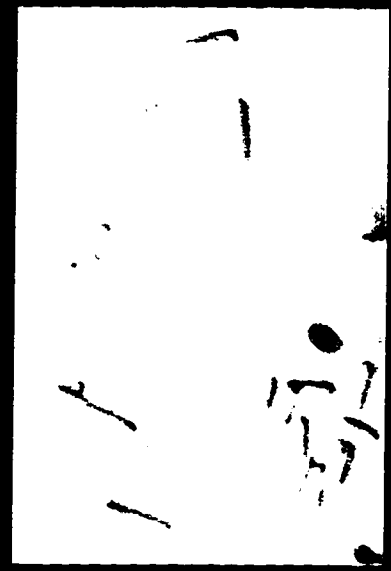
**A**



**B**



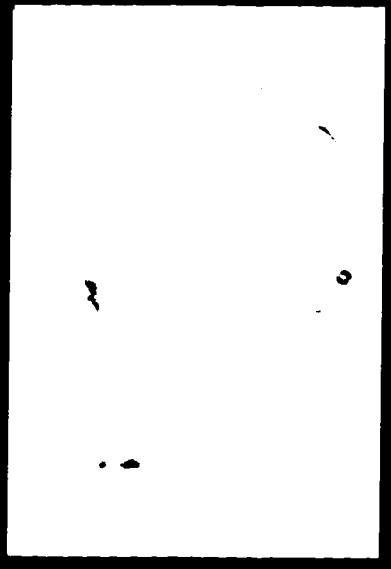
**C**



**D**



**E**



**F**

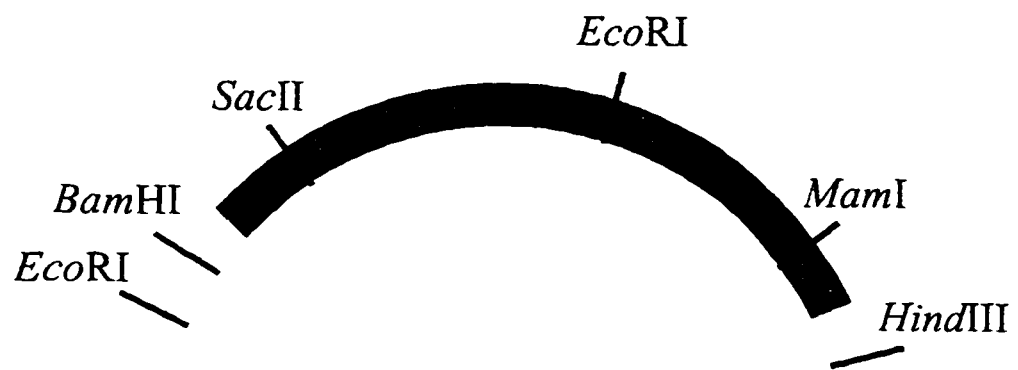


added. The chloramphenicol acetyltransferase (*cat*) gene was successfully amplified from pACYC184, and used for the insertion into pAR1. pAR4 (Figure 16) contains the cloned partial *ftsZ* gene with a deletion of 179 bp and the insertion of the 1.4 kb *cat* cassette. The deletion and insertion is from bp 493 to bp 672 of the *ftsZ* coding sequence, and removes 60 aa of FtsZ which lies downstream of the GTP binding domain. The uptake sequences on pAR4 should allow its transformation into *N. gonorrhoeae*, and the *ftsZ* with the *cat* insertion should recombine with the chromosomal *ftsZ* to create a knock-out mutant. Transformations of *N. gonorrhoeae* with this plasmid construct were tried, but no transformants were obtained upon selection with chloramphenicol. The transformation control showed that the *N. gonorrhoeae* cells used were competent because the streptomycin resistance was acquired when the chromosomal DNA of a streptomycin resistant strain was applied. The inability to obtain a transformant with an insertion in the *ftsZ* gene indicates that an insertion into the gene is lethal and that the *ftsZ* gene is essential in *N. gonorrhoeae*.

### 3.9 Identification of *ftsZ* in *Chlamydia trachomatis*

For interest, preliminary investigation of the *ftsZ* gene was initiated in *C. trachomatis*, another STD pathogen with a different cell wall structure. The degenerate primers ar1 and ar2 (shown in Table 2) for amplification of a part of *ftsZ* were used to amplify a homologue of *ftsZ* from *C. trachomatis*. The PCR resulted in an approximately 220 bp amplicon which is of expected size based on the primer positions on the *E. coli* sequence. The amplicon was sequenced (Figure 17A) and a BlastN sequence similarity search revealed similarity to *ftsZ*. The translation of this partial sequence was aligned with FtsZ of *N. gonorrhoeae* and

FIGURE 16. The plasmid pAR4 containing a partial gonococcal *ftsZ* gene with a *cat* insertion cloned in pTAg. The partial *ftsZ* gene is shown in red, with the gonococcal uptake sequence on the primers shown in yellow, the *cat* gene insertion is in green, the *lac* promoter in green, and the ampicillin resistance gene in grey. Relevant restriction sites are also indicated.



pAR4  
(5.9 kb)

FIGURE 17. Nucleotide and amino acid sequence of the partial *C. trachomatis ftsZ*. Figure 17A. The nucleotide sequence of the 220 bp amplicon obtained using degenerate *ftsZ* primers ar1 and ar2, with the amino acid translation indicated below. Figure 17B. Manual alignment of the partial chlamydial *ftsZ* translation protein (Ctr) with the FtsZ of *N. gonorrhoeae* (Ngo) and *E. coli* (Eco). Identical residues between the FtsZ of *C. tracomatis* and either *N. gonorrhoeae* or *E. coli* FtsZ are highlighted in yellow.

**A**

1 GTACTTAAAGTATCCAAAGCAGATGAGCGTATTCAATTAGGTAAGAAGTTGACTCGAGGA  
V L K V S K A D E R I Q L G K K L T R G

61 TTAGGTGCAGGCGCTAAACCAGCTATTGGTAAGCAAGCAGCACTCGAATCTGAGGATGAT  
L G A G A K P A I G K Q A A L E S R D D

121 ATTAGAGAAGTACTATCTGATGCAGATATGGTTTTTATTACTGCTGGTATGGGTGGTGGT  
I R E V L S D A D M V F I T A G M G G G

191 ACCGGCACA  
T G T

**B**

Ngo MEFV----YDVAESAVSPAVIKVIGLGGGGCNAINNMVANNVRS-----  
Eco M-----FEPME-LTNDAVIKVIGVGGGGGNAVEHMRERIEG-----

Ngo -----VEFISANTDAQSLAKNHAAKRIQLGTNLTRGLGAGAN  
Eco -----VEFFAVNTDAQALRKTAVGQTIQIGSGITKGLGAGAN  
Ctr **VLKVS KADERIQLGKKLTRGLGAGAK**

Ngo **PDIGRAAAQEDREAIEEAIRGANMLFITTMGGGGTGTGSAPVVAEIAKSL**  
Eco **PEVGRNAAEDRDALRAALEGADMVFLAAGMGGGTGTGAAPVVAEVAKDL**  
Ctr **PAIGKQALESRDDIREVLSDADMVFI TAGMGGGTGT**

Ngo GILTVAVVTRPFSYEGK-RVHVAQAGLEQLKEHVDSLIIIPNDKLM TALG  
Eco GILTVAVVTKPFNFEGKKRMAFAEQGITELSKHVNSLITIPNDKLLKVLG

Ngo EDVTMREAFRAADNVLRDAVAGISEVVTCPSEIINLDFADVKTVM SNRGI  
Eco RGISLLDAFGAANDVLKGA VQGI AELITRPG-LMNVD FADV RTVM SEMGH

Ngo AMMGSGYA---QGIDRARMATDQAISSPLLDVTLDGARGVLVNIITAPG  
Eco AMMGSGVASGE---DRAEEAAEMAISSPLLEDIDL SGARGVLVNI TAGFD

Ngo CLKMSELSEVMKIVNQS AHPDLECKFGAAEDETMS EDAIRITIIATGLKE  
Eco -LRLDEFETVGNTIRAFASDNATVVIGTSLDPDMNDE-LRVTVVATGIG-

Ngo KGAVDPTPER-----EVEAVAPSKQE QSHI-VEGMIRTNRGIRTMNL  
Eco MDRRPEITLV-----TNKQVQQ-----PVM DRYQQHGMAPL

Ngo TAAFDNQSVL-----DDFEIPAILRRQHNSDK  
Eco TQE--QKPVAKVVNDNAPQTA---KEPDYLDIPAF LRKQAD---

*E. coli* (Figure 17B). The chlamydial FtsZ is 59% and 56% identical to FtsZ of *N. gonorrhoeae* and *E. coli* in the region highlighted (Figure 17B). The GTP-binding sequence is also present at the end of the determined sequence and it is conserved. This partial sequence confirms the presence of a *ftsZ* homolog in *C. trachomatis*, and will allow for the amplification of the remainder of the gene using an inverse PCR strategy.

## 4. DISCUSSION

Our hypothesis that a homolog of the *ftsZ* gene exists in *N. gonorrhoeae* was confirmed using a combination of PCR strategies and sequencing. Having obtained the entire nucleotide sequence of the gene, we began initial characterization of the gonococcal *ftsZ* by analysis of the amino acid translation. The gonococcal FtsZ is a protein sharing conserved regions of amino acids with the other FtsZ as indicated by Blast search of the protein database. One highly conserved region that was found is the glycine rich amino acid sequence GGGTGTG which has been identified as the GTP binding motif due to homology to the eukaryotic tubulin signature sequence (RayChoudhuri and Park, 1992). The conservation of the GTP binding motif strongly suggests that the gonococcal protein might also be a GTP binding protein with GTPase activity as has been shown for *E. coli* and *B. subtilis* FtsZ (deBoer *et al.*, 1992; Wang and Lutkenhaus, 1993). Since this GTPase activity is essential for the protein's function in septum initiation and formation, in our laboratory we plan to show this biochemical function of the gonococcal FtsZ once the protein has been purified.

Alignment of the gonococcal FtsZ with other selected FtsZ proteins (Figure 9) revealed that the gonococcal FtsZ shares greatest homology in the amino terminal two thirds of the protein, which includes the GTP binding motif region. The carboxy segment of all the FtsZ proteins are not very conserved, being variable in amino acid composition and in size. Other published alignments of FtsZ proteins also found this variable carboxy terminus (McCormick *et al.*, 1994; Margolin *et al.*, 1996). Work on *E. coli* FtsZ has shown that the carboxy segment is not required for GTPase activity *in vitro*, but is essential for complementation of an *ftsZ* mutant *in vivo* (Wang and Lutkenhaus, 1996a). The function of

this region remains to be determined, but recently work with FtsZ-GFP fusion proteins with C-terminal truncations indicate that this region may play a role in the stability of polymerized FtsZ tubules (Ma *et al.*, 1996).

The relatively high homology (72% identity) between FtsZ of *N. gonorrhoeae* and that of *E. coli* allowed for the detection of FtsZ in gonococcal lysates using anti-*E. coli* FtsZ antiserum. We have shown that the anti-*E. coli* FtsZ antiserum detected an immunoreactive protein in *N. gonorrhoeae* cell lysates which was larger than the 40 kDa *E. coli* FtsZ. The immunoreactive protein in gonococcal lysates migrated at a size of 43 kDa, which corresponded to the protein seen in the *in vitro* transcription/translation of the gonococcal *ftsZ* gene amplicon, but is migrating larger than the 41.5 kDa predicted by sequence. This antisera has been used previously to detect the presence of FtsZ homologs in various eubacteria (Corton *et al.*, 1987), and they found that in the *Enterobacteriaceae*, the immunoreactive FtsZ protein was conserved in size. Our analysis of various *Neisseria* species indicates a FtsZ homolog conserved in size in *N. gonorrhoeae* strains CH811 and MS11 and the two commensals *N. lactamica* and *N. cinerea*. Recently in our laboratory, *N. gonorrhoeae* CH811 *ftsZ* sequence specific primers were used to amplify the *ftsZ* gene from various *Neisseria* species. An amplicon conserved in size (and of expected size) was obtained from the chromosomal DNA of the various gonococcal strains, *N. meningitidis* strains, and some commensal species including *N. lactamica* and *N. cinerea* (Russo and Radia, unpublished data).

The detection of an immunoreactive band corresponding to FtsZ indicates that the *ftsZ* gene is expressed in *N. gonorrhoeae*. Some of the results obtained provide presumptive

evidence for a promoter upstream of *ftsZ* which would likely have a role in gene expression. The *in vitro* transcription/translation results, in which only the *ftsZ* amplicon containing 200 bp of upstream region was provided as a template, supports the presence of a promoter just upstream of the gene. A translation product was obtained, indicating that a native promoter for transcription of *ftsZ* must be present on the amplicon and that it was recognized by the *E. coli* transcription machinery.

Our attempts to clone the gonococcal *ftsZ* containing 200 bp of upstream region in an *E. coli* host were unsuccessful. However, an amplicon without this region was easily cloned downstream of a repressed *lac* promoter. Our results suggest that a gonococcal promoter which is active in *E. coli* is present in the region upstream of *ftsZ*, and no transformants were obtained because the expression of the gene was lethal to *E. coli*. Similarly, when cloning of the *ftsZ* of *B. subtilis* with an upstream region which they presumed contained promoters was attempted, no transformants were obtained (Beall *et al.*, 1988). Upon removal of the upstream region allowed cloning of the gene in *E. coli* host, which suggested that overproduction of the *B. subtilis* FtsZ by the promoter(s) was lethal to *E. coli* (Beall *et al.*, 1988). In *C. crescentus*, a native *ftsZ* promoter was identified in the 200 bp region upstream of the gene (Quarokos *et al.*, 1996), and in *H. salinarium* a putative promoter has been identified just upstream of the *ftsZ* gene based on the presence of a consensus halobacterial promoter box A sequence (Margolin *et al.*, 1996).

The confirmation of the putative gonococcal *ftsZ* promoter will require promoter mapping by primer extension, and this is currently underway in our laboratory. The promoter mapping and identification of *ftsZ* transcripts by Northern hybridization will aid in determining

if the expression of *ftsZ* is controlled by many promoters as is seen in *E. coli*. The identification of promoters of *ftsZ* in the gonococcus is of interest because the study of the regulation of *ftsZ* is required to fully understand the role of FtsZ in the control of cell division.

Our difficulties in cloning the gonococcal *ftsZ* gene with its putative native promoter in *E. coli* indicated that expression of the gene product was lethal to *E. coli*. To study this further, we were able to clone the gonococcal *ftsZ* gene under the control of a vector borne *lac* promoter. Upon induction of the *E. coli* host carrying this plasmid, the level of expression of the gonococcal *ftsZ* gene was such that it led to *E. coli* cell filamentation. The smooth filaments that were observed indicate that the gonococcal FtsZ is interrupting the *E. coli* cell division at an early stage, maybe in the formation of the FtsZ ring. The gonococcal FtsZ may be aggregating at the division site, interacting with the *E. coli* FtsZ, and disrupting its interaction with the other proteins involved in septation. A similar phenotype was observed when the *ftsZ* genes from *B. subtilis* (Beall et al., 1988), *R. meliloti* (Beall and Lutkenhaus, 1991), and *M. pulmonis* (Wang and Lutkenhaus, 1996) were expressed in *E. coli*, and the filamentation could be overcome by increasing the levels of the *E. coli* FtsZ by using a plasmid copy of the gene. Recent studies using green fluorescent protein tagged FtsZ of *R. meliloti* found that it could polymerize in the *E. coli* host, and that it copolymerized with the *E. coli* FtsZ forming non-functional tubules which leads to cell filamentation (Ma et al., 1996).

We have begun to investigate the biological function of FtsZ in *N. gonorrhoeae*. Using a cloned copy of a partial *ftsZ* gene, we have deleted 179 bp in the middle of the gene coding sequence and have inserted a *cat* cassette. The insertion is downstream of the GTP binding

region coding sequence. This construct was used to try and knock-out the *ftsZ* gene on the chromosome by transformation and recombination. The inability to obtain transformants suggests that *ftsZ* may be essential for cell viability. Insertions in other regions of *ftsZ* will be constructed to determine if any viable mutants can be obtained. Other organisms in which *ftsZ* was shown to be essential for cell division and viability include *E. coli*, *R. meliloti*, *B. subtilis*, *Anabena* sp., and *C. crescentus* (Dai and Lutkenhaus, 1991; Beall and Lutkenhaus, 1991; Margolin and Long, 1994; Zhong *et al.*, 1995; Quardokus *et al.*, 1996). To positively show that the gonococcal *ftsZ* gene is essential, the construction of a mutation on the chromosomal copy of the gene in the presence of a wild type copy in trans is required. It is also of interest to control the level of expression of *ftsZ* in *N. gonorrhoeae* to determine the effects of various levels of FtsZ on cell division and morphology. A *B. subtilis* strain was constructed in which the chromosomal copy of *ftsZ* was under the control of an inducible *spac* promoter, and this strain was used to show the role of FtsZ in vegetative growth and sporulation (Beall and Lutkenhaus, 1991).

In order to extensively characterize the gonococcal FtsZ, purification of the protein is required. Obtaining purified FtsZ would allow for testing the GTP binding and GTPase activity of the protein. In addition, the generation of polyclonal antibodies would be possible and these would serve as important tools in the immunolocalization of FtsZ in *N. gonorrhoeae*. Another area of future work is to determine if epitopes mapped on *E. coli* FtsZ are present and conserved on the gonococcal FtsZ using monoclonal antibodies (Voskuil *et al.*, 1994). Of long term interest is the protein modelling of FtsZ, which could lead to development of drugs that could inhibit its function in the process of cell division.

The identification of the *ftsZ* gene has resulted in the identification of numerous other cell division gene homologs by our laboratory (Victor and Francis, unpublished data) using the newly released raw DNA sequence data made available through the Gonococcal Genome Sequencing Project (Dr. B.A. Roe, Dr. S. Clifton and Dr. W.Dyer, University of Oklahoma, OK). Using the sequence derived from the *ftsZ* of strain CH811, our analysis of the genome raw sequence from *N. gonorrhoeae* strain FA1090 has led to the identification of the *ftsZ* gene in this strain. The sequence in the two strains is 99% identical, and the few mismatches could be because the FA1090 sequence is still unpolished. We have found that *ftsZ* lies within a cluster of cell division and growth gene homologs (Victor and Francis, unpublished data). The organization of the cluster is similar to that seen in *E. coli* (Figure 1A), although some differences exist in the gene sizes and intergenic spaces. Of notable difference is the presence of a hypothetical protein ORF just downstream of *ftsZ*, whereas in *E. coli* the *envA* protein is found. With respect to gene expression, the gonococcal cluster appears to differ from that of *E. coli* because several putative transcriptional terminators have been identified within the cluster. Many gene homologs can now be amplified from the chromosome and can be studied with regards to their function in the cell and their interaction with FtsZ. The genome project of *H. influenzae* also reveals a cluster of cell division and growth genes very similar to *E. coli* (Fleischmann *et al.*, 1995), and in *P. aeruginosa* gene mapping indicates *ftsZ* is clustered with a few cell division and cell growth genes (Liao *et al.*, 1996).

The identification of a chlamydial *ftsZ* was possible using degenerate primer PCR. An amplicon was obtained from *C. trachomatis* DNA, and subsequent sequencing indicated a partial *ftsZ* gene. Degenerate primers have been previously used to amplify *ftsZ* homologs

from *R. meliloti* (Margolin *et al.*, 1991), *Anabaena sp.* (Zhang *et al.*, 1995), and *M. pulmonis* (Wang and Lutkenhaus, 1996). The entire chlamydial *ftsZ* gene can now be obtained using an inverse PCR strategy as we have done for the *N. gonorrhoeae ftsZ*. To my knowledge this is the first report of the identification of a cell division gene in the obligate intracellular pathogen *C. trachomatis*. This is an interesting organism in which to study cell division, as in its life cycle it undergoes two cellular types: the reticulate body which divides, and the elementary body which does not undergo division (Beatty *et al.*, 1994). The control and regulation of the intracellular development remains largely unknown (Beatty *et al.*, 1994), but further characterization of a cell division protein like FtsZ may provide new insight into the chlamydial life cycle. Chlamydiae, like Mycoplasma species, do not have a cell wall composed of peptidoglycan (Beatty *et al.*, 1994). It will be interesting to determine if any of the other cell division gene homologs exist in this organism because in the genome of *M. genitalium* no other *fts* gene homologs have been identified (Fraser *et al.*, 1995).

In conclusion, this work reports the identification of the essential cell division gene *ftsZ* in *N. gonorrhoeae*. The initial characterization of the gonococcal FtsZ using multiple sequence alignments indicates that it shares many conserved sequences with other FtsZ, including the GTP binding motif. A homolog of FtsZ was also detected in cell extracts of Neisserial species using an anti-*E. coli* FtsZ antisera. An amplicon of the gonococcal *ftsZ* gene was transcribed and translated in an *in vitro* system, indicating the presence of a native promoter upstream of the gene. This work shows that the expression of the gonococcal *ftsZ* in *E. coli* leads to filamentation of the cells, indicating a disruption of the cell division process. The identification of *ftsZ* has also led to the identification of a large number of cell division

and growth genes in the gonococcus. This work on the gonococcal *ftsZ* gene provides a basis on which to further study the molecular biology of cell division in *N. gonorrhoeae*, which will also contribute to the model of cell division in bacteria.

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