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Molecular cloning and characterization of the *ftsEX* genes of *Neisseria gonorrhoeae* CH811 encoding a putative ABC transporter and identification of their flanking genes.

Clonage et caractérisation moléculaire des gènes *ftsEX* chez *Neisseria gonorrhoeae* CH811 codant pour un présumé transporteur ABC et identification des gènes voisins.

by/par

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Abstract

ABC (ATP-binding cassette) transporters are involved in import and export processes in all living cells. These transporters couple ATP hydrolysis to the translocation of a specific substrate across the cellular membrane. ABC transporters carry substrates ranging from ions to proteins. They are composed of two core domains, the ATP-binding domain that binds and hydrolyzes ATP, and a membrane domain that serves as a membrane anchor for the ATP-binding domain.

Cell division is an essential process in any living cell. In *Escherichia coli*, the majority of cell division genes are clustered at two loci on the chromosome. The *ftsY*, *ftsE* and *ftsX* genes constitute one of these clusters. FtsY has been shown to be involved in protein transport, but there is no experimental evidence to link FtsE and FtsX in this process. The status of *ftsE* as a cell division gene is controversial as the filamentous phenotype of an *E. coli ftsE* thermosensitive (Ts) mutant was observed only in rich medium at the restrictive temperature, while Ts mutants of other cell division genes filamented both in rich and in minimal media.

The Gram-negative bacterium *Neisseria gonorrhoeae* is the causative agent of the sexually-transmitted disease (STD) gonorrhoea. Most of the research efforts on *N. gonorrhoeae* have been directed at the study its mechanisms of infection and pathogenicity. Other cellular processes such as cell division remain uncharacterized. A project involving the molecular characterization of cell division genes in *N. gonorrhoeae* was initiated in our laboratory. The hypothesis of this Ph.D. research project was that the *ftsY*, *ftsE* and *ftsX* genes are present and clustered in *N. gonorrhoeae*, and that their gene products are involved in an uncharacterized aspect of cell division.

The plasmid pSB19 was isolated while screening a genomic library of *N. gonorrhoeae* strain CH811. The analysis of the DNA sequence of the insert of pSB19 showed a complete open-reading frame (ORF) showing sequence similarity with the *ftsX* gene of *E. coli*. Two other partial ORFs respectively encoding a protein similar to 3-phosphoglycerate kinase and a protein similar *E. coli* FtsE were identified downstream and upstream of *ftsX*. The partial *ftsE* homologue

overlapped *ftsX* by 4 base pairs (bp). To fully characterize the gonococcal *ftsE* and *ftsX* genes, the complete *ftsE* gene was required. A DNA fragment containing the 5'-section of *ftsE* with 2.3 kilobases of sequence upstream of *ftsE* was amplified by inverse PCR, cloned and sequenced.

The gonococcal *ftsE* gene comprised 651 bp and encoded a polypeptide of 216 amino acid (aa) residues. The gonococcal FtsE protein shared 60 to 71% similarity and 32 to 49% identity with other known bacterial FtsE homologues, and shared significant aa sequence similarities with numerous other ATP-binding domains of ABC transporters. The gonococcal *ftsX* gene included 918 bp encoding a protein of 305 aa residues that shared 47 to 55% similarity and 19 to 29% identity with its bacterial homologues, and did not share significant similarity to other protein sequences included in the public databases. Sequence analyses performed on *N. gonorrhoeae* FtsE and FtsX and on the five other known bacterial homologues of FtsE and FtsX predicted that FtsE did not contain transmembrane segments while FtsX was predicted to contain four of them. The size of FtsX varied between bacterial species and this variation appeared attributable to the amino-terminal section of the protein. This section of FtsX could fulfill a species-specific function. *N. gonorrhoeae* FtsX was predicted to adopt a membrane topology that would locate both its amino- and carboxy-terminal ends in the cytoplasm. Identical topologies were predicted for the other known FtsX homologues.

The presence of a *cat* cassette inside the chromosomal copy of *ftsX* did not affect the viability of *N. gonorrhoeae*, indicating that *ftsX* was not an essential gene. The morphology of *N. gonorrhoeae* was not affected by the presence of an insertion in *ftsX*, suggesting that *ftsX* was not a cell division gene. The minimal inhibitory concentrations of the antibiotics tested on the *ftsX* mutant were identical to those of its wild-type parent, suggesting that FtsX was not part of a membrane system to extrude antibiotics from gonococci. Viable counts of *N. gonorrhoeae* CS19, the *ftsX* mutant, were 6.5 to 10 times lower than those of the parental strain CH811 on GCMB medium containing 100 mM KCl or 400 mM sucrose. These results suggested that the putative FtsEX transporter may be involved in a process of maintenance of the osmotic balance in balance in the gonococcus.

The presence of *ftsE* and *ftsX* was verified in ten other *Neisseria* species by Southern hybridization. *ftsE* and *ftsX* probes hybridized restriction fragments in each species, suggesting that *ftsE* and *ftsX* were present in each species tested. The presence of *ftsE* and *ftsX* in the same ten species was also verified by PCR amplification. *ftsE* amplicons of 282 bp, the size of the *ftsE* amplicon in *N. gonorrhoeae*, were generated from five of the ten species. *ftsX* amplicons were generated in all ten species but their size varied from 894 bp, the size of the *ftsX* amplicon in *N. gonorrhoeae*, to 1.6 Kb. The five *ftsE* amplicons hybridized the gonococcal *ftsE* amplicon, suggesting that these amplicons were indeed *ftsE* amplicons. The 894 bp *ftsX* amplicons alone hybridized the gonococcal *ftsX* amplicon, suggesting that only those were genuine *ftsX* amplicons. Taken together, these results indicated that the *ftsE* and *ftsX* genes were present in various *Neisseria* species but that their nucleotide sequence may diverge from that of *N. gonorrhoeae*.

The 4 bp overlap observed between *ftsE* and *ftsX* suggested that these genes were co-transcribed. The co-transcription of *ftsE* and *ftsX* was confirmed by *in vitro* transcription/translation experiments. A polypeptide of the expected molecular weight (Mw) for FtsX was synthesized only if a promoter was provided upstream of *ftsX*. Proteins of the expected Mw for FtsE and FtsX were synthesized if a promoter was provided upstream of *ftsE* whether *ftsE* and *ftsX* were present on a plasmid or on an amplicon. These results confirmed that *ftsE* and *ftsX* were co-transcribed. These genes would hereafter be referred to as *ftsEX*.

Overproduction of the gonococcal FtsE, FtsX alone or together had no effect on the morphology of *E. coli* in rich or minimal media. Overexpression of other cell division genes have been shown to cause filamentation in *E. coli*. However, the absence of filamentation could not suffice to dismiss *ftsEX* as cell division genes as overexpression of other cell division genes did not have morphological effects in *E. coli*.

The *ftsX* gene of *N. gonorrhoeae* could not complement *E. coli* JS10, a *ftsX* Ts mutant. This result could be attributable to the fact that FtsX contained species-specific attributes absent from its homologues from other species.

The gonococcal FtsE protein was purified as a fusion protein with six histidinyl residues at its amino-terminal end (6HISFtsE) using the QIAexpressionist system from QIAGEN. The pure 6HISFtsE protein will be used to raise polyclonal anti-6HISFtsE antibodies.

The *tlpA* gene, encoding a thioredoxin-like protein, *arsC*, encoding arsenate reductase, and *gltX*, encoding glutamyl-$_T$RNA synthetase were identified upstream of *ftsE*. The six genes identified in this project displayed an identical genetic organization in *N. gonorrhoeae* strain FA1090. Contrary to what had been observed in *E. coli* and *Haemophilus influenzae*, a homologue of *ftsY* was not present immediately upstream of *ftsE* in *N. gonorrhoeae*.

Together, the results obtained suggest that FtsE and FtsX respectively constitute the ATP-binding and membrane domains of a putative ABC transporter that transports an unidentified substrate from the cell. It cannot be excluded that this transporter participates in the cell division process, but it appears not to be essential since a mutant in which *ftsX* had been disrupted was viable while other cell division genes were shown to be essential.

Résumé

Les transporteurs ABC (ATP-binding cassette) sont impliqués dans des processus d'importation et d'exportation dans toute cellule vivante. Ces transporteurs associent l'hydrolyse d'ATP à la translocation d'un substrat spécifique par-delà la membrane de la cellule. Les transporteurs ABC transportent des substrats variés, allant d'ions jusqu'à des protéines. Ils sont constitués de deux domaines principaux, un domaine qui lie et hydrolyse l'ATP, et un domaine membranaire servant d'ancre membranaire au domaine liant l'ATP.

La division cellulaire est un processus essentiel pour toute cellule. Chez *Escherichia coli*, la majorité des gènes de division cellulaire sont groupés en deux loci sur le chromosome. Les gènes *ftsY*, *ftsE* et *ftsX* constituent l'un de ces amas. Il a été démontré que FtsY était impliqué dans le transport protéique mais il n'existe aucune preuve expérimentale impliquant FtsE et FtsX dans ce même processus. Le rôle de *ftsE* en tant que gène de division cellulaire est controversé parce que le phénotype filamenteux d'un mutant thermosensible (Ts) de *ftsE* chez *E. coli* n'était observable qu'en milieu riche à température restrictive, contrairement à des mutants Ts d'autres gènes de division cellulaire qui formaient des filaments en milieu riche ou minimal.

La bactérie Gram-négative *Neisseria gonorrhoeae* est l'agent causal de la gonorrhée, une maladie transmise sexuellement (MTS). La majorité des recherches sur *N. gonorrhoeae* sont concentrées sur l'étude des mécanismes d'infection et de pathogénicité. D'autres processus cellulaires tels la division cellulaire n'ont pas été caractérisés. Un projet sur l'étude des gènes de division cellulaire de *N. gonorrhoeae* a été lancé dans notre laboratoire. L'hypothèse de ce projet de doctorat est que les gènes *ftsY*, *ftsE* et *ftsX* sont présents et regroupés chez *N. gonorrhoeae*, et que les protéines codées par ces gènes sont impliquées dans un aspect non caractérisé de la division cellulaire.

Le plasmide pSB19 fut isolé au cours du criblage d'une banque d'ADN de la souche CH811 de *N. gonorrhoeae*. L'analyse de la séquence d'ADN de l'insert de pSB19 révéla la présence d'un cadre de lecture ouvert (CLO) complet présentant une similarité de séquence avec le gène *ftsX* de *E. coli*. Deux autres CLOs incomplets, codant respectivement pour une protéine

similaire à FtsE de *E. coli* et à une protéine similaire à la 3-phosphoglycérate kinase, furent identifiés en amont et en aval de *ftsX*. Il y avait un chevauchement de 4 paires de bases (pb) entre le gène *ftsE* incomplet et le gène *ftsX*. Pour pouvoir caractériser les gènes *ftsE* et *ftsX* du gonocoque, le gène *ftsE* complet devait être disponible. Un fragment d'ADN contenant la partie 5' de *ftsE* et 2.3 Kb en amont de *ftsE* fut amplifié par PCR inverse, cloné, et séquencé.

Le gène *ftsE* de *N. gonorrhoeae* comprend 651 pb codant pour un polypeptide de 216 acides aminés (aa). La protéine FtsE du gonocoque partage 60 à 71% de similarité et 32 à 49% d'identité de séquence avec les autres homologues bactériens connus de FtsE, et partage une similarité significative avec de nombreux domaines liant l'ATP chez les transporteurs ABC. Le gène *ftsX* de *N. gonorrhoeae* comprend 918 pb codant pour une protéine de 305 aa partageant 47 à 55% de similarité et 19 à 29% d'identité de séquence avec ses homologues bactériens sans partager de similarité significative avec aucune séquence protéique présente dans les bases de données publiques. Les analyses des séquences de FtsE et FtsX de *N. gonorrhoeae* et des cinq homologues bactériens connus prédisent que FtsE ne contiendrait pas d'hélices transmembranaires tandis que FtsX en contiendrait quatre. La taille de FtsX varie entre espèces bactériennes et cette variation semble due à la taille de la section amino-terminale de la protéine. Cette partie de FtsX pourrait remplir une fonction spécifique à chaque espèce bactérienne. Il fut prédit que FtsX de *N. gonorrhoeae* adoptait une topologie membranaire telle que ses extrémités amino- et carboxy-terminales étaient situées dans le cytoplasme. Une topologie identique à celle prédite pour le FtsX gonococcal est prédite pour les autres FtsX bactériens.

La présence d'une cassette *cat* à l'intérieur de la copie chromosomale de n'affecte pas la viabilité de *N. gonorrhoeae*, indiquant que *ftsX* n'était pas un gène essentiel. La morphologie de *N. gonorrhoeae* n'est pas affectée par la présence d'une insertion dans *ftsX*, suggérant que *ftsX* n'est pas un gène de division cellulaire. Les concentrations minimales d'inhibition du mutant *ftsX* étaient identiques à celles de la souche parentale, suggérant que FtsX ne faisait pas partie d'un système d'extrusion d'antibiotiques. Les comptes de cellules viables Des comptes de cellules viables de la souche CS19, le mutant *ftsX*, étaient 6.5 à 10 fois inférieurs à ceux de la souche

parentale, CH811, en milieu de culture contenant du KCl 100 mM ou du sucrose 400 mM. Ceci indique que le présumé transporteur FtsEX pourrait être impliqué dans un processus de maintien de l'équilibre osmotique dans le gonocoque.

La présence des gènes *ftsE* et *ftsX* chez dix autres espèces de *Neisseria* fut vérifiée par hybridation moléculaire Southern. Les sondes *ftsE* et *ftsX* s'apparièrent à des fragments de restriction chez chacune des espèces, suggérant que les gènes *ftsE* et *ftsX* étaient présents dans chaque espèce étudiée. La présence des gènes *ftsE* et *ftsX* chez ces mêmes espèces fut également vérifiée par PCR. Des amplicons *ftsE* de 282 pb, la taille de l'amplicon *ftsE* chez *N. gonorrhoeae*, furent générés chez cinq des dix espèces étudiées. Des amplicons *ftsX* furent générés chez les dix espèces testées, mais leur taille variait de 894 pb, la taille de l'amplicon *ftsX* chez *N. gonorrhoeae*, à 1.6 Kb. Les cinq amplicons *ftsE* hybridèrent l'amplicon *ftsE* de *N. gonorrhoeae*, suggérant que ces amplicons sont effectivement des amplicons *ftsE*. Seuls les amplicons *ftsX* de 894 pb hybridèrent la sonde *ftsX* de *N. gonorrhoeae*, suggérant que seuls ces amplicons étaient d'authentiques amplicons *ftsX*. Pris ensembles, ces résultats indiquent que les gènes *ftsE* et *ftsX* sont présents chez diverses espèces de *Neisseria* mais que leur séquence nucléotidique peut diverger de celle de *N. gonorrhoeae*.

Le chevauchement de 4 pb observé entre *ftsE* et *ftsX* suggère que ces gènes sont co-transcrits. La co-transcription de *ftsE* et *ftsX* fut confirmée par la réalisation d'expériences de transcription/traduction *in vitro*. Un polypeptide ayant le poids moléculaire (Pm) attendu pour FtsX ne fut synthétisé que si un promoteur était présent en amont du gène *ftsX*. Des protéines ayant les Pm attendus pour FtsE et FtsX furent synthétisées si un promoteur était présent en amont de *ftsE*, que *ftsE* et *ftsX* étaient présents sur un plasmide ou sur un amplicon. Ces résultats confirment donc que *ftsE* et *ftsX* sont co-transcrits. Ces deux gènes furent donc désignés *ftsEX*.

La surproduction de FtsE et\ou de FtsX de *N. gonorrhoeae* n'a aucun effet sur la morphologie cellulaire de *E. coli* en milieu riche et en milieu minimal. La surexpression d'autres de gènes de division cellulaire causait la filamentation chez *E. coli*. Cependant, l'absence de filamentation ne pouvait suffire pour conclure que *ftsEX* n'étaient pas des gènes de division

cellulaire car la surexpression d'autres gènes de division cellulaire était sans effet sur la morphologie cellulaire de *E. coli*.

Le gène *ftsX* de *N. gonorrhoeae* ne put complémenter *E. coli* JS10, un mutant thermosensible de *ftsX*. Ce résultat pourrait être dû au fait que FtsX possède des caractéristiques propres à chaque bactérie, lesquelles seraient absentes chez ses homologues d'autres espèces bactériennes.

La protéine FtsE de *N. gonorrhoeae* fut purifiée sous forme d'une protéine de fusion comprenant six résidues histidinyl à son extrémité amino-terminale (6HISFtsE) en utilisant le système "the QIAexpressionist" de la compagnie QIAGEN. La protéine 6HISFtsE purifiée sera utilisée ultérieurement pour provoquer la formation d'anticorps polyclonaux anti-6HISFtsE.

Les gènes *tlpA*, codant pour une protéine ressemblant à la thiorédoxine, *arsC*, codant pour l'arsénate réductase, et *gltX*, codant pour l'ARN_T-synthétase furent identifiés en amont de *ftsE*. Les six gènes identifiés dans ce projet présentaient une organisation génétique identique dans la souche FA1090 de *N. gonorrhoeae*. Contrairement à ce qui fut observé chez *E. coli* et *Haemophilus influenzae*, un homologue de *ftsY* n'est pas présent immédiatement en amont de *ftsE*.

Pris ensembles, les résultats obtenus suggèrent que FtsE et FtsX seraient respectivement le domaine liant l'ATP et le domaine membranaire d'un transporteur ABC transportant un substrat non-identifié vers l'extérieur de la cellule. Il n'est pas exclus que ce transporteur participe au processus de division cellulaire, mais il ne serait pas essentiel vu qu'un mutant dans lequel *ftsX* a été inactivé est viable alors que les autres gènes de division cellulaire sont essentiels.

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À Marie-Claude, ma Grande Blonde,

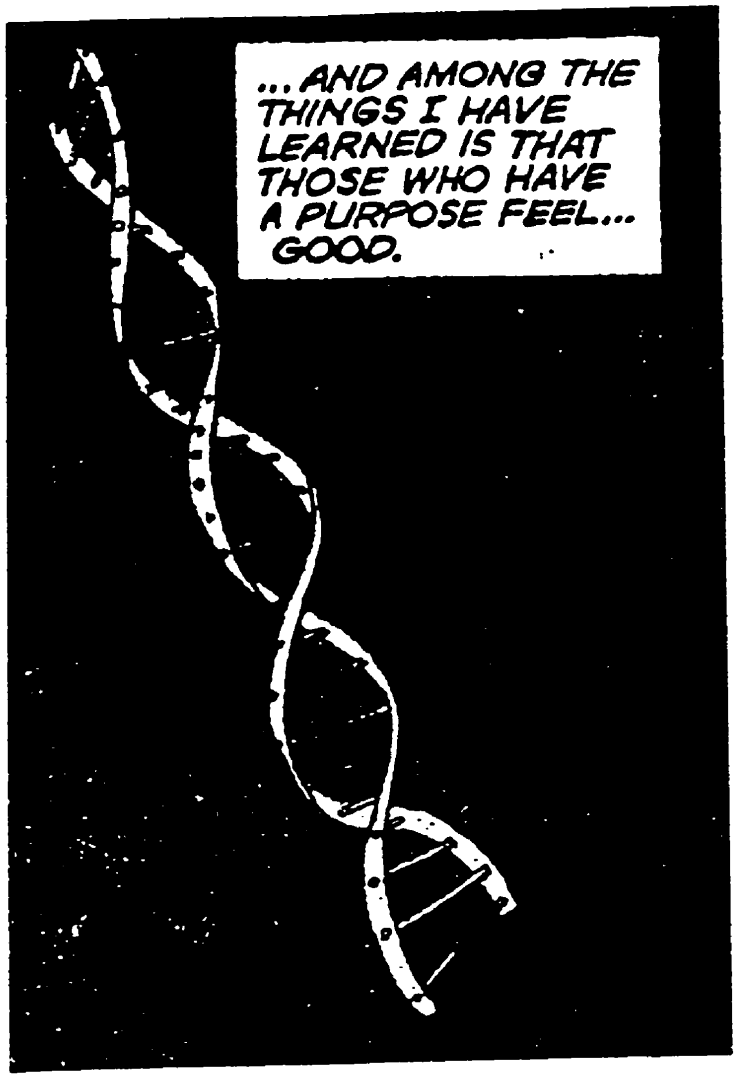
et à p'pa pis m'man (encore!).

Tous les matins, je courais au laboratoire mettre en train mes expériences. Une boulimie. Un délire. Comme un gosse à qui l'on vient de donner un jouet inattendu. Avec les bactéries et les virus, sur lesquels je travaillais, on préparait une expérience le matin. On la réalisait l'après-midi. Le résultat, on l'avait le lendemain matin. Juste à temps pour préparer une nouvelle expérience qu'on éxécutait dans la journée et ainsi de suite. Un rythme d'enfer. Une course sans fin. La pousuite éperdue du lendemain. Plus que les réponses importaient les questions et la manière de les formuler car, dans le meilleur des cas, la réponse obligeait à poser de nouvelles questions. Un système à concocter de l'expectation. Une machine à fabriquer de l'avenir. Pour moi, ce monde de questions et de provisoire, cette chasse à une réponse toujours reportée au lendemain, c'était l'euphorie. Je vivais au futur. J'attendais le résultat du lendemain. Mon anxiété, j'en avais fait mon métier.

François Jacob, La statue intérieure, pp.: 14-15 Folio, 1987.

Every morning I ran to the laboratory to set up my experiments. It was a sort of bulimia; of delirium; like a kid unwrapping an unexpected toy. In the morning, I prepared the bacteria and the viruses I was working on and ran the experiments in the afternoon. The next morning I got the results just in time to put together a further experiment to run later the same day, and so on. A fiendish pace. A race without end. The mad pursuit of the day after this one. What mattered more than the answers were the questions and how they were formulated; for in the best of cases, the answer led to new questions. It was a system for concocting expectation; a machine for making the future. For me, this world of questions and the provisional, this chase after an answer that was always put off to the next day, all that was euphoric. I lived in the future. I always waited for the results of tomorrow. I had turned my anxiety into my profession.

François Jacob, The statue within, pp. 8-9. Basic Books, Inc., Publishers. New York, 1988.



The Uncanny X-Men Vol.1, issue 202 (1986). Artist: John Romita Jr, Writer: Chris Claremont. TM & © Marvel Characters, Inc. All rights reserved.

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aa	amino acid(s)
aaRS	aminoacyl-tRNA synthetase
ABC	ATP-binding cassette
ADK	adenylate kinase
AF	accessory factor
AIDS	acquired immunodeficiency syndrome
Amp	ampicillin
AP	alkaline phosphatase
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CFTR	cystic fibrosis conductance regulator protein
cfu	colony-forming unit
Clr	chloramphenicol
Da	Dalton(s)
DTT	1, 4-dithio-DL-threitol
EDTA	ethylenediamine tetraacetate
EM	electron microscopy
Ffh	fifty-four homologue protein
FHCS	Fitz-Hugh and Curtis syndrome
fts	filamentation temperature sensitive
GCMB	GC medium base
GDP	guanosine diphosphate
GluRS	glutamyl-tRNA synthetase
GTP	guanosine triphosphate

HIV	human immunodeficiency virus
iPCR	inverse PCR
IPTG	isopropylthiogalactoside
Kan	kanamycin
Kb	kilobase(s) (1000 bp)
KDa	kiloDalton
L	Luria broth
LB	Luria-Bertani broth
LOS	lipooligosaccharide
MBP	maltose-binding protein
MIC	minimal inhibitory concentration
min.	minute(s)
mL	milliliter
Mw	molecular weight
Na(As[III])	sodium arsenite
Na(As[V])	sodium arsenate
Ni-NTA	nickel nitrilo-tri-acetic acid
nm	nanometer(s)
OD	optical density
OMP	outer membrane protein
ORF	open reading frame
PCR	polymerase chain reaction
Pgk	3-phosphoglycerate kinase
pI	isoelectric point
PID	pelvic inflammatory disease
PPNG	Penicillinase-producing *N. gonorrhoeae*
RBP	ribose-binding protein

RBS	ribosome-binding site
RNP	ribonucleoprotein
rpm	rotation per minute
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
sec.	second(s)
6HISFtsE	histidine-tagged FtsE protein of *N. gonorrhoeae*
SRα	α-subunit of the mammalian docking protein
SRP	signal recognition particle
SRP54	54 KDa polypeptide part of the SRP
SSC	standard sodium citrate
STD	sexually transmitted disease
Strep.	streptomycin
TBS	tris-buffered saline
TE	tris-EDTA buffer
Tet	tetracycline
Tm(°C)	melting temperature (in degrees Celsius)
Ts	thermosensitive
TTBS	TBS+Tween 20
US	uptake sequence
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

PART 1. INTRODUCTION

1.1. PREAMBLE TO THE INTRODUCTION

The scope of this research project is the identification and characterization of the *ftsE* and *ftsX* cell division genes in *Neisseria gonorrhoeae* strain CH811 and their flanking genes. In other bacteria, the protein encoded by one of these genes, FtsE, displays features specific to the ATP-binding domain of ABC (ATP-binding cassette) transporters, which is not the case for other cell division proteins. FtsE shares amino acid (aa) sequence similarity with the ATP-binding domain of ABC transporters, while FtsX has been predicted to contain transmembrane segments, suggesting it is an integral membrane protein. Because of these features of FtsE and FtsX, the introduction has been divided into three sections that respectively review ABC transporters, bacterial cell division, and aspects of *N. gonorrhoeae*.

ABC transporters are discussed in the first section (section A). Their biological function, the genetic organization of their genes and the general organization of the domains of ABC transporters are reviewed. The two core domains of ABC transporters, the ATP-binding and membrane domains are also reviewed. This section ends with the description of the two ABC transporters identified to date in the *Neisseriaceae*.

Bacterial cell division, with a focus on the *Escherichia coli* model, is reviewed in the second section (section B). The isolation of *ftsE* and *ftsX* thermosensitive (Ts) mutants in *E. coli* to the characterization of the genes, their overall genetic organization and the features of their gene product is first described. The genetic organization of the main cluster of cell division genes is also briefly reviewed, followed by a description of cell division in *E. coli*. This section is completed with the current understanding of cell division in *N. gonorrhoeae* derived from electron microscopic observations and the putative cell division genes that have been identified.

In the last section of the introduction (section C), a short historical account of gonorrhea is presented, followed by the importance of *N. gonorrhoeae* as a bacterial pathogen world-wide and in Canada. A description of the disease, and a summary of the continual problem caused by its acquisition of novel antibiotic resistance are provided. The current knowledge of the gonococcal

chromosome is also reviewed as the determination of the nucleotide sequence of the chromosome of *N. gonorrhoeae* is almost completed.

A. ABC transporters

1.2. ABC transporters

Members of the ATP-binding cassette (ABC; Hyde *et al.*, 1990) family are present in prokaryotes as well as eukaryotes (Higgins, 1992). From the initial observation that a few bacterial proteins contained the Walker A and B nucleotide-binding motifs (section 1.2.2.; Higgins *et al.*, 1988, 1986; Gill *et al.*, 1986; Higgins, 1985), a number of proteins have now been found to contain these motifs (Boos and Lucht, 1996; Fath and Kolter, 1993; Higgins, 1992). More than 50 members of this family have been identified in prokaryotes (Boos and Lucht, 1996; Fath and Kolter, 1993; Higgins 1992), and at least 25 eukaryotic ABC transporters have been identified (Dean and Allikmets, 1995).

ABC transporters are also designated traffic ATPases (Doige and Ames, 1993; Ames *et al.*, 1992). They couple ATP hydrolysis to the import or export of a specific ion or molecule to or from the cell. The substrates transported by these transporters are varied (Fath and Kolter, 1993; Higgins, 1992). In bacteria, ABC transporters for ions such as Na^{+}, Fe^{3+}, and Mg^{2+} have been identified (Cheng *et al.*, 1997; Adhikari *et al.*, 1996; Bartsevich and Pakrasi, 1996). They may also transport small molecules: aa (e.g., histidine and arginine), sugars (e.g., maltose), bacteriocins (e.g., colicin V; Wissenbach *et al.*, 1995; Fath *et al.*, 1992; Dassa and Hofnung, 1985a; Froshauer and Beckwith, 1984; Gilson *et al.*, 1982; Higgins *et al.*, 1982). Even macromolecules may be transported by ABC transporters: antibiotics in *Streptomyces* species, α-hemolysin in *E. coli* and other bacteria, capsular polysaccharides in *E. coli* and *Neisseria meningitidis* (Bliss and Silver, 1996; Fernández *et al.*, 1996; Olano *et al.*, 1995; Fath and Kolter,

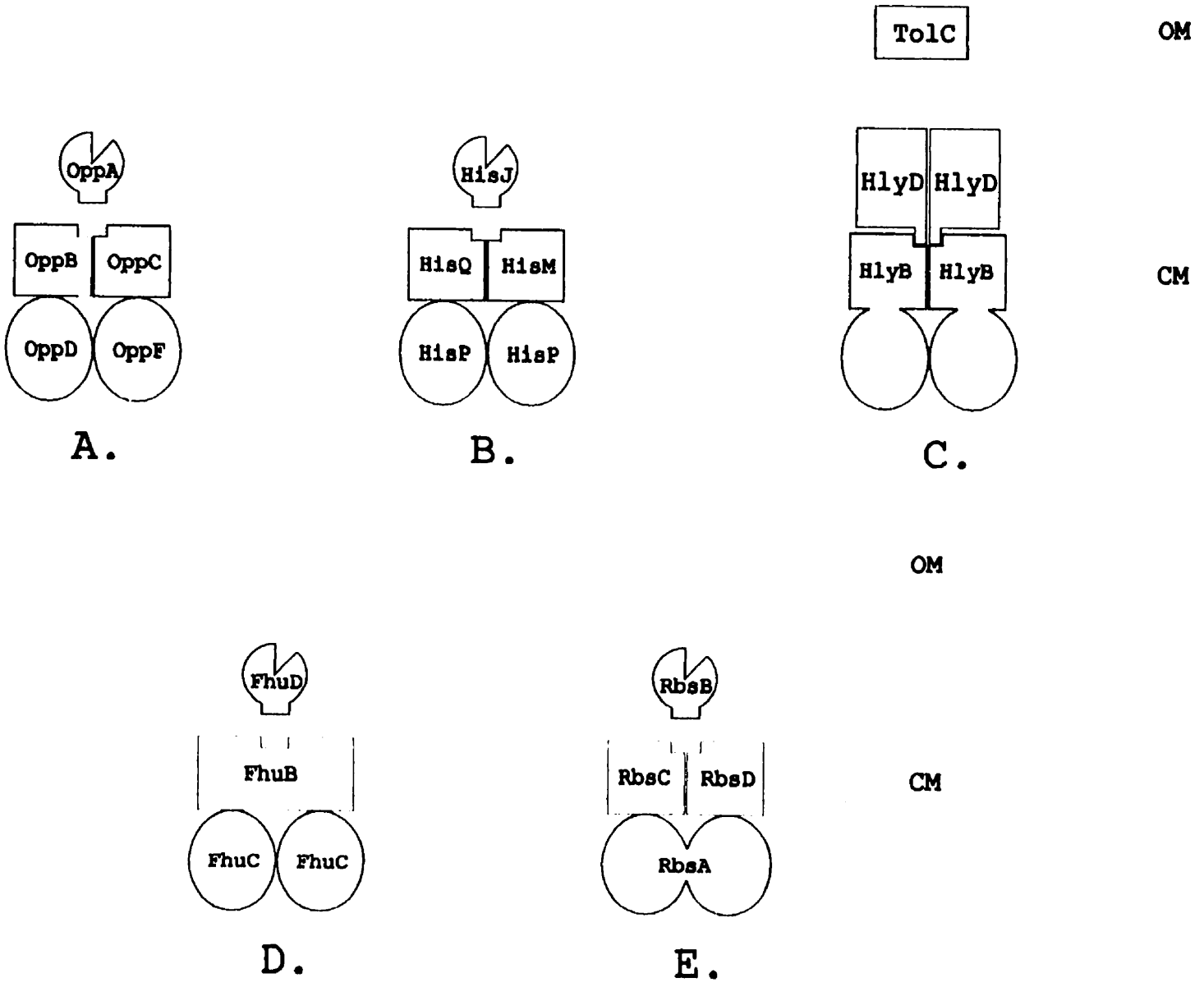
1993; Frosch *et al.*, 1991). ABC transporters may be involved in more complex functions such as plant attachment in *Agrobacterium tumefasciens* (Matthysse *et al.*, 1996).

The human ABC transporter ABC1 is expressed in macrophages engaged in the engulfment and clearance of dead cells (Luciani and Chimini, 1996). The small number of eukaryotic ABC transporters identified have been associated with clinically relevant phenotypes (Luciani and Chimini, 1996). The P-glycoprotein in humans is amplified and/or overexpressed in cell lines selected for resistance to cytotoxic drugs. They are also overexpressed in certain human tumors, most commonly those that relapse following chemotherapic treatments (Dean and Allikmets, 1995). P-glycoprotein expression is enhanced following infection with the human immunodeficiency virus (HIV), which could contribute to the resistance of HIV-infected cells to antiviral drugs (Gupta and Gollapudi, 1993). P-glycoproteins homologues are overexpressed or their gene is even amplified in certain drug-resistant parasites (Dean and Allikmets, 1995). Pgh1, an ABC transporter of the malarial parasite *Plasmodium falciparum*, is linked to chloroquine resistance (Higgins, 1995). Mutations in the *CFTR* gene encoding the cystic fibrosis conductance regulator protein (CFTR) cause cystic fibrosis (Riordan *et al.*, 1989; section 1.2.2.). Other diseases that have been linked to human ABC transporters include adrenoleukodistrophy and Zellweger's syndrome, two diseases linked to the function or the formation of peroxisomes (Dean and Allikmets, 1995). The receptor of sulfonylureas, oral hypoglycemics used to treat non-insulin-dependent diabetes mellitus to stimulate insulin release from pancreatic islet β cells, is an ABC transporter (Aguilar-Bryan *et al.*, 1995).

1.2.1. Organization of ABC transporters

ABC transporters are typically composed of two ATP-binding domains and two hydrophobic membrane domains (Higgins, 1992). The individual domains of ABC transporters are often expressed as separate polypeptides, particularly in prokaryotes, for instance in the oligopeptide permease and in the histidine permease of *Salmonella typhimurium* (Fig. 1A and B; Higgins, 1992). However, all possible organizations of the domains have been observed (Fig. 1):

Figure 1: Organization of the domains of selected ABC transporters. The membrane domains are represented by blue boxes, the ATP-binding domains are represented by red circles. Other components of some ABC transporters such as the periplasmic substrate-binding proteins (in A, B, D and E) are represented by green circles; the accessory factor (in C) is represented by a purple box, and the outer membrane protein (in C) is represented by a black box. The cytoplasmic membrane (CM) and the outer membrane (OM) are represented by double black lines. The ABC transporters appearing in this figure are (A), the oligopeptide permease of *S. typhimurium*; (B), the histidine permease of *S. typhimurium*; (C), the α-hemolysin exporter of *E. coli*; (D), the iron-hydroxamate transporter of *E. coli*; and (E), the ribose transporter of *E. coli*. This figure is based on Fath and Kolter (1993), and Higgins (1992).


TolC
OM
OppA
OppB
OppC
OppD
OppF
A.
HisJ
HisQ
HisM
HisP
HisP
B.
HlyD
HlyD
HlyB
HlyB
CM
C.
OM
FhuD
FhuB
FhuC
FhuC
D.
RbsB
RbsC
RbsD
RbsA
CM
E.

HlyB, a component of the α-hemolysin exporter of *E. coli* has one ATP-binding domain fused to a single transmembrane domain (Fig. 1C); the membrane domains are fused in the iron-hydroxamate transporter FhuB of *E. coli* (Fig. 1D); the two ATP-binding domains are expressed as a single polypeptide in the ribose transporter protein RbsA in *E. coli* (Fig. 4E). In other ABC transporters, all four domains are fused such as in the human multidrug resistance P-glycoprotein and in the CFTR protein (Fath and Kolter, 1993; Higgins, 1992; Higgins *et al.*, 1990).

Transporters involved in import processes also include a third component, a periplasmic protein that binds specifically to the ion or molecule to be imported in the cell (Boos and Lucht, 1996; Doige and Ames, 1993; Higgins, 1992). Such periplasmic substrate-binding proteins include OppA from the oligopeptide transporter of *S. typhimurium* (Fig. 1A), HisJ from the histidine importer of *S. typhimurium* (Fig. 1B), FhuD from the iron-hydroxamate importer of *E. coli* (Fig. 1D), and RbsA from the ribose transporter of *E. coli* (Fig. 1E).

Many ABC exporters require an additional protein for proper function, the accessory factor (AF), and the gene encoding the AF is always linked to the genes encoding the domains of the ABC transporter (Fath and Kolter, 1993). For example, HlyD is the AF for HlyA export in *E. coli* (Fig. 1C; Fath and Kolter, 1993). Other Gram negative bacterial ABC exporters, especially those involved in the export of nonprotein substrates, do not include an AF (Fath and Kolter, 1993). In such systems, the ultimate destination of the exported product is the periplasmic space or the outer membrane.

Some ABC transporters require an additional component in the form of an outer membrane protein (OMP) to complete the translocation of the product (Fath and Kolter, 1993). For example, the TolC protein facilitates the passage of HlyA and colicinV through the outer membrane (Figure 1C; Fath and Kolter, 1993; Fath *et al.*, 1992). The gene encoding the OMP can be tightly linked to the other genes encoding the domains of the ABC exporter, as is the case for the *prtF* gene (encoding the OMP) that is linked with *prtD* and *prtE* (encoding the domains of the ABC transporter) in the protease synthesis and export system of *Erwinia chrysanthemi* (Fath and Kolter, 1993). Alternatively, it may not be linked, as is the case with the TolC protein that is

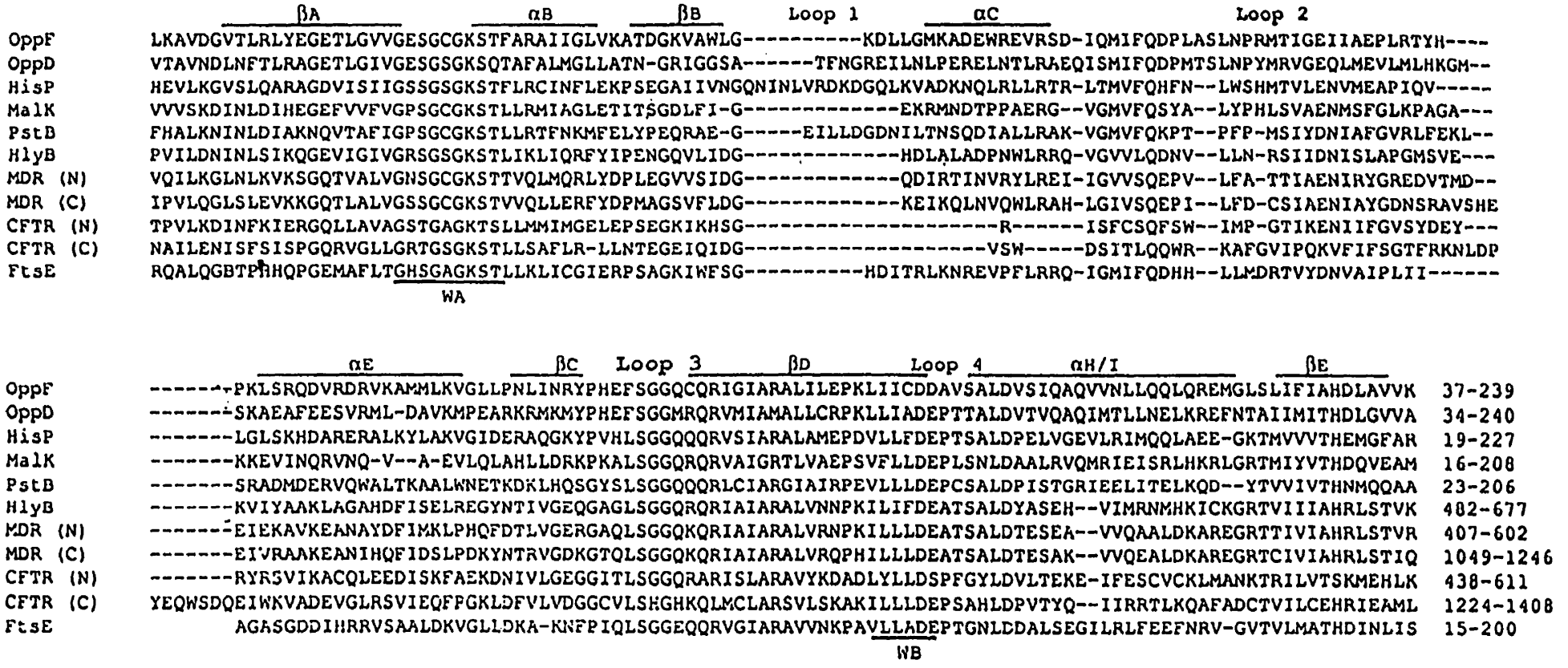
involved in colicinV and α-hemolysin export while *tolC* (encoding the OMP TolC) is unlinked with either *cvaAB* (encoding the domains of the ABC exporter for colicin V, Fath *et al.*, 1992) or the *hlyCABD* genes (encoding the domains and the AF of the ABC exporter for α-hemolysin, Fath and Kolter, 1993).

1.2.2. The ATP-binding domain

The ATP-binding domains of ABC transporters contain two nucleotide-binding motifs known as the Walker motifs A and B (Fig. 2; Walker A consensus: GX_4GKS/T, Walker B consensus: $R/K_{6-8}LHyd_4D$; Walker *et al.*, 1982). These motifs were initially identified in the α- and β-subunits of ATP synthase, myosin, phosphofructokinase and adenylate kinase (ADK; Walker *et al.*, 1982). The importance of both Walker motifs for the activity of the ATP-binding domain has been demonstrated by site-directed mutagenesis in several ATP-binding domains such as HisP from *S. typhimurium* (Shyamala *et al.*, 1991), HlyB of *E. coli* (Koronakis *et al.*, 1995), KpsT of *E. coli* (Pavelka *et al.*, 1994), MalK of *E. coli* (Panagiotidis *et al.*, 1993), OleB of *Streptomyces antibioticus* (Aparicio *et al.*, 1996), CFTR (Logan *et al.*, 1994; Anderson and Welsh, 1992; Cheng *et al.*, 1990), and murine MDR1 (Azzaria *et al.*, 1989). The results obtained for these studies indicated that the ABC transporter, regardless of its domain organization or if it was an importer or an exporter, was always disabled when an invariant or highly-conserved aa residue was altered in any of the conserved motifs (Walker motifs A and B or the linker peptide; Koronakis *et al.*, 1995).

A model for the three-dimensional structure of the ATP-binding domain of ABC transporters was developed based on similarities between the predicted secondary structures of ATP-bonding domains and the known three dimensional structure of ADK (Fig. 2; Hyde *et al.*, 1990). The core of the model was a nucleotide-binding fold involving five hydrophobic β-sheets (βA-βE, Fig. 2) and the Walker motif A properly positioned to interact with ATP (Fig. 2). Two loops (loops 2 and 3, Fig. 2) constituted the differences between the predicted structure of the ABC proteins and that of ADK. Loop 2 (Fig. 2) had no equivalent in ADK and was thought to

Figure 2: Alignment of the amino acid sequences of the ATP-binding domains of ABC transporters. The α-helices and β-sheets are identified after Hyde *et al.* (1990) and are predicted to form the nucleotide-binding fold (Hyde *et al.*, 1990). The Walker motifs A (WA) and B (WB) are indicated on the figure, and the linker peptide is indicated as loop 3. OppF and OppD are part of the oligopeptide importer of *S. typhimurium*, HisP is part of the histidine importer of *S. typhimurium*, MalK is part of the maltose importer of *S. typhimurium*, PstB is part of the phosphate importer of *E. coli*, HlyB is part of the hemolysin exporter of *E. coli*, MDR (N) and MDR (C) are the amino- and carboxy-terminal domains of the murine multidrug resistance protein, CFTR (N) and CFTR (C) are the amino- and carboxy-terminal domains of the human CFTR protein, and FtsE is a putative *E. coli* cell division protein (section 1.3.2.). The aa sequence of CFTR is from by Riordan *et al.* (1989), the one of *E. coli* FtsE is from Gill *et al.* (1986). See Higgins (1992) and Hyde *et al.* (1990) for references for the other sequences. This figure was taken from Higgins (1992).


βA αB βB Loop 1 αC Loop 2
OppF LKAVDGVTLRLYEGETLGVVGESGCGKSTFARAIIGLVKATDGKVAWLG----------KDLLGMKADEWREVRSD-IQMIFQDPLASLNPRMTIGEIIAEPLRTYH----
OppD VTAVNDLNFTLRAGETLGIVGESGSGKSQTAFALMGLLATN-GRIGGSA------TFNGREILNLPERELNTLRAEQISMIFQDPMTSLNPYMRVGEQLMEVLMLHKGM--
HisP HEVLKGVSLQARAGDVISIIGSSGSGKSTFLRCINFLEKPSEGAIIVNGQNINLVRDKDGQLKVADKNQLRLLRTR-LTMVFQHFN--LWSHMTVLENVMEAPIQV-----
MalK VVVSKDINLDIHEGEFVVFVGPSGCGKSTLLRMIAGLETITSGDLFI-G----------EKRMNDTPPAERG--VGMVFQSYA--LYPHLSVAENMSFGLKPAGA---
PstB FHALKNINLDIAKNQVTAFIGPSGCGKSTLLRTFNKMFELYPEQRAE-G-----EILLDGDNILTNSQDIALLRAK-VGMVFQKPT--PFP-MSIYDNIAFGVRLFEKL--
HlyB PVILDNINLSIKQGEVIGIVGRSGSGKSTLIKLIQRFYIPENGQVLIDG----------HDLALADPNWLRRQ-VGVVLQDNV--LLN-RSIIDNISLAPGMSVE---
MDR (N) VQILKGLNLKVKSGQTVALVGNSGCGKSTTVQLMQRLYDPLEGVVSIDG----------QDIRTINVRYLREI-IGVVSQEPV--LFA-TTIAENIRYGREDVTMD--
MDR (C) IPVLQGLSLEVKKGQTLALVGSSGCGKSTVVQLLERFYDPMAGSVFLDG----------KEIKQLNVQWLRAH-LGIVSQEPI--LFD-CSIAENIAYGDNSRAVSHE
CFTR (N) TPVLKDINFKIERGQLLAVAGSTGAGKTSLLMMIMGELEPSEGKIKHSG----------R------ISFCSQFSW--IMP-GTIKENIIFGVSYDEY---
CFTR (C) NAILENISFSISPGQRVGLLGRTGSGKSTLLSAFLR-LLNTEGEIQIDG----------VSW-----DSITLQQWR--KAFGVIPQKVFIFSGTFRKNLDP
FtsE RQALQGBTPHHQPGEMAFLTGHSGAGKSTLLKLICGIERPSAGKIWFSG----------HDITRLKNREVPFLRRQ-IGMIFQDHH--LLMDRTVYDNVAIPLII------
WA
αE βC Loop 3 βD Loop 4 αH/I βE
OppF ------PKLSRQDVRDRVKAMMLKVGLLPNLINRYPHEFSGGQCQRIGIARALILEPKLIICDDAVSALDVSIQAQVVNLLQQLQREMGLSLIFIAHDLAVVK 37-239
OppD -------SKAEAFEESVRML-DAVKMPEARKRMKMYPHEFSGGMRQRVMIAMALLCRPKLLIADEPTTALDVTVQAQIMTLLNELKREFNTAIIMITHDLGVVA 34-240
HisP -------LGLSKHDARERALKYLAKVGIDERAQGKYPVHLSGGQQQRVSIARALAMEPDVLLFDEPTSALDPELVGEVLRIMQQLAEE-GKTMVVVTHEMGFAR 19-227
MalK -------KKEVINQRVNQ-V--A-EVLQLAHLLDRKPKALSGGQRQRVAIGRTLVAEPSVFLLDEPLSNLDAALRVQMRIEISRLHKRLGRTMIYVTHDQVEAM 16-208
PstB -------SRADMDERVQWALTKAALWNETKDKLHQSGYSLSGGQQQRLCIARGIAIRPEVLLLDEPCSALDPISTGRIEELITELKQD--YTVVIVTHNMQQAA 23-206
HlyB -------KVIYAAKLAGAHDFISELREGYNTIVGEQGAGLSGGQRQRIAIARALVNNPKILIFDEATSALDYASEH--VIMRNMHKICKGRTVIIIAHRLSTVK 482-677
MDR (N) -------EIEKAVKEANAYDFIMKLPHQFDTLVGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEA--VVQAALDKAREGRTTIVIAHRLSTVR 407-602
MDR (C) -------EIVRAAKEANIHQFIDSLPDKYNTRVGDKGTQLSGGQKQRIAIARALVRQPHILLLDEATSALDTESAK--VVQEALDKAREGRTCIVIAHRLSTIQ 1049-1246
CFTR (N) -------RYRSVIKACQLEEDISKFAEKDNIVLGEGGITLSGGQRARISLARAVYKDADLYLLDSPFGYLDVLTEKE-IFESCVCKLMANKTRILVTSKMEHLK 438-611
CFTR (C) YEQWSDQEIWKVADEVGLRSVIEQFPGKLDFVLVDGGCVLSHGHKQLMCLARSVLSKAKILLLDEPSAHLDPVTYQ--IIRRTLKQAFADCTVILCEHRIEAML 1224-1408
FtsE AGASGDDIHRRVSAALDKVGLLDKA-KNFPIQLSGGEQQRVGIARAVVNKPAVLLADEPTGNLDDALSEGILRLFEEFNRV-GVTVLMATHDINLIS 15-200
WB

assume a role specific to the ABC domain rather than to be involved in ATP binding. Loop 3 (Fig. 2) was also believed to serve an ABC domain-specific function as its sequence was conserved between ABC domains. A well-conserved motif rich in glycine and glutamine, the linker peptide, has been identified in loop 3 (Fig. 2; prokaryotic consensus: LSGGQQQ; Ames *et al.*, 1992). This motif was proposed to serve as a linker to join separate domains within proteins (Doige and Ames, 1993; Ames *et al.*, 1992; Argos, 1990). The ATP-binding domains of ABC transporters were predicted to have a tightly folded core structure that bound and hydrolyzed ATP while their loops 2 and 3 were proposed to couple ATP-dependent conformational changes to the transport process, possibly through an interaction with the membrane domains of the transporter (Higgins, 1992; Hyde *et al.*, 1990).

Amino acid substitutions in the linker peptide of *E. coli* FtsE were responsible for cell filamentation at 42°C (Gibbs *et al.*, 1992). Several of the mutations inside the CFTR protein were identified in the linker peptide (Cheng *et al.*, 1990; Cutting *et al.*, 1990), again suggesting a role for this sequence motif. Other aa residues than those of the linker motif may be involved in the process of joining the two Walker motifs. The aa residues preceding the linker motif in the first nucleotide-binding domain of the murine Mdr3 protein were proposed to serve a transporter-specific structural role, perhaps with the linker domain (Beaudet and Gros, 1995).

The distinguishing feature of the ATP-binding domains of ABC transporters over ATP-binding proteins is that the sequence similarity between the ATP-binding domains of ABC transporters extended over the whole length of the domain (about 200 aa residues; Higgins, 1992), whereas ATP-binding proteins shared sequence similarities around the nucleotide-binding motifs only, namely around the Walker motifs A and B (Higgins, 1992).

1.2.3. The transmembrane domain

The transmembrane domain of ABC transporters typically contains six transmembrane segments (for a total of twelve transmembrane segments per transporter). However, transporters in which the transmembrane domain did not conform to the "two-times-six" structure have been

identified: the ArtQ and ArtM of the arginine importer of *E. coli* were respectively predicted to contain four and three transmembrane segments; the MalF protein of the maltose transporter in *E. coli* contains eight transmembrane segments; the HisQ and HisM proteins from the histidine transporter of *S. typhimurium* were each predicted to contain five transmembrane segments (Wissenbach *et al.*, 1995; Froshauer *et al.*, 1988; Higgins *et al.*, 1982).

Whereas the ATP-binding proteins contained highly conserved aa motifs (section 1.2.1.), little or no aa sequence similarities were found among the transmembrane domains of the transporters, suggesting that many aa combinations could satisfy the constraints required for the proper function of the transmembrane domains (Higgins, 1992). Subsequent analyses revealed the presence of a conserved sequence in the membrane domain. This short sequence, designated the EAA loop (consensus sequence: $EAA(X)_3G(X)_9IXLP$, where X represents any amino acid; Dassa and Hofnung, 1985a), was identified in the last cytoplasmic loop of several membrane domains of ABC importers, between 95 to 114 positions from the carboxy-end of the polypeptide (Boos and Lucht, 1996; Saurin *et al.*, 1994; Dassa and Hofnung, 1985a, 1985b). The EAA loop appeared to be absent from the membrane domains of ABC exporters (Saurin *et al.*, 1994). Based on the sequence similarities between various membrane domains, the proteins could be subdivided in groups (Saurin *et al.*, 1994). Transporters specific for a group of similar substrates such as certain sugars, ions or iron-siderophores shared more sequence similarities in the region of the EAA loop, and consensus sequences were deduced (Saurin *et al.*, 1994). These consensus sequences will be useful to help characterize other membrane proteins and the substrate of ABC transporters. The EAA loop has been proposed to constitute a recognition site for a ligand or a partner common to protein-binding dependent permeases (Dassa and Hofnung, 1985b), which could be the most conserved component in such permeases, namely the ATP-binding proteins (Kerppola and Ames, 1992). Mutations in the EAA loop of the MalG and MalF proteins from the maltose importer of *E. coli* were shown to affect the formation of the transporter, indicating that the EAA loop of the membrane domain functionally interacted with the nucleotide-binding domain (i.e., MalK; Mourez *et al.*, 1997).

The mechanisms of transport by ABC transporters, their substrate specificity, and all other aspects have been the subject of several reviews (Boos and Lucht, 1996; Dean and Allikmets, 1995; Doige and Ames, 1993; Fath and Kolter, 1993; Ames *et al.*, 1992; Higgins 1992; Higgins *et al.*, 1990) and will not be discussed here .

1.2.4. ABC transporters in *Neisseria* species

Only two ABC transporters have been identified in the *Neisseria*: an importer of ferric ions in *N. gonorrhoeae* (Adhikari *et al.*, 1996), and an export complex involved in the export of capsular polysaccharides in *N. meningitidis* (Frosch *et al.*, 1991). The ferric ions importer complex is encoded by the *fbpABC* genes that respectively encode a periplasmic ferric-iron-binding protein, a membrane domain containing 11 predicted membrane-spanning segments and an ATP-binding domain (Adhikari *et al.*, 1996). The capsular polysaccharides export complex comprises the *ctrABCD* genes, with *ctrA* encoding a putative lipoprotein involved in export across the outer membrane, *ctrB* and *ctrC* encoding membrane proteins, and *ctrD* encoding the ATP-binding domain (Frosch *et al.*, 1991).

It is to be expected that many other ABC transporters will be identified in *N. gonorrhoeae* and in *N. meningitidis* once their respective genome projects are completed. In the genome of *E. coli*, 80 ABC transporters have been identified (Blattner *et al.*, 1997); in *Mycoplasma pneumoniae*, 34 genes encoding predicted domains of ABC transporters have been identified (Himmelreich *et al.*, 1996).

B. BACTERIAL CELL DIVISION

1.3. Overview of the current state of knowledge on cell division in *Escherichia coli*

The bacterial cell cycle can be divided in two steps (Rothfield and Justice, 1997). The first step includes the replication of the bacterial chromosome and chromosome segregation. The

second step comprises cell division, which culminates in cytokinesis (the separation of the cytoplasm into two compartments bounded by the cytoplasmic membrane (Donachie, 1993), and the separation of the daughter cells. During cell division, the cell must identify the midcell site at which division will later occur, differentiate this site in preparation for cytokinesis, and finally form the division septum by the coordinated growth of the cytoplasmic membrane, the cell wall, the synthesis of septal peptidoglycan and the outer membrane in Gram-negative bacteria (section 1.6.; Lutkenhaus and Addinall, 1997; Lutkenhaus and Mukherjee, 1995).

Cell division genes are considered essential for two reasons: first, cell division is a vital process for any cellular organism; second, cell division genes were identified by the isolation of conditionally lethal temperature sensitive (Ts) mutants in *E. coli* (Bi and Lutkenhaus, 1991a). Such conditional lethal Ts mutants were phenotypically normal at their permissive temperature but presented a different phenotype at their restrictive temperature (Bi and Lutkenhaus, 1991a). Some of these Ts mutants of *E. coli* formed filaments at their restrictive temperature but were not affected in DNA segregation as they contained regularly distributed nucleoids, while other filamentous mutants had defects in DNA replication or DNA partitioning (Lutkenhaus and Mukherjee, 1995; Hirota *et al.*, 1968). Filamentation without any visible noticeable effect on DNA segregation suggested that such mutants were specifically affected in septum formation and the mutated genes in such mutants were designated *fts* (filamentation temperature sensitive; Lutkenhaus and Mukherjee, 1995; Bi and Lutkenhaus, 1991a).

In most of the bacteria examined to date, the cell division genes were found to share a similar overall genetic organization. Most cell division genes were clustered at two loci on the bacterial chromosome: the *ftsYEX* cluster, and the morphogene (or *dcw*, for division-cell wall (Sánchez *et al.*, 1994)) cluster. The genes contained in each cluster and the function of their gene products is reviewed in the following sections.

1.4. The *ftsYEX* cluster

This gene cluster includes three genes: *ftsY*, *ftsE* and *ftsX*. The three gene products, FtsY, FtsE and FtsX are associated with the inner membrane in *E. coli* (Gill and Salmond, 1987), and FtsY is also present in the soluble cell fraction (Luirink *et al.*, 1994). These three proteins are believed to be involved in a transport function that is important for cell division (Gill and Salmond, 1990, 1987; Gill *et al.*, 1986), perhaps as part of the septalsome (a multiprotein complex responsible for cell septation and cell separation; Holland and Jones, 1985). There has been more interest in the biological function of FtsY since it was shown to share sequence similarity with the α-subunit of the mammalian signal recognition particle (SRP, a ribonucleoprotein complex involved in protein transport; Appendix 1) docking protein (SRα, the endoplasmic reticulum membrane receptor of the SRP; Appendix 1). The *E. coli* FtsE shares aa sequence similarity with the ATP-binding domain of ABC transporters while FtsX is predicted to contain four transmembrane segments (sections 1.5.1. and 1.5.2.).

As was the case for other *E. coli fts* genes, *ftsE* and *ftsX* were first identified by the characterization of Ts mutants following ultraviolet mutagenesis of *E. coli* cells (Sturgeon and Ingram, 1978; Ricard and Hirota, 1973a, 1973b). The first *ftsE* Ts mutant to be examined (strain MFT1181) had an immediate block in septation following a shift to the restrictive temperature, and the block was not due to the arrest of DNA or RNA synthesis (Ricard and Hirota, 1973a). The strain MFT1181 showed no increased sensitivity to sodium deoxycholate at the permissive or the restrictive temperatures, and did not exhibit increased resistance to penicillin G when compared to its parental strain (Ricard and Hirota, 1973a). These observations suggested that there was no alteration in the integrity of the cell envelope. The block in cell division was RecA-independent (Ricard and Hirota, 1973a).

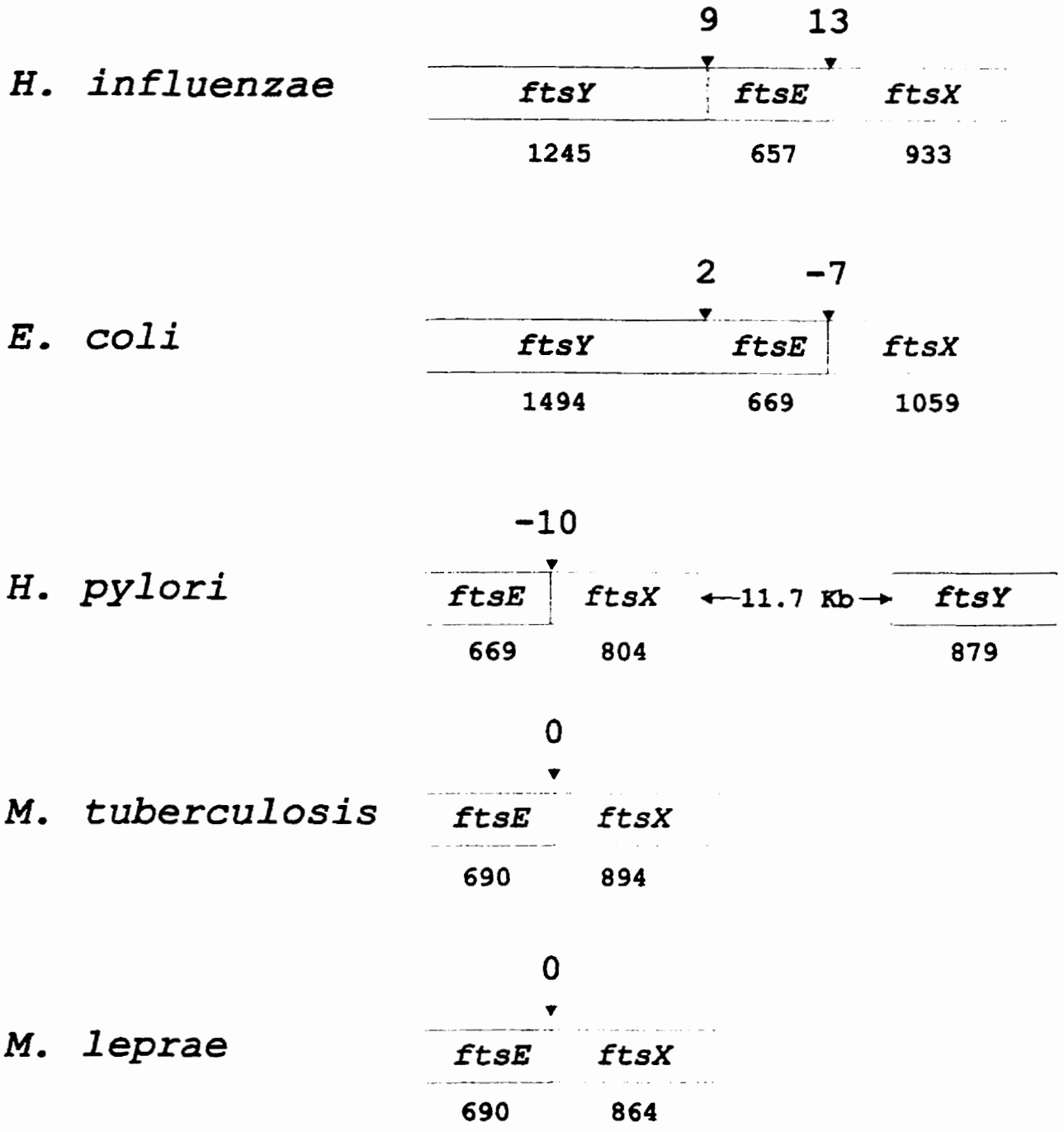
The *ftsX* locus of *E. coli* was also identified following ultraviolet mutagenesis of *E. coli* cells, but the cultures were enriched for long cells by repeated cycles of bacterial growth in liquid medium, filtration of the cultures to isolate filamentous cells, and using these filamentous cells to grow a new bacterial culture (Sturgeon and Ingram, 1978). The mutant strain JS10 was cold-

sensitive: the bacteria appeared as short rods at 37°C but formed filaments at 30°C (Sturgeon and Ingram, 1978). The filamentous cells of the strain JS10 did not show constrictions, suggesting that the block in cell division in strain JS10 occurred at a point preceding the assembly of the septum (Sturgeon and Ingram, 1978). The presence of constrictions in the filaments would have indicated that the early events of cell division occurred normally and the defect would have been in a gene involved in the late stages of cell division. Compared to the parental strain χ462, the mutant strain JS10 showed excessive sensitivity to sodium deoxycholate (as opposed to what had been observed in *E. coli* MFT1181, a *ftsE* Ts mutant), displayed increased sensitivity to several antibiotics (including penicillin) that affected cell wall biosynthesis or protein synthesis at its restrictive temperature, and released ribonuclease from the periplasm. All these observations were indicative of alterations of the cell envelope (Sturgeon and Ingram, 1978), which was unaffected in *E. coli* MFT1181. JS10 did not display any impairment in DNA or RNA synthesis at either temperature (Sturgeon and Ingram, 1978). The locus conferring the temperature sensitive phenotype was mapped between 74 and 79 minutes on the chromosome (Sturgeon and Ingram, 1978). It was considered possible that the mutation in strain JS10 could be allelic to *ftsE*, or that it could be in a new cell division gene (Sturgeon and Ingram, 1978).

Through the use of generalized transduction and transducing lambda phages, the mutation in strain JS10 was subsequently mapped to *ftsE* and the mutation was designated *ftsE2000* (Salmond and Plakidou, 1984). These analyses also identified a new cell division locus that was named *ftsS* (Salmond and Plakidou, 1984). The two genes mapped at approximately 76 minutes on the *E. coli* chromosome (Salmond and Plakidou, 1984).

A 4.5 kilobase (Kb) *Hin*dIII fragment of the wild-type *E. coli* chromosome was able to complement strain MFT1181. The complete nucleotide sequence of this fragment was determined and the analysis of its DNA sequence revealed the presence of four genes in this region: *ftsS*, *ftsY*, *ftsE* and *ftsX*, with *ftsS* being divergently transcribed from *ftsY*, *ftsE* and *ftsX* (Gill *et al.*, 1986). *ftsY* and *ftsE* were separated by two base pairs (bp), *ftsE* and *ftsX* overlapped by seven bp, and *ftsY* and *ftsS* were separated by 150 bp (Fig. 3; Gill *et al.*, 1986).

Figure 3: Genetic organization of *ftsY*, *ftsE* and *ftsX* in selected bacteria. The cluster of *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Gill *et al.*, 1986), *H. pylori* (Tomb *et al.*, 1997), *M. tuberculosis* (Devlin *et al.*, 1997, Tyagi *et al.*, 1996), and *M. leprae* (Parkhill *et al.*, 1997, 1997b) are represented. Each gene is represented by a colored box with the size (in bp) indicated underneath. The number above the boxes indicates the spacing (in bp) between the genes (negative numbers indicate overlaps, and zero indicates that the genes are contiguous without overlapping).


H. influenzae
9
13
ftsY
ftsE
ftsX
1245
657
933
E. coli
2
-7
ftsY
ftsE
ftsX
1494
669
1059
H. pylori
-10
ftsE
ftsX
11.7 Kb
ftsY
669
804
879
M. tuberculosis
0
ftsE
ftsX
690
894
M. leprae
0
ftsE
ftsX
690
864
1 Kb

Insertions of Tn*1000* in *ftsY* or *ftsE* abolished complementation of strains MFT1181 and JS10 while an insertion inside of *ftsX* allowed complementation of strain MFT1181 but not of strain JS10 (Gill *et al.*, 1986). These observations indicated that the mutation in strain JS10 was located within the *ftsX* gene. These data also showed that *ftsY*, *ftsE* and *ftsX* likely formed an operon in *E. coli* because of the polar effect of the insertion of Tn*1000* on the genes downstream (Gill *et al.*, 1986). Further characterization of *ftsY* showed that one of the Tn*1000* insertions initially believed to be in *ftsY* (Gill *et al.*, 1986) was actually in the first codon of *ftsE* (Gill and Salmond, 1990), and it was found that an insertion in *ftsY*, preventing synthesis of FtsY, did not abolish the synthesis of FtsE and FtsX as the strains MFT1181 and JS10 could be complemented (Gill and Salmond, 1990). Thus, an insertion in *ftsE* has a polar effect on *ftsX*, but the polar effect of an insertion in *ftsY* on *ftsE* and *ftsX* is less clear, and more investigation is required to show the polar effect of a mutation in *ftsY* on *ftsE* and *ftsX*. The *ftsY*, *ftsE* and *ftsX* genes were located at 77.6 minutes on the most recent map of the *E. coli* chromosome (Berlyn *et al.*, 1995).

Through genome projects, *ftsE*, *ftsX* and *ftsY* have been identified in several other bacteria and there is variability in their genetic organization (Fig. 3): *ftsY*, *ftsE* and *ftsX* are also clustered in *Haemophilus influenzae*: *ftsY* lies 9 bp upstream of *ftsE* which lies 13 bp upstream from *ftsX* (Fleischmann *et al.*, 1995). In *Helicobacter pylori*, *ftsE* and *ftsX* overlap by 1 bp but *ftsY* is located 10 Kb downstream from *ftsX* (Tomb *et al.*, 1997). A homologue of *ftsY* has been identified in *Mycobacterium leprae* but is not within 26.8 Kb upstream of *ftsE* (Parkhill *et al.*, 1997a, 1997b). A homologue of *ftsY* remains to be identified in *Mycobacterium tuberculosis*, and it is not located within 25 Kb upstream of *ftsE* and *ftsX* (Devlin *et al.*, 1997). Homologues of *ftsE* and *ftsX* have been identified in *M. tuberculosis* and in *M. leprae* and they are immediately contiguous in each bacterium (Parkhill *et al.*, 1997a; Tyagi *et al.*, 1996). Only *ftsY* is present in the genomes of *Mycoplasma genitalium*, *M. pneumoniae*, and the cyanobacterium *Synechocystis* sp. 6083 (Himmelreich *et al.*, 1996; Kaneko *et al.*, 1996; Fraser *et al.*, 1995). *ftsY* has been identified in *Mycoplasma mycoides*, in which it is followed by a gene encoding a protein involved in transport (Macao *et al.*, 1997). None of the three genes is present in the methanogenic archaeon

Methanococcus jannaschii (Bult *et al.*, 1996). A homologue of FtsY has been identified in the genome of another archaea, *Sulfolobus solfataricus* (Ramirez and Matheson, 1991). This putative *S. solfataricus ftsY* gene homologue is flanked by genes encoding proteins that do not share aa sequence similarity with FtsE and FtsX (Ramirez and Matheson, 1991). The presence of *ftsE* and *ftsX* has not been verified in this archaea.

There has been little work done on the regulation of the expression of the *ftsY*, *ftsE* and *ftsX* genes. There are differences in the regulation of the expression of these genes between *E. coli* and *M. tuberculosis*. Two promoters whose exact location has not been determined have been identified for *ftsY*, *ftsE* and *ftsX* in *E. coli*: a first promoter (P_Y) located upstream of *ftsY* from which all three genes were expressed, and a second promoter located upstream of *ftsE* in the 3'-section of *ftsY* (P_E) from which *ftsE* and *ftsX* were expressed (Gill and Salmond, 1990). A *ftsX*-specific promoter has been identified in *M. tuberculosis* (Tyagi *et al.*, 1996; section 1.5.2.). The regulation of the expression of *ftsY*, *ftsE* and *ftsX* has not been investigated in other bacteria.

1.5. The FtsE, FtsX and FtsY proteins

1.5.1. The FtsE protein

The FtsE protein is associated with the inner membrane in *E. coli* (Gill and Salmond, 1987). Based on the sizes of the known FtsE polypeptides, the average size of FtsE is 224 aa, which is smaller than FtsY and FtsX (Table 1). The first FtsE homologue to be characterized was from *E. coli* (Gill *et al.*, 1986). Sequence similarity searches indicated it shared similarity with other proteins involved in transport processes and that the similarities were the highest around two regions of the polypeptides, which were presumed to be nucleotide-binding motifs (Gill *et al.*, 1986; Higgins *et al.*, 1986). These two motifs, the Walker motifs A and B, have since been recognized to be the signature sequence of the members of the ABC protein family (sections 1.2. and 1.2.1.). Based on these results, FtsE was considered to be an ATP-binding protein involved in an uncharacterized aspect of cell division (Gill *et al.*, 1986.). Studies of *E. coli* FtsE by random

mutagenesis showed the importance of the linker peptide for FtsE function, as mutations of aa residues part of the linker peptide rendered the bacteria Ts (Gill *et al.*, 1992; section 1.2.2.).

Table 1. Reported sizes in amino acids of the FtsY, FtsE and FtsX proteins in bacteria.

Bacterium	FtsY (aa)	FtsE (aa)	FtsX (aa)	Reference
E. coli	497	222	352	Gill *et al.*, 1986
H. influenzae	414	218	310	Fleischmann *et al.*, 1995
H. pylori	293	223	268	Tomb *et al.*, 1997
M. genitalium	346	NP[1]	NP[1]	Fraser *et al.*, 1995
M. leprae	430	229	287	Parkhill *et al.*, 1997a, 1997b
M. mycoides	411	ND[2]	ND[2]	Macao *et al.*, 1997
M. pneumoniae	346	NP[1]	NP[1]	Himmelreich *et al.*, 1996
M. tuberculosis	ND[2]	229	297	Devlin *et al.*, 1997; Tyagi *et al.*, 1996
S. solfataricus	369	ND[2]	ND[2]	Ramirez and Matheson, 1991
Synechocystis	504	NP[1]	NP[1]	Kaneko *et al.*, 1996

[1]NP: not present in this organism. In all cases, the complete genome sequence of the organism has been determined through a genome project.

[2]ND: not determined.

The status of *ftsE* as a cell-division gene is controversial since it has been shown that the filamentous phenotype of *E. coli* strain MFT1181, a *ftsE* Ts mutant, was medium-dependent (Taschner *et al.*, 1988). Indeed, MFT1181 cells could only form filaments in rich medium at their

restrictive temperature; in minimal medium of low osmolarity, they formed small chains of swollen cells. This swelling was suppressed in minimal medium of higher osmolarity but failed to restore the filamentous phenotype. In contrast, Ts mutants of other known cell division genes (*ftsZ*, *ftsQ*, *ftsA* and *ftsI*) all formed filaments at their restrictive temperature in either type of culture medium (Taschner *et al.*, 1988). However, two *E. coli* mutants blocked in the earliest stages of cell division were found to be mutated in *ftsE* (Begg *et al.*, 1995). Also, FtsE and FtsX were found to interact with the SRP (Appendix 1) in *E. coli* (Ulbrandt *et al.*, 1997). Since it had been shown that disruption of the SRP pathway by preventing the expression of *ffh* (encoding the fifty-four homologue protein (Ffh), the bacterial homologue of SRP54, Römisch *et al.*, 1989; Appendix 1) led to cell filamentation in *E. coli* (Patel and Austen, 1995; Phillips and Silhavy, 1992), it suggested that both genes encoded cell division proteins.

1.5.2. The FtsX protein

The FtsX protein is also associated with the inner membrane in *E. coli* (Gill and Salmond, 1987). FtsX encodes a protein larger than FtsE but is more heterogeneous in size, the largest consisting of 352 aa residues (*E. coli*) and the shortest comprising 268 aa residues (*H. pylori*), with an average size of 305 aa residues, based on the sizes of the known FtsX polypeptides (Table 1). The biological function of FtsX has not been investigated. From aa sequence analyses, the FtsX proteins from *M. tuberculosis* and *E. coli* were predicted to contain four transmembrane segments, suggesting they were located in the bacterial membrane (Tyagi *et al.*, 1996). These results correlated well with the membrane-associated location for FtsX observed in *E. coli* (Gill and Salmond, 1987). The *ftsE/ftsX* gene products interact with the SRP, like many other integral membrane proteins (Ulbrandt *et al.*, 1997). FtsX homologues do not share significant amino acid sequence similarities with other protein sequences in the databases.

In *M. tuberculosis*, the expression of *ftsX* was found maximized in the log phase of growth in liquid cultures and was negligible in a 20 day stationary culture, correlating well with the division state of the bacteria in each case (Tyagi *et al.*, 1996). The possible correlation of *ftsX*

expression with the growth phase has not been investigated in other bacteria. The filamentous phenotype of *E. coli* JS10, the *ftsX* Ts mutant, has not been investigated in different types of culture media as has been done with *E. coli* MFT1181 (section 1.5.1.).

1.5.3. The FtsY protein

The size of FtsY varies between bacteria, from 293 aa residues in *H. pylori* to 504 aa residues in the *Synechocystis* sp. 6083 (Table 1). The size difference between the bacterial FtsY homologues was attributed to the size of its amino-terminal portion (Macao *et* al., 1997): the amino-terminal portion of FtsY comprised 197 aa residues in *E. coli*, compared to 100 aa residues in *M. mycoides* and about 40 aa residues in *M. genitalium* and *M. pneumoniae*. The amino-terminal region of FtsY was characterized by an abundance of charged aa residues: they accounted for as much as 50% of the aa residues in *M. mycoides* FtsY (Macao *et* al., 1997), and 30% of the aa residues in *E. coli* FtsY were acidic (Gill and Salmond, 1990).

A putative nucleotide-binding site was identified in the carboxy-terminal region of *E. coli* FtsY (Gill *et al.*, 1986). *E. coli* FtsY was later found to share aa sequence similarity with SRα, suggesting that FtsY may have a role in protein transport (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). The region of FtsY containing the nucleotide-binding motif was located within the region sharing the highest aa similarity with SRα (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). *E. coli* FtsY shared aa sequence similarity with members of the GTPase superfamily, and was found to be closest in aa sequence to proteins involved in protein transport such as a 54 KiloDalton (Kda;) polypeptide (SRP54, a component of the eukaryotic SRP; Appendix 1) identified in mice, and *E. coli* Ffh. Based on aa sequence similarities with other members of the GTPase family, four regions of FtsY were predicted to be important for GTP binding; the first of which corresponded to the nucleotide-binding motif initially identified by Gill *et al.* (1986) (Bourne *et al.*, 1991). The introduction of mutations in the nucleotide-binding motifs of *E. coli* and *M. mycoides* FtsY altered or abrogated GTP binding (Macao *et al.*, 1997; Ulbrandt *et al.*, 1997; Kusters *et al.*, 1995).

FtsY is now considered to be a component of the bacterial SRP receptor (Luirink and Dobberstein, 1994; Wolin, 1994). FtsY was predicted to lack transmembrane segments (Gill and Salmond, 1990) and is therefore associated with the inner membrane through interactions with another protein.

Powers and Walter (1997) proposed that the function of the NH_2-terminal domain of FtsY was to increase the association of FtsY with the cytoplasmic membrane. The amino-terminal domain of FtsY serves to target the nucleotide-binding domain of FtsY to the membrane of the cell (Zelazny *et al.*, 1997); this was shown by the inability of FtsY mutants with deleted amino-terminal domains to support bacterial growth. Similar results were obtained by using TrpE-FtsY fusions, in which the nucleotide-binding domain of FtsY was fused to the amino-terminal domain of the cytoplasmic protein TrpE (Zelazny *et al.*, 1997). In contrast, LacY-FtsY fusions, in which the nucleotide-binding domain of FtsY was fused to the amino-terminal domain of the membrane protein LacY, were able to support bacterial growth (Zelazny *et al.*, 1997). All these data also showed that FtsY was required to be located at the inner membrane to properly perform its biological function.

Evidence for the involvement of FtsY in protein transport was provided when *E. coli* FtsY was shown to bind a complex formed by Ffh and 4.5S RNA, two other components of a protein transport pathway in bacteria (Miller *et al.*, 1994; Appendix 1). This interaction between FtsY, Ffh and 4.5S RNA was shown by affinity chromatography and was dependent on GTP hydrolysis (Miller *et al.*, 1994). The GTP hydrolysis activity of *E. coli* FtsY was shown to be dependent on the formation of a complex between FtsY and Ffh-4.5S RNA (Miller *et al.*, 1994). Similar results were obtained with *M. mycoides* FtsY (Macao *et al.*, 1997). Other studies showed that FtsY had interactions with the SRP in *E. coli*, which fit in well with the role of FtsY in protein transport (Ulbrandt *et al.*, 1997). FtsY from *E. coli* bound GTP, and while FtsY could bind Ffh-4.5S RNA in the presence of a GTP analogue, FtsY mutants with a lowered affinity for GTP could only bind Ffh-4.5S RNA with reduced efficiency or not at all (Kusters *et al.*, 1995). Other studies showed that FtsY and Ffh-4.5S RNA stimulated each other's GTPase activity if both proteins were in a

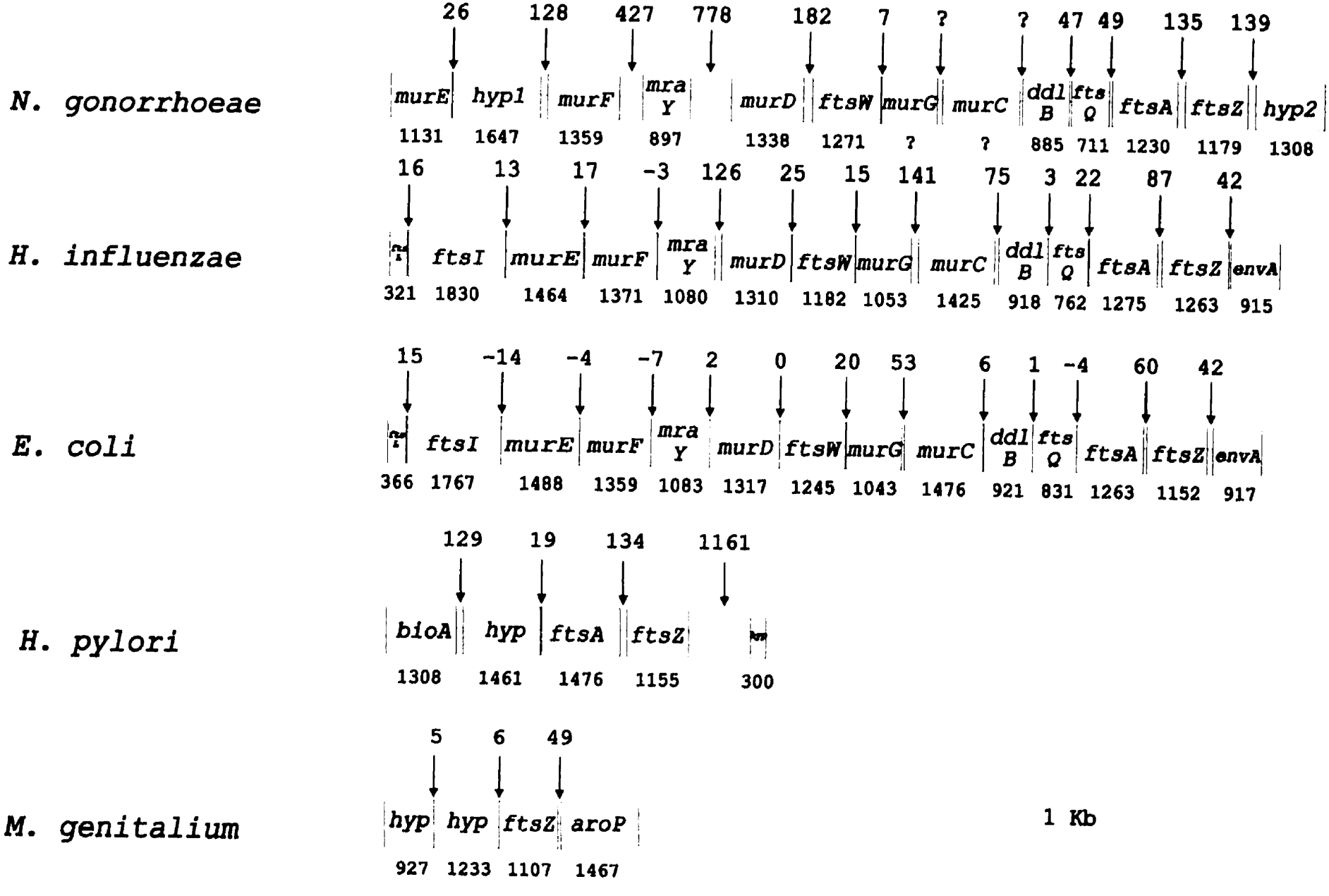
GTP-bound state (Powers and Walter, 1995), and that protein targeting by FtsY and Ffh-4.5S RNA took place only if GTP was available (Powers and Walter, 1997).

Also supporting a role for FtsY in protein transport, FtsY was shown to affect the transport of some proteins in *E. coli* although the evidence was contradictory in some cases: overproduction of FtsY caused an accumulation of pre-β-lactamase but not of pre-OmpA (Luirink *et al.*, 1994). Depletion of FtsY in *E. coli* in a strain where *ftsY* was under the control of an inducible promoter abolished the translocation of the ribose-binding protein (RBP), β-lactamase, and OmpF without affecting the processing of pre-OmpA, pre-OmpC and pre-MBP (maltose-binding protein; Luirink *et al.*, 1994). In contrast, OmpA and RBP export were not affected and export of β-lactamase was only delayed in cells that overproduced a FtsY mutant with a dominant lethal phenotype (Ulbrandt *et al.*, 1997). FtsY was shown to be essential in *E. coli* for the expression of SecY and *lac* permease, two membrane proteins; FtsY affected only weakly the synthesis of β-lactamase and had no effect on β-galactosidase, a cytoplasmic protein (Seluanov and Bibi, 1997). Since *lac* permease and β-galactosidase are encoded by genes part of the same operon, Seluanov and Bibi (1997) deduced that the difference in their expression pattern was a reflection of post-translational, FtsY-related events.

1.6. The *dcw* cluster

The so-called *dcw* cluster contains the majority of the cell growth and cell division genes. This cluster includes sixteen genes located at two minutes on the chromosome of *E. coli* (Fig. 4; Berlyn *et al.*, 1995; Donachie, 1993). A similar cluster was found on the chromosome of *H. influenzae* but the spacing between the genes was different to that in *E. coli* (Fig. 4; Fleischmann *et al.*, 1995). There were more differences in the genetic organization of this cluster in *N. gonorrhoeae* (Fig. 4) as found through analyses of the DNA sequences generated through the Gonococcal Genome Project (Dyer *et al.*, 1996) conducted in our laboratory (section 1.8.1.; Radia *et al.*, unpublished data). This genetic organization was not ubiquitous among microorganisms.

Figure 4: Genetic organization of the *dcw* gene cluster in selected bacteria. The *dcw* clusters of *N. gonorrhoeae* (Radia *et al.*, unpublished data), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), *H. pylori* (Tomb *et al.*, 1997) and *M. genitalium* (Fraser *et al.*, 1995) are represented. Each gene is represented by a box with the size in bp indicated underneath. Genes encoding proteins that have been shown to participate in cell division are in red, and other genes are in black. The number above the boxes indicates the spacing between the genes (negative numbers indicate overlaps, and zero indicates that the genes are contiguous without overlapping). *hyp* indicates hypothetical genes. The size of the gonococcal *murG* and *murC* genes, and the intergenic spaces between *murG* and *murC* and between *murC* and *ddlB* could not be determined with the available data, and are indicated by question marks (?). On this figure, gene sizes of *murG* and *murC* and intergenic spaces between *murG* and *murC* and between *murC* and *ddlB* were determined arbitrarily by using the gene size and intergenic spaces from *E. coli* and *H. influenzae* and calculating the average.


N. gonorrhoeae
26 128 427 778 182 7 ? ? 47 49 135 139
murE hyp1 murF mra Y murD ftsW murG murC ddl B fts Q ftsA ftsZ hyp2
1131 1647 1359 897 1338 1271 ? ? 885 711 1230 1179 1308
H. influenzae
16 13 17 -3 126 25 15 141 75 3 22 87 42
ftsI murE murF mra Y murD ftsW murG murC ddl B fts Q ftsA ftsZ envA
321 1830 1464 1371 1080 1310 1182 1053 1425 918 762 1275 1263 915
E. coli
15 -14 -4 -7 2 0 20 53 6 1 -4 60 42
ftsI murE murF mra Y murD ftsW murG murC ddl B fts Q ftsA ftsZ envA
366 1767 1488 1359 1083 1317 1245 1043 1476 921 831 1263 1152 917
H. pylori
129 19 134 1161
bioA hyp ftsA ftsZ
1308 1461 1476 1155 300
M. genitalium
5 6 49
hyp hyp ftsZ aroP
927 1233 1107 1467
1 Kb

For instance *H. pylori* did not contain all the cell division genes identified in *E. coli* and *H. influenzae* and those present were not clustered (Fig. 4; Tomb *et al.*, 1997). Only *ftsA* and *ftsZ* were found clustered in *H. pylori* (Fig. 4), while *ftsI*, *ftsK* and *ftsW* were located elsewhere on the chromosome (Tomb *et al.*, 1997). Only the *ftsZ* gene was present in the genomes of *M. genitalium* (Fig. 4; Fraser *et al.*, 1995) and *M. pneumoniae* (not shown; Himmelreich *et al.*, 1996). The complete *fts-mur* locus has been studied in *Pseudomonas aeruginosa*, and its organization appeared very similar to that of *E. coli* and other bacterial species (not shown; Sanschagrin *et al.*, 1997). A cluster of cell division genes in *C. crescentus* appeared similarly organized as their homologues in *E. coli* (not shown; Ohta *et al.*, 1997). Only a few cell division genes have been identified in *S. typhimurium*: *divA*, *divC*, *divD*, *ftsZ*, *lkyD* and *min* (not shown; Sanderson *et al.*, 1995). In *S. typhimurium*, the *divC* gene that is involved in septum initiation and *ftsZ* were located close on the genetic map (not shown; Sanderson *et al.*, 1995). The other cell division genes were found scattered on the chromosome (Sanderson *et al.*, 1995).

A similar genetic organization to that found in *E. coli* and *H. influenzae* was observed in Gram-positive bacteria. In *Bacillus subtilis*, the putative cell division genes *ftsL*, *ftsA*, *ftsZ*, 4 *mur* genes, and *mraY* are clustered between 130 and 135 minutes on the genomic map (not shown; Biaudet *et al.*, 1996). The genes *yllB*, *yllC*, *yllD*, *ftsL*, *pbpC*, *mraY*, *murD*, *murG*, *div1B*, *ftsA* and *ftsZ* were clustered in *Enterococcus faecalis* and in *Staphylococcus aureus* (not shown; Pucci *et al.*, 1997). Preliminary indications suggest that several cell division genes are clustered in *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Pucci *et al.*, 1997).

In *Synechocystis* sp.6083, *ftsZ* and other putative cell division genes, *minCDE* (section 1.7.5.), *maf*, and one other uncharacterized cell division gene have been identified, but all were scattered over the genome (not shown; Kaneko *et al.*, 1996). In the archaeon *M. jannaschii*, the cell division gene were not clustered although homologues of *ftsZ*, *ftsJ*, and three homologues of *minD* have been identified (not shown; Bult *et al.*, 1996).

The *zipA* gene that encodes the membrane receptor for FtsZ (see section 1.7.1.) lies outside of the morphogene cluster, at 52 minutes on the *E. coli* chromosome (Hale and de Boer,

1997). Similarly, the *H. influenzae zipA* gene was not located close to the cluster of cell division genes (Hale and de Boer, 1997). A *zipA* homologue was identified in *S. typhimurium*, but none was found in *M. genitalium* or in *M. jannaschii* (Hale and de Boer, 1997). Similarity searches done in our laboratory on the gonococcal genome data base could not identify a *zipA* homologue in *N. gonorrhoeae* strain FA1090 (Victor and Dillon, unpublished data).

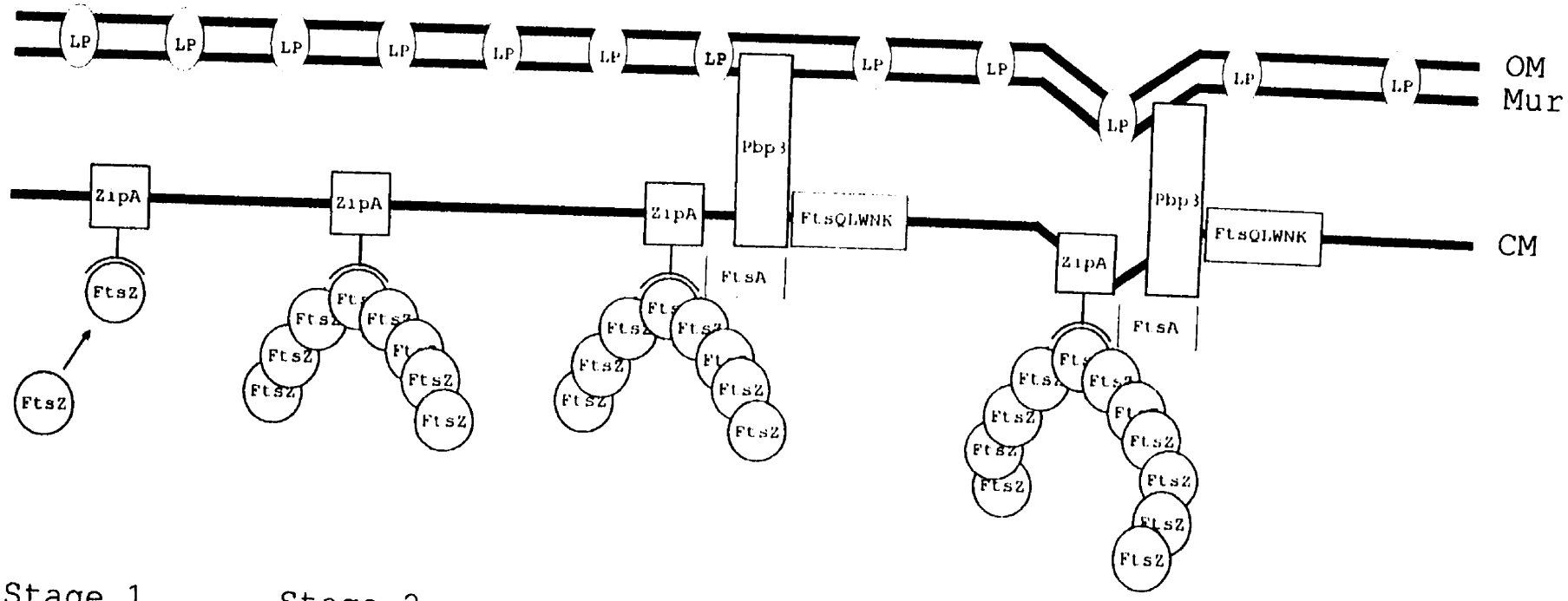
1.7. *E. coli* cell division occurs in four stages

According to the latest models, cell division in *E. coli* may be divided in four stages (Fig. 5, Rothfield and Justice, 1997): binding of FtsZ to its membrane anchor ZipA (Stage 1), formation of the FtsZ ring (Stage 2), assembly of the cytokinesis machinery (Stage 3), and cytokinesis (Stage 4). Each of the four steps are briefly described in this section. The putative cell division proteins FtsE and FtsX proteins are not part of this model, as the exact role of each protein in cell division has not been determined.

1.7.1. FtsZ-ZipA binding (Stage 1)

The early events of cell division (Fig. 5, Stage 1) involve the localization of FtsZ to the inner membrane, where it remains associated with the invaginating septum during cytokinesis (Rothfield and Justice, 1997). ZipA, the probable membrane receptor for FtsZ, was predicted to be a bitopic membrane protein, with its amino-terminal end crossing the inner membrane once with the rest of the protein in the cytoplasm, and was shown essential for septum formation (Hale and de Boer, 1997). *zipA* is an essential gene, and its overexpression caused the formation of unconstricted filaments, suggesting that ZipA acted early in cell division. The filamentous phenotype caused by *zipA* overexpression was relieved by *ftsZ* overexpression, suggesting interactions between ZipA and FtsZ. ZipA was found to be distributed in a ring at the site of cell division; this ring was always located at the site of constriction. It remains unclear as to which protein recruits the other to the future septation site (Hale and de Boer, 1997).

Figure 5: Proposed sequence of events during cell division in *E. coli* (from Rothfield and Justice, 1997). OM: outer membrane, LP: lipoprotein, Mur: murein layer (peptidoglycan, CM: cytoplasmic (inner) membrane.


LP
OM
Mur
CM
ZipA
FtsZ
Pbp3
FtsQLWNK
FtsA
Stage 1
Stage 2
Stage 3
Stage 4

1.7.2. FtsZ and the FtsZ ring (Stage 2)

The level of FtsZ was found critical for cell division in *E. coli*, as overproduction by two to seven-fold caused the formation of minicells whereas lowering the levels of FtsZ either by limitation of *ftsZ* expression using antisense RNA or by growing null mutants of *ftsZ* in which *ftsZ* was carried on a plasmid with a Ts replication defect inhibited cell division and caused the formation of filaments (Tétart *et al.*, 1992; Dai and Lutkenhaus, 1991; Ward and Lutkenhaus, 1985). FtsZ proteins from *E. coli* and from other microorganisms bind GTP and/or possess a GTPase activity (Wang *et al.*, 1997; Wang and Lutkenhaus, 1996a, 1996b; Mukherjee *et al.*, 1993; de Boer *et al.*, 1992; RayChaudhuri and Park, 1992). Associated with the inner membrane through interactions with ZipA (section 1.7.1.), FtsZ molecules form an annular structure, the FtsZ ring (Bi and Lutkenhaus, 1991b), extending around the circumference of the cell (Fig. 5, Stage 2). The FtsZ ring is believed to be formed by polymerization of FtsZ molecules in a GTP-dependent fashion, and may consist of one or more polymers extending completely around the cell up to twenty times, or alternatively could be a series of shorter polymers organized to form a continual annulus (Rothfield and Justice, 1997; Erickson *et al.*, 1996; Mukherjee and Lutkenhaus, 1994). FtsZ contains a highly conserved sequence motif (GGGTGTG) that is similar to the tubulin signature motif ((G/A)GGTG(S/A)G; Mukherjee and Lutkenhaus, 1994; Mukherjee *et al.*, 1993; de Boer *et al.*, 1992a; RayChaudhuri and Park, 1992): FtsZ polymers were remarkably similar to tubulin polymers (Erickson *et al.*, 1996), and FtsZ was assembled in filaments and tubules (Lutkenhaus and Addinall, 1997; Erickson, 1995). The constriction of the FtsZ ring plays a central role in triggering cytokinesis events (section 1.7.4.). The FtsZ ring may serve as a cytoskeletal element that provides the energy for the constriction of the cytoplasmic membrane and/or to act as a mooring to recruit other cell division proteins to the division site (Lutkenhaus and Addinall, 1997).

1.7.3. Assembly of the cytokinesis machinery (Stage 3)

The other cell division proteins (PBP3 (FtsI), FtsA, FtsL, FtsN, FtsQ, FtsW, FtsK) are thought to assemble at the future division site following the formation of the FtsZ ring (Fig. 5, stage 3). This is at least true for FtsA, FtsI, FtsQ and FtsN, as the FtsZ ring was shown to form in *ftsA*(Ts), *ftsI*(Ts) and *ftsQ*(Ts) mutants (Addinall and Lutkenhaus, 1996a) and in cells in which the FtsN level was severely reduced (Addinall *et al.*, 1997). These observations indicated that FtsA, FtsI, FtsQ and FtsN were not required for the formation of the FtsZ ring (Addinall *et al.*, 1997; Addinall and Lutkenhaus, 1996a).

The functions of other proteins involved in cytokinesis have been determined: FtsA may act as a link between FtsZ and other cell division proteins or may enhance the dynamics of the FtsZ ring (Addinall and Lutkenhaus, 1996b), or may link the FtsZ ring to septal specific peptidoglycan synthesis (Lutkenhaus and Mukherjee, 1995). PBP3, the product of the *ftsI* gene, is specifically required for the synthesis of septal peptidoglycan (Botta and Park, 1981; Spratt, 1977, 1975). Bitopic membrane proteins such as FtsQ, FtsL and FtsN are potential candidates for signaling molecules that could coordinate periplasmic processes such as peptidoglycan assembly to cytoplasmic events such as DNA synthesis or chromosome segregation (Guzman *et al.*, 1992). FtsW was proposed to be involved in the stabilization of the FtsZ ring (Boyle *et al.*, 1997). FtsK may be directly involved in the completion of the septa and to be a peptidoglycan-modifying enzyme (Begg *et al.*, 1995).

1.7.4. Cytokinesis (Stage 4)

The last stage of cell division involves the invagination of the septum (Fig. 5, Stage 4). This complex process involves the circumferential ingrowth of three layers of the cell envelope and is currently explained by two models (Rothfield and Justice, 1997). In the first model, the so-called PUSH model, the ingrowth of the peptidoglycan layer (perpendicular to the long axis of the bacterium) is triggered by a cell cycle signal transmitted via the division machinery at the midpoint of the cell (Rothfield and Justice, 1997). This ingrowth of the peptidoglycan then

"pushes" the inner membrane inward. In the second model, the PULL model, the constriction of the FtsZ ring "pulls" on the cytoplasmic membrane, thereby initiating the synthesis of septal murein by sending a signal to the septal murein-synthesizing machinery (Rothfield and Justice, 1997). FtsZ is present in bacteria with and without a cell wall (*Mycoplasma* species) and in archaea, in which the cell wall is chemically different that of Gram-negative bacteria (Madigan *et al.*, 1997). If FtsZ plays the same role in cell division apparatus in these organisms as it does in *E. coli*, it would imply that constriction of the FtsZ ring can occur independently of the ingrowth of the peptidoglycan, thus favoring the PULL model (Rothfield and Justice, 1997). The constriction of the FtsZ is likely an important triggering event in septum formation, with ingrowth of septal murein being a secondary or maybe parallel process responding to the same signal that activates the constriction of the FtsZ ring.

1.7.5. Other events of cell division

The selection of the proper site at midcell for the formation of the division septum requires three gene products: MinC, MinD and MinE. MinC and MinD act together to form the MinCD cell division inhibitor. The MinCD division inhibitor is required to prevent septation at the potential division sites at the cell poles, since loss of either MinC or MinD leads to the production of minicells (de Boer *et al.*, 1989). MinE is an anti-MinCD factor that provides topological specificity to the MinCD division inhibitor as it is capable of suppressing the activity of MinCD at midcell but not at the cell poles (Zhao *et al.*, 1995). The selection of the division site through MinCDE has recently been reviewed (Lutkenhaus and Addinall, 1997).

Other events of cell division (DNA segregation, peptidoglycan synthesis, cell separation) have not been reviewed here but have been reviewed elsewhere (van Heijenoort, 1996; Lutkenhaus and Mukherjee, 1995; Donachie, 1993).

1.8. Cell division in *Neisseria gonorrhoeae*

N. gonorrhoeae is a pathogenic bacterium causing the sexually transmitted disease gonorrhea (section 1.9.). The majority of the research efforts have been directed at elucidating the mechanisms of infection and pathogenicity (e.g., *pil* and *opa* genes, lipooligosaccharide (LOS) biosynthesis genes; Seifert, 1996; van Putten and Robertson, 1995; Koomey, 1994; Meyer *et al.*, 1994; Robertson and Meyer, 1992; Seifert, 1992; Meyer, 1990a, 1990b) and identifying a suitable candidate for the development of a vaccine (section 1.10.). The majority of other cellular and metabolic processes remain largely uncharacterized. Cell division is one major cellular event that has not received much attention.

1.8.1. Gonococcal cell division genes

Three cell division genes from *N. gonorrhoeae*, *ftsZ*, *penA* and *tpc*, have been identified and characterized by conventional cloning methods (see below). The genes flanking *ftsZ* and all the other cell division genes clustered with *ftsZ* in *N. gonorrhoeae* have been identified in our laboratory through the analysis of the *N. gonorrhoeae* sequences generated through the Gonococcal Genome Project (section 1.6.). The analyses done in our laboratory showed that the gene order of the main cell division cluster in *N. gonorrhoeae* was similar to that in *E. coli* and *H. influenzae* (Fig. 4; section 1.6.). The same analyses also showed that in *N. gonorrhoeae*, there were no overlaps between genes, and the spacing between genes was greater than what had been observed in *E. coli* and *H. influenzae* (Fig. 4; section 1.6.). In addition, the *ftsI* homologue was missing in the cluster in *N. gonorrhoeae*, there was a hypothetical gene (*hyp1*) inserted between *murE* and *murF*, and *envA*, the last gene of the cluster in *E. coli* and *H. influenzae*, was replaced by a hypothetical gene (*hyp2*; Fig. 4; Radia *et al.*, unpublished data).

The gonococcal homologue of *ftsZ* has been cloned and characterized in our laboratory (Radia *et al.*, unpublished data; Radia, 1997). The gonococcal *ftsZ* gene comprised 1179 bp encoding a protein of 392 aa with a predicted molecular weight (Mw) of 41 542 Daltons (Da) and contained the conserved motifs important for GTP binding (Radia, 1997). It shared 72%

similarity with the other known prokaryotic FtsZ proteins (Radia, 1997). Overexpression of the gonococcal *ftsZ* caused filamentation in *E. coli* as it had been observed when *ftsZ* from other bacteria was overexpressed in *E. coli* (Radia, 1997). The *ftsZ* gene is probably essential in the gonococcus as it is in other bacteria as all attempts to construct isogenic mutants of *ftsZ* failed (Radia, 1997). However, direct evidence of the participation of the gonococcal FtsZ protein in cell division in this bacterial species remains to be demonstrated.

Another putative gonococcal cell division gene, *penA*, encodes the PBP2 protein (Spratt, 1988). This protein is the gonococcal homologue of *E. coli* PBP3. PBP2 displayed 40% amino acid sequence similarity with PBP3 (Spratt, 1988). The *penA* gene was not located within the large cluster of cell division and cell growth genes identified in our laboratory (see above, and Fig. 4), contrary to *ftsI* that was located in this cluster in *E. coli* and *H. influenzae* (section 1.6.). This gene is likely to be widespread in the *Neisseriaceae* as it has been identified in *Neisseria perflava/sicca* (Perez-Castillo *et al.*, 1994) and in *N. meningitidis* (Spratt *et al.*, 1992, 1989). The *penA* gene has only been investigated as part of projects pertaining to antibiotic resistance and the role of PenA in gonococcal cell division remains to be demonstrated.

The third putative gonococcal cell division gene is the *tpc* (*tetrapac*) gene (Fussenegger *et al.*, 1996a). It is an ORF of 1040 bp encoding a protein of 346 aa with a predicted Mw of about 37 KDa (Fussenegger *et al.*, 1996a). The aa sequence of Tpc did not display any similarity to protein sequences available in the public databases (Fussenegger *et al.*, 1996a). Its only notable feature was a leader sequence that showed perfect identity with lipoprotein leader sequences. This leader sequence was shown to be essential for export and function of the Tpc protein although the lipoprotein nature of Tpc was unclear. *tpc* mutants showed diminished murein hydrolase activity, suggesting that Tpc might be a murein hydrolase, therefore the primary defect of *tpc* mutants likely relates to disfunction of bacterial cell division and cell separation. *tpc* mutants also appeared tetracoccal in shape (Fussenegger *et al.*, 1996a; section 1.8.2.). The *tpc* gene is preceded by the *folC* gene that encodes folylpoly-(γ)-glutamate-dihydrofolate synthetase, an enzyme

involved in the metabolism of one carbon molecules (Fussenegger *et al.*, 1996a, 1996b). It is possible that *folC* and *tpc* form a transcriptional unit (Fussenegger *et al.*, 1996b).

1.8.2. Electronic microscopic observations

Early investigations on gonococcal cell division were conducted by electron microscopy (EM) of dividing gonococci (Westling-Häggström *et al.*, 1977; Reyn *et al.*, 1970; Fitz-James, 1964). The earliest indications of division was a hemispherical constriction deeper on one side than the other (Fitz-James, 1964). These folds were present at the division plane. Observation of gonococci on agar slides indicated that the first expansion occurred perpendicular to the division plane, then ceased, and that subsequent growth occurred parallel to the existing septum to finally produce a tetrad of cells (Westling-Häggström *et al.*, 1977). Expansion of individual gonococci proceeded in only one dimension throughout virtually the entire cell cycle, but expansion in two dimensions was observed in some cells at the end of the division period. Growth in the second dimension began slightly before the distance from one pole to the division plane equaled cell width.

Active growth occurred in the septal region as treatment of growing gonococci with penicillin caused distortions in the septal region (Westling-Häggström *et al.*, 1977). *N. gonorrhoeae* was observed to divide by septation rather than by constriction. Cell division was initiated by an ingrowth of cytoplasmic membrane enclosing a fold of peptidoglycan that gave rise to complete septal structures. Septal peptidoglycan was often visualized as two separate layers (Westling-Häggström *et al.*, 1977). The completion of the cell wall was observed to occur much later in cell development (Fitz-James, 1964). Microscopic observation suggested that the cell wall material between two daughter cells matured slowly as it was not as dense as the cell wall surrounding the cell. Hence, a delay in separation of already divided cells accounted for the high proportion of apparently dividing duplex forms seen in cultures of gonococci (Fitz-James, 1964). This pattern of bidimensional growth was also observed for other *Neisseria* species such as *N. meningitidis* and *Neisseria pharyngitis* (Westling-Häggström *et al.*, 1977).

N. gonorrhoeae strains with an inactivated *tpc* gene, encoding a protein involved in late stages of gonococcal cell division were also observed by EM (Fussenegger *et al.*, 1996a; section 1.8.1.). Gonococci from such strains appeared tetracoccal in shape (i.e., two diplococci connected by a double murein layer at their interface). The inner membrane was separated and the outer membrane was continuous around the whole tetrapac. The outer membrane also appeared to bulge out and form blebs because invagination at the septum was blocked by the double murein layer. Tetrapacs were observed to use a third possible division plane and divided through an octapac intermediate; these octapac intermediates were rarely observed in cultures of *tpc* mutants. The *comL* gene, involved in competence for transformation, is also believed to encode a murein hydrolase (Fussenegger *et al.*, 1996c). Unlike *tpc* mutants, *comL* mutants had a diplococcal morphology but the cells are approximately two times smaller than wild-type gonococci (Fussenegger *et al.*, 1996c).

C. *Neisseria gonorrhoeae*

1.9. A brief history of gonorrhea

Gonorrhea is a disease that has been long known to man; there are references to gonococcal infection in old Chinese, Japanese, Egyptian and Vedic sources, as well as in the Bible (Kiple, 1993; McGrew, 1985). The first scientific observations on gonorrhea were made by Hippocrates (460-355 B.C.), who associated the infection with "the pleasures of Venus" (Palella *et al.*, 1997). Gonococcal infections were also described by the Greek physician Galen (120-200 A.D.) who coined the term gonorrhea ("flow of seed"), which originates from two greek words, "gonos" (seed), and "rheîn" (flow) (Jephcott, 1990). The term "clap", a colloquialism sometimes used to designate a gonococcal infection, first appeared in 1378 A.D. and probably referred to Les Clappiers or Clappoir, a district of Paris where prostitutes commonly worked at the time (Palella *et al.*, 1997). Following the great syphilis endemic that began in the last decade of the fifteenth

century, gonorrhea and syphilis were regularly confused and gonorrhea was widely considered to be the onset stage of syphilis (Kiple, 1993; McGrew, 1985). In 1767, the Scottish surgeon John Hunter inoculated himself with pustular discharge of a patient believed to have gonorrhea but developed syphilis (Kiple, 1993; McGrew, 1985); this experiment postponed scientific understanding of the two diseases by decades. In 1838, gonorrhea was finally shown to be a separate disease from syphilis by Philippe Ricord in Paris through a series of clinical observations and direct experiments (Kiple, 1993; McGrew, 1985). The bacterium responsible for the sexually transmitted disease gonorrhea was isolated in 1879 by Albert Neisser at the University of Breslau in Germany (Kiple, 1993). Ernst von Bumm, one of Neisser's research associates, later cultivated the bacterium, named *Neisseria gonorrhoeae*, and inoculated human volunteers to prove its pathogenicity in pure culture (Kiple, 1993).

1.10. *N. gonorrhoeae* is a pathogen of world-wide importance

The World Health Organization (WHO) estimated that there would be 250 million new cases of sexually transmitted diseases (STD) occurring throughout the world in 1995, and that 25 million of these would be cases of gonorrhea (Pan American Health Organization, 1994). Gonorrhea is an STD of world-wide importance and is extremely common in developing areas of the world (Morse, 1991). The incidence in some African countries was estimated to be between 3000 and 10 000 cases per 100 000 population (Kiple, 1993; Morse, 1991). Such rates were much higher than those observed in industrialized countries; for instance there were 21 cases per 100 000 population in Canada in 1994 (Canada Communicable Disease Report, 1996.), 14 cases per 100 000 population in Sweden in 1989 (Danielsson, 1990) and 168 cases per 100 000 population in the United States in 1994 (Division of STD Prevention. Sexually Transmitted Disease Surveillance, 1994). Nonetheless, gonorrhea remains one of the most important bacterial STDs in industrialized countries (Gillespie, 1994).

In Canada in 1994, gonococcal infections ranked sixth among the forty-six reportable infections and were ranked the second-most common bacterial sexually transmitted infection

(Canada Communicable Disease Report, 1996). The number of reported cases has been steadily decreasing in Canada: from more than 56 000 reported cases in 1982 to 6167 reported cases in 1994 (Canada Communicable Disease Report, 1996). The incidence has decreased almost eight-fold since 1985, from 162.1 cases per 100 000 population in 1985 to 21 cases per 100 000 population in 1994 (Canada Communicable Disease Report, 1996). The incidence of gonorrhea is the highest among females aged between 15 to 24 years old and men aged between 20 to 24 years old (Canada Communicable Disease Report, 1996).

Humans are the only natural host for *N. gonorrhoeae*; gonococcal infections are acquired almost exclusively by sexual contact. It therefore depends entirely on human behavior for successful transmission. This becomes especially important considering that up to 50% of males and females with gonococcal infections may be asymptomatic (Canada Communicable Disease Report, 1992). The consequences of these asymptomatic infections can be a chronic carriage of the bacterium and sexual contacts will also be more likely to be asymptomatic (Canada Communicable Disease Report, 1992).

The incubation time of the disease varies between two to seven days (Canada Communicable Disease Report, 1992; Jephcott, 1990). In men, the most common symptom of uncomplicated gonorrhea is a discharge that may range from scanty, clear or cloudy fluid to one that is copious and purulent and often accompanied by difficulty or pain in urination (Morse, 1991). The infection may spread to the prostate, the seminal vesicles and Cowper's glands; the epididymes may also become infected and in such cases, inflammation may result in sterility (Jephcott, 1990). Endocervical infection is the most common form (i.e., that does not spread beyond the cervix) of gonorrhea in women. Such infections are usually characterized by discharge and sometimes dysuria (Morse, 1991). Gonococci in women may ascend form the endocervical canal through the endometrium to the fallopian tubes and finally to the peritoneum, causing complications such as endometritis, salpingitis or peritonitis (Morse, 1991). These infections can cause pelvic and abdominal pain, fever, chills, and cervical motion tenderness, all which are symptoms of pelvic inflammatory disease (PID; Morse, 1991). More serious complications may

arise from PID such as tubo-ovarian abscesses, pelvic peritonitis, or the Fitz-Hugh and Curtis syndrome (FHCS), an inflammation of Glisson's capsule of the liver (Lopez-Zeno *et al.*, 1985). FHCS is an extrapelvic manifestation of PID. Ultimately, PID may lead to involuntary infertility or ectopic pregnancy (Salyers and Whitt, 1994; Morse, 1991). The importance of bacterial STDs as human pathogens rests in part on their adverse effects on reproductive health (Sparling *et al.*, 1994), and the brunt of these adverse effects is borne by women (Adimora *et al.*, 1994).

Bacterial STDs, including gonorrhea, facilitate the heterosexual transmission of HIV (Wasserheit, 1992; Aral and Holmes, 1991; Cameron *et al.*, 1989). Gonococcal infections are believed to increase HIV transmission by a factor of 3.5 to 8.9 among women and by less than a two-fold factor in homosexual men (Wasserheit, 1992). STDs may increase the heterosexual transmission of HIV by producing genital inflammation in HIV-infected people, thereby increasing the shedding of HIV in genital secretions, or by producing genital inflammation in individuals who are sexually exposed to HIV, thus enhancing susceptibility to HIV acquisition (Holmes, 1994; Sparling *et al.*, 1994). These observations led the WHO to conclude that an effective strategy to control HIV should include the development of effective programs to achieve the control of bacterial STDs (Sparling *et al.*, 1994). Long lasting effective strategies will require the availability of vaccines to prevent bacterial STDs (Sparling *et al.*, 1994).

N. gonorrhoeae isolates constantly develop novel antibiotic resistance. The first drugs used to treat gonococcal infections were sulfonamides, and these were replaced by penicillin when it became widely available, because gonococcal isolates had rapidly developed resistance to sulfonamides (Kiple, 1993). By the early 1970s, strains with low-level chromosomally-mediated penicillin resistance were observed in may parts of the world, especially in Southeast Asia (Palella *et al.*, 1997). Penicillinase-producing isolates of *N. gonorrhoeae* (PPNG) were first reported in 1976 in strains originating from Africa and the Far East (Elwell *et al.*, 1977; Roberts *et al.*, 1977). PPNG isolates were subsequently reported worldwide (Sehgal and Srivatsava, 1987). This penicillin resistance is caused by a family of several structurally related plasmids (Aman, 1994; Dillon and Yeung, 1989). Tetracycline resistance, emerging resistance to

ciprofloxacine, and reduced susceptibility to ceftriaxone have been observed in clinical isolates of *N. gonorrhoeae*.

Gonococcal infections are treatable with several new antibiotics (fluroquinolones such as ciprofloxacin or 3rd generation cephalosporins such as ceftriaxone), but the ever-evolving resistance of *N. gonorrhoeae* to antibiotics constantly necessitates newer, more expensive antibiotics, which are often unavailable in developing countries (Sparling *et al.*, 1994). The development of vaccines against bacterial STDs has thus been advocated by the WHO as a necessary tool for an effective strategy against HIV since bacterial STDs sometimes play an accessory role in the spread of HIV. The development of a vaccine against *N. gonorrhoeae* has received the greatest attention over the years because of its importance as a pathogen (Adimora *et al.*, 1994), and the need for this vaccine was first recognized in 1963 (WHO expert committee on gonococcal infections, 1963). However, since the first immunization attempts using autolyzed gonococci (Greenberg *et al.*, 1971) and many efforts in the following years, there is still no vaccine to cure or prevent gonococcal infections (Blake and Wetzler, 1995; Adimora *et al.*, 1994; Sparling *et al.*, 1994). The principal cause of failure to develop a successful vaccine is partly due to the choice of targets for vaccine development. It was discovered that the gonococcal proteins chosen as vaccine targets were subject to intense antigenic variation, pilin proteins being a good example (Seifert, 1996; Koomey, 1994; Meyer *et al.*, 1994; Robertson and Meyer, 1992a; Seifert, 1992; Meyer, 1990a, 1990b). Attempts were made to raise a vaccine against the LOS but it was discovered that the genes encoding components of the LOS were regulated by mechanisms allowing the expression of variants (van Putten and Robertson, 1995), as is the case for pilin proteins. Few attempts have been made to create vaccines raised from Opacity (Opa) proteins, as they are subject to antigenic variation as well (Meyer *et al.*, 1994; Sparling *et al.*, 1994; Robertson and Meyer, 1992a; Seifert, 1992; Meyer, 1990a, 1990b)).

1.11. Characterization of the chromosome of *N. gonorrhoeae*

The structure and organization of the gonococcal chromosome has been under investigation for several years. The initial work was a literature review of the 75 known loci that had been identified by mutational analysis with phenotypical changes and complementation, and of gene products that had been well characterized by other means than mutagenesis (West and Clark, 1989). Later, macrorestriction maps of *N. gonorrhoeae* strains FA1090 (40 loci) and MS11 (with over 60 loci) were constructed and genes were localized on this map by means of hybridizations (Dempsey and Cannon, 1994, 1991; Bilhmaier *et al.*, 1991). The last genetic map of *N. gonorrhoeae* FA1090 to be published included 68 mapped loci (Dempsey and Cannon, 1994). However, these macrorestriction maps will soon be obsolete for the purpose of mapping genes as the determination of complete nucleotide sequence of the genome of *N. gonorrhoeae* strain FA1090 was undertaken. The complete revised sequence of the gonococcal genome is expected to be completed at the end of 1997 (Dyer *et al.*, 1996).

The gonococcal uptake sequence (US) is a well-known important sequence feature of *N. gonorrhoeae*. The gonococcal US is a 10 bp sequence (5'-GCCGTCTGAA-3') that was proposed to play a significant role in the regulation of gene expression (Goodman and Scocca, 1988). The US are often located downstream of genes in the form of inverted repeats, and may form a stem structure that could act as a transcriptional terminator (Barber *et al.*, 1994; Goodman and Scocca, 1988). The gonococcal US was proposed to be present at one copy per Kb of chromosomal DNA (Goodman and Scocca, 1991). The gonococcal US was also shown to be important for bacterial transformation, as double- and single-stranded DNA, as well as circular or linear plasmid DNA could be transformed in to *N. gonorrhoeae* as long as they contained the gonococcal US (Stein, 1991). Only DNA molecules containing the gonococcal US were specifically internalized by gonococci (Elkins *et al.*, 1991; Goodman and Scocca, 1988).

D. Hypotheses and goals

The molecular biology of gonococcal cell division remains largely uncharacterized and the cell division process in coccal bacteria has not received much attention with the focus being on *E. coli* (section 1.7.). The gonococcal *ftsZ* gene was characterized in our laboratory and we used the data generated by the gonococcal genome project (Dyer *et al.*, 1996) to identify most of the cell division genes in *N. gonorrhoeae* by performing similarity searches using the sequences of known cell division genes from other bacteria (Radia *et al.*, unpublished data; section 1.8.1.). The presence in *N. gonorrhoeae* of the majority of the cell division genes identified in *E. coli* and their similar overall organization suggests that the overall process of cell division in *N. gonorrhoeae* and *E. coli* is similar.

In the initial stages of this project, we sequenced plasmid clones selected from a *N. gonorrhoeae* genomic bank and one of these contained a complete homologue of the *E. coli ftsX* gene. Because *ftsX* had been proposed to be involved in cell division, and a project involving the molecular characterization of gonococcal cell division genes was being initiated in our laboratory, the identification and molecular characterization of the gonococcal homologues of the *E. coli ftsYEX* genes became the focus of this study.

This project was initiated before the gonococcal genome project was launched, when only *penA* had been identified as a putative cell division gene in *N. gonorrhoeae* (section 1.8.1.). The starting hypothesis is thus that the *ftsY*, *ftsE* and *ftsX* genes are present and clustered in *N. gonorrhoeae* as in *E. coli* and *H. influenzae*, and that the gene products of these three genes are involved in an uncharacterized aspect of cell division (section 1.4. and 1.5.). FtsE from *E. coli* was found to share aa sequence similarities with the ATP-binding domains of ABC transporters (section 1.2.1.); based on the existing knowledge on the organization of genes encoding ABC transporters, it is also hypothesized that *ftsY*, *ftsE* and *ftsX* encode the subunits of an ABC transporter. FtsY could be a protein peripherally associated with this putative ABC transporter, as there is considerable evidence for the role of FtsY in protein transport (section 1.5.3.).

The first goal of this project is to identify and characterize the putative *ftsY*, *ftsE* and *ftsX* cell division gene cluster in the gonococcus and to study their expression.

The biological function of FtsE and FtsX has not been determined (section 1.5.). The second goal of this project is to determine the biological function of these proteins, and to confirm their involvement in cell division. Mutations in *ftsE* or *ftsX* in *E. coli* render the bacterium thermosensitive and they form filaments at their restrictive temperature (section 1.4.). The *ftsX* gene will be insertionally inactivated in *N. gonorrhoeae* to verify if it is an essential gene. The mutation in *E. coli* JS10 altered the antibiotic resistance of the strain. The effect of inactivating *ftsX* on the antibiotic resistance profile of *N. gonorrhoeae* will also be verified.

The *ftsE* and *ftsX* genes are not present in all bacterial genera (section 1.4.). The third goal of this project is to investigate the prevalence of the *ftsE* and *ftsX* genes in the *Neisseriaceae*.

As a last objective, the genes flanking *ftsY*, *ftsE* and *ftsX* will be identified and characterized.

PART 2. MATERIALS AND METHODS

2.1. Bacterial strains, growth conditions, plasmids and DNA

The bacterial strains and plasmids used in this work are listed in Table 2, and their construction is described in the appropriate section of the methods. *E. coli* DH5α was used for the construction of a bank of *N. gonorrhoeae* CH811 DNA. *E. coli* ligATor cells (R&D Systems, Abingdon, United Kingdom) were used as hosts for all constructs made using the pTAg plasmid unless otherwise specified. *E. coli* INVαF' was used as the host strain for all other cloning procedures.

All *N. gonorrhoeae* isolates were grown on half-strength GC medium base (GCMB; Difco Laboratories, Detroit, Michigan) supplemented with modified Kellogg's Defined Supplement for 18-24 hours at 35°C in a humid environment supplemented with 5% CO_2 (Picard and Dillon, 1989a). GCMB was supplemented as needed with 100 mM KCl, 400 mM sucrose or 300 mM urea. GCMB with varying concentrations of NaCl (0-2%) was prepared from its basic components as described (Difco Manual, 1990) and the required amount of NaCl. The pH of the GCMB was adjusted as needed using 5 N NaOH or 10 M HCl when needed. *N. gonorrhoeae* strains CH811 and CS19 were grown anaerobically on GCMB with Kellogg's defined supplement onto which a sterile disk (Schleicher & Schuell, Keene, NH) inoculated with 40 µL of 2.4 M $NaNO_2$ had been centrally placed. Plates were incubated overnight in a humid environment consisting of 5% CO_2, 7% H_2, and 88% N_2 at 35°C in an anaerobic chamber (Forma Scientific, Marietta, OH). The strains were subcultured twice before being used for colony counts, and all subculturing was performed within the anaerobic chamber to ensure that no residual oxygen was present. GCMB and *N. gonorrhoeae* chemically defined medium (Hendry and Stewart, 1979) plates were pre-reduced for at least 24 h in the anaerobic chamber before use.

Table 2. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Source/Ref.
N. gonorrhoeae CH811	prototrophic strain/serovar IB-2/plasmid free	Garcia-Moreno *et al.* (1987)
N. gonorrhoeae CH811Str^R	derivative of *N. gonorrhoeae* CH811 that is resistant to streptomycin	Li and Dillon (unpublished data)
N. gonorrhoeae CS19	derivative of CH811, its *ftsX* gene is disrupted by a 2.9 Kb blunted *Acc*I-*Ava*I fragment containing *cat* from pACYC184 cloned in the *Nru*I site	This work
E. coli AT3141	Hfr *thr*-1 *leu*B6 *pro*A30 *lac*Z4 *gln*V44 LAM$^-$ *rps*L8 *arg*D37 *thi*-1 Mu+	Taylor, 1970
E. coli DH5α	F$^-$*deo*R *end*A1 *gyr*A96 *hsd*R17 (r_k^- r_m^+) *rec*A1 *rel*A1 *sup*E44 *thi*-1 *lac*U169 (ϕ80*lac*ZM15)	V.N. Iyer
E. coli GY3428	*gln*V44? λ$^-$ *rec*A431 *cysJ*43 *rel*A1? *Thy*A61 *lys*A22 *rps*E2339(Spec^R) *mal*T1(λ^R) *xyl*A7 *mtl*A2	M.K. Berlyn; Morand *et al.*, 1977
E. coli H1230	*thr*-20 *leu*-32 *fhu*A48 *pro*A35 *arg*F58 λ$^-$ *rel*A1 *spo*T1 *arg*I60	M.K. Berlyn
E. coli INVαF'	F' *end*A1 *rec*A1 *hsd*R17 (r_k^- r_m^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZM15	Invitrogen Corp.
E. coli ligATor cells	*end*A1 *hsd*R17 (r_{12}^- r_{12}^+) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*[F'*pro*A$^+$B$^+$ *lac*I^{q}ZΔM15::Tn10(Tet^R)]	R&D Systems
E. coli JS10	*ara leu azi ton*A *pro*A *lac*Z *rps*L *xyl mtl met*E *thi* λ nonlysogen *ftsX*	Sturgeon and Ingram, 1978

Table 2 (continued) Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Source/Ref.
E. coli K146	*fhu*A2? *Lac*Y1 or *lac*Z4 or *lac*-20 *tsx*-7 *gln*V44? *gal*-6 λ⁻? *his*G1 *rec*A1 *rps*L8 or *rps*L9 or *rps*L17 *mal*T1(λ^R) *kdg*K1 *met*B1 Δ*arg*H1 *thi*-1	M.K. Berlyn
E. coli KL723	*thr*-1 *ara*-14 *leu*B6 Δ(*gpt-pro*A)62 *lac*Y1 *tsx*-33 *gln*V44 *gal*K2 λ⁻ rac⁻ *his*G4 *rfb*D1 *mgl*-51 *rec*A13 *rps*L31(Str^R) *kdg*51 *xyl*A5 *mtl*-1 *argE*3 *thi*-1	M.K. Berlyn; Low, 1972
E. coli 30SOMA4	Hfr *lac*Z43 LAM⁻ *rel*A1 *arg*B62 *thi*-1	Glansdorff, 1965
E. coli UQ27	F⁻ *arg*G75 *pro*S127 *lac*Z4	Bohmann and Isaksson, 1980
E. coli W3421	F⁻ *gal*T23 LAM⁻ *arg*A21 IN(*rrn*D-*rrn*E)1	B.J. Bachmann
E. coli W3678	*gal*T23 λ⁻ IN(*rrn*D-*rrn*E)1 *arg*H56	M.K. Berlyn
E. coli W3679	F⁻ *gal*T23 LAM⁻ IN(*rrn*D-*rrn*E)1 *arg*C24	B.J. Bachmann
E. coli χ462	*ara leu azi ton*A *pro*A *lac*Z *rps*L *xyl mtl met*E *thi* λ nonlysogen	Sturgeon and Ingram, 1978
pACYC184	Tet^R, Clr^R	New England Biolabs
pAR2	pTAg with a *N. gonorrhoeae fisZ* amplicon under *lac* promoter control	Radia, 1997
pBluescript KS+	cloning vector (Amp^R)	Stratagene
pCRII	cloning vector for amplicons (Amp^R)	Invitrogen Corp.
pGEM-T	cloning vector for amplicons (Amp^R)	Promega Corp.
pHL1	pGEM-T containing the amplified *fisEX* genes from *N. gonorrhoeae* with their upstream region	Li, unpublished data

Table 2 (continued) Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Source/Ref.
pQE30	cloning vector used to generate histidine-tagged proteins (Amp^R, Clr^R)	QIAGEN
pREP4	pACYC184 derivative overexpressing the lac repressor (Kan^R)	QIAGEN
pSB19	pKS+ containing a 1.8 Kb *Sau*3AI DNA fragment *of N. gonorrhoeae* containing partial *fisE* and *pgk* genes and a complete *fisX* gene; *fisX* and the vectorborne *lac* promoter are in opposite orientations	This work
pSB190	pKS+ containing the blunted *Eco*RI-*Xba*I fragment from pSB19 subcloned in the *Sma*I site of pKS+; *N. gonorrhoeae fisX* is under *lac* promoter control	This work
pSB193	derivative of pSB19 in which the *fisX* gene is disrupted by a 2.9 Kb blunted *Acc*I-*Ava*I fragment containing *cat* from pACYC184 cloned in the unique *Nru*I site of *fisX*	This work
pSB195	derivative of pSB19 in which the *fisX* gene is disrupted by a 2.9 Kb blunted *Acc*I-*Ava*I fragment containing *cat* from pACYC184 cloned in the unique *Sty*I site of *fisX*	This work
pSB1900	pCRII containing a 2.8 Kb amplicon of gonococcal DNA including the 5'-section of *fisE* and complete *tlpA*, *arsG* and a partial *gltX*	This work
pSB1910	pTAg with a *N. gonorrhoeae fisE* amplicon under *lac* promoter control	This work
pSB1911	pTAg with a *N. gonorrhoeae fisE* amplicon and the *lac* promoter from pTAg in opposite orientations	This work
pSB1931	pQE30 containing a truncated *N. gonorrhoeae fisE* gene cloned in the *Bam*HI-*Pst*I site of the vector	This work
pTAg	clonign vector for amplicons (Amp^R, Kan^R)	R&D Systems

E. coli strains were grown in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989), in Luria (L) broth (Miller, 1972) or in minimal medium M9 (Sambrook *et al.*, 1989) with shaking or on tryptic soy agar (TSA; Difco) at 37°C unless otherwise indicated. The arginine defect of arginine mutants of *E. coli* were confirmed by growth on M9 medium containing the product (positive growth control) or the substrate (negative growth control) of the enzymatic reaction catalyzed by each *arg* gene product (Fig. A2-1). While confirming arginine biosynthesis gene defects, all other nutritional requirements for the strains tested were added to the M9 medium. M9 medium was supplemented with thiamine to a final concentration of 50 μg mL^{-1}, amino acids and intermediates of arginine biosynthesis were added as needed to a final concentration of 100 μg mL^{-1}.

All *N. gonorrhoeae* strains were verified by Gram staining and by colony morphology on GCMB. *E. coli* strains were verified by the confirmation of their growth requirements on minimal medium M9. All bacterial strains were stored at -70°C in bovine heart infusion (BHI; Difco) with 20% glycerol.

When required, antibiotics were added to LB broth and TSA plates at the following concentrations: 100 μg milliliter $(mL)^{-1}$ for ampicillin (Amp), 30 μg mL^{-1} for chloramphenicol (Clr), 15 μg mL^{-1} for kanamycin (Kan), 100 μg mL^{-1} for streptomycin (Strep), and 15 μg mL^{-1} for tetracycline (Tet). For the growth of *N. gonorrhoeae* with arsenical compounds, sodium arsenate (Na(As[V]); dibasic heptahydrate, $Na_2HAsO_4 \cdot 7H_2O$; Sigma Chemical Co., St-Louis, MO) was used at 512 μg mL^{-1} and sodium arsenite (Na(As[III]); $NaAsO_2$; Sigma Chemical Co.) was used at 2 μg mL^{-1}. When required, arginine was supplemented at 100 μg mL^{-1}.

DNA from *Neisseria meningitidis* 1527, *Neisseria flava* 1682, *Neisseria subflava* 1683, *Neisseria elongata* 1684, *Neisseria mucosa* 1685, *Neisseria lactamica* 1688, *Neisseria*

perflavalsicca 1691, *Neisseria flavescens* 1693, *Neisseria polysaccharеae* 1696 and *Neisseria cinerea* 1699 was purified as described elsewhere (Lawson *et al.*, 1995). The DNA preparations used throughout this work were stored in water at -20°C.

2.2. Determination of minimal inhibitory concentration, auxotype, and viable counts of *N. gonorrhoeae*

The minimal inhibitory concentrations (MIC) to penicillin (Wyeth-Ayerst Canada Inc., St-Laurent, Québec), tetracycline (Pfizer Canada, Pointe-Claire, Québec), spectinomycin (Upjohn Co. of Canada, Don Mills, Ontario), ceftriaxone (Hoffman-LaRoche, Mississauga, Ontario), ciprofloxacin (Bayer Leverkusen, Leverkusen, Germany), azithromycin (Pfizer Canada, Pointe-Claire, Québec) of *N. gonorrhoeae* strains CH811 and CS19 were determined following the agar dilution method using GC medium base (Difco) supplemented with 1% Kellogg's defined supplement and serial twofold dilutions of antibiotic (NCCLS, 1997). Bacterial inocula were prepared in auxotyping minimal medium (Dillon *et al.*, 1987). The same procedures were followed for the determination of the MIC to Na(As[V]) and Na(As[III]). Control strains included WHO III, WHO V, WHO VII (Reyn *et al.*, 1980), ATCC 49226 (NCCLS, 1997) and the PPNG strain GCI-182 from our strain collection. MIC interpretative criteria for each antimicrobial were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The MIC for an antibiotic or an arsenic compound was defined as the lowest concentration of a compound at which there was no bacterial growth. All MICs were done in duplicate.

The auxotype of *N. gonorrhoeae* CH811 and CS19 was determined by a method (Dillon, 1983) based on the procedure of Hendry and Stewart (1979). To determine viable counts, *N.*

gonorrhoeae cells from overnight cultures were resuspended in auxotyping diluent (Hendry and Stewart, 1979) to a McFarland Equivalence Turbidity Standard of 0.5 (Oxoid Inc., Nepean, Ont.). The bacteria were vortexed for 30 seconds (sec.) to remove clumps. Serial tenfold dilutions (10^{-1} to 10^{-7}) were prepared and mixed in auxotyping diluent, and 25 µL from each dilution and from the undiluted bacterial suspension were spotted in duplicate on supplemented GCMB plates. The plates were incubated as described in section 2.1. The number of colony forming units (cfus) was determined after 18-24 hours on GCMB or after 48 hours on minimal (auxotyping) medium. These experiments were repeated twice (four counts).

2.3. Microscopy techniques

For light microscopy to observe bacterial morphology, *E. coli* cells were recovered by centrifugation (5 min./6000 rotations per minute (rpm) using a F28/Micro rotor (Sorvall)/4°C), resuspended and fixed in 2% glutaraldehyde for 30 minutes (min.) at room temperature. The fixed bacteria were then pelleted by centrifugation (5 min./6000 rpm (F28/Micro rotor)/4°C) and resuspended in the same volume of 0.9% NaCl. Aliquots of fixed cells were subsequently observed using a Leitz Laborlux K light microscope (Wild Leitz, Willowdale, Ont.).

For electronic microscopy (EM), *Neisseria gonorrhoeae* cells from a fresh overnight culture were collected (1/4 loopful) and resuspended in sterile PBS to a McFarland Equivalence Turbidity Standard 0.5 (1.5×10^8 bacteria mL^{-1}; Oxoid Inc.). The bacteria were vortexed during resuspension to remove clumps. One drop of the bacteria in solution was placed on a piece of parafilm. Formvar coated grids (M400; for grid preparation, see below) were used to lift the bacteria, followed by staining in uranyl acetate (Marivac Ltd., Halifax, Nova Scotia; 10 µL of 21.2% uranyl acetate in 0.5 M HCl [made in 90% EtOH] to 10 mL 90% EtOH). The grids were

air dried before viewing under the electron microscope. In some instances, the grids were rinsed by dipping in 90% EtOH for 10 sec. before air drying. Alternately, the bacteria were stained in 10% phosphotungstic acid (PTA; Marivac Ltd.) before rinsing and air drying. The grids were viewed using a Philips EM201 microscope. Pictures were taken using a 1 sec. exposure, and the plates (negatives) were developed on Ilford MGX-0.1M (glossy finish) paper.

The copper grids (300 and/or 400 mesh; Marivac Ltd.) were coated using a novel procedure (B. Luck, personal communication). A tissue (Optical Lens Tissue; Marivac Ltd.) cleaned microscope slide (VWR Scientific, Media, PA) was dipped into 30 mL of formvar solution (0.3% in ethylene dichloride; Formvar and Ethylene Dichloride obtained from Marivac Ltd.) and allowed to dry. The edges of the slide were etched using cleaned forceps to cut the formvar layer. The slide was then carefully placed into a Petri dish containing sterile water at a 45^0 angle to float the formvar layer onto the water phase. The copper grids were placed onto the formvar layer, and the layer was collected using a piece of parafilm (i.e. the lift was done with the side of parafilm which contains the paper). Once dried, the parafilm was placed in a Petri plate.

2.4. DNA manipulations

2.4.1. DNA restriction and other manipulations

Restriction enzymes were purchased from Boehringer Mannheim (Laval, Québec), Promega (Madison, Wisconsin) or Pharmacia Biotech Inc. (Baie d'Urfé, Québec); T4 DNA ligase and T4 DNA polymerase were purchased from Pharmacia Biotech Inc., and all enzymes were used according to the instructions of the manufacturers. DNA was electrophoresed through 1% agarose gels unless otherwise specified and visualized by staining in 0.5 μg ethidium bromide mL^{-1} followed by exposure to ultraviolet light (Model 3-3000 transilluminator, Fotodyne Inc.,

New Berlin, Wisconsin), rinced 5-10 min. in double-distilled water and photographed using the GelPrint 2000i digital imaging system (Bio Photonics Corp., Ann Arbor, Michigan). DNA fragments were purified from agarose gels using the GlassMAX DNA isolation matrix system (Gibco BRL). Preparation of competent *E. coli* for transformation was done using the calcium chloride method (Sambrook *et al.*, 1989). When *E. coli* transformants were selected on M9 medium, the cells were pelleted by centrifugation (12 000g/30 sec./4°C in a Sorvall MC12V centrifuge) following the 1 hour growth in rich medium. The cells were carefully resuspended in 1 mL of liquid M9 medium and centrifuged again and resuspended in 200 µL of liquid M9, and were spread on the plates. *N. gonorrhoeae* was transformed as described in section 2.13.2.

2.4.2. Plasmid DNA purification

Plasmid DNA used for cloning, manual sequencing or for *in vitro* transcription/translation studies was purified on cesium chloride gradients (Sambrook *et al.*, 1989). Plasmid DNA used for automated sequencing was purified using QIAGEN-tip 20 columns (QIAGEN, Chatsworth, California), and plasmid DNA used for routine procedures such as restriction digests was purified using Wizard Minipreps columns (Promega).

2.5. Construction of the library of *Neisseria gonorrhoeae* CH811 DNA and isolation of plasmid clones

Genomic DNA from *N. gonorrhoeae* CH811 was purified as previously described (Ng and Dillon, 1993). This DNA was partially digested with *Sau*3AI and fragments up to 6 kilobases in size were fractionated by ultracentrifugation (50 000 rpm/10°C/20 hours in a Vti50.1 rotor) on sucrose gradients (Ausubel *et al.*, 1989; Sambrook *et al.*, 1989). Fragments between 2 and 4

kilobases (Kb) were recovered, dephosphorylated and ligated into the *Bam*HI site of pBluescript KS+. Ligation mixtures were transformed into *E. coli* DH5α following a procedure previously described (Chung *et al.*, 1989) and transformants were selected on TSA + Amp. plates containing 75 μg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) mL^{-1}. Recombinant clones were restreaked twice on the same type of medium and were then pooled in groups of fifty and stored at -70°C as described in section 2.1. Aliquots from these pools were grown in liquid cultures and the plasmids were purified following a minilysis procedure (Brun *et al.*, 1991). The purified plasmids were transformed in *E. coli* UQ27, a strain containing a mutation in the *argG* gene which encodes argininosuccinate synthetase (Table 2). Transformants capable of growing in the absence of arginine were selected on M9 medium for 2 days at 37°C. As a transformation control, *E. coli* UQ27 was transformed with pYN87, a plasmid containing the wild-type *argG* gene of *E. coli* (Table 2). Transformants were selected on M9 medium; *E. coli* UQ27 was also grown on M9 supplemented with arginine as a viability control.

2.6. Construction of deletion clones and DNA sequencing procedures

Deletion clones of pSB19 were constructed using the Erase-a-Base System (Promega) following the instructions of the manufacturer. The nucleotide sequences of the inserts of pSB19 and pSB1900 were determined in both directions using a combination of manual and automated procedures using dideoxynucleotides (Sanger *et al.*, 1977). T3 and T7 primers were obtained from Promega. Other primers used for primer extension to fill in sequence gaps were selected using the Primer Designer program and were purchased from the University of Ottawa Biotechnology Research Institute. The oligonucleotides used as primers for DNA sequencing are listed in Table 3.

Table 3. Oligonucleotides used in this work

Oligonucleotide	Sequence	Coordinates on Figure 6B
4D1	5'-GGTTGAACATCGGCTGGCGT-3'	3769-3789
6AL1R	5'-CGCGTTCCTTGAGTTCTTGG-3'	4380-4361
9C1	5'-TTCTACGTCGGCGTAACTCG-3'	3793-3812
9C2R	5'-ACAAGCCACGCGCCGAATAC-3'	3863-3844
9C3	5'-AGTAGAAGGCGACGGACACC-3'	4058-4077
9C4R	5'-GTCCGTCGCCTTCTACTTCG-3'	4074-4055
19-1	5'-CGTATCCTGTGCCTCTCGAA-3'	2954-2973
19-2R	5'-GACGACGATGACGGTGGTTC-3'	2917-2898
19-3R	5'-CCAACCGTCTTCCTTGCCGA-3'	3295-3275
19-4	5'-TCGACAACATCCGCTTCATC-3'	3257-3276
19-4R	5'-AGTCGGATGGTGTTGTGTGC-3'	3578-3559
19-5R	5'-CGGATTCGACCATAGGCAGC-3'	3451-3432
19-7	5'-CGACGTGCAGCGAGAAGTAG-3'	3019-3000
19-8	5'-GTCCGTCGCCTTCTACTTCG-3'	4074-4055
1900-5	5'-CGCATCGCTTCCAATTCTTC-3'	82-101
1900-6	5'-AATTGGAAGCGATGCGCGAG-3'	97-78
1900-7	5'-CATGCTCGGCTTGGTGATGC-3'	2506-2487
1900-9	5'-CTCCTGTAATGGTCTGCCTG-3'	2086-2067
1900-10	5'-AATCCACCGTCCTAAGCTGA-3'	2460-2479
1900-11	5'-GCGGATACAGGCAGACCATT-3'	2060-2079
1900-12	5'-GCTTGAATCTCGCCGCAATC-3'	1670-1689
1900-13	5'-GATTGCGGCGAGATTCAAGC-3'	1689-1670
1900-14	5'-GGCGTTGCGACCACCTTGTT-3'	359-340
1900-15	5'-ACAATCGGACGCTCCAACAG-3'	1247-1266

Table 3 (continued). Oligonucleotides used in this work

Oligonucleotide	Sequence	Coordinates on Figure 6B
1900-16	5'-CCTGTTGGAGCGTCCGATTG-3'	1267-1248
1900-17	5'-CACTTCGCGCAACACTTATG-3'	811-792
1900-18	5'-CATAAGTGTTGCGCGAAGTG-3'	792-811
1900-19	5'-CCTCAATCAGACAGCCATCC-3'	529-548
1900-20	5'-AACGCATCAACCACGCCATC-3'	971-952
1900-21	5'-TTCCACAATCCGCGTTGCAG-3'	1521-1502
1900-28	5'-CTTCCTTACAGGACCTCCTA-3'	2320-2339
*ftsE*A	5'-GGATCGTCCAATTCTCGTCC-3'	2742-2723
*ftsE*HIS1	5'-[CGGGATCC]ATCCGTTTCGAACAAGTTTC-3' [1]	2342-2361 [2, 3]
*ftsE*HIS2	5'-[AACTGCAG]TGCGAGTCGTCCTT TCGAGA-3' [1]	2986-2967 [2, 4]
*ftsX*A	5'-CAGAGCAGGTGTTGTGTGGT-3'	3884-3865
*ftsX*B	5'-GCATCATCCACTACTTCTCG-3'	2990-3009
SA-2	5'-GCGACGGCAAGCATTATGAG-3'	3098-3079
SA-3	5'-CACAGGCGTTGTTGTTCACC-3'	2778-2759
SA-4	5'-TCATCCACTACTTCTCGCTG-3'	3012-2993
T3	5'-ATTAACCCTCACTAAAG-3'	N/A
T7	5'-AATACGACTCACTATAG-3'	N/A

[1] Primer containing a 5'-extension. In all cases the extension is between brackets.

[2] Primer coordinates exclude the 5'-extension.

[3] Extension contains a *Bam*HI restriction site.

[4] Extension contains a *Pst*I restriction site.

2.6.1. Manual sequencing

For manual sequencing, the sequencing reactions were prepared using [α-^{35}]dATP (1000 Ci/mmol) as described for the Taqtrack Sequencing system (Promega). The reactions were

electrophoresed on denaturing 8% polyacrylamide gels as indicated in the manufacturer's instructions. Polyacrylamide gels were dried in a Model 583 Gel Dryer (BIO-RAD, Hercules, California) following the instructions of the manufacturer. Kodak X-OMAT™ AR films were exposed overnight to the dried gel and were developed.

2.6.2. Automated sequencing procedures

For automated sequencing, the Taq Dyedeoxy™ and ABI Prism™ Terminator Cycle Sequencing kits (PE Applied Biosystems, Mississauga, Ontario), and Centri-Sep Spin Columns (Princeton Separation, Adelphia, New Jersey) were used as per the manufacturer's instructions. The sequencing reactions were electrophoresed using an Automated DNA Sequencer System Model 373 (ABI).

2.7. Nucleotide and amino acid sequence analysis

Nucleotide and amino acid sequence similarities and features were investigated using the software package (version 7) of the Genetics Computer Group (Madison, Wisconsin) and Blast (Altschul *et al.*, 1989). Predictions of integral membrane protein helices and membrane-associated alpha helices were done using PSORT (Nakai and Kanehisa, 1991) through the Internet (http://psort.nibb.ac.jp) as well as the algorithms available in the PC Gene software package (Intelligenetics, Mountain View, California). The program TopPredII 1.3 (Claros and von Heijne, 1994; von Heijne, 1992) used for predictions of membrane protein topology was downloaded from an anonymous ftp site (ftp.ebi.ac.uk) and used from a Apple McIntosh IIci computer. All multiple aa sequence alignments were done with Clustal W 1.7 (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). All the programs used for

sequence analysis during this work were used at their default settings unless otherwise specified. The DNA sequence of the genome of *N. gonorrhoeae* strain FA1090 was accessed through the site of the University of Oklahoma *Neisseria gonorrhoeae* Genome Blast Server (http://dna1.chem.uoknor.edu/gono_blast.html).

The complete nucleotide sequence of *ftsEX* from *N. gonorrhoeae* CH811 and their flanking genes is available in Genbank under the accession number U76418.

2.8. DNA transfers and hybridizations

2.8.1. Labeling of DNA fragments and oligonucleotides

All probes used in this work were labelled with digoxygenin. Gonococcal DNA and any fragment larger than 100 bp were labeled using the Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim) and oligonucleotides were labeled with the DNA Tailing Kit (Boehringer Mannheim) according to the instructions of the manufacturer.

2.8.2. Southern transfers of DNA to nylon membranes

DNA was first separated on 1% agarose gels and was subsequently transferred to nylon membranes (Boehringer Mannheim) by Southern transfer (Sambrook *et al.*, 1989). Vacuum transfers were done using a Model 785 Vacuum Blotter (BIO-RAD) following the manufacturer's instructions and overnight alkaline capillary transfers were done following the method of Chomczynski and Qasba (1984), in which NaOH 0.4 N is used as a transfer solution. Following vacuum or capillary transfers, the DNA was crosslinked to the nylon support by exposure to ultraviolet light for 1 min. using a Model 3-3000 transilluminator (Fotodyne Inc.).

2.8.3. Hybridizations and detection

Hybridizations to confirm the gonococcal origin of DNA fragments were done at 68°C. The melting temperature (Tm(°C)) of oligonucleotides was determined according to the formula of Suggs *et al.* (1981; Tm(°C) = 2x(A+T) + 4x(G+C)) and hybridizations using labelled oligonucleotides were done at five degrees below its Tm(°C). Hybridizations to *Neisseria* DNA using *ftsE* and *ftsX* amplicons from *N. gonorrhoeae* as probes were first done at 60°C (Lawson *et al.*, 1995) and at 50°C. Hybridizations using oligonucleotide probes were done for 2 hours at the hybridization temperature while hybridizations using any other types of probes (DNA fragments from plasmids, genomic DNA, amplicons) were performed for 12-16 hours at the hybridization temperature. All hybridizations were done in hybridization solution (5x SSC, 0.02% SDS, 0.1% N-lauroylsarcosine, 1% blocking reagent) at the appropriate temperature. Post-hybridization washes consisted in two washes in 2x SSC and 0.1% SDS of 5 min. at room temperature and in two washes in 0.1x SSC and 0.1% SDS for 15 min. at the hybridization temperature. Non-radioactive detection was done by colorimetry using the Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim) as described by the manufacturer.

2.9. Polymerase chain reaction procedures and cloning of amplicons

2.9.1. Amplification procedures to generate amplicons for cloning and other procedures

The polymerase chain reaction (PCR) Core Kit (Boehringer Mannheim) with the following reaction conditions was used for all amplification reactions: 1x PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer and 2.5 U of *Taq* DNA polymerase per 100 μL reaction. All the oligonucleotide primers used for amplification procedures are listed in

Table 3. All amplifications were done in a Perkin-Elmer 9600 Thermocycler (Perkin-Elmer Corp., Norwalk, Connecticut).

The *ftsEX* amplicon used for *in vitro* transcription/translation was amplified using the primers 1900-11 and 19-8 with the following thermal profile: 3 min. at 94°C, 30 cycles consisting of 15 sec. at 94°C, 15 sec. at 57°C, 2.5 min. at 72°C; 5 min. at 72°C. The resulting amplicon was cleaned using the QIAquick PCR Purification Kit (QIAGEN) and was used directly in *in vitro* transcription/translation experiments (section 2.10.). *ftsEX* without its upstream region was amplified using the primers 1900-28 and 19-8 with the following thermal profile: 3 min. at 94°C, 30 cycles consisting of 15 sec. at 94°C, 15 sec. at 55°C, 2 min. at 72°C; 5 min. at 72°C. The amplicon was cleaned as described and was cloned in pTAg and one plasmid containing *ftsEX* in the same orientation as the vectorborne *lac* promoter was designated pSB5696. The amplicon was resequenced to assure sequence integrity.

A promoterless *ftsE* was generated using the primers 1900-28 and 19-7 with the following thermal profile: 30 cycles consisting of 15 sec. at 94°C, 15 sec. at 55°C, 1 min. at 72°C; 5 min. at 72°C. The resulting amplicon was cleaned as described above and was cloned in pTAg. One plasmid containing *ftsE* in the same orientation as the vectorborne *lac* promoter was designated pSB1910, and another plasmid containing *ftsE* and the *lac* promoter in opposite orientations was called pSB1911. To verify the orientation of the insert in each plasmid, the inserts were amplified using primers T7 and 19-7 and T7 and 1900-28 with a thermal profile consisting in 30 cycles of 15 sec. at 94°C, 15 sec. at 44°C, 1 min. at 72°C; 5 min. at 72°C.

ftsE and *ftsX* amplicons from *Neisseria* species were generated using the primer pairs *ftsE*A and 1900-10 for *ftsE* or *ftsX*A and *ftsX*B for *ftsX*. The thermal profile consisted of 30 cycles of 15 sec. at 94°C, 15 sec. at 55°C, 1 min. at 72°C; 5 min. at 72°C.

An amplicon containing a truncated *ftsE* gene from *N. gonorrhoeae* was generated using the primers 6HISFtsE1 and 6HISFtsE2. The thermal profile consisted of 30 cycles of 15 sec. at 94°C, 15 sec. at 51°C, 1 min. at 72°C; 5 min. at 72°C. This amplicon was subsequently processed as described in section 2.13.1. to construct the plasmid pSB1931.

The insertion of the blunted 1.9 Kb *Acc*I-*Ava*I fragment in the chromosome of *N. gonorrhoeae* strain CS19 was verified by using the primers *ftsX*A and *ftsX*B in a thermal profile consisted of 35 cycles of 15 sec. at 94°C, 15 sec. at 60°C, 3.5 min. at 72°C; 5 min. at 72°C. In these experiments, cells from an overnight culture *N. gonorrhoeae* CS19 were grown on GCMB with streptomycin and chloramphenicol were resuspended in water and used directly in the amplification reaction.

2.9.2. Inverse PCR

Inverse PCR (iPCR; Hartl and Ochman, 1994; Silver, 1991; Ochman *et al.*, 1990) was used to amplify the DNA upstream of the partial *ftsE* cloned in pSB19. DNA from *N. gonorrhoeae* CH811 was digested to completion with *Pst*I and *Nru*I, the ends of the DNA fragments were blunted with T4 DNA polymerase and ligated o/n at 16°C in a final volume of 500 µL containing 0.08 U of T4 DNA ligase μL^{-1}. Ligations were done with 0.2, 0.3 and 0.5 µg of DNA mL^{-1} to favor the circularization of monomeric molecules (Hartl and Ochman, 1994; Silver, 1991; Ochman *et al.*, 1990). The religated DNA was subsequently cleaned by organic extractions, precipitated, resuspended in sterile water and used for the PCR. The thermal profile for the PCR using the religated DNA was as follows: 3 min. at 94°C, 30 cycles consisting of 30 sec. at 94°C, 15 sec. at 62°C, 4 min. at 72°C; 3 min. at 72°C using the primers SA-3 and SA-4. This amplicon was purified on low-melting agarose, cleaned using the Glassmax® Isolation

Matrix System (Gibco BRL, Bethesda, Maryland) and was cloned in pCRII™ to generate the plasmid pSB1900.

2.9.3. Reverse transcriptase PCR

For reverse transcriptase PCR (RT-PCR), total RNA was isolated from gonococci using the RNeasy Total RNA kit (QIAGEN). Any residual traces of DNA were removed using RQ1 DNase (Promega). This RNA was reverse transcribed using Superscript™ II reverse transcriptase (Gibco BRL) with the primer 1900-9 (*tlpA*) or 1900-15 (*arsC*). The cDNA was then used in subsequent PCR reactions. The thermal profile consisted of 30 cycles of 15 sec. at 94°C, 15 sec. at 57°C, 1 min. at 72°C to generate a *tlpA*-specific amplicon with the primers 1900-9 and 1900-12. To generate the *arsC*-specific amplicon, the thermal profile comprised 30 cycles of 15 sec. at 94°C, 15 sec. at 57°C, 1 min. at 72°C using the primers 1900-15 and 1900-21.

2.10. *In vitro* transcription/translation

The *E. coli* S30 Extract System for Circular DNA Templates (Promega) was used with undigested plasmid DNA and the *E. coli* S30 Extract System for Linear DNA Templates was used with amplicons according to the manufacturer's instructions. [^{35}S]-methionine (15 mCi mL^{-1}) (Amersham Canada, Oakville, Ontario) and 1 μL of RNAguard (Pharmacia Biotech Inc.) were also added to each reaction. The reaction products were prepared for electrophoresis and were separated on denaturing 10% polyacrylamide-SDS gels (section 2.15.1.). Gels were dried in a Model 583 Gel Dryer (BIO-RAD) and Kodak X-OMAT™ AR films were exposed overnight to the dried gel and developed.

2.11. Overexpression of gonococcal genes in *E. coli*

Overexpression of *fts* genes from *N. gonorrhoeae* in *E. coli* DH5α was done in two types of culture media: LB broth was used for growth in rich medium and minimal medium M9 (Sambrook *et al.*, 1989) supplemented with 0.2 % casamino acids (Difco) was used for growth in minimal medium. Fresh cultures of *E. coli* INVαF' cells containing pSB19, pSB190 or pSB5696 were inoculated in fresh culture medium from overnight cultures and were grown four hours (six hours in M9) at 37°C with shaking. Each culture was then divided in two, the inducer isopropylthiogalactoside (IPTG) was added to one of the cultures to a final concentration of 1 mM, and were grown for another four hours (six hours in M9) at 37°C with shaking. In each experiment, INVαF' cells containing pAR2, a plasmid containing the *ftsZ* gene from *N. gonorrhoeae* under the control of the *lac* promoter (Table 2) was included as a filamentation positive control.

2.12. Filamentation assay and complementation of *E. coli* JS10

Cultures of *E. coli* JS10 and χ462 (Table 2) were grown in LB and L broth at 30°C and 37°C and aliquots of cells were taken after 1, 2, 4 and 6 hours of growth. The cells were harvested, fixed and observed using the light microscope as described in section 2.3.

For complementation of JS10, duplicate cultures of *E. coli* JS10, *E. coli* χ462 and *E. coli* JS10+pSB190 (Table 3) were grown in LB medium at 30°C and 37°C. After one hour of growth, the cells from one culture were induced with IPTG to a final concentration of 1 mM. Aliquots of cells from the cultures were taken immediately before induction, and 1, 3 and 5 hours after induction. The cells were subsequently harvested, fixed and observed using the light microscope as described in section 2.3.

2.13. Insertion of a *cat* gene in the *ftsX* gene of *N. gonorrhoeae* CH811

2.13.1. Construction of the plasmid pSB193

The plasmid pACYC184 containing a *cat* gene (encoding chloramphenicol acetyltransferase; Table 2) was digested with *Acc*I and *Ava*I. The 1919 bp fragment containing the *cat* gene was recovered and purified, blunted with T_4 DNA polymerase, and cloned in the *Nru*I site (positions 3095-3100, Fig. 6A and 6B) of pSB19, 111 base pairs (bp) from the 5'-end of the *ftsX* gene. *E. coli* transformants were selected on ampicillin and chloramphenicol. The plasmids were isolated and confirmed by their *Eco*RI, *Hin*cII, *Sca*I, and *Sty*I digestion patterns. One recombinant plasmid with the *cat* gene inserted in the same orientation as *ftsX* was designated pSB193. The plasmid pSB195 was generated following the same cloning procedure, except that the blunted 1.9 Kb *Acc*I-*Ava*I fragment was inserted in the *Sty*I site (positions 3740-3745, Fig. 6A and 6B) of pSB19, 756 bp from the 5'-end of the *ftsX* gene, and the plasmid was verified by its *Hin*cII, *Sca*I and *Xba*I restriction profile.

2.13.2. Construction of *N. gonorrhoeae* strain CS19

A streptomycin-resistant derivative of *N. gonorrhoeae* CH811 was first obtained by growing the isolate on GCMB supplemented with streptomycin using the gradient plate technique (Carpenter, 1967). One streptomycin-resistant colony, designated strain CH811Str^R was used for further manipulations.

Pure cultures of T2 colonies of strain CH811Str^R were subsequently obtained by repeated subculture every 18 to 20 hours on GCMB plates supplemented with streptomycin. *N. gonorrhoeae* was transformed using the method of Janik *et al.* (1976): cells from a fresh overnight culture of CH811Str^R were harvested and restreaked on a supplemented GCMB plate.

Thirty micrograms of pSB193 in 35μL of water were spread on the inoculum and the plate was incubated for six hours at 35°C in a humid environment with 5% CO_2. Following the incubation period, the cells were harvested with a sterile loop and spread on GCMB plate supplemented with streptomycin and Clr and incubated overnight. One transformant was designated CS19. The presence of the *cat* gene within the *ftsX* gene on the gonococcal chromosome was confirmed by PCR (section 2.9.1.).

2.14. Purification of the gonococcal FtsE protein

A histidine-tagged gonococcal FtsE protein was generated and purified. This pure protein will be used to generate rabbit polyclonal anti-6HISFtsE antibodies. The strategy of purification involved the construction of a fusion gene that would encode the gonococcal FtsE protein preceded by six consecutive histidines using the QIAexpress type IV kit from QIAGEN (The QIA*expressionist*, 1992). The fusion protein, 6HISFtsE, will contain seventeen additional amino acids; eleven will precede the FtsE sequence, and seven more will follow it. The fusion protein is overproduced and purified by affinity chromatography using Ni-NTA resin (The QIA*expressionist*, 1992).

2.14.1. Construction of pSB1931 expressing a histidine-tagged FtsE (6HISFtsE)

A truncated *ftsE* gene was generated by PCR using the primers *ftsE*HIS1 and *ftsE*HIS2 that respectively contained a *Bam*HI and a *Pst*I site (section 2.9.1.). This amplicon was subsequently restricted with *Bam*HI and *Pst*I, ligated in the *Bam*HI and *Pst*I sites of the plasmid pQE30 (QIAGEN; see Table 2), and transformed in *E. coli* M15 (pREP4). Transformants were selected using ampicillin and kanamycin.

The plasmids from ampicillin- and kanamycin-resistant colonies were purified and their size was verified by electrophoresis. Positive clones were confirmed by amplification of the truncated *ftsE* gene using the primers 6HISFtsE-1 and 6HISFtsE-2, and also by *Bam*HI and *Pst*I restriction patterns. One plasmid was designated pSB1931 and was used for all subsequent manipulations.

2.14.2. Small-scale expression of 6HISFtsE

This protocol is described in the QIAexpressionist user's manual (The QIA*expressionist*, 1992). A 10 mL culture of *E. coli* M15 (pSB1931, pREP4) inoculated with 500 µL of cells from an overnight culture was grown in LB containing ampicillin and kanamycin at 37°C with shaking until the OD_{600} reached 0.7-0.9, and IPTG was added to a final concentration of 2 mM. The culture was grown for five more hours and 1 mL aliquots of cells were taken at every hour and kept on ice. A 1 mL aliquot was taken just before the addition of the IPTG to serve as an uninduced control. The cells were harvested by centrifugation for 3 min./3000 rpm/4°C using a F-28/MICRO rotor).

The cells were resuspended in 200 ul of buffer B (8M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0), and the cells were lysed by gentle vortexing and avoiding frothing. The lysate was then centrifuged (10 min/15 000 rpm/4°C using a F-28/MICRO rotor) to pellet the cellular debris. The supernatant was collected and 50 µL of a 50% slurry of nickel- nitrilo-tri-acetic acid (Ni-NTA) resin which was added to each tube, and incubated for 30 min. at room temperature. The solution was centrifuged (10 sec./15000 rpm/4°C using a F-28/MICRO rotor) and the supernatant was discarded. The resin was then washed three times with 1 mL of buffer C (8M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 6.3). The bound proteins were eluted from the

resin by adding 20 µL of buffer C/EDTA 100 mM followed by incubation of 2 min. at room temperature with gentle mixing. The resin was pelleted by centrifugation (10 sec./15000 rpm/4°C using a F-28/MICRO rotor), and the supernatant was collected in a clean tube. The eluate was verified by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels (see section 2.15.).

2.14.3. Large-scale expression and purification of 6HISFtsE

The procedure described in section 2.14.2. was scaled up to accommodate cultures of 50 mL. Induction was done with 2 mM of IPTG and the cultures were grown for three hours after induction. The cells were collected by centrifugation (10 min/4000rpm/4°C in a SS-34 rotor). Cell pellets were frozen in a mixture of dry ice and ethanol and were stored at -70°C until use. Cell pellets were slowly thawed on ice prior to use.

The 6HISFtsE proteins eluted from the Ni-NTA resin were separated on large SDS-polyacrylamide gels using a PROTEAN®II xi Cell apparatus (BIO-RAD). To elute 6HISFtsE from the SDS-polyacrylamide gel, the method of Hager and Burgess (1980) was used: following electrophoresis, the gel was rinsed with water and was stained 5 min. in ice-cold 0.25 M KCl and 1 mM dithiothreitol (DTT), rinsed again with water and destained for 60 min. in water with 1 mM DTT. The gel was cut to separate the band of 6HISFtsE, and the pieces of gel were soaked twice for 15 min. in water with 1 mM DTT. The slice of gel was then cut in small pieces and the pieces were incubated in 1 mL of elution buffer (Tris-HCl 0.05 M pH 7.9, 0.1% SDS, 0.1 mM ethylenediamine tetraacetate (EDTA), 5 mM DTT, 0.1 mg/mL BSA, 0.15 M NaCl) for 12 hours at room temperature. The solution was centrifuged (10 min./10 000 rpm/4°C in a SS-34 rotor) to pellet the gel pieces, and the supernatant was recovered in a clean tube. The eluted 6HISFtsE was

precipitated for 30 min. in a dry-ice ethanol bath or overnight at -20°C with four volumes of cold (-20°C) acetone. The 6HISFtsE proteins were pelleted by centrifugation (10 min./10 000 rpm/4°C in a SS-34 rotor). The 6HISFtsE proteins were washed with a solution of 80% ice-cold acetone and 20% elution buffer to remove the last traces of SDS, and were dried at room temperature. The 6HISFtsE pellet was resuspended in 20 ul of 6 M guanidine-HCl in dilution buffer (Tris-HCl 0.05 M pH 7.9, 20% glycerol, 0.1 mg/mL bovine serum albumin (BSA), 0.15 M NaCl, 1 mM DTT, 0.1 mM EDTA). Following resuspension, the 6HISFtsE proteins were left at room temperature for 15 min., and were then diluted 50 times with dilution buffer. The eluted 6HISFtsE was left at room temperature 12 hours to allow renaturation. Purified stocks of 6HISFtsE were always verified on polyacrylamide gels before being used for other manipulations.

2.15. Gel electrophoresis of proteins

Sample buffer (20% glycerol, 2% SDS, 125 mM Tris pH6.8, 0.025% (w/v) bromophenol blue, 715 mM β-mercaptoethanol) was added to the samples and they were heated for 3 min. in boiling water. The samples were then briefly centrifuged and kept on ice until they were loaded on the SDS-polyacrylamide gels Proteins were separated on 10% SDS-polyacrylamide gels using a Mini-PROTEAN® II apparatus (BIO-RAD) for 45 min. at 200 V in running buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH8.3) unless otherwise specified. SDS-PAGE standards Broad Range molecular weight (BIO-RAD) were electrophoresed alongside the samples for size estimation of polypeptides.

Separated proteins were visualized by staining with Coomassie blue or SYPRO™ ORANGE (BIO-RAD). For visualization using Coomassie Blue, the SDS-polyacrylamide gel

was stained in 0.25% (w/v) Coomassie blue R250, 25% isopropanol, 10% glacial acetic acid and stained overnight with gentle agitation. The proteins were subsequently visualized by destaining the gel in the destaining solution (7% acetic acid in water). For visualization using SYPRO™ ORANGE, the gel was stained in a 1:5000 dilution of SYPRO™ ORANGE in 7.5% (v/v) acetic acid for 30 min. and was examined under ultraviolet light (Model 3-3000 Transilluminator, Fotodyne Inc.).

PART 3. RESULTS

3.1. Isolation of the plasmid pSB19 and cloning of the *ftsX* gene.

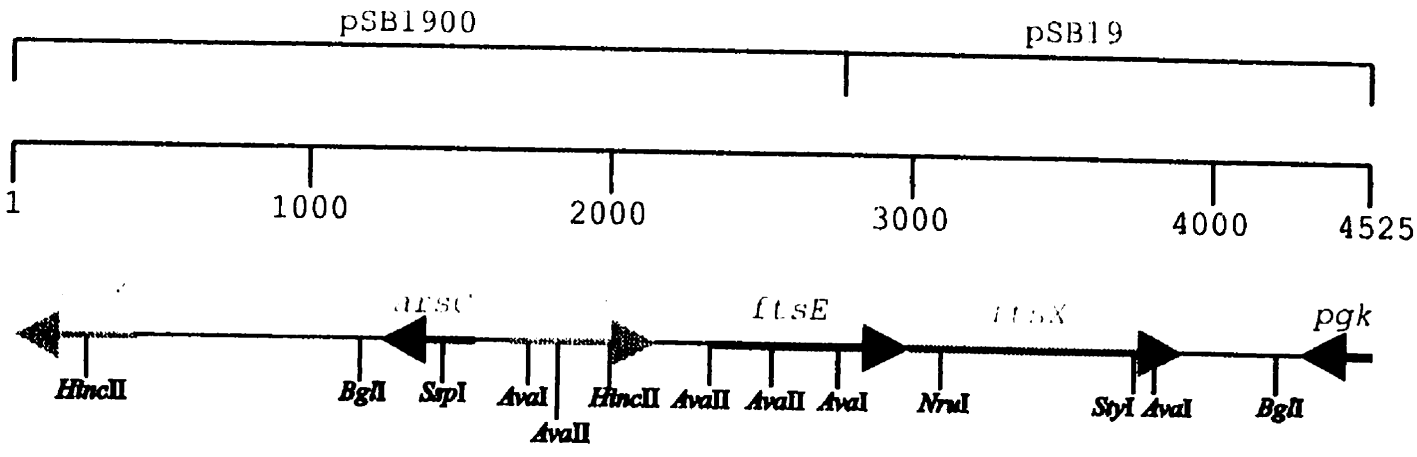
The plasmid pSB19 was isolated while screening a genomic bank of *N. gonorrhoeae* CH811 and its insert characterized by DNA sequencing (Appendix 2). The complete nucleotide sequence of the insert of pSB19 comprised 1789 bp (positions 2779-4526, Fig. 6B). One complete open reading frame (ORF) and two partial ORFs were identified in the insert of pSB19. The complete ORF (positions 2986-3903, Fig. 6B) shared 55% aa sequence similarity with the FtsX protein of *E. coli* (section 3.3.2.). One of the partial ORFs shared 71% aa sequence similarity to the FtsE protein of *E. coli*, and was in the same orientation as the putative *ftsX* gene and overlapped it by 4 bp (positions 2779-2989, Fig. 6B). The other partial ORF (positions 4526-4296, Fig. 6B) shared 83% aa sequence similarity 3-phosphoglycerate kinase (Pgk; section 3.10.4) of *E. coli*. Together, these data suggested that the insert of pSB19 contained a complete gonococcal homologue of the *E. coli ftsX* gene, and that this *ftsX* homologue was flanked by partial *ftsE* and *pgk* gene homologues (Fig. 6A). It further showed that the *ftsE* and *ftsX* genes were overlapping on the gonococcal chromosome as had been found at the time in *E. coli* (Gill *et al.*, 1986). The 4 bp overlap observed between *ftsE* and *ftsX* in *N. gonorrhoeae* suggested these genes were co-transcribed. These gonococcal homologues of *E. coli ftsX* and *ftsE* were further investigated as part of the research project of cell division genes in our laboratory.

3.2. Cloning of the 5'-section of the gonococcal *ftsE* gene and its upstream region.

The 5'-region of the *ftsE* gene was cloned using an iPCR strategy (Hartl and Ochman, 1994; Silver, 1991; Ochman *et al.*, 1990; Fig. 7). The first step involved the identification of a DNA fragment of a size amenable to ligation preceding the other steps of iPCR. Genomic DNA from *N. gonorrhoeae* CH811 was digested overnight with the restriction enzymes *Acc*I, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I and *Sma*I, as the analysis of the nucleotide sequence of the insert of pSB19 showed that it contained no recognition sites for any of these restriction enzymes. The restricted DNA was subsequently hybridized at 55°C with the labeled oligonucleotide 19-4R (positions

Figure 6. (A) Genetic and physical map of the *ftsEX* region of *N. gonorrhoeae* CH811. (B) The complete nucleotide sequence of the *ftsEX* genes and their flanking regions as generated from the plasmids pSB1900 (positions 1-2778) and pSB19 (positions 2779-4526). (A) Each gene is indicated by an arrow whose orientation indicates the direction of transcription. Some restriction enzyme sites are also indicated. Gene names appear in italics. (B) The initiation codon for each gene is boxed in green, with a green arrow extending from the box indicating the direction of transcription, and the stop codon is boxed in red. When identified, ribosome-binding sites (RBS) are circled. Gonococcal uptake sequences (US) and the putative -10 box of promoters (-10) are boxed in black. Mismatches in the US from the consensus sequence are indicated by asterisks. The stems of the predicted transcriptional terminators are indicated by arrows. Relevant restriction enzyme sites are indicated.

A.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1000
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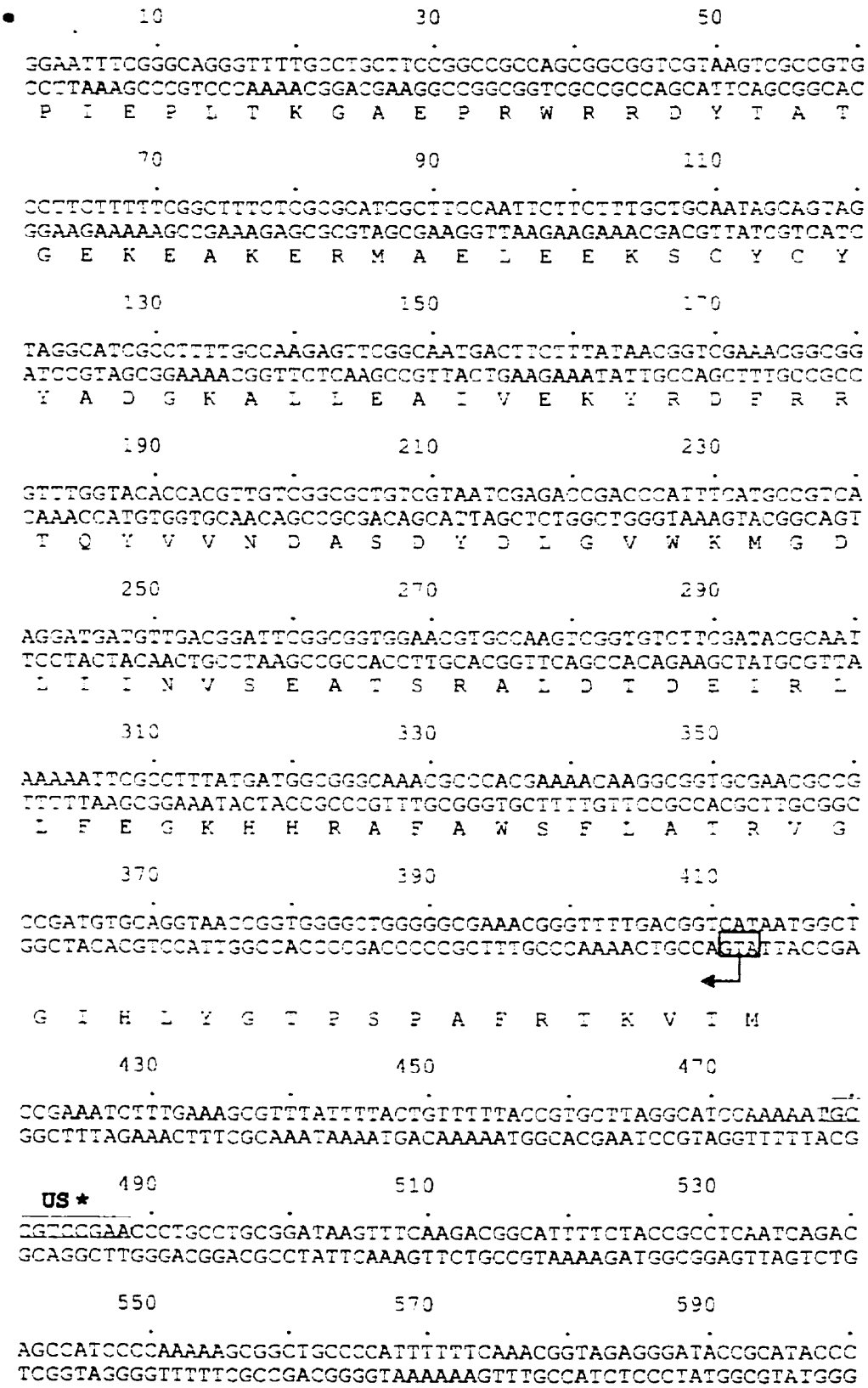
B.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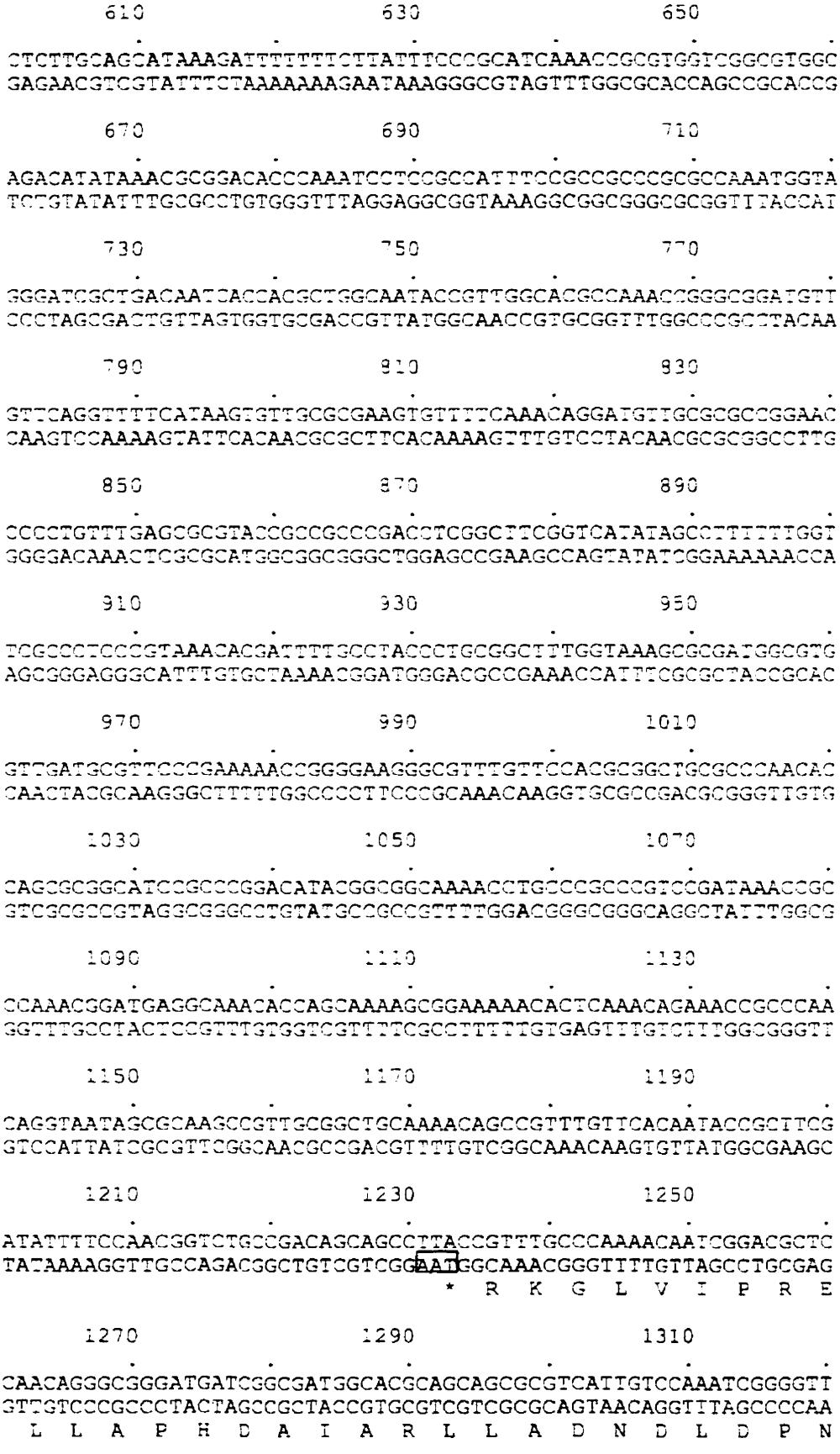
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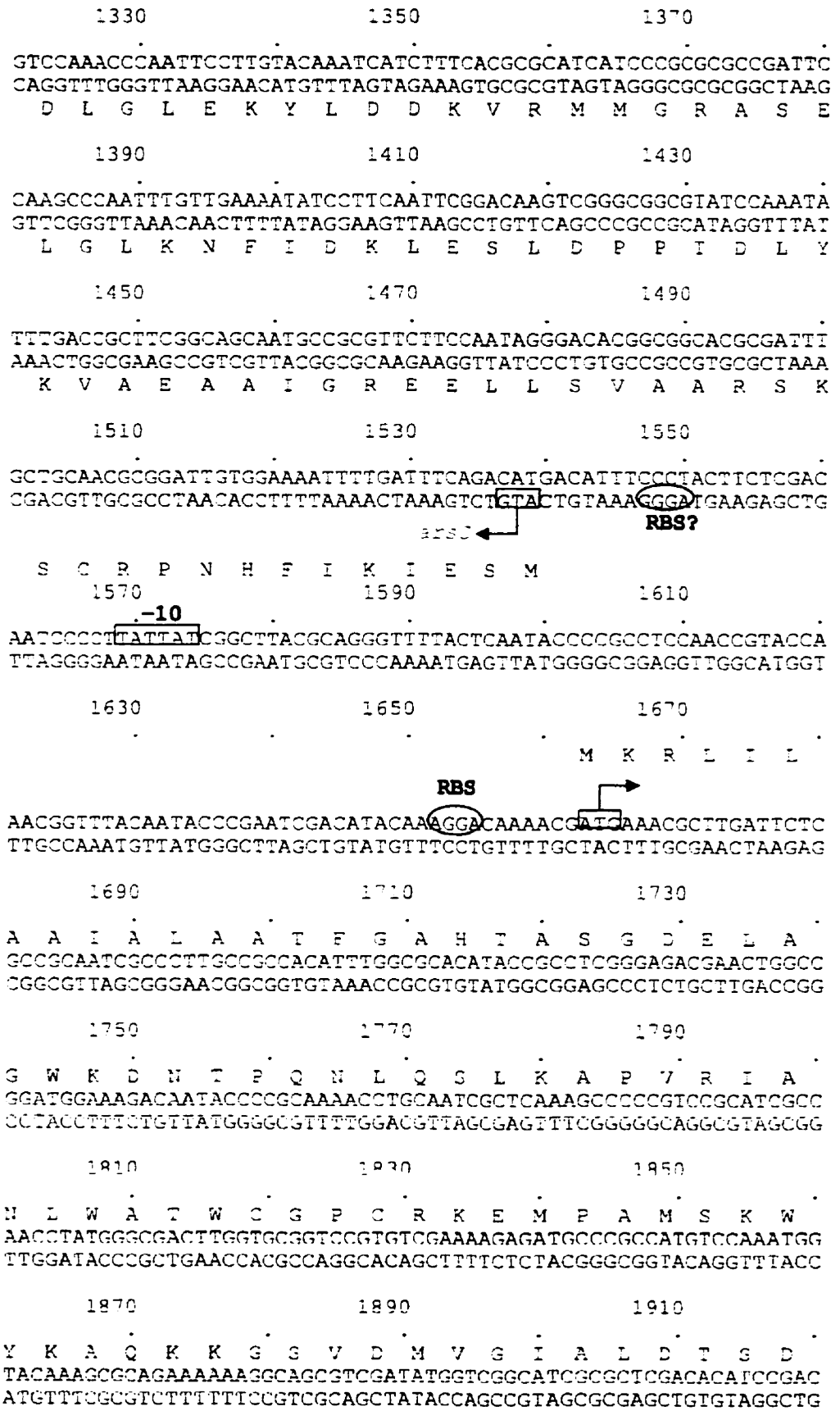
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RBS?
S C R P N H F I K I E S M
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1590
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1830
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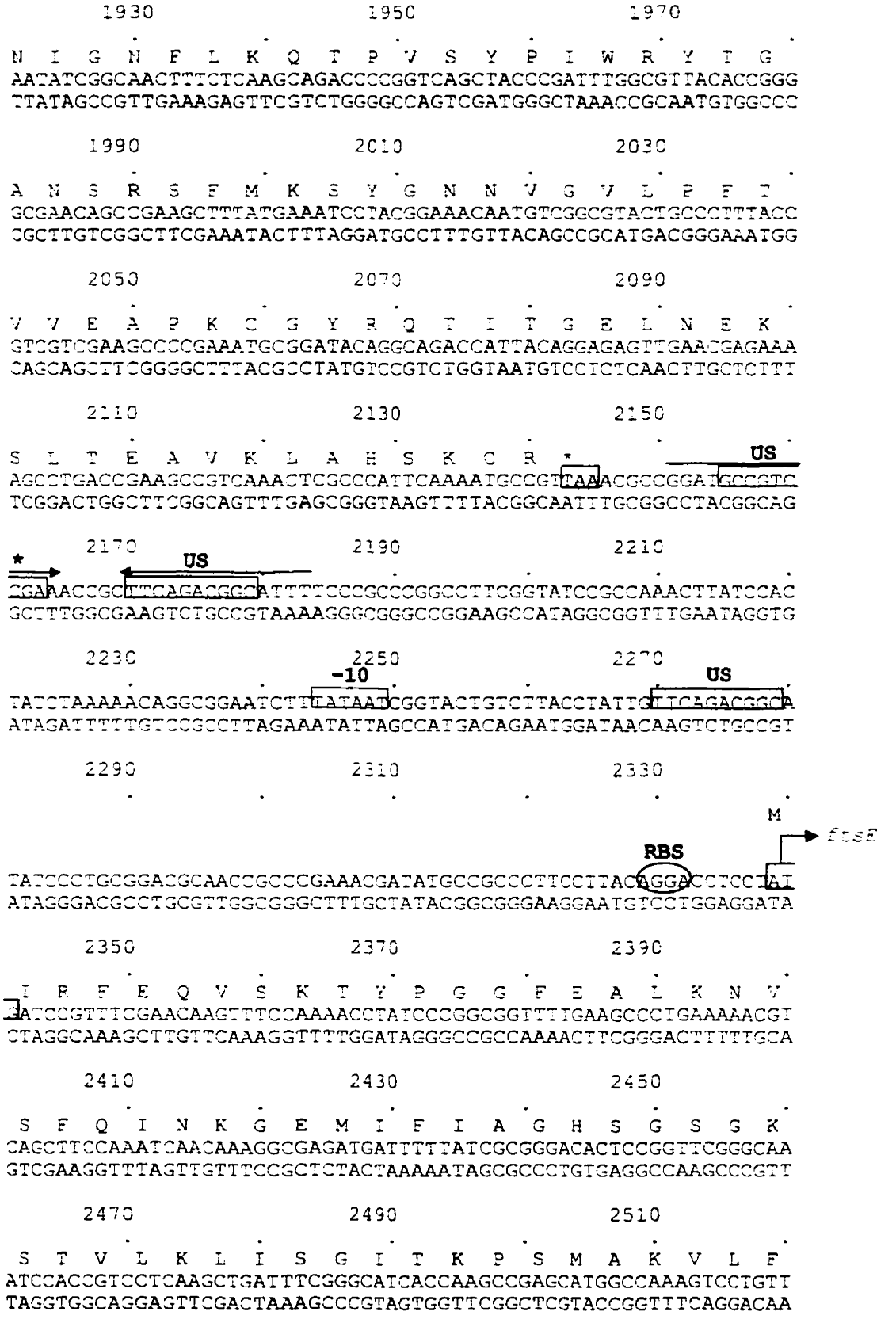
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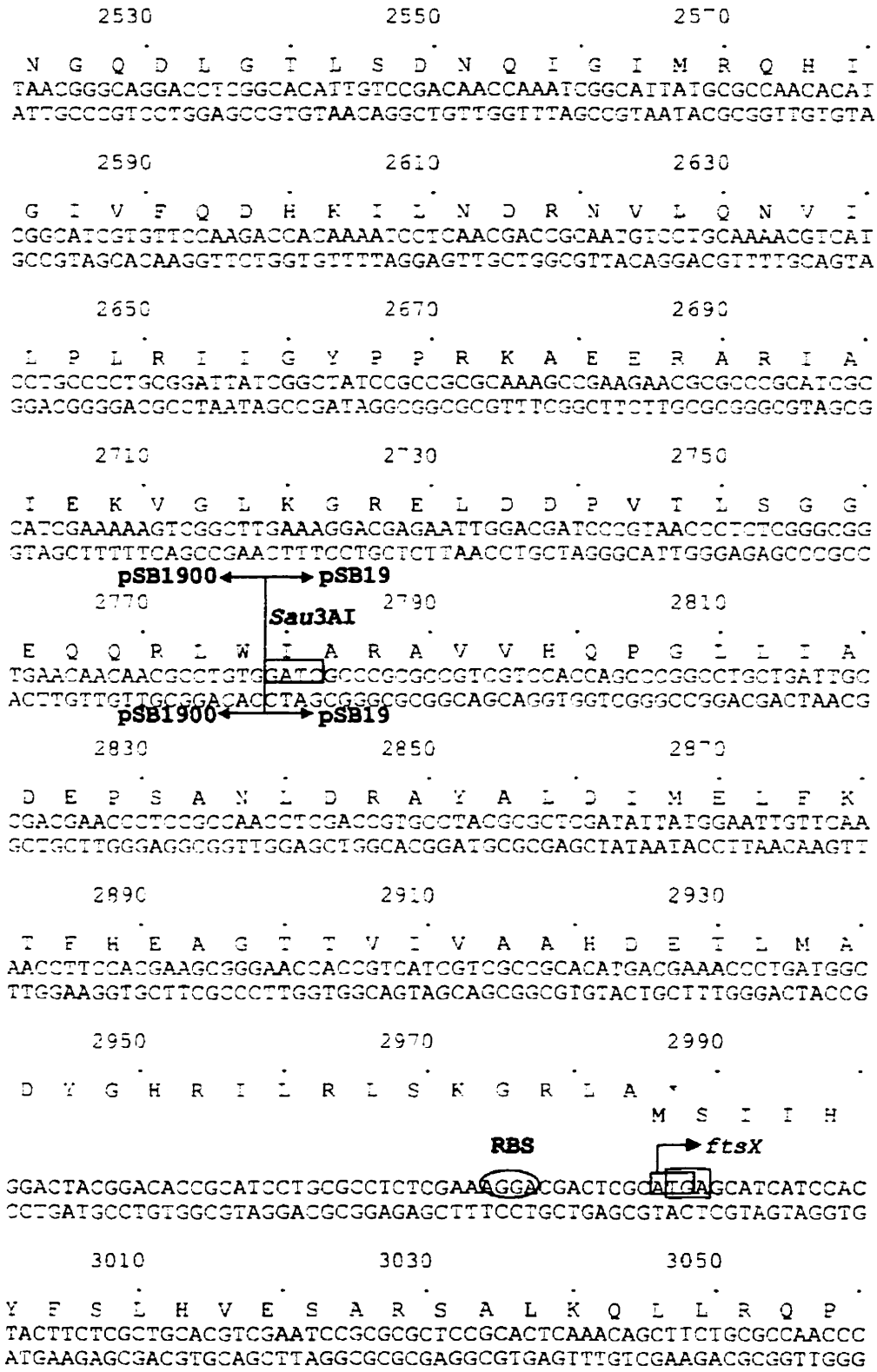
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pSB19
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Sau3AI
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M S I I H
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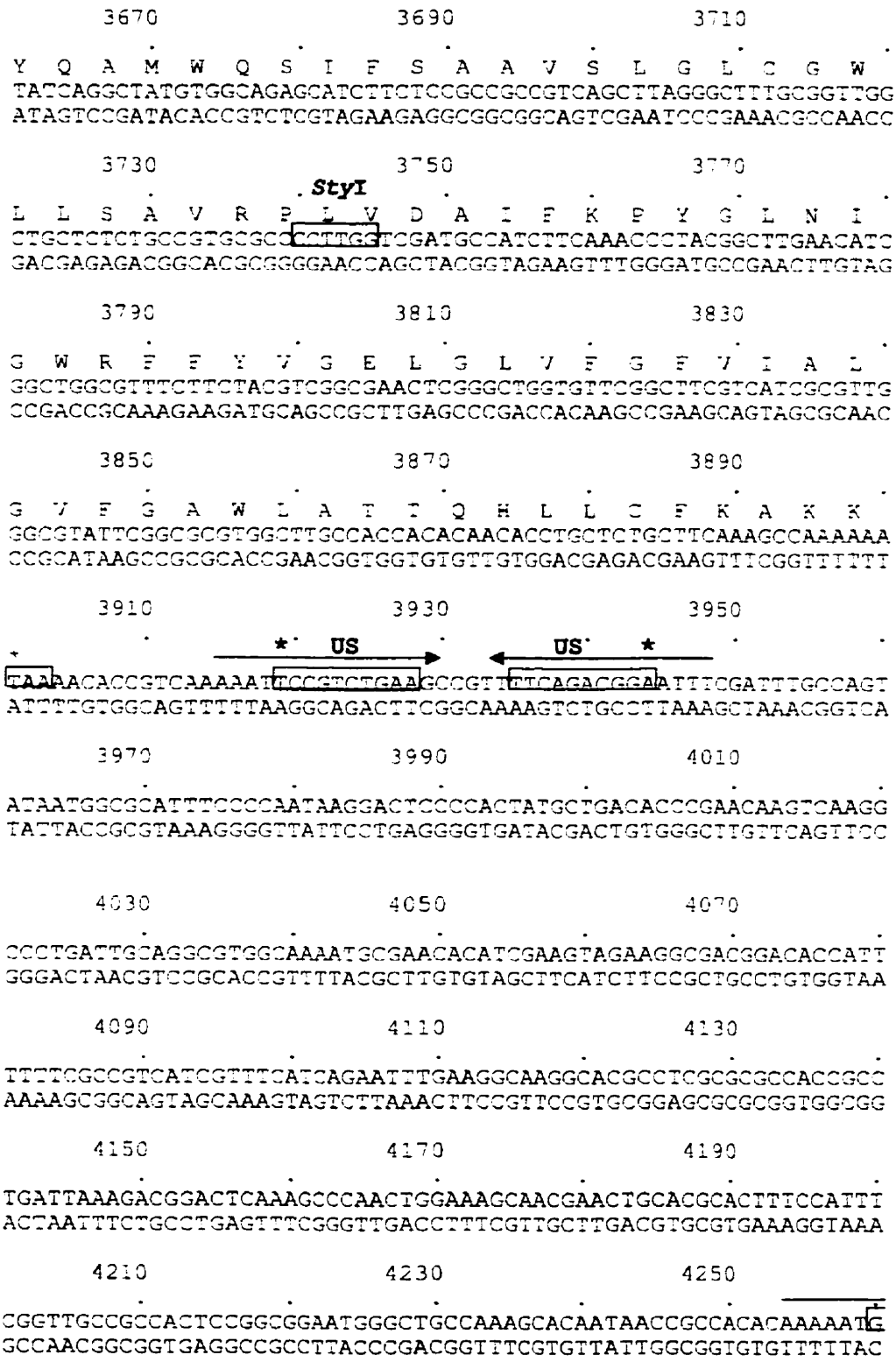
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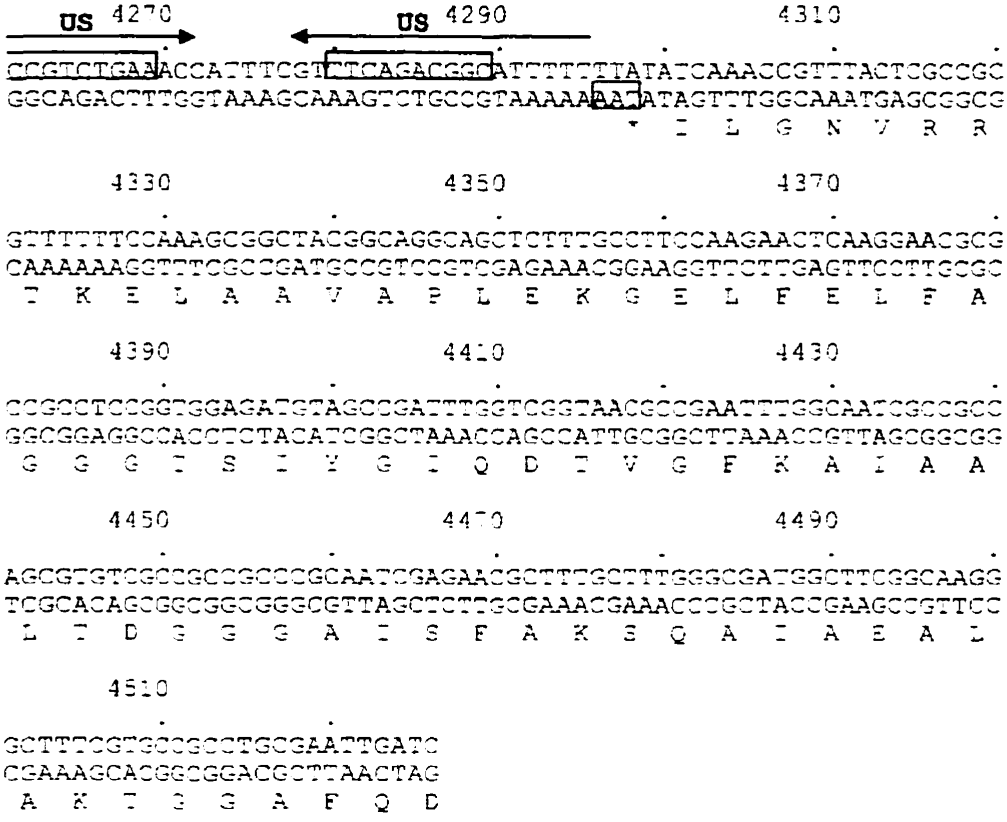
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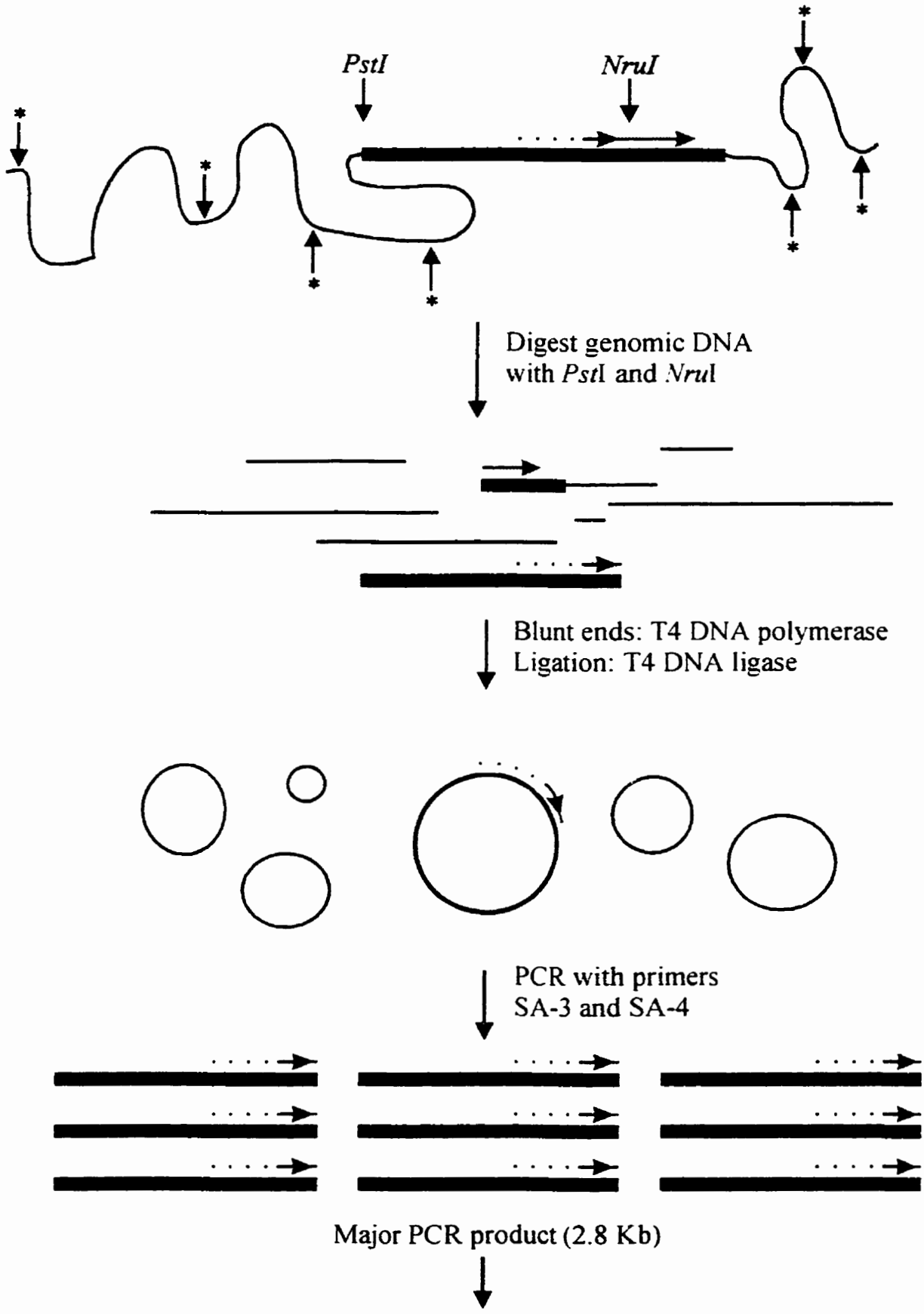
A K I G G A F Q D

Figure 7. Inverse PCR strategy to clone the 5'-region of the *N. gonorrhoeae ftsE* gene and the region upstream of *ftsE*. The thin black line represents the gonococcal chromosome, the thick black line represents the 1.8 Kb *Sau*3AI fragment cloned into pSB19, and the thick green line symbolizes the region to be amplified by iPCR. The blue arrow represents the *ftsX* gene; the red arrow represents the partial *ftsE* gene cloned in pSB19 and the dotted red line represents the section of *ftsE* to be amplified by iPCR. The *Pst*I and *Nru*I restriction sites used for the iPCR are indicated by red arrows, and other *Pst*I and *Nru*I restriction sites are indicated by black arrows with asterisks. The primers SA-3 and SA-4 used for the amplification are indicated by yellow arrows. The drawing is not to scale.



Amplicon is cloned in pCRII to generate pSB1900

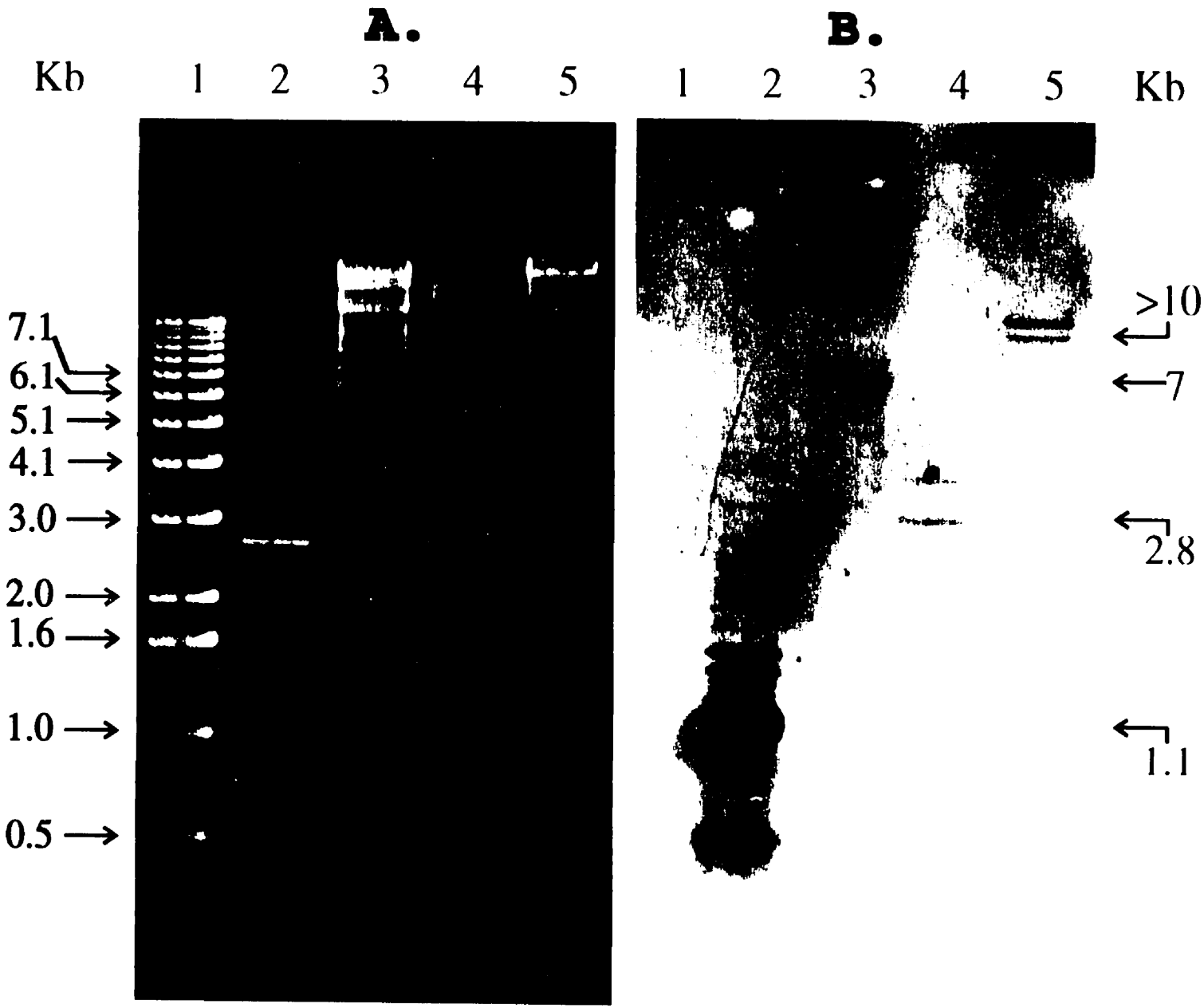
3578-3559, Table 3), one of the oligonucleotides used as a primer for the sequencing of the gonococcal *ftsX* gene (Table 3).

Restriction fragments of sizes from 6.9 Kb to 8.5 Kb hybridized the probe 19-4R (data not shown), the 6.9 Kb restriction fragment being obtained from the *Pst*I digestion. As a control, the insert of pSB19 also hybridized the probe (data not shown). Because the 6.9 Kb *Pst*I fragment was the smallest of those identified, this *Pst*I fragment was used for the next step of the strategy to clone the gonococcal *ftsE* gene.

Restriction enzyme analyses of pSB19 and computer analyses of its nucleotide sequence showed that *Pst*I did not restrict the 1789 bp insert (Fig. 6A). It was inferred that the 6.9 Kb *Pst*I fragment that hybridized the 19-4R oligonucleotide probe (data not shown) contained the 1.8 Kb insert of pSB19 together with 5.1 Kb of flanking sequences. Since only the sequence containing the 5'-section of the *ftsE* gene was needed, the cloning strategy was optimized to amplify only the region upstream of the partial *ftsE* gene already cloned in pSB19.

Based on the analysis of its DNA sequence, a unique *Nru*I site is present in the insert of pSB19 in the 5' region of *ftsX* (positions 3095-3100, Fig. 6A, B). It was reasoned that the 6.9 Kb *Pst*I fragment would be smaller if it was also restricted with *Nru*I. A shorter fragment would facilitate the circularization step that precedes the amplification step of iPCR. To test this, gonococcal DNA was digested with *Nru*I and *Pst*I and was probed with the oligonucleotide SA-2 (positions 3098-3079, Table 3; Fig. 8), designed from the sequence immediately 5' to the *Nru*I restriction site. In the *Pst*I digest, the 6.9 Kb *Pst*I chromosomal fragment hybridized the probe as had been observed in the first hybridization using the probe 19-4R (Fig. 8B, lane 3). Two large *Nru*I fragments also hybridized the probe (Fig. 8B, lane 5). Since one restriction fragment per digest was expected to hybridize the probe, the presence of two signals for this digest suggested the *Nru*I digest was not complete. Similar to what had been obtained with the *Nru*I digest, two *Nru*I-*Pst*I fragments of 2.8 and 3.0 Kb hybridized the probe (Fig. 8B, lane 4). The presence of two signals from both the *Pst*I-*Nru*I and the *Nru*I digests, but not from the *Pst*I digest, suggested again that the *Nru*I digest was incomplete. As for the hybridization to pSB19 (Fig. 8B, lane 2),

Figure 8. Hybridization of the oligonucleotide probe SA-2 to gonococcal DNA digested with *Pst*I, *Nru*I, and *Pst*I-*Nru*I. (A) Restriction enzyme digestions of the chromosomal DNA, (B) hybridization of the DNA from (A) with the oligonucleotide SA-2. Lane 1, 1 Kb ladder; lane 2, pSB19 digested with *Bss*HII; lane 3, *N. gonorrhoeae* DNA digested with *Pst*I; lane 4, *N. gonorrhoeae* DNA digested with *Pst*I and *Nru*I; lane 5, *N. gonorrhoeae* DNA digested with *Nru*I. The sizes (in bp) of the size markers and the size of the fragments having hybridized the probe are indicated are indicated to the left and to the right of the figure, respectively.


A.
B.
Kb
1
2
3
4
5
7.1
6.1
5.1
4.1
3.0
2.0
1.6
1.0
0.5
>10
7
2.8
1.1

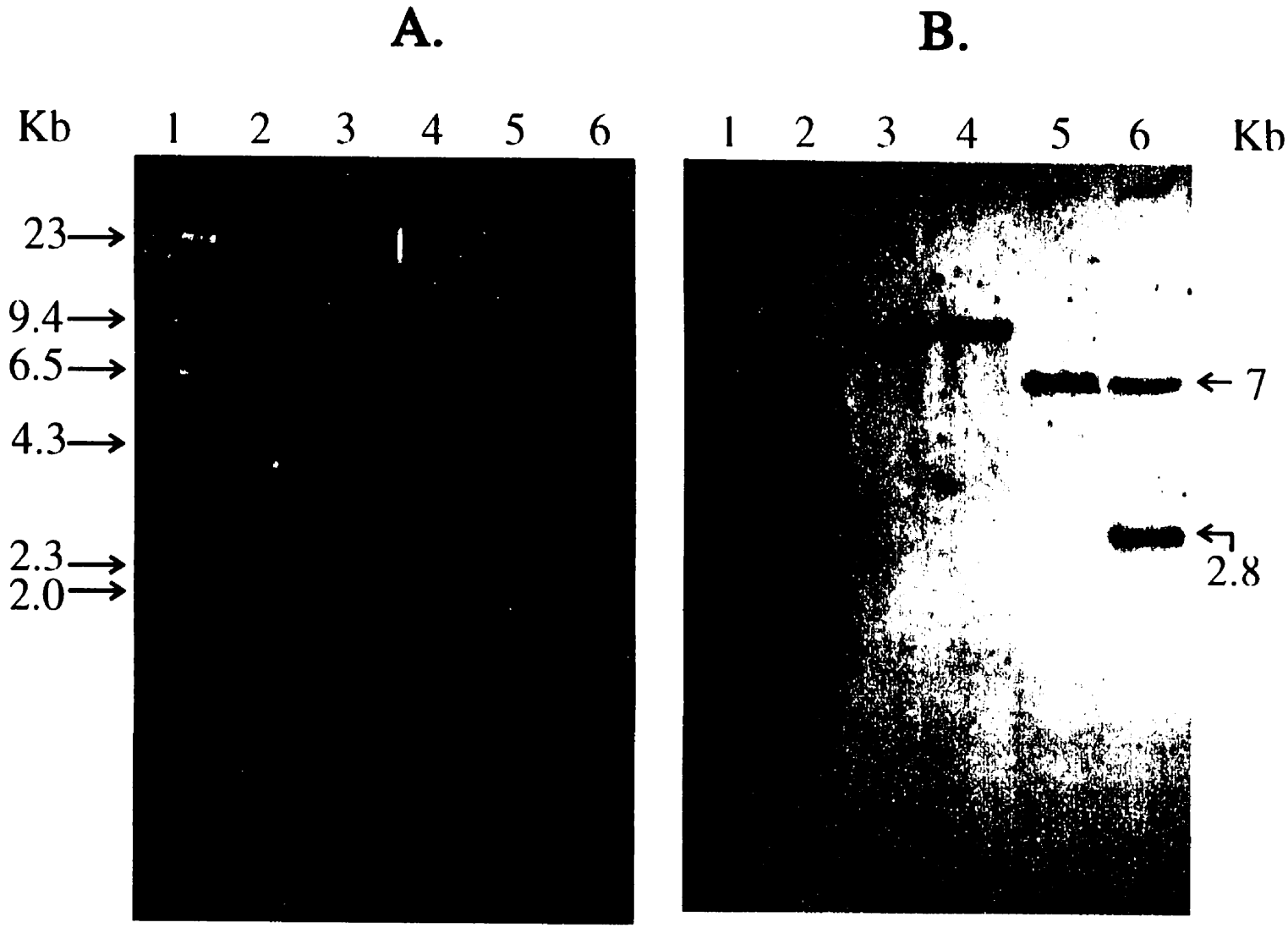
two fragments of 0.5 Kb and 1.1 Kb were expected to hybridize the probe as both contained insert sequences. The other hybridization signals present above the 1 Kb hybridization signal could also be due to an incomplete digestion of pSB19.

The 2.8 Kb *Pst*I-*Nru*I fragment was used as a template for iPCR. Gonococcal DNA was digested with *Pst*I-*Nru*I and the digested DNA was used in the steps of iPCR (Fig. 7; section 2.9.2.). The amplification generated a 2.8 Kb amplicon that was ligated with the plasmid pCRII™, a vector designed to efficiently clone amplicons generated with *Taq* DNA polymerase, to generate the plasmid pSB1900 (Fig. 7; section 2.9.2.).

The gonococcal origin of the amplicon cloned in pSB1900 was confirmed by hybridization at 68°C to *N. gonorrhoeae* CH811 DNA restricted with *Eco*RI, *Hin*dIII, *Pst*I, and *Nru*I-*Pst*I (Fig. 9). Two *Nru*I-*Pst*I fragments hybridized the insert of pSB1900 (Fig. 9B, lane 6) the expected 2.8 Kb *Nru*I-*Pst*I fragment and a 7 Kb fragment that was likely the *Pst*I fragment that had not been restricted with *Nru*I. This was likely due to the small amount of *Nru*I that had been used in the restriction digest. There was also hybridization with a 6.9 Kb *Pst*I fragment (Fig. 9B, lane 5), the same size as the larger fragment from the *Nru*I-*Pst*I digest (Fig. 9B, lane 7), confirming that this fragment had probably not been digested by *Nru*I in the double digest. The 6.9 Kb *Nru*I-*Pst*I fragment (Fig. 9B, lane 5) was of identical size to the *Pst*I fragment initially identified in the first set of hybridizations using the probe 19-4R. In addition, an *Eco*RI restriction fragment of 8.0 Kb (Fig. 9B, lane 3), and a *Hin*dIII restriction fragment of 8.2 Kb (Fig. 9B, lane 4) also hybridized the insert of pSB1900. The size of these *Eco*RI and *Hin*dIII fragments were the same as those that had hybridized the oligonucleotide probe 19-4R in the initial hybridization (data not shown). The insert of pSB1900 hybridized the probe (Fig. 10B, lane 2), but there was also a signal with the vector part of pSB1900.

The nucleotide sequence of the insert of pSB1900 was determined in both directions. The analysis of the DNA sequence of the insert of pSB1900 indicated that the amplicon was 2778 bp in size (positions 1-2778, Fig. 6B), and that it contained the nucleotide sequence of the 5'-end of *ftsE* (positions 2339-2778, Fig. 6B) as well as 2338 bp of sequence upstream of *ftsE*. This

Figure 9. Hybridization of the insert of the plasmid pSB1900 to digested *N. gonorrhoeae* DNA. (A) Restriction enzyme digestions of the chromosomal DNA, (B) hybridization of the DNA from (A) with the amplicon cloned in pSB1900. Lane 1, λ/*Hin*dIII; lane 2, pSB1900 digested with *Eco*RI; lane 3, *N. gonorrhoeae* DNA digested with *Eco*RI; lane 4, *N. gonorrhoeae* DNA digested with *Hin*dIII; lane 5, *N. gonorrhoeae* DNA digested with *Pst*I; lane 6, *N. gonorrhoeae* DNA digested with *Pst*I and *Nru*I. The sizes (in bp) of the size markers and the size of the fragments having hybridized the probe are indicated are indicated to the left and to the right of the figure, respectively.


A.
B.
Kb
1 2 3 4 5 6
23
9.4
6.5
4.3
2.3
2.0
1 2 3 4 5 6 Kb
7
2.8

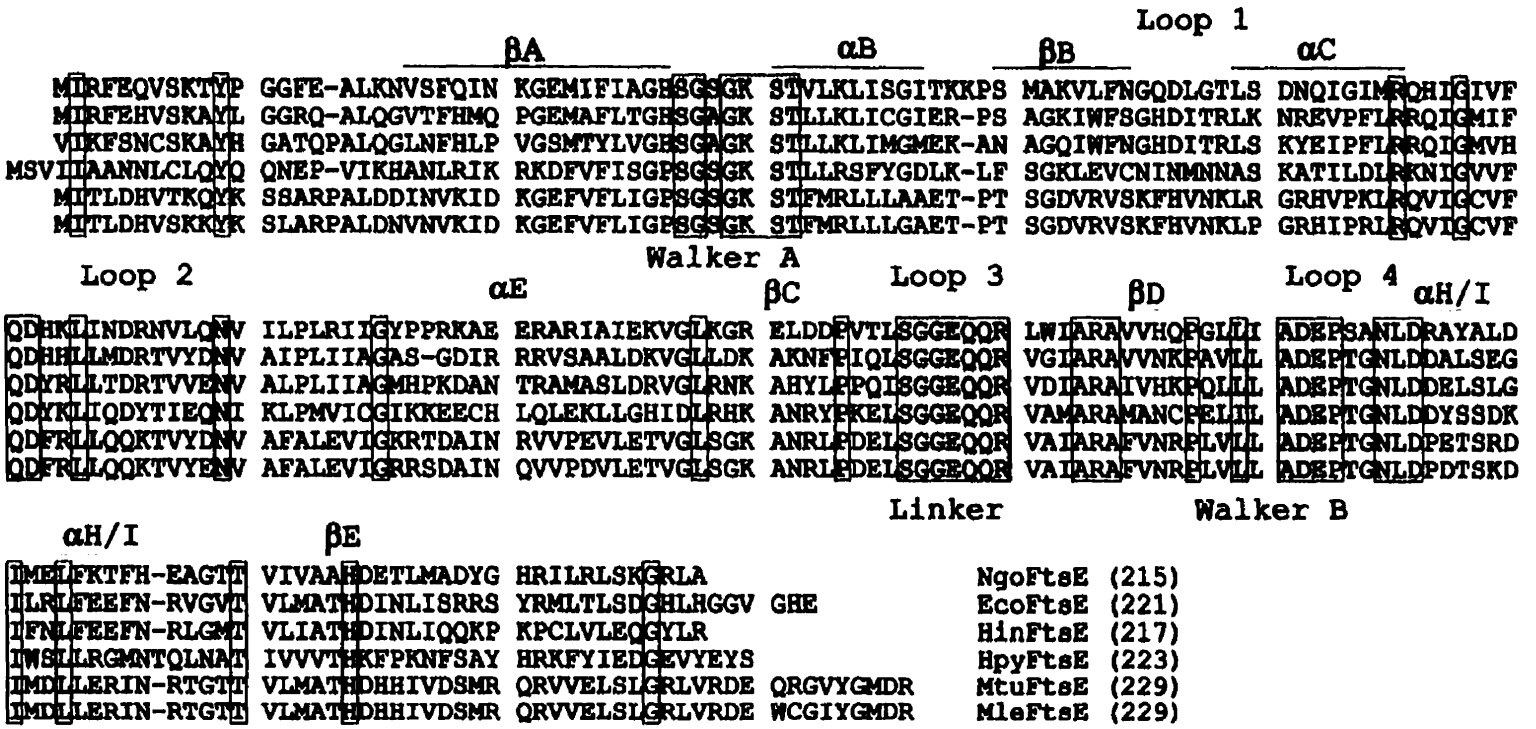
analysis indicated that no *ftsY* gene was located close upstream of *ftsE* in *N. gonorrhoeae* (Fig. 6A). Instead, complete *tlpA* and *arsC* gene homologues as well as a partial *gltX* gene homologue were identified upstream upstream of *ftsE* (Fig. 6A; sections 3.10.1. to 3.10.3.). The complete 4526 bp nucleotide sequence of pSB1900 and pSB19 including the *ftsE* and *ftsX* genes and their flanking regions was submitted to Genbank and is available under the accession number U76418.

3.3. Analysis of the *ftsEX* genes of *N. gonorrhoeae* strain CH811

3.3.1. The *ftsE* gene

The complete gonococcal *ftsE* gene comprised 651 bp (positions 2339-2989, Fig. 6B). It was preceded by a putative consensus RBS (AGGA, positions 2329 to 3232, Fig. 6B) located 7 bp upstream from the ATG initiation codon. A perfectly conserved -10 box (TATAAT, positions 2244 to 2249, Fig. 6B) was identified at positions -95 to -90 from *ftsE* and could be part of a promoter controlling the expression of *ftsE*. The stem-loop of a putative transcriptional terminator could not be located immediately downstream from *ftsE*. The nucleotide sequence of *ftsE* encoded a protein of 216 aa with a predicted Mw of 23 885 Da. Alignments using BESTFIT showed that the gonococcal FtsE displayed varying degrees of sequence similarity to other prokaryotic FtsE proteins available in Genbank (Fig. 10). The gonococcal FtsE was more similar to its homologues from other Gram negative bacteria than those from Gram-positive bacteria. It shared 71% similarity and 49% identity with FtsE from *E. coli* (Gill *et al.*, 1986), 66% similarity and 45% identity with FtsE from *H. influenzae* (Fleischmann *et al.*, 1995), 54% similarity and 32% identity with FtsE from *H. pylori* (Tomb *et al.*, 1997), 62% similarity and 41% identity with FtsE from *M. leprae* (Parkhill *et al.*, 1997a), and 60% similarity and 39% identity with the FtsE from *M. tuberculosis* (Devlin *et al.*, 1997). The important motifs in ABC transporters, namely the Walker motifs A and B involved in ATP binding and the linker peptide (section 1.2.2.), were well-conserved in the gonococcal FtsE (Fig. 10). Other predicted structural features of the ATP-binding domain were also well conserved between the gonococcal FtsE and the other FtsE homologues (Fig. 10). It also shared high similarity (over 60% from Blast similarity searches)

Figure 10. Alignment of the amino acid sequence of the *N. gonorrhoeae* FtsE with other prokaryotic FtsE proteins. Positions perfectly conserved in all six proteins are boxed. The sequences of the Walker A and B motifs and of the linker motif are boxed in gray, other positions identical between the sequences shown are boxed. The α-helices, the β-sheets and the loops are identified as in Fig. 2 based on Higgins (1992).


Loop 1
βA
αB
βB
αC
Walker A
Loop 2
αE
βC
Loop 3
βD
Loop 4
αH/I
αH/I
βE
Linker
Walker B
NgoFtsE (215)
EcoFtsE (221)
HinFtsE (217)
HpyFtsE (223)
MtuFtsE (229)
MleFtsE (229)

with other members of the ABC protein family, with the sequence similarities centered around the ATP-binding domains and the linker peptide (data not shown).

Computer predictions of the amino acid sequence of *N. gonorrhoeae* FtsE showed that it did not contain membrane-spanning helices, and identical results were obtained for FtsE from the other five bacterial FtsE proteins (data not shown). These results suggested that if FtsE was located to the inner membrane as is the case in *E. coli* (section 1.2.), it would require an association with another protein.

3.3.2. The *ftsX* gene

There was a 4 base pair overlap between *ftsE* and *ftsX*, the ORF located immediately downstream of *ftsE* (section 3.1.). The *ftsX* ORF contained 918 bp (positions 2986-3903 in Fig. 6B) and was preceded by a consensus putative RBS (AGGA, positions 2974 to 2977, Fig. 6B) located 8 bp upstream of the ATG initiation codon. Two inverted copies of the gonococcal US (positions 3919-3928 and 3935-3944, Fig. 6B) were identified downstream of the *ftsX* ORF. They were predicted to be part of the stem of a predicted transcriptional terminator (positions 3919-3944, Fig. 6B) located downstream of *ftsX*. Both copies of the US contain one mismatch with the consensus sequence: in the first US, a guanosine was replaced by a thymidine (position 3919, Fig. 6B), and in the second US, a guanosine was replaced by an adenosine (position 3944, Fig. 6B). The gonococcal US had been shown to be essential for efficient uptake of DNA by gonococci and had been identified as a transcriptional terminator for gonococcal genes (section 1.11.).

The nucleotide sequence of *ftsX* translated into a polypeptide of 305 aa with a predicted Mw of 34 213 Da. Alignments using BLAST and BESTFIT showed the gonococcal FtsX displayed similar degrees of sequence similarity with only the five known prokaryotic FtsX proteins (Fig. 11). Similarly to what had been observed for the gonococcal FtsE, the gonococcal FtsX shared a lower degree of similarity with FtsX from *M. tuberculosis* and *M. leprae*. The gonococcal FtsX shared 55% similarity and 29% identity with FtsX from *E. coli* (Gill *et al.*, 1986), 55% similarity and 26% identity with FtsX from *H. influenzae* (Fleischmann *et al.*, 1995),

47% similarity and 23% identity with FtsX from *H. pylori* (Tomb *et al.*, 1997), and 52% similarity and 20% identity with FtsX from *M. tuberculosis* (Devlin *et al.*, 1997; Tyagi *et al.*, 1996), 49% similarity and 19% identity with FtsX from *M. leprae* (Devlin *et al.*, 1997a). No significant similarity was detected with other protein sequences in the databases.

There were only six perfectly conserved positions in the six FtsX sequences (Fig. 11): Pro 124, Leu191, Leu212, Gly214, Ala215 and Leu225 (positions in *N. gonorrhoeae* FtsX). Five of the six conserved positions were located closer to one another (Leu191, Leu212, Gly214, Ala215 and Leu225, Fig. 11), between the end of the second transmembrane segment and the beginning of the third transmembrane segment. This is the first report on the identification of conserved positions in FtsX homologues as alignments of multiple FtsX sequences had not been possible until recently. Several other positions were also conserved in three or four proteins. The alignment of the six FtsX proteins presented in Fig. 11 shows the diversity in size of this protein. With 305 aa residues, the *N. gonorrhoeae* FtsX was 47 and 2 aa residues shorter than FtsX from *E. coli* and *H. influenzae*, respectively. It was also 8 aa residues longer than *M. tuberculosis* FtsX (297 aa), 18 residues longer than *M. leprae* FtsX (287 aa) and 37 aa residues longer than *H. pylori* FtsX (268 aa).

Analyses of the aa sequence of the gonococcal FtsX using the algorithms available in PCGene, PSORT and TopPredII predicted it contained four transmembrane segments (Fig. 11). Each FtsX homologue was also predicted to contain four transmembrane segments, and these segments were identified at similar locations on the alignment of the six FtsX aa sequences (Fig. 11). This suggested that FtsX was an integral membrane protein with four transmembrane segments, and that despite the size differences between species, there were structural similarities between the bacterial FtsX proteins. Tyagi *et al.* (1996) performed similar computer-based predictions on *M. tuberculosis* and *E. coli* FtsX, but this report constitutes the first analysis of multiple FtsX homologues. The present predictions and those of Tyagi *et al.* (1996) corroborate well with the results of Gill *et al.* (1987) that showed that FtsX was an integral membrane protein in *E. coli*.

Figure 11. Alignment of the amino acid sequence of the *N. gonorrhoeae* FtsX with other prokaryotic FtsX proteins. Positions perfectly conserved in all six proteins are boxed. The aa residues predicted to be part of a transmembrane segments are indicated in bold type, and each of the four transmembrane segments is identified.


Transmb. segment 1
Transmb. segment 2
Transmb. segment 3
Transmb. segment 4
NgoFtsX (305)
EcoFtsX (352)
HinFtsX (310)
HpyFtsX (268)
MtuFtsX (297)
MleFtsX (287)

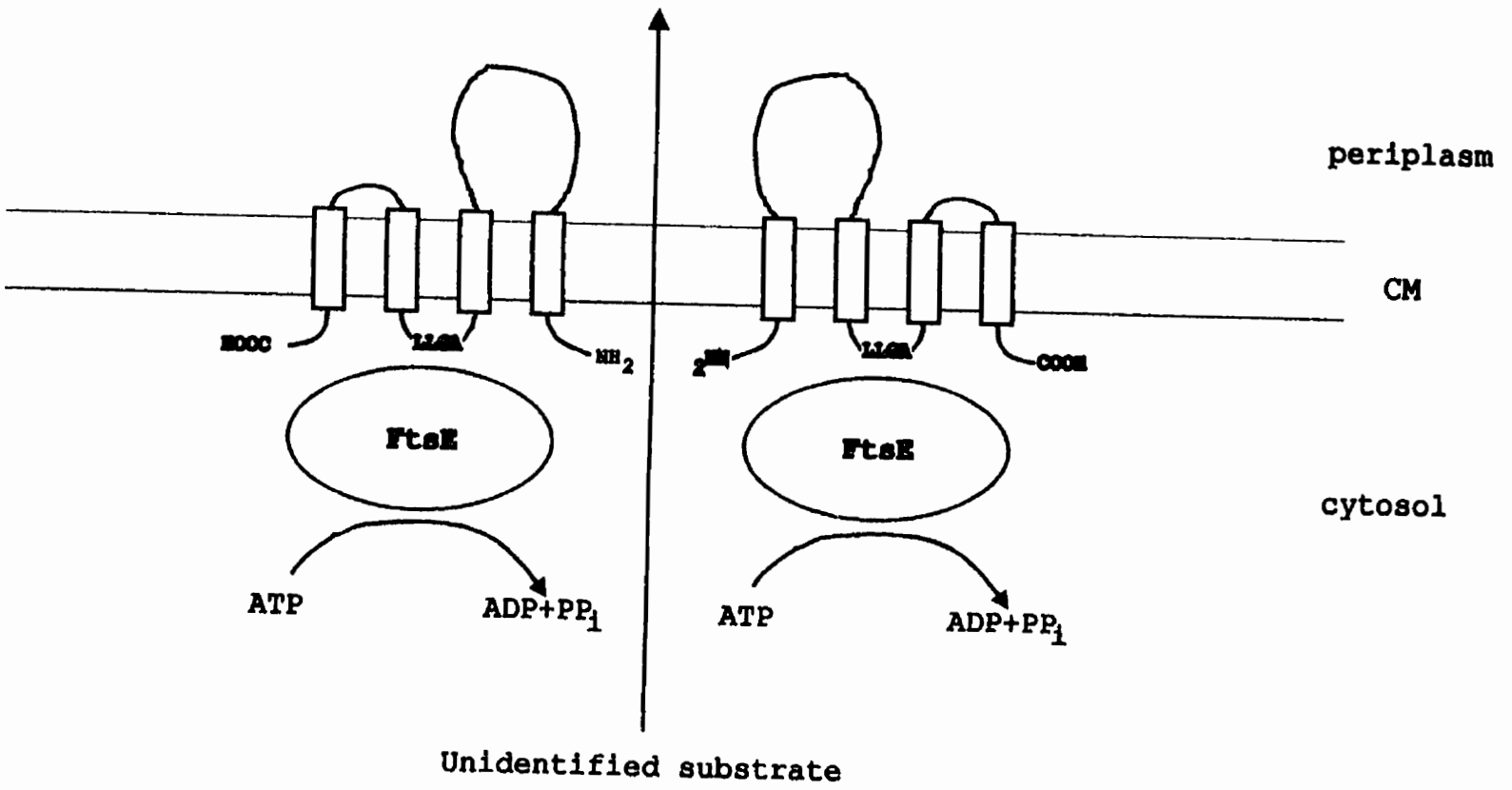
The alignment shown on Fig. 11 also suggested that the length of the amino-terminal domain of FtsX varied considerably. *E. coli* FtsX had the longest amino-terminal domain (71 aa residues) compared to the other FtsX sequences. Any possible structural and/or functional implications for this difference are unknown at this time. The linker between the first and second transmembrane segments in all four bacterial FtsX was much larger than the other linkers and could be a functional domain. Five of the six perfectly conserved positions are located at the end of the second transmembrane segment and in the second linker between the second and third predicted transmembrane domains. These regions, especially the second linker domain, may have structural or functional importance.

ABC transporters typically comprise two ATP-binding domains and two hydrophobic domains containing membrane-spanning helices that can be encoded by different genes (section 1.2.1.). The present analysis suggested that FtsE was an ATP-binding protein and that FtsX was the transmembrane protein of a putative ABC transporter. Since the linker between the first and second transmembrane domains was predicted to be larger, this domain could be the site of interactions between FtsE and FtsX.

To test this hypothesis, the membrane topology of the FtsX protein from *N. gonorrhoeae* was predicted by using TopPredII 1.3 (section 2.7.). *N. gonorrhoeae* FtsX was predicted to contain four transmembrane segments by TopPredII, which correlated perfectly with our previous analyses of FtsX. It was predicted to have its amino- and carboxy-terminal termini on the cytoplasmic side of the membrane. This implicated that the large linker domain between the first two transmembrane segments was periplasmic, and could not serve as the site of FtsE-FtsX interactions. For comparison, the membrane topologies of the other five known FtsX homologues were also predicted with TopPredII. All five FtsX homologues were predicted to present a membrane topology identical to that predicted for *N. gonorrhoeae* FtsX.

A schematic representation of the putative ABC transporter formed by FtsE and FtsX is shown in Fig. 12, with its two ATP-binding domains and two hydrophobic domains each containing 4 transmembrane segments.

Figure 12. Schematic representation of the putative ABC transporter formed by FtsE and FtsX. The membrane domain (FtsX) is represented in blue with the four predicted transmembrane segments as boxes. The amino- and carboxy-terminal ends as well as the LLGA conserved motif are shown. The ATP-binding domain that binds and hydrolyses ATP (FtsE) is represented in red. The putative FtsEX transporter is proposed to export an unidentified substrate out of the bacterial cytosol, as indicated.


periplasm
CM
cytosol
FtsE
FtsE
ATP
ADP+PPi
ATP
ADP+PPi
Unidentified substrate

3.4. *ftsX* is not an essential gene

3.4.1. Construction of a *ftsX* mutant of *N. gonorrhoeae*

A strain of *N. gonorrhoeae* in which the *ftsX* gene was insertionally inactivated was constructed to verify if *ftsX* was an essential gene. The strategy consisted in inserting a *cat* cassette within the coding sequence of *ftsX*, which would disrupt the coding sequence of *ftsX*. The construction of *N. gonorrhoeae* strain CS19 is described in section 2.13.2. If no Clr^R colonies were obtained following transformation, it would indicate that *ftsX* was an essential gene.

Following the transformation of *N. gonorrhoeae* $CH811Str^R$ with pSB193, Clr^R colonies were obtained. The presence of the *cat* gene within *ftsX* in the strain CS19 were confirmed by PCR (section 2.9.1.). The expected amplicon of 894 bp was generated from the strain CH811, showing that the *ftsX* gene was intact (Fig. 13, lane 2). In *N. gonorrhoeae* CS19, an amplicon of 2.8 Kb was generated from 2 colonies of *N. gonorrhoeae* CS19 (Fig. 13, lanes 3 and 4). This corresponded to the size of *ftsX* containing an insertion of 2.9 Kb, the size of the blunted *Acc*I-*Ava*I fragment containing the *cat* gene. The viability of the strain CS19 and the confirmation of an insertion within *ftsX* strain indicated that *ftsX* was not an essential gene.

3.4.2. Morphology of *N. gonorrhoeae* strain CS19

Cell division mutants of *E. coli* may exhibit aberrant morphologies (section 1.3.). *N. gonorrhoeae tpc* mutants have also been shown to present abnormal morphologies (section 1.8.2.). Cells from *N. gonorrhoeae* strain CS19 were observed by electron microscopy and their morphology was compared to that of *N. gonorrhoeae* strain CH811, its wild-type parent.

The cells shown in Fig. 14 are representative of each of the three strains observed (strains CH811, $CH811Str^R$, and CS19). Cells from *N. gonorrhoeae* strains CH811 and $CH811Str^R$ appeared as round, non piliated diplococci (Fig. 14A and B). *N. gonorrhoeae* CS19 cells also appeared as round, non piliated diplococci (Fig. 14C and D). The cells presumably lost their pili during the vortexing step of sample processing prior to the microscopic observation (section 2.3.); free pili are visible on the pictures presented in Fig. 14. The dimensions of the individual

Figure 13. PCR amplification of the *cat* gene inserted in the *ftsX* gene in *N. gonorrhoeae* CS19. Lanes 1 and 6, 1 Kb ladder; lane 2, *N. gonorrhoeae* CH811; lanes 3 and 4, *N. gonorrhoeae* CS19; lane 5, negative control. The sizes of the Mw marker and of the *ftsX* amplicons are indicated to the left and right of the figure, respectively.

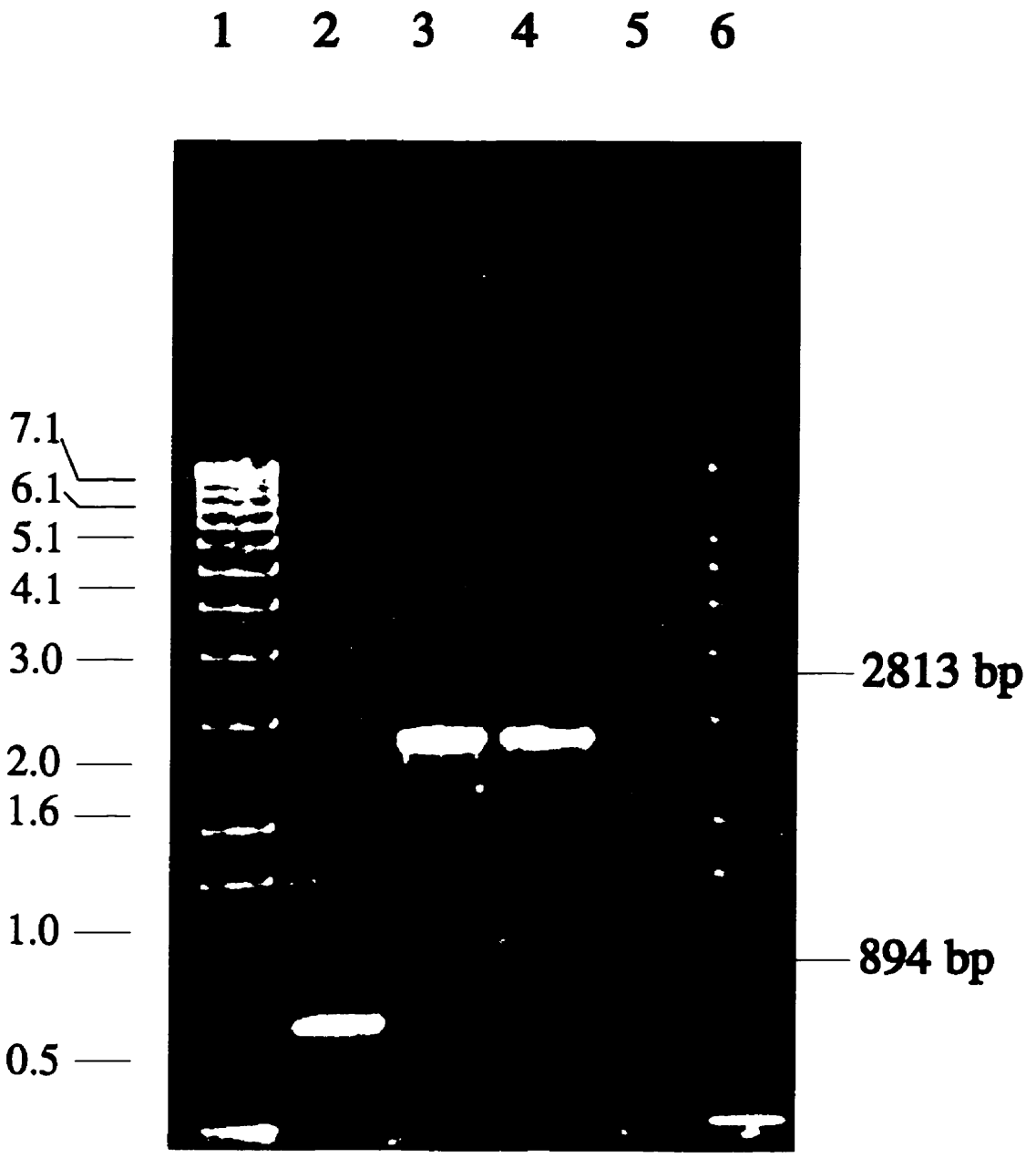
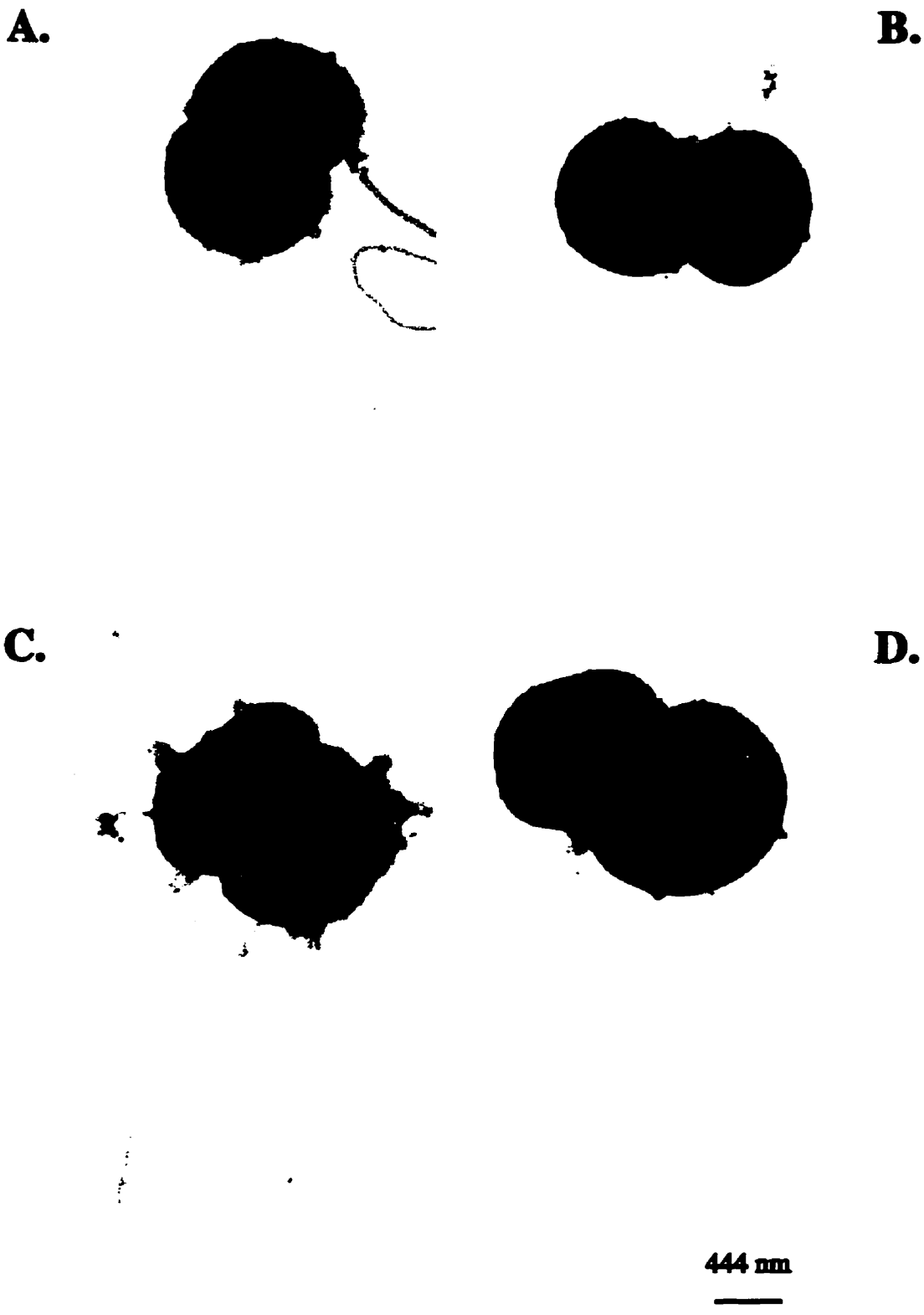

1
2
3
4
5
6
7.1
6.1
5.1
4.1
3.0
2.0
1.6
1.0
0.5
2813 bp
894 bp

Figure 14. Morphological observation of *N. gonorrhoeae* strains by electron microscopy. (A), *N. gonorrhoeae* CH811; (B), *N. gonorrhoeae* CH811Str^{R}; (C and D), *N. gonorrhoeae* CS19. All observations were done at a magnification factor of 22 550X. The scale (in nanometers (nm)) is indicated to the lower right corner of the figure.


A.
B.
C.
D.
444 nm

cocci from the three strains were then measured (Table 4).

Table 4. Dimensions of individual gonococci from different strains

N. gonorrhoeae strain[1]	Length (nm)	Width (nm)
CH811 (8)	750±150	940±80
CH811Str^R (10)	750±55	950±90
CS19 (4)	820±60	1150±75

[1] The number in parentheses following each strain indicates the number of gonococci whose dimensions were measured.

When the dimensions of individual gonococci were measured, no differences were found between *N. gonorrhoeae* CH811, CH811Str^R and the *ftsX* mutant CS19. The dimensions measured in Table 4 correspond with the published dimensions of *N. gonorrhoeae* cells (600 to 1000 nm in diameter; Gillespie, 1994; Wilfert *et al.*, 1992)

3.4.3. MICs, auxotype and viable counts of *N. gonorrhoeae* strains CH811 and CS19

In *E. coli* JS10, the Ts mutant of *ftsX*, the sensitivity to several antibiotics including penicillin was increased at the restrictive temperature when compared to its parent strain (section 1.4.). To verify if this was also the case between *N. gonorrhoeae* strains CS19 and CH811, the MIC of both strains for penicillin and other antibiotics were determined (Table 5).

Table 5. Minimal inhibitory concentrations of various antibiotics on *N. gonorrhoeae* CH811 and CS19

Antibiotic	MIC (µg/mL) of *N. gonorrhoeae* CH811	*N. gonorrhoeae* CS19
penicillin	0.063	0.063
tetracyclin	0.5	0.5
spectinomycin	16.0	16.0
ceftriaxone	0.004	0.004
ciprofloxacine	0.004	0.004
azithromycin	0.5	0.5

The data shown in Table 5 indicated there was no difference between the MIC profiles of *N. gonorrhoeae* strains CS19 and CH811 for the six antibiotics tested.

N. gonorrhoeae CS19 was found to be a non-requiring strain, as had been shown to be the case with *N. gonorrhoeae* CH811 (Lawson *et al.*, 1995). To verify if the presence of the *cat* insertion within *ftsX* affected the growth of *N. gonorrhoeae* CS19 compared to CH811, the two were grown on GCMB plates containing various supplements and the cfus were determined (Table 6).

Table 6. Colony forming units of *N. gonorrhoeae* CH811 and CS19 in various environmental conditions

		N. gonorrhoeae CH811	*N. gonorrhoeae* CS19
NaCl	0%	$2.04 \times 10^8 \pm 0.91 \times 10^8$	$6.65 \times 10^7 \pm 6.35 \times 10^7$
	0.25%	$5.40 \times 10^8 \pm 3.88 \times 10^8$	$1.46 \times 10^8 \pm 1.32 \times 10^8$
	0.50%	$4.15 \times 10^8 \pm 2.39 \times 10^8$	$2.23 \times 10^8 \pm 0.90 \times 10^8$
	1.0%	$5.2 \times 10^7 \pm 4.4 \times 10^7$	$1.1 \times 10^7 \pm 0.27 \times 10^7$
	1.5%	0	0
	2.0%	0	0
pH	8.0	$2.85 \times 10^8 \pm 2.85 \times 10^8$	$8.4 \times 10^7 \pm 6.7 \times 10^7$
	10.0	$5.3 \times 10^7 \pm 1.2 \times 10^7$	$1.31 \times 10^7 \pm 1.35 \times 10^7$
KCl[1]	100 mM	$6.2 \times 10^7 \pm 1.4 \times 10^7$	$5.4 \times 10^6 \pm 1.98 \times 10^6$
Sucrose	400 mM	$2.39 \times 10^7 \pm 1.4 \times 10^7$	$2.1 \times 10^6 \pm 1.1 \times 10^6$
Anaerobiosis[2]	GCMB	$1.7 \times 10^8 \pm 0.76 \times 10^8$	$1.2 \times 10^8 \pm 0.31 \times 10^8$
	minimal medium	0	0

[1] Based on two readings.

[2] The bacterial suspensions used to prepare the dilutions under anaerobiosis are different from those used to prepare the dilutions under aerobiosis, but both suspensions were adjusted to a McFarland Equivalence Turbidity Standard of 0.5 (section 2.2.).

Both strains grow up to a NaCl concentration of 1%. There were no significant differences observed between CH811 and CS19 when grown with varying concentrations of NaCl or at pH 8.0 or pH 10.0, suggesting that the insertion in *ftsX* did not affect the growth of strain CS19 in these conditions (Table 6). More important differences were noted on GCMB with 100 mM KCl (6.5-fold) or with 400 mM sucrose (10-fold) between the two strains in these conditions (Table 6). Under anaerobiosis the two strains had similar viable counts on GCMB, but neither strain grew on minimal medium (Table 6). These results suggested that the *ftsX* gene product was not important under anaerobiosis.

3.5. Prevalence of *ftsE* and *ftsX* in the *Neisseriaceae*.

3.5.1. Investigation of the *ftsEX* genes by hybridization

The presence of *ftsE* and *ftsX* in ten *Neisseria* species (and in *N. gonorrhoeae* as a positive control) was investigated by Southern blotting. Labeled *ftsE* and *ftsX* amplicons from *N. gonorrhoeae* were hybridized to *Pst*I-digested genomic DNA from *Neisseria* species at 60°C (data not shown). Under these conditions, the *ftsE* and *ftsX* probes hybridized to restriction fragments in *N. meningitidis*, *N. lactamica* and *N. cinerea*. The *ftsX* probe hybridized to a restriction fragment in *N. subflava* and *N. flavescens*, while the *ftsE* probe did not hybridize any *Pst*I restriction fragment in the same species (data not shown). Both probes hybridized a 7 Kb fragment of *N. gonorrhoeae* DNA as expected (data not shown). These results suggested sequence divergence among the *ftsE* and *ftsX* genes between *Neisseria* species. The hybridization was then repeated at lower stringency (50°C), and in these conditions *Pst*I fragments from all species hybridized both probes (Fig. 15 and 16).

In *N. gonorrhoeae*, a 7 Kb *Pst*I restriction fragment hybridized the *ftsE* probe (Fig. 15B, lane 2), which was the same size as the *Pst*I fragment identified following the hybridizations done prior to the cloning of the 5'-section of the gonococcal *ftsE* gene and its upstream region (section 3.2). The *ftsE* probe hybridized DNA fragments of high Mw (higher than 16 Kb) in *N. meningitidis*, (Fig. 15B, lane 3), *N. elongata* (Fig. 15B, lane 6), *N. lactamica* (Fig. 15B, lane 8), *N. perflava/sicca* (Fig. 15B, lane 9) and *N. polysaccharea* (Fig. 15B, lane 11). In *N. flava* and in *N. mucosa*, the *ftsE* probe hybridized restriction fragments of 6 Kb, (Fig. 15B, lanes 4 and 7). In *N. subflava* the *ftsE* probe hybridized a restriction fragment of 2.6 Kb (Fig. 15B, lane 5); and it hybridized a restriction fragment of 9 Kb in *N. flavescens* (Fig. 15B, lane 10). Finally, the *ftsE* probe hybridized a restriction fragment of 7 Kb in *N. cinerea* (Fig. 15B, lane 12). In seven of the eleven species tested, the *ftsX* probe hybridized a fragment of the same size as that hybridized by the *ftsE* probe (Fig. 16B, lanes 2, 3, 6, 8, 9, 11, 12). In *N. gonorrhoeae* and *N. cinerea*, the *ftsX* probe hybridized to a 7 Kb restriction fragment (Fig. 16B, lanes 2 and 12). The *ftsX* probe hybridized DNA fragments of high Mw (higher than 16 Kb) in *N. meningitidis*, (Fig. 16B, lane 3), *N. elongata* (Fig. 16B, lane 6), *N. lactamica* (Fig. 16B, lane 8), *N. perflava/sicca* (Fig. 16B, lane 9) and *N. polysaccharea* (Fig. 16B, lane 11). The *ftsX* probe hybridized a restriction fragment of a different size in the remaining *Neisseria* species: in *N. flava* and *N. mucosa*, the *ftsX* probe hybridized a restriction fragment of 5 Kb (Fig. 16B, lanes 4 and 7). In *N. subflava*, it hybridized a restriction fragment of 10 Kb (Fig. 16B, lane 5). Finally, the *ftsX* probe hybridized a restriction fragment of 6 Kb in *N. flavescens* (Fig. 16B, lane 10).

For *N. flava* and in *N. mucosa*, the observed size difference between the fragments that hybridized each probe were minor, and are probably in fact of the same size. Taken together, these data suggested that the *ftsE* and *ftsX* genes were present in all nine *Neisseria* species investigated. However, no conclusion could be drawn regarding the genetic organization of these genes.

Figure 15. Identification of the *ftsE* gene in the *Neisseriaceae* by Southern blotting at 50°C. (A), *Pst*I restriction enzyme digests of chromosomal DNA from all species. (B), hybridization of the *ftsE* probe to the restricted DNA from (A). All lanes are identified above the lane number. The sizes of the molecular weight marker are indicated to the left of the figure.

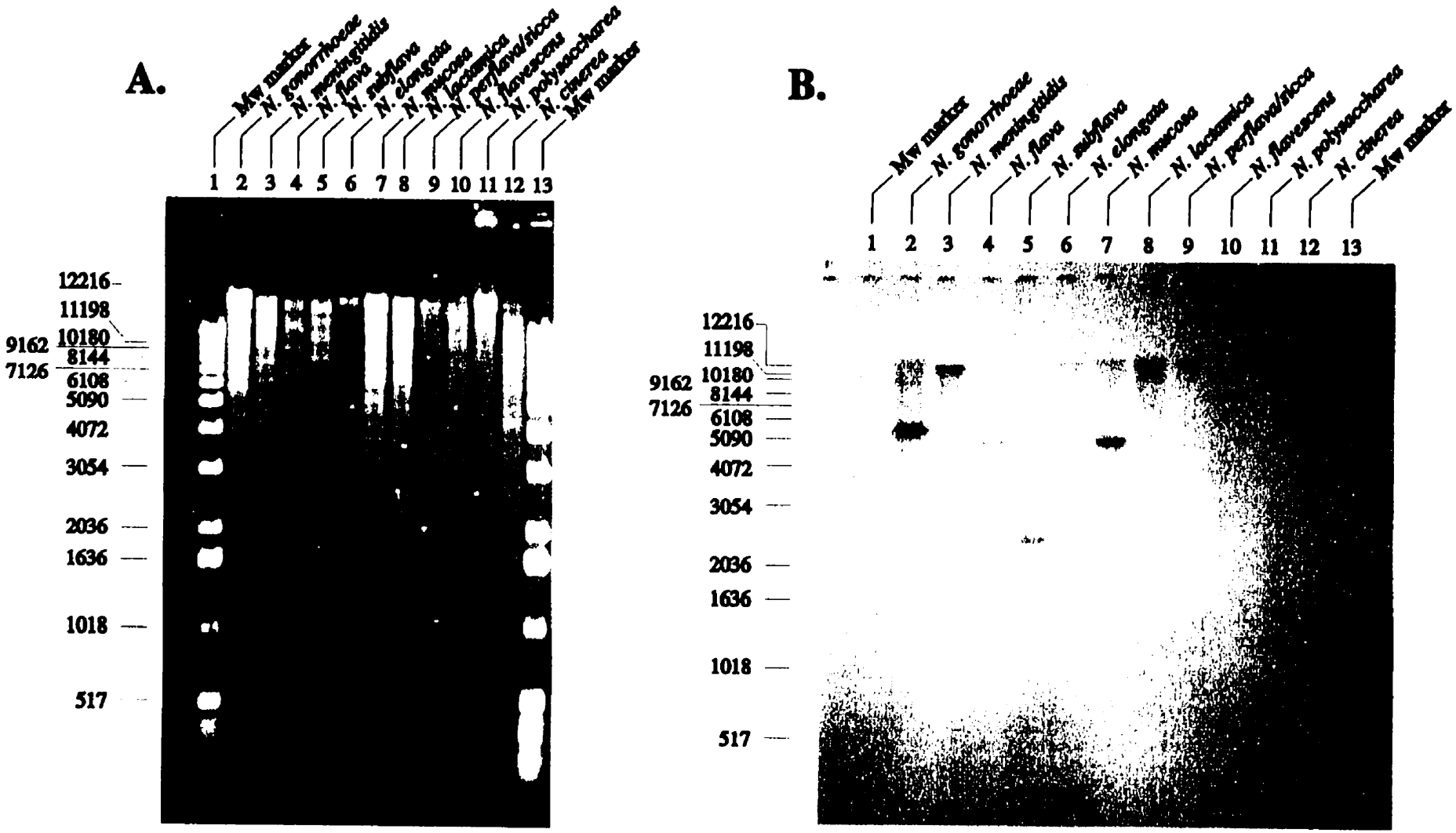
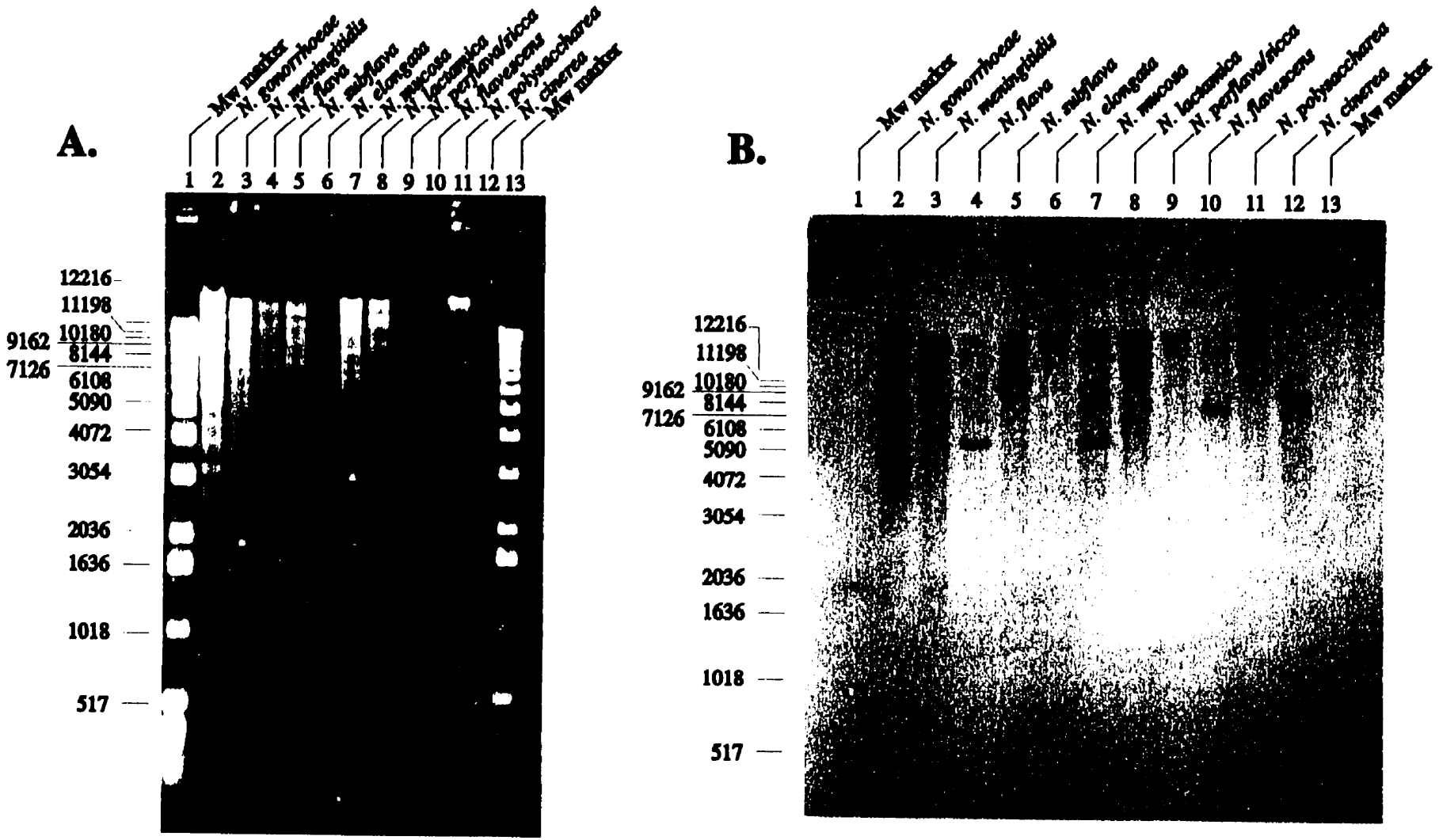

A.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflava/sicca
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
12216
11198
9162 10180
8144
7126 6108
5090
4072
3054
2036
1636
1018
517
B.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflava/sicca
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
12216
11198
9162 10180
8144
7126 6108
5090
4072
3054
2036
1636
1018
517

Figure 16. Identification of the *ftsX* gene in the *Neisseriaceae* by Southern blotting at 50°C. (A), *Pst*I restriction enzyme digests of chromosomal DNA from all species. (B), hybridization of the *ftsX* probe to the restricted DNA from (A). All lanes are identified above the lane number. The sizes of the molecular weight marker are indicated to the left of the figure.


A.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflava/sicca
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
12216
11198
9162 10180
8144
7126 6108
5090
4072
3054
2036
1636
1018
517
B.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflava/sicca
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
12216
11198
9162 10180
8144
7126 6108
5090
4072
3054
2036
1636
1018
517

3.5.2. Investigation of the *ftsEX* genes by PCR

In addition to the investigation by Southern blotting of *Neisseria* DNA, the presence of *ftsE* and *ftsX* was investigated in the same ten *Neisseria* species (and in *N. gonorrhoeae* as a positive control) by PCR as described (section 2.9.1.).

ftsE amplicons were detected in six of ten *Neisseria* species tested (Fig. 17A). The *ftsE* amplicons were generated from *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. perflava/sicca*, *N. polysaccharea*, and *N. cinerea* (Fig. 17A, lanes 3, 8, 9, 11 and 12). The amplicons were of the same size (282 bp; Fig. 17A). The absence of *ftsE* amplicons in *N. flava*, *N. subflava*, *N. elongata*, *N. mucosa* and *N. flavescens* was attributed to sequence divergence and not to the absence of *ftsE* because the results from the preceding experiment suggested *ftsE* was present in each of these *Neisseria* species (section 3.5.1.).

The *ftsX* amplicons were obtained in all eleven species tested but were heterogeneous in size: the amplicons from *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. perflava/sicca*, *N. polysaccharea*, and *N. cinerea* were of the same size (894 bp; Fig. 18A, lanes 2, 3, 8, 9, 11 and 12); *N. subflava* and *N. flavescens* generated amplicons of 1.4 Kb (Fig. 18A, lanes 5 and 10); *N. flava* and *N. mucosa* generated amplicons of 1.5 Kb (Fig. 18A, lanes 4 and 7), and *N. elongata* produced a 1.6 Kb amplicon (Fig. 18A, lane 6). These results suggested that the *ftsX* gene varied in size between *Neisseria* species. The five *Neisseria* species from which *ftsX* amplicons 894 bp were obtained were the same that generated *ftsE* amplicons. These results suggested that the *ftsE* and *ftsX* genes from these five species had nucleotide sequences more similar to those from *N. gonorrhoeae*. However, further investigation was necessary as the absence of *ftsE* amplicon and/or the variability in size of the *ftsX* amplicon in some species could be attributable to the conditions of the PCR assay.

The *ftsE* and *ftsX* amplicons from the *Neisseria* species were further investigated by hybridization with the same *ftsE*-and *ftsX*-specific probes used for the hybridizations described in section 3.5.1. The five *ftsE* amplicons hybridized the *ftsE* probe at 60°C (Fig. 17B), which provided additional evidence that these amplicons were *ftsE*-specific. In the case of the *ftsX*

Figure 17. Prevalence of the *ftsE* gene in the *Neisseriaceae* as investigated by PCR. (A) *ftsE* amplicons generated from *Neisseria* species. (B), hybridization of the *ftsE* amplicon from *n. gonorrhoeae* to the *ftsE* amplicons from (A). All lanes are identified above the lane number, and the hybridization signal corresponding to the *ftsE* amplicon is indicated on the right side of (B). The sizes of the molecular weight marker are indicated to the left of the figure.

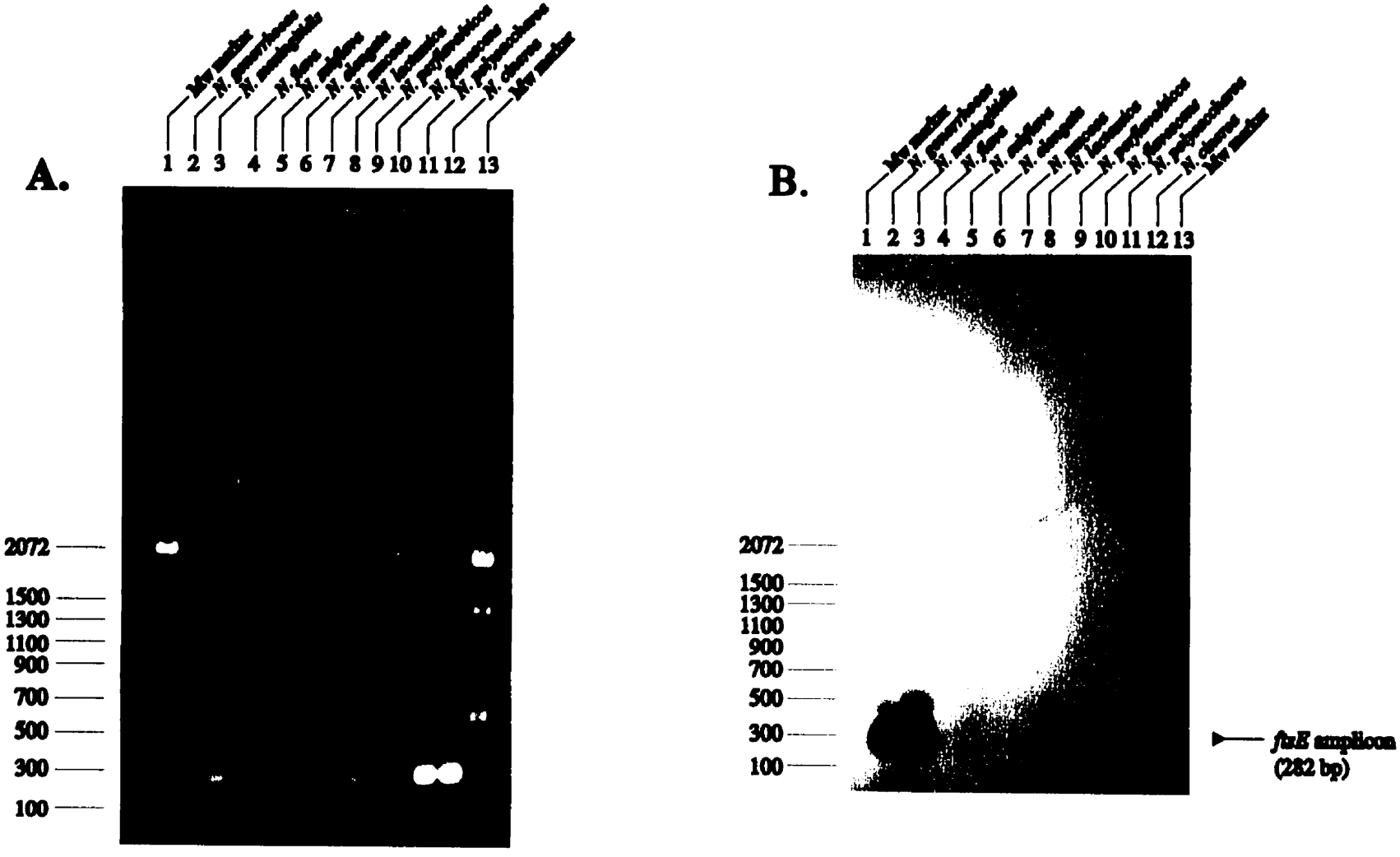
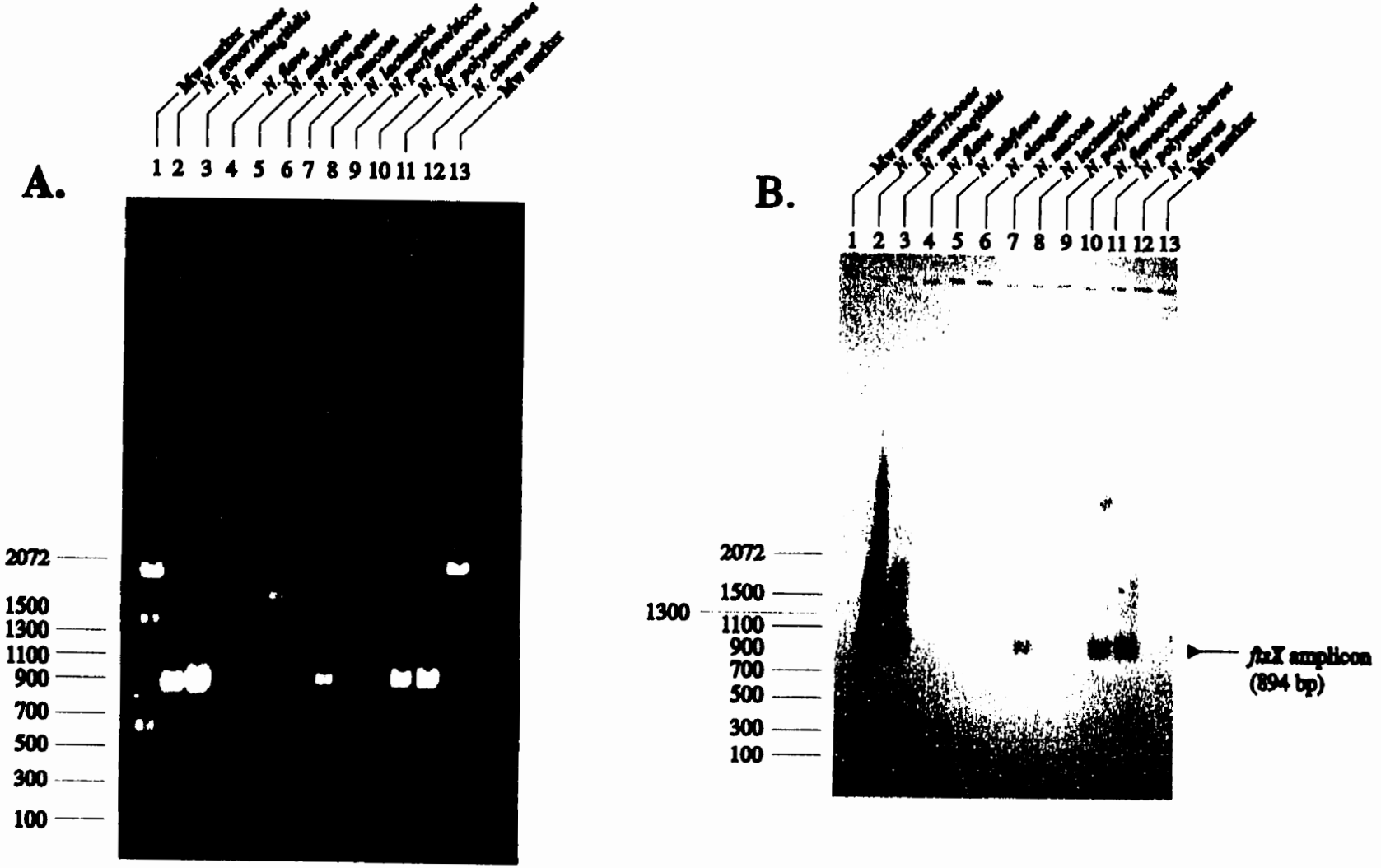

A.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. sulfaro
N. elongata
N. mucosa
N. lactamica
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
2072
1500
1300
1100
900
700
500
300
100
B.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. sulfaro
N. elongata
N. mucosa
N. lactamica
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
2072
1500
1300
1100
900
700
500
300
100
fxsE ampliconn
(282 bp)

Figure 18. Prevalence of the *ftsX* gene in the *Neisseriaceae* as investigated by PCR. (A) *ftsX* amplicons generated from *Neisseria* species. (B), hybridization of the *ftsX* amplicon from *n. gonorrhoeae* to the *ftsX* amplicons from (A). All lanes are identified above the lane number, and the hybridization signal corresponding to the *ftsX* amplicon is indicated on the right side of (B). The sizes of the molecular weight marker are indicated to the left of the figure.


A.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflavescens
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
2072
1500
1300
1100
900
700
500
300
100
B.
Mw marker
N. gonorrhoeae
N. meningitidis
N. flava
N. subflava
N. elongata
N. mucosa
N. lactamica
N. perflavescens
N. flavescens
N. polysaccharea
N. cinerea
Mw marker
1 2 3 4 5 6 7 8 9 10 11 12 13
2072
1500
1300
1100
900
700
500
300
100
ftsX amplicon
(894 bp)

amplicons, only the six amplicons of 894 bp hybridized the *ftsX* probe at 60°C (Fig. 18B). Similar results were obtained when the hybridizations were performed at 50°C (data not shown). These results suggested that the amplicons obtained in *N. meningitidis*, *N. lactamica*, *N. perflava/sicca*, *N. polysaccharea* and *N. cinerea* were more likely *ftsX*-specific. The other amplicons might be *ftsX*-specific, but their nucleotide sequences would be more divergent to that of *N. gonorrhoeae*.

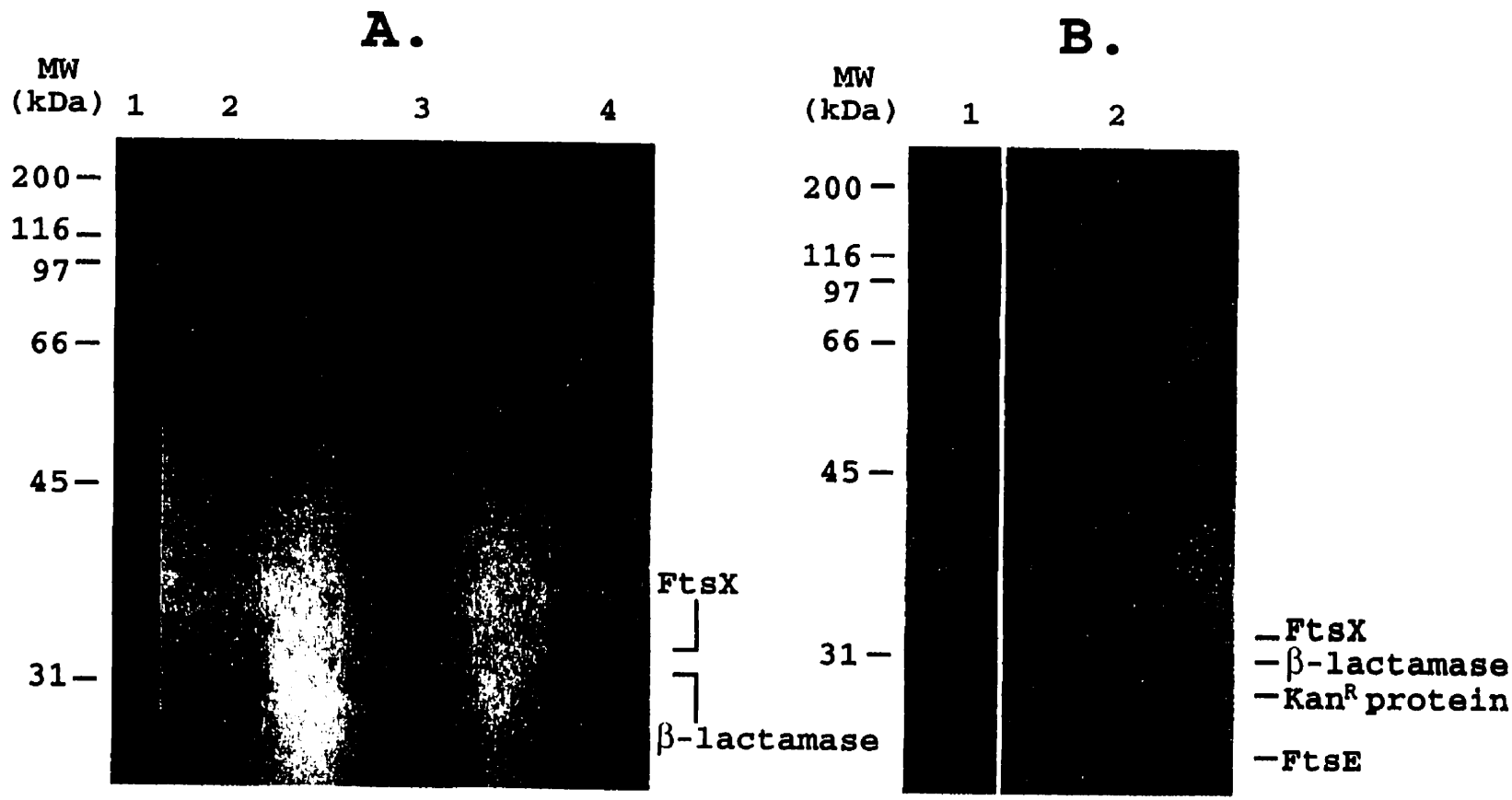
3.6. Transcription of *ftsE* and *ftsX*.

The 4 bp overlap between *ftsE* and *ftsX* and the presence of a putative transcriptional terminator downstream of *ftsX* but not downstream of *ftsE* suggested that the two genes were co-transcribed (sections 3.3.1. and 3.3.2.). If this was the case, the promoter regulating the expression of *ftsE* and *ftsX* would be located within the 194 bp intergenic region between *ftsE* and *tlpA*, since the presence of a putative transcriptional terminator downstream of *tlpA* (section 3.10.1.) suggested that *tlpA* and *ftsE* were independently transcribed.

Experimental evidence for co-transcription of *ftsE* and *ftsX* was obtained by conducting *in vitro* transcription/translation experiments using the plasmids pSB19 and pSB190. The plasmid pSB19 contains the complete *ftsX* gene with partial *ftsE* and *pgk* genes (section 3.1. and Table 2) while pSB190 contained the insert of pSB19 cloned in the opposite orientation to that in pSB19 so that *ftsX* was under the control of the vectorborne *lac* promoter (Table 2). It was hypothesized that if *ftsE* and *ftsX* were co-transcribed, a promoter would be located upstream of *ftsE* and not upstream of *ftsX*; in these conditions, the FtsX protein would not be synthesized from pSB19. On the other hand, if *ftsE* and *ftsX* were separately transcribed, there would be a promoter located upstream of *ftsX* and this promoter could be present in the 3'-section of the *ftsE* gene.

When the synthesis of FtsX was monitored from pSB19 and pSB190, a polypeptide of the expected Mw for FtsX (34 KDa) was synthesized from pSB190 in the *in vitro* transcription/translation system (Fig. 19A, lane 3). In comparison, this 34 KDa protein was not synthesized from pSB19, suggesting that FtsX could not be synthesized from pSB19 (Fig. 19A,

Figure 19. Identification of the gonococcal FtsE and FtsX proteins *in vitro*. (A) Identification of FtsX *in vitro*. Lane 1, pBluescript KS+; lane 2, pSB19; lane 3, pSB190. (B) Identification of FtsE and FtsX from pSB5696. Lane 1, molecular weight marker; lane 2, pSB5696. The sizes of the Mw markers are indicated on the left side of each figure, and the protein bands are identified to the right of each figure.


A.
MW
(kDa)
1
2
3
4
200
116
97
66
45
31
FtsX
β-lactamase
B.
MW
(kDa)
1
2
200
116
97
66
45
31
FtsX
β-lactamase
Kan^R protein
FtsE

lane 2). As expected, this 34 KDa protein was not synthesized from pKS+ (Fig. 19A, lane 1). A polypeptide with a Mw of 31 KDa corresponding to the vectorborne β-lactamase was synthesized from all three plasmids (Fig. 19A, lanes 1-3). Collectively, these results indicated that, as expected, *ftsX* did not possess its own promoter.

To verify if *ftsE* and *ftsX* could be co-transcribed from a promoter located upstream of *ftsE*, a second *in vitro* transcription/translation experiment was performed using the plasmid pSB5696. This plasmid contains the gonococcal *ftsE* and *ftsX* genes under the control of the vectorborne *lac* promoter (Table 2). Proteins of the expected Mw for FtsE and FtsX, 23 and 34 KDa respectively, were synthesized from pSB5696 (Fig. 19B, lane 2). Vector-encoded proteins of 31 and 28 KDa corresponding to β-lactamase and kanamycin nucleotidyltransferase, respectively, were also synthesized (Fig. 19B). These results indicated that *ftsE* and *ftsX* were co-transcribed if a promoter was provided upstream of *ftsE*. This result provided more evidence favoring the co-transcription of *ftsE* and *ftsX*.

Finally, to verify if FtsE and FtsX were synthesized when *ftsE* and *ftsX* were transcribed from a promoter located in the intergenic region between *ftsE* and *tlpA*, another *in vitro* transcription/translation experiment was accomplished using a 2014 bp amplicon that contained complete *ftsE* and *ftsX* genes, the 194 bp intergenic space between *ftsE* and *tlpA* and the last 85 nucleotides of *tlpA* (positions 2060-4074, Fig. 6B; section 2.9.1.). As expected, polypeptides of the expected Mw for FtsE and FtsX were synthesized (data not shown). These results also supported the co-transcription of *ftsE* and *ftsX*.

Taken together, all these data indicated that *ftsE* and *ftsX* were co-transcribed because *ftsX* did not possess its own promoter, and that both genes could be transcribed together if a promoter was provided upstream of *ftsE*. The results further indicated that a promoter controlling the expression of *ftsE* and *ftsX* lies within 279 bp upstream of *ftsE*, more likely within the 194 bp *tlpA-ftsE* intergenic region. A possible promoter for these genes could be the TATAAT box located within the *ftsE-tlpA* intergenic region (section 3.3.1.). These experiments also indicated that at least one type of gonococcal promoter was recognized by the *E. coli* transcriptional

apparatus. These two genes therefore constitute an operon in the gonococcus and will be hereafter be designated *ftsEX*.

3.7. Overexpression of the gonococcal *ftsE*, *ftsX* and *ftsEX* genes in *E. coli*.

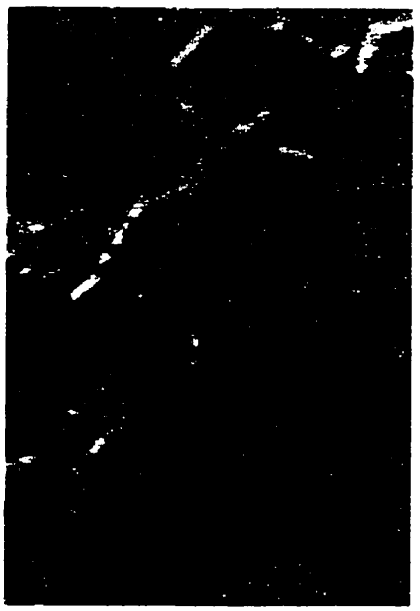
The gonococcal *ftsEX* genes were overexpressed in *E. coli* to investigate if they could cause morphological changes such as filamentation, as it has been observed following overexpression of other bacterial cell division genes in an *E. coli* background (Margolin and Long, 1994; Quardokus *et al.*, 1996). INVαF' cells overexpressing *ftsX* from the plasmid pSB190 in which *ftsX* was under the control of a *lac* promoter, overexpressing *ftsE* from the plasmid pSB1910 in which *ftsE* was under the control of a *lac* promoter, or overexpressing *ftsEX* from the plasmid pSB5696 in which *ftsEX* were under the control of a *lac* promoter (Table 2) were grown in rich medium. In all cases, the cells presented a typical, short rod-like morphology in the absence or presence of IPTG (Fig. 20A-C). In contrast, INVαF' containing pAR2, a plasmid in which the *N. gonorrhoeae ftsZ* gene is under *lac* promoter control (Table 2), formed filaments following induction with IPTG (Fig. 20D). In minimal medium, INVαF' cells overexpressing *ftsE*, *ftsX* or *ftsEX* from the plasmids pSB1911, pSB190 or pSB5696 had again a typical, short rod-like morphology (Fig. 20E). Collectively, these data suggested that overexpression of the *N. gonorrhoeae ftsE*, *ftsX* or *ftsEX* genes did not cause morphological aberrations in *E. coli*.

3.8. Functional complementation of an *E. coli ftsX* mutant

The ability of the gonococcal *ftsX* gene to complement the *ftsX* defect in *E. coli* strain JS10 was verified. *E. coli* JS10 is a derivative of χ462 that contains an uncharacterized mutation in its *ftsX* gene; JS10 cells present a normal morphology at 37°C but form filaments at 30°C (section 1.4.). If JS10 cells containing the gonococcal *ftsX* gene on a plasmid presented a normal morphology at 30°C, this would indicate that the gonococcal *ftsX* was capable of complementing the *ftsX* defect of the host strain.

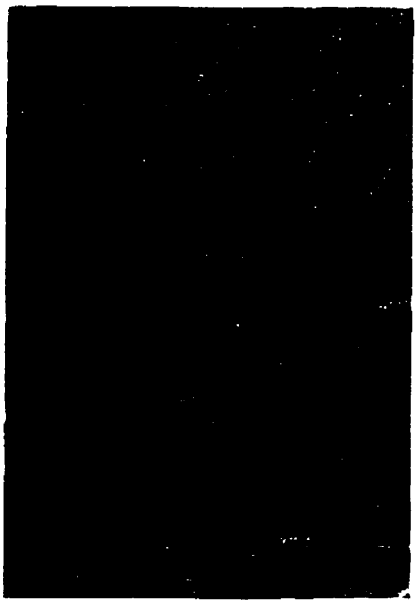
Figure 20. Overexpression of the *N. gonorrhoeae ftsE*, *ftsX* and *ftsEX* genes in *E. coli* in rich (A-D) or minimal (E) culture media. (A), *E. coli* DH5α+pSB190 (overexpressing *N. gonorrhoeae ftsX*); (B), *E. coli* DH5α+pSB1910 (overexpressing *N. gonorrhoeae ftsE*); (C and E), *E. coli* DH5α+pSB5696 (overexpressing *N. gonorrhoeae ftsEX*); (D), *E. coli* DH5α+pAR2 (overexpressing *N. gonorrhoeae ftsZ*).

A.



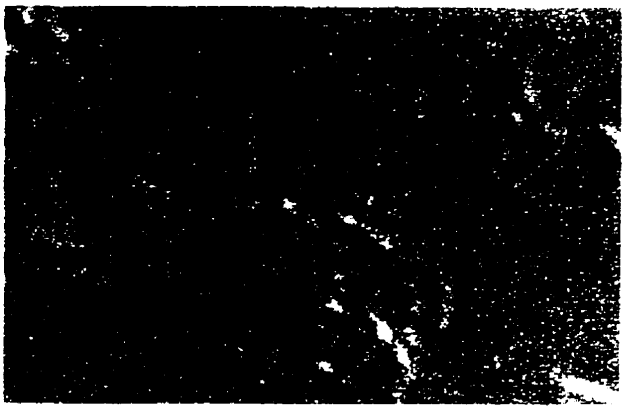
B.

C.



D.

E.

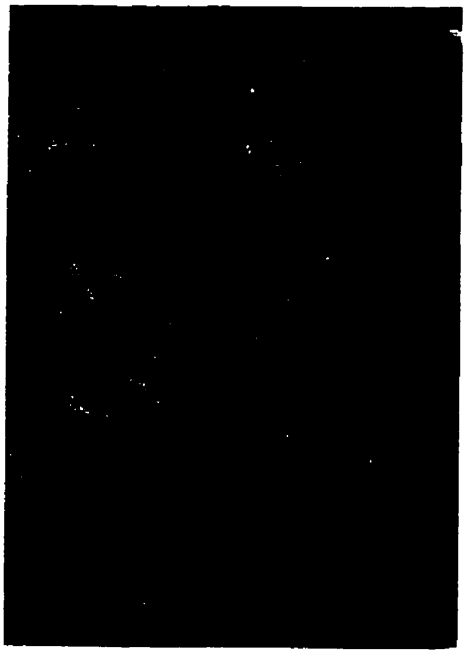


First, proper culture conditions in which JS10 cells formed filaments needed to be established. *E. coli* strains JS10 and χ462 were grown in L and LB broths, harvested, fixed and observed as indicated (section 2.12.). As expected, *E. coli* χ462 did not form filaments in either type of culture medium at either temperature (Fig. 21, A-D). *E. coli* JS10 produced a few filaments in L broth but generated more filaments in LB broth (Fig. 21, E-H). *E. coli* JS10 produced filaments at both temperatures, but there were more filaments at 30°C than at 37°C (Fig. 21, F and H). An important observation noted during this work was that the filamentous phenotype of *E. coli* JS10 was lost over time so that bacteria presented a normal, short rod-like morphology after six hours of growth. Therefore, to verify if the gonococcal *ftsX* gene would be capable of complementing the *ftsX* defect of *E. coli* JS10, the bacteria were to be grown at 30°C in LB broth and the morphology of the cells would be verified after two hours of growth, or one hour after induction.

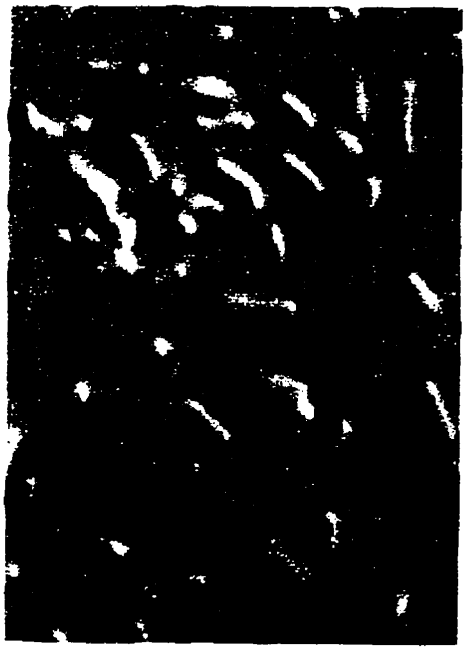
The *E. coli* strains χ462, JS10 and JS10+pSB190 were then grown in LB broth with or without induction at 30°C and 37°C as indicated in the section 2.12. χ462 cells, serving as a control, presented a normal morphology and were likewise unaffected by the presence of IPTG in the culture medium (data not shown). JS10 cells formed filaments as previously observed, regardless of the presence of inducer (data not shown). As expected, uninduced JS10 cells containing pSB190 presented a filamentous morphology (Fig. 22A), and induced JS10 cells containing pSB190 exhibited a similar morphology (Fig. 22B). These results suggested that the induction of the expression of the gonococcal *ftsX* could not complement the mutation in the *ftsX* gene of *E. coli* JS10 despite the induction of the gonococcal *ftsX* gene from the plasmid.

Figure 21. Morphology of *E. coli* strains χ462 and JS10 following 2.5 hours of growth in liquid culture. (A) *E. coli* χ462 in L broth at 37°C; (B), *E. coli* χ462 in LB broth at 37°C; (C), *E. coli* χ462 in L broth at 30°C; (D), *E. coli* χ462 in LB broth at 30°C; (E), *E. coli* JS10 in L broth at 37°C; (F) *E. coli* JS10 in L broth at 37°C; (G) *E. coli* JS10 in L broth at 30°C; (H), *E. coli* JS10 in LB broth at 30°C.

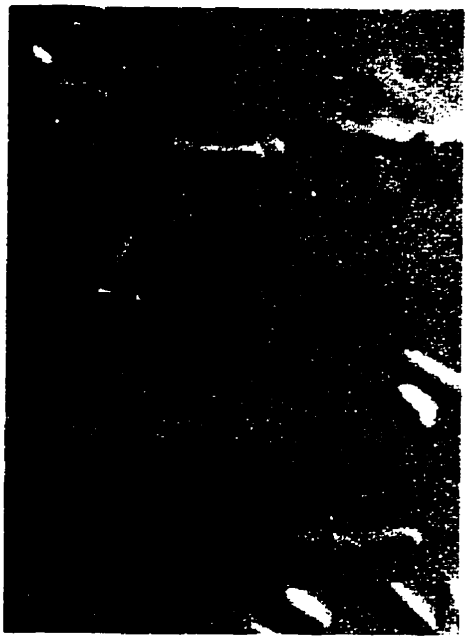
A.



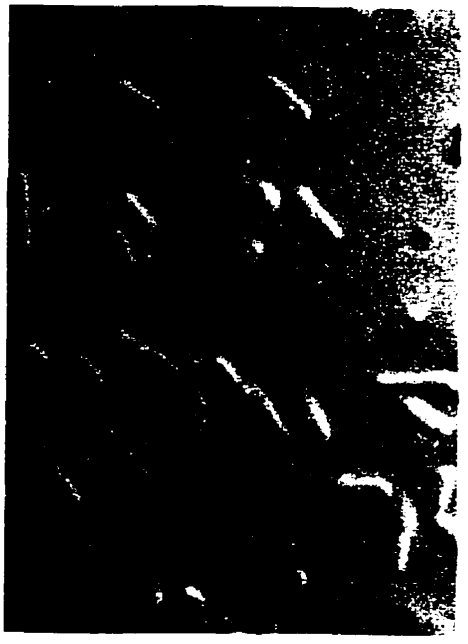
B.



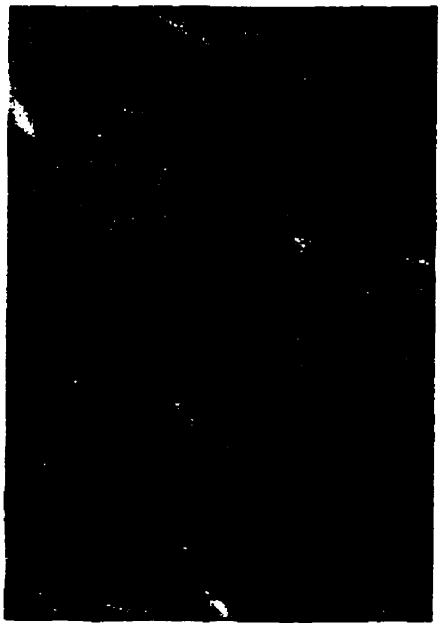
C.



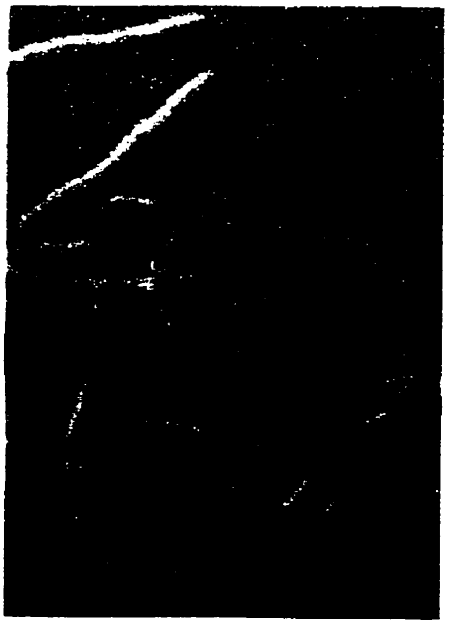
D.



E.



F.



G.

H.

Figure 22. Complementation of *E. coli* JS10 with the *ftsX* gene of *N. gonorrhoeae* in LB medium at 30°C. The cells were observed after 2.5 hours of growth, (A), 1.5 hour following the addition of inducer and (B), without addition of inducer.

A.

B.

3.9. Production of a histidine-labelled FtsE protein.

There currently is no available anti-FtsE antibody. The availability of such antibodies would be useful for subcellular studies of FtsE.

3.9.1. Contruction of the plasmid pSB1931

The *ftsE* gene from *N. gonorrhoeae* lacking its initiation and termination codons was amplified by PCR and cloned in pQE30 to generate the plasmid pSB1931 as described in section 2.14.1. In pSB1931, the *ftsE* gene is under the control of a vectorborne *lac* promoter (Table 2) and was therefore inducible by IPTG. The *ftsE* gene on pSB1931 encoded a protein with predicted Mw of 26 040 Da comprising 233 aa, 214 aa from the original *N. gonorrhoeae* FtsE (positions 2-214), preceded by an amino-terminal extension of 12 aa including six consecutive histidinyl residues, and followed by a carboxy-terminal extension of 7 aa. The plasmid pSB1931 was confirmed by PCR (Fig. 23), and by its *Pst*I and *Bam*HI restriction patterns (data not shown). The primers *ftsE*HIS1 and *ftsE*HIS2 amplified a DNA fragment of the expected size from the plasmid purified from one of the bacterial clones (Fig. 23, lane 3), and this amplicon was of the same size as the fragment amplified from *N. gonorrhoeae* DNA using the same primer pair (Fig. 23, lane 4) the expression of the histidine-tagged FtsE protein (6HISFtsE) was verified through a small-scale expression procedure (section 2.14.2.). The purification strategy was based on the selective binding of proteins containing six consecutive histidinyl residues to Ni-NTA resin. Following the binding to the Ni-NTA resin, the bound proteins were eluted by washing the resin.

A protein of the expected Mw for 6HISFtsE (26 Kda) was purified through this procedure (Fig. 24). A small amount of protein purified from cells harvested one hour following induction was visible on the gel (Fig. 24, lane 4), and greater amounts of this protein were present in cells harvested two to four hours after induction (Fig. 24, lanes 5-7). Less protein was purified from cells harvested five hours following induction (Fig. 24, lane 8). No protein with a Mw of 26 KDa was purified from uninduced cells harvested after one or six hours of growth (Fig. 24, lanes 2-3), confirming that the purified protein had been produced from an induced gene. Together, these

Figure 23: Amplification of the insert of pSB1931 by PCR. Lanes 1 and 6, 100 bp Mw marker; lane 2, plasmid purified from another colony; lanes 3, pSB1931; lane 4, *N. gonorrhoeae* CH811 DNA; lane 5, negative control. The sizes of the Mw marker and of the truncated *ftsE* amplicon are indicated to the left and right of the figure, respectively.

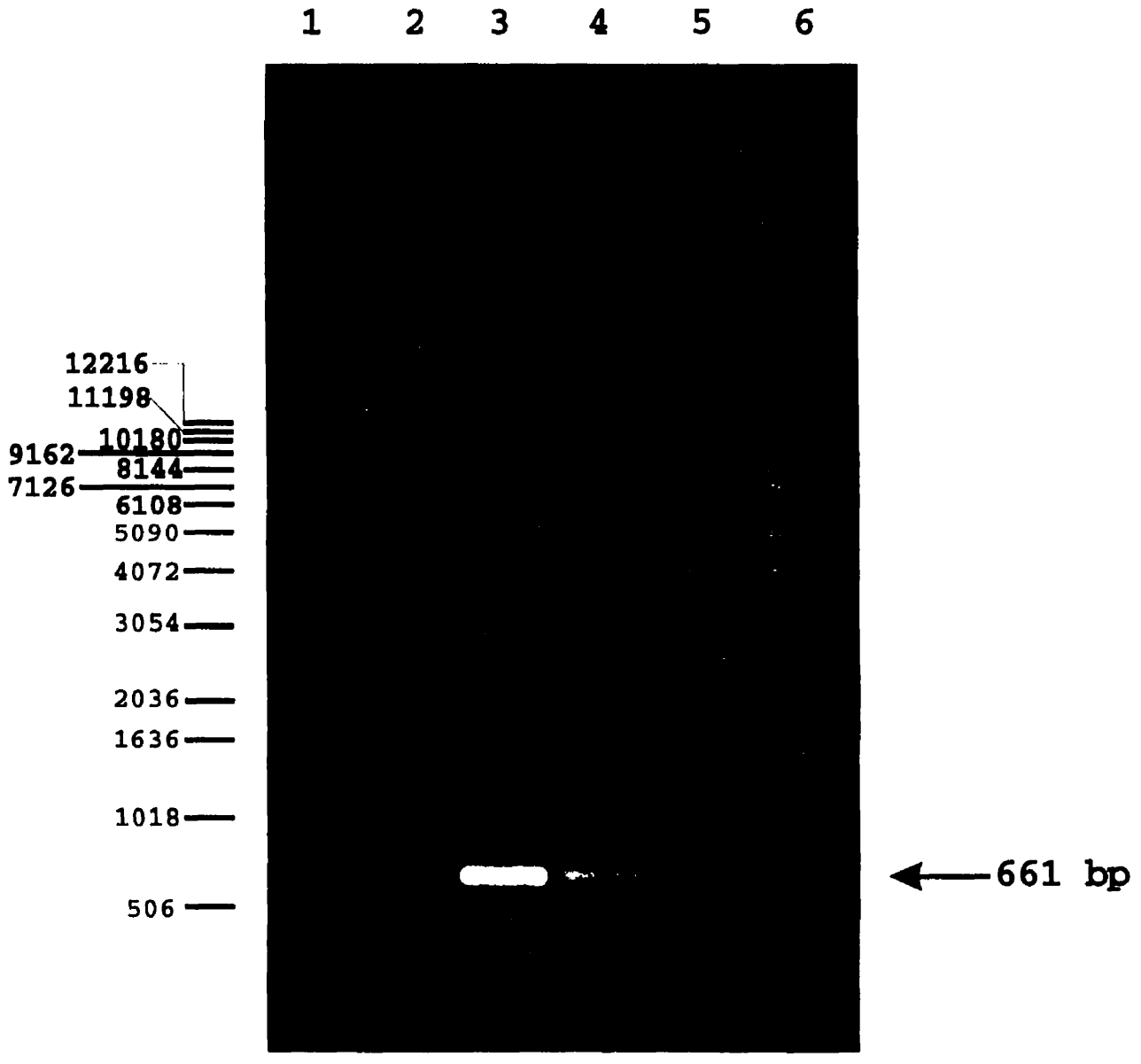

1
2
3
4
5
6
12216
11198
10180
9162
8144
7126
6108
5090
4072
3054
2036
1636
1018
506
661 bp

Figure 24. Purification of the histidine-labeled gonococcal FtsE (6HISFtsE) from *E. coli*. The bacterial cultures were grown and 6HISFtsE was purified by affinity binding to the Ni-NTA resin as described, and the protein fractions were examined on a 10% polyacrylamide-SDS gel colored with SYPRO™ ORANGE. Lanes 1 and 9, broad range Mw marker; lane 2, uninduced cells after 1 hour of growth; lane 3, uninduced cells after 6 hours of growth; lane 4, induced cells, 1 hour after induction; lane 5, 2 hours after induction; lane 6, 3 hours after induction; lane 7, 4 hours after induction; lane 8, 5 hours after induction. The sizes of the Mw markers are indicated to the left side of the figure, and position of 6HISFtsE is indicated to the right side of the figure.


KDa
1
2
3
4
5
6
7
8
9
200
116
97
66
45
31
21
6HISFtsE

data indicated that the synthesis of a protein of 26 KDa, the expected Mw for 6HISFtsE, could be induced from the plasmid pSB1931, and that maximal amounts of protein were synthesized two to four hours after induction. From these results, it was decided that for all subsequent manipulations, the cells would be harvested after three hours of induction.

To purify larger amounts of 6HISFtsE that will be used later to raise polyclonal anti-6HISFtsE rabbit antibodies, the purification procedure was scaled up as described in section 2.13.3. No other proteins were present with the purified 6HISFtsE protein (data not shown).

3.10. Analysis of the genes flanking *ftsEX* in *N. gonorrhoeae* CH811

3.10.1. The *tlpA* gene

The first gene identified in the region upstream of *ftsE* lay 193 bp upstream and in the same orientation as *ftsE* (Fig. 6A and 6B). This gene contained 483 nucleotides (positions 1665-2145, Fig. 6B) encoding a protein of 160 aa with a predicted Mw of 17.5 KDa that shared significant aa sequence similarity with the thioredoxin-like protein (TlpA) of *Bradyrhizobium japonicum* (Loferer *et al.*, 1993). The gene was accordingly named *tlpA*. This gonococcal *tlpA* gene homologue was preceded by a putative RBS (positions 1652-1655, Fig. 6B). Also located 89 bp upstream of the start codon of *tlpA* was a putative -10 box that could be part of the promoter of *tlpA* (TATTAAT, positions 1569-1574, Fig. 6B). The gene was followed by a sequence capable of forming a stem-loop structure (positions 2155-2179, Fig. 6B) containing inverted repeats of the gonococcal US (positions 2155-2164 and 2170-2179, Fig. 6B). The first US contained one mismatch from the consensus sequence: a guanosine was replaced by a thymidine (position 2161, Fig. 6B). This stem-loop could be part of the transcriptional terminator of the *tlpA* gene. The presence of a putative transcriptional terminator downstream of *tlpA* suggested that it was transcribed separately from *ftsE*. A third, perfectly conserved, copy of the gonococcal US (positions 2270-2279, Fig. 6B) was located in the intergenic region between *tlpA* and *ftsE*. No ORF was identified in the region between *tlpA* and *ftsEX*.

The polypeptide encoded by *N. gonorrhoeae tlpA* shared 53% aa sequence similarity and 31% aa sequence identity with TlpA from *B. japonicum* but it was 61 aa shorter than the *B. japonicum* TlpA (predicted Mw of 23.2 KDa). Other similarity analyses with *B. japonicum* TlpA, with bacterial thioredoxins and other thioredoxin-like proteins (HelX, CycY) as well as experimental evidence for the transcription of *tlpA* are presented in Appendix 3.

3.10.2. The *arsC* gene

arsC, the second gene located in the region upstream of *ftsEX*, was located 123 bp upstream and in the opposite orientation to that of *tlpA* (Fig. 6A and 6B). The gonococcal *arsC* gene contained 309 bp (positions 1539-1231, Fig. 6B) encoding a putative polypeptide of 102 aa. No consensus RBS was identified upstream of the initiation codon but a purine-rich region (positions 1547-1552, Fig. 6B) located 8-10 bp upstream of the initiation codon may contain the RBS. No sequences capable of forming the stem-loop structure of a transcriptional terminator, and no gonococcal US were identified downstream of *arsC*. A partial *gltX* gene was located 817 bp downstream and in the opposite orientation to that of *arsC* (section 3.10.3.). No ORF was identified in the 817 bp intergenic region between *arsC* and *gltX*. The only distinctive feature of the 817 bp spacer is the presence of a copy of the gonococcal US (positions 479-488, Fig. 6B) containing one mismatch from the consensus sequence, a substitution of a thymidine by a cytosine (position 485, Fig. 6B). These results indicated that *N. gonorrhoeae arsC* was not located within an *ars* operon, similar to what was found in *H. influenzae* Rd and *Synechocystis* PCC6083 (Fleischmann *et al.*, 1995; Kaneko *et al.*, 1996).

The gonococcal ArsC was closest in size and in aa sequence similarity to ArsC from *H. influenzae* (116 aa); the two polypeptides shared 66% similarity and 50% identity. The gonococcal ArsC also shared between 45% and 64% similarity and between 18% and 39% identity with other bacterial arsenate reductases (Neyt *et al.*, 1997; Bruhn *et al.*, 1996; Kaneko *et al.*, 1996; Diorio *et al.*, 1995; Fleischmann *et al.*, 1995; Ji and Silver, 1992; Rosenstein *et al.*, 1992; Chen *et al.*, 1986). Data on the sequence similarities between the gonococcal ArsC and its

bacterial homologues, the expression of the *arsC* gene and the MIC of *N. gonorrhoeae* for Na(As[V]) and Na(As[III]) are presented in Appendix 4.

3.10.3. The *gltX* gene

A partial *gltX* gene was identified 817 bp and in the opposite orientation to *arsC* (Fig. 6A; positions 413-1, Fig. 6B). A consensus RBS could not be identified upstream of *gltX*. This partial ORF comprised 413 nucleotides (positions 413-1, Fig. 6B) encoding the amino-terminal 138 aa of a protein sharing significant similarity with glutamyl-$_T$RNA synthetase (GluRS). A brief analysis of the partial *N. gonorrhoeae* GluRS sequence and an alignment with other GluRSs is presented in Appendix 5.

3.10.4. The *pgk* gene

A partial homologue of *pgk* was located 391 bp and in the opposite orientation to *ftsEX* (Fig. 6A; positions 4526-4296, Fig. 6B). The 321 bp of the *pgk* gene encoded the carboxy-terminal 76 aa of a protein that shared similarity to Pgk. Inverted copies of gonococcal US were identified downstream of the *pgk* gene (positions 4260-4269 and 4280-4289, Fig. 6B). The Pgk enzyme catalyzes the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate with production of one molecule of ATP (Mayes, 1985). The partial sequence of the gonococcal Pgk shares high aa sequence similarity with other Pgk enzymes from all kingdoms. The analysis of the partial aa sequence of *N. gonorrhoeae* Pgk and an alignment of the partial gonococcal Pgk enzyme with other Pgk enzymes are presented in Appendix 6.

3.11. Genetic organization of the *gltX-pgk* region in *N. gonorrhoeae* strain FA1090

The determination of the complete sequence of the genome of *N. gonorrhoeae* strain FA1090 is currently in its last stages but is not yet completed (section 1.11.; Dyer *et al.*, 1996). The most recent release of the genome of *N. gonorrhoeae* strain FA1090 was investigated to verify if the genetic organization of the *gltX-arsC-tlpA-ftsE-ftsX-pgk* genes would be similar to

what had been determined in *N. gonorrhoeae* strain CH811. The gene order *gltX-arsC-tlpA-ftsE-ftsX-pgk* was also found in the strain FA1090 (data not shown).

When the aa sequences of the proteins encoded by these genes were compared, the FtsX proteins were found to share 96% identity and 97% similarity; the TlpA proteins shared 96% identity and 98% similarity; the ArsC proteins shared 100% identity. The partial GluRSs shared 99% identity and 100% similarity, the partial Pgks shared 98% identity and 100% similarity. The sequence of the *ftsE* gene from the strain FA1090 is not completed, but partial aa sequence comparisons indicated that the first 32 aa residues were identical between FtsE in the two strains, and that there was 98% identity and 99% similarity over the positions 81-216. The two FtsE proteins can be expected to be highly similar, as was the case for the other polypeptides identified during this work.

The presence of a gonococcal *ftsY* gene in the vicinity of *ftsE* and *ftsX* was investigated by identifying the genes located downstream of *gltX* and upstream of *pgk*. In *N. gonorrhoeae* FA1090, a gene encoding a hypothetical protein was located downstream of *gltX* (data not shown). A homologue of the *murZ* gene was located upstream of *pgk* (data not shown). In *E. coli*, the *murZ* gene encodes UDP-N-acetylglucosamine enolpyruvyltransferase, a protein involved in murein biosynthesis (Marquardt *et al.*, 1992). The polypeptide showing the closest similarity to FtsY was PilA (data not shown), a GTP-binding protein part of a putative two component transducer system (Taha *et al.*, 1991, 1988). The similarity between FtsY and PilA was the strongest in the region of the nucleotide-binding site, and little amino acid similarity was found between the amino-terminal domains of the polypeptides (data not shown; Taha *et al.*, 1991). The presence of a gonococcal *ftsY* gene remains to be verified, as the nucleotide sequence of the genome is not completed. However, PilA has already been proposed to be the gonococcal homologue of FtsY (Fyfe and Davies, 1996).

PART 4. DISCUSSION

This work is among the first devoted to the identification and characterization of cell division genes in *N. gonorrhoeae*. This important cell process remains largely uncharacterized in the gonococcus as most research has focused on its pathogenicity and virulence processes. Recently, the cloning and initial characterization of the *ftsZ* gene of *N. gonorrhoeae* has been reported in our laboratory along with the identification of the other genes of the *dcw* cluster (section 1.8.1.; Radia, 1997). The determination of the complete sequence of the genome of *N. gonorrhoeae* (Dyer *et al.*, 1996) has already greatly facilitated the initial identification of cell division homologues in the gonococcus (section 1.8.1.), but the regulation of the expression of these genes as well as the characterization of the biological function of the cell division proteins will still be required. Once their function in the cell division process is understood, the potential of each protein to become a potential therapeutic target will be considered. The present work reports the identification and biological characterization of two genes, *ftsE* and *ftsX*, that were initially believed to participate in cell division (Gill *et al.*, 1986). These genes are now proposed to encode the domains of an ABC transporter, and they are not involved in cell division.

From the analysis of the aa sequences, the gonococcal FtsE was found to contain the Walker A and B motifs and the linker peptide, three aa motifs characteristic of the ATP-binding domain of ABC transporters. Besides its sequence similarities to other known FtsE homologues and other ATP-binding domains of ABC transporters, other polypeptides have been reported to share sequence similarity with FtsE. A protein designated ORF-C containing the two conserved Walker motifs and that shared 36% identity with FtsE from *E. coli* was identified in *Buchnera aphidicola*, a prokaryotic endosymbiont of the aphid *Schizaphis graminum*. The *orfC* gene of *B. aphidicola* was not followed by a gene encoding a FtsX homologue (Kolibachuk and Baumann, 1995). ORF-C shared 59% aa sequence similarity and 35% aa sequence identity with *N.*

gonorrhoeae FtsE, and between 57-61% aa sequence and between 34-37% aa sequence identity with the other bacterial FtsE homologues (data not shown). The absence of a *ftsX* homologue downstream of *orf-C* argued against *orf-C* encoding a FtsE homologue, since *ftsE* and *ftsX* have been found clustered in other bacteria. A putative homologue of FtsE (designated B65F) was likewise identified in *B. subtilis* (Yoshida *et al.*, 1994), but the poor aa sequence similarity shared between the ORF following that of B65F and FtsX sheds doubt on the status of B65F as a legitimate FtsE homologue (see below).

The linker peptide of the ATP-binding domains of ABC transporters has been proposed to serve as a hinge bringing the two Walker motifs together to form the ATP-binding pocket (section 1.2.2.). The aa residues preceding those of the linker domain are not conserved among the ATP-binding domains of ABC transporters (Fig. 2), and those positions are not perfectly conserved between the known FtsE sequences with the exception of a prolyl residue (Pro136 in *N. gonorrhoeae* FtsE; Fig. 10). Prolyl residues are often breakers of regular secondary structures (α-helices and β-sheets), and occur in turns, in non-repetitive structures and at the end of strands and helices (Richardson and Richardson, 1989). Considering these properties of proline, the conserved prolyl residue in FtsE could also contribute to the function of the linker domain. Other evidence supporting the potential importance of this prolyl residue is that its substitution by a seryl residue in *E. coli* FtsE rendered the bacteria Ts (Gibbs *et al.*, 1992). FtsE was shown to be associated with the inner membrane in *E. coli* (Gill and Salmond, 1987), but the analyses of *N. gonorrhoeae* FtsE and the other known bacterial FtsE sequences suggested that they did not contain transmembrane segments. Therefore, for FtsE to be associated with the membrane as in *E. coli*, it necessarily required an interaction with a membrane protein. Further investigation is required to determine the nature of the association of FtsE with the inner membrane.

Other polypeptides were reported to share sequence similarity with FtsX from *E. coli*. The gene located immediately downstream of the gene encoding B65F, the putative FtsE in *B. subtilis* (see above), encoded a protein designated B65G that shared sequence similarity with FtsX (Yoshida *et al.*, 1994). However, our analyses indicated B65G is predicted to contain at least 7 predicted transmembrane helices and that it showed no significant similarity to *N. gonorrhoeae* FtsX (data not shown). The genetic organization of the genes encoding B65F and B65G was suggestive of an ABC transporter although they may not constitute FtsE and FtsX homologues, mainly because B65G did not share the predicted organization of the six known bacterial FtsX polypeptides. The NS3-2 gene of the plasmid pOAD2 of *Flavobacterium* sp. was reported to encode a polypeptide of 293 aa residues that shared 22% similarity with *E. coli* FtsX (Kato *et al.*, 1995). Further investigation would be required to confirm that the NS3-2 protein is a genuine FtsX homologue, as it is less similar to *E. coli* FtsX than other bacterial FtsX homologues, and also because the NS3-2 gene is not flanked by a gene encoding a FtsE homologue.

The results from this work suggest that FtsX protein is an obvious candidate to anchor FtsE to the cytoplasmic membrane. FtsE from *N. gonorrhoeae* and other bacteria shared aa sequence similarity with the ATP-binding domain of ABC transporters (section 3.3.1.), and FtsX from *N. gonorrhoeae* and other bacteria were to be predicted to be integral membrane proteins (section 3.3.2.; Tyagi *et al.*, 1996). The involvement of the FtsE and FtsX polypeptides in the same cellular process was also suggested by the co-transcription of their genes in *N. gonorrhoeae* (section 3.6.). Our model in which FtsE and FtsX are the domains of a putative ABC transporter correlated well with the experimental results of Gill and Salmond (1987) showing that FtsE and FtsX were associated with the inner membrane in *E. coli*.

The binding site for FtsE on the FtsX polypeptide remains to be identified. The linker domain between the first two transmembrane segments of FtsX could have been a good candidate to be the FtsE binding site because of its larger size compared to the other linkers. However, the topological predictions of FtsX done using the TopPredII program predicted that the amino- and carboxy-terminal ends of the FtsX polypeptide were cytoplasmic. Consequently, the linker domain between the first two transmembrane segments of FtsX is periplasmically located and cannot be involved in binding FtsE. Since the correct membrane topology of 23 out of 24 (96%) prokaryotic membrane proteins were correctly predicted using TopPredII (von Heijne, 1992), the predicted topology of FtsX could well be what occurs *in vivo*. If this prediction is true, FtsE could not bind FtsX through interactions with this linker, as FtsE is cytoplasmically located.

A possibly important site for FtsE-FtsX interactions is the LLGA sequence identified in this work. This sequence was found extremely conserved among the six FtsX protein sequences examined (section 3.3.2.). According to the topological model for FtsX predicted by the TopPredII algorithm, this conserved sequence would be cytoplasmically located, inside the linker between the second and third transmembrane domains, at an average 87 positions from the carboxy-terminal end of the polypeptide. Thus, like the EAA loop, the LLGA sequence is cytoplasmically located and lies approximately 100 positions from the carboxy-terminal end of the polypeptide. The LLGA motif could fill a function analogous to that of the EAA loop in the FtsX protein, and could be important for FtsE-FtsX interactions.

The *ftsX* gene was found non-essential in *N. gonorrhoeae*, as gonococci with a disrupted *ftsX* gene were viable (section 3.4.1). Preliminary observations of *N. gonorrhoeae* CH811, CH811Str^R and CS19 by electronic microscopy showed that CS19 did not exhibit a different morphology from that of its parental strain. The dimensions of the individual gonococci from

each strain indicated that there were no differences between the strains (Table 4). The bacteria from the strains CH811 and CH811Str^R appear symmetrical (Fig. 14 (A) and (B)), which is not the case with *N. gonorrhoeae* CS19 (Fig. 14 (C) and (D)). The possible significance of this difference cannot be assessed with certainty with the available data because of the small number of bacteria observed for the strain CS19. More cells from each strain will need to be observed and their dimensions measured to verify the observations reported here, i.e., the similar sizes of gonococci from the strains CS19 and CH811, and also to verify if the asymmetry of the diplococci of the strain CS19 is a characteristic of this strain.

The filamentous phenotype of some *E. coli ftsE* and *ftsA* Ts mutants was found reversible by altering the concentration of sodium chloride in the culture medium (Ricard and Hirota, 1973a, 1973b, 1969). The variation in the concentration of sodium chloride was proposed to help the mutated protein to maintain its three-dimensional structure (Ricard and Hirota, 1973b). This indicated that the mutation in *ftsE* or *ftsA* in these strains (MFT99 and MFT123 for *ftsE*; MFT1, MFT96, MFT1182, PAT84 for *ftsA*; Ricard and Hirota, 1973a, 1973b) in such strains was likely a point mutation. The mutation in *ftsX* in *E. coli* JS10 is likely a point mutation as well, as the bacteria were initially irradiated with ultraviolet light before the isolation of long cells (section 1.4.; Sturgeon and Ingram, 1978). The finding from the present work that the concentration of sodium chloride affected the filamentous phenotype of *E. coli* JS10 as it formed more filaments in LB medium (0.5% NaCl) than in L medium (0.05% NaCl) also suggested that the mutation affecting *ftsX* in *E. coli* was a point mutation (section 3.8.). It would be interesting to identify the mutation in *ftsX* of *E. coli* JS10 to see if its location and nature could be correlated with the analyses and speculations on the structure of the FtsX protein developed in this discussion. For instance, if the mutation caused a change in one of the six conserved positions, or more

specifically in the LLGA motif, this would indicate that it was indeed important for the proper structure-function of FtsX. Compared to the culture conditions of *E. coli* JS10, *N. gonorrhoeae* CS19 was grown on half strength GC medium base (section 2.1.) which contained 0.25% NaCl (Difco Manual, 1990). To verify the effect of the salt concentration on the bacterial morphology of the *ftsX* insertional mutant of *N. gonorrhoeae*, the strain CS19 could be verified in cells grown with various concentrations of sodium chloride in the culture medium, starting at 0.5% NaCl, the concentration at which more filaments were observed in *E. coli* JS10. However, it can be predicted that the salt concentration should not affect the morphology of *N. gonorrhoeae* CS19 cells as the *ftsX* was inactivated by an insertion, effectively disrupting the reading frame of *ftsX*.

The MICs of *N. gonorrhoeae* CS19 were identical to those of *N. gonorrhoeae* CH811 for six antibiotics tested (Table 5). This indicated that FtsX, and by extension the ABC transporter formed by FtsE and FtsX, was not involved in a transport process of antibiotics from the cytosol, contrary to the case with the Mdr or P-glycoproteins. Sturgeon and Ingram (1978) found that *E. coli* JS10 displayed increased sensitivities to antibiotics interfering with synthesis of the cell wall (bacitracin, penicillin), protein synthesis (kanamycin, neomycin) or with DNA gyrase (nalidixic acid). The antibiotics tested in this study had similar modes of action to those used by Sturgeon and Ingram (1978): penicillin and ceftriaxome both interfere with cell wall biosynthesis; tetracycline, spectinomycin and azithromycin interfere with protein synthesis, and ciprofloxacine inhibits subunit A of DNA gyrase.

The non-requiring auxotype of strain CS19 indicated that FtsX was not essential for the synthesis of precursors during gonococcal growth. When the viable counts of the strain CS19 were compared to those of strain CH811 in various environmental conditions, no significant differences were observed when the strains were grown on GCMB with various concentrations of

NaCl, at alkaline pH (8.0 and 10.0), or on GCMB under anaerobiosis (Table 6). However, the 10-fold difference observed on GCMB with 400 mM sucrose and the 6.5-fold difference on GCMB with 100 mM KCl indicated that the strain CS19 was affected under these conditions (Table 6).

The growth of *N. gonorrhoeae* under various environmental conditions has not been much investigated. Other investigators have reported that high osmolarity (300 mM NaCl (1.75%), 300 mM KCl, or 600 mM sucrose), iron depletion, 400 mM urea, increased temperature from 37°C to 41°C, and pH 6.0 reduced the growth of *N. gonorrhoeae* MS11 and derivatives by approximately 90% while anaerobic growth or temperatures between 30°C and 37°C reduced growth by 50% (Larribe *et al.*, 1997). In this work, it was found that acidic pH (pH 6.0), 150 mM KCl, 1.5% NaCl and 500 mM sucrose inhibited growth of both strains (data not shown). In addition, growth trials indicated that 350 mM urea reduced growth significantly. The results presented in this work are different from the findings reported by Larribe *et al.* (1997). *N. gonorrhoeae* strains might possess varying degrees of tolerance to any of these substances, which could explain the discrepancies noted here.

The differences in the viable counts between the two strains on GCMB containing KCl and sucrose suggest that FtsX, and by extension the FtsEX transporter might be involved in processes related to osmotic equilibrium. The putative transporter could be involved in the transport of a solute to the outside of the cell.

The effect of prevailing environmental conditions on the expression level of *ftsEX* could be examined, which may help to understand the biological function of FtsE and FtsX. For example, the level of transcription of *ftsEX* could be measured in *N. gonorrhoeae* grown on 100 mM KCl or 400 mM sucrose, the conditions found to affect the viable counts the most. If FtsX (and by extension the FtsEX transporter) is required for growth in these conditions, it is possible

that the level of transcription of *ftsEX* will be higher. Primer extension analyses done using RNA purified from anaerobically grown *N. gonorrhoeae* CH811 indicated that *ftsEX* were expressed under anaerobiosis (Francis, 1998), but possible differences in expression levels were not investigated.

The genomes of other *Neisseria* species were investigated for the presence of the *ftsE* and *ftsX* genes (section 3.5.). Hybridizations done on the whole genome of *Neisseria* species suggested that *ftsE* and *ftsX* genes were present in all the species tested (section 3.5.1.). There were variations as to the sizes of the restriction fragments that hybridized each probe (section 3.5.1.). When *ftsE* and *ftsX* were investigated by PCR, the six *ftsE* amplicons obtained were of the same size, while there was more variation in *ftsX* amplicon size (section 3.5.2.). The *ftsX* amplicon obtained from five *Neisseria* species were larger than that obtained from *N. gonorrhoeae* (section 3.5.2.). However, only the *ftsX* amplicons that were of the same size as the *N. gonorrhoeae ftsX* amplicon hybridized the *ftsX* probe (section 3.5.2.), suggesting that the larger amplicons were not *ftsX* amplicons. The determination of the nucleotide sequence of these larger amplicons would confirm whether or not they were partial *ftsX* homologues. Other primer pairs could also be used to verify if amplicons would be generated in all these species, and also if those amplicons would also have various sizes as was observed in the present work. In this study, the species in which the *ftsE* and *ftsX* probes hybridized restriction fragments of similar sizes in the hybridization with chromosomal DNA were the same as those in which the *ftsE* and *ftsX* amplicons were of the same size as that from *N. gonorrhoeae*.

No conclusions can be drawn regarding the genetic organization of *ftsE* and *ftsX* based on these data, but since *ftsE* and *ftsX* are overlapping in *N. gonorrhoeae*, they can reasonably be expected to be overlapping in other *Neisseria* as well. The *ftsE* and *ftsX* probes hybridized

restriction fragments at higher stringency (60°C) in *N. meningitidis*, *N. lactamica* and *N. cinerea*, and the amplicons produced for each gene in these three species were the same size as those from *N. gonorrhoeae*. The result was not surprising in the case of *N. meningitidis*, since *N. meningitidis* and *N. gonorrhoeae* are closely related as shown by the very high (over 98%) similarity between their housekeeping genes (Zhou and Spratt, 1992). The results obtained in these studies suggest that *N. lactamica* and *N. cinerea* are also more closely related to *N. gonorrhoeae*.

The *ftsE* and *ftsX* genes were shown to be co-transcribed in *N. gonorrhoeae*, as both gene products were synthesized when a promoter (the *lac* promoter or the native *ftsEX* promoter) was provided upstream of *ftsE* (section 3.6.). FtsX was synthesized only if a promoter was provided upstream of *ftsX*, indicating that *ftsX* did not possess its own promoter (section 3.6.). The co-transcription of *ftsEX* has also been demonstrated by RT-PCR (Francis, 1998). The co-transcription of *ftsX* with *ftsE* in *N. gonorrhoeae* implied that *ftsX* did not have its own promoter. This differed from the observations in *M. tuberculosis*, in which a weak promoter for *ftsX* was identified (Tyagi *et al.*, 1996). The TATAAT sequence identified as a possible promoter for *ftsEX* in *N. gonorrhoeae* (section 3.3.1.) was recently shown to be part of the first (P_E1) of two promoters for *ftsEX* (Francis, 1998). The second promoter (P_E2) for *ftsEX* appeared weaker than P_E1 and was located inside the *tlpA* gene. The current hypothesis to explain the difference in promoter strength is that the sequences of the -10 box of P_E2 was more divergent from the consensus promoter sequences than that of P_E1 (Francis, 1998). In addition, a putative -35 box was identified in P_E1 while none could be identified for P_E2 (Francis, 1998). The mechanisms that regulate the expression of *ftsEX* from P_E1 and P_E2 are currently under investigation (Francis, 1998).

The overexpression in rich or minimal medium of *N. gonorrhoeae ftsX*, *ftsE* or *ftsEX* in *E. coli* did not cause cell filamentation (section 3.7.). In comparison, overexpression of *N. gonorrhoeae ftsZ* caused cell filamentation in similar conditions (this work; Radia, 1997; Radia *et al.*, unpublished data). Although overproduction of FtsE and/or FtsX was not confirmed by examining the protein profiles of cell lysates on polyacrylamide gels, the *in vitro* transcription/translation data indicated that the proteins were synthesized from pSB190 and pSB5696 (Fig. 19). In addition, the vector pBluescript KS+ has a high copy number, implying that there is a much higher number of copies of *ftsE*, *ftsX* or *ftsEX* from *N. gonorrhoeae* in the cells, compared to the chromosomal copy of the host cell.

The absence of filamentation following overexpression of the gonococcal *ftsE* and *ftsX* in *E. coli* does not exclude them as cell division genes. When overexpressed in *E. coli*, cell division genes may have different effects on the cellular morphology. Overexpression of *E. coli ftsQ* or *ftsL* had no effect in rich medium and caused filamentation in minimal medium (Dai *et al.*, 1996, 1993; Guzman *et al.*, 1992; Carson *et al.*, 1991; Storts *et al.*, 1989). Overexpression of *ftsZ* from *E. coli* and other species in rich medium caused filamentation (sections 1.6.2.; Quardokus *et al.*, 1996; Margolin and Long, 1994; Margolin *et al.*, 1991; Ward and Lutkenhaus, 1985) and overexpression of *E. coli ftsN* caused short filamentation and cell lysis (Dai *et al.*, 1993). Filamentation can be induced by blocking the expression of genes such as *ffh* (encoding Ffh; Appendix 1) or *ffs* (encoding 4.5S RNA; Appendix 1) in *E. coli* (Phillips and Silhavy, 1992; Ribes *et al.*, 1990). Expression of a negative-dominant mutant of *kpsT*, which encodes the ATP-binding domain of the ABC exporter for capsular polysaccharide in *E. coli* K1 also caused cell filamentation (Bliss *et al.*, 1996). Neither *ffh*, *ffs* or *kpsT* are cell division genes, but interestingly enough, each gene encodes a protein involved in a transport process.

E. coli JS10 produced more filamentous cells in LB than in L medium (LB contains 0.5% NaCl while L medium contains 0.05% NaCl; Sambrook *et al.*, 1989; Miller, 1972). This observation has not been previously documented, as the cells were only grown in L medium in the previous study on this mutant strain (Sturgeon and Ingram, 1978). In addition, the phenotype of JS10 was not observed beyond two hours of growth in L broth. The disappearance of the filamentous phenotype of *E. coli* JS10 over time also constitutes a novel observation.

The *ftsX* gene from *N. gonorrhoeae* could not complement the unknown *ftsX* defect in *E. coli* JS10 (section 3.8.). The failure of the complementation could not be attributed to the absence of expression of the gonococcal *ftsX* gene under the experimental conditions since the gonococcal *ftsX* was expressed from the plasmid pSB190 as shown by *in vitro* transcription/translation (section 3.6.), but the production of the gonococcal FtsX could have been verified in cell extracts fractionated on polyacrylamide gels. Interestingly, attempts to complement *E. coli* JS10 with *ftsX* from *M. tuberculosis* have likewise been unsuccessful (Tyagi *et al.*, 1996). So far, *E. coli* JS10 has only been successfully complemented with the wild-type *E. coli ftsX* (Gill *et al.*, 1986). Together, these data suggested that FtsX possessed species-specific characteristics; this was hypothesized as a possible explanation for the failed complementation of *E. coli* JS10 with *ftsX* gene from *M. tuberculosis* (Tyagi *et al.*, 1996). The comparison of the aa sequences of the six known FtsX polypeptides has shown considerable size variation between the FtsX homologues, and the analyses done in this thesis suggest that this size variability was attributable to the size of the amino-terminal region of the protein. The *M. tuberculosis* FtsX comprises 268 aa residues (Tyagi *et al.*, 1996) compared to 352 in *E. coli* (Gill *et al.*, 1986) and 305 in *N. gonorrhoeae* (section 3.3.2.). Based on the analyses presented in the section 3.3.2., the size of the amino-terminal domain of *M. tuberculosis* FtsX is 17 aa, compared to 74 in *E. coli*, and 28 in *N.*

gonorrhoeae. It can be speculated that the size of this region varies between homologues to serve a species-specific function. The importance of the amino-terminal region of FtsX could be verified by constructing a series of deletions in the 5'-region of the *E. coli ftsX* gene and to use these deletions to attempt relieving the filamentous phenotype of *E. coli* JS10 at its restrictive temperature. The carboxy-terminal region of FtsX also appears important for proper function of FtsX, as *E. coli* FtsX truncated by its 38 carboxy-terminal aa residues (starting at Ile315 of *E. coli* FtsX; Fig. 11), effectively removing 9 aa residues from the linker between the third and fourth predicted transmembrane segments, the fourth transmembrane segment and the carboxy-terminal domain, was incapable of complementing the defect in *E. coli* JS10 (Gill *et al.*, 1986).

The purified 6HISFtsE protein will be used to generate anti-6HIFtsE polyclonal antibodies. These antibodies will then be used for subcellular localization studies of FtsE in *N. gonorrhoeae* by immunoelectron microscopy as has been done for FtsZ in *E. coli* (Bi and Lutkenhaus, 1991b). These antibodies could also be used to confirm any interaction between FtsE and FtsX: in a wild-type *N. gonorrhoeae* strain, FtsE is predicted to be located to the inner side of the cytoplasmic membrane by binding FtsX; therefore anti-6HISFtsE polyclonal antibodies would also be located to the inner side of the cytoplasmic membrane. If the same experiment was done using *N. gonorrhoeae* CS19, in which *ftsX* has been disrupted, the hypothesized consequence would be that FtsE could not be located close to the membrane and the polyclonal antibodies would be located in the cytosol. If FtsE was found to be localized to the inner membrane despite the absence of FtsX, this would be indicative of the existence of at least another possible membrane anchor for FtsE.

The absence of *ftsY* immediately upstream of *ftsE* and *ftsX* was unexpected (sections 3.2. and 3.11.). At the time the genetic organization of *ftsE* and *ftsX* was being determined in *N.*

gonorrhoeae, these genes had only been identified and characterized in *E. coli* and *H. influenzae* and *ftsY*, *ftsE* and *ftsX* were clustered in both bacteria (section 1.4.; Fleischmann *et al.*, 1995; Gill *et al.*, 1986). *N. gonorrhoeae* became the first bacterium in which *ftsY*, *ftsE* and *ftsX* were found not to be clustered (Bernatchez *et al.*, 1996). The genetic organization of *ftsEX* in *N. gonorrhoeae*, combined with the information available on the genetic organization of *ftsY*, *ftsE* and *ftsX* from *M. tuberculosis* (Devlin *et al.*, 1997), *M. leprae* (Parkhill *et al.*, 1997a, 1997b), *H. pylori* (Tomb *et al.*, 1997), *M. pneumoniae* (Himmelreich *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *M. genitalium* (Fraser *et al.*, 1995), and *E. coli* (Gill *et al.*, 1986) has now indicated that the genetic organization of these genes varies between bacterial species (this work; section 1.4.).

The presence of *ftsY* and the absence of *ftsE* and *ftsX* in *M. genitalium* and *M. pneumoniae* (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995) suggested that *ftsY* was essential in bacteria while *ftsE* and *ftsX* were not. This hypothesis was supported by the observation that *ftsY* was found essential in *E. coli*, while *ftsE* or *ftsX* mutants of *E. coli* were viable (Luirink *et al.*, 1994; Gibbs *et al.*, 1992; Gill and Salmond, 1990; Sturgeon and Ingram, 1978).

The *pilA* gene has been proposed to be the gonococcal homologue of *ftsY* (Fyfe and Davies, 1996). This hypothesis was based on significant amino acid sequence similarity between PilA and FtsY in their carboxy-terminal sections and on the fact that both *pilA* and *ftsY* are essential genes (Fyfe and Davies, 1996; Taha *et al.*, 1991, 1988). The similarity between PilA and FtsY started at position 121 in the PilA aa sequence; its amino-terminal domain (positions 1-120) contains 32% of charged aa residues (including 21% of acidic aa residues, data not shown), which was less than the ratio of charged aa residues observed in the amino-terminal domains of *E. coli* FtsY (section 1.5.3.). PilA was shown to hydrolyze GTP (Arvidson and So, 1995a), as does FtsY

(section 1.5.3.). The amino-terminal domain of PilA was predicted to contain an α-helix-turn-α-helix motif characteristic of a DNA-binding site (Taha *et al.*, 1988). PilA was shown to bind DNA containing the promoter region of the *pilE* gene and to be involved in the regulation of the *pilE* gene in *N. gonorrhoeae* (Arvidson and So, 1995b; Taha *et al.*, 1995). Although FtsY was shown to be involved in protein transport in *E. coli* (section 1.5.3.), the available experimental evidence for PilA argued against its involvement in protein transport in *N. gonorrhoeae*: while FtsY of *E. coli* was shown to interact with 4.5S RNA (section 1.5.3.), no interaction between PilA and the gonococcal homologue of the 4.5S RNA could be detected (Taha *et al.*, 1992). PilA has been proposed to be a pleiotropic regulator involved in the modulation of growth rate in response to various environmental stimuli (Larribe *et al.*, 1997; Taha *et al.*, 1992). More investigation on the biological function of PilA is required to resolve the controversy concerning its role. In this work, the similarity searches in the *N. gonorrhoeae* FA1090 genome database using FtsY from *E. coli* only detected PilA sequences (section 3.11.). From these analyses, it appeared that no *ftsY* gene was present in the genome of *N. gonorrhoeae*. The question concerning the presence of a gonococcal homologue of the *ftsY* gene in the chromosome of *N. gonorrhoeae* will be resolved on further investigation of the entire sequence of the genome and of PilA function.

The effect of inactivating *ftsE* in *N. gonorrhoeae* remains to be assessed. Point mutations in *ftsE* that rendered *E. coli* Ts have been characterized (section 1.2.2.; Gibbs *et al.*, 1992). It would be interesting to selectively inactivate *ftsE* without affecting *ftsX* expression, and also to inactivate *ftsEX* to verify the effect of disabling the putative transporter formed by FtsE and FtsX on the viability and morphology of *N. gonorrhoeae*. Since *ftsX* is not an essential gene (section 3.4.), it can be supposed that *ftsE* is not essential, and that the putative FtsEX transporter itself may not essential. The MIC profile of the *ftsE* mutant should also be determined for comparison

to that of *N. gonorrhoeae* CS19. The MIC for ampicillin of both mutant strains when they contain a plasmid containing a *bla* gene (encoding β-lactamase) should be verified. These results would then be compared with the observations of Luirink *et al.* (1994) who found that β-lactamase translocation was not affected in an *E. coli ftsE* Ts mutant, suggesting that FtsE was not involved in the translocation process. This observation on the non-involvement of FtsE in β-lactamase translocation can be predicted to be also valid for FtsX and for the putative FtsEX transporter.

The classification of *ftsE* as a cell division gene has been previously questioned since filamentation of a *ftsE* mutant of *E. coli* was medium-dependent (section 1.5.1.; Taschner *et al.*, 1988). However, two mutants of *E. coli* blocked at the earliest stage of cell division were found to be mutated in *ftsE* (section 1.5.1.; Begg and Donachie, 1995). This suggested that FtsE, and by inference the ABC transporter formed by FtsE and FtsX, could be involved in early stages of cell division, perhaps in the translocation of cell division proteins. Further characterization of the function of FtsE will be required to determine its role. FtsX was believed to be involved in cell division in *M. tuberculosis* based on the observation that *ftsX* expression was correlated with the division status in this bacterium (section 1.5.2.; Tyagi *et al.*, 1996). As with FtsE, further characterization of FtsX is needed to determine its biological function.

The only ABC transporters identified so far in the *Neisseriaceae* have been a transporter involved in iron uptake in *N. gonorrhoeae* and a transporter involved in export of the capsule polysaccharide in *N. meningitidis* (section 1.2.4.). The membrane proteins of each of these ABC transporters were also predicted to differ from the standard structure: FbpB from the gonococcal iron importer was predicted to have 11 transmembrane segments (Adhikari *et al.*, 1996), while CtrB and CtrC from the meningococcal liposaccharide exporter were predicted to be hydrophobic proteins (Frosch *et al.*, 1991) containing 2 and 5 transmembrane segments, respectively (data not

shown). As was determined in this work for *ftsEX*, the *fbpABC* and the *ctrABCD* genes encoding the domains of these two other transporters are organized in operons (Adhikari *et al.*, 1996; Frosch *et al.*, 1991). No additional protein is expected to be part of the putative ABC transporter identified in the present work as only *ftsE* and *ftsX* were found to be part of the same transcriptional unit.

ABC transporters are involved in import or export processes (section 1.2.). Based on the data gathered from this work, the putative ABC transporter formed by FtsE and FtsX is predicted to participate in an export process. This prediction is based on two observations. First, ABC importers always comprise a third protein (the periplasmic substrate-binding protein; section 1.2.1.), encoded by a gene clustered with the genes encoding the domains of the ABC transporter (Fath and Kolter, 1993). By contrast, some ABC exporters require an AF, but the gene encoding that third protein may be linked or not linked with those encoding the domains of the ABC transporter (section 1.2.1.). No other gene appears to be transcribed with *ftsEX*, but the putative FtsEX transporter may require a third protein encoded by an unlinked gene. Second, the EAA loop is absent from the aa sequence of the FtsX protein. This sequence is present in a cytoplasmic loop of the membrane domain of ABC importers (section 1.2.3.). In addition, earlier sequence comparisons showed that FtsE belonged to a group comprising ABC exporters and importers in which the ATP-binding and membrane domains were found on separate polypeptides (Fath and Kolter, 1993; Fath *et al.*, 1992).

A plethora of questions need to be answered to confirm the existence of the ABC transporter formed by FtsE and FtsX. Experimental evidence will be required to show that FtsE binds ATP. This constitutes a vital question, as binding and hydrolysis of ATP by the ATP-binding domain is intimately linked to the mechanism of substrate transport by the transporter.

FtsE and FtsX will need to be shown to interact. This interaction could be verified by using polyclonal anti-FtsE antibodies as described once they are available (see above), or by using the yeast two-hybrid system which has already been used to show interactions between FtsA and FtsZ (Wang *et al.*, 1997) and also between MinC and MinD (Huang *et al.*, 1996). An experimental approach similar to that followed by Hale and de Boer (1997) to identify ZipA, the membrane receptor for FtsZ (section 1.7.1.) could also be used to identify FtsX. In this experiment, FtsE (or 6HISFtsE) would be labeled with a radioactive isotope and used to probe the proteins of a membrane fraction of *N. gonorrhoeae*.

The topological predictions of *N. gonorrhoeae* FtsX need to be experimentally verified. There are several systems to determine the membrane topology of a membrane protein by means of fusions with alkaline phosphatase (AP), β-galactosidase or β-lactamase (Froshauer *et al.*, 1988; Broome-Smith and Spratt, 1986; Manoil and Beckwith, 1985). Fusions between FtsX and AP would be preferred in the present case as there is a system allowing the determination of the AP activity of the fusion in *N. gonorrhoeae* (Boyle-Vavra and Seifert, 1995). The importance of the LLGA motif for FtsX-FtsE interactions could be assessed by mutating the aa residues and verifying if FtsE and FtsX are still able to form a complex using a yeast two-hybrid system. In addition, an extensive search of the aa sequence of the membrane domains of other ABC exporters will be required to verify if the LLGA motif is present and conserved.

One of the main unanswered questions is the nature of the ion or molecule transported by FtsE/FtsX. An interesting possibility would be that one of the murein biosynthesis proteins that need to be periplasmically located as a substrate for FtsE and FtsX. This possibility is especially interesting considering the absence of *ftsE* and *ftsX* in *M. pneumoniae* and *M. genitalium*, two bacteria that do not have cell walls, and that are consequently devoid of the *mur* genes

(Himmelreich *et al.*, 1996; Fraser *et al.*, 1995). However, the *mur* genes are present in *Synechocystis* PCC6803 notwithstanding the absence of *ftsE* and *ftsX* in this cyanobacterium (Kaneko *et al.*, 1996). Gill *et al.* (1986) suggested Ca^{2+} or Mg^{2+} cations as potential candidates for the FtsEX transporter. The results of the viable counts under various environmental conditions (section 3.4.3.) suggest that the FtsEX transporter could be involved in osmotic equilibrium so that more investigation in that direction could be worthwhile. The participation of a possible third protein (like TolC for colicinV or α-hemolysin export; section 1.2.1.) in the process in which the FtsE/FtsX transporter is involved will also need to be verified.

FtsY has been shown to be involved in protein transport in *E. coli* (section 1.5.3.). Based on the clustering of their genes with *ftsY*, FtsE and FtsX were proposed to be part of the same cellular process with FtsY; however, in contrast to what had been observed following FtsY depletion in *E. coli*, there was no accumulation of β-lactamase in an *E. coli ftsE* (Ts) strain (Luirink *et al.*, 1994; section 1.5.3.). Results from the present work argue against a role for FtsE and FtsX in the SRP pathway: other known components of the SRP pathway (FtsY, Ffh, 4.5S RNA; Appendix 1) have been shown to be essential for cell viability (Luirink *et al.*, 1994; Phillips and Silhavy, 1992; Brown and Fournier, 1984); in contrast the viability of the *ftsX* mutant *N. gonorrhoeae* CS19 indicated that *ftsX* was not an essential gene. Morphological aberrations were caused by overexpression of *ftsY* in *E. coli* (Luirink *et al.*, 1994; while the overexpression of the gonococcal *ftsE*, *ftsX* or *ftsEX* in *E. coli* had no observable effect (section 3.7.).

The genetic organization of the other gene homologues identified in this study varied between bacterial species. In *E. coli*, *arsC* was located at 78.6 minutes (Berlyn *et al.*, 1995) and is the closest to *ftsYEX* among the other gene homologues identified in this work; *pgk* and *gltX* are located elsewhere on the chromosome while no *tlpA* gene has been identified (Berlyn *et al.*,

1995). In *H. influenzae*, *arsC*, *gltX* and *pgk* were not linked between themselves or with *ftsYEX* (Fleischmann *et al.*, 1995). It is however noteworthy that *arsC* is alone on the chromosome of *H. influenzae*, as is the case in *N. gonorrhoeae*. The *arsC* gene is part of the *ars* operon that comprises genes involved in arsenic resistance in *E. coli*, and plasmids of diverse bacteria such as *E. coli*, *Yersinia*, and *Staphylococci* species (Berlyn *et al.*, 1995; Fleischmann *et al.*, 1995). In *H. pylori*, there was no *arsC* gene, and *gltX* and *pgk* were unlinked between themselves and were not linked with *ftsE* and *ftsX* (Tomb *et al.*, 1997). In *M. genitalium*, *pgk* and *ftsY* were separated by 4.1 Kb (Fraser *et al.*, 1995); *gltX* was located elsewhere on the chromosome while *arsC* and *tlpA* were not present (Fraser *et al.*, 1995). In *M. jannaschii*, only *gltX* and *pgk* were present and they were not linked (Bult *et al.*, 1996).

PART 5. CONCLUSIONS

The *ftsE* and *ftsX* genes of *N. gonorrhoeae* and their flanking genes have been cloned and characterized. The gonococcal FtsE contained the Walker A and B domains characteristic of the ATP-binding protein domains of ABC transporters. The gonococcal FtsX only shared significant aa sequence similarity with its known bacterial homologues. Analyses of the aa sequence of the six known bacterial FtsX showed that the size of the amino-terminal region varied between FtsX homologues and that it contained four putative transmembrane helices. These results suggested that FtsE and FtsX were the ATP-binding and transmembrane domains of an uncharacterized ABC transporter. The putative FtsEX transporter was predicted to be involved in an export process; first because no other gene was part of the *ftsEX* transcription unit, and also because the EAA loop was not present in FtsX.

The *ftsX* gene was shown to be non-essential for bacterial viability since *N. gonorrhoeae* was viable following the disruption of the *ftsX* reading frame. The size of gonococci from the mutant strain did not differ from that of the wild-type parental strain, and no morphological aberrations were observed. Since the absence of other known cell division genes in *E. coli* has been shown to be lethal, this suggested that *ftsX*, and by inference the FtsEX transporter, was not involved in cell division.

The *ftsE* and *ftsX* genes were identified in ten other *Neisseria* species by Southern blotting and PCR, suggesting that they were ubiquitous in the Neisseriaceae. The *ftsE* and *ftsX* genes overlapped by 4 bp suggesting co-transcription. *ftsE* and *ftsX* were shown to be co-transcribed, as synthesis of the FtsE and FtsX proteins occurred when a promoter was present upstream of *ftsEX*, but FtsX was not synthesized from *ftsX* alone if no promoter was provided. A putative -10 sequence identified upstream of *ftsE* could be part of the *ftsEX* promoter.

While overexpression of some cell division genes caused morphological aberrations in *E. coli*, overexpression of *ftsE*, *ftsX* or *ftsEX* had no observable effect. Complementation attempts of the *E. coli* JS10 *ftsX* Ts mutant were not successful. This may be due to species-specific features of the FtsX protein, particularly in its amino-terminal region. However, the absence of morphological aberrations upon overexpression of *ftsEX* does not constitute sufficient

experimental evidence to dismiss *ftsEX* as cell division genes, as other genes caused morphological aberrations if their regular pattern of expression was altered.

Contrary to the case in *E. coli* and *H. influenzae*, a homologue of the *ftsY* was not present immediately upstream of *ftsEX*. Gonococcal genes encoding a putative thioredoxin-like protein, arsenate reductase and glutamyl-$_T$RNA synthetase were identified upstream of *ftsEX*, and a gene encoding 3-phosphoglycerate kinase was identified downstream of *ftsEX*. Similarity searches in the genome database of *N gonorrhoeae* FA1090 using FtsY from *E. coli* failed to identify a gonococcal FtsY homologue.

The putative FtsEX transporter, or any ABC transporters, are not included in the current model of bacterial cell division. The viability of the *ftsX* mutant of *N. gonorrhoeae* suggested that *ftsX*, and by extension *ftsE*, were not cell division genes. A role for the FtsEX transporter in the SRP pathway is also unlikely, again based on the viability of the *ftsX* insertion mutant while the depletion of other known components of the SRP pathway (Ffh, 4.5S RNA, FtsY) in *E. coli* were ultimately lethal. The biological function of the FtsEX transporter remains to be determined, but may be linked to osmotic equilibrium since the *ftsX* mutant had colony counts 6.5 and 10 times lower than those of the wild-type parental strain on media containing 100 mM KCl or 400 mM sucrose.

Additional investigation of FtsE and FtsX will necessitate the characterization of the FtsEX transporter, starting with the identification of the substrate of the transporter. The identification of the substrate would confirm the role of FtsE and FtsX in transport and would also allow to understand the biological function of the FtsEX transporter. However, since the inactivation of *ftsX* did not affect cell viability, suggesting *ftsX* and the FtsEX transporter were not essential, the identification of the substrate could be difficult. Based on the results from the viable counts, the first series of possible substrates to investigate would be molecules involved in the maintenance of osmotic equilibrium. The nature of the substrate may also help to determine if there is an additional component to the transporter, such as an OMP.

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PART 7. APPENDICES

Appendix 1. The SRP pathway of protein transport.

There are two pathways for protein transport across or integration into the membrane of the endoplasmic reticulum (ER) in eukaryotic cells (Rapoport *et al.*, 1996). In the co-translational pathway, the transport takes place while the polypeptide is being synthesized on a ribosome bound to the ER membrane, while in the post-translational pathway, the synthesis of the polypeptide is completed before it is transported (Rapoport *et al.*, 1996).

The co-translational pathway begins as the signal sequence of a nascent polypeptide emerges from the ribosome and is bound by the signal recognition particle (SRP). The SRP is a ribonucleoprotein complex consisting of one RNA molecule, 7S RNA, and six polypeptides with Mws of 9, 14, 19, 54, 68 and 72 KDa (Lütcke, 1995). The nascent polypeptide is bound through one of the domains of the 54 KDa polypeptide of the SRP (SRP 54; Rapoport *et al.*, 1996; Lütcke, 1995). The binding of the SRP to the signal sequence causes a temporary arrest in the elongation of the polypeptide (Lütcke, 1995). The complex formed by the SRP and the ribosome then binds the SRP receptor (SR, also called the docking protein) located on the membrane of the ER. The SR is constituted by two subunits (SRα and SRβ, both of which possess a GTPase activity; Lütcke, 1995); the interaction of SR with the SRP occurs through SRα. The SRP is then released from the signal sequence and the ribosome, and the nascent polypeptide is transferred into the lumen of the ER (Rapoport *et al.*, 1996). The release of the SRP from its receptor occurs through hydrolysis of GTP, and once the SRP is free in the cytosol once more, a new cycle can begin.

In bacteria, protein transport is mostly handled through the general secretory pathway (Arkowitz and Bassilana, 1994; Pugsley, 1993). However, several bacterial gene products have been identified as homologues of components of the mammalian SRP, suggesting the existence of a SRP pathway in bacteria. (Lütcke, 1995; Dobberstein, 1994; Luirink and Dobberstein, 1994; Wolin, 1994; Rapoport, 1991). For instance, the product of the *ffs* gene, a 4.5S RNA, was found to possess a structure similar to that of 7S RNA (Poritz *et al.*, 1988); Ffh, a 48 KDa polypeptide encoded by the *ffh* gene shared significant aa sequence similarity with the 54 KDa polypeptide of

the mammalian SRP (Bernstein *et al.*, 1989; Römisch *et al.*, 1989); the FtsY protein was found to share sequence similarity with SRα (Bernstein *et al.*, 1989; Römisch *et al.*, 1989; section 1.5.3.).

Following the identification of these homologues of the constituents of the SRP in *E. coli*, Ffh and 4.5S RNA were shown to form a complex, with indicated that a SRP existed in *E. coli* (Poritz *et al.*, 1990; Ribes *et al.*, 1990). This bacterial SRP would only consist of the Ffh polypeptide and of the 4.5S RNA. The Ffh polypeptide was shown to be essential for bacterial viability and for the efficient export of several proteins in *E. coli* (Phillips and Silhavy, 1992). The evidence involving FtsY in protein transport, more specifically in the SRP pathway, is reviewed in section 1.5.3. Also similar to the eukaryotic SRP, the *E. coli* SRP was shown to act co-translationally (Powers and Walter, 1997).

It is believed that the SRP pathway increases the targeting efficiency of a subset of *E. coli* presecretory proteins in an uncharacterized process (Schatz and Dobberstein, 1996; Wolin, 1994).

Appendix 2. The initial isolation of the plasmid pSB19.

A strategy to clone gonococcal arginine biosynthesis genes consisted of the genetic complementation of *E. coli* arginine auxotrophs on minimal medium. Lytic complementation of *E. coli* arginine auxotrophs with genes from *N. gonorrhoeae* had previously been accomplished using the recombinant λ bacteriophages from a bank of *N. gonorrhoeae* CH811 DNA (Picard and Dillon, 1989a). For the present work, the strategy involved transforming the plasmid pools from the genomic bank constructed in the plasmid pBluescript KS+ (section 2.5.) into *E. coli* strain UQ27, which harbors a defective *argG* gene (Table 2). This gene encodes the enzyme argininosuccinate synthetase, which catalyzes the seventh step of the arginine biosynthetic pathway (Glansdorff, 1996; Fig. A2-1.). The transformants were selected on minimal medium lacking arginine as described in section 2.5. The growth of *E. coli* UQ27 on minimal medium lacking arginine required the presence of a gonococcal gene able to compensate the function of the defective *argG* gene of the *E. coli* host strain. Twenty-five bacterial colonies (bacterial clones SB1 to SB25) were obtained following the genetic complementation experiment, and the ability of each colony to grow in the absence of arginine was confirmed by restreaking on minimal medium lacking arginine.

The plasmids from these 25 clones were purified (plasmid clones pSB1 to pSB25) and divided in eight groups based on their banding pattern and on the size of their insert. The plasmid groups comprised from one to seven plasmids (Table A2-1.). The rationale for the grouping of the plasmids was that all inserts of the same size were expected to be identical since all the plasmids had complemented the arginine defect of *E. coli* UQ27 on minimal medium lacking arginine.

Since the groups 4, 5, 6 and 8 included 20 of the 25 bacterial clones obtained from the complementation experiment, the plasmids from these four groups were the first ones to be investigated. One or several plasmid clones from each of the groups 4, 5, 6 and 8 were randomly selected for further characterization (nine plasmid clones in total; Table

A2-1.). The plasmids were restricted with *Pvu*II to free the inserts from the vector and the gonococcal origin of the insert of each of the nine plasmid clones was successfully confirmed by hybridization with gonococcal DNA at 68°C (data not shown).

Table A2-1. Plasmids complementing the arginine requirement of *E. coli* strain UQ27 on minimal medium M9 lacking arginine.

Plasmid group	Number of plasmids in the group	Insert size (Kb)	Plasmid clones[1]
1	1	1.9	pSB1
2	1	2.2	pSB2
3	1	1.6	pSB16
4	4	1.0	**pSB3**, pSB4, pSB13, pSB25
5	7	1.9	**pSB5**, **pSB14**, pSB17, pSB18, **pSB22**, pSB23, **pSB24**
6	4	1.8	**pSB6**, **pSB11**, pSB15, **pSB19**
7	2	1.0	pSB7, pSB8
8	5	1.6	pSB9, pSB10, pSB12, pSB20, **pSB21**

[1] The plasmids in bold are those that were chosen for further characterization.

The presence of the *argG* gene in the nine plasmids was then investigated by hybridization using the *argG* gene from *E. coli* as a probe. The *argG* gene from *E. coli* was present on a 5.3 Kb *Sal*I-*Bgl*II fragment that had been originally cloned in the plasmid pYN87 (Table 2; Kurihara and Nakamura, 1983), and the complete nucleotide sequence of *argG* had been determined (Van Vliet *et al.*, 1990). The plasmid pYN87 contained a functional *argG* gene since it could complement *E. coli* UQ27 on minimal

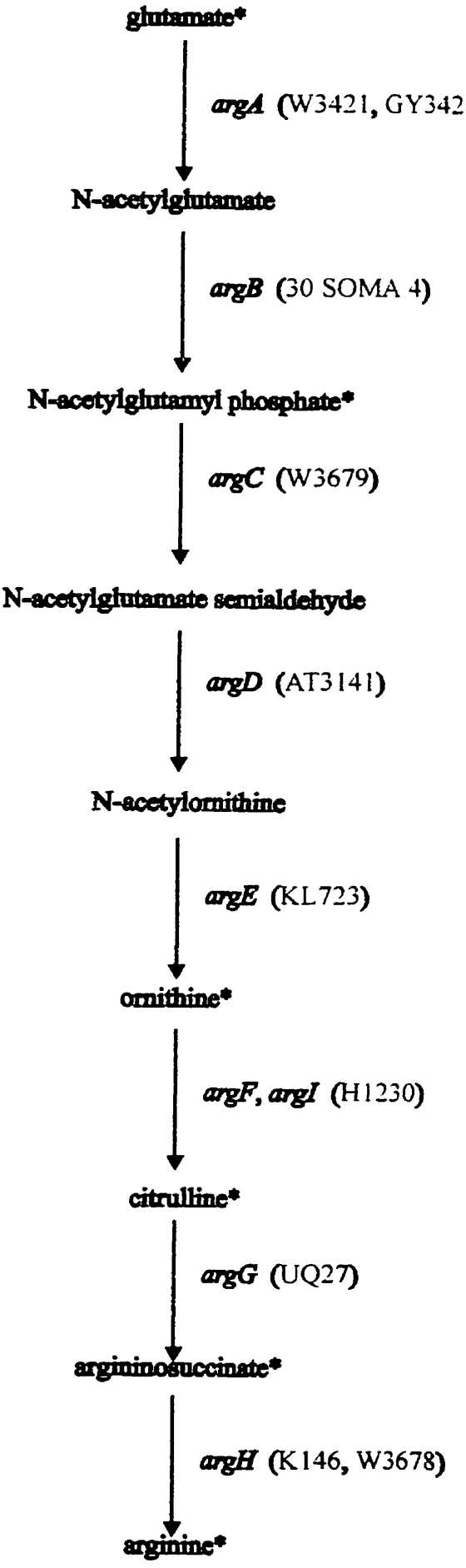
medium lacking arginine. Of the nine plasmids investigated (Table A2-1.), only the insert of pSB19 weakly hybridized the *E. coli argG* gene probe at 60°C (data not shown). This result suggested that only the insert of pSB19 shared nucleotide sequence similarity with the *argG* gene from *E. coli*. The hybridization signal was stronger at 50°C, but was still weak (data not shown). The results of these hybridizations, combined with the fact that the plasmid pSB19 allowed growth of *E. coli* UQ27 on minimal medium lacking arginine, suggested that the insert of the plasmid pSB19 contained the gonococcal *argG* gene. However, the weak hybridization signal was obtained between the two DNA fragments even at relatively low stringency (50°C) suggested that the nucleotide sequence of the putative gonococcal *argG* gene would be divergent from that of *E. coli*. The complementation of *E. coli* UQ27 with pSB19 was successfully repeated once more before proceeding with the determination of the nucleotide sequence of the insert of pSB19. The plasmid pSB19 was digested with *Sac*I and *Xba*I to liberate the insert and nested deletions were constructed to facilitate the sequencing step, and the insert of pSB19 was completely sequenced in both orientations.

The analysis of the nucleotide sequence of the insert of pSB19 showed it contained one complete gene sharing sequence similarity to the *ftsX* gene from *E. coli*. Two other partial genes that respectively shared nucleotide sequence similarity to the *ftsE* and *pgk* genes were also identified (section 3.1.). There was no sequence similarity with the *argG* gene of *E. coli* or of other bacteria.

The possible complementation of any other step of arginine biosynthesis by the gonococcal *ftsX* gene was verified by transforming pSB19 into *E. coli* strains with defects in arginine biosynthesis (Fig. A2-1 and Table 2). Before the mutants were transformed with pSB19, the defective step of arginine biosynthesis was confirmed by nutritional requirements as described in section 2.1. The defects in first two steps of arginine biosynthesis (strains W3421 and GY3428 for *argA*, strain 30 SOMA 4 for *argB*) could not be confirmed in the strains used as they did not grow on M9 medium supplemented

with N-acetylglutamate and ornithine, respectively. The defects in arginine biosynthesis were confirmed for strains mutated in each of the other six steps of the biosynthetic pathway (*argC*, *argD*, *argE*, *argF*, *argG* and *argH*; Fig. A2-1.), and the corresponding mutant strains were transformed with pSB19 and pHL1 (a pGEM-T derivative containing amplified *ftsEX* genes from *N. gonorrhoeae* with the upstream region containing the promoter; Table 2). None of the seven arginine mutants tested (Fig. A2-1.) grew on minimal medium supplemented with the substrate of each defective enzyme following transformation with pSB19 or pHL1, including strain UQ27. The fact that the initial complementation of *E. coli* UQ27 by pSB19 could not be reproduced, combined with the absence of complementation of other arginine auxotrophs of *E. coli* by pSB19, indicated that *ftsX* plays no role in arginine biosynthesis and that the initial observation was a spurious result. Together, the results from the complementation attempts indicated that the *ftsE* and *ftsX* gene products played no role in the 3rd (*argC*), 4th (*argD*), 5th (*argE*), 6th (*argG*) and 7th (*argH*) steps of the arginine biosynthesis pathway. Also, based on these results, it can be predicted that pSB19 and pHL1 would not complement the defects in mutants of the 1st (*argA*) or 2nd (*argB*) steps of arginine biosynthesis. Thus, *ftsX* (and *ftsE*) have no role in this pathway.

Figure A2-1.: The pathway of arginine biosynthesis in *E. coli*. The asterisks accompanying some of the intermediates indicate they were used for the confirmation of the defect in arginine biosynthesis in the mutants. For instance, the defect in strain AT3141 was confirmed by growth on M9 medium with ornithine, and the absence of growth of AT3141 was confirmed on M9 containing N-acetylglutamate. The strains used for each gene are indicated in parentheses next to each gene; green characters indicate the strain was tested, red characters indicate the strain was not tested.

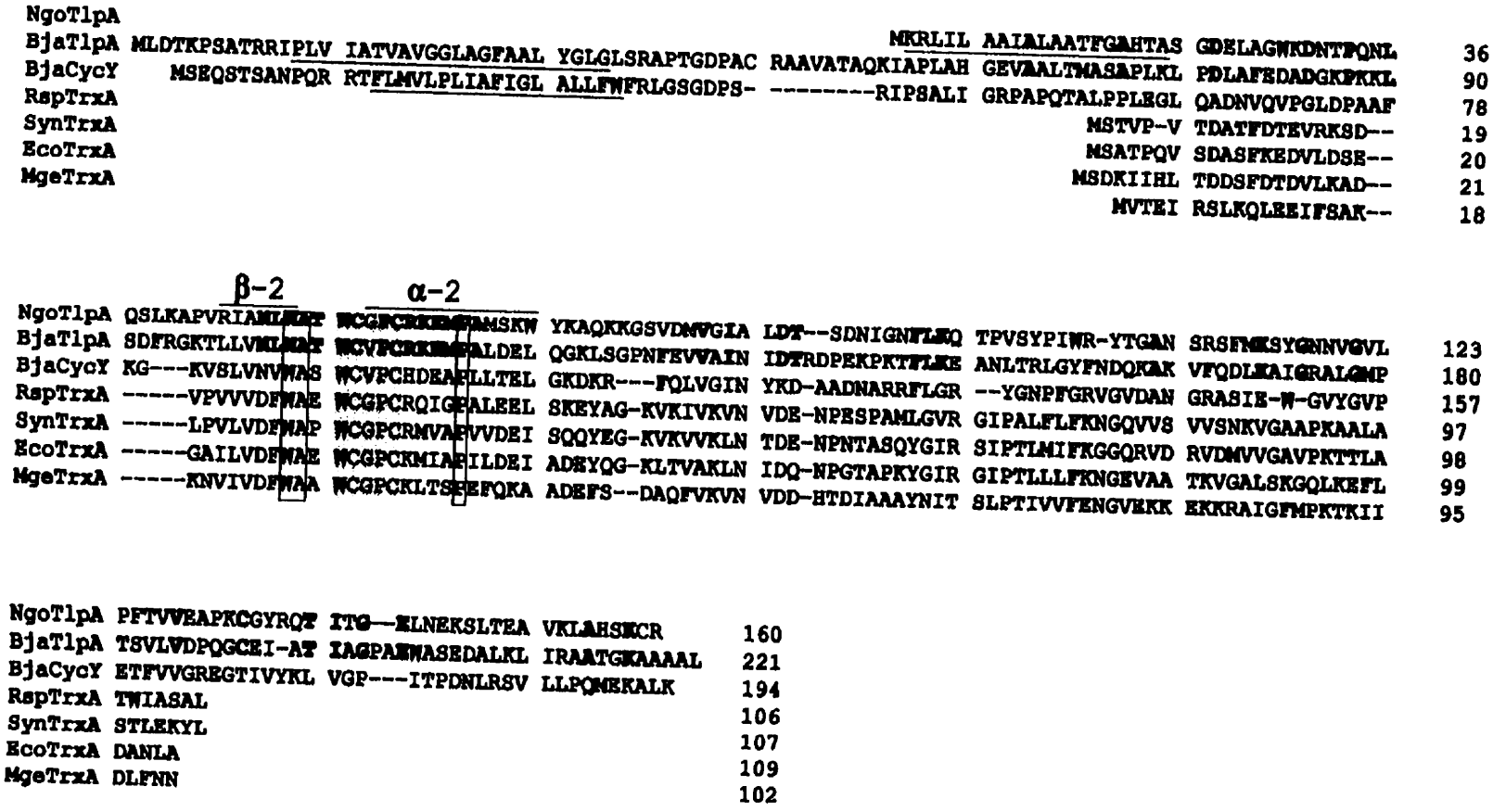

glutamate*
argA (W3421, GY3428)
N-acetylglutamate
argB (30 SOMA 4)
N-acetylglutamyl phosphate*
argC (W3679)
N-acetylglutamate semialdehyde
argD (AT3141)
N-acetylornithine
argE (KL723)
ornithine*
argF, argI (H1230)
citrulline*
argG (UQ27)
argininosuccinate*
argH (K146, W3678)
arginine*

Appendix 3. Other analyses of the *tlpA* gene.

Besides the aa sequence similarities with *B. japonicum* TlpA (52.6% similarity and 31.2% identity), the gonococcal TlpA had other similar features to *B. japonicum* TlpA. The two polypeptides were predicted to have alkaline isoelectric points (pI): the predicted pI for *N. gonorrhoeae* TlpA was 9.63, compared to a predicted pI of 8.97 for *B. japonicum* TlpA (Loferer *et al.*, 1993). Also, a putative transmembrane segment was identified near the amino-terminal end of the gonococcal polypeptide (positions 2 to 20, Fig. A3-1), as is the case in *B. japonicum* TlpA (Fig. A3-1; (Loferer *et al.*, 1993)). This putative transmembrane segment contained two positively charged amino acid residues at its beginning, suggesting that its amino terminus is located in the cytoplasm and that it contained a large periplasmic domain, as had been reported for *B. japonicum* TlpA (Loferer *et al.*, 1993). However, the gonococcal TlpA was 61 aa shorter than the *B. japonicum* TlpA.

B. japonicum TlpA was reported to share about 30% identity with various bacterial thioredoxins (Loferer and Hennecke, 1994; Loferer *et al.*, 1993). In comparison, *N. gonorrhoeae* strain CH811 TlpA shared between 46.7% and 52.5% aa similarity and between 23.6% and 27.6% aa identity with the thioredoxins from *E. coli*, *Rhodobacter sphaeroides*, *S. typhimurium*, *M. genitalium*, and *Synechocystis* sp. 6083. An alignment of TlpA from *N. gonorrhoeae* and *B. japonicum* with the thioredoxins from *E. coli*, *S. typhimurium* and *M. genitalium* is shown in Fig. A1-1. The amino acid residues of the active site of bacterial thioredoxins (consensus sequence: WCGPC (Loferer and Hennecke, 1994)) are perfectly conserved in the gonococcal TlpA (positions 52 to 56, Fig. A3-1). As had been observed with *B. japonicum* TlpA (Loferer *et al.*, 1993), the aa residues forming β-sheet 2 and α-helix 2 that precede and follow the cysteinyl residues of the active site in the thioredoxins (Ellis *et al.*, 1992) are well conserved in the gonococcal TlpA. There are eleven identities and three conservative changes between the gonococcal and *B. japonicum* TlpA polypeptides over the nineteen amino acid residues part of β-sheet 2 and α-helix 2 (Fig. A3-1). This suggests there exist some structural similarities between *N. gonorrhoeae* TlpA, *B. japonicum* TlpA and the thioredoxins.

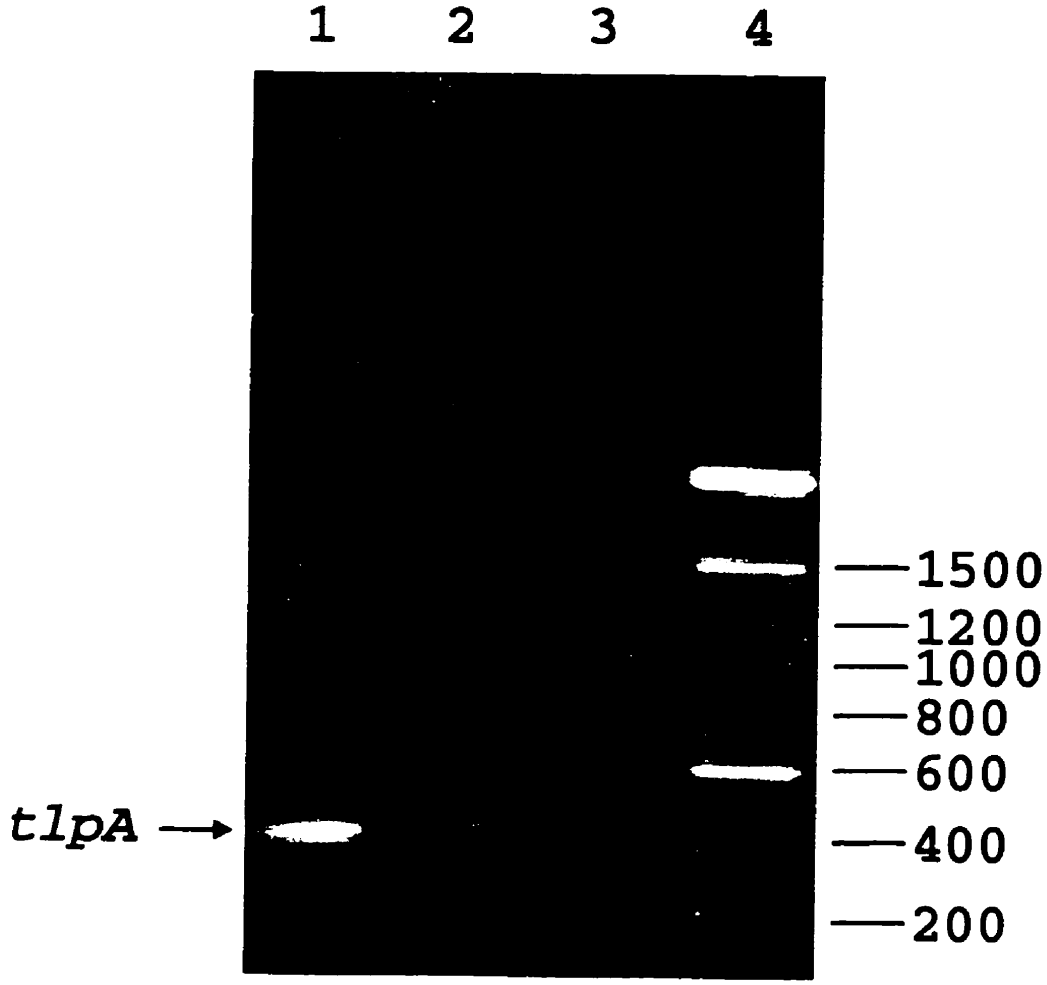
Figure A3-1. Alignment of the amino acid sequences of bacterial thioredoxin-like proteins and thioredoxins. Included in this alignment are the thioredoxin-like proteins of *N. gonorrhoeae* (NgoTlpA; this work) and *B. japonicum* (BjaTlpA; Loferer *et al.*, 1993), the CycY protein from *B. japonicum* (BjaCycY; Fabianek *et al.*, 1997), and the thioredoxins of *R. sphaeroides* (RspTrxA, Genbank accession number M33806), *Synechocystis* PCC6083 (SynTrxA, Genbank accession number X80486), *E. coli* (EcoTrxA; Genbank accession number M10424), and *M. genitalium* (MgeTrxA, Genbank accession number U39691). The active site of thioredoxin enzymes (WCGPC) is boxed in gray. Amino acid residues conserved in all five enzymes are boxed, and those conserved between *N. gonorrhoeae* and *B. japonicum* are in bold. The β_2 strand and α-helix 2 are also indicated based on Ellis *et al.*, 1992. The transmembrane regions of TlpA from *N. gonorrhoeae* and TlpA and CycY from *B. japonicum* are underlined.


β-2
α-2

The *N. gonorrhoeae* TlpA also shared 48.3% and 53.1% aa sequence similarity and 25.5% and 24.5% aa sequence identity with CycY, a protein that has been identified in *B. japonicum* (Fabianek *et al.*, 1997) and in *Rhizobium leguminosarum* (Vargas *et al.*, 1994) and is related to TlpA. There were five identities and 5 conservative changes between *N. gonorrhoeae* TlpA and either CycY protein among the nineteen aa residues forming β-sheet 2 and α-helix 2.

The expression of *tlpA* was verified by reverse transcriptase PCR (RT-PCR; section 2.9.3.). An amplicon of 415 bp was generated from gonococcal RNA (Fig. A3-2, lane 1), and an amplicon of the same size was generated from gonococcal DNA (Fig. A3-2, lane 2). These results indicated that the *tlpA* gene was transcribed in *N. gonorrhoeae*. The presence of a putative transcription terminator in the form of two inverted copies of the gonococcal US immediately downstream of *tlpA* suggested that it constituted a single transcription unit.

Figure A3-2. Reverse transcriptase PCR of the *tlpA* gene. The expected amplicon for *tlpA* was 415 bp in size. Lane 1, gonococcal RNA, lane 2, gonococcal DNA (positive control), Lanes 3, 100 bp molecular weight marker (Gibco BRL). The *tlpA*-specific amplicon is indicated on the left of the picture, and sizes of the molecular weight markers are indicated to the right.


1
2
3
4
1500
1200
1000
800
600
400
200
tlpA

Appendix 4. Other analyses of the *arsC* gene.

The aa sequence similarity and identity scores between the gonococcal ArsC, which encodes a putative arsenate reductase enzyme and its homologues from other bacteria was variable (Fig. A4-1). Similarity scores varied between 63% and 66% and identity scores varied between 37% and 50% with the enzymes from Gram-negative bacteria. Comparatively, similarity (45% and 47%) and identity (19% and 20%) scores were lower with arsenate reductases of the plasmids pSX267 and pI258 from staphylococcal species. These observations were consistent with the results of other investigators that showed low similarity scores between arsenate reductases from Gram-negative and Gram-positive bacteria (Neyt *et al.*, 1997; Bruhn *et al.*, 1996; Diorio *et al.*, 1995; Ji and Silver, 1992; Rosenstein *et al.*, 1992). Lower similarity (51%) and identity (18%) scores were also observed with ArsC from *Synechocystis* PCC6083. One of the few positions conserved in all nine enzymes on the alignment presented in Fig. A2-1 is the cysteinyl residue at position 12 that has been shown essential for catalysis (Liu *et al.*, 1995)

N. gonorrhoeae CH811 was shown to have sodium arsenate and sodium arsenite minimal inhibitory concentrations (MIC) of 1024 μg/mL and 4 μg/mL, respectively (Table A4-1). The MIC for arsenate was considerably higher than that measured in *Staphylococcus carnosus* (61 μg/mL; Rosenstein *et al.*, 1992) and *Yersinia enterolitica* (94 μg/mL; Neyt *et al.*, 1997) and was in the range of the concentrations that inhibited *E. coli* JM109 (pUC19) (936 μg/mL; Ji and Silver, 1992). The MIC for arsenite was in the same range than what was measured in *Y. enterolitica* and *S. carnosus* (9 μg/mL; Neyt *et al.*, 1997; Rosenstein *et al.*, 1992), but was much lower than what had been measured in *E. coli* (400 μg/mL for *E. coli* 40 and 194 μg/mL for *E. coli* JM109 (pUC19) ; Diorio *et al.*, 1995; Ji and Silver, 1992), *Bacillus subtilis* (260 μg/mL) and *Staphylococcus aureus* (104 μg/mL; Ji and Silver, 1992). This large difference between the MICs for the two arsenical compounds could be a reflection of the higher toxicity of arsenite over arsenate. It also suggested that *N. gonorrhoeae* may not possess the arsenite pump (ArsB) to exclude arsenite ions from the cells since it is much more sensitive to arsenite than to arsenate.

Figure A4-1. Alignment of the amino acid sequences of bacterial arsenate reductase enzymes. Included in this alignment are the enzymes from *N. gonorrhoeae* CH811 (NgoArsC), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (EcoArsC; Diorio *et al.*, 1995), plasmid R773 (R773ArsC; Chen *et al.*, 1986), plasmid R46 (R46ArsC; Bruhn *et al.*, 1996), pSX267 of *S. xylosus* (pSX267ArsC; Rosenstein *et al.*, 1992), plasmid pI258 from *S. aureus* (pI258ArsC; Ji and Silver, 1992), plasmid pYV of Yersiniae species (YspArsC; Neyt *et al.*, 1997), and *Synechocystis* PCC6083 (SynArsC; Kaneko *et al.*, 1996). Amino acid residues conserved in all nine enzymes are boxed, those conserved in eight enzymes are indicated by bold asterisks, those conserved in six or seven enzymes are indicated by an asterisk. Amino acid residues in bold are identical in the enzymes of *N. gonorrhoeae* and *H. influenzae*. The conserved cysteinyl residue essential for enzyme activity (Liu *et al.*, 1995) is also identified.

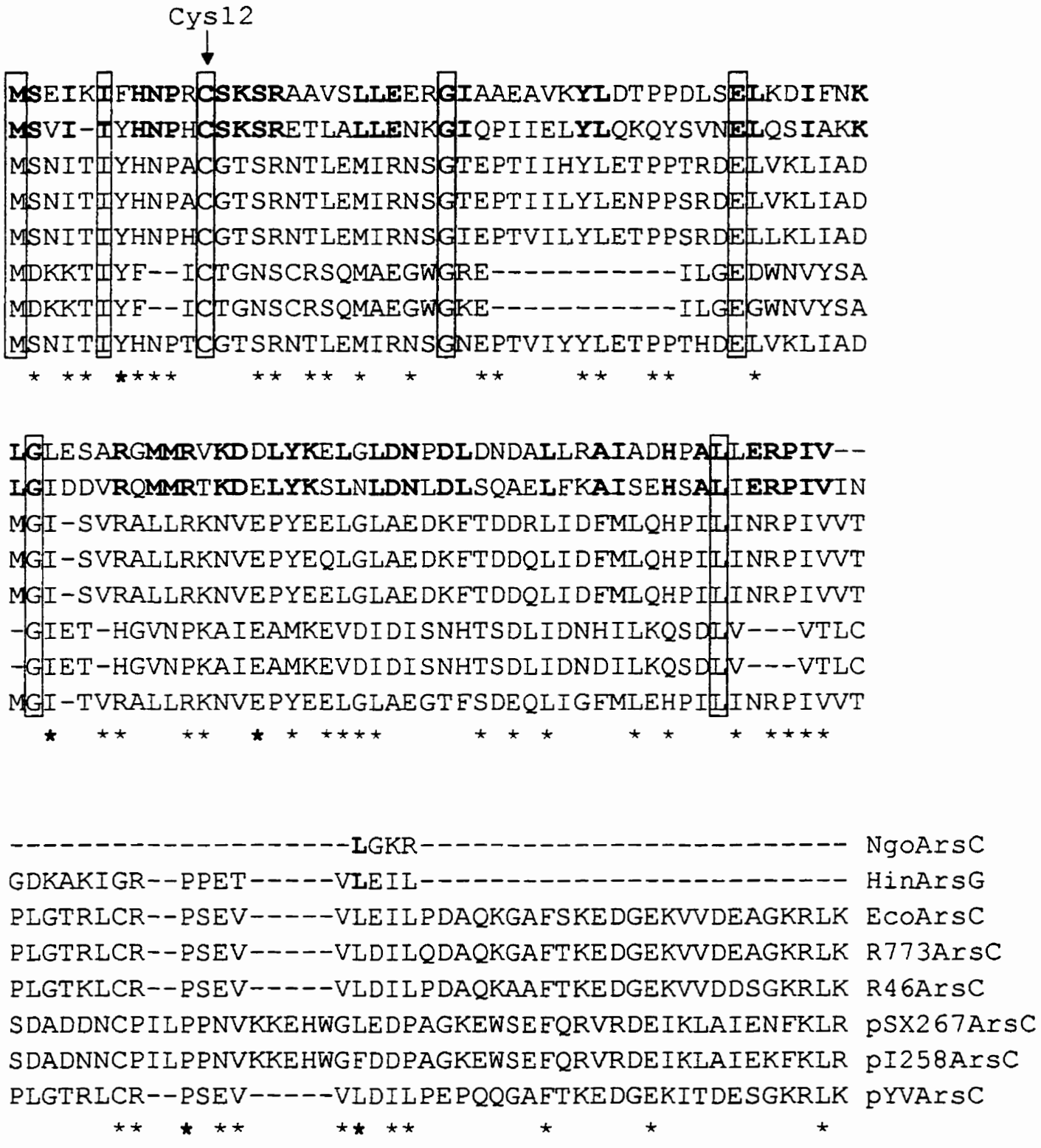

Cys12
MSEIKIFHNPRCSKSRAAVSLLEERGIAAEAVKYLDTPPDLSELKDIFNK
MSVI-TYHNPHCSKSRETLALLENKGIQPIIELYLQKQYSVNELQSIAKK
MSNITIYHNPACGTSRNTLEMIRNSGTEPTIIHYLETPPTRDELVKLIAD
MSNITIYHNPACGTSRNTLEMIRNSGTEPTIILYLENPPSRDELVKLIAD
MSNITIYHNPHCGTSRNTLEMIRNSGIEPTVILYLETPPSRDELLKLIAD
MDKKTIYF--ICTGNSCRSQMAEGWGRE----------ILGEDWNVYSA
MDKKTIYF--ICTGNSCRSQMAEGWGKE----------ILGEGWNVYSA
MSNITIYHNPTCGTSRNTLEMIRNSGNEPTVIYYLETPPTHDELVKLIAD
LGLESARGMMRVKDDLYKELGLDNPDLDNDALLRAIADHPALLERPIV--
LGIDDVRQMMRTKDELYKSLNLDNLDLSQAELFKAISEHSALIERPIVIN
MGI-SVRALLRKNVEPYEELGLAEDKFTDDRLIDFMLQHPILINRPIVVT
MGI-SVRALLRKNVEPYEQLGLAEDKFTDDQLIDFMLQHPILINRPIVVT
MGI-SVRALLRKNVEPYEELGLAEDKFTDDQLIDFMLQHPILINRPIVVT
-GIET-HGVNPKAIEAMKEVDIDISNHTSDLIDNHILKQSDLV---VTLC
-GIET-HGVNPKAIEAMKEVDIDISNHTSDLIDNDILKQSDLV---VTLC
MGI-TVRALLRKNVEPYEELGLAEGTFSDEQLIGFMLEHPILINRPIVVT
----------LGKR---------- NgoArsC
GDKAKIGR--PPET-----VLEIL---------- HinArsG
PLGTRLCR--PSEV-----VLEILPDAQKGAFSKEDGEKVVDEAGKRLK EcoArsC
PLGTRLCR--PSEV-----VLDILQDAQKGAFTKEDGEKVVDEAGKRLK R773ArsC
PLGTKLCR--PSEV-----VLDILPDAQKAAFTKEDGEKVVDDSGKRLK R46ArsC
SDADDNCPILPPNVKKEHWGLEDPAGKEWSEFQRVRDEIKLAIENFKLR pSX267ArsC
SDADNNCPILPPNVKKEHWGFDDPAGKEWSEFQRVRDEIKLAIEKFKLR pI258ArsC
PLGTRLCR--PSEV-----VLDILPEPQQGAFTKEDGEKITDESGKRLK pYVArsC

Table A4-1. Minimal inhibitory concentrations (μg/mL) of arsenic compounds on *N. gonorrhoeae* CH811 and CS19

Antibacterial compound	MIC (μg/mL) of *N. gonorrhoeae* CH811[1]	*N. gonorrhoeae* CS19[2]
sodium arsenate	1024	1024
sodium arsenite	4.0	4.0

[1] Wild-type parent strain

[2] Strain with an insertion in its *ftsX* gene.

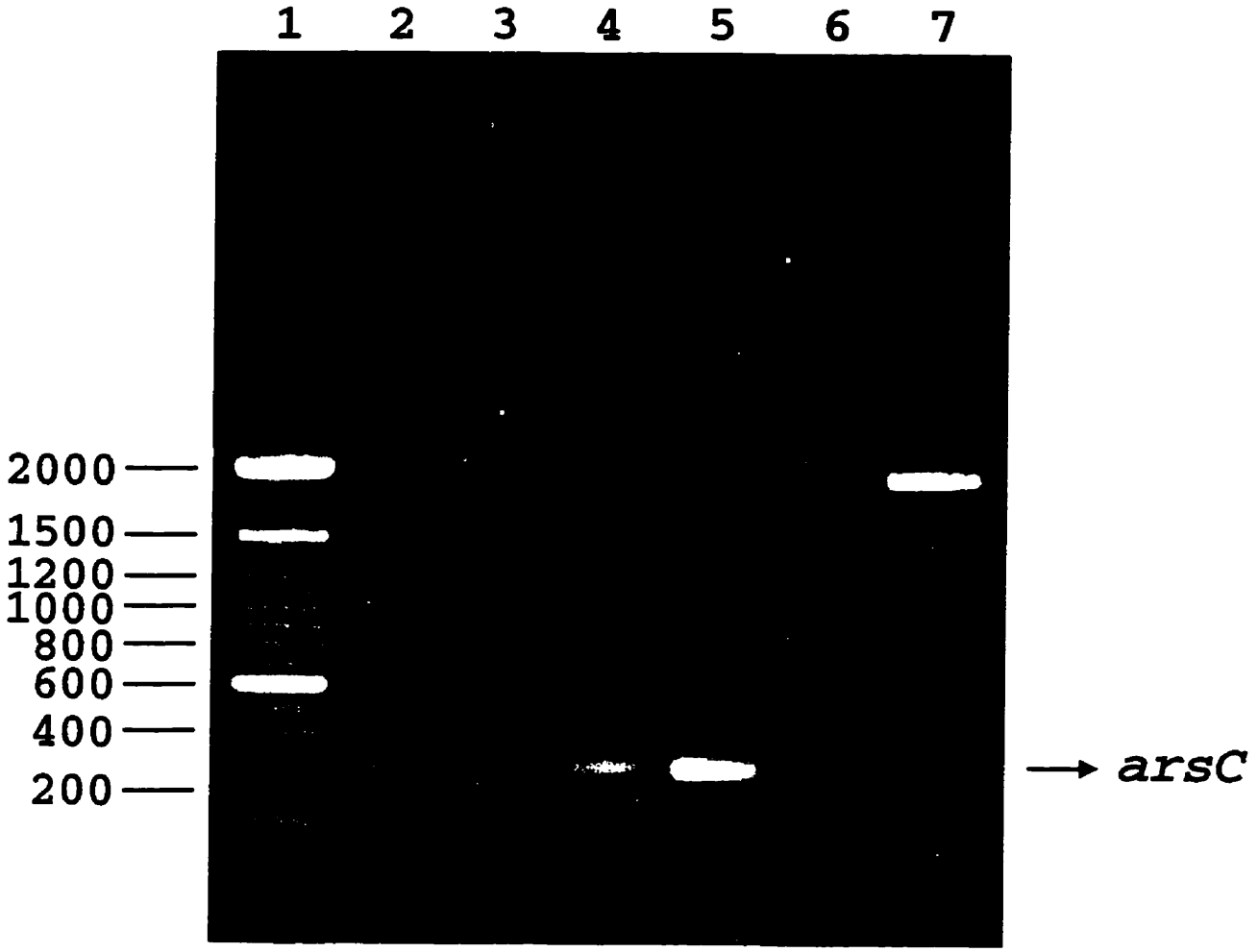
The products of the *ftsEX* genes are predicted to form a membrane complex involved in transport (section 3.3.2.), and the ion or molecule transported by the putative FtsEX transporter has not been identified (section 4.). The *ftsEX* genes were located close to *arsC* on the gonococcal chromosome (sections 3.2. and 3.10.). To verify if *ftsEX* is linked to arsenic extrusion from the *N. gonorrhoeae* cytoplasm, the MICs of the wild-type *N. gonorrhoeae* CH811 and *N. gonorrhoeae* strain CS19, which contained an insertion in its *ftsX* gene, for sodium arsenate and sodium arsenite were determined. The MICs of the strain CS19 are identical to those of its wild-type parent, strain CH811, for both arsenic compounds (Table A4-1.). These results indicated that FtsX, and by extension the *ftsEX* genes, are not involved in the transport of arsenite or arsenate from the cells.

With 102 aa, the putative gonococcal ArsC is the smallest of the known arsenate reductases. In comparison, the *H. influenzae* enzyme includes 116 aa (Fleischmann *et al.*, 1995), those from the plasmids pI258 and pSX267 of *Staphylococcus* species and that of *Synechocystis* PCC6083 comprise 131 aa (Kaneko *et al.*, 1996; Rosenstein *et al.*, 1992; Ji and Silver, 1992), and those from *E. coli*, *E. coli* plasmids R46 and R773, and *Yersinia* all contain 141 aa (Neyt *et al.*, 1997; Bruhn *et al.*, 1996; Diorio *et al.*, 1995; Chen *et al.*, 1986). ArsC could be a truncated enzyme or *arsC* could be an inactive gene since the putative ArsC polypeptide is shorter than its known homologues and there are no clear regulatory elements (RBS, transcription terminator)

flanking *arsC* (section 3.10.2.). To verify if the gonococcal *arsC* gene was transcribed, its expression was investigated by RT-PCR (section 2.9.3.). An amplicon of 275 bp was generated from RNA isolated from gonococci grown in the absence of sodium arsenate or sodium arsenite (Fig. A4-2, lane 2). This result indicated that the gonococcal *arsC* gene was expressed. Expression of the *arsC* gene in the absence of arsenic had been previously observed in *E. coli* (Carlin *et al.*, 1995). When the RT-PCR was repeated using RNA isolated from *N. gonorrhoeae* grown in the presence of sodium arsenate or sodium arsenite, the *arsC* gene was also expressed (Fig. A4-2, lanes 3 and 4). In *E. coli*, arsenite has been shown to be an inducer of *ars* gene expression (Cai and DuBow, 1996; Carlin *et al.*, 1995). In the present case, the level of induction was not measured as the amount of RNA used in each RT-PCR reaction was not quantified.

The presence of the other known *ars* genes in *N. gonorrhoeae* FA1090 was investigated using the known bacterial ArsR, ArsD, ArsA, ArsB and ArsH protein sequences. No other *ars* gene was identified in the strain FA1090, suggesting that *N. gonorrhoeae* contains only an *arsC* gene, as is the case in *H. influenzae* and *Synechocystis* (Kaneko *et al.*, 1996; Fleischmann *et al.*, 1995). When these analyses were made, about ninety percent (88.9%) of the genome sequence was available so the presence of the other *ars* genes in *N. gonorrhoeae* cannot be excluded. However, the possible absence of *arsB*, which encodes the arsenite pump, would explain the low MIC for arsenite in *N. gonorrhoeae*.

Figure A4-2. Reverse transcriptase PCR of the *arsC* gene. The expected size of the *arsC* amplicon was 275 bp. Lanes 1 and 5, 100 bp molecular weight marker, lane 2, *N. gonorrhoeae* RNA, lane 3, *N. gonorrhoeae* RNA (isolated from cells grown on sodium arsenate), lane 4, *N. gonorrhoeae* RNA (isolated from cells grown on sodium arsenite), lane 5, *N. gonorrhoeae* DNA (positive control). Sizes of the molecular weight markers are indicated to the left of the picture, the *arsC*-specific amplicon is indicated on the right.


1
2
3
4
5
6
7
2000
1500
1200
1000
800
600
400
200
arsC

Appendix 5. Other analyses of the *gltX* gene.

The 5'-end of the *gltX* gene encoding the NH_2-terminal 138 aa residues of GluRS was cloned during the characterization of the region upstream of *ftsEX* in *N. gonorrhoeae* (sections 3.2. and 3.10.3.). The catalytic attachment of an amino acid to its cognate tRNA by the aminoacyl-tRNA synthetase (aaRS) takes place in a two-step reaction (Söll and Schimmel, 1974):

$$\text{aaRS} + \text{amino acid} + \text{ATP} \Leftrightarrow \text{aaRS}\bullet\text{aminoacyl-AMP} + \text{PPi} \qquad (1)$$

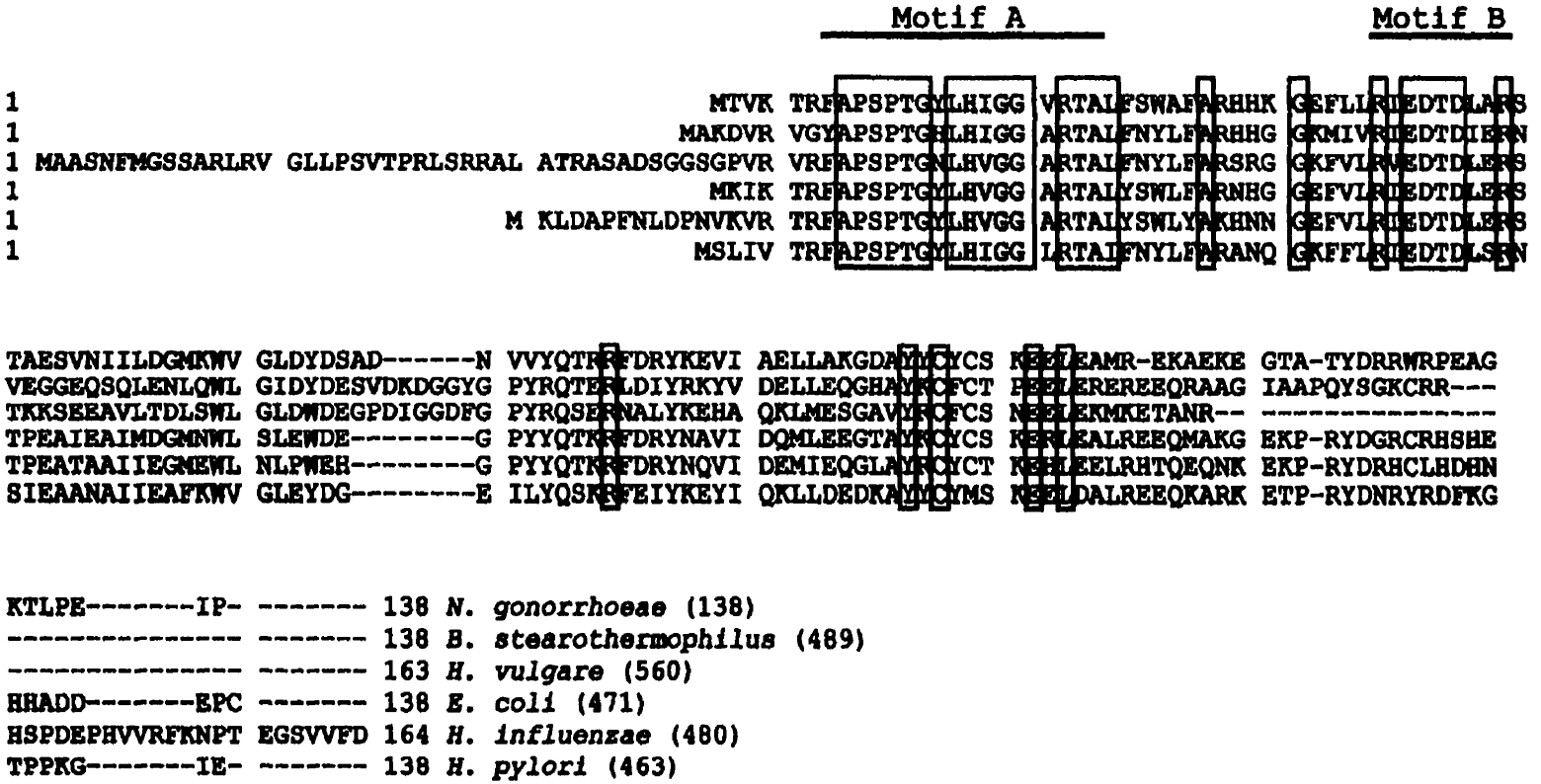
$$\text{aaRS}\bullet\text{aminoacyl-AMP} + \text{tRNA} \Leftrightarrow \text{aminoacyl-tRNA} + \text{AMP} + \text{aaRS} \qquad (2)$$

GluRS belongs to the first class of aaRSs due to the presence of the signature sequence of class I aaRS in its primary sequence (consensus His-Ile-Gly-His; Schimmel, 1987). The signature sequence His-Ile-Gly-Gly was identified at positions 16 to 19 in the amino-terminal section of the gonococcal GltX, (Fig. 6B). The partial gonococcal GltX possesses the a and b motifs identified and conserved in several bacterial aminoacyl-tRNA synthetases that have been proposed to participate in glutamate binding (Fig. A5-1; Breton *et al.*, 1990).

An alignment of the 138 aa residues of the gonococcal GluRS with five other GluRSs is presented in Fig. A5-1. The similarity of *N. gonorrhoeae* GluRS with other GluRSs was more important over the first 75 aa residues (that contains the signature sequence and the conserved motif A). Among these sequences included in Fig. A5-1 for the first 75 aa residues, the gonococcal GluRS shared the least identity (52%) with *Bacillus stearothermophilus* GluRS, the highest identity (69%) was shared with the enzyme from *E. coli*. Similarity scores ranged from 75% (*Hordeum vulgare*, *B. stearothermophilus*) to 86% (*H. pylori*). A second region of similarity covering positions 75 to 120 (containing the conserved motif B) of the gonococcal enzyme; the identity ranged from 41% (*B. stearothermophilus*) to 60% (*E. coli*) and the similarity ranged from 56% (*B. stearothermophilus*) to 82% (*H. pylori*).

Besides the GluRS sequences displayed in Fig. A5-1, the *N. gonorrhoeae* GluRS also shared aa sequence similarities with other GluRS enzymes. Most of the sequences were of

Figure A5-1. Alignment of the amino acid sequences of bacterial and eukaryotic glutamyl-$_T$RNA synthetases. Included in this alignment are the GluRSs from *N. gonorrhoeae*, *B. stearothermophilus*, *H. vulgare*, *E. coli*, *H. influenzae*, and *H. pylori*. The total number of amino acid residues of each GluRs homologue is indicated in parentheses. The motifs A and B are indicated, and the sequence signature of class I aaRSs is boxed in gray.


Motif A
Motif B
1 MTVK TRFAPSPTGYLHIGG VRTALFSWAFARHHK GEFLLRIEDTDLARS
1 MAKDVR VGYAPSPTGHLHIGG ARTALFNYLFARHHG GKMIVRIEDTDIERN
1 MAASNFMGSSARLRV GLLPSVTPRLSRRAL ATRASADSGGSGPVR VRFAPSPTGNLHVGG ARTALFNYLFARSRG GKFVLRIEDTDLERS
1 MKIK TRFAPSPTGYLHVGG ARTALYSWLFARNHG GEFVLRIEDTDLERS
1 M KLDAPFNLDPNVKVR TRFAPSPTGYLHVGG ARTALYSWLYAKHNN GEFVLRIEDTDLERS
1 MSLIV TRFAPSPTGYLHIGG LRTALFNYLFARANQ GKFFLRIEDTDLSRN
TAESVNIILDGMKWV GLDYDSAD------N VVYQTRFDRYKEVI AELLAKGDAYYCYCS KEELEAMR-EKAEKE GTA-TYDRRWRPEAG
VEGGEQSQLENLQWL GIDYDESVDKDGGYG PYRQTERLDIYRKYV DELLEQGHAYRCFCT PEELEREREEQRAAG IAAPQYSGKCRR---
TKKSEEAVLTDLSWL GLDWDEGPDIGGDFG PYRQSERNALYKEHA QKLMESGAVYRCFCS NEELEKMKETANR-- ----------
TPEAIEAIMDGMNWL SLEWDE--------G PYYQTKRFDRYNAVI DQMLEEGTAYRCYCS KEELEALREEQMAKG EKP-RYDGRCRHSHE
TPEATAAIIEGMEWL NLPWEH--------G PYYQTKRFDRYNQVI DEMIEQGLAYRCYCT KEELEELRHTQEQNK EKP-RYDRHCLHDHN
SIEAANAIIEAFKWV GLEYDG--------E ILYQSRRFEIYKEYI QKLLDEDKAYYCYMS KEELDALREEQKARK ETP-RYDNRYRDFKG
KTLPE-------IP- ------- 138 N. gonorrhoeae (138)
--------------- ------- 138 B. stearothermophilus (489)
--------------- ------- 163 H. vulgare (560)
HHADD-------EPC ------- 138 E. coli (471)
HSPDEPHVVRFKNPT EGSVVFD 164 H. influenzae (480)
TPPKG-------IE- ------- 138 H. pylori (463)

bacterial origin, but significant similarity was also noted with the enzyme from barley (*H. vulgare*).

Appendix 6. Other analyses of the *pgk* gene.

The 321 bp from the partial ORF located downstream and in the opposite orientation to that of *ftsX* in pSB19 could be translated into 76 aa residues that shared considerable aa sequence similarity with prokaryotic and eukaryotic 3-phosphoglycerate kinases (section 3.10.4.). Pgk catalyzes the following reaction (Mayes, 1985):

1,3-diphosphoglycerate + ADP ⇔ 3-phosphoglycerate + ATP

With its important role in the catabolism of glucose, and based on its importance in all living organisms, the structure of Pgk was expected to be very conserved during evolution (Mori *et al.*, 1986). The aa sequence of Pgk is highly conserved, as is indicated by the alignment of the partial gonococcal Pgk with the same region of ten other Pgk sequences (Fig. A6-1). Among the partial Pgk sequences presented in Fig. A6-1, the gonococcal Pgk shared the least identity (49%) with the *M. jannaschii* enzyme; in all other cases the identity was at least 56% (*Gallus gallus*), up to 72% (*E. coli*) and 76% (*Alcaligenes eutrophus*). The similarity scores between the gonococccal enzyme and the other sequences displayed in Fig. A6-1 was at least 69% (*G. gallus*), up to 83% (*E. coli*) and 93% (*A. eutrophus*). The extent of sequence conservation of 3-phophoglycerate spans all kingdoms: prokaryotes (Gram-negative bacteria (*A. eutrophus*, *E. coli*, *H. influenzae*) and Gram-positive bacteria (*Bacillus stearothermophilus*)), archaeae (*M. jannaschii*); eukaryotes (yeast (*Saccharomyces cerevisiae*), fungi (*Trichoderma viride*), higher plants (wheat, *Triticum aestivum*), mammals (mouse, *Mus musculus*), and birds (chicken, *G. gallus*). Amino acid sequence similarity was observed with a great number of other Pgk sequences for all kingdoms when the similarity search was conducted (data not shown).

The structure of Pgk from several organisms has been resolved, and the structure is found to be very conserved. Pgk is a symmetrical two-domain protein with each domain mainly consisting in six repeats of a α/β structural motif (Mori *et al.*, 1986). Upon combining the primary sequence information with the three-dimensional structure of Pgk from mouse, human, horse, yeast and two trypanosomes, it was observed that most of the conserved positions were

part of twelve β-sheets and adjacent peptide regions that formed the inner loops surrounding the ATP and 3-phophoglycerate-binding cleft (Mori *et al.*, 1986). The carboxy-terminal domain, more conserved, serves as the ATP-binding domain, and the 3-phosphoglycerate-binding site is located in α-helix 14 (α14; Watson *et al.*, 1982). The conserved β-sheets 11 and 12 (β11 and β 12) and α13 and α14 are part of the substrate-binding cleft in the carboxy-terminal end of the enzyme and they are conserved in the partial *N. gonorrhoeae* Pgk sequence (Fig. A6-1).

Figure A6-1. Alignment of the amino acid sequences of bacterial and eukaryotic phosphoglycerate kinases. Included in this alignment are the enzymes from *N. gonorrhoeae* (this work, U76418), *A. eutrophus* (I39554), *E. coli* (X14436), *H. influenzae* (U32734), *S. cerevisiae* (X59720), *T. viride* (S25381), *M. musculus* (M17299), *G. gallus* (L37101), *T. aestivum* (X15233), *B. stearothermophilus* (X58059), and *M. jannaschii* (U67512). The numbers in parentheses are the Genbank accession number of each sequence displayed. The numbers in parentheses on the figure indicate the total number of amino acids of the Pgk homologue. The beta helices 11 and 12 (β11, β12), and the α-helices (α13, α14) part of the substrate-binding cleft of Pgk are also indicated (Mori *et al.*, 1986).

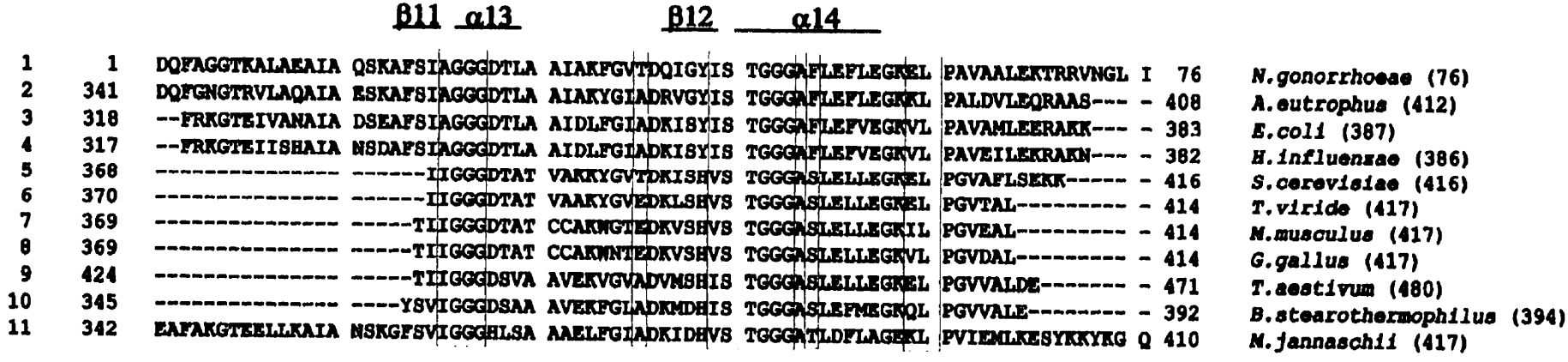
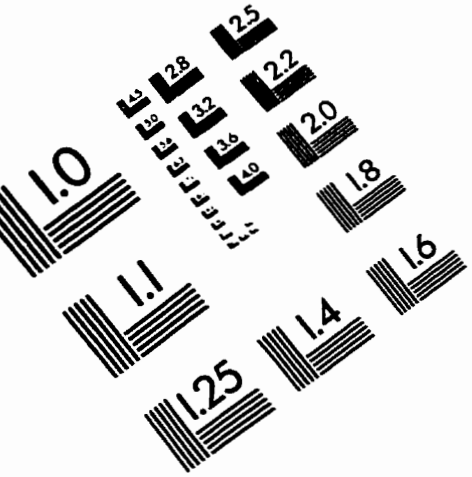
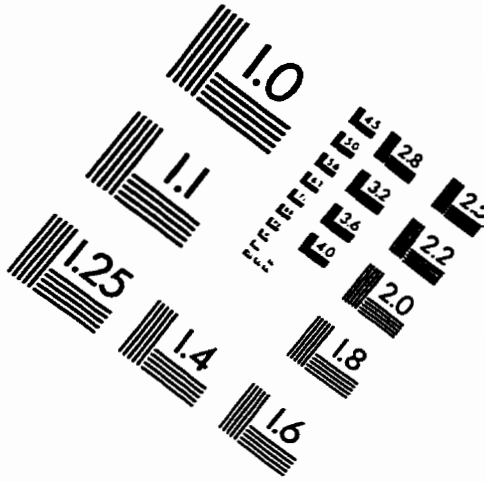
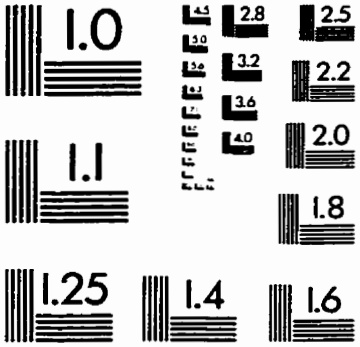
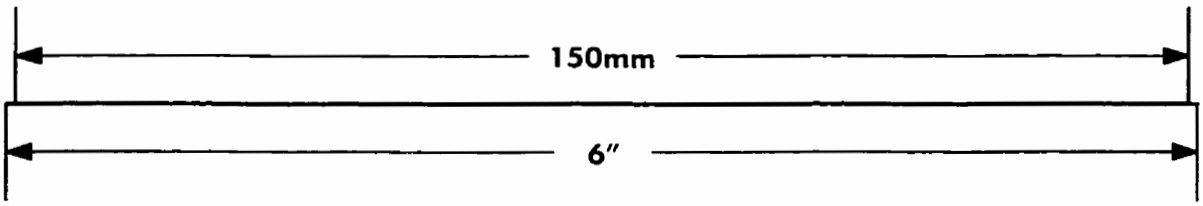

β11 α13
β12 α14
1 1 DQFAGGTKALAEAIA QSKAFSIAGGGDTLA AIAKFGVTDQIGYIS TGGGAFLEFLEGKEL PAVAALEKTRRVNGL I 76 N.gonorrhoeae (76)
2 341 DQFGNGTRVLAQAIA ESKAFSIAGGGDTLA AIAKYGIADRVGYIS TGGGAFLEFLEGKKL PALDVLEQRAAS--- - 408 A.eutrophus (412)
3 318 --FRKGTEIVANAIA DSEAFSIAGGGDTLA AIDLFGIADKISYIS TGGGAFLEFVEGKVL PAVAMLEERAKK--- - 383 E.coli (387)
4 317 --FRKGTEIISHAIA NSDAFSIAGGGDTLA AIDLFGIADKISYIS TGGGAFLEFVEGKVL PAVEILEKRAKN--- - 382 H.influenzae (386)
5 368 ---------- ------IIGGGDTAT VAKKYGVTDKISHVS TGGGASLELLEGKEL PGVAFLSEKK----- - 416 S.cerevisiae (416)
6 370 ---------- ------IIGGGDTAT VAAKYGVEDKLSHVS TGGGASLELLEGKEL PGVTAL--------- - 414 T.viride (417)
7 369 ---------- -----TIIGGGDTAT CCAKWGTEDKVSHVS TGGGASLELLEGKIL PGVEAL--------- - 414 M.musculus (417)
8 369 ---------- -----TIIGGGDTAT CCAKWNTEDKVSHVS TGGGASLELLEGKVL PGVDAL--------- - 414 G.gallus (417)
9 424 ---------- -----TIIGGGDSVA AVEKVGVADVMSHIS TGGGASLELLEGKEL PGVVALDE------- - 471 T.aestivum (480)
10 345 ---------- ----YSVIGGGDSAA AVEKFGLADKMDHIS TGGGASLEFMEGKQL PGVVALE-------- - 392 B.stearothermophilus (394)
11 342 EAFAKGTEELLKAIA NSKGFSVIGGGHLSA AAELFGIADKIDHVS TGGGATLDFLAGEKL PVIEMLKESYKKYKG Q 410 M.jannaschii (417)

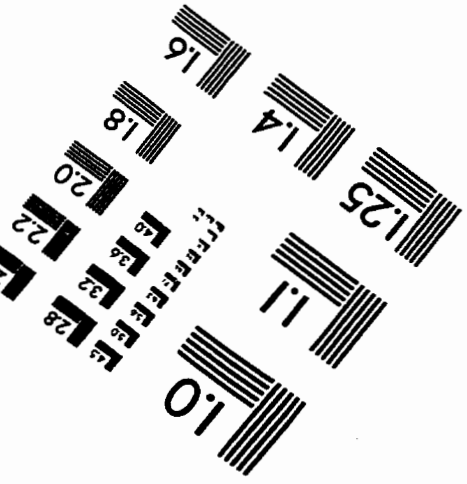
IMAGE EVALUATION
TEST TARGET (QA-3)

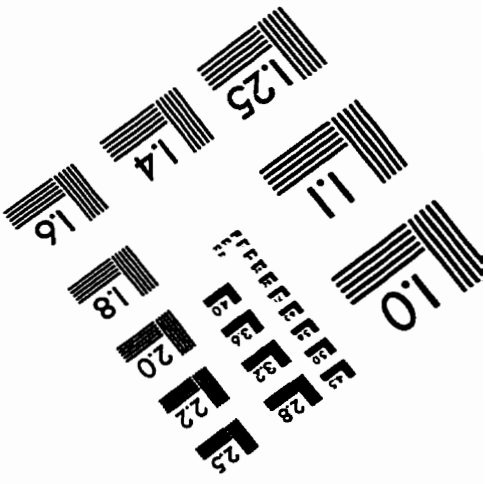












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