The *Neisseria gonorrhoeae* cell division interactome and the roles of FtsA and N-terminus of FtsI in cell division and antimicrobial resistance

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By

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Abstract

Bacterial cell division is an essential biological process which is driven by the formation of a ring-like structure at the division site. Cell division proteins that form the ring-like structure vary in different bacterial species. *Neisseria gonorrhoeae* (Ng) encodes eight essential cell division proteins, i.e. $FtsZ_{Ng}$, $FtsA_{Ng}$, $ZipA_{Ng}$, $FtsK_{Ng}$, $FtsQ_{Ng}$, $FtsL_{Ng}$, $FtsI_{Ng}$, $FtsW_{Ng}$ and $FtsN_{Ng}$. This research investigated the nature of the *N. gonorrhoeae* cell division interactome by ascertaining cell division protein-protein interactions; and characterized the roles of $FtsA_{Ng}$ and the N-terminal domain of $FtsI_{Ng}$ in cell division and antimicrobial resistance in *N. gonorrhoeae*.

Nine interactions among seven gonococcal cell division proteins were observed, using a combination of biological and biophysical methods. $ZipA_{Ng}$ did not interact with any cell division protein tested. Comparison between the gonococcal cell division interactome and two other established interactomes from *Escherichia coli* (Ec) and *Streptococcus pneumoniae* (Sp) revealed two common (FtsZ-FtsA and FtsZ-FtsK) and two unique interactions (FtsA-FtsW and FtsK-FtsN) in *N. gonorrhoeae*. These results show that *N. gonorrhoeae* forms a distinct cell division interactome.

Expression of $ftsA_{Ng}$ in *E. coli* disrupted its cell division. Fluorescence microscopy showed that 37% of $FtsA_{Ng}$ localized to *E. coli* cell poles or the division site, whereas 63% of $FtsA_{Ng}$ was dispersed throughout the cytoplasm. $FtsA_{Ng}$ failed to complement an *E. coli ftsA* mutant strain and only interacted with $FtsN_{Ec}$ as compared to $FtsA_{Ec}$. This interaction was mediated by the 2A and 2B subdomains of $FtsA_{Ng}$. These data indicate that the function of $FtsA_{Ng}$ is species-specific.

Three conserved residues, Arg75, Arg167 and Glu193, were identified at the N-terminal non-catalytic periplasmic region of $FtsI_{Ng}$, which forms a conserved Arg-Arg-Glu linker structure that connects both the C-terminal and N-terminal domains. Mutations of these residues affected the interaction of $FtsI_{Ng}$ with $FtsW_{Ng}$. Alterations of Arg75 and Arg167 also impaired the penicillin binding capacity of $FtsI_{Ng}$, whereas a mutation at Glu193 had no influence. Circular dichroism analysis indicated that the E193G mutant altered the secondary structure and stability of $FtsI_{Ng}$, while both R75G and R167G mutants had no significant impact on conformation. Attempts to introduce an unmarked R167G mutation on chromosomal $ftsI_{Ng}$ was not successful since the insertional mutagenesis led to a heterodiploid genotype. This research shows that the conserved residues at the N-terminal periplasmic region of $FtsI_{Ng}$ are necessary for protein interaction and may influence antimicrobial resistance.

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Yinan

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List of abbreviations

aa	amino acids
AMP	ampicillin
Arg	Arginine
ATP	adenosine 5‟ triphosphate
B2H	Bacterial Two-Hybrid
bp	Base pair
Bs	*Bacillus subtilis*
Cc	*Caulobacter crescentus*
CD	Circular dichroism
CIP	Calf Intestinal
DAPI	4',6-diamidino-2-phenylindole
dcw	division cell wall cluster
dca	division cluster associated
DIC	differential interference contrast
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Ec	*Escherichia coli*
ECT	electron cryo-tomography
FRAP	Fluorescent recovery after photobleaching
FRET	fluorescence resonance energy transfer
Fts	filamentous temperature sensitive

Glu	Glutamic acid
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	Guanosine 5′-triphosphate
HIV	human immunodeficiency virus
HMW	high-molecular-weight
HTH	helix-turn-helix
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
kDa	Kilodalton
LB	Luria broth
LMW	low-molecular-weight
MIC	minimum inhibitory concentration
MTS	membrane-targeting sequence
Ng	*Neisseria gonorrhoeae*
NO	nucleoid occlusion
OD	Optical density
ONPG	o-nitro-phenyl-D-galactopyranoside
ORF	Open reading frame
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PG	peptidoglycan
POTRA	polypeptide-transport-associated
rpm	rotations per minute
SBS	SlmA binding site
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEDS	shape, elongation, division, and sporulation
Sp	*Streptococccus pneumoniae*
SPR	surface plasmon resonance
TEM	transmission electron microscopy
TIRF	total internal reflection fluorescence
Tm (1)	*Thermotoga maritima*
Tm (2)	Melting temperature
WHO	World Health Organization
Y2H	Yeast Two-Hybrid assay

Chapter 1 General introduction

Cell division is an essential process for bacterial growth, development, and reproduction. During cell division, a division plane appears between two segregated chromosomes at the division site to ensure that the mother cell splits into two progeny cells, each with one copy of the chromosome. Assembly of the division machinery, also known as the divisome, is initiated by the formation of a ring-like structure called the Z-ring. The Z-ring structure is composed of polymers of FtsZ, which is a bacterial homolog of tubulin, that is present in most bacterial species (Bi and Lutkenhaus, 1991). Localization of the Z-ring is modulated by regulatory proteins that either positively or negatively regulate FtsZ. The formation of the divisome continues as the Z-ring recruits various cell division proteins to the division site followed by constriction along with the cell wall.

Divisome formation has been extensively investigated in two model organisms (i.e., Gram-negative *Escherichia coli* (Ec) and Gram-positive *Bacillus subtilis* (Bs)) due to the great availability of genetic tools for both species. These studies have focused on the division cell wall (*dcw*) cluster which contains most cell division genes. In contrast, cell division in non-model organisms is poorly understood. Recent studies in cell division of non-model organisms, such as *Chlamydia trachomatis*, clearly indicate that the knowledge of cell division obtained from model organisms may not apply to the non-model bacteria (Abdelrahman *et al.*, 2016).

In the first section of the general introduction, an overview of bacterial cell division and key proteins involved in divisome formation in model organisms will be provided. In the second and third sections, cell division and key proteins involved in divisome formation

will be reviewed in both model organisms, *E. coli* and *B. subtilis*, and non-model organisms, *Chlamydiales* and *Streptococcus pneumoniae,* respectively. The section will be followed by the fourth section which describes the current knowledge of cell division in *Neisseria gonorrhoeae*.

1.1. An overview of bacterial cell division

1.1.1. The division cell wall (dcw) cluster

In *E. coli*, the model organism for cell division in Gram-negative bacteria, genes encoding proteins that are directly involved in cell division are named as filamentous temperature sensitive (*fts*) genes. These genes were initially identified when treating *E. coli* K12 strains with the chemical mutagenic agent *N*-methyl-*N*'-nitrosoguanidine. Mutants were able to divide normally at the permissive temperature, but not at non-permissive temperature. Several mutants were isolated independently (van de Putte *et al.*, 1964). Mutations were mapped to a region, which was later named as the Division and Cell Wall (*dcw*) cluster (Ayala et al., 1994).

The organization of genes in the *dcw* cluster varies in different bacterial species. In *E. coli*, 16 genes were identified: *mraZ*$_{Ec}$, *mraW*$_{Ec}$, *ftsL*$_{Ec}$, *ftsI*$_{Ec}$, *murE*$_{Ec}$, *murF*$_{Ec}$, *mraY*$_{Ec}$, *murD*$_{Ec}$, *ftsW*$_{Ec}$, *murG*$_{Ec}$, *murC*$_{Ec}$, *ddlB*$_{Ec}$, *ftsQ*$_{Ec}$, *ftsA*$_{Ec}$, *ftsZ*$_{Ec}$ and *envA*$_{Ec}$ (Fig. 1.1) (Ayala et al., 1994; Mingorance et al., 2004). At least six internal promoters associated with *ftsZ*$_{Ec}$ are present (Dewar and Dorazi, 2000). *mraZ* is a highly conserved transcriptional regulator in bacteria and *E. coli mraW* is the antagonist of *mraZ*$_{Ec}$ (Eraso *et al.*, 2014). The *mur* genes, together with *mraY*$_{Ec}$ and *ddlB*$_{Ec}$, are a group of genes essential for the synthesis of peptidoglycan precursors (Pilhofer *et al.*, 2008).

Fig. 1.1. Schematic representation of the *dcw* clusters of *N. gonorrhoeae* (Francis *et al*., 2000), *E. coli* (Ayala *et al.*, 1994; Mingorance *et al.*, 2004), *B. subtilis* (Mingorance et al., 2004; Real and Henriques, 2006), and *S. pneumoniae* (Vicente *et al.*, 2004). Transcriptional terminators are indicated as stem loops.

The Gram-positive diplococcus *S. pneumoniae* has a distinctive *dcw* cluster that differs from that of *E. coli* (Massidda *et al.*, 1998). The streptococcal *dcw* cluster is distributed into three separate regions on the chromosome (Fig. 1.1) and composes 17 genes (Massidda et al., 1998). The first region, *dcw1*, contains eight genes including the *pbp2b*$_{Sp}$, *recM*$_{Sp}$, *ddl*$_{Sp}$, *murF*$_{Sp}$, *mutT*$_{Sp}$, *orf1*, *ftsA*$_{Sp}$ and *ftsZ*$_{Sp}$. Five genes, *murG*$_{Sp}$, *divIB*$_{Sp}$, *pyrF*$_{Sp}$, and *pyrE*$_{Sp}$, were identified in the second region of the streptococcal *dcw* cluster. The third region, *dcw3*, is composed of the *yllC*$_{Sp}$, *yllD*$_{Sp}$, *pbp2x*$_{Sp}$, and *mraY*$_{Sp}$ genes. Moreover, four putative genes are located downstream of *ftsZ*$_{Sp}$ in *S. pneumoniae dcw* cluster (Massidda et al., 1998). The protein encoded by the last gene shares 65% of amino acid similarity of *B. subtilis* DivIVA which is involved in Gram-positive bacteria cell division (Edwards and Errington, 1997; Cha and Stewart, 1997).

In *B. subtilis*, the Gram-positive model organism for cell division (Harwood, 2007), 17 genes (*mraZ*$_{Bs}$, *mraW*$_{Bs}$, *ftsL*$_{Bs}$, *ftsI*$_{Bs}$, *spoVD*$_{Bs}$, *murE*$_{Bs}$, *murF*$_{Bs}$, *mraY*$_{Bs}$, *murD*$_{Bs}$, *ftsW*$_{Bs}$, *murG*$_{Bs}$, *murB*$_{Bs}$, *ftsQ*$_{Bs}$, *ylxW*$_{Bs}$, *ylxX*$_{Bs}$, *ftsA*$_{Bs}$ and *ftsZ*$_{Bs}$) were identified in the *dcw* cluster (Fig. 1.1) (Mingorance et al., 2004; Real and Henriques, 2006). The organization of the *B. subtilis dcw* cluster is similar to that in *E. coli*, except for *spoVD*$_{Bs}$. *spoVD*$_{Bs}$ encodes a sporulation-specific protein for endospore cortex PG synthesis, which shares 33% identity with the upstream *ftsI*$_{Bs}$ (Vicente et al., 2004; Daniel *et al.*, 1994). The other difference is that there is an internal transcription terminator between *ftsI*$_{Bs}$ and *spoVD*$_{Bs}$.

1.1.2. Cell division site selection

Spatial regulation of cell division in bacteria is mainly mediated by two regulatory systems: nucleoid occlusion (NO) and the Min systems.

The NO system is based on the observation that the cell division process is inhibited near the bacterial nucleoid (Fig. 1.2A) (Woldringh *et al.*, 1990). The bacterial nucleoid is highly condensed and lacks nuclear membranes that separate the chromosome from the cytosol. In rod-shaped bacteria, the NO system prevents Z-ring formation over the chromosome, so that the assembly of this ring structure will not initiate until the division site (the midcell) is clear of the nucleoid. (Wu and Errington, 2011). Various proteins that regulate the NO system have been identified in different species. For example, Noc is a non-specific DNA binding protein that inhibits the division process in *B. subtilis* (Wu and Errington, 2004). In *E. coli*, SlmA is recognized as a cell division inhibitor that competes with other cell division proteins to bind FtsZ (Bernhardt and de Boer, 2005). In *C. crescentus*, the nucleoid occlusion system involves MipZ, which is a FtsZ inhibitor (Erickson *et al.*, 2010). It dynamically interacts with the DNA binding protein ParB at both poles and leaves the mid-cell area suitable for Z-ring formation (Thanbichler and Shapiro, 2006).

Min is another system that regulates cell division site selection in bacteria. A typical Min system, such as in *E. coli*, has three Min proteins: $MinC_{Ec}$, $MinD_{Ec}$, and $MinE_{Ec}$ (Fig. 1.2B). $MinC_{Ec}$ is an inhibitor which prevents Z ring formation. $MinD_{Ec}$ is an ATPase which interacts with $MinC_{Ec}$ (Koonin, 1993). In Gram-negative bacteria, the ATP binding capacity of $MinD_{Ec}$ triggers oligomerization and localization to the inner membrane (Lutkenhaus, 2007). However, $MinD_{Ec}$ from Gram-positive bacteria may be ATP-independent. Several studies have confirmed that GFP-$MinD_{Ec}$ oscillated from pole-to-pole with $MinE_{Ec}$, the third Min proteins, was present in *E. coli* (Raskin and de Boer, 1999; Rowland *et al.*, 2000). This oscillation pattern generates a higher density of $MinC_{Ec}$ at both

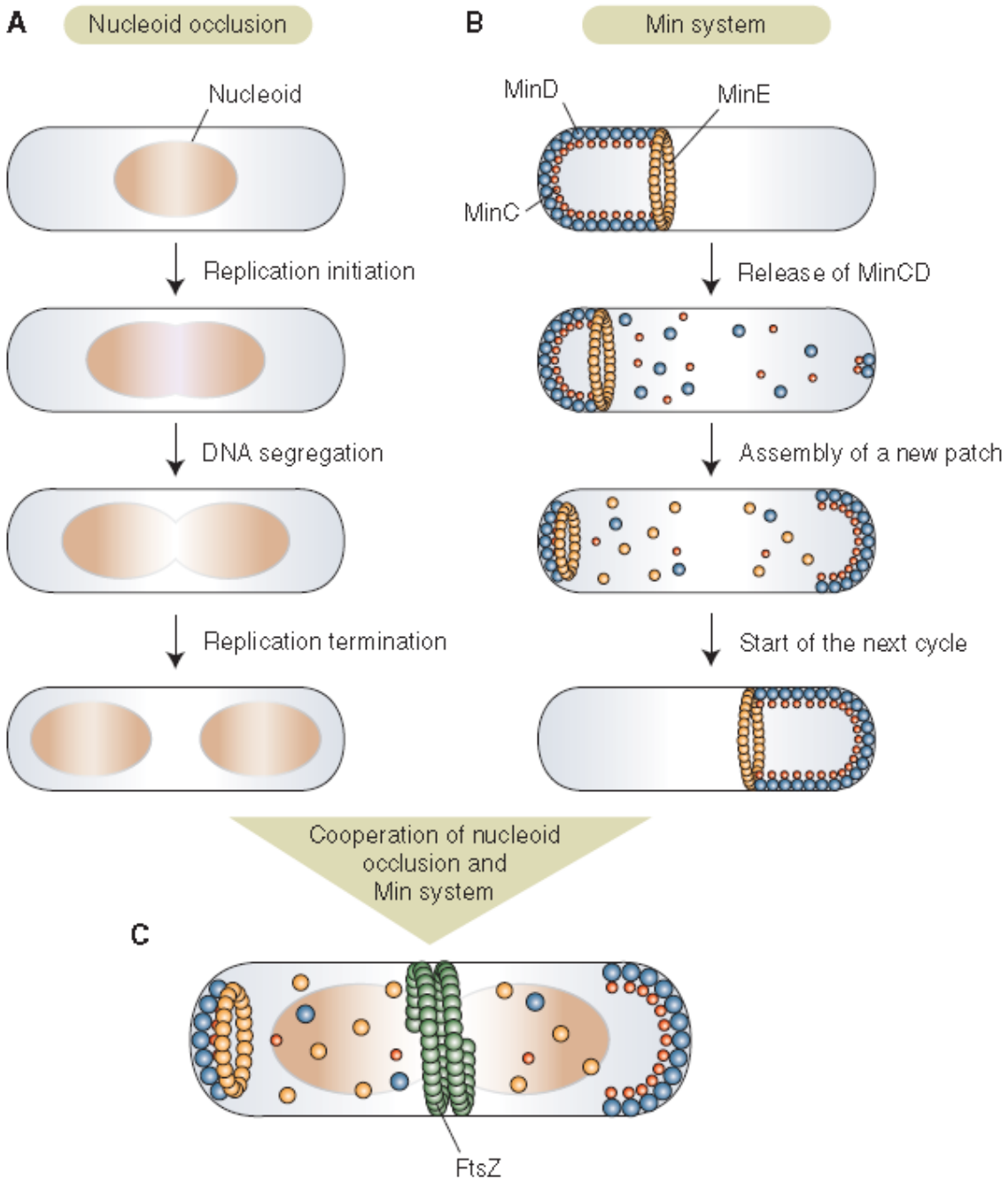

Fig. 1.2. Division site selection mediated by nucleoid occlusion and Min system in *E. coli*. (A) Temporal and spatial regulation of division process by nucleoid occlusion. (B) Negative regulation of FtsZ-ring formation at cell poles by MinCDE system (Thanbichler, 2010).

poles and a lower density at the mid-cell area, allowing the formation of the Z-ring only at the mid-cell. The composition of specific Min proteins varies in different bacterial species. In *B. subtilis*, $MinE_{Bs}$ is absent for division site selection (Pinho *et al.*, 2013). Instead, a unique division protein, DivIVA, is only present in Gram-positive bacteria. In *S. pneumoniae*, all Min proteins are absent, but DivIVA is present. *C. crescentus* does not contain any Min protein nor DivIVA. The differences in the Min system in bacteria indicate that the cell division site selection is a dynamic process.

1.1.3. Divisome formation

The bacterial divisome is defined as a complex of cell division proteins that form a ring-like structure involved in the cell division process. Formation of the divisome during cell division involves a series of proteins that are essential for cell division as well as a number of non-essential proteins (Goehring and Beckwith, 2005; Gamba et al., 2009; Goley *et al.*, 2011). Although the essential proteins involved in divisome formation differ in bacteria, there are some common proteins found in all bacterial species. In *E. coli*, for example, FtsZ, the last *fts* gene in the *E. coli dcw* cluster, is a highly conserved cell division protein in almost all bacterial species, expect for *Chlamydia trachomatis* (Ct) (Stephens *et al.*, 1998). A homolog of FtsZ is also found in eukaryotic cells and encodes tubulin. FtsZ is a GTPase. By hydrolyzing GTP into GDP, $FtsZ_{Ec}$ monomers polymerize to form filaments that interact with another highly conserved cell division protein, FtsA (Loose and Mitchison, 2014). In *E. coli*, the $FtsZ_{Ec}$-$FtsA_{Ec}$ interaction localizes $FtsZ_{Ec}$ filaments to the inner membrane and recruits other essential cell division proteins including $FtsK_{Ec}$, $FtsQ_{Ec}$, $FtsB_{Ec}$, $FtsL_{Ec}$, $FtsI_{Ec}$, $FtsW_{Ec}$, and $FtsN_{Ec}$.

In *E. coli*, the divisome assembly follows a concerted model (Fig. 1.3). Firstly, there is evidence suggesting a two-step assembly of the divisome. Three cell division proteins, $FtsZ_{Ec}$, $FtsA_{Ec}$, and $ZipA_{Ec}$, that formed the proto-ring were defined as early divisome proteins (Aarsman *et al.*, 2005). Recruitment of the remaining cell division proteins, called late divisome proteins including $FtsK_{Ec}$, $FtsQ_{Ec}$, $FtsB_{Ec}$, $FtsL_{Ec}$, $FtsW_{Ec}$, $FtsI_{Ec}$ and $FtsN_{Ec}$, took place after 14 to 21 minutes (Aarsman et al., 2005). Secondly, other studies suggested that upstream cell division proteins can be back-recruited by their downstream proteins under certain conditions. For example, $FtsQ_{Ec}$ could back-recruit $FtsK_{Ec}$ to the division site when $FtsA_{Ec}$ was absent (Goehring *et al.*, 2005). In another study, $FtsQ_{Ec}$, $FtsB_{Ec}$, and $FtsL_{Ec}$ failed to localize to the division site when the upstream protein $FtsK_{Ec}$ was depleted, but their localization could be restored when GFP-$FtsN_{Ec}$ was overexpressed (Goehring *et al.*, 2007). This implies that $FtsN_{Ec}$, as the last essential division protein, can back-recruit its upstream cell division proteins, possibly by its interaction with $FtsA_{Ec}$. Mutations found in $ftsB_{Ec}$ and $ftsL_{Ec}$ could bypass $ZipA_{Ec}$, $FtsK_{Ec}$, $FtsN_{Ec}$ and partially $FtsA_{Ec}$, but the mechanism was still unclear (Tsang and Bernhardt, 2015; Liu *et al.*, 2015; Pichoff *et al.*, 2015). Thirdly, it is clear now that some proteins form complexes before being assembled to the divisome. For example, $FtsQ_{Ec}$, $FtsB_{Ec}$ and $FtsL_{Ec}$ formed a complex that acted as a signal transmitter for cell wall synthesis and constriction before their incorporation into the divisome (Buddelmeijer and Beckwith, 2004; Tsang and Bernhardt, 2015). Fraipont et al (2011) identified another protein complex formed during cell division in *E. coli* by $FtsW_{Ec}$ and $FtsI_{Ec}$. Taken together, these data suggest that cell division proteins assemble to the divisome in a complex and cooperative way.

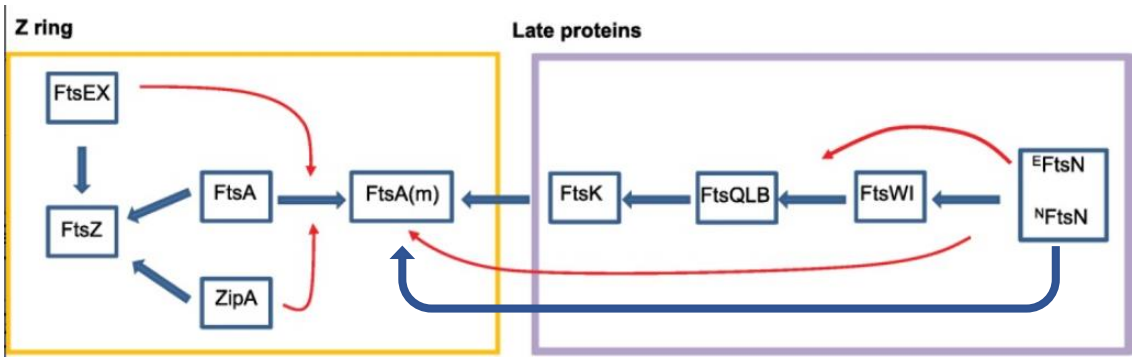

Fig. 1.3. A concerted model of divisome assembly in *E. coli* (Du and Lutkenhaus, 2017). Yellow box indicates early stage of divisome assembly. Blue box indicates late stage of divisome assembly. Blue arrows indicate the recruitment dependency of cell division proteins. Red arrows indicate regulatory interactions. $FtsEX_{Ec}$ and $ZipA_{Ec}$ convert $FtsA_{Ec}$ polymers into monomers. The last cell division protein $FtsN_{Ec}$ activates peptidoglycan synthesis through the interaction with monomer $FtsA_{Ec}$ via ^{N}FtsN and the interaction with FtsQLB complex via ^{E}FtsN. FtsA(m): $FtsA_{Ec}$ monomers; ^{E}FtsN: $FtsN_{Ec}$ essential domain; ^{N}FtsN: $FtsN_{Ec}$ N-terimal domain.

1.2. Cell division in model organisms and essential divisome proteins

1.2.1. Cell division in *Escherichia coli*

Bacterial cell division has been primarily studied in *E. coli* which is a Gram-negative rod-shaped model organism. The division site usually localizes at the mid-cell area that is perpendicular to the long axis of the cell. Assembly of the Z-ring at the division site allows recruiting other cell division proteins to form a complete divisome (Aarsman et al., 2005; Gamba et al., 2009; Goley et al., 2011). The maturation of the divisome activates peptidoglycan synthesis as well as the constriction process which ultimately splits the mother cell into two daughter cells. In total, ten essential cell division proteins were identified for the divisome formation in *E. coli*: $FtsZ_{Ec}$, $FtsA_{Ec}$, $ZipA_{Ec}$, $FtsK_{Ec}$, $FtsQ_{Ec}$, $FtsB_{Ec}$, $FtsL_{Ec}$, $FtsW_{Ec}$, $FtsI_{Ec}$ and $FtsN_{Ec}$ (Errington *et al.*, 2003; Goehring *et al.*, 2006; Grenga *et al.*, 2013).

1.2.1.1. FtsZ

FtsZ is the most abundant and most highly conserved cell division protein in bacteria (Löwe and Amos, 1998; Erickson, 1995). The tertiary structure of FtsZ is highly conserved, which is the homolog of eukaryotic tubulin (Löwe and Amos, 1998; Erickson, 1995). *E. coli* FtsZ contains four domains (Vaughan *et al.*, 2004): a short and poorly conserved N-terminal region (~15 residues), a highly conserved domain (approximately 300 residues) that is homologous with tubulin, a poorly conserved linker (~50 residues) and a short, conserved C-terminal domain (~15 residues). The C-terminus has been well studied and is the binding site for two other proteins: $FtsA_{Ec}$ and $ZipA_{Ec}$ (Ma and Margolin, 1999). Similar to tubulin, $FtsZ_{Ec}$ is also a GTPase (de Boer *et al.*, 1992). GTPase activity

is mediated by the highly conserved N-terminal region, which is also required for self-assembly (Mukherjee *et al.*, 2001; Scheffers *et al.*, 2002). The GTPase activity of $FtsZ_{Ec}$ is also the energy source of $FtsZ_{Ec}$ polymerization (Mukherjee and Lutkenhaus, 1994; Erickson *et al.*, 1996). *In vitro*, the configuration of $FtsZ_{Ec}$ polymers is highly dependent on GTP hydrolysis (Osawa *et al.*, 2008; Lu *et al.*, 2000). Initially, when $FtsZ_{Ec}$ binds to GTP, this interaction induced $FtsZ_{Ec}$ monomers to polymerize into straight, thin protofilaments (Mukherjee and Lutkenhaus, 1994; Erickson et al., 1996; Mukherjee and Lutkenhaus, 1998; Lu et al., 2000). Assembly then triggered GTP hydrolysis, which initiated the disassembly of the protofilaments (Mukherjee and Lutkenhaus, 1998; Mukherjee and Lutkenhaus, 1999). These protofilaments further assembled into higher-order structures promoted by cations such as Mg^{2+}, $Ca^{2+,}$ and DEAE-dextran (Erickson et al., 1996; Lu et al., 2000; Yu and Margolin, 1997). In addition, the cell division protein $ZipA_{Ec}$ could also reorganize $FtsZ_{Ec}$ protofilaments into bundles and prolong polymer presence by reducing $FtsZ_{Ec}$ GTPase activity (Mukherjee and Lutkenhaus, 1999).

Quantitative fluorescence methods show that only 30–40% of total FtsZ in the cell was located in the Z-ring structure in both *B. subtilis* and *E. coli* (Anderson *et al.*, 2004; Geissler *et al.*, 2007). FRAP (Fluorescent Recovery After Photobleaching) studies suggested that the subunits of the Z-ring underwent rapid turnover ($T_{1/2}$ of 8–10 s) (Erickson et al., 2010; Stricker *et al.*, 2002). *In vivo* immunogold labeling showed that $FtsZ_{Ec}$ formed a contractile ring at the midcell, which constricts during the division process (Bi and Lutkenhaus, 1991). Evidence obtained using electron cryo-tomography (ECT) suggested that FtsZ polymers multiply aligned continually along the membrane in *E. coli* and *C. crescentus* (Szwedziak *et al.*, 2014). However, multiple imaging techniques showed

that the Z-ring was a patchy, loosely arranged structure in multiple species (Strauss *et al.*, 2012; Rowlett and Margolin, 2014; Tsui *et al.*, 2014). Due to the controversy *in vivo* evidence, it is crucial to obtain more data using different methods to determine the structure of the Z-ring.

1.2.1.2. FtsA

$FtsA_{Ec}$ is a highly conserved protein for bacterial cell division, which belongs to the actin/MreB family (van den Ent and Lowe, 2000; Sánchez *et al.*, 1994). The crystal structures of $FtsA_{Ec}$ from both *Thermotoga maritima* and *Staphylococcus aureus* showed that they had a similar structure to the bacterial actin homolog, MreB, which has the classic two-domain structure of the actin family. Each domain can be further divided into two subdomains: 1A, 1C, 2A and 2B (Fig. 1.4) (Fujita *et al.*, 2014; van den Ent and Lowe, 2000; Szwedziak *et al.*, 2012). The novel subdomain 1C of $FtsA_{Ec}$ is in the place of the 1B subdomain of MreB as well as actin (Busiek and Margolin, 2015). The 1C domain mediates polymerization and recruitment of the late divisome proteins (Rico *et al.*, 2004; Krupka *et al.*, 2014). The 1A and 2A subdomains form a nucleotide-binding cleft that is required for ATPase activity, while 1C and 2B subdomains are located on each side of the cleft (van den Ent and Lowe, 2000).

$FtsA_{Ec}$ belongs to the actin/MreB family which undergoes ATPase activity for polymerization (Bork *et al.*, 1992; Esue *et al.*, 2006). In *S. pneumoniae* and *Deinococcus radiodurans*, FtsA polymerizes in an ATP-dependent manner, but ATPase activity is not necessary (Modi and Misra, 2014; Krupka *et al.*, 2012; Lara *et al.*, 2005). The truncated C-terminal region of FtsA from *S. pneumoniae* had a more rapid polymerization process than wild-type without any measurable ATPase activity (Lara et al., 2005). Several studies

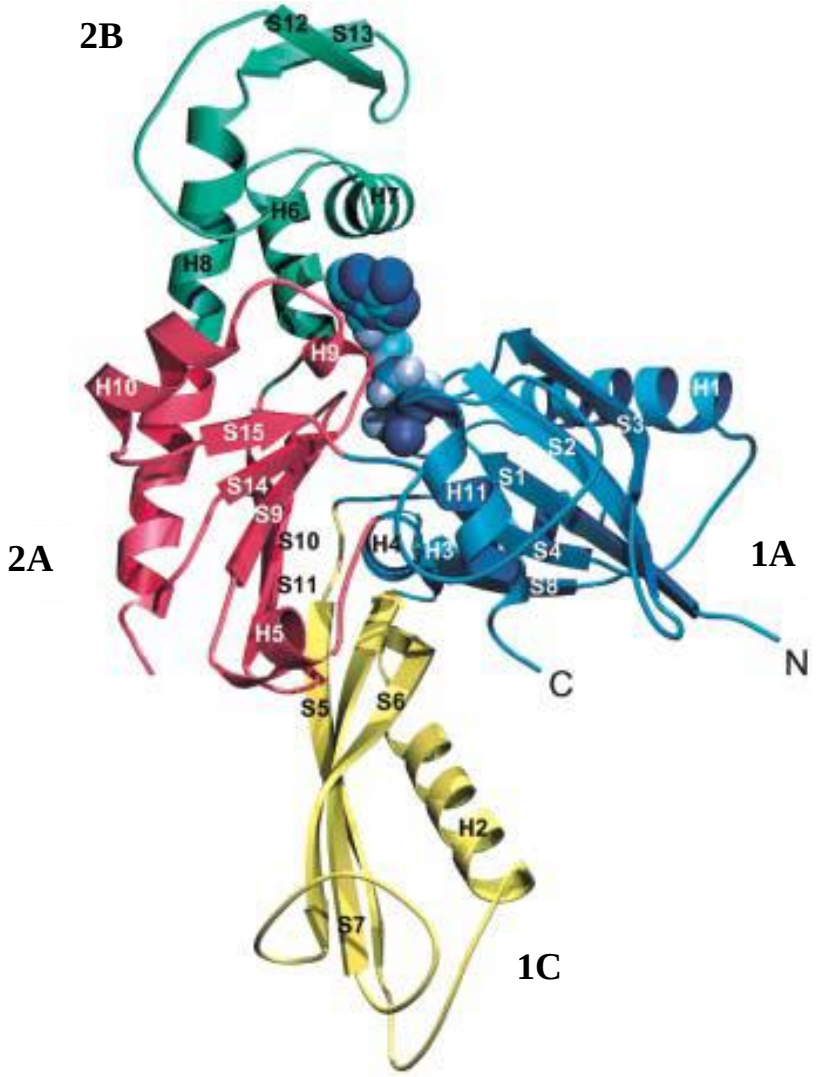

Fig. 1.4. Crystal structure of FtsA from *Thermotoga maritima* (van den Ent and Lowe, 2000). FtsA contains four subdomains: 1A (Blue, right), 2A (red, left), 2B (green, top), and a unique 1C (yellow, bottom).

suggested that FtsA did not need ATPase activity to interact with FtsZ (Pichoff and Lutkenhaus, 2002; Pichoff and Lutkenhaus, 2005; Beuria *et al.*, 2009; Szwedziak et al., 2012). This is in accordance with studies on FtsA ATPase activity in other bacterial species, such as *D. radiodurans* (Modi and Misra, 2014). Thermosensitive mutations in or near the *E. coli* FtsA ATP binding pocket resulted in loss of ATP binding and hydrolysis capacity, which led to impaired cell division at the non-permissive temperature (Herricks *et al.*, 2014). In addition, suppressors of these $FtsA_{Ec}$ mutants were located in the $FtsA_{Ec}$-$FtsA_{Ec}$ subunit interface, suggesting that allosteric effects may influence ATP binding and/or hydrolysis activity (Herricks et al., 2014). In *B. subtilis*, the ATPase activity was readily detected *in vitro* using purified $FtsA_{Bs}$ (Feucht *et al.*, 2001), whereas *S. aureus* FtsA exhibited very weak ATPase activity (Fujita et al., 2014). All these differences in FtsA ATPase activity suggest that this protein may have a species-specific function.

One of the functions of FtsA in cell division is to anchor FtsZ to the inner membrane. In *E. coli*, this interaction is mediated by the 2B subdomain of $FtsA_{Ec}$. Deletion of this region resulted in the disruption of $FtsA_{Ec}$-$FtsZ_{Ec}$ interaction, which further led to a filamentous phenotype (Rico et al., 2004). Together with another early divisome protein, $ZipA_{Ec}$, $FtsA_{Ec}$ associated with $FtsZ_{Ec}$ on the inner membrane via its conserved C-terminal amphipathic helix, which was known as the membrane targeting sequence (Pichoff and Lutkenhaus, 2005). A recent study suggested that a gain-of-function $FtsA_{Ec}$ mutant (R286W) could curve and shorten $FtsZ_{Ec}$ polymers when ATP was bound, indicating a negative regulatory role of $FtsA_{Ec}$ in the Z-ring formation process (Herricks et al., 2014; Beuria et al., 2009). In addition to this mutation, a number of $FtsA_{Ec}$ mutants could completely bypass the need for the membrane. More recently, the $FtsA_{Ec}$-$FtsZ_{Ec}$ interaction

was reconstituted *in vitro* using a supportive lipid bilayer (Fig. 1.5) (Loose and Mitchison, 2014). Using total internal reflection fluorescence (TIRF) microscopy, these authors found that a large-scale rearrangement of FtsZ from its polymerization dynamics was mediated by the $FtsA_{Ec}$ and the presence of ATP (Loose and Mitchison, 2014). They also found dual roles of $FtsA_{Ec}$ regarding its interaction with $FtsZ_{Ec}$ (i.e. tethering $FtsZ_{Ec}$ polymers to the membrane and destabilizing $FtsZ_{Ec}$ polymers).

The *in vivo* FtsZ:FtsA ratio is crucial for proper cell division. In *E. coli*, overexpression of either $FtsZ_{Ec}$ or $FtsA_{Ec}$ led to the disruption in cell division due to a deficiency of their interacting partner (Dai and Lutkenhaus, 1992; Ward and Lutkenhaus, 1985). The impaired cell division could be suppressed by increasing the level of either $FtsA_{Ec}$ or $FtsZ_{Ec}$, suggesting that *E. coli* is susceptible to the ratio of $FtsZ_{Ec}$ to $FtsA_{Ec}$ (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992). The average number of $FtsZ_{Ec}$ molecules in *E. coli* is from 3,200 to 5,000 molecules per cell, depending on the strain types (Rueda *et al.*, 2003; Pla *et al.*, 1991). The average number of FtsA in *E. coli* is 740, giving the ratio of FtsZ: FtsA as 5:1 (Rueda et al., 2003). The ratio can be different in other species. In *S. pneumoniae*, the ratio of FtsZ: FtsA is 1.5:1, while *B. subtilis* has a ratio of 5:1 (Lara et al., 2005; Feucht et al., 2001).

In addition to the requirement of FtsA for FtsZ localization, it is also required for downstream cell division protein recruitments. In *E. coli*, $FtsA_{Ec}$ interacted with a series of late divisome proteins, including $FtsQ_{Ec}$, $FtsI_{Ec}$, and $FtsN_{Ec}$ (Di Lallo et al., 2003; Karimova et al., 2005). Using a polar recruitment assay, $FtsA_{Ec}$ was found to recruit $FtsI_{Ec}$ and $FtsN_{Ec}$ to the cell poles independently of the Z ring (Corbin *et al.*, 2004). Further investigation suggested that these interactions were mediated by the 1C subdomain of $FtsA_{Ec}$

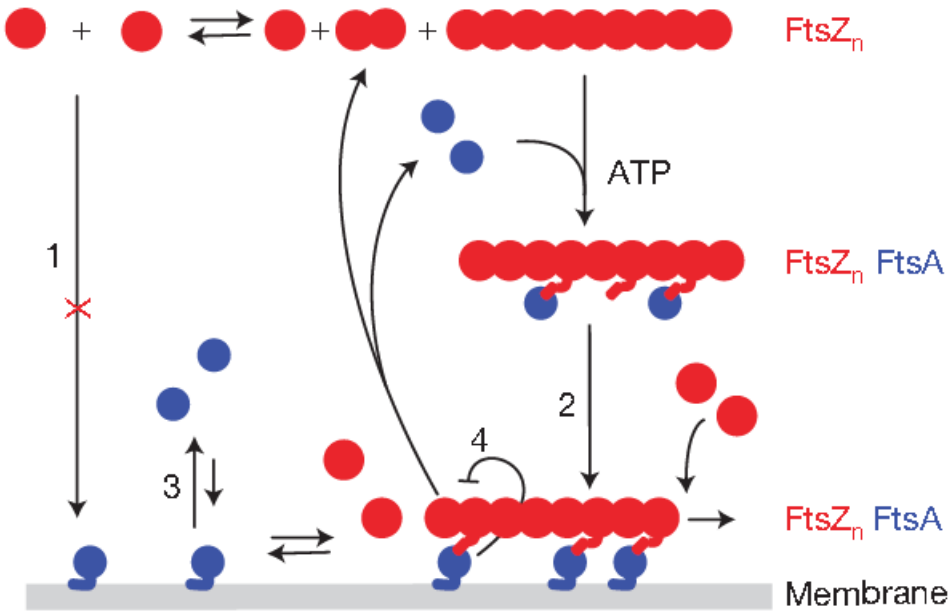

Fig. 1.5. A *in vitro* model of dynamic FtsZ-FtsA interaction (Loose and Mitchison, 2014). FtsZ is not able to interact with FtsA until it polymerizes (1). FtsA interacts with FtsZ polymers (2) and anchors FtsZ filaments to an artificial membrane support (3). At the same time, the FtsA-FtsZ interaction destabilize the FtsZ filaments, converting long polymers to short filaments or monomers (4). Red dots: FtsZ monomers; Blue dots: FtsA monomers

(Busiek *et al.*, 2012). When a truncated $FtsA_{Ec}$ lacking its 1C subdomain was present, $FtsQ_{Ec}$ failed to localize at the division ring, indicating that the recruitment of $FtsQ_{Ec}$ was dependent on $FtsA_{Ec}$ (Rico et al., 2004). The 1C subdomain is a unique region in $FtsA_{Ec}$ that does not have a clear homology with any protein from the actin/MreB family, and which occupies the position corresponding to the 1B subdomain of eukaryotic actin (van den Ent and Lowe, 2000; Szwedziak et al., 2012). These data suggested that the 1C subdomain of $FtsA_{Ng}$ may play a novel role in bacterial cell division. Recently, the $FtsA_{Ec}$-$FtsN_{Ec}$ interaction was proposed as a crucial interaction for cell division (Liu et al., 2015; Pichoff et al., 2015; Busiek and Margolin, 2014). The details will be discussed in the FtsN section.

1.2.1.3. ZipA

ZipA is a less conserved protein compared to FtsZ and FtsA and is only present in Gammaproteobacteria (Haeusser and Margolin, 2016). *E. coli* ZipA is a bitopic protein composed of a transmembrane anchor, a long proline-glutamine linker region and a large, globular C-terminal domain (Hale and de Boer, 1997; Mosyak *et al.*, 2000). The N-terminal anchor also tethered $FtsZ_{Ec}$ to the inner membrane, as does the C-terminal region of $FtsA_{Ec}$ (Hale and de Boer, 1997). In contrast to $FtsA_{Ec}$, $ZipA_{Ec}$ could recruit both monomeric and polymeric $FtsZ_{Ec}$ to the membrane *in vitro* (Haeusser and Margolin, 2016; Loose and Mitchison, 2014). However, a recent study suggested that the $FtsZ_{Ec}$ monomers recruited by $ZipA_{Ec}$ *in vitro* were actually polymers which were polymerized at high concentrations of magnesium, even without the presence of GTP (Du *et al.*, 2015). Although $ZipA_{Ec}$ was necessary for the recruitment of several proteins, including $FtsK_{Ec}$, $FtsQ_{Ec}$, $FtsL_{Ec}$, and $FtsN_{Ec}$ (Pichoff and Lutkenhaus, 2002; Hale and de Boer, 2002), a number of studies

suggested that mutations in $FtsZ_{Ec}$ and $FtsA_{Ec}$ could bypass the need for $ZipA_{Ec}$ (Pichoff *et al.*, 2012; Haeusser *et al.*, 2015; Geissler *et al.*, 2003). In addition, overexpression of the last divisome protein, $FtsN_{Ec}$, also bypassed the requirement for $ZipA_{Ec}$ (Pichoff et al., 2015). All of these evidences suggested that $ZipA_{Ec}$ is not as crucial as other cell division proteins, such as $FtsA_{Ec}$, in divisome formation.

1.2.1.4. FtsK

FtsK is a multifunctional protein that is widely distributed in bacterial species. It has three functional domains: an N-terminal 200 amino acid domain containing four trans-membrane helices, a proline-glutamine rich linker region of various lengths among different species and a 500 amino acid C-terminal region (Aussel *et al.*, 2002; Massey *et al.*, 2006; Sherratt *et al.*, 2001; Yu *et al.*, 1998; Steiner *et al.*, 1999; Wang and Lutkenhaus, 1998). In *E. coli*, the N-terminal region of $FtsK_{Ec}$ was critical for proper cell division by localizing FtsZ to the division apparatus (Grainge, 2010; Draper *et al.*, 1998). Using a bacterial two-hybrid (B2H) assay, the N-terminal domain of $FtsK_{Ec}$ was shown to be involved in interactions with $FtsQ_{Ec}$ and $FtsL_{Ec}$ (Grenga *et al.*, 2008). The proline-glutamine rich linker region is a spacer that separates the N- and C-terminal domains. Later evidence suggested that it was also necessary for proper cell division and chromosome segregation (Dubarry *et al.*, 2010). Surprisingly, this region was also required for the interaction of $FtsK_{Ec}$ with $FtsI_{Ec}$ and $FtsZ_{Ec}$, as determined by B2H assays (Grenga et al., 2008). The C-terminal region of $FtsK_{Ec}$ is well known as a DNA translocase, which is a member of the RecA-fold ATPase family (Aussel et al., 2002; Sherratt et al., 2001; Massey et al., 2006). This region recognizes short DNA motifs that are oriented such that FtsK translocate toward a specific chromosome site called *dif*, which is in the replication

terminus (Grainge, 2010). Once $FtsK_{Ec}$ reached the *dif* site, it interacted with the XerCD recombinase and activated XerCD-*dif* site-specific recombination (Grainge, 2010). This recombination process converted a dimeric chromosome into monomers. In addition, the C-terminal region of $FtsK_{Ec}$ was found to interact with $FtsI_{Ec}$ in *E. coli* (Grenga et al., 2008).

1.2.1.5. FtsQ, FtsB, and FtsL

$FtsQ_{Ec}$ is a bitopic protein that contains a conserved polypeptide-transport-associated domain (POTRA) domain, belonging to a class of β-barrel outer-membrane proteins, which was proposed to be related to the polypeptide translocation process (Sánchez-Pulido *et al.*, 2003; Molloy, 2007). Using B2H and co-immunoprecipitation assays, the POTRA domain of $FtsQ_{Ec}$ was found to be involved in interactions with $FtsI_{Ec}$, $FtsW_{Ec}$, $FtsN_{Ec}$, and self-interacted (D'Ulisse *et al.*, 2007). Residues between 136 and 202 of $FtsQ_{Ec}$ were involved in its interaction with $FtsB_{Ec}$, while the extreme C-terminal region of $FtsQ_{Ec}$ was essential for its interactions with $FtsL_{Ec}$, $FtsK_{Ec}$, $FtsI_{Ec}$, $FtsN_{Ec}$ (D'Ulisse et al., 2007).

E. coli $FtsL_{Ec}$ and $FtsB_{Ec}$ are both bitopic proteins which have nearly identical domain organization, suggesting that they may originate from a common ancestor. Both $FtsL_{Ec}$ and $FtsB_{Ec}$ contain a short N-terminal tail, a trans-membrane domain and a juxtamembrane coiled coil, and a C-terminal periplasmic region (Condon *et al.*, 2018). *In vitro*, the trans-membrane region of both proteins formed a stable higher-order oligomer with equal amounts of $FtsL_{Ec}$ and $FtsB_{Ec}$, suggesting that this region mediated protein complex formation between these two proteins (Khadria and Senes, 2013). Also, $FtsQ_{Ec}$, $FtsB_{Ec}$, and $FtsL_{Ec}$ proteins interacted with each other and formed a protein complex (Di Lallo et al., 2003; Buddelmeijer and Beckwith, 2004). Interestingly, the FtsLB subcomplex

could assemble in the absence of $FtsQ_{Ec}$ *in vivo* (Goehring et al., 2006). Upon subcomplex formation, the FtsLB complex interacted with the C-terminal region of $FtsQ_{Ec}$ via their C-terminal tails, which allowed $FtsQ_{Ec}$ to recruit FtsLB to the division site (Buddelmeijer and Beckwith, 2004; Gonzalez and Beckwith, 2009; Gonzalez *et al.*, 2010).

Since the N-terminal cytoplasmic tails of both *E. coli* FtsL and FtsB were necessary for the recruitment of $FtsW_{Ec}$ and $FtsI_{Ec}$, the role of the FtsQBL complex was considered as a linker that connects the upstream and downstream division proteins and possibly acting as a scaffold for proper divisome assembly (Gonzalez and Beckwith, 2009). Recent evidence suggested that the FtsQLB complex played a critical role in triggering cell constriction (Tsang and Bernhardt, 2015; Liu et al., 2015).

1.2.1.6. FtsW and FtsI

$FtsW_{Ec}$ is a polytopic membrane protein with ten predicted transmembrane domains that belong to the SEDS (Shape, Elongation, Division, and Sporulation) family (Lara and Ayala, 2002). In addition to $FtsW_{Ec}$, and RodA from *E. coli* and SpoVE from *B. subtilis* are also members of this family and are required for peptidoglycan synthesis (Henriques *et al.*, 1992; Henriques *et al.*, 1998). The function of $FtsW_{Ec}$ in *E. coli* cell division was to stabilize the Z-ring and mediate peptidoglycan synthesis with another protein called $FtsI_{Ec}$ (penicillin-binding protein 3). $FtsW_{Ec}$ acted as a flippase that translocated the final cell wall precursor lipid II across the inner membrane (Mohammadi *et al.*, 2011; Mohammadi *et al.*, 2014). Previously, using B2H assays, $FtsW_{Ec}$ was found to interact with $FtsI_{Ec}$. The direct interaction between these two proteins was confirmed using Fluorescence Resonance Energy Transfer (FRET) and co-immunoprecipitation assays (Fraipont et al., 2011). In addition, Fraipont et al (2011) reported that these two proteins could form a protein

complex independent of any other cell division protein. This interaction was mediated by a periplasmic loop between transmembrane (TM) section 9 and TM10 of $FtsW_{Ec}$ and the K2-V42 peptide of $FtsI_{Ec}$ (Fraipont et al., 2011). The periplasmic loop between TM7 and TM8 was identified as the region for cell wall synthesis, whereas the cytoplasmic tail of $FtsW_{Ec}$ was necessary for its interaction with $FtsQ_{Ec}$ (Mohammadi et al., 2014).

FtsI, also known as penicillin-binding protein 3 in *E. coli*, is a transpeptidase for septal peptidoglycan cross-linking (Adam *et al.*, 1997; Botta and Park, 1981). It contains three domains: an N-terminal cytoplasmic domain, a transmembrane region and a long periplasmic domain (Sauvage *et al.*, 2014). Localization studies showed that the transmembrane region of $FtsI_{Ec}$ and some residues near this region were required for its localization to the division site (Wissel *et al.*, 2005; Wissel and Weiss, 2003). The noncatalytic domain in the periplasmic region was necessary for the recruitment of $FtsN_{Ec}$ because mutations in this region led to the failure of $FtsN_{Ec}$ recruitment (Wissel and Weiss, 2003). The periplasmic region of $FtsI_{Ec}$ was also essential for its interaction with other cell division proteins including $FtsA_{Ec}$, $FtsQ_{Ec}$, $FtsL_{Ec}$, and $FtsW_{Ec}$ (Corbin et al., 2004; Wissel and Weiss, 2003). Inactivation of $FtsI_{Ec}$ led to unconstricted Z-ring, suggesting that it was crucial for cell constriction (Pogliano *et al.*, 1997).

1.2.1.7. FtsN

E. coli FtsN is the last essential cell division protein that is recruited to the division site (Goehring et al., 2005). It has a short N-terminal cytoplasmic domain, a single transmembrane region and a periplasmic SPOR domain at the C-terminus (Addinall *et al.*, 1997; Busiek and Margolin, 2014). Several studies suggested that the overexpression of $FtsN_{Ec}$ could bypass mutations in several cell division proteins (Dai *et al.*, 1993; Draper et

al., 1998; Goehring et al., 2007; Geissler and Margolin, 2005; Reddy, 2007). This is supported by evidence that $FtsN_{Ec}$ was recruited to the divisome following a self-enhanced process (Fig. 1.6)(Gerding *et al.*, 2009). In this model, very few $FtsN_{Ec}$ molecules were initially recruited to the division site via their interactions with $FtsA_{Ec}$ (Busiek and Margolin, 2014). These $FtsN_{Ec}$ triggered peptidoglycan synthesis and remodeling via a small essential domain in the periplasm. This process further recruited more $FtsN_{Ec}$ to the division site via its SPOR domain. At a certain point, the accumulation of $FtsN_{Ec}$ passed a signal for cell constriction. The essential region of $FtsN_{Ec}$ interacted with the FtsQBL complex, which led to a conformational change of the protein complex (Tsang and Bernhardt, 2015). The signal was then passed to the FtsIW complex to stimulate cell wall remodeling and cell constriction (Tsang and Bernhardt, 2015). Evidence from a study using an $FtsL_{Ec}$ mutant showed that this mutant could bypass the function of the essential region of $FtsN_{Ec}$, suggesting that $FtsN_{Ec}$ played a role in transmitting signals to the FtsQBL complex, which further promotes cell wall synthesis and cell constriction (Liu et al., 2015).

1.2.1.8. FtsE and FtsX

E. coli also encodes several non-essential cell division proteins, such as $FtsE_{Ec}$ and $FtsX_{Ec}$ (Crickmore and Salmond, 1986). $FtsE_{Ec}$ is the ATP-binding cassette (ABC) component, while $FtsX_{Ec}$ is the membrane component (Schmidt *et al.*, 2004). $FtsE_{Ec}$ and $FtsX_{Ec}$ were shown to interact with each other and form a protein complex that acted as an ABC transporter in *E. coli* (Schmidt et al., 2004). Depletion of FtsE and/or FtsX in *E. coli* resulted in a mild filamentous phenotype, and an FtsEX null mutant was viable in nutrient-rich media (de Leeuw *et al.*, 1999; Schmidt et al., 2004). Interestingly, the division defects caused by the FtsEX null mutation were suppressed by the introduction of osmotic

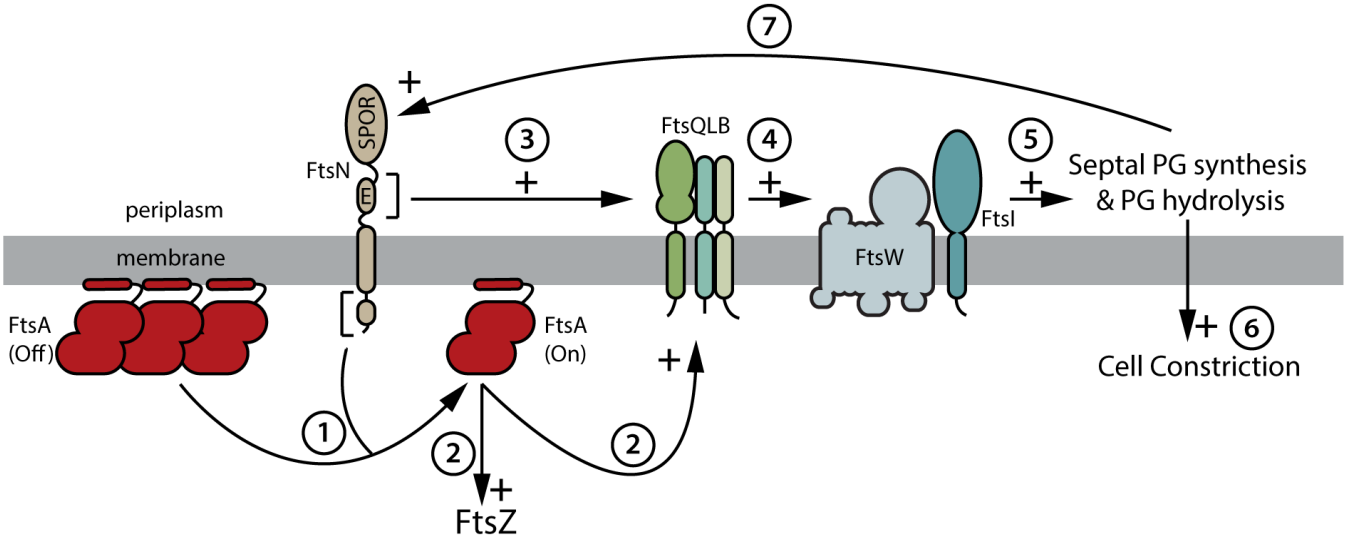

Fig. 1.6. A potential signaling model for controlling peptidoglycan synthesis and cell constriction (Tsang and Bernhardt, 2015). Step 1: *E. coli* FtsN competes with FtsA polymers to release monomeric FtsA; Step 2: FtsA may promote alteration of the Z-ring structure and directly or indirectly signal the FtsQLB complex. Step 3: The essential (E) domain of FtsN may also interact with the FtsQLB complex. Step 4, 5, and 6: The interaction of the FtsQLB complex with FtsN may cause a conformation changes and signal the downstream proteins, including FtsIW complex to initiate septal peptidoglycan synthesis and cell constriction. Step 7: The newly synthesized cell wall recruits more FtsN to the division site by a positive feedback involving the SPOR domain of FtsN.

protectants, such as a high salt condition (Reddy, 2007). Taken together, these results suggested the *E. coli* FtsE and FtsX were not essential for cell division in nutrient-rich media but were conditionally essential in a low osmolarity environment.

1.2.1.9. The Z-ring regulators

Despite the essential cell division proteins, the divisome assembly also involves several proteins that regulates the Z-ring.

ZapA is a Z-ring regulator which is short for Z-ring associated protein A. In *E. coli*, ZapA is recruited to the mid-cell area at the early stage of cell division, probably by the interaction with FtsZ (Gueiros-Filho and Losick, 2002). The overexpression of ZapA in *B. subtilis* can suppress the lethal effect induced by overexpression of MinD (Gueiros-Filho and Losick, 2002). Deletion of ZapA results in no phenotypic changes in wildtype *E. coli*, but it can be lethal when the level of FtsZ is artificially reduced (Gueiros-Filho and Losick, 2002; Johnson et al., 2004). The role of ZapA in cell division is to enhance initial polymerization of FtsZ and stabilize the FtsZ polymers by inhibiting the GTPase activity of FtsZ (Low *et al.*, 2004; Small *et al.*, 2007). One model suggests that the inhibitory effect on FtsZ GTPase activity is because the binding site for ZapA to close the active site for GTP, which further affects the GTP hydrolysis (Small et al., 2007; Marrington *et al.*, 2004). A second model proposed that ZapA may promote FtsZ bundle formation by cross-linking protofilaments (Gueiros-Filho and Losick, 2002; Low et al., 2004). This ability has been found in orthologues of ZapA from several species (Gueiros-Filho and Losick, 2002; Low et al., 2004; Small et al., 2007).

ZapB is another Z-ring regulator which is also recruited to the division site at the early stage of cell division. Deletion of ZapB in *E. coli* results in slight elongation and reduction in frequency of the Z-ring formation (Adams and Errington, 2009). Recently, a study shows that ZapA and ZapB form a FtsZ-independent structure that may be associated with chromosome replication and segregation (Buss *et al.*, 2017). Given the fact that both ZapA and ZapB are positive Z-ring regulators, it is proposed that ZapA and ZapB may also involves in proper Z-ring placement (Buss et al., 2017).

1.2.1.10. *E. coli* cell division interactome

Using B2H assays, a cell division interactome, a protein-protein interaction network, was ascertained among nine *E. coli* essential cell division proteins (Fig. 1.7), including $FtsZ_{Ec}$, $FtsA_{Ec}$, $ZipA_{Ec}$, $FtsK_{Ec}$, $FtsQ_{Ec}$, $FtsB_{Ec}$, $FtsL_{Ec}$, $FtsI_{Ec}$, $FtsW_{Ec}$, and $FtsN_{Ec}$ (Di Lallo et al., 2003; Karimova et al., 2005). A total 20 interactions were identified among *E. coli* cell division proteins as follows: $FtsZ_{Ec}$ with $FtsA_{Ec}$, $ZipA_{Ec}$, $FtsK_{Ec}$; $FtsA_{Ec}$ with $FtsI_{Ec}$, $FtsN_{Ec}$, $FtsQ_{Ec}$; $FtsK_{Ec}$ with $FtsI_{Ec}$, $FtsQ_{Ec}$; $FtsQ_{Ec}$ with $FtsB_{Ec}$, $FtsL_{Ec}$, $FtsI_{Ec}$, $FtsN_{Ec}$, $FtsW_{Ec}$; $FtsB_{Ec}$ with $FtsL_{Ec}$, $FtsI_{Ec}$; $FtsL_{Ec}$ with $FtsI_{Ec}$, $FtsW_{Ec}$; $FtsI_{Ec}$ with $FtsW_{Ec}$, $FtsN_{Ec}$; and $FtsW_{Ec}$ with $FtsN_{Ec}$.

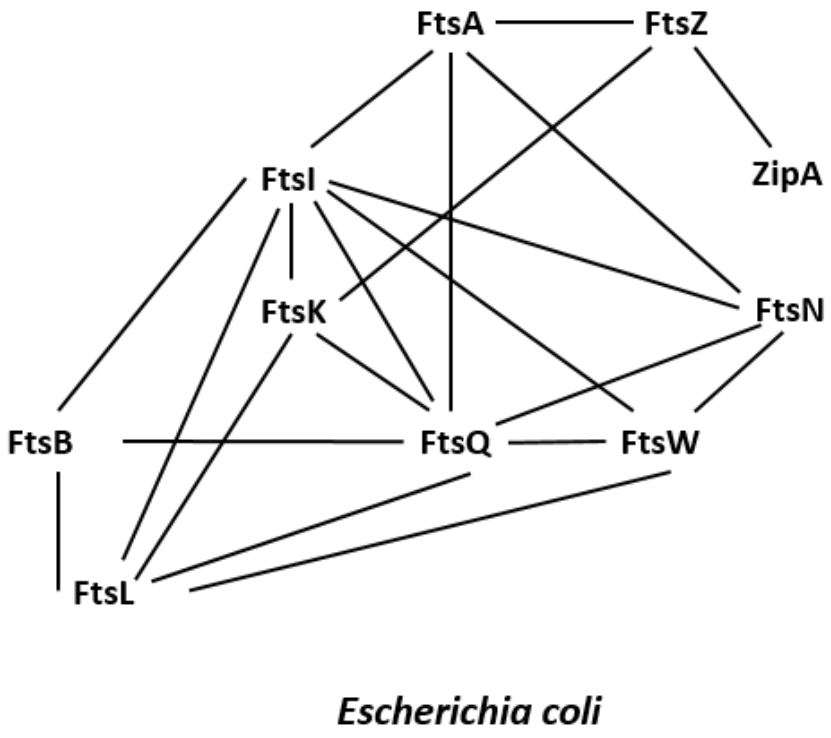

Fig. 1.7. The cell division interactome of *E. coli* (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005).

1.2.2. Cell division in *Bacillus subtilis*

Cell division in *B. subtilis* has two different mechanisms: vegetative cell division at the mid-cell area and sporulation.

1.2.2.1. Vegetative cell division

Vegetative cell division in *B. subtilis* is mediated by a group of essential cell division proteins that are similar to those in *E. coli*. Using time-series and time-lapse microscopy, a two-step assembly of the divisome was observed, and which was separated by at least 20% of the cell cycle (Gamba et al., 2009). Early assembly involves $FtsZ_{Bs}$, $FtsA_{Bs}$, $ZapA_{Bs}$, and $EzrA_{Bs}$, while the late assembly includes $SepF_{Bs}$, $GpsB_{Bs}$, $FtsL_{Bs}$, $DivIB_{Bs}$ (FtsQ), $FtsW_{Bs}$, $PBP1_{Bs}$ and $PBP2B_{Bs}$ (FtsI) (Fig. 1.8). The *B. subtilis* FtsZ polymerized in a GTP-dependent manner (Lutkenhaus and Addinall, 1997). $FtsA_{Bs}$ then anchored $FtsZ_{Bs}$ to the inner membrane (Jensen *et al.*, 2005). The function of $ZapA_{Bs}$ in *B. subtilis* was to promote Z-ring assembly, which was similar to that in *E. coli* (Gueiros-Filho and Losick, 2002). $EzrA_{Bs}$ is a negative regulator for $FtsZ_{Bs}$ polymerization (Margolin, 2000). The absence of $EzrA_{Bs}$ in *B. subtilis* resulted in abnormal formation of multiple Z-rings at cells poles (Levin *et al.*, 1999; Haeusser *et al.*, 2004). Similar to $EzrA_{Bs}$, $GpsB_{Bs}$ is also a negative regulator in *B. subtilis* and is involved in controlling the cell-elongation division cycle (Levin et al., 1999; Wu and Errington, 2004; Claessen *et al.*, 2008). $SepF_{Bs}$ is a Z-ring associated protein which is highly conserved in Gram-positive bacteria (Hamoen *et al.*, 2006; Ishikawa *et al.*, 2006). Depletion of SepF resulted in a deformed division septum, indicating that it was involved in *B. subtilis* cell division (Gundogdu *et al.*, 2011). Overexpression of $sepF_{Bs}$ could restore the depletion of $ftsA_{Bs}$ in a *B. subtilis ftsA*-null mutant, suggesting an overlapping function between $FtsA_{Bs}$ and

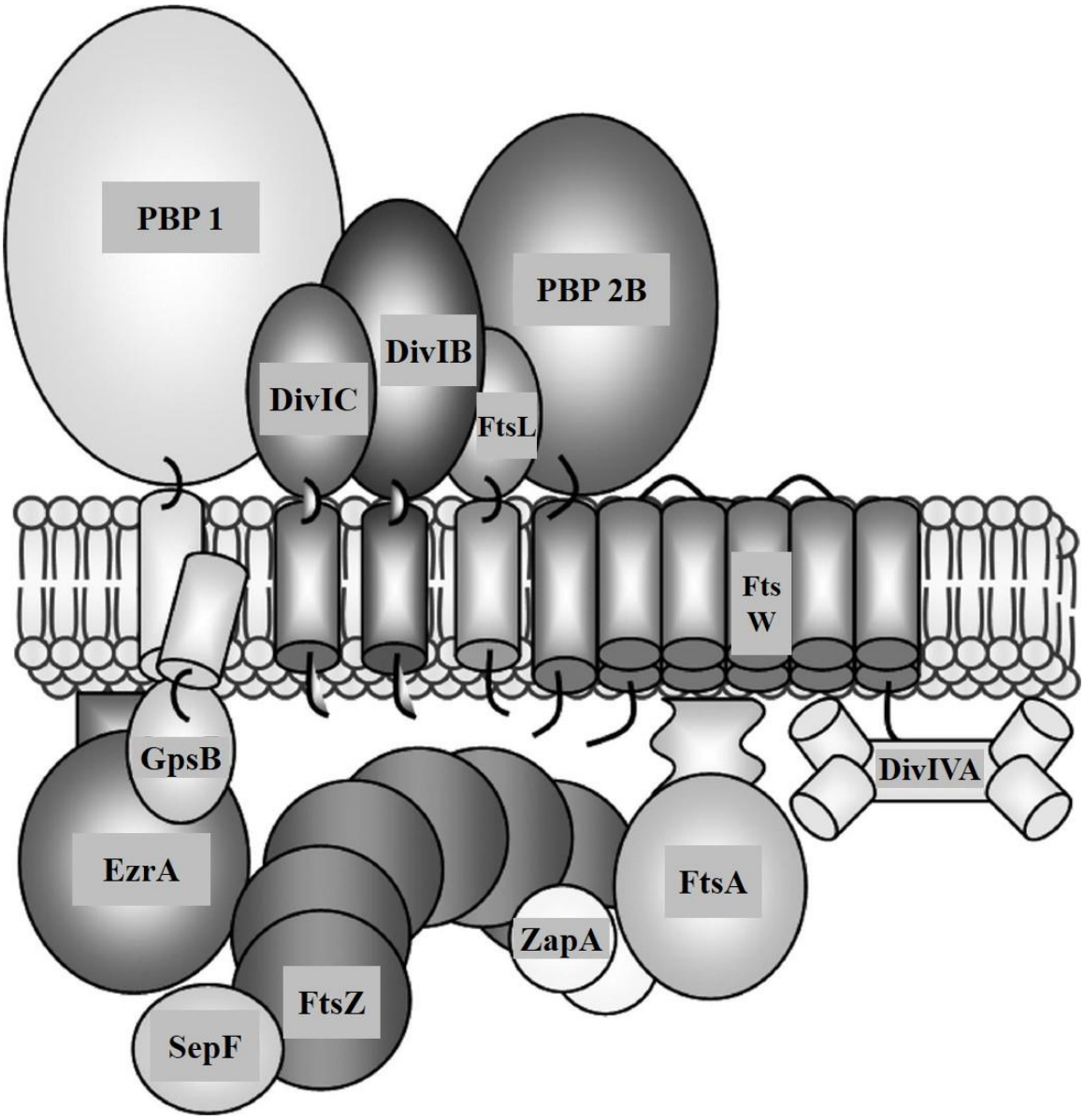

Fig. 1.8. Schematic representation of the *B. subtilis* divisome (Gamba *et al.*, 2009). Proteins involved in *B. subtilis* divisome assembly are FtsZ; FtsA; FtsL; FtsW; DivIB(IB); DivIC; DivIVA; GpsB, EzrA, SepF, ZapA, PBP1, and PBP2B.

SepF$_{Bs}$ (Gundogdu et al., 2011). A homolog of FtsK$_{Bs}$, named SpoIIIE$_{Bs}$, is also present in *B. subtilis*. It has a highly conserved C-terminal region and an N-terminal region that mediates its localization at the division site. The C-terminal region has an ATP-dependent DNA binding capacity (Bath *et al.*, 2000). However, SpoIIIE$_{Bs}$ was only required in the sporulation process and was not involved in vegetative cell division (Bath et al., 2000). *B. subtilis* also contains DivIB$_{Bs}$, FtsL$_{Bs}$, and DivIC$_{Bs}$, which are the homologs of FtsQ, FtsL and FtsB in *E. coli* (Daniel *et al.*, 1998; Daniel *et al.*, 2006; Errington et al., 2003). All three proteins were independently required for assembly, suggesting that the divisome assembly in *B. subtilis* was also following a concerted manner, rather than a linear model (Errington et al., 2003). Using a yeast trihybrid method, DivIB$_{Bs}$, FtsL$_{Bs}$ and DivIC$_{Bs}$ were found to interact with each other, suggesting the formation of a complex, which had been observed in other species, such as *E. coli* and *S. pneumoniae* (Buddelmeijer and Beckwith, 2004; Noirclerc-Savoye *et al.*, 2005). In addition to this, DivIB$_{Bs}$ could stabilize FtsL$_{Bs}$ but destabilize DivIC$_{Bs}$ when FtsL$_{Bs}$ is absent (Daniel et al., 2006). The depletion of the FtsI$_{Ec}$ homolog, PBP2B$_{Bs}$, in *B. subtilis* impaired the cell division process, but constriction was not affected (Daniel *et al.*, 2000). Immunofluorescence microscopy showed that PBP2B was localized at the division site during cell division, suggesting that it was involved in the vegetative cell division process in *B. subtilis*. PBP1, encoded by *ponA*, is another penicillin-binding protein that is involved *B. subtilis* cell division. Knockout of *ponA*$_{Bs}$ resulted in a slower cell growth rate, increased cell length and decreased cell width, while the sporulation efficiency was greatly affected, suggesting that PBP1 was required for sporulation (Popham and Setlow, 1996). *B. subtilis spoVD* is located at the same position in the *dcw* cluster as *ftsW*$_{Ec}$ is in *E. coli*. However, this protein was specifically required

for sporulation but not vegetative cell division (Henriques et al., 1992; Errington et al., 2003). Interestingly, *B. subtilis* encodes another FtsW homolog, YlaO, which is essential for septation (Henriques et al., 1998; Süel *et al.*, 2007). A recent study suggests that the localization of FtsW (YlaO) was dependent on the presence of PBP2B, while the dependency relationship was opposite in *E. coli* (Mercer and Weiss, 2002; Gamba *et al.*, 2016). The last essential cell division protein – FtsN – is not present in *B. subtilis* and other Gram-positive bacteria.

1.2.2.2. Sporulation

Cell division in *B. subtilis* normally occurs at the mid-cell area. However, when nutrients are limited, cells may undergo asymmetric cell division to form a highly resistant endospore (Hilbert and Piggot, 2004). During sporulation, the Z-ring was formed at the cell poles instead of the mid-cell area (Levin and Losick, 1996). This switch was mediated by a master transcription factor called Spo0A. Spo0A mediated *B. subtilis* sporulation by increasing the expression of $FtsZ_{Bs}$ and the sporulation protein $SpoIIE_{Bs}$ (Dworkin, 2014). $Spo0A_{Bs}$ also regulated the localization of the Z-ring from the mid-cell area to the poles during sporulation (Levin and Losick, 1996). SpoIIIE, a homolog of FtsK in *E. coli*, is necessary to separate the replicated chromosome and pump it into the forespore (Dworkin, 2014).

1.3. Cell division in non-model organisms and essential divisome proteins

Studies on cell division in non-model organisms are greatly restricted due to the limited availability of genetic tools. Recently, variations of cell division mechanisms in non-model organisms, with different cell shapes or more complex cell cycles compared to the model organisms, have been reported (Eswara and Ramamurthi, 2017). For example, Gram-negative alphaproteobacterium *Agrobacterium tumefaciens* (At) has three copies of *ftsZ* (Eswara and Ramamurthi, 2017). One copy of $ftsZ_{At}$ is the homolog of *E. coli ftsZ* located between $ftsQ_{At}$ and *ftsA* in the *dcw* cluster. After the regular cell division at the midcell site, $FtsZ_{At}$ and $FtsA_{At}$ were relocated at one cell pole and facilitated polar growth, which was not present in *E. coli* and *B. subtilis* (Brown *et al.*, 2012; Zupan *et al.*, 2013). *S. aureus*, a Gram-positive spherical bacterium, divides through three division planes which are perpendicular to each other (Pinho et al., 2013). Division site selection in *S. aureus* is mediated by the NO system because the Min system is absent. The homolog of *B. subtilis* DivIVA, which regulates the Min system, is also present in *S. aureus* (Eswaramoorthy *et al.*, 2011). However, knockout of $divIVA_{Sa}$ did not affect cell division and chromosome segregation (Pinho and Errington, 2004). Surprisingly, $DivIB_{Sa}$, a homolog of *E. coli* FtsQ, was speculated to be crucial for division site selection because depletion of $DivIB_{Sa}$ in *S. aureus* resulted in misplaced division septums (Bottomley *et al.*, 2014). *Chlamydia trachomatis* is an obligate human pathogen that does not possess FtsZ. How cell division is processed in *C. trachomatis* is unknown. An recent observation suggested that peptidoglycan was only transiently, but predominantly present at the division site (Liechti *et al.*, 2014). Two cell wall synthesis related proteins, MreB and RodZ, were found to localize at cell division sites (Kemege *et al.*, 2015). These studies suggested that cell

division in FtsZ-less bacteria, such as *C. trachomatis*, might be driven by peptidoglycan synthesis. Recent progess in cell division in non-model organisms suggests that cell division mechanisms in model bacteria may not be reflected in non-model species. Here, I reviewed cell division in the *Chlamydiales* order and *S. pneumoniae* as two non-model systems.

1.3.1. Cell division in *Chlamydiales*

Chlamydiales is an order of obligate intracellular bacteria including the members of the *Chlamydiaceae* family and the *Chlamydia*-related bacteria. For instance, *C. trachomatis* causes urogenital infections and trachoma; *C. pneumoniae* and *C. psittacci* cause pneumonia (Greub, 2009); and *Waddlia chondrophila* (Wc), a Chlamydia-related bacterium, was suggested to be associated with abortion in bovines and miscarriage in humans (Rurangirwa *et al.*, 1999; Baud *et al.*, 2007; Baud *et al.*, 2008; Baud *et al.*, 2011).

Bacterial species from the *Chlamydiales* order have a distinct biphasic developmental cycle: the extracellular, infectious elementary bodies with reduced metabolic activity and the intracellular, non-infectious reticulate bodies that can divide (Sixt *et al.*, 2013; Friis, 1972; Abdelrahman and Belland, 2005).

As a non-model bacterial order, *Chlamydiales* undergoes an atypical cell division process that lacks FtsZ (Bertelli *et al.*, 2010; Stephens et al., 1998). It possesses several cell division proteins, including MreB, RodZ, FtsK, FtsW, FtsQ, and FtsI, while FtsA, FtsB, and FtsN are absent (Ouellette *et al.*, 2012; Jacquier *et al.*, 2014; Shiomi *et al.*, 2008; Alyahya *et al.*, 2009; Jacquier *et al.*, 2015; Ouellette *et al.*, 2015).

In rod-shaped bacteria, MreB, an actin homolog, is a key protein in cell wall synthesis in cell elongation (Divakaruni *et al.*, 2005). Treatments of antibodies targeting *C. trachomatis* $MreB_{Ct}$ resulted in an enlarged cell phenotype, indicating its role in the cell division process (Ouellette et al., 2012). Similar results were also observed in *C. pneumoniae* and *W. chondrophila* (Ouellette et al., 2012; Jacquier et al., 2014). Using B2H assays, two proteins were identified as interacting partners of chlamydial MreB: RodZ, FtsK (Ouellette *et al.*, 2014; Ouellette et al., 2012). Localization of *W. chondrophila* MreB revealed that it was only present at medium and late division stages, suggesting that MreB might act as a replacement of FtsZ in other species which functioned as an organizer of early division events (Jacquier et al., 2014).

In contrast, the localization of $RodZ_{Wc}$ in *W. chondrophila* showed that it localized at the early stage of cell division (Jacquier et al., 2014). In *E. coli*, RodZ was characterized as a rod shape determinant, which was a bitopic protein with a cytoplasmic helix-turn-helix domain (Jacquier et al., 2014; Shiomi et al., 2008; Alyahya et al., 2009; van den Ent *et al.*, 2001). Because RodZ interacted with MreB in *C. trachomatis* (Ouellette et al., 2012), it was proposed that the RodZ might act as the division organizer in chlamydial cell division. Interestingly, Jacquier *et al* (2014) showed that MreB is required for proper localization of RodZ in *W. chondrophila*, although it appears at the division site later than the RodZ.

PBP3, a homolog of *E. coli* FtsI, is present in *C. trachomatis*, although it shares little homology within the first 150 amino acids of *E. coli* FtsI (Ouellette et al., 2012). β-lactam treated chlamydiae exhibited an enlarged cell phenotype, indicating the disruption of cell division (Ouellette et al., 2012). Immunofluorescence analysis revealed that $PBP3_{Ct}$ localized in the cell following a punctate pattern with no more than one copy per cell

(Ouellette et al., 2012). B2H results showed that $FtsI_{Ct}$ interacted with $FtsK_{Ct}$, but not $FtsW_{Ct}$.

In addition to *ftsZ*, the homolog of *ftsA* is also absent in the chlamydial *dcw* cluster (Ouellette et al., 2015). However, a putative gene, *ct764*, present in the opposite direction compared to other genes in the *dcw* cluster, is identified as the homolog of *E. coli* FtsQ (Ouellette et al., 2015). The putative FtsQ in *C. trachomatis* shares structural homology with *E. coli* FtsQ, although they do not have similarity in their primary sequences. Localization of GFP-Ct764 showed a punctate pattern in *C. trachomatis*, and overexpression of GFP-Ct764 resulted in abnormal cell morphology, indicating that Ct764 was involved in *C. trachomatis* cell division. B2H results showed that Ct764 interacted with $MreB_{Ct}$, $RodZ_{Ct}$, $FtsK_{Ct}$, and self-interacted but did not with $FtsI_{Ct}$ and $FtsW_{Ct}$ (Ouellette et al., 2015). Expression of Ct764 in an *E. coli ftsQ* temperature sensitive mutant suggested that it did not complement the loss of $ftsQ_{Ec}$ at the non-permissive temperature, indicating that the function of *C. trachomatis* FtsQ differs from its *E. coli* homolog (Ouellette et al., 2015).

FtsK homologs have been identified in *C. trachomatis*, *C. pneumoniae* and *W. chondrophila*, sharing 46.04%, 44.84% and 40.91% of identities with their *E. coli* homolog, respectively (Jacquier et al., 2015). Interactions of chlamydial FtsK with MreB and FtsI were identified using B2H assays (Ouellette et al., 2012; Ouellette et al., 2014). Similarly, FtsW is also present in the chlamydial species mentioned above. B2H assays failed to identify any protein interaction between FtsW and any other chlamydial cell division protein (Ouellette et al., 2015). Thus, the function of FtsW in chlamydial cell division is still unclear.

1.3.2. Cell division in *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive bacterium that causes bacterial pneumonia and meningitis (Carapetis *et al.*, 2005). Cell division in *S. pneumoniae* relies on a membrane protein called MapZ (Fleurie *et al.*, 2014). It has two major functions in *S. pneumoniae* cell division. First, it formed a ring-like structure in dividing cells and helped localize the Z-ring formed by $FtsZ_{Sp}$ polymers at the division site by direct interaction with $FtsZ_{Sp}$ (Holeckova *et al.*, 2014; Fleurie et al., 2014). During cell elongation, the $MapZ_{Sp}$ ring splited into two rings and moved away from the division site at the same speed as cell elongation (Fleurie et al., 2014). Second, a third $MapZ_{Sp}$ ring appeared as the cell elongated and remained at the division site until the mother cell was completely divided into two daughter cells (Fleurie et al., 2014).

S. pneumoniae ftsZ encodes a 40 kDa protein that hybridizes with an *E. coli ftsZ* probe (Corton *et al.*, 1987). Overexpression of $ftsZ_{Sp}$ in *Enterobacteriaceae* resulted in the formation of minicells, indicating a role of $FtsZ_{Sp}$ in cell division. *In vitro* analysis of $FtsZ_{Sp}$ revealed that it polymerized into high molecular mass polymers via its GTPase activity (Salvarelli *et al.*, 2015). Using photoactivated localization microscopy (PALM), the Z-ring formed by $FtsZ_{Sp}$ polymers in *S. pneumoniae* was visualized as a bead-collar-like structure with regions of varied densities, indicating the ring-like structure was composed of heterogeneous clusters of loose protofilaments (Jacq *et al.*, 2015).

S. pneumoniae FtsA is essential for cell viability (Lara et al., 2005). An interaction of $FtsZ_{Sp}$ with $FtsA_{Sp}$ was ascertained in a Y2H assay (Lara et al., 2005). Immunostaining of both $FtsZ_{Sp}$ and $FtsA_{Sp}$ showed that they co-localized at the division site in dividing cells, supporting the Y2H result (Lara et al., 2005). In addition, ATP binding and hydrolysis

assays showed that $FtsA_{Sp}$ polymerized *in vitro* in an ATP-dependent manner, but no hydrolytic activity was observed (Lara et al., 2005). The 1C and 2B subdomains of $FtsA_{Sp}$ were associated with its polymerization activity (Krupka et al., 2012). Complete depletion of $FtsA_{Sp}$ in *S. pneumoniae* resulted in the delocalization of the Z-ring and further led to cell lysis, indicating that $FtsA_{Sp}$ localized the Z-ring at the division site with $MapZ_{Sp}$ (Mura *et al.*, 2016). $FtsA_{Sp}$ also interacted with many other cell division proteins in *S. pneumoniae*, as determined using B2H assays, including $FtsK_{Sp}$, $FtsL_{Sp}$, $FtsI_{Sp}$, $ZapA_{Sp}$ and self-interacted (Maggi et al., 2008).

S. pneumoniae encodes a cell division protein, DivIVA, which is only present in Gram-positive bacteria. *divIVA*$_{Sp}$ is downstream of *ftsZ*$_{Sp}$ in the streptococcal *dcw* cluster (Fadda *et al.*, 2003). Inactivation of *divIVA*$_{Sp}$ led to a significant inhibition in growth rate and cells exhibited as chains of unseparated cells, indicating that cell division and constriction were affected (Fadda et al., 2003). DAPI staining revealed that the chromosome was not correctly segregated when *divIVA*$_{Sp}$ was knocked out in streptococcal cells, suggesting it was also required for chromosome segregation (Fadda et al., 2003). Microscopic analysis demonstrated a unique localization pattern of $DivIVA_{Sp}$ as it was present in both the division site and cell poles simultaneously (Fadda *et al.*, 2007). None of the proteins of the streptococcal division machinery was dependent on $DivIVA_{Sp}$ for localization, although a few of them interacted with $DivIVA_{Sp}$, including $FtsZ_{Sp}$, $ZapA_{Sp}$, $FtsA_{Sp}$, $FtsK_{Sp}$, $FtsL_{Sp}$, $FtsB_{Sp}$, $FtsQ_{Sp}$, $FtsW_{Sp}$, and self-interacted (Fadda et al., 2007).

FtsQ, also known as DivIB, is a conserved cell division protein in most eubacteria and forms a protein complex with two other cell division proteins, $FtsB_{Sp}$ (DivIC) and $FtsL_{Sp}$, in *S. pneumoniae* (Noirclerc-Savoye et al., 2005). Immunofluorescence microscopy

showed that $FtsB_{Sp}$ was always localized at the division site in streptococcal cells, whereas the $FtsQ_{Sp}$-$FtsL_{Sp}$ subcomplex only co-localized with $FtsB_{Sp}$ during cell division (Noirclerc-Savoye et al., 2005). The interaction of $FtsQ_{Sp}$-$FtsL_{Sp}$ was identified as a crucial interaction for cell division (Le Gouellec *et al.*, 2008). Unlike *E. coli*, *S. pneumoniae* FtsQ was not essential when cells were grown in rich medium and was required in chemically defined medium (Le Gouellec et al., 2008). Cells grown in rich medium displayed as chains of unseparated cells as well as a small population of enlarged cells, indicating the cell division process is interrupted (Le Gouellec et al., 2008). Further analysis showed that $FtsL_{Sp}$ rapidly degraded without the presence of $FtsQ_{Sp}$, suggesting a stabilizing role of $FtsQ_{Sp}$ for $FtsL_{Sp}$ (Le Gouellec et al., 2008).

S. pneumoniae FtsI is necessary for cell viability as insertional inactivation of its encoding gene, *pbp2x*, was lethal to streptococcal cells (Kell *et al.*, 1993). Immunofluorescence microscopy revealed that $FtsI_{Sp}$ had the same localization pattern as $FtsW_{Sp}$, a cell division protein that was reported to form a functional complex with $FtsI_{Sp}$ in other species (Mercer and Weiss, 2002; Gamba et al., 2016). A B2H assay also showed that there was a direct interaction between $FtsI_{Sp}$ and $FtsW_{Sp}$ (Maggi et al., 2008). A recent study suggested that GFP-$FtsI_{Sp}$ was localized at the division site in *S. pneumoniae* and depletion of $FtsI_{Sp}$ resulted in severe defects in both cell growth and division, confirming its involvement in the streptococcal cell division process (Peters *et al.*, 2014). Moreover, it was determined that the localization of $FtsI_{Sp}$ did not reply on its C-terminal transpeptidase domain, leaving its N-terminal domain as the only candidate for localization (Peters et al., 2014).

A cell division interactome describing the interaction network among *S. pneumoniae* essential cell division proteins was established by Maggi *et al* (2008)(Fig. 1.9). Using a combination of B2H and co-immunoprecipitation assays, a total of 17 interactions were determined among nine division proteins (Maggi et al., 2008). These interactions were: $FtsZ_{Sp}$ with $FtsA_{Sp}$, $FtsK_{Sp}$, $FtsQ_{Sp}$, $FtsB_{Sp}$, $FtsL_{Sp}$, and $FtsW_{Sp}$; $FtsA_{Sp}$ with $FtsK_{Sp}$, $FtsL_{Sp}$, and $FtsL_{Sp}$; $FtsK_{Sp}$ with $FtsQ_{Sp}$, $Ftsl_{Sp}$, and $FtsW_{Sp}$; $FtsQ_{Sp}$ with $FtsL_{Sp}$, and $FtsW_{Sp}$; $FtsB_{Sp}$-$FtsW_{Sp}$; and $FtsL_{Sp}$ with $FtsI_{Sp}$, and $FtsW_{Sp}$. Compared to *E. coli* cell division interactome, eight of these interactions were unique in *S. pneumoniae*, suggesting the distinction of cell division between these two organisms (Maggi et al., 2008; Di Lallo et al., 2003; Karimova et al., 2005).

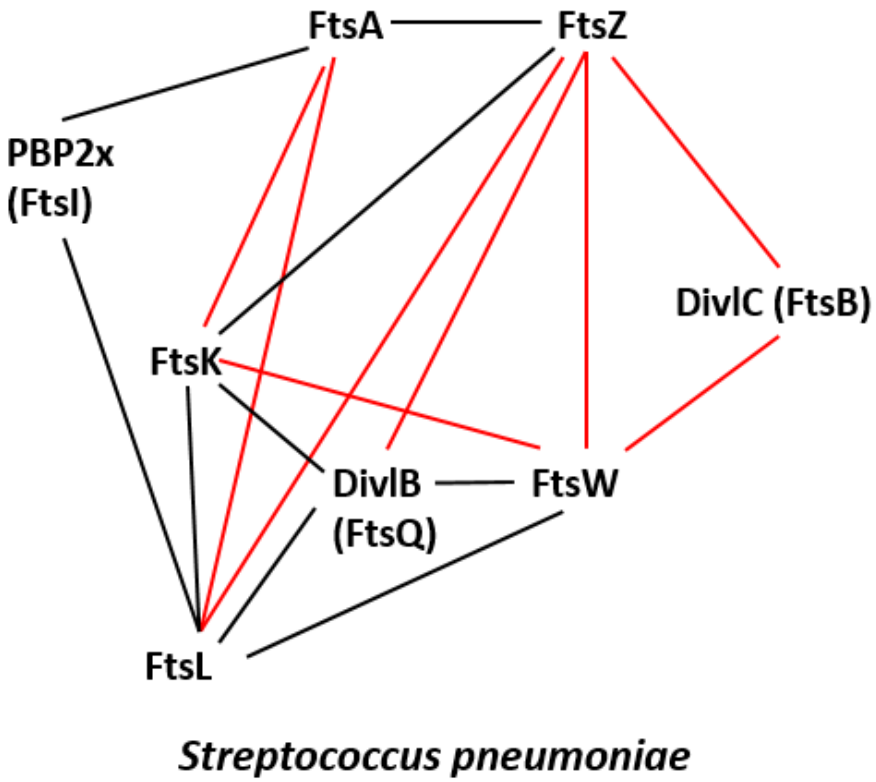

Fig. 1.9. The cell division interactomesof *S. pneumoniae* (Maggi *et al.*, 2008). Red lines indicate unique interactions in *S. pneumoniae* compared to *E. coli*.

1.4. Current knowledge of cell division in *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative coccus which often appears as a single, pair or tetrad of cells. Clinically, gonococcal cells are often seen as diplococci. As a non-traditional organism for cell division study, *N. gonorrhoeae* divides in two dimensions and produces two cell division planes that are perpendicular to each other, which is different from the cell division processes in both *E. coli* and *B. subtilis* (Westling-Häggström *et al.*, 1977; Fitz-James, 1964). Cell division in *N. gonorrhoeae* has been well studied in division site selection. Homologs of the *E. coli* Min system have been well characterized (Ramirez-Arcos *et al.*, 2001b; Szeto *et al.*, 2001; Ramirez-Arcos *et al.*, 2002; Szeto *et al.*, 2004; Ramirez-Arcos *et al.*, 2004; Szeto *et al.*, 2005; Ramos *et al.*, 2006; Ghasriani *et al.*, 2010). Differences in essentialities of Min proteins from *N. gonorrhoeae* and *E. coli* suggested that cell division mechanisms in both species might not be the same (Ramirez-Arcos et al., 2001b; Szeto et al., 2001).

1.4.1. Medical importance of *N. gonorrhoeae*

N. gonorrhoeae is a Gram-negative obligate human pathogen that causes gonorrhea, the second most common sexually transmitted infection in North America and worldwide (Piszczek *et al.*, 2015; WHO, 2016). Based on data from the World Health Organization, there were 26.8 million new cases of gonorrhea infections in adults aged 15–49 worldwide in 2012 (Looker *et al.*, 2015). The prevalence of reported gonococcal infections in Canada was increased by 65.4% from 2010 to 2015 (Choudhri *et al.*, 2018). In the United States, 468,514 new cases of gonococcal infections were reported in 2016, which was an 18.5% increase compared to 2015.

The symptoms of gonorrhea in males include urethral discharge, dysuria, and testicular pain, while females can suffer from vaginal discharge, dysuria, dyspareunia, abnormal uterine bleeding and lower abdominal pain. However, approximately two-thirds of all infected men and more than half of infected women are asymptomatic and become a major source of infection spread (Rice *et al.*, 2017). In women, untreated infections can lead to pelvic inflammatory disease, infertility, ectopic pregnancy and chronic pain, while men can suffer from epididymal-orchitis, reactive arthritis and, rarely, infertility with untreated infections (Diseases, 2013). Moreover, gonococcal infections also increase the risk of acquisition and transmission of HIV (Cohen *et al.*, 1997).

Due to the lack of a suitable animal model, as well as frequent antigenic or phase variation in *N. gonorrhoeae*, there is no good vaccine candidate against gonococcal infections (Jerse *et al.*, 2014). Therefore, treatment of gonococcal infection is purely dependent on the use of antibiotics, starting with the sulfonamides in 1938, followed by penicillins, tetracyclines, spectinomycin, quinolones, macrolides and third-generation cephalosporins (Rice et al., 2017). Unfortunately, resistance to these antibiotics appeared soon after the applications of these drugs. Due to the increasing number of strains with cefixime resistance, which is known as the last highly effective antimicrobial drug in a single dose for gonorrhea treatment, the WHO recommended a dual therapy of ceftriaxone and azithromycin to treat gonococcal infections (WHO, 2016). Recently, a ceftriaxone/azithromycin resistant strain was reported (Fifer *et al.*, 2016), which brings the warning that there may not be an effective antimicrobial drug for gonorrhea treatment. Seeking new drugs for the treatment of gonococcal infections is extremely urgent.

1.4.2. The dcw cluster of *N. gonorrhoeae*

Our laboratory has pioneered in gonococcal cell division studies. Investigation of the *dcw* cluster of *N. gonorrhoeae* revealed 17 genes: *mraZ*$_{Ng}$ *mraW*$_{Ng}$, *ftsI*$_{Ng}$, *murE*$_{Ng}$, *hyp1*, *murF*$_{Ng}$, *mraY*$_{Ng}$, *hyp2*, *murD*$_{Ng}$, *ftsW*$_{Ng}$, *murG*$_{Ng}$, *murC*$_{Ng}$, *ddl*$_{Ng}$, *ftsQ*$_{Ng}$, *ftsA*$_{Ng}$, *ftsZ*$_{Ng}$, and *hyp3* (Fig. 1.1)(Francis *et al.*, 2000). The gene order of gonococcal *dcw* cluster was similar to that in *E. coli* (Ayala et al., 1994; Mingorance et al., 2004). Three hypothetical genes were identified in this cluster, named *hyp1*, *hyp2*, and *hyp3*. The *hyp1* gene product was highly similar (47% in amino acid sequence) with YbiP in *E. coli*. Hyp2 shared 61% similarity with an outer membrane protein, YebT, in *E. coli* (Isom *et al.*, 2017), while Hyp3 was a homolog of YieG in *E. coli*, sharing 60% similarity. Compared with *E. coli*, the gonococcal *dcw* cluster did not contain *envA* and *ftsL* genes, which were located downstream of *ftsZ* and *mraW*, respectively in *E. coli* genome (Vicente *et al.*, 1998). The homolog of *envA* was present elsewhere in *N. gonorrhoeae* (Francis et al., 2000). *E. coli ftsL* is located downstream of *mraW*$_{Ec}$ and upstream of *ftsI*$_{Ec}$ (Mingorance et al., 2004). An open reading frame (ORF) encoding an 86 aa protein was identified in the region in the gonococcal *dcw* cluster, which only shared 22% similarity with *E. coli* FtsL (Francis et al., 2000). Additional bioinformatical analysis using the PCGene suggested that this putative region was not a coding ORF. Thus, this region was not considered as *N. gonorrhoeae ftsL* (Francis et al., 2000). However, others suggested that the gene product of this putative ORF was *N. gonorrhoeae* FtsL, regardless of the low similarity with *E. coli* FtsL (Snyder *et al.*, 2001). Moreover, the *hyp1* defined by Francis (2000) was proposed to be involved in phase variation and natural competence, which was re-named as *dca* (Division cluster Competence Associated) (Snyder et al., 2001).

Transcriptional analysis showed that the *N. gonorrhoeae dcw* cluster was divided into five different transcripts by four functional internal transcriptional terminators (Fig 1.1)(Francis et al., 2000). Among the four internal terminators, three consist of inverted repeats of the gonococcal uptake sequence (5‘-GCCGTCTGAA-3’) required for transformation and the other contained the Correia element (Francis et al., 2000). Locations of three internal terminators with gonococcal uptake sequence were between *mraY*$_{Ng}$-*murD*$_{Ng}$, *murD*$_{Ng}$-*ftsW*$_{Ng}$, and *murG*$_{Ng}$-*murC*$_{Ng}$, while a Correia element was located between *murF*$_{Ng}$ and *mraY*$_{Ng}$ (Francis et al., 2000).

1.4.3. *N. gonorrhoeae* Min system

Studies on division site selection with *N. gonorrhoeae* revealed three homologs of the Min system (i.e. *minC, minD,* and *minE*) present on the *N. gonorrhoeae* FA1090 genome (Ramirez-Arcos et al., 2001b). These genes were clustered in a 17kb region on the gonococcal chromosome, which was flanked by gonococcal DNA uptake sequences (Ramirez-Arcos et al., 2001b). Gene products of *minCDE* in *N. gonorrhoeae* share 36% (MinC$_{Ng}$), 73% (MinD$_{Ng}$), and 42% (MinC$_{Ng}$) identities with their *E. coli* homologs, respectively (Ramirez-Arcos et al., 2001b).

N. gonorrhoeae MinC is a 237-aa protein that is necessary for proper cell division. Depletion of MinC$_{Ng}$ caused abnormal cell division which further led to cell lysis (Ramirez-Arcos et al., 2001b). Overexpression of MinC$_{Ng}$ in *E. coli* resulted in a filamentous phenotype, indicating that the cell division was disrupted. In addition, expression of *minC*$_{Ng}$ could compensate for the lack of functional *minC*$_{Ec}$ in an *E. coli* MinC mutant, suggesting that *N.* MinC$_{Ng}$ also acted as a cell division inhibitor, as its homolog did in *E. coli* (Ramirez-Arcos et al., 2001b). Further investigation, using B2H

assays, showed that the N-terminal region, particularly the 13th amino acid, was critical for the interaction of $MinC_{Ng}$ with $FtsZ_{Ng}$ (Greco-Stewart *et al.*, 2007). The C-terminal conserved glycine residues at positions 135, 154, and 171 of $MinC_{Ng}$ were required for the interaction with the α-7 helix of $MinD_{Ng}$ (Ramirez-Arcos et al., 2004).

$MinD_{Ng}$ is a 271-aa protein that shares 73% and 39% similarity with its homologs in *E. coli* and *B. subtilis*, respectively (Ramirez-Arcos et al., 2001b). Overexpression of $MinD_{Ng}$ together with $MinC_{Ng}$ resulted in significant enlargement of gonococcal cells, indicating the disruption of cell division (Szeto et al., 2001). Depletion of $MinD_{Ng}$ caused a reduction in cell viability and abnormal cell morphology (Szeto et al., 2001). Similar to $MinC_{Ng}$, overexpression of *N. gonorrhoeae* MinD led to cell filamentation in wild-type *E. coli* and compensated for the loss of native $MinD_{Ec}$ in an *E. coli* MinD mutant, suggesting a similar function of MinD in both species (Szeto et al., 2001). Due to the lack of appropriate genetic tools, localization studies of $MinD_{Ng}$ were mainly performed in an *E. coli* background. Expression of GFP-$MinD_{Ng}$ demonstrated a similar pole-to-pole oscillation pattern in *E. coli* as its *E. coli* homolog when $MinE_{Ng}$ was co-expressed (Ramirez-Arcos et al., 2002). In round-shape *E. coli* cells, $MinD_{Ng}$ oscillated in a pattern parallel to the septum, which was predicted as the pattern in *N. gonorrhoeae* (Ramirez-Arcos et al., 2002). In *E. coli*, the ATPase activity of $MinD_{Ec}$ is stimulated by the interaction with $MinE_{Ec}$ (Hu and Lutkenhaus, 1999). However, mutations in the N-terminus of $MinD_{Ng}$ abolished the dependency on $MinE_{Ng}$ for ATPase activity and led to a higher ATPase activity and a faster oscillation cycle from pole to pole (Szeto et al., 2004; Szeto et al., 2005; Eng *et al.*, 2006).

N. gonorrhoeae MinE is a small protein of 87 residues. An early study on $MinE_{Ng}$ indicated that it could form a ring structure in a similar manner to its *E. coli* homolog and oscillate from pole to pole in the presence of *E. coli* MinD (Ramirez-Arcos et al., 2002; Fu *et al.*, 2001; Hale *et al.*, 2001). A similar structure was observed formed by $MinE_{Ng}$-GFP in *N. gonorrhoeae* (Eng, 2007). However, no oscillation of the $MinE_{Ng}$-GFP ring was detected, suggesting that the function of $MinE_{Ng}$ was different from its *E. coli* homolog. Overexpression of wild-type $MinE_{Ng}$ resulted in a minicell phenotype in *E. coli*, indicating the disruption of cell division regulation (Eng et al., 2006). An R30D mutation in N-terminus of $MinE_{Ng}$ resulted in a faster oscillation cycle for $MinD_{Ng}$ in *E. coli* (Ramirez-Arcos et al., 2002). In addition, a gonococcal MinE mutant with mutations at the 18^{th} and 22^{nd} residues of its N-terminus failed to interact with $MinD_{Ng}$, suggesting that the N-terminal region of $MinE_{Ng}$ was not involved in the oscillation pattern, but was required for the protein interaction with $MinD_{Ng}$. Interestingly, mutations in the C-terminal region of $MinE_{Ng}$ also led to the reduction of $MinD_{Ng}$ binding capacity, suggesting that both N- and C-terminal regions of $MinE_{Ng}$ were required for its interaction with $MinD_{Ng}$ (Eng et al., 2006). Furthermore, $MinD_{Ng}$ ATPase stimulation was positively correlated with the affinity of $MinD_{Ng}$-$MinE_{Ng}$ interaction since the stronger inhibitory effect on MinD-MinE resulted in a lower rate of $MinD_{Ng}$ ATPase activity (Eng et al., 2006).

1.4.4. Divisome proteins in *N. gonorrhoeae*

Although most cell division genes found in *E. coli* are present in the *N. gonorrhoeae* genome, only a few of those gene products have been characterized (Salimnia et al., 2000; Ramirez-Arcos *et al.*, 2001a; Du and Arvidson, 2003; Bernatchez *et al.*, 2000).

Gonococcal *ftsZ* is located at the 3' end in the *dcw* cluster (Snyder et al., 2001; Francis et al., 2000). $FtsZ_{Ng}$ is a 392-aa protein which shares similarities from 57% to 67% with its homologs in various bacterial species (Salimnia et al., 2000). Expression of $FtsZ_{Ng}$ in *E. coli* led to a filamentous phenotype with no signs of constriction, suggesting that host cell division was interrupted at an early stage (Salimnia et al., 2000). When $FtsZ_{Ng}$-GFP was expressed at low levels in *E. coli*, microscopy analysis showed that the $FtsZ_{Ng}$-GFP fusion protein localized at the mid-cell area in a high percentage of cells (Fig. 1.10A), suggesting that $FtsZ_{Ng}$ might have a cross-species function. Expression of $FtsZ_{Ng}$-GFP in *N. gonorrhoeae* F62 resulted in the formation of insoluble fusion proteins, and no specific localization of $FtsZ_{Ng}$ was detected (Fig. 1.10B)(Salimnia et al., 2000). A previous study in our laboratory using immunogold microscopy showed that, using a monoclonal anti-$FtsZ_{Ng}$ antibody, $FtsZ_{Ng}$ in *N. gonorrhoeae* was visualized along the septum, which was the first evidence of $FtsZ_{Ng}$ localization in its native environment (Szeto, 2004).

ZipA is a less conserved cell division protein, which is only present in Gammaproteobacteria (Haeusser and Margolin, 2016). *zipA* in *N. gonorrhoeae* was first found as an unknown ORF located between the *ligA*, a gene encoding DNA ligase, and *ampD*, a gene encoding N-acetylmuramyl-L-alanine amidase (Lehman, 1974). Since *E. coli zipA* is also located downstream of *ligA* in the *E. coli* K-12 genome, the corresponding ORF in *N. gonorrhoeae* as considered as the homolog of $ZipA_{Ec}$ (Hale and de Boer, 1997). $ZipA_{Ng}$ is a 428-aa protein with a hydrophobic N-terminal region, a short region with basic residues, and a large cytoplasmic domain at the C-terminal. It was suggested that *E. coli* ZipA, a 328-aa protein, anchored $FtsZ_{Ec}$ to the inner membrane (Hale and de Boer, 1997). Although the primary amino acid sequence of $ZipA_{Ng}$ showed no similarity with its *E. coli*

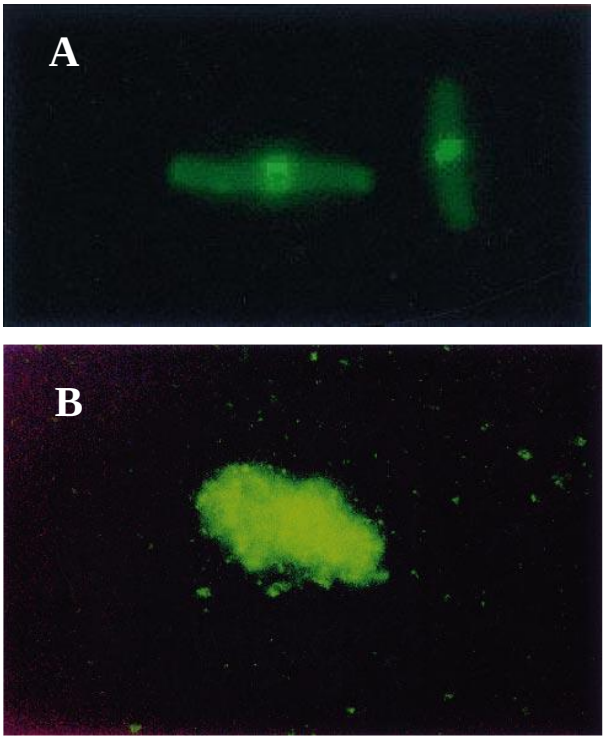

Fig. 1.10. Fluorescent microscopy analysis of $FtsZ_{Ng}$-GFP localization in *E. coli* HB101 (A) and *N. gonorrhoeae* F62 (B) (Salimnia *et al.*, 2000).

homolog, they did share a similar predicted secondary structure (Du and Arvidson, 2003). In addition, $ZipA_{Ng}$ has a proline-glutamine rich region which is required for membrane anchoring in *E. coli* (Hale and de Boer, 1997). Comparison of the C-terminal domain of $ZipA_{Ng}$ with ZipA from *E. coli* and *Haemophilus influenzae* suggested that the C-terminus was highly similar among these three proteins, indicating a similar function of ZipA across different species (Du and Arvidson, 2003). Expression of $ZipA_{Ng}$ in an *E. coli* ZipA temperature-sensitive mutant could complement the loss of $ZipA_{Ec}$ at non-permissive temperatures (Du and Arvidson, 2003). However, the filamentous phenotype caused by inactivation of $ZipA_{Ec}$ was only partially alleviated by $ZipA_{Ng}$, suggesting that, although $ZipA_{Ng}$ was structurally closely related with $ZipA_{Ec}$, the function of $ZipA_{Ng}$ was not identical as its *E. coli* homolog (Du and Arvidson, 2003).

N. gonorrhoeae ftsE and *ftsX* genes were first identified by random sequencing from a plasmid bank of *N. gonorrhoeae* CH811 (Bernatchez et al., 2000). These genes were flanked by *tlpA* (Thioredoxin-LeProtein), *arsC* (AeReductase) and a partial *gltX* (Glutamyl-RNA synthetase gene) upstream of $ftsE_{Ng}$ and a partial *pgk* (PhosphoGeKee) gene. $FtsE_{Ng}$ consists of 216 amino acids and shares 54–71% similarity with its homologs from various species, which all contain a conserved ABC domain. The Walker motifs A and B were involved in ATP binding (Gibbs *et al.*, 1992; Fleischmann *et al.*, 1995; Tomb *et al.*, 1997; Walker *et al.*, 1982). $FtsX_{Ng}$ is 305-aa protein which shares 23–29% similarities with its homologs from various species (Gibbs et al., 1992; Fleischmann et al., 1995; Tomb et al., 1997). Although the similarity between FtsX homologs was very low, all homologs contain a conserved motif (Leu-hydrophobic aa-Gly-Ala/Gly) located between the second and third transmembrane region (Bernatchez et al., 2000). $ftsE_{Ng}$ and

ftsX$_{Ng}$ were co-transcribed from two promoters, P_E1 and P_E2, in both aerobic and anaerobic conditions (Bernatchez et al., 2000). Inactivation of either $FtsE_{Ng}$ or $FtsX_{Ng}$ in *N. gonorrhoeae* did not affect cell viability, and growth curves of each mutant were similar to wild-type cells, indicating that both proteins were not essential for *N. gonorrhoeae* cell division (Bernatchez et al., 2000).

Aside from the gonococcal cell division proteins mentioned above, homologs of FtsA, FtsK, FtsQ, FtsI, FtsW, and FtsN also have been identified on the *N. gonorrhoeae* chromosome (Francis et al., 2000; Salimnia et al., 2000).

1.4.5. *N. gonorrhoeae* FtsI (PBP2)

Based on the function of penicillin-binding proteins, members from this family can be categorized into three classes: class A, B, and C. Both class A and B PBPs have transpeptidase functions which cross-link peptides of adjacent peptidoglycan strands. However, class A PBPs have an additional transglycosylase function at their N-terminal region which polymerizes peptidoglycan strands. Class C is a group of PBPs that have carboxypeptidase activity, which possibly mediates the level of peptidoglycan cross-linking by excluding the terminal D-Ala of the peptide. In addition, PBPs can also be differentiated by their molecular mass, which further divides them into high- and low-molecular-mass groups. Regardless of classification, all PBPs are molecular targets of β-lactam antibiotics (Wang *et al.*, 2003).

N. gonorrhoeae possesses four penicillin-binding proteins: two high molecular mass transpeptidases, class A $PBP1_{Ng}$ and class B $PBP2_{Ng}$, and two low molecular mass PBPs, $PBP3_{Ng}$ and $PBP4_{Ng}$, which are both class C PBPs (Barbour, 1981; Stefanova *et al.*,

2003). Both PBP1 $_{Ng}$ and PBP2 $_{Ng}$ were shown to be essential for *N. gonorrhoeae* cell viability, while depletion of both PBP3 $_{Ng}$ and PBP4 $_{Ng}$ were not lethal to cells (Stefanova et al., 2003; Ropp and Nicholas, 1997; Barbour, 1981).

1.4.5.1. Crystal structure of FtsI

FtsI, or penicillin-binding protein 2 (PBP2) in *N. gonorrhoeae,* is a protein comprising 582 amino acids encoded by *penA* (Zapun *et al.*, 2016). The crystal structure of FtsI $_{Ng}$ revealed that the protein contains two domains, an N-terminal domain and a C-terminal transpeptidase domain which was also known as a penicillin-binding domain (Powell et al., 2009). The N-terminal domain featured several β-strands and a subdomain containing short strands and small helices (Fig. 1.11A)(Powell et al., 2009). The N-terminal subdomain was proposed to interact with a protruding loop at the C-terminal region, as observed in other high-molecular-mass penicillin-binding proteins (Contreras-Martel *et al.*, 2006; Lim and Strynadka, 2002).

The C-terminal transpeptidase domain of FtsI$_{Ng}$ can be divided into two subdomains: an α-subdomain and an α/β-subdomain (Powell et al., 2009). Helices (α2, α4-α6 and α8) from the α-subdomain lie at the top of the molecule and form one side of the active site groove (Powell et al., 2009). One of the common mutations at D345 in the *penA* allele was found in the β-strand ribbon located between the α2 and α4 helices (Dowson *et al.*, 1989). The α/β-subdomain consists of a set of anti-parallel β-strands (β1- β5) surrounded by several long helices (α1, α9, α10, and α11) at both sides. Both N- and C-terminal domains contain disordered structures at residues 91–163 and 502–512, respectively (Fig. 1.8A) (Powell et al., 2009).

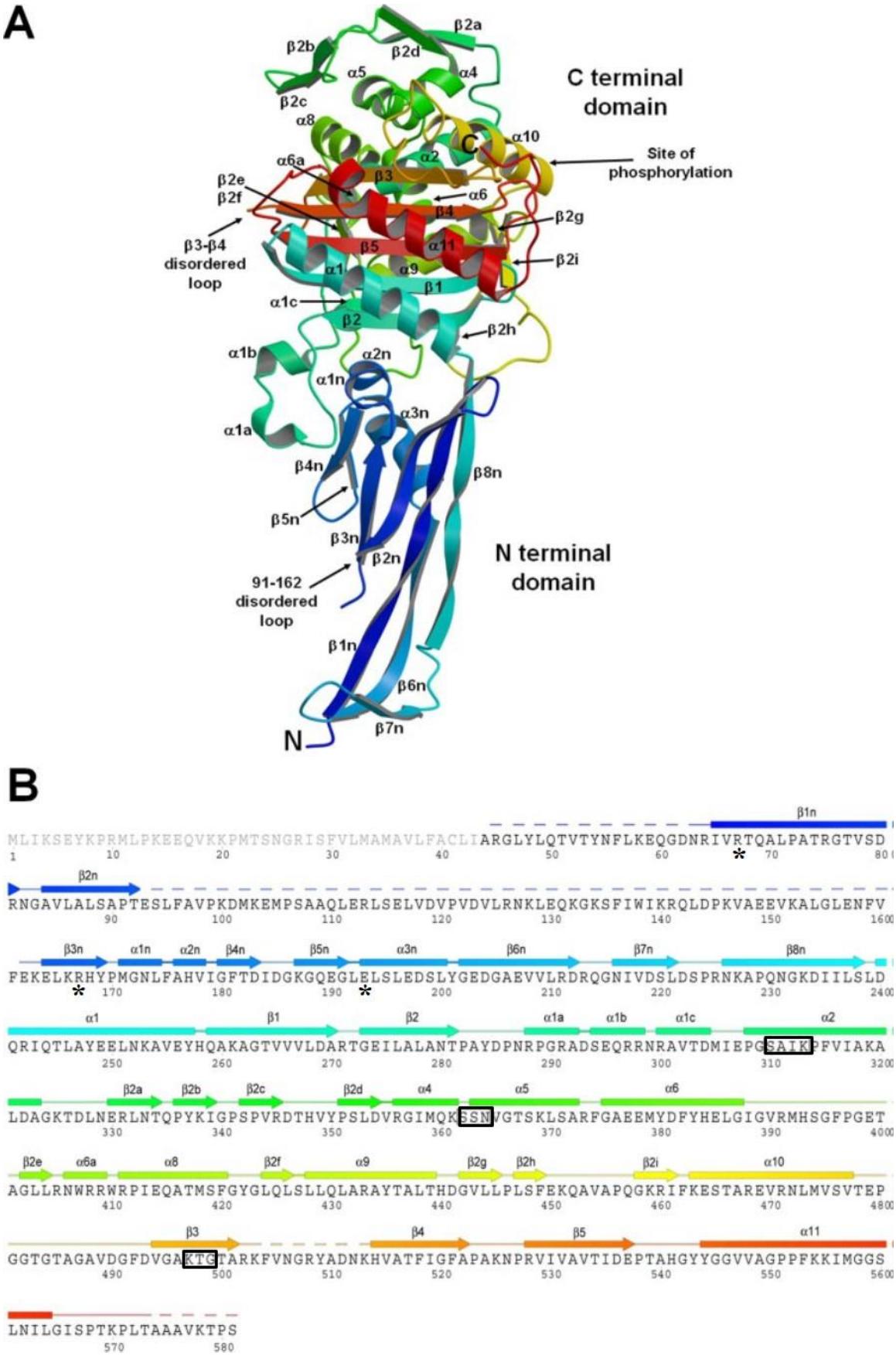
A
C terminal domain
N terminal domain
Site of phosphorylation
β3-β4 disordered loop
91-162 disordered loop
N
C
B

Fig. 1.11. The structure of FtsI from *N. gonorrhoeae* (Powell *et al.*, 2009). (A) The structure is color-ramped *blue*-to-*red* in the N-terminal to C-terminal direction. (B) the primary amino acid sequence of *N. gonorrhoeae* FtsI. The C-terminal conserved motifs are highlighted in boxes.

$FtsI_{Ng}$ contains three active sites which are conserved in almost all penicillin-binding proteins (Fig. 1.11B)(Ghuysen, 1991). The SAIK motif is in an α2 helix with two critical residues, S310 and K313, for catalysis. The SSN motif resides on the loop that connects α4 and α5 helices, while the KTG motif locates on the β3 strand (Powell et al., 2009). Key residues in these active sites are within the distance of hydrogen bonding.

1.4.5.2. The role of FtsI in antimicrobial resistance and cell division

$FtsI_{Ng}$ is a primary target of penicillin and is inhibited at 10-fold lower penicillin concentrations compared to $PBP1_{Ng}$ (Barbour, 1981; Powell et al., 2009). Traditionally, *ftsI*$_{Ng}$ contains five to nine mutations that decrease the acylation rate of $FtsI_{Ng}$, resulting in the reduction of susceptibility to penicillin by 16 fold. Since these mutations are located in the chromosomal *ftsI*$_{Ng}$, the resistance to penicillin is called chromosomally mediated penicillin resistance (Ropp and Nicholas, 1997; Powell et al., 2009; Sparling *et al.*, 1975). These mutations include a codon insertion at the 345th residue and four (F504L, A510V, A516G and H541N) to eight (F504L, A510V, A516G, H541N, P552V, K555Q, I556V and I566V) mutations located at the C-terminal region (Whiley *et al.*, 2007; Ohnishi *et al.*, 2011).

In addition to confering resistance to penicillin mutations, $FtsI_{Ng}$ also confers resistance to extended-spectrum cephalosporins, such as cefixime and ceftriaxone (Ohnishi et al., 2011; Ito *et al.*, 2004; Whiley et al., 2007; Lee *et al.*, 2010; Liao *et al.*, 2009; Allen *et al.*, 2011). The decreased susceptibility to the third-generation cephalosporins is caused by alteration of *penA*, which has incorporated mosaic sequences that contain up to 60 to 70 different amino acids at the C-terminal hypervariable transpeptidase domain (Unemo and Nicholas, 2012). It is believed that the incorporation of mosaic sequences was caused by

horizontal gene transfer from other commensal *Neisseria* species including *Neisseria perflava*, *Neisseria sicca*, *Neisseria polysaccharea*, *Neisseria cinerea* and/or *Neisseria flavescens* (Unemo and Nicholas, 2012; Ameyama *et al.*, 2002; Ito *et al.*, 2005; Osaka *et al.*, 2008)**.** The acquisition of a *penA* allele is considered the most common mechanism of increased resistance to third-generation cephalosporins (Ameyama et al., 2002; Unemo and Nicholas, 2012). To date, 39 polymorphism patterns have been described (Ohnishi et al., 2011; Ito et al., 2004; Whiley et al., 2007; Lee et al., 2010; Martin *et al.*, 2012).

Three mutations (G545S, I312M, and V316T) are present in the *penA* allele in gonococcal isolates with increased resistance or susceptibility to cefixime (Osaka et al., 2008; Takahata *et al.*, 2006). The I312M and V316T mutations are located close to the S310 residue of the SAIK active site, while G545S is involved in binding to the T498, located within the KTG active site motif (Unemo and Nicholas, 2012). Reversion of these mutations in an isolate with increased resistance or susceptibility to cefixime results in dramatic decrease in resistance to extended-spectrum cephalosporins. However, introduction of these mutations in wild-type *penA* does not significantly affect the *N. gonorrhoeae* antimicrobial resistance to ceftriaxone and cefixime, suggesting that these mutations require additional mutations in the mosaic *penA* allele to increase resistance to third-generation cephalosporins (Unemo and Nicholas, 2012; Tomberg *et al.*, 2010).

Compared to the well-characterized C-terminal transpeptidase domain, the function of the N-terminal domain of $FtsI_{Ng}$ is unclear. Evidence from its *E. coli* homolog suggested that the N-terminal domain of $FtsI_{Ec}$ might be involved in an interaction with *E. coli* FtsW and localization at the division site (Wissel and Weiss, 2003; Wissel et al., 2005). Due to the comprehensive research of the C-terminal transpeptidase domain of $FtsI_{Ng}$ on

antimicrobial resistance, knowledge about the potential impact of its N-terminal domain on both cell division and antimicrobial resistance will provide more profound insights in this non-model human pathogen.

1.4.6. The role of FtsA in cell division

N. gonorrhoeae FtsA is 415-aa protein that shares 40% of similarity with $FtsA_{Ec}$ (Li, 2011). Predicted structure of $FtsA_{Ng}$ showed that it has a similar structure with *Thermotoga maritima* FtsA, which contains 1A, 2A, 1B, and 1C subdomains (Fig. 1.4)(Li, 2011). Using a quantitative western blotting method, the ratio of $FtsA_{Ng}$ to $FtsZ_{Ng}$ was determined in three different gonococcal strains (i.e. *N. gonorrhoeae* CH811, FA1090, and F62), giving ratios of 1:24 to 1:33 which are different from that in *E. coli*, *B. subtilis*, and *S. pneumoniae* (Rueda et al., 2003; Lara et al., 2005; Feucht et al., 2001).

Interactions of $FtsA_{Ng}$ with other gonococcal cell division proteins were also ascertained using B2H assays. A total of five cell division proteins were found to interact with $FtsA_{Ng}$, including $FtsZ_{Ng}$, $FtsK_{Ng}$, $FtsQ_{Ng}$, $FtsW_{Ng}$, and $FtsN_{Ng}$.

Overexpression of $FtsA_{Ng}$ in *E. coli* resulted in a filamentous phenotype, indicating that cell division was disrupted (Li, 2011). Since $ftsA_{Ng}$ was expressed from a high copy plasmid under a strong promoter (T7), high concentration of $FtsA_{Ng}$ may cause stoichiometry issues and artificially lead to an abnormal cell morphology (Sopko *et al.*, 2006). Thus, it is necessary to re-evaluate inhibitory effect of $FtsA_{Ng}$ in *E. coli* cell division by expressing $ftsA_{Ng}$ at a more physiologically appropriate level. Using a *E. coli ftsA*-temperature sensitive mutant, $FtsA_{Ng}$ was found not to complement the loss of $FtsA_{Ec}$ at a non-permissive temperature, suggesting that functions of FtsAs from *N. gonorrhoeae* and

E. coli might not be the same (Li, 2011). Thus, understanding how $FtsA_{Ng}$ affected *E. coli* cell division will broaden our knowledge of the function of $FtsA_{Ng}$ in *N. gonorrhoeae* cell division.

1.5. Hypothesis and objectives

1.5.1. Hypothesis

Studies of bacterial cell division mechanisms are mainly focused on the model organisms, such as *E. coli* and *B. subtilis*. An interactome describing the interaction network among *E. coli* essential cell division proteins was also established. The introduction of the second cell division interactome from a non-traditional organism, *S. pneumoniae*, shows that cell division protein-protein interactions in Gram-positive diplococci vary from *E. coli*. Nothing is known about how the divisome is assembled in *N. gonorrhoeae*, a non-traditional organism for studying cell division. An indepth study of *N. gonorrhoeae* cell division protein-protein interactions will provide the knowledge of the divisome formation process in this obligate human pathogen.

Based on the current knowledge of gonococcal cell division, the hypothesis of my thesis is that *N. gonorrhoeae* possess a distinct cell division interactome that differs from the other two established interactomes from *E. coli* and *S. pneumoniae*.

Previous studies on FtsA from other bacterial species have proved that it plays a vital role in bacterial cell division. In addition, the function of FtsA varies in different species. It is hypothesized that *N. gonorrhoeae* FtsA has a species-specific function in *N. gonorrhoeae* cell division that differs from its homolog in *E. coli*.

FtsI, also known as penicillin-binding protein 2 (PBP2) encoded by *penA*, is the primary target of β-lactam antibiotics. Extensive investigation has demonstrated the mutations in the C-terminal transpeptidase domain of *N. gonorrhoeae* FtsI confer resistance to penicillin and third-generation cephalosporins. In contrast, the function of the

N-terminal domain, containing a linker structure in the periplasmic region of $FtsI_{Ng}$, has not been characterized. It is hypothesized that the N-terminal of *N. gonorrhoeae* FtsI is involved in the cell division process and possibly in antimicrobial resistance.

1.5.2. Objectives

1. To establish a cell division interactome of *N. gonorrhoeae* by:
    a. Testing *N. gonorrhoeae* cell division protein-protein interactions using B2H assays and re-analyze previous B2H data (Li, 2011).
    b. Verifying the interactions of $FtsA_{Ng}$-$FtsZ_{Ng}$, $FtsA_{Ng}$-$FtsN_{Ng}$, and $FtsA_{Ng}$-$FtsQ_{Ng}$ using GST pull-down assays.
    c. Ascertaining the interactions of $FtsA_{Ng}$-$FtsZ_{Ng}$, $FtsA_{Ng}$-$FtsN_{Ng}$, and $FtsA_{Ng}$-$FtsQ_{Ng}$ using surface plasmon resonance.
2. To exploit an *E. coli* expression model to study the effects of $FtsA_{Ng}$ on cell division by:
    a. Ascertaining the morphological changes of *E. coli* when $FtsA_{Ng}$ is expressed by differential interference contrast (DIC) microscopy.
    b. Determining the localization of $FtsA_{Ng}$ in *E. coli* by confocal microscopy.
    c. Ascertaining the interaction between $FtsA_{Ng}$ and *E. coli* divisome proteins by B2H and GST pull-down assays.
    d. Determining the domains of $FtsA_{Ng}$ involving in the interaction with *E. coli* divisome proteins by B2H and GST pull-down assays.
3. To investigate the function of N-terminal region of $FtsI_{Ng}$ on *N. gonorrhoeae* cell division by:

a. Identifying conserved amino acids on N-terminal region of $FtsI_{Ng}$ by bioinformatical analysis.
b. Testing the interaction between wild-type $FtsW_{Ng}$ and $FtsI_{Ng}$ mutant by B2H assay
c. Determining the conformational change and thermal stability of the FtsI mutant using circular dichroism.
d. Investigating the morphological changes in *N. gonorrhoeae*, caused by N-terminal conserved amino acid substitution, using electron microscopy.
e. Investigating the effects of mutations in the N-terminus of $FtsI_s$ on penicillin binding activity using BOCILLIN FL penicillin.

Chapter 2 The distinctive cell division interactome of *Neisseria gonorrhoeae*

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Running title: cell division interactome of *N. gonorrhoeae*

2.1. Abstract

Background: Bacterial cell division is an essential process driven by the formation of a Z-ring structure, as a cytoskeletal scaffold at the mid-cell, followed by the recruitment of various proteins which form the divisome. The cell division interactome reflects the complement of different interactions between all divisome proteins. To date, only two cell division interactomes have been characterized, in *Escherichia coli* and in *Streptococcus pneumoniae*. The cell divison proteins encoded by *Neisseria gonorrhoeae* include FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsI, FtsW, and FtsN. The purpose of the present study was to characterize the cell division interactome of *N. gonorrhoeae* using several different methods to identify protein-protein interactions. We also characterized the specific subdomains of FtsA implicated in interactions with FtsZ, FtsQ, FtsN and FtsW.

Results: Using a combination of bacterial two-hybrid (B2H), glutathione S-transferase (GST) pull-down assays, and surface plasmon resonance (SPR), nine interactions were observed among the eight gonococcal cell division proteins tested. ZipA did not interact with any other cell division proteins. Comparisons of the *N. gonorrhoeae* cell division interactome with the published interactomes from *E. coli* and *S. pneumoniae* indicated that FtsA-FtsZ and FtsZ-FtsK interactions were common to all three species. FtsA-FtsW and FtsK-FtsN interactions were only present in *N. gonorrhoeae*. The 2A and 2B subdomains of $FtsA_{Ng}$ were involved in interactions with FtsQ, FtsZ, and FtsN, and the 2A subdomain was involved in interaction with FtsW.

Conclusions: Results from this research indicate that *N. gonorrhoeae* has a distinctive cell division interactome as compared with other microorganisms.

Keywords: cell division, interactome, *N. gonorrhoeae*, protein-protein interaction, bacterial two-hybrid assay, surface plasmon resonance, GST pull-down, FtsA domains

2.2. Introduction

Cell division is essential for bacterial survival. In *Escherichia coli* (Ec), normal cell division is driven by the formation of an FtsZ-ring at the division site (Lutkenhaus and Addinall, 1997), followed by the recruitment of other essential proteins, which together form the divisome (Margolin, 2000). Genes encoding most cell division proteins are located in a conserved region, the *d*ivision and *c*ell *w*all (*dcw*) cluster (Ayala et al., 1994). *dcw* clusters have been identified in most bacterial species, including *E. coli*, *Bacillus subtilis* (Bs), *Streptococcus pneumoniae* (Sp), *Caulobacter crescentus* (Cc) and *Neisseria gonorrhoeae* (Ng) (Lara et al., 2005; Real and Henriques, 2006; Massidda et al., 1998; Francis et al., 2000). Although the gene organization of the *dcw* cluster varies in different bacteria species (Tamames *et al.*, 2001), proteins involved in the cell division process are relatively conserved (Lutkenhaus *et al.*, 2012; Haeusser and Margolin, 2016).

E. coli encodes ten essential cell division proteins, including FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI, and FtsN (Errington et al., 2003; Grenga et al., 2013). Assembly of the FtsZ-ring structure is initiated with the polymerization of FtsZ, driven by GTP hydrolysis, at the mid-cell (Mukherjee and Lutkenhaus, 1998). FtsA and ZipA are recruited by FtsZ and anchor FtsZ to the inner membrane (Hale and de Boer, 1997). After the recruitment of FtsK, a DNA translocase involved in DNA segregation (Goehring et al., 2006; Begg *et al.*, 1995; Yu et al., 1998), the protein complexes FtsQ-FtsB-FtsL and FtsW-FtsI are localized to the septal ring, sequentially (Goehring et al., 2006; Buddelmeijer and Beckwith, 2004). Recent studies showed that the FtsQ-FtsB-FtsL complex serves as a signal sensor which promotes cell wall remodeling necessary for cell constriction (Tsang and Bernhardt, 2015). FtsI is a high-molecular-weight transpeptidase that cross-links

glycan strands. The FtsW-FtsI complex is part of the peptidoglycan synthesis machinery, and FtsW, a lipid II flippase, transports the cell wall precursor across the membrane (Mohammadi et al., 2011; Fraipont et al., 2011). FtsN is recruited as the last essential division protein that initiates cell constriction (Vicente and Rico, 2006).

Using a bacterial two-hybrid (B2H) assay, an *E. coli* cell division protein-protein interaction network, the cell division interactome, which included 16 interactions between 10 cell divison proteins, was identified (Karimova et al., 2005; Di Lallo et al., 2003). The cell division interactome of *S. pneumoniae* was also characterized using a combination of B2H and co-immunoprecipitation assays (Maggi et al., 2008). A total of 17 interactions was observed among nine cell division proteins of *S. pneumoniae* which included FtsZ, FtsA, FtsK, DivlB, DivlC, FtsL, FtsW, and PBP2x (Maggi et al., 2008). To date, *E. coli* and *S. pneumoniae* are the only two organisms with characterized cell division interactomes (Karimova et al., 2005; Di Lallo et al., 2003; Maggi et al., 2008).

N. gonorrhoeae is a Gram-negative diplococcus that causes gonorrhea in humans (Tapsall *et al.*, 2009). Previous studies on *N. gonorrhoeae* cell division focused on its Min system which localizes FtsZ to the mid-cell (Salimnia et al., 2000; Ramirez-Arcos et al., 2001b; Szeto et al., 2001). *N. gonorrhoeae* also contains a *dcw* cluster which encodes 5 cell division proteins - FtsZ, FtsA, FtsQ, FtsW, and FtsI (Francis et al., 2000). Other non-*dcw* cluster divisome proteins encoded by *N. gonorrhoeae* include ZipA, FtsK, and FtsN. As compared to *E. coli*, *N. gonorrhoeae* lacks FtsB and FtsL (Francis et al., 2000).

To investigate the cell division interactome in *N. gonorrhoeae*, its cell division protein interactions were identified using a combination of B2H and glutathione S-transferase (GST) pull-down assays, as well as surface plasmon resonance (SPR). We

identified nine interactions among the eight cell division proteins tested. We also identified the subdomains of $FtsA_{Ng}$ involved in its interaction with $FtsQ_{Ng}$, $FtsZ_{Ng}$, $FtsN_{Ng}$, and $FtsW_{Ng}$. Comparison of the cell division interactomes of *E. coli*, *S. pneumoniae* and *N. gonorrhoeae* indicates that *N. gonorrhoeae* possesses a distinctive cell division interactome.

2.3. Materials and methods

2.3.1. Strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 2.1. *E. coli* DH5α and XL1-Blue were used as hosts for cloning. *E. coli* BL21 (DE3) and C41 (DE3) were used as hosts for protein purification. *E. coli* R721 was used in B2H assays (Di Lallo *et al.*, 2001). *E. coli* DH5α, XL-1Blue, BL21(DE3) and C41 (DE3) were grown in Luria-Bertani (LB) medium (BD Difco™, Sparks, MD), for 16-18 hours (hr), at 37 °C. *E. coli* R721 was grown under the same conditions and incubated at 34 °C, as described previously (Di Lallo et al., 2003).

N. gonorrhoeae CH811 was grown on GC medium base agar (GCMB, Oakville, ON), supplemented with Kellogg's defined supplement (GCMBK, 40 g D-glucose, 1 g glutamine, 10 ml of 0.5% ferric nitrate and 1 ml of 20% cocarboxylase), at 35 °C, in a humid environment, with 5% CO_2, for 18 to 24 h (Kellogg *et al.*, 1963).

When required, the following concentrations of antibiotics were added to LB medium: 100 μg/ml ampicillin (Sigma, Oakville, ON) or 50 μg/ml kanamycin (Sigma). For B2H assays, 34 μg/ml chloramphenicol (Sigma), 30 μg/ml kanamycin, and 50μg/ml ampicillin were added to LB medium.

2.3.2. DNA manipulations

N. gonorrhoeae CH811 genomic DNA was purified using a QIAamp® genomic DNA kit (Qiagen, Mississauga, Ontario, Canada). DNA samples were stored at -20°C. Oligonucleotides for polymerase chain reaction (PCR) amplifications were synthesized by Invitrogen (Table 2.2; Burlington, Ontario, Canada). PCRs were performed in a

Table 2.1 Bacterial strains used in this study

Stain	Relevant characteristics	Source/reference
E. coli DH5α	*supE44 ΔlacU169* (*80lacZΔM15*) *hsdR17 endA1 gyrA96 thi-1 relA1*	Gibco
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB laclq ZΔM15*] Tn10	Stratagene
E. coli BL21(DE3)	F^-*, dcm Δ, ompT, hsdS* (r^-_B m^-_B)*, gal, λ(DE3)*	Stratagene
E. coli C41 (DE3)	F^- *ompT hsdSB* (r^-_B m^-_B) *gal dcm (srl-recA)* 306::Tn10 (Tet^r) (*DE3*)	(Miroux and Walker, 1996)
E. coli R721	*supE thy D*(*lac-proAB*) F' [$proAB^+$ $lacI^q$ *lac*ZDM15] *glpT*::O-$_{P434/P22}$*lacZ*	(Di Lallo et al., 2001)
N. gonorrhoeae CH811	Auxotype (A)/serotype (S)/plasmid content (P) class: nonrequiring/IB-2/plasmid-free, Str^r	(Picard and Dillon, 1989)

Table 2.2 Primers designed in this study

Primer name		**Sequences (5'-3')**
P1	FtsA-reBamHI	GCGCGGATCCTCAGAGGTTGTTTTCAATCC
P2	FtsA-fwSalI	GCGCGTCGACCATGGAACAGCAGAAAAGATAC
P3	fwSalI-ftsK	GCGCGTCGACCATGTTTTGGATAGTTTTGATCGTTAT
P4	reBamHI-ftsK	CGCGGGATCCTCAAGCATTGTCCAAGGGGACGAG
P5	fwSalI-ftsQ	GCGCGTCGACCATGTGGGATAATGCCGAAGCGATG
P6	reBamHI-ftsQ	CGCGGGATCCCTATTCTTCGGATTCTTTTTCGGG
P7	fwSalI-ftsI	GCGCGTCGACCATGTTGATTAAAAGCGAATATAAGCC
P8	reBamHI-ftsI	CGCGGGATCCTTAAGACGGTGTTTTGACGGCTGC
P9	fwSalI-ftsW	GCGCGTCGACCATGAAGATTTCGGAAGTATTGGTAAA
P10	reBamHI-ftsW	CGCGGGATCCTTACTCCACCCGGTAACCGCGCAT
P11	fwSalI-ftsN	GCGCGTCGACCATGTTTATGAACAAATTTTCCCAATC
P12	reBamHI-ftsN	CGCGGGATCCTTATTTGCCTTCAATCGCACGGAT
P13	fwBglII-ZipA	GCGCGAGATCTGATGATTTACATCGTACTGTTCCTC

P14	reBamHI-ZipA	CGCGGGATCCTTATGAAAACAGGCGCAGGGC
P15	FtsA-reEcoRI-pET30a	ATATCGAATTCTCAGAGGTTGTTTTCAATCCACC
P16	FtsA-fwBglII-pET30a	AGCCCAGATCTGATGGAACAGCAGAAAAGATACATC
P17	fwBglII-FtsQ- pET30a	AGCCCAGATCTGATGTGGGATAATGCCGAAGCGATG
P18	reEcoRI-ftsQ- pET30a	ATATCGAATTCCTATTCTTCGGATTCTTTTTCGGG
P19	FtsZ-fwBgl II-pET30a	AGCCCAGATCTGATGGAATTTGTTTACGACGTGGCA
P20	FtsZ-ReEcoRI-pET30a	AGCCCGAATTCTTATTTGTCTGAATTGTGTTGACG
P21	fwFtsA-BamHI-GST	CGCGGGATCCATGGAACAGCAGAAAAGATACATC
P22	fwEcoRI-FtsN	GACGAATTCATGTTTATGAACAAATTTTCCCAATCC
P23	reXhoI-FtsN	GACCTCGAGTTATTTGCCTTCAATCGCACG

GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) as follows: 4 minutes (min) at 94°C, 30 cycles of denaturation for 1 min at 94°C, annealing for 4seconds (s) at 55°C, extension for 1.5 mins at 72°C, and 10 mins at 72°C. PCRs were carried out in 100-µl (final volume) mixtures comprising 71.5 µl double-distilled H_2O (ddH_2O), 10 µl of 10x PCR buffer [15 mM $MgCl_2$, 4 µl of 10 mM deoxynucleoside triphosphate (dNTP), 2 µl of each primer (0.2 µg/ml), 0.5 µl of Taq DNA polymerase (5 U/µl; New England BioLabs, Ontario, Canada)], and, 10 µl of purified *N. gonorrhoeae* CH811 genomic DNA suspension.

2.3.3. Bacterial Two-Hybrid assays

The method developed by Di Lallo et al. (2003) was used for all B2H assays. *ftsA*, *ftsK*, *ftsQ*, *ftsI*, *ftsW*, and *ftsN* were amplified from *N. gonorrhoeae* CH811 by PCR using the primer pairs P1/P2, P3/P4, P5/P6, P7/P8, P9/P10, and P11/P12 (Table 2.2), respectively. PCR amplicons were digested with BamHI and SalI and ligated into previously digested pcI_{p22} and pcI_{434} vectors, to produce pcl_{p22}-A, pcI_{p22}-K, pcI_{p22}-I, pcI_{p22}-W, pcI_{p22}-Q, pcI_{p22}-N, pcI_{434}-A, pcI_{434}-K, pcI_{434}-I, pcI_{434}-W, pcI_{434}-Q, and pcI_{434}-N (Table 2.3). $zipA_{Ng}$ was amplified from *N. gonorrhoeae* CH811 genomic DNA using the primer pair P13/P14 (Table 2.2); the PCR amplicon was digested with BglII and BamHI, and ligated into pre-digested pcI_{p22} and pcI_{434} to produce pcI_{p22}-ZipA and pcI_{434}-ZipA. pcI_{p22}-Z and pcI_{434}-Z constructs were generated previously (2007).

The expression of $ftsA_{Ng}$, $ftsZ_{Ng}$ and $zipA_{Ng}$ from B2H constructs was verified by Western blot analysis using appropriate antibodies. These proteins were expressed from the vectors under the conditions tested (data not shown). The expression of these proteins

Table 2.3 Plasmids used in this study

Plasmid	Relevant genotype	Source/Reference
pcI_{p22}	pC132 derivative carrying N-terminal end of P22 repressor	(Di Lallo et al., 2001)
pcI_{434}	pACYC177 derivative carrying N-terminal end of 434 repressor	(Di Lallo et al., 2001)
pcI_{p22}-A	$pcIp_{22}$ derivative carrying the $ftsA_{Ng}$ gene	This study
pcI_{434}-A	pcI_{434} derivative carrying the $ftsA_{Ng}$ gene	This study
pcI_{p22}-K	$pcIp_{22}$ derivative carrying the $ftsK_{Ng}$ gene	This study
pcI_{434}-K	pcI_{434} derivative carrying the $ftsK_{Ng}$ gene	This study
pcI_{p22}-Q	$pcIp_{22}$ derivative carrying the $ftsQ_{Ng}$ gene	This study
pcI_{434}-Q	pcI_{434} derivative carrying the $ftsQ_{Ng}$ gene	This study
pcI_{p22}-I	$pcIp_{22}$ derivative carrying the $ftsI_{Ng}$ gene	This study
pcI_{434}-I	pcI_{434} derivative carrying the $ftsI_{Ng}$ gene	This study
pcI_{p22}-W	$pcIp_{22}$ derivative carrying the $ftsW_{Ng}$ gene	This study

pcI$_{434}$-W	pcI$_{434}$ derivative carrying the *ftsW*$_{Ng}$ gene	This study
pcI$_{p22}$-N	pcIp$_{22}$ derivative carrying the *ftsN*$_{Ng}$ gene	This study
pcI$_{434}$-N	pcI$_{434}$ derivative carrying the *ftsN*$_{Ng}$ gene	This study
pcI$_{p22}$-Z	pcIp$_{22}$ derivative carrying the *ftsZ*$_{Ng}$ gene	(Greco-Stewart et al., 2007)
pcI$_{434}$-Z	pcI$_{434}$ derivative carrying the *ftsZ*$_{Ng}$ gene	(Greco-Stewart et al., 2007)
pcI$_{p22}$-AT1	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-162	(Zou *et al.*, 2017b)
pcI$_{p22}$-AT2	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-194	(Zou et al., 2017b)
pcI$_{p22}$-AT3	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-230	(Zou et al., 2017b)
pcI$_{p22}$-AT4	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 231-301	(Zou et al., 2017b)
pcI$_{p22}$-AT5	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 302-414	(Zou et al., 2017b)
pcI$_{p22}$-AT6	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 351-414	(Zou et al., 2017b)
pcI$_{434}$-AT1	pcI$_{434}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-162	(Zou et al., 2017b)
pcI$_{434}$-AT2	pcI$_{434}$ derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-194	(Zou et al., 2017b)

pcI_{434}-AT3	pcI_{434} derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-230	(Zou et al., 2017b)
pcI_{434}-AT4	pcI_{434} derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 231-301	(Zou et al., 2017b)
pcI_{434}-AT5	pcI_{434} derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 302-414	(Zou et al., 2017b)
pcI_{434}-AT6	pcI_{434} derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 351-414	(Zou et al., 2017b)
pET30a	Kan^R P_{T7}::6Xhis	EMD Millipore, Billerica, MA
pETA	pET30a derivative carrying the *ftsA*$_{Ng}$ gene	This study
pETQ	pET30a derivative carrying the *ftsQ*$_{Ng}$ gene	This study
pETZ	pET30a derivative carrying the *ftsZ*$_{Ng}$ gene	This study
pGEX2T	$Amp^R P_{tac}$::*gst*::*lacIq*	Amersham Bioscience, Uppsala, Sweden
pGEXA	pGEX2T derivative carrying the *ftsA*$_{Ng}$ gene	This study
pGEXN	pGEX2T derivative carrying the *ftsA*$_{Ng}$ gene	This study

indicates that any negative B2H interactions involving them was not a function of lack of expression.

To ascertain what subdomains of $FtsA_{Ng}$ interacted with gonococcal cell division proteins $FtsZ_{Ng}$, $FtsQ_{Ng}$, $FtsW_{Ng}$, or $FtsN_{Ng}$, six previously created truncations of the protein (T1, T2, T3, T4, T5, and T6; Fig. 2.1) were used (Zou et al., 2017b). Plasmid constructs for B2H assays were previously generated (Zou et al., 2017b).

B2H assays were performed as described previously (Di Lallo et al., 2003). This assay is based on the reconstitution of a chimeric repressor that binds to the 434/P22 hybrid operator and represses the expression of a downstream *lacZ* gene in *E. coli* R721. Each gene tested for a potential interaction was cloned into pcI_{p22} and pcI_{434} and recombinant constructs were transformed into *E. coli* R721 either singly or in combination. *N. gonorrhoeae* FtsZ self-interaction was used as positive control. R721 without plasmids and single plasmid transformants were used as negative controls. R721 without plasmids had a β-galactosidase activity of 2504±34 Miller units. The β-galactosidase activity of each combination was compared to that of R721. Values of less than 50% (<1250 Miller Units) of full β-galactosidase activity indicate a positive interaction between two proteins, while values of more than 50% (>1250 Miller Units) indicate a negative interaction. Statistical analyses were performed using the unpaired Student t-test. Standard deviations were determined for the mean value of Miller units where three independent experiments were performed.

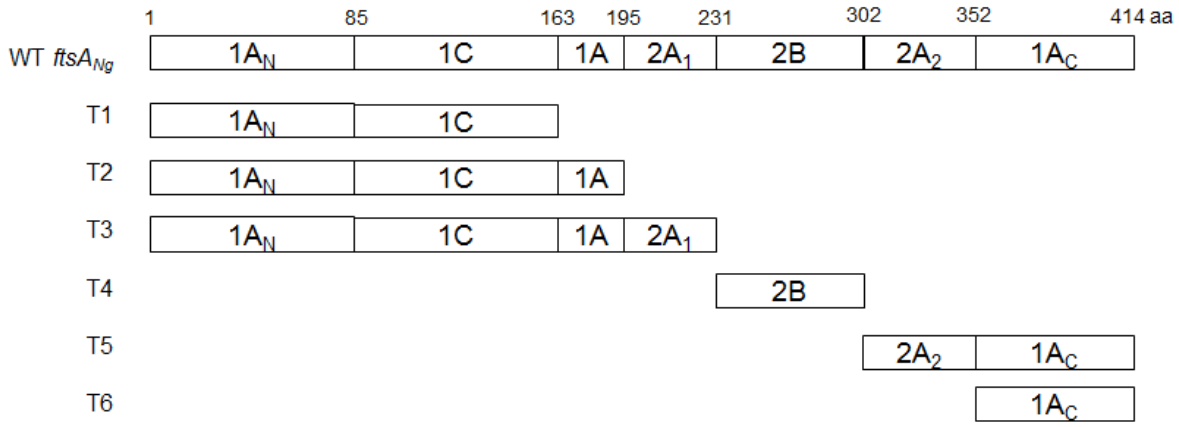

Fig. 2.1. Schematic representation of *N. gonorrhoeae ftsA* and its truncations. T1 (162aa, Met1-Ala162) contained the N-terminal 1A and 1C domains of $ftsA_{Ng}$. T2 (194aa, Met1-Val194) included the N-terminal 1A, 1C and 1A domains of $ftsA_{Ng}$. T3 (230aa, Met1-Ile230) included the N-terminal 1A, 1C, 1A and $2A_1$ domains of $ftsA_{Ng}$. T4 (71aa, Pro231-Glu301) contained the 2B domain of $ftsA_{Ng}$. T5 (114aa, Ile301-Leu414) contained the $2A_2$ and 1A C-terminal domains of $ftsA_{Ng}$. T6 (64aa, Ala351-Leu414) contained the 1A C-terminal domain of $ftsA_{Ng}$.

2.3.4. Construction and purification of His-fusion proteins

For His-fusion constructs, full-length *ftsA*, *ftsQ*, and *ftsZ* were PCR-amplified from *N. gonorrhoeae* CH811 genomic DNA using primer pairs P15/P16, P17/P18 and P19/P20 (Table 2.2), respectively. PCR amplicons were digested with EcoRI and BglII and ligated into pre-digested pET30a, to create pETA, pETQ, and pETZ (Table 2.3). Plasmid pETA was transformed into *E. coli* C41 (DE3) and plasmids pETQ and pETZ were transformed into *E. coli* BL21 (DE3). The overexpression of all fusion proteins was induced with 400µM IPTG, at 30 °C, for 2 hours. Cells were centrifuged at 6000rpm for 10 minutes at 4 °C and resuspended in Binding Buffer (5 mM Imidazole; 0.5 M NaCl; 20 mM Tris-HCl, pH 7.9). Cells were lysed by sonic disruption and centrifuged for 15 minutes at 11 000 *g* at 4 ºC. The supernatant containing His-tagged proteins was purified by metal affinity chromatography on a nickel column (His·Bind Resin; Novagen) including an elution step with 300 mM of imidazole in a 0.5 M NaCl; and 20 mM Tris-HCl (pH 7.9) buffer. Eluted proteins were dialyzed into PBS buffer for long term storage. Integrity and purity of proteins were checked by SDS–PAGE and quantified by the Bradford method with a commercial assay (Bio-Rad).

Purified His-$FtsZ_{Ng}$ was further treated with thrombin protease (EMD Millipore, Billerica, MA), overnight, at 4 °C, to cleave the N-terminal His tag. Thrombin was removed using 100 µl of p-aminobenzamidine-agarose (Sigma #A7155). FtsZ was dialyzed against MES buffer (50mM MES, 300mM KCl, 10mM $MgCl_2$, pH 7.5) prior to use in FtsZ polymerization experiments (Krol and Scheffers, 2013).

2.3.5. GST pull-down assay

For GST fusion constructs, full-length *ftsA* and *ftsN* were PCR-amplified, from *N. gonorrhoeae* CH811, using primer pairs P21/P18 and P22/P23 (Table 2.2), respectively. The *ftsA* amplicon was digested with BamHI and EcoRI and ligated into pre-digested pGEX2T, to create pGEXA (Table 2.3). The *ftsN* amplicon was digested with EcoRI and XhoI and ligated into pre-digested pGEX2T, producing pGEXN (Table 2.3). Plasmids pGEXA and pGEXN were transformed into *E. coli* C41 (DE3) and *E. coli* BL21 (DE), respectively. Overexpression of GST-FtsA and GST-FtsN was accomplished by induction with either 400µM or 800µM of IPTG, respectively, at 30 °C, for 2 hours. Purification of GST-FtsA and GST-FtsN was carried out using GST•Bind™ Resin (EMD Millipore, Billerica, MA), following the manufacturer's instructions.

Purified GST-fusion and His-fusion proteins were incubated with pre-equilibrated GST•Bind™ Resin in phosphate buffered saline (PBS) buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 0.5% Triton-X100, 1mM DTT, pH 7.9) at 4°C overnight. Pre-purified GST was used as a negative control. The pre-bound resin was collected by centrifugation and washed in PBS three times. Bound proteins were dissociated from resin by adding 5X Laemmli buffer, separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and identified by Western blot using polyclonal anti-GST or anti-6×His antibodies (Thermo Scientific; Waltham, MA), sequentially.

For $FtsA_{Ng}$-$FtsZ_{Ng}$ interactions, the GST pull-down assay was performed in MES buffer (50mM MES-NaOH, 50mM KCl, 10mM $MgCl_2$, 0.5% Triton-X100, 1mM ATP, 2mM GTP, pH 7.5) (Krol and Scheffers, 2013). To promote the polymerization of $FtsZ_{Ng}$ necessary for this interaction, $FtsZ_{Ng}$ was treated with 2mM GTP and 1mM ATP, as

described previously (Krol and Scheffers, 2013), before mixing with GST-FtsA$_{Ng}$ and GST•Bind™ Resin.

All GST pull-down assays were performed minimally in duplicate.

2.3.6. FtsZ polymerization assays

FtsZ$_{Ng}$ polymerization was measured by 90° angle light scattering using a Dynapro-MS800 instrument (Protein Solutions) with a wavelength of 310 nm and a slit width of 0.5 mm. MES buffer is optimal for FtsZ polymerization which is required to observe an FtsA-FtsZ interaction (Loose and Mitchison, 2014; Krol and Scheffers, 2013). FtsZ$_{Ng}$ (~6µM) in MES buffer (50mM MES-NaOH, 50mM KCl, 10mM $MgCl_2$, pH 7.5) was injected into a 45 ul quartz cuvette and warmed to 30°C, prior to the measurement. Data were collected, for 4 min, from unpolymerized FtsZ$_{Ng}$ to establish a baseline. GTP was then added to a final concentration of 2mM and data were collected every 5 seconds for 25 min. Data were recorded and analyzed using Dynamics v5 software.

Negative stain electron microscopy was used to visualize FtsZ$_{Ng}$ polymers. 5 µl of FtsZ (6µM) with, or without, GTP (final concentration 2mM) was incubated, at 30°C, for 5 min. The mixture was placed on a carbon-coated copper grid (400 mesh size) for 2 min and then blot dried. The grid containing FtsZ$_{Ng}$ was stained with 1% uranyl acetate, blotted, and air-dried for 3 hours. Polymers were visualized and photographed using a Hitachi transmission electron microscopy HT7700.

2.3.7. Surface plasmon resonance (SPR)

Protein interactions were examined by SPR using a Bio-Rad XPR36 (Bio-Rad Laboratories) instrument and a ProteOn™ HTE Sensor Chip (Bio-Rad Laboratories). The

chip surface was regenerated by injection of 0.5% SDS, 50mM NaOH, 100mM HCl and 300mM EDTA, at a flow rate of 30 µl/min, for 120 seconds. Activation was performed using 500µM of $NiSO_4$.

For $FtsA_{Ng}$-$FtsN_{Ng}$ and $FtsA_{Ng}$-$FtsQ_{Ng}$ SPR experiments, ligands (i.e. His-$FtsN_{Ng}$ for $FtsA_{Ng}$-$FtsN_{Ng}$, and His-$FtsQ_{Ng}$ for $FtsA_{Ng}$-$FtsQ_{Ng}$ interactions) were immobilized onto the sensor chip at a concentration of 200nM. A two-fold dilution series of the analyte ($FtsA_{Ng}$), in PBS buffer with Tween-20 (PBST; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 0.1% BSA, 0.05% Tween-20, pH 7.9), was injected at a flow rate of 30 µl/min over the surface of the chip for 120 seconds. This was followed by an injection of PBST buffer for 300 seconds. Negative controls comprised a reference channel flowed with PBST buffer, and a chip surface immobilized with either $FtsQ_{Ng}$ or $FtsN_{Ng}$ flowed with GST in PBST.

For the $FtsA_{Ng}$-$FtsZ_{Ng}$ interaction, the SPR binding assay was performed using MES buffer, to which 0.1% BSA, 0.05% Tween-20, 1mM ATP were added with the pH adjusted to 7.5. $FtsA_{Ng}$ was immobilized on the chip surface as described above. Each 120-second injection of polymerized $FtsZ_{Ng}$ was followed by an injection of supplemented MES buffer for 300 seconds for dissociation. Negative controls included a reference channel which was flowed with MES buffer containing 2mM GTP, and the $FtsA_{Ng}$-immobilized chip surface flowed with GST in supplemented MES instead of polymerized $FtsZ_{Ng}$.

All SPR data were analyzed with ProteOn Manager™ (Bio-Rad Laboratories). The sensorgram (i.e. a graph of the response unit versus time) was first subtracted by the response units (RU) of the reference channel, with no immobilized ligands, to reduce the

non-specific binding signals between analyte and empty chip surface. Then, the sensorgram was subtracted with the RU signal with running buffer and ligand immobilized on the chip. Association and disassociation constants were obtained using the Langmuir 1:1 kinetic fit model, by nonlinear regression, using ProteOn Manager™. Each protein pair was tested minimally in duplicate.

2.4. Results

2.4.1. Identification of *N. gonorrhoeae* cell division protein interactions by bacterial two-hybrid assay

Using B2H assays, we investigated 28 potential interactions among eight gonococcal divisome proteins including FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsI, FtsW, and FtsN. The results (Table 2.4) show that nine interactions, FtsZ-FtsA, FtsZ-FtsK, FtsZ-FtsW, FtsA-FtsK, FtsA-FtsQ, FtsA-FtsW, FtsA-FtsN, FtsI-FtsW, and FtsK-FtsN, displayed a residual β-galactosidase activity lower than 50%, indicating a positive interaction between these proteins in *N. gonorrhoeae*. The interaction between $FtsA_{Ng}$ and $FtsN_{Ng}$ had the lowest residual β-galactosidase activity (24%), indicating the strongest interaction. This was followed by $FtsA_{Ng}$-$FtsK_{Ng}$ (30%), $FtsN_{Ng}$-$FtsK_{Ng}$ (31%), $FtsI_{Ng}$-$FtsW_{Ng}$ (35%), $FtsZ_{Ng}$-$FtsW_{Ng}$ (39%), $FtsA_{Ng}$-$FtsZ_{Ng}$ (40%), $FtsZ_{Ng}$-$FtsK_{Ng}$ (41%), $FtsA_{Ng}$-$FtsW_{Ng}$ (45%), and $FtsA_{Ng}$-$FtsQ_{Ng}$ (48%) interactions. $ZipA_{Ng}$ did not directly interact with other cell division proteins as the residual β-galactosidase activity of all interactions was above 50% (Table 2.4).

2.4.2. GST pull-down of $FtsA_{Ng}$-$FtsQ_{Ng}$, $FtsA_{Ng}$-$FtsZ_{Ng}$ and $FtsA_{Ng}$-$FtsN_{Ng}$ interactions

To confirm the results of the selected B2H assays, we examined several interactions (i.e. $FtsQ_{Ng}$-$FtsA_{Ng}$, $FtsA_{Ng}$-$FtsN_{Ng}$, $FtsA_{Ng}$-$FtsZ_{Ng}$) using GST pull-down assays. GST pull-down results (Fig. 2.2A) showed that His-$FtsQ_{Ng}$ was pulled down by GST-$FtsA_{Ng}$, but not GST itself (negative control), indicating an interaction between $FtsA_{Ng}$ and $FtsQ_{Ng}$.

Table 2.4 Interactions between eight cell division proteins in *N. gonorrhoeae* as determined by B2H assay.

pcI$_{P22}$	**pcI$_{434}$**						
	ZipA	FtsZ	FtsA	FtsK	FtsI	FtsQ	FtsW
FtsZ	71±2.62%	28±0.27%					
FtsA	77±1.67%	40±2.46%*					
FtsK	78±2.45%	41±2.06%*	30±2.53%*				
FtsI	83±3.21%	91±3.92%	83±3.10%	76±2.00%			
FtsQ	100±4.04%	73±2.15%	48±2.59%NS	88±3.09%	90±2.82%		
FtsW	87±2.95%	39±2.66%*	45±3.29%*	100±2.62%	32±1.31%*	100±4.72%	
FtsN	100±2.78%	76±1.25%	24±1.66%*	31±0.67%*	100±4.82%	88±3.21%	97±2.10%

By comparison to positive controls (*E. coli* R721 without plasmids), interactions with less than 50% of residual ß-galactosidase activity (framed) were considered as positive, $FtsZ_{Ng}$ self-interaction was used as a positive control. The numbers represent percentage of mean ß-galactosidase activity ± standard deviation*: Statistically significant ($P \leq 0.05$); NS: not statistically significant ($P > 0.05$).

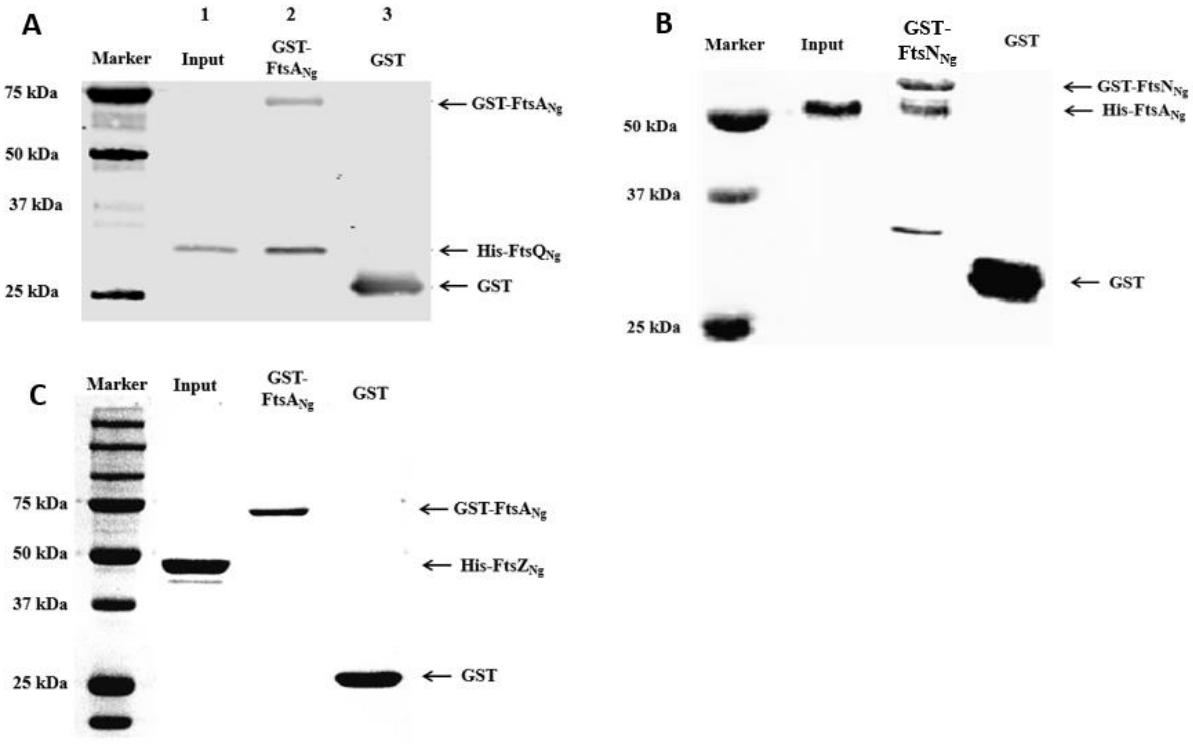

Fig. 2.2 Interactions of $FtsA_{Ng}$ with $FtsQ_{Ng}$, $FtsN_{Ng}$ and $FtsZ_{Ng}$ by GST pull-down. (A): GST pull down result between His-$FtsQ_{Ng}$ and GST-$FtsA_{Ng}$. Lane 1: His-$FtsQ_{Ng}$ input; Lane 2: GST-$FtsA_{Ng}$ and His-$FtsQ_{Ng}$ mixture; Lane 3: GST and His-$FtsQ_{Ng}$ mixture; (B): GST pull down result between His-$FtsA_{Ng}$ and GST-$FtsN_{Ng}$. Lane 1: His-$FtsA_{Ng}$ input; Lane 2: GST-$FtsN_{Ng}$ and His-$FtsA_{Ng}$ mixture, GST-$FtsN_{Ng}$ was loaded with GST and GST-$FtsN_{Ng}$ degradation products; Lane 3: GST and His-$FtsA_{Ng}$ mixture; (**C):** GST pull down result between His-$FtsZ_{Ng}$ and GST-$FtsA_{Ng}$. Lane 1: His-$FtsZ_{Ng}$ input; Lane 2: GST-$FtsA_{Ng}$ and His-$FtsZ_{Ng}$ mixture; Lane 3: GST and His-$FtsZ_{Ng}$ mixture; His-tagged fusion proteins were visualized using anti-6×His antibody; GST and GST-tagged fusion proteins were visualized using anti-GST antibody.

Using similar evaluation criteria, we ascertained that His-$FtsA_{Ng}$ was pulled down by GST-$FtsN_{Ng}$, indicating an interaction between these two proteins (Fig. 2.2B).

The interactions of $FtsA_{Ng}$ and $FtsZ_{Ng}$ from *E. coli in vitro* requires the presence of both ATP and GTP (Loose and Mitchison, 2014). GTP promotes FtsZ polymerization, and ATP is necessary for FtsA to interact with FtsZ, but not for FtsZ polymerization (Pichoff and Lutkenhaus, 2005; Beuria et al., 2009). The presence of $FtsZ_{Ng}$ polymers in MES buffer was determined by transmission electron microscopy (TEM) and dynamic light scattering (DLS; Fig. 2.3). GST pull-down assay did not detect an interaction between $FtsA_{Ng}$ and $FtsZ_{Ng}$ in the presence of 1mM ATP and 2mM GTP (Fig. 2.2C). This result was unexpected, given our B2H results and the commonality of FtsA-FtsZ interaction in other bacterial species (Di Lallo et al., 2003; Maggi et al., 2008; Jensen et al., 2005; Yan *et al.*, 2000), as ascertained by different *in vivo* assays (i.e. B2H, yeast two-hybrid, chemical cross-linking with co-immunoprecipitation).

2.4.3. Surface plasmon resonance evaluation of $FtsA_{Ng}$-$FtsQ_{Ng}$, $FtsA_{Ng}$-$FtsZ_{Ng}$ and $FtsA_{Ng}$-$FtsN_{Ng}$ interactions

Surface plasmon resonance (SPR) was used to confirm selected gonococcal cell division protein-protein interactions in real-time. SPR was used to evaluate the interactions of $FtsA_{Ng}$ with $FtsZ_{Ng}$ because of the conflicting results observed with B2H and GST pull-down assays. GTP was added to promote $FtsZ_{Ng}$ polymerization (Fig. 2.3). The sensorgram indicated that $FtsZ_{Ng}$ interacted with $FtsA_{Ng}$ at concentrations of 6µM and 12µM (Fig. 2.4A), but not at concentrations lower than 6µM (data not shown). Kinetic analysis showed that the $FtsA_{Ng}$-$FtsZ_{Ng}$ interaction had a slow association ($ka=3.56 \times 10^{2}\ M^{-1}s^{-1}$) and a significant disassociation activity ($kd=5.31 \times 10^{-3}\ s^{-1}$), giving a KD value of 14.9µM. This

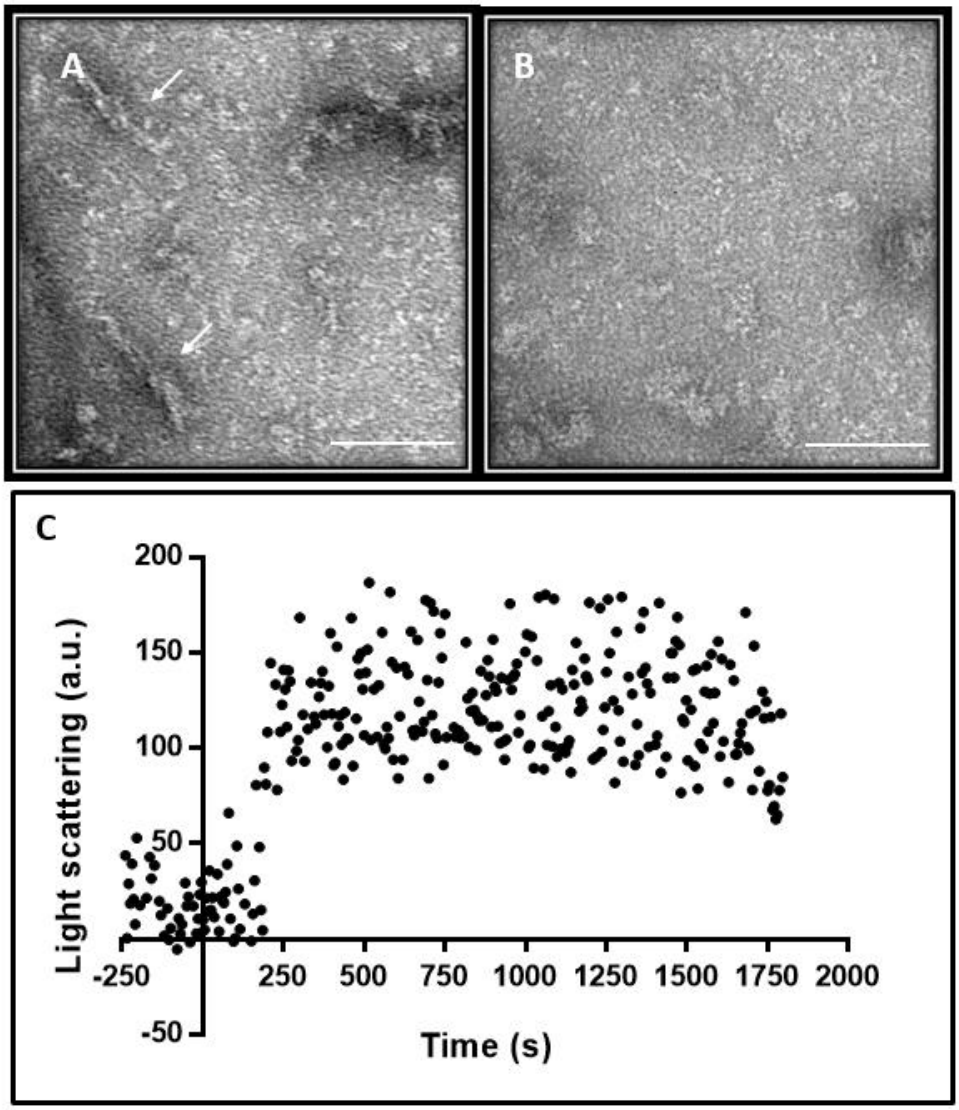

Fig. 2.3 $FtsZ_{Ng}$ polymerization assays. $FtsZ_{Ng}$ polymers visualized by transmission electron microscope with(A) or without (B) 2 mM GTP in MES buffer (50mM MES-NaOH, 50mM KCl, 10mM $MgCl_2$, pH 7.5) at 30°C. Solid arrows indicate $FtsZ_{Ng}$ polymers. Scale bar indicates 100 nm. (C) Light scattering of $FtsZ_{Ng}$ polymerization (6 μM) in MES buffer.

suggested that the interaction between $FtsA_{Ng}$ and $FtsZ_{Ng}$ was likely transient. When GTP was absent from the $FtsZ_{Ng}$ protein solution, no binding was detected between $FtsA_{Ng}$ and $FtsZ_{Ng}$ (data not shown). The sensorgram of the interaction between $FtsA_{Ng}$ and the negative control (GST) also showed no binding activity (Fig. 2.4B), indicating the specificity of the SPR results for the interaction of $FtsA_{Ng}$ with $FtsZ_{Ng}$.

For the SPR analysis of the $FtsA_{Ng}$-$FtsQ_{Ng}$ interaction, $FtsA_{Ng}$ was tested using various concentrations (from 31.25 nM to 250 nM; Fig. 2.4C). The association of $FtsA_{Ng}$ and $FtsQ_{Ng}$ was observed immediately following injection of the $FtsA_{Ng}$ solution onto the $FtsQ_{Ng}$-labeled chip surface, with a rapid increase of response units (ka=2.72 x 10^5 $M^{-1}s^{-1}$; Fig. 2.4C). This indicated a fast binding event between the two proteins. Disassociation between $FtsA_{Ng}$ and $FtsQ_{Ng}$ was not significant (kd=4.09 x $10^{-}3$ s^{-1}), suggesting this interaction was strong and stable (KD=15.1nM). The negative control, using non-interacting GST, did not cause any change in the response units (Fig. 2.4D).

The $FtsA_{Ng}$-$FtsN_{Ng}$ interaction was observed with an increasing concentration of $FtsA_{Ng}$ (62.5nM, 125nM, 250nM and 500nM; Fig. 2.4E). His-$FtsN_{Ng}$ had a binding affinity (KD) of 53.3nM with $FtsA_{Ng}$. The association and disassociation constants were 1.15 x 10^5 $M^{-1}s^{-1}$, and 6.16 x 10^{-3} s^{-1}, respectively (Fig. 2.4E), indicating a strong interaction between $FtsA_{Ng}$ and $FtsN_{Ng}$. The injection of non-interacting GST onto the $FtsN_{Ng}$ immobilized chip surface did not cause any change in the response units (Fig. 2.4F).

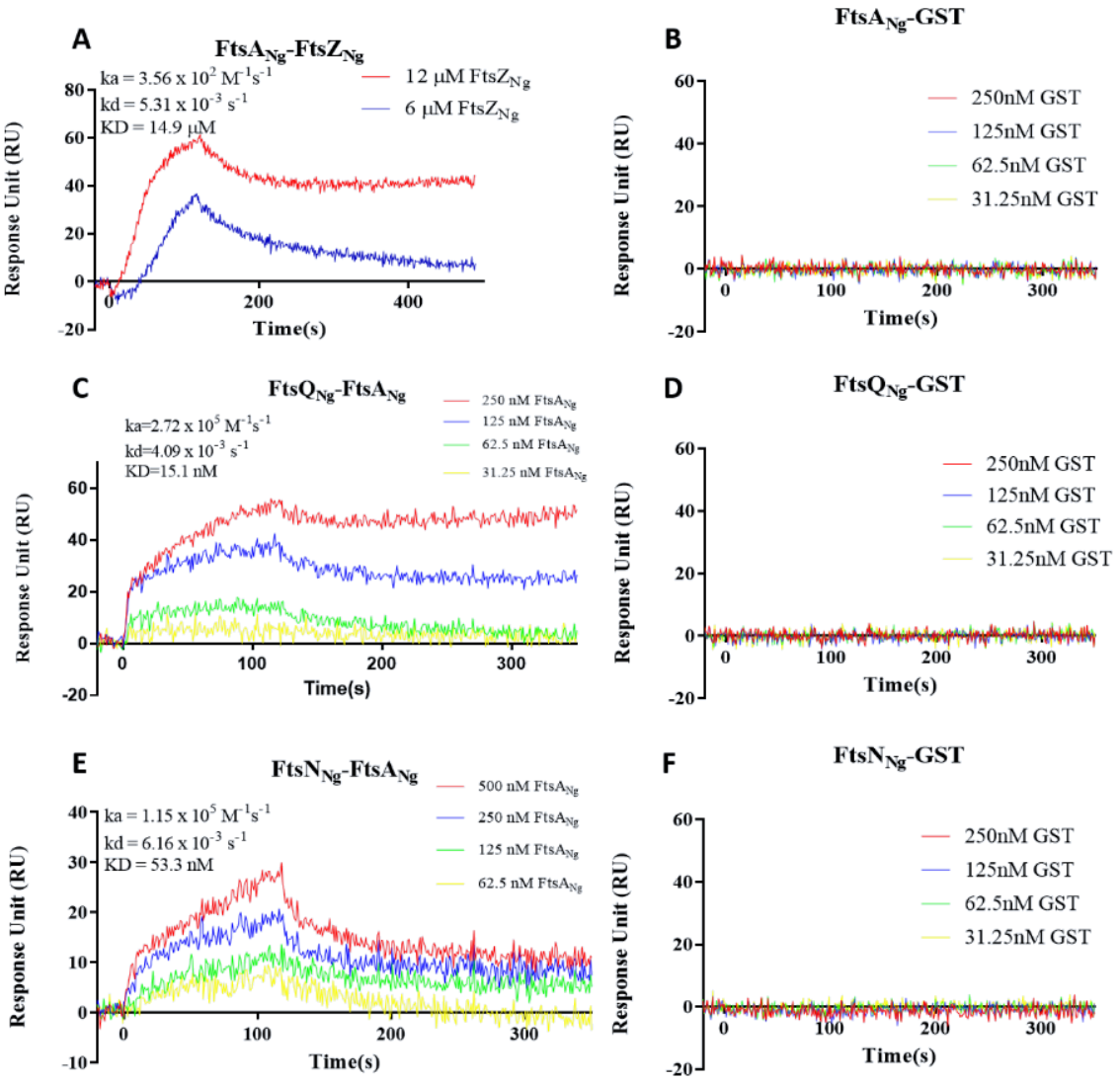

Fig. 2.4 SPR measurement for *N. gonorrhoeae* FtsA-FtsZ, FtsQ-FtsA and FtsA-FtsN interactions. (A) 6 and 12 µM of $FtsZ_{Ng}$ were analyzed for interaction with $FtsA_{Ng}$; (B) Negative interaction between $FtsA_{Ng}$ and GST; (C) $FtsA_{Ng}$ at different concentrations (31.25, 62.5, 125 and 250 nM) were measured for binding affinity to $FtsQ_{Ng}$; (D) Negative interaction between $FtsQ_{Ng}$ and GST; (E) $FtsN_{Ng}$ at different concentrations (62.5, 125, 250 and 500 nM) was analyzed for interaction with $FtsA_{Ng}$; (F) Negative interaction between $FtsN_{Ng}$ and GST. Association and disassociation constants were obtained using the Langmuir 1:1 kinetic fit model by nonlinear regression using ProteOn Manager™ (Bio-Rad Laboratories).

2.4.4. The 2A and 2B subdomains of $FtsA_{Ng}$ interacts with $FtsZ_{Ng}$, $FtsN_{Ng}$, $FtsW_{Ng}$ and $FtsQ_{Ng}$

Since $FtsA_{Ng}$ interacted with $FtsZ_{Ng}$, $FtsQ_{Ng}$, $FtsW_{Ng}$, and $FtsN_{Ng}$, we further examined the interaction regions of $FtsA_{Ng}$ with these four proteins using B2H assays. Based on $FtsA_{Ng}$ homology modeling, six $FtsA_{Ng}$ truncations (T1-T6) were created (Fig. 2.1), which contained one or more $FtsA_{Ng}$ subdomains (Zou et al., 2017b). $FtsZ_{Ng}$ self-interaction was used as a positive control. And negative controls included *E. coli* R721 without plasmids or carrying each single recombinant B2H vector in which the gene of interest had been cloned. $FtsA_{Ng}$ truncations T3, T4, and T5 interacted with $FtsZ_{Ng}$ and $FtsN_{Ng}$ (Fig. 2.5 & 2.6, blue bars). $FtsA_{Ng}$ truncations T1, T2, and T6 did not show an interaction with these proteins (Fig. 2.5 & 2.6, green bars). The T4 and T5 truncations included the 2B and $2A_2$ subdomains of $FtsA_{Ng}$, suggesting that these subdomains of $FtsA_{Ng}$ interacted with both $FtsZ_{Ng}$ and $FtsN_{Ng}$. The T3 construct also contained the $2A_1$ subdomain of $FtsA_{Ng}$, as compared to truncations T1 and T2, indicating that this subdomain was also involved in interactions with $FtsZ_{Ng}$ and $FtsN_{Ng}$. $FtsQ_{Ng}$ interacted only with the T4 and T5 truncations of $FtsA_{Ng}$ (Fig. 2.7, blue bars), indicating that the 2B and $2A_2$ subdomains, but not the $2A_1$ subdomain, were required for the $FtsA_{Ng}$-$FtsQ_{Ng}$ interaction. Only the T5 truncation of $FtsA_{Ng}$ interacted with $FtsW_{Ng}$, suggesting that $2A_2$ subdomain was involved in the interaction with $FtsW_{Ng}$ (Fig. 2.8). In summary, these results showed that the $2A_1$, $2A_2$ and 2B subdomains of $FtsA_{Ng}$ are required for its interaction with $FtsN_{Ng}$ and $FtsZ_{Ng}$. The $FtsA_{Ng}$ $2A_2$ and 2B subdomains are required for interaction with $FtsQ_{Ng}$, and the $2A_2$ subdomain is involved in the interaction with $FtsW_{Ng}$.

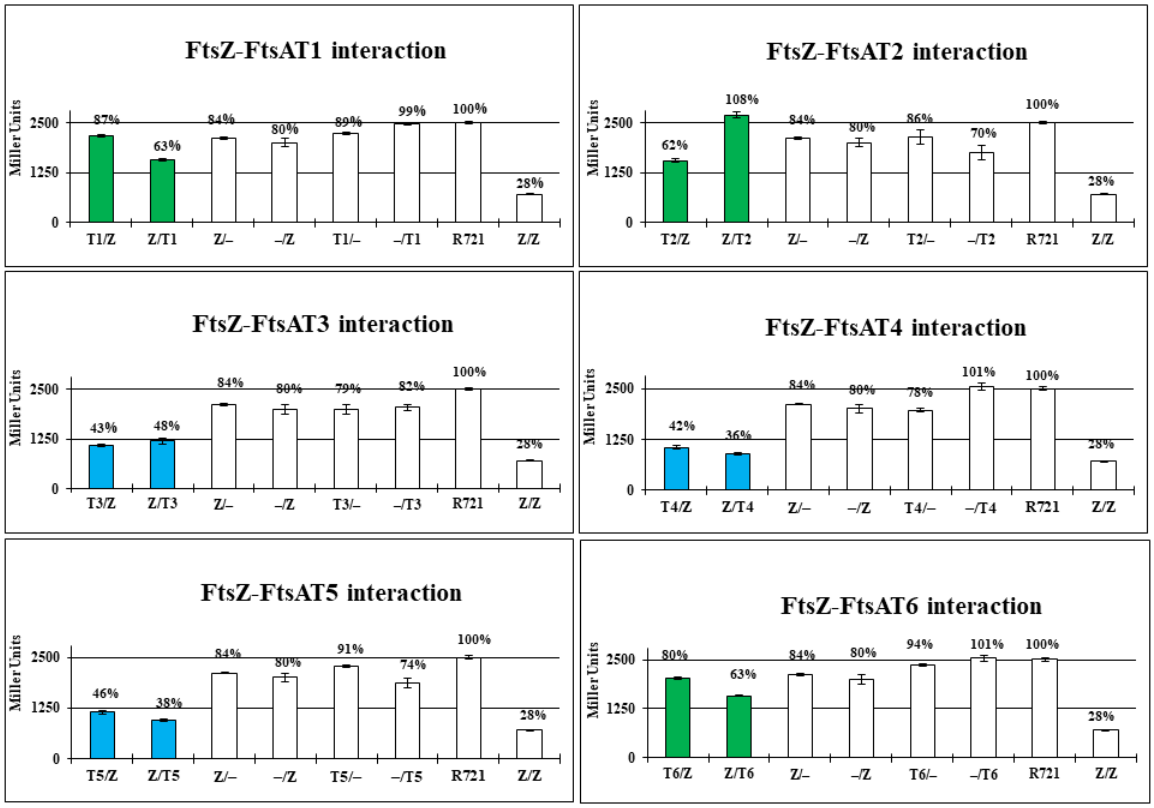

Fig. 2.5 Interactions between $FtsA_{Ng}$ truncations (T1, T2, T3, T4, T5 and T6) and $FtsZ_{Ng}$ (Z) by B2H assays. R721 without plasmids and single transformants were used as negative controls. R721 without plasmids had a β-galactosidase activity of 2504±34 Miller units. $FtsZ_{Ng}$ self-interaction was used as a positive control. Values of less than 50% (<1250 Miller Unites) indicate a positive interaction between two proteins (blue bars) while values of more than 50% (>1250 Miller Unites) indicate a negative interaction (green bars).

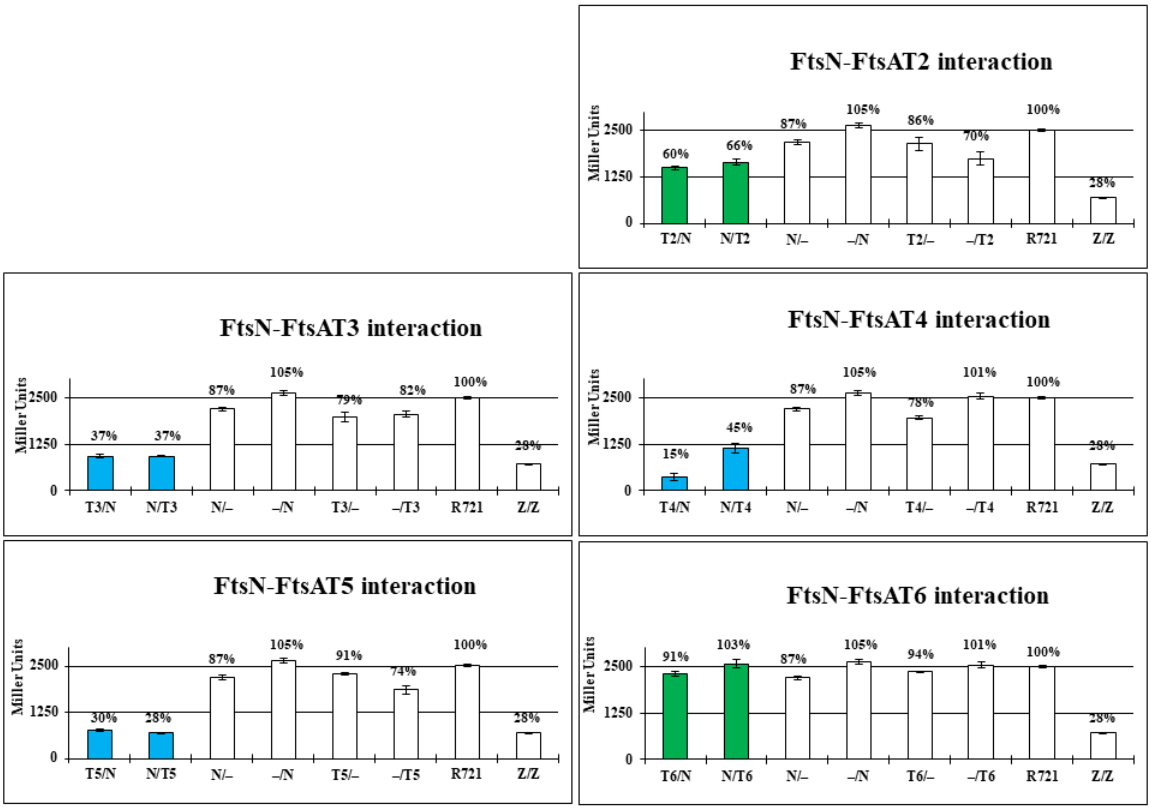

Fig. 2.6 Interactions between $FtsA_{Ng}$ truncations (T2, T3, T4, T5 and T6) and $FtsN_{Ng}$ (N) by B2H assays. Values of less than 50% (<1250 Miller Unites) indicate a positive interaction (blue bars) while values of more than 50% (>1250 Miller Unites) indicate a negative interaction (green bars).

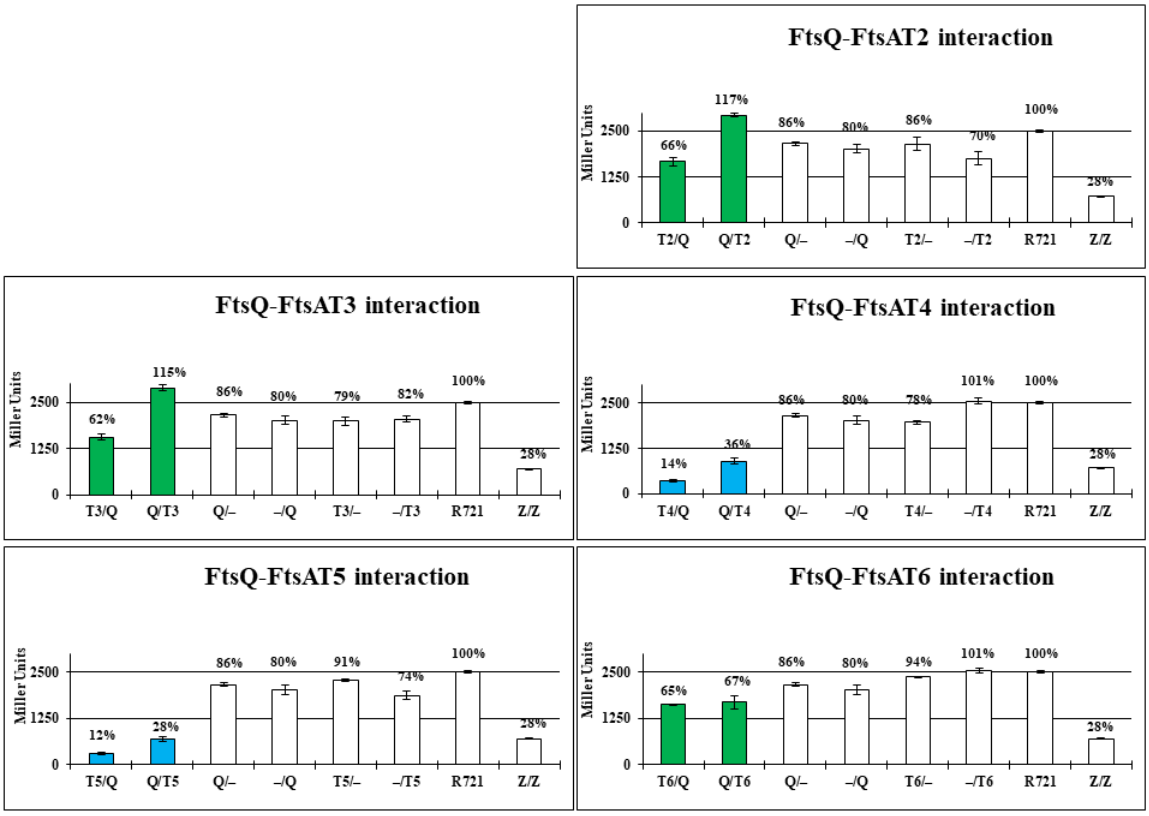

Fig. 2.7 Interactions between $FtsA_{Ng}$ truncations (T2, T3, T4, T5 and T6) and $FtsQ_{Ng}$ (Q) by B2H assays. Values of less than 50% (<1250 Miller Unites) indicate a positive interaction between two proteins (blue bars) while values of more than 50% (>1250 Miller Unites) indicate a negative interaction between the two proteins (green bars).

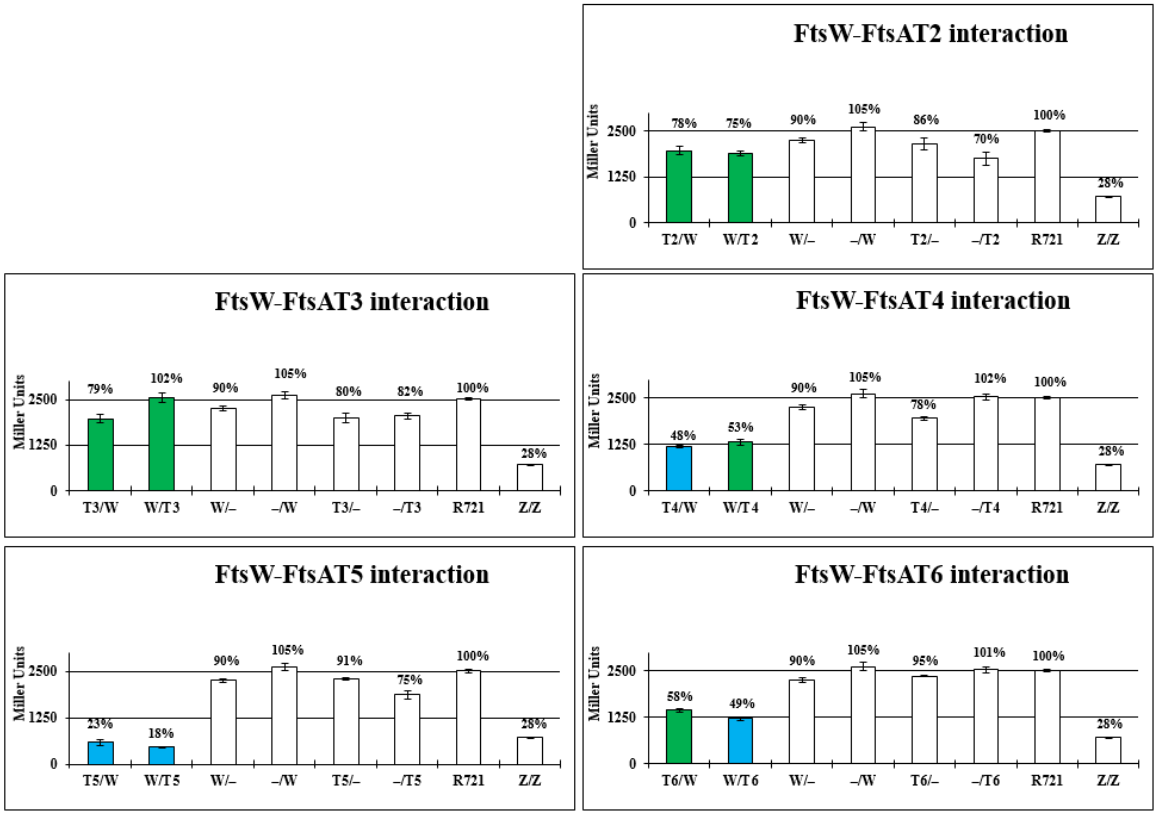

Fig. 2.8 Interactions between $FtsA_{Ng}$ truncations (T2, T3, T4, T5 and T6) and $FtsW_{Ng}$ (W) by B2H assay. Values of less than 50% (<1250 Miller Units) indicate a positive interaction between two proteins (blue bars) while values of more than 50% (>1250 Miller Units) indicate a negative interaction (green bars).

2.5. Discussion

The *N. gonorrhoeae* cell division interactome described in our study is the third cell division interaction network identified in bacteria, in addition to *E. coli* and *S. pneumoniae* (Fig. 2.9A) (Karimova et al., 2005; Di Lallo et al., 2003; Maggi et al., 2008). Compared to the other two interactomes (Fig. 2.9B and C), fewer interaction protein pairs are identified in *N. gonorrhoeae* (Fig. 2.9A). Only nine interactions are present among the eight divisome proteins tested in *N. gonorrhoeae*, while *E. coli* and *S. pneumoniae* have 21 and 17 interactions among ten and eight divisome proteins, respectively (Di Lallo et al., 2003; Maggi et al., 2008).

The development of all three cell division interactomes was based on interaction data obtained from the same B2H system (Di Lallo et al., 2003; Maggi et al., 2008) The *E. coli* interactome was developed using B2H results exclusively and the *S. pneumoniae* study also applied co-immunoprecipitation to verify selected B2H positive interaction pairs (Di Lallo et al., 2003; Maggi et al., 2008). In our study, we used a combination of GST pull-down and surface plasmon resonance to further study selected positive B2H interactions.

Two interactions, FtsA-FtsZ and FtsZ-FtsK, are conserved in the cell division interactomes of *N. gonorrhoeae*, *E. coli* and *S. pneumoniae* (Fig. 2.9, red lines). The FtsA-FtsZ interaction is a common interaction in prokaryotes (Di Lallo et al., 2003; Maggi et al., 2008; Yan et al., 2000; Gamba et al., 2009; Din *et al.*, 1998). Both our B2H and SPR results confirmed this interaction in *N. gonorrhoeae*. A proper ratio between FtsA and FtsZ is crucial for the interaction in *E. coli* (Rueda et al., 2003) and our SPR results support this finding; $FtsA_{Ng}$ interacts with $FtsZ_{Ng}$ only when its concentration is higher than 6 μM (Fig.

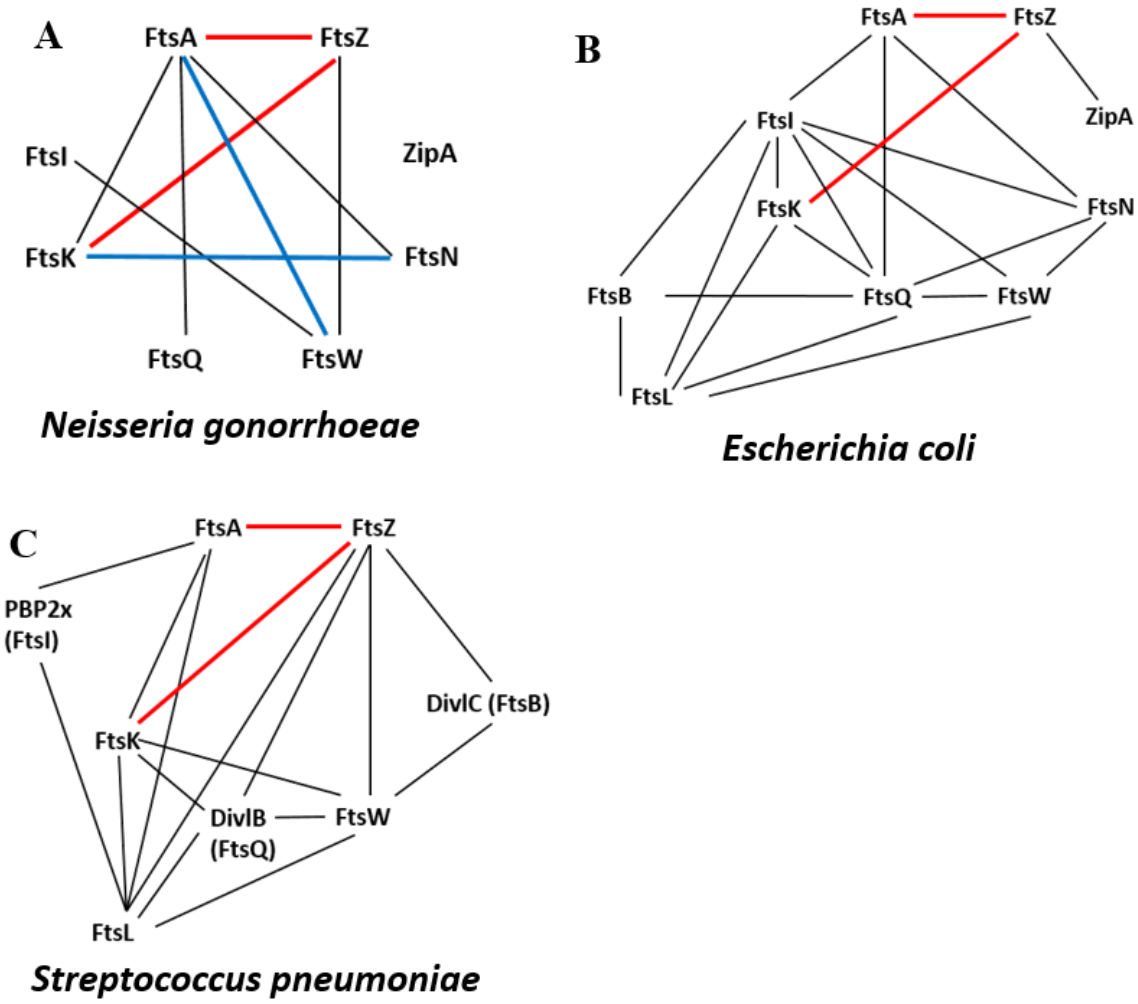

Fig. 2.9 Cell division interactomes of A) *N. gonorrhoeae*, B) *E. coli* (Karimova et al., 2005; Di Lallo et al., 2003), and C) *S. pneumoniae* (Maggi et al., 2008)[8]. Red lines indicate common interactions; blue lines indicate unique interactions in *N. gonorrhoeae*.

2.4B), indicating that the interaction requires a critical concentration threshold. Our SPR results further showed that interaction between $FtsA_{Ng}$ and $FtsZ_{Ng}$ was transient, a result warranting further study to fully understand its implications for divisome formation in *N. gonorrhoeae*. Unexpectedly, the GST pull-down assay, an *in vitro* assay, did not detect an $FtsA_{Ng}$-$FtsZ_{Ng}$ interaction. We believe that this "false negative" *in vitro* result was caused by the requirement of a membrane/solid surface support for the interaction to anchor FtsA (Loose and Mitchison, 2014; Osawa *et al.*, 2009; Arumugam *et al.*, 2012).

The interaction of FtsZ with FtsK has been observed in *N. gonorrhoeae, E. coli, S. pneumoniae, B. subtilis* and *C. crescentus* (Di Lallo et al., 2003; Maggi et al., 2008; Wang *et al.*, 2006; Biller and Burkholder, 2009). The C-terminus of FtsK is required for proper DNA segregation in *E. coli* (Sherratt *et al.*, 2010). The absence of an FtsZ-FtsK interaction in both *E. coli* and *C. crescentus* caused abnormal chromosome segregation and cell filamentation (Wang et al., 2006; Grenga et al., 2008). This suggests that the FtsZ-FtsK interaction connects the cell division process with chromosome segregation, by ensuring that the replicated chromosome is cleared from the division site.

The FtsA-FtsW interaction has been observed only in *N. gonorrhoeae* (Fig. 2.9, blue lines). Since FtsW is a membrane protein and difficult to purify, we did not verify the interaction by GST pull-down and SPR assays. However, we performed additional B2H assays to identify which subdomains of FtsA were involved in its interaction with FtsW and showed that the $2A_2$ subdomain of FtsA strongly interacts with FtsW (Fig. 2.8). FtsW, an inner membrane protein, is required in *E. coli* for the recruitment of FtsI and the translocation of the cell well precursor, lipid II (Mohammadi et al., 2011; Fraipont et al., 2011; Mercer and Weiss, 2002; Mohammadi et al., 2014). An FtsI-FtsW protein interaction

has been observed in *E. coli*, *Streptomyces coelicolor*, and *Mycobacterium tuberculosis* (Fraipont et al., 2011; Mistry *et al.*, 2008; Datta *et al.*, 2006). Interestingly, we discovered that $FtsI_{Ng}$ only interacts with $FtsW_{Ng}$, suggesting that its localization may depend on this protein.

The importance of the unique $FtsK_{Ng}$-$FtsN_{Ng}$ interaction in *N. gonorrhoeae*, as determined by B2H, is not clear (Fig. 2.9, blue lines). In *E. coli*, FtsN is the last protein, of ten essential cell division proteins, recruited to the division site to initiate cell constriction (Gerding et al., 2009; Rico *et al.*, 2010). A previous study suggested that *E. coli* FtsN and FtsK stabilize the Z-ring cooperatively, without direct interactions (Goehring et al., 2007). Since the FtsK-FtsN interaction is present in *N. gonorrhoeae*, their joint involvement in gonococcal cell division requires further investigation.

$ZipA_{Ng}$ did not interact with any other tested gonococcal cell division protein. In *E. coli*, ZipA only interacts with FtsZ, and is required for downstream protein recruitment, including FtsK, FtsQ, FtsL, and FtsN (Di Lallo et al., 2003; Hale and de Boer, 2002). One report suggested that $ZipA_{Ng}$ is a homologue of the *E. coli* protein with high similarity in its key domains (Du and Arvidson, 2003). Although $ZipA_{Ng}$ complemented a conditional *zipA* mutant in *E. coli*, it did not fully restore a wildtype phenotype in this strain (Du and Arvidson, 2003). Given these data, the role of ZipA in gonococcal cell division remains to be elucidated.

In *N. gonorrhoeae*, the existence of $FtsL_{Ng}$ is unclear due to its low homology with *E. coli* FtsL (Snyder et al., 2001). An open reading frame (ORF) located between *mraW* and *ftsI* in the *dcw* cluster of *N. gonorrhoeae* was reported by Francis et al. (Francis et al., 2000) and they reported that it was not a coding ORF. Snyder et al. (2001) named the same

ORF *ftsL*. Because this ORF shares only 17% amino acid similarity to its *E. coli* homologue, we considered that it was not a functional ORF and did not test its interaction with other gonococcal cell division proteins.

N. gonorrhoeae lacks FtsB (Francis et al., 2000); thus, the protein complex FtsQ-B-L, present in other species, such as *E. coli*, *S. pneumoniae* and *B. subtilis*, would not be formed in *N. gonorrhoeae* (Daniel et al., 2006; Noirclerc-Savoye et al., 2005; Robichon *et al.*, 2008). This protein complex has been described as a bridge connecting FtsK and the FtsI-FtsW complex in *E. coli* (Buddelmeijer and Beckwith, 2004). A recent study suggests that the *E. coli* FtsQ-B-L complex acts as a signal transmitter for cell wall remodeling and constriction, which is mediated by direct interactions with the FtsI-W complex and FtsN (Tsang and Bernhardt, 2015). In *S. pneumoniae*, the FtsQ homologue, DivIB, interacts with $FtsK_{Sp}$, $FtsL_{Sp,}$ and $FtsW_{Sp}$ (Maggi et al., 2008). Interestingly, our B2H data show that $FtsQ_{Ng}$ only interacts with $FtsA_{Ng}$, suggesting that the function of $FtsQ_{Ng}$ in cell division in *N. gonorrhoeae* may be distinct.

There are several models for bacterial cell constriction. One *E. coli* model suggests that the force that drives constriction comes from septal peptidoglycan synthesis (Eswara and Ramamurthi, 2017). In this model, the $FtsA_{Ec}$-$FtsN_{Ec}$ interaction activates peptidoglycan synthesis by direct or indirect interaction with $FtsI_{Ec}$ (Liu et al., 2015). Another *E. coli* model suggests that the energy generated from FtsZ-mediated GTP hydrolysis drives cell constriction (Osawa et al., 2009). We observed an $FtsA_{Ng}$-$FtsN_{Ng}$ interaction in *N. gonorrhoeae*. However, there is no further evidence supporting either model of cell constriction in *N. gonorrhoeae* at this time.

The non-essential proteins, $FtsE_{Ng}$ and $FtsX_{Ng}$, are also implicated in cell division in *N. gonorrhoeae* (Bernatchez et al., 2000). Similarly, in *E. coli*, FtsE and FtsX are non-essential for cell division under conditions of high osmotic pressure (Corbin *et al.*, 2007). Gonococcal FtsE and FtsX have high similarity in amino acid sequence to known homologues in other species (Bernatchez et al., 2000). In *E. coli*, the interaction between FtsE and FtsZ has a regulatory effect on the Z-ring (Corbin et al., 2007). Future research could focus on revealing the effects of $FtsE_{Ng}$ and $FtsX_{Ng}$ on cell division in *N. gonorrhoeae*.

The major issue interpreting B2H assay results is the empirical cut-off of 50% residual ß-galactosidase activity used to discriminate positive and negative interactions. In particular, values close to the cut-off could be interpreted as either false positive or negative results. To validate our B2H results, we used other B2H interactions to test which subdomains of $FtsA_{Ng}$ interacted with $FtsZ_{Ng}$, $FtsN_{Ng}$, and $FtsQ_{Ng}$. We determined that the 2A and 2B subdomains of $FtsA_{Ng}$ interacted with $FtsZ_{Ng}$, $FtsQ_{Ng}$, and $FtsN_{Ng}$. We also evaluated some positive interactions obtained by B2H using SPR and GST pull-down assays. The SPR method detects and measures weak or transient interactions, in real-time, with high sensitivity (Ngounou Wetie *et al.*, 2013). The SPR method showed a transient $FtsA_{Ng}$-$FtsZ_{Ng}$ interaction. GST pull-down assays, on the other hand, are ideal in detecting strong protein-protein interactions, as weak interactions may dissociate during the assay (Bruckner *et al.*, 2009). We consider this to be a reasonable explanation for our failure to confirm when the interaction of $FtsA_{Ng}$ with $FtsZ_{Ng}$ when using a GST pull-down assay.

To date, most studies on cell division have been focused on model organisms (i.e. the Gram-negative rod *E. coli* and the Gram-positive rod *B. subtilis*) due to the abundant availability of tools for genetic manipulation (Eswara and Ramamurthi, 2017). Research

on cell division in non-model organisms is expanding, and this includes studies with *N. gonorrhoeae* (Francis et al., 2000; Salimnia et al., 2000). For example, *Chlamydia trachomatis*, which lacks FtsZ, requires an actin-like protein, MreB, for cell division (Liechti *et al.*, 2016). A gene cluster encoding three cell division proteins, named MldA, MldB, and MldC, were identified only in *Clostridium difficile* and its closely related bacteria (Ransom *et al.*, 2014). Results from studies using non-model organisms suggest that cell division mechanisms are complex and vary in different organisms, reflecting vast biological diversity.

2.6. Conclusions

In our research, we discovered that nine interactions among eight cell division proteins defined the cell division interactome of *N. gonorrhoeae*. In comparison with the published cell division interactomes of *E. coli* and *S. pneumoniae*, FtsA-FtsZ and FtsZ-FtsK interactions were common to all three bacteria. FtsK-FtsN and FtsA-FtsW interactions were only present in *N. gonorrhoeae*, suggesting that they play different roles in the cell division of this microorganism. $ZipA_{Ng}$ did not interact with any other cell division proteins tested in this study, indicating that its role may differ as compared to its *E. coli* homologue. We also determined that the subdomains of $FtsA_{Ng}$ which interacted with $FtsQ_{Ng}$, $FtsZ_{Ng}$, $FtsW_{Ng}$, or $FtsN_{Ng}$, differed from its *E. coli* homologue. This suggests that *N. gonorrhoeae* possesses a distinctive cell division interactome, and likely a different mechanism of cell division as compared to *E. coli* and other organisms.

Chapter 3 An *Escherichia coli* expression model reveals the species-specific function of FtsA from *Neisseria gonorrhoeae* in cell division

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Running title: Expression of *gonococcal ftsA in E. coli*

3.1. Abstract

Escherichia coli (Ec) has been used to study the function of cell division proteins from different microorganisms, especially when genetic tools are limited for studying these proteins in their native hosts. The expression of *ftsA* from *N. gonorrhoeae* (Ng) disrupted cell division in *E. coli* resulting in a significant increase in cell length. In some cells, $FtsA_{Ng}$ localized to the division site and the poles of *E. coli* cells, but the majority of cells showed no specifical localization. $FtsA_{Ng}$ did not complement an *E. coli ftsA* mutant strain. Bacterial two-hybrid and GST pull-down assays indicated that $FtsA_{Ng}$ interacted with $FtsN_{Ec,}$ but no other cell division proteins from *E. coli*. This interaction was mediated through the 2A and 2B subdomains of $FtsA_{Ng.}$ This evidence suggests that the function of $FtsA_{Ng}$ is species-specific.

Keywords: FtsA; protein interaction; cell division; *Neisseria gonorrhoeae*; divisome; *E. coli*

3.2. Introduction

FtsA, an essential bacterial cell division protein, is a member of the actin/Hsp70/sugar kinase ATPase superfamily (Sánchez et al., 1994). It shares a universal structure containing 1A, 1C, 2A and 2B subdomains (Fig. S1A; van den Ent and Lowe, 2000; Fujita et al., 2014). In *Escherichia coli* (Ec), the 1C domain of $FtsA_{Ec}$ is necessary for self-interaction, polymerization, membrane association and late divisome protein recruitment (Rico et al., 2004; Krupka et al., 2014). The 2B subdomain is required for the interaction with the cell division protein $FtsZ_{Ec}$ (Pichoff and Lutkenhaus, 2007). In *E. coli* $FtsA_{Ec}$ interacts with several cell division proteins including $FtsZ_{Ec}$, $FtsQ_{Ec}$, $FtsI_{Ec}$, $FtsN_{Ec}$ and $FtsA_{Ec}$ itself (Karimova et al., 2005; Di Lallo et al., 2003). Normal cell division in *E. coli* is driven by the formation of a Z-ring at the division site (Lutkenhaus and Addinall, 1997). This is followed by the recruitment of other essential proteins which form the divisome including FtsA, ZipA, FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI and FtsN (Errington et al., 2003; Goehring et al., 2006; Grenga et al., 2013). FtsN, the last protein recruited to the divisome, initiates FtsZ-ring constriction (Goehring et al., 2006; Gerding et al., 2009; Rico et al., 2010). The focus of our present study is FtsA from *Neisseria gonorrhoeae* (Ng) for which there is published information about interactions with other proteins.

E. coli has been used as a host for studying the impacts of heterologous proteins on cell division often because the functionality of the protein in question is difficult to evaluate in its native host. Expression of several heterologous cell division genes in *E. coli* often produce a filamentous cell phenotype, including *Mycoplasma pulmonis* FtsZ (Wang and Lutkenhaus, 1996), *Bacillus subtilis* FtsZ (Beall *et al.*, 1988), *Chlamydia trachomatis* FtsQ (Ouellette et al., 2015) and *C. crescentus* FtsN (Moll and Thanbichler, 2009). These

heterologous cell division proteins were not able to complement mutations in their corresponding *E. coli* homologues, indicating their species-specific functions. However, not all cell division proteins have species-specific functionality. For example, *ftsL* from *Bacillus licheniformis* can complement a *Bacillus subtilis ftsL* null strain (Sievers and Errington, 2000). An *E. coli* model has also been used to reveal the subcellular architecture and function of *Neisseria gonorrhoeae* Min proteins (Ramirez-Arcos et al., 2002; Ramirez-Arcos et al., 2001b). A filamentous phenotype caused by overexpression of $minC_{Ng}$ and successful complementation of an *E. coli* MinC mutant by $MinC_{Ng}$ suggested that *E. coli* can be a suitable model for investigating function and localization of gonococcal cell division proteins.

The role of FtsA from various species (e.g. *Agrobacterium tumefaciens*, *Rhizobium meliloti, Staphylococcus aureus*) has been investigated using *E. coli* as an expression host (Ma *et al.*, 1997; Yan et al., 2000). Here, we report on the functional characterization of FtsA from *N. gonorrhoeae* exploiting an *E. coli* expression model for its advantages in cell size, easy visualization and relevant genetic tools. We determined that $FtsA_{Ng}$, disrupted cell division in *E. coli* cells and increased cell length. $FtsA_{Ng}$ did not complement an *E. coli ftsA* mutant strain. Using bacterial two-hybrid (B2H) and GST pull-down assays, $FtsA_{Ng}$ did not interact with any other divisome protein of *E. coli*, except FtsN. These results indicate that $FtsA_{Ng}$ is not a strong inhibitor of cell division in *E. coli* and has species-specific functionality.

3.3. Materials and methods

3.3.1. Strains and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 3.1. *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco; Franklin Lakes, NJ). The following antibiotics were added to the medium as appropriate: ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}), and chloramphenicol (30 μg mL^{-1}). *N. gonorrhoeae* was grown on GC medium base agar (Difco; Franklin Lakes, NJ) supplemented with Kellogg's defined supplement (Kellogg et al., 1963).

3.3.2. Plasmids construction for microscopy, bacterial two hybrid (B2H) analysis and GST pull-down assay

For $FtsA_{Ng}$ localization studies, $ftsA_{Ng}$ was PCR amplified from *N. gonorrhoeae* CH811 with primers P1/P2 (Table 3.2), PCR amplicon was digested with EcoRI and BamHI and ligated into EcoRI/BamHI-digested pDSW209 [which contained GFP derived from pGFPmut2 (Cormack *et al.*, 1996)], creating pDSW209-A_{Ng} (Table 3.1).

For complementation assays, primers P3/P4 were used to PCR amplify $ftsA_{Ec}$ from *E. coli* PB103 (Table 3.2). The amplicon was was digested with BamHI and SalI and ligated into BamHI/SalI-digested pDSW209 (Weiss *et al.*, 1999), yielding pDSW209-A_{Ec} (Table 3.1).

$ftsZ_{Ec}$, $ftsA_{Ec}$, $ftsQ_{Ec}$, $ftsN_{Ec}$, and $ftsI_{Ec}$ were PCR amplified from *E. coli* PB103 using primers P5/P6, P7/P8, P9/P10, P11/P12, and P13/P14, respectively (Table 3.2). The PCR products were digested with SalI/BamHI and ligated into pre-digested B2H vectors to create the plasmids listed in Table 3.1. The $ftsQ_{Ec}$ amplicon was digested with SalI and

Table 3.1. Bacterial strains and plasmids

Strain	**Relevant characteristics**	**Source/reference**
E. coli XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB laclq ZΔM15] Tn10	Stratagene
E. coli C41 (DE3)	F- ompT hsdSB (r-B m-B) gal dcm Δ (srl-recA) 306::Tn10 (Tetr) (DE3)	(Miroux and Walker, 1996)
E. coli BL21 (DE3)pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) pLysS(cm^R)	(Rico et al., 2004)
E. coli PB103	dadRI trpE61 trpA62 tna-5 purB+	(de Boer *et al.*, 1988)
E. coli P163	CH2 (recA::Tn10 ftsA0)/pDB280 (repAts ftsA+)	(Hale and De Boer, 1999)
E. coli R721	71/18 glpT::O-P434/p22 lacZ	(Di Lallo et al., 2001)
N. gonorrhoeae CH811	Auxotype/serovar/plasmid content class: nonrequiring NR/IB-2/plasmid-free	(Picard and Dillon, 1989)
Plasmid	**Relevant genotype**	**Source/Reference**
pDSW209	pGFPmut2 derivative carrying the *gfp* gene	(Weiss et al., 1999)
pDSW209-A_{Ec}	*ftsA*$_{Ec}$ in pDSW209	This study
pDSW209-A_{Ng}	*ftsA*$_{Ng}$ in pDSW209	This study

pSEB306-D242E	*ftsA*$_{Ec}$D242E in pSEB306	(Pichoff and Lutkenhaus, 2007)
pcIp$_{22}$	pC132 derivative carrying N-terminal end of P22 repressor	(Di Lallo et al., 2001)
pcI$_{434}$	pACYC177 derivative carrying N-terminal end of 434 repressor	(Di Lallo et al., 2001)
pcIp22-A	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene	This study
pcIp22-AT1	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-162	This study
pcIp22-AT2	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-194	This study
pcIp22-AT3	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-230	This study
pcIp22-AT4	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 231-301	This study
pcIp22-AT5	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 302-414	This study
pcIp22-AT6	pcIp22 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 351-414	This study
pcI434-A	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene	This study

pcI434-AT1	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-162	This study
pcI434-AT2	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-194	This study
pcI434-AT3	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 1-230	This study
pcI434-AT4	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 231-301	This study
pcI434-AT5	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 302-414	This study
pcI434-AT6	pcI434 derivative carrying the *ftsA*$_{Ng}$ gene fragment encoding amino acids 351-414	This study
pcIp$_{22}$-Z	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ng}$ gene	(Greco-Stewart et al., 2007)
pcI$_{434}$-Z	pcI$_{434}$ derivative carrying the *ftsZ*$_{Ng}$ gene	(Greco-Stewart et al., 2007)
pcIp$_{22}$-Z$_{Ec}$	pcIp$_{22}$ derivative carrying the *ftsZ*$_{Ec}$ gene	This study
pcI$_{434}$-Z$_{Ec}$	pcI$_{434}$ derivative carrying the *ftsZ*$_{Ec}$ gene	This study
pcIp$_{22}$-A$_{Ec}$	pcIp$_{22}$ derivative carrying the *ftsA*$_{Ec}$ gene	This study
pcI$_{434}$-A$_{Ec}$	pcI$_{434}$ derivative carrying the *ftsA*$_{Ec}$ gene	This study

pcIp$_{22}$-Q$_{Ec}$	pcIp$_{22}$ derivative carrying the *ftsQ*$_{Ec}$ gene	This study
pcI$_{434}$-Q$_{Ec}$	pcI$_{434}$ derivative carrying the *ftsQ*$_{Ec}$ gene	This study
pcIp$_{22}$-N$_{Ec}$	pcIp$_{22}$ derivative carrying the *ftsN*$_{Ec}$ gene	This study
pcI$_{434}$-N$_{Ec}$	pcI$_{434}$ derivative carrying the *ftsN*$_{Ec}$ gene	This study
pcIp$_{22}$-I$_{Ec}$	pcIp$_{22}$ derivative carrying the *ftsI*$_{Ec}$ gene	This study
pcI$_{434}$-I$_{Ec}$	pcI$_{434}$ derivative carrying the *ftsI*$_{Ec}$ gene	This study
pET30a	Kan^{R} P$_{T7}$::6×his	Novagen
pETZ	Kan^{R} P$_{T7}$::*ftsZ*$_{Ng}$-6×His	This study
pETA	Kan^{R} P$_{T7}$::*ftsA*$_{Ng}$-6×His	This study
pGEX2T	Amp^{R} P^{tac}::*gst*::*lacI*^{q}	Amersham
pGEXA	Amp^{R} P$_{tac}$::*gst-ftsA*$_{Ng}$::*lacI*^{q}	This study
pMGV1	Kan^{R} P$_{T7}$::*ftsN*$_{Ec}$-6×His	(Rico et al., 2004)
pETAT2	KanR P$_{T7}$::*ftsA*$_{Ng}$ (T2)-6×His	This study
pETAT3	KanR P$_{T7}$::*ftsA*$_{Ng}$ (T3)-6×His	This study

pETAT4	KanR P_{T7}::*ftsA*$_{Ng}$ (T4)-6×His	This study
pETAT5	KanR P_{T7}::*ftsA*$_{Ng}$ (T5)-6×His	This study
pETAT6	KanR P_{T7}::*ftsA*$_{Ng}$ (T6)-6×His	This study
pGEXN$_{Ec}$	Amp^R P_{tac}::*gst-ftsN*$_{Ec}$::*lacI*^{q}	This study

BglII and ligated into each pre-digested B2H vector, yielding pcIp22-Q_{Ec} and pcIp434-Q_{Ec} (Table 3.1). pcI_{p22}-Z and pcI_{434}-Z plasmids were previously constructed (Greco-Stewart et al., 2007).

The construction of $FtsA_{Ng}$ truncations was based on structure homology modeling of FtsA from *T. maritima* (Fig. 2.1)(van den Ent and Lowe, 2000). Six truncated $ftsA_{Ng}$, covering different regions of $FtsA_{Ng}$ (Fig. 2.1), were PCR amplified from *N. gonorrhoeae* CH811 with the following primers (Table 3.2): T1- P20/P22; T2- P20/P23; T3- P20/P24; T4- P25/P26; T5- P25/P27; T6- P25/P21. Amplicons were with SalI and BamHI and ligated into B2H vectors to produce various recombinant plasmids (Table 3.1). pcI_{p22}-Z and $pcIp_{434}$-Z plasmids were previously constructed (Greco-Stewart et al., 2007).

For GST pull-down assays, pGEXA and $pGEXN_{Ec}$ (Table 3.1) were constructed by amplifying $ftsA_{Ng}$ and $ftsN_{Ec}$ from *N. gonorrhoeae* CH811 and *E. coli* PB103 with primers P15/P16 and P18/19 (Table 3.2). PCR amplicons were digested with BamHI/EcoRI and ligated into pre-digested pGEX2T plasmids.

Truncated $ftsA_{Ng}$ (T2, T3, T4, T5 and T6) were amplified from *N. gonorrhoeae* CH811 with the following primers: T2-P17/P29; T3-P17/P30; T4-P31/P32; T5-P33/P15; T6-P34/P15 (Table 3.2). Amplicons were digested with BglII and EcoRI, followed by ligation into pET30a, yielding pETAT2, pETAT3, pETAT4, pETAT5, and pETAT6 (Table 3.1), respectively.

Gene integrity and fidelity for all constructs were verified by DNA sequence analysis (Eurofins MWG Operon; Louisville, KY).

Table 3.2. Primers designed in this study

Primer name		Sequences (5'-3')
P1	fwEcoRI-ftsA$_{Ng}$-GFP	CGCCGGAATTCATGGAACAGCAGAAAAGATACATC
P2	reBamHI-ftsA$_{Ng}$-GFP	CCCGGGGATCCTCAGAGGTTGTTTTCAATCCACC
P3	fwBamHI-ftsA$_{Ec}$-GFP	CGCGGGATCCATGATCAAGGCGACGGACAGAAAA
P4	reSalI-ftsA$_{Ec}$-GFP	GCGCGTCGACTTAAAACTCTTTTCGCAGCCAACT
P5	fwSalI-ftsZ$_{Ec}$-B2H	GCGCGTCGACCATGTTTGAACCAATGGAACTTACC
P6	reBamHI-ftsZ$_{Ec}$-B2H	CGCGGGATCCTTAATCAGCTTGCTTACGCAGGAA
P7	fwSalI-ftsA$_{Ec}$-B2H	GCGCGTCGACCATGATCAAGGCGACGGACAGAAAA
P8	reBamHI-ftsA$_{Ec}$-B2H	CGCGGGATCCTTAAAACTCTTTTCGCAGCCAACT
P9	fwSalI-FtsQ$_{Ec}$-B2H	CGGTCGACCATGTCGCAGGCTGC
P10	reBglII-FtsQ$_{Ec}$-B2H	GAAGATCTTCATTGTTGTTCTGCCTGTG
P11	fwSal-FtsN$_{Ec}$-B2H	GCGTCGACAGTGGCACAACGAGATTATG
P12	reBamHI-FtsN$_{Ec}$-B2H	CGGGATCCTCAACCCCCGGCGGCG
P13	fwSal-FtsI$_{Ec}$-B2H	GCGTCGACCATGAAAGCAGCGGCGAAAAC

P14	reBamHI-FtsI$_{Ec}$-B2H	CGGGATCCTTACGATCTGCCACCTGTC
P15	fwFtsA-BamHI-GST	CGCGGGATCCATGGAACAGCAGAAAAGATACATC
P16	FtsA-reEcoRI-pET30a	ATATCGAATTCTCAGAGGTTGTTTTCAATCCACC
P17	FtsA-fwBglII-pET30a	AGCCCAGATCTGATGGAACAGCAGAAAAGATACATC
P18	fwBamHI-ftsN$_{Ec}$-GST	GCGCGGATCCATGGCACAACGAGATTATGTACG
P19	reEcoRI-ftsN$_{Ec}$-GST	GCGCGAATTCTCAACCCCCGGCGGC
P20	fwSalI-ftsA$_{Ng}$-B2H	GCGCGTCGACCATGGAACAGCAGAAAAGATAC
P21	reBamHI-ftsA$_{Ng}$-B2H	GCGCGGATCCTCAGAGGTTGTTTTCAATCC
P22	reBamHI-ftsAT1$_{Ng}$-B2H	CGCGGGATCCTCATGCACCGGTAATGATGTGCACCCG
P23	reBamHI-ftsAT2$_{Ng}$-B2H	CGCGGGATCCTCACACCGCCTGCCCGCTTGCCAACGG
P24	reBamHI-ftsAT3$_{Ng}$-B2H	CGCGGGATCCTCAAATGACGGACGTATGGCGGATGGC
P25	fwSalI-ftsAT4$_{Ng}$-B2H	GCGCGTCGACCCCGGCCGGTGGTAATCTGATTACC
P26	reBamHI-ftsAT4$_{Ng}$-B2H	CGCGGGATCCTCACTCCTGAATACGTGCGCTGATGAT
P27	fwSalI-ftsAT5$_{Ng}$-B2H	GCGCGTCGACCATTTTTGGCGTAGTGCTGGGCGAACTGCAA
P28	fwSalI-ftsAT6$_{Ng}$-B2H	GCGCGTCGACCGCGCCCCAAGAAATGGGCGGTTTGTCCGAC

P29	reEcoRI-ftsA$_{Ng}$T2-pET30a	ATATCGAATTCTCACACCGCCTGCCCGCTTGCCAACGG
P30	reEcoRl-ftsA$_{Ng}$T3-pET30a	ATATCGAATTCTCAAATGACGGACGTATGGCGGATGGC
P31	fwBglll-ftsA$_{Ng}$T4-pET30a	AGCCCAGATCTGCCGGCCGGTGGTAATCTG ATTACC
P32	reEcoRI-ftsA$_{Ng}$T4-pET30a	ATATCGAATTCTCACTCCTGAATACGTGCGCTGATGAT
P33	fwBglll-ftsA$_{Ng}$T5-pET30a	AGCCCAGATCTGATTTTTGGCGTAGTGCTGGGCGAACTGCAA
P34	fwBglll-ftsA$_{Ng}$T6-pET30a	AGCCCAGATCTGGCGCCCCAAGAAATGGGCGGTTTGTCCGAC

3.3.3. Protein purification

pGEXA and pGEXN$_{Ec}$ were transformed into *E. coli* BL21 (DE3) pLysS for expression of *gst-gstA*$_{Ng}$ and *gst-gstN*$_{Ec}$, respectively. Cells were grown in LB medium supplemented with ampicillin at 37 ºC overnight followed by a 1:100 dilution into a fresh LB medium until the OD_{600} reached 0.4. The expression of both fusion proteins was induced with 400 µM IPTG for two hours 37 ºC. Cells were harvested by centrifugation and resuspended in GST Bind/Wash Buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2 PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3). Cells were lysed by sonic disruption and centrifuged for 15 minutes at 11 000 *g* at 4 ºC. The soluble fusion proteins in the supernatant were applied on a GST column (GST·Bind Resin; Novagen) and purified with 10 mM reduced glutathione. Purified proteins were further dialyzed into PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.9) for long term storage. Integrity and purity of proteins were checked by SDS–PAGE and quantified by the Bradford method with a commercial assay (Bio-Rad).

His-FtsN$_{Ec}$ was purified using conditions described by Rico *et al* (2004). *E. coli* BL21(DE3) pLysS with pMGV1 plasmid (containing *6xhis-ftsN*$_{Ec}$) were grown at 37 ºC in LB medium supplemented with kanamycin and chloramphenicol until the OD_{600} reached 0.4. Overexpression of the fusion protein was induced with 2 mM IPTG for two hours. Cells were harvested by centrifugation and resuspended in Binding Buffer (5 mM Imidazole; 0.5 M NaCl; 20 mM Tris-HCl, pH 7.9) supplemented with 1% Triton X100. Cells were lysed by sonic disruption and centrifuged for 15 minutes at 11 000 *g* at 4 ºC. Pellets containing the His-FtsN$_{Ec}$ were resuspended in Binding Buffer supplemented with 1% Triton X-100 and 6 M Urea followed by centrifugation for 10 minutes at 11 000 *g* at 4

ºC. The denatured His-tagged proteins in the supernatant were purified by metal affinity chromatography on a nickel column (His·Bind Resin; Novagen) including an elution step with 300 mM of imidazole in a 0.5 M NaCl; 6 M Urea and 20 mM Tris-HCl (pH 7.9) buffer. Eluted proteins were dialyzed into PBS buffer to refold denatured His-$FtsN_{Ec}$. Integrity and purity of proteins were checked by SDS–PAGE and quantified by the Bradford method with a commercial assay (Bio-Rad).

The condition for overexpression of *his-ftsA*$_{Ng}$ and its truncations was the same as for GST fusion proteins. His-tagged fusion proteins were purified under denaturing conditions (6M urea) following the manufacturer's instructions (Novagen). Purified proteins were renatured by dialysis with a step gradient of imidazole (0.3M, 0.1M and 0M) and urea (4M, 2M, 1M and 0M).

3.3.4. Production of anti-$FtsA_{Ng}$ antibodies

Female New Zealand White rabbits were injected with 300 μm of purified $FtsA_{Ng}$ in Freund's adjuvant (Sigma; v/v=1:1) and boosted once on day 21 after the initial injection (Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan). The antiserum was tested for anti-$FtsA_{Ng}$ antibody using Western blot assays and purified as described previously (Ramirez-Arcos et al., 2001b).

3.3.5. Microscopy

The morphology of *E. coli* PB103 harboring pDSW209-A_{Ng} (Table 3.1) was ascertained using an Olympus BX61 microscope (Olympus Canada Inc.) as described previously (Ramirez-Arcos et al., 2001b). The average length between IPTG induced and

non-induced samples was statistically analyzed using the unpaired Student's *t*-test ($P < 0.05$).

For the localization of $FtsA_{Ng}$ in *E. coli*, samples were prepared as described previously (Ramirez-Arcos et al., 2001b) with an additional staining step using ProLong® Diamond anti-fade mountant with DAPI (Thermo Fisher Scientific; Lenexa, KS). Images were captured with a Leica TCS SP5 confocal microscope (Leica Microststems Inc; Buffalo Grove, IL) with a fluorescein isothiocyanate filter set for GFP signal and a 49,6-diamidino-2-phenylindole (DAPI) filter for nucleoid visualization. Deconvolution and image analysis was done as described previously (Ekanayake *et al.*, 2015).

3.3.6. Complementation of *E. coli* P163

P163 is a *E. coli ftsA* mutant that grows at 30ºC (permissive temperature) and 42ºC (non-permissive temperature) with $ftsA_{Ec}$ compensation. Plasmids pDSW209-A_{Ec}, pDSW209-A_{Ng} and pSEB306-D242E were transformed into *E. coli* P163 (Hale and De Boer, 1999), individually. Overnight cultures of *E. coli* P163 harboring the indicated plasmids were diluted 1:100 and either incubated at 30ºC or 42ºC for 2 hours. 20 µl of serial 10-fold dilutions was spotted onto LB agar at each concentration of IPTG and incubated overnight at either 30 ºC or 42 ºC. Growth was examined after 14-18 hours. The experiment was replicated 3 times.

3.3.7. Bacterial two-hybrid assays

B2H assays were performed as described previously (Di Lallo et al., 2003). This assay is based on the reconstitution of a chimeric repressor that binds to the 434/P22 hybrid operator and represses the expression of a downstream *lacZ* gene in *E. coli* R721. Each

gene tested for a potential interaction was cloned into pcI_{p22} and pcI_{434} and recombinant constructs were transformed into *E. coli* R721 either singly or in combination and grown in the LB medium with appropriate antibiotics for 14-16 hours at 37 °C. *N. gonorrhoeae* FtsZ self-interaction was used as positive control. R721 without plasmids and single plasmid transformants were used as negative controls. Overnight cultures were then diluted 1:100 into fresh LB medium and incubated for 1 hour at 37 °C. Gene expression was induced by IPTG at a concentration of 0.1mM for 2 hours. Cells were then placed on ice for 10 minutes and approximately 1.5 ml of total cell culture was harvested by centrifugation at 6000 rpm for 10 minutes. Cell density was standardized to an OD_{600} of 0.4 by resuspending cell pellets in Z-buffer (60 mM Na_2HP0_4, 40 mM $NaH_2P0_4H_20$, 2 mM KCl, 1 mM $MgS0_4$-$7H_20$, 0.28% β-mercaptoethanol, pH 7). Cell resuspension was further diluted at a ratio of 1:10 into Z-buffer followed by addition of 0.1 ml of chloroform and 0.05 ml of 0.1% SDS to permeabilize the cells. Cells were vortexed and incubated for 5 minutes at room temperature. To start the reaction, 0.2 ml of 4 mg/ml) of ONPG in 0.1 M phosphate buffer (pH 7) was added into permeabilized cells and time is counted as 0. Reaction was kept undergoing at room temperature until a sufficient yellow color appeared, whereas controls which did not contain cells stayed transparent. The reaction was stopped by addition of 0.5 ml of Na_2CO_3 into the reaction the stop time was immediately recorded. Cells were centrifuged for 5 minutes at 12000 rpm and the OD_{420} and OD_{550} of the supernatant were measured. The average β-galactosidase activity was calculated using the equation described by Miller (1972). R721 without plasmids had a β-galactosidase activity of 2504±34 Miller units. The β-galactosidase activity of each combination was compared to that of R721. Values of less than 50% (<1250 Miller Units) indicate a positive interaction

between two proteins, while values of more than 50% (>1250 Miller Units) indicate a negative interaction. Statistical analyses were performed using the unpaired Student t-test. Standard deviations were determined for the mean value of Miller units where three independent experiments were performed.

3.3.8. GST pull-down assays

Both GST-$FtsA_{Ng}$ and His-$FtsN_{Ec}$ were incubated with pre-equilibrated GST•Bind™ Resin (Novagen) in PBS buffer at 4 ºC overnight. Pre-purified GST was used as a negative control in the pull-down assay. The pre-bound resin was collected by centrifugation at 800 g for 2 minutes and washed with PBS buffer three times. Bound proteins were dissociated by adding 5X Laemmli buffer and were analyzed using 10% SDS-PAGE and Western blotting with polyclonal anti-GST and anti-6×His antibodies (Thermo Scientific; Waltham, MA).

3.4. Results

3.4.1. Expression of *gfp-ftsA$_{Ng}$* disrupts *E. coli* cell division

E. coli PB103 harbouring pDSW209-A$_{Ng}$ exhibited typical *E. coli* short rod morphology with no IPTG induction (Fig. 3.1A). When 350 cells were randomly measured, the average length of uninduced control cells was 3.75 ± 0.05328 μm. By contrast, the average cell length of 350 induced cells excluding long filaments (>8 μm) was 3.95 ± 0.05819 and this was statistically significant (P = 0.0124; Fig. 3.1B). Only 9 (2.5%) of 350 induced cells produced long filaments (i.e >8 μm); 5 (1.4%) were extremely filamentous with average cell length of 48.65 ± 7.799 μm (P < 0.0001, Fig. 3.1C) and 4 (1.1%) had an average cell length of 11.5 ± 1.06 μm (P < 0.0001, Fig. 3.1C). Using anti-FtsA$_{Ng}$ antibody, Western blot analysis confirmed that *ftsA*$_{Ng}$ was expressed in *E. coli* harbouring pDSW209-FtsA$_{Ng}$ (Fig. 3.1D, lane 2). Therefore, we named those cell with extremely long cell length (>8 μm) as 'long filaments' and cells that shorter than 8μm as 'slightly elongated".

3.4.2. FtsA$_{Ng}$ localizes at the division site in slightly elongated cells of *E. coli*

Constructs with *gfp* fused at the either N-terminus or C-terminus of *ftsA*$_{Ng}$ were developed to determine their localization in *E. coli*. No differences in FtsA$_{Ng}$ localization were noted with either N-terminal or C-terminal GFP tagged FtsA$_{Ng}$ constructs (data not shown). *E. coli* PB103 expressing only *gfp* from pDSW209, induced with 300 μM IPTG, were of normal size and showed non-specific GFP localization (data not shown. When *gfp-ftsA$_{Ng}$* expression were induced with 300 μM IPTG in *E. coli*, cells appeared as two different phenotypes: long filaments (2.5%) and slightly elongated cells (98.6%). In the filamentous cells, GFP-FtsA$_{Ng}$ was mostly detected as a punctate pattern along the

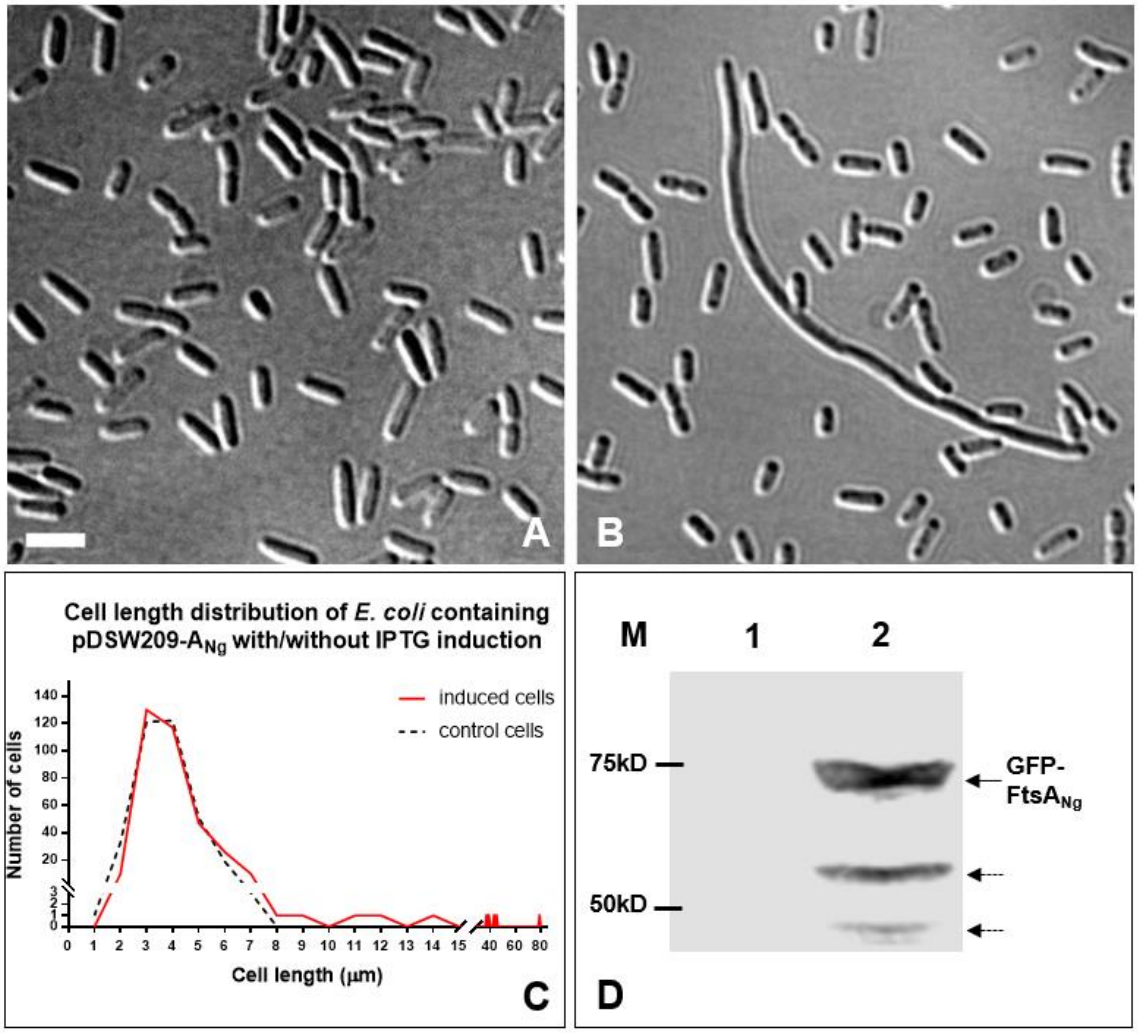

Fig. 3.1. Effect of GFP-FtsA$_{Ng}$ in *E. coli* PB103 on cell morphology. (A) *E. coli* PB103 transformed with pDSW209-A$_{Ng}$ without IPTG induction. (B) *E. coli* PB103 transformed with pDSW209-FtsA$_{Ng}$ and induced with 300 μM IPTG. Scale bars represent 2 μm. (C) Cell length distribution of *E. coli* PB103 containing pDSW209-A$_{Ng}$ with/without IPTG induction. Red line indicates cell length distribution of IPTG induced cells containing FtsA$_{Ng}$; black dash line indicates distribution of un-induced cells without *ftsA$_{Ng}$* expression. (D) Western blot using anti-FtsA$_{Ng}$ antibody for *E. coli* PB103 containing pDSW209-A$_{Ng}$ (lane 1) without IPTG induction and *E. coli* PB103 with pDSW209-A$_{Ng}$ induced with 300μM of IPTG (lane 2). Solid arrow indicates GFP-FtsA$_{Ng}$ with (70kDa); dashed arrows indicate GFP-FtsA degradation products.

membrane (Fig. 3.2A2 and A3). In the slightly elongated cells, 32% (192/582) of GFP-$FtsA_{Ng}$ localized at the cell poles (dashed arrow, Fig. 3.2B2 and B3), 4.3% (25/582) localized at mid-cell (solid arrow, Fig. 3.2B2 and B3), and 63% (365/582) had no specific localization in the cytoplasm (data not shown).

3.4.3. *N. gonorrhoeae* FtsA does not complement an *E. coli* ftsA temperature sensitive strain

Plasmids pDSW209-A_{Ng} ($FtsA_{Ng}$), pDSW209-A_{Ec} ($FtsA_{Ec}$), or pSEB306-D242E ($FtsA_{Ec}$D242E mutant at its 2B subdomain) were transformed into *E. coli* P163, an *ftsA*$_{Ec}$ temperature sensitive strain (Hale and De Boer, 1999). At 30°C, all three strains grew at various culture dilutions without IPTG induction (Fig. 3.3A). When the same strains were incubated on LB plates with IPTG ranging from 7.5-200µM at 42 °C (Fig. 3.3B-F), *E. coli* P163 expressing *ftsA*$_{Ec}$ grew well when induced with a concentration of IPTG from 7.5 to 200µm (Fig. 3.3B, lane 2), demonstrating that $FtsA_{Ec}$ can complement the *ftsA* deficiency in *E. coli* P163. However, neither *E. coli* P163 expressing *ftsA*$_{Ng}$ (Fig. 3.3B-F, lane 1) nor the FtsAD242E mutant (Fig. 3.3B-F, lane 3) grew under these conditions, indicating that *N. gonorrhoeae* FtsA did not complement the loss of $FtsA_{Ec}$ in *E. coli* P163.

3.4.4. *N. gonorrhoeae* FtsA interacts with FtsN from *E. coli*

Since $FtsA_{Ec}$ interacts with $FtsZ_{Ec}$, $FtsQ_{Ec}$, $FtsI_{Ec}$, $FtsN_{Ec}$ and $FtsA_{Ec}$ itself (Di Lallo et al., 2003; Karimova et al., 2005), we investigated whether $FtsA_{Ng}$ interacted with these proteins? B2H assays showed that residual β-galactosidase activity for $FtsA_{Ng}$ interaction with $FtsZ_{Ec}$, $FtsQ_{Ec}$, $FtsI_{Ec}$, and $FtsA_{Ec}$ was 100%, 88%, 89% and 73%, respectively (Table

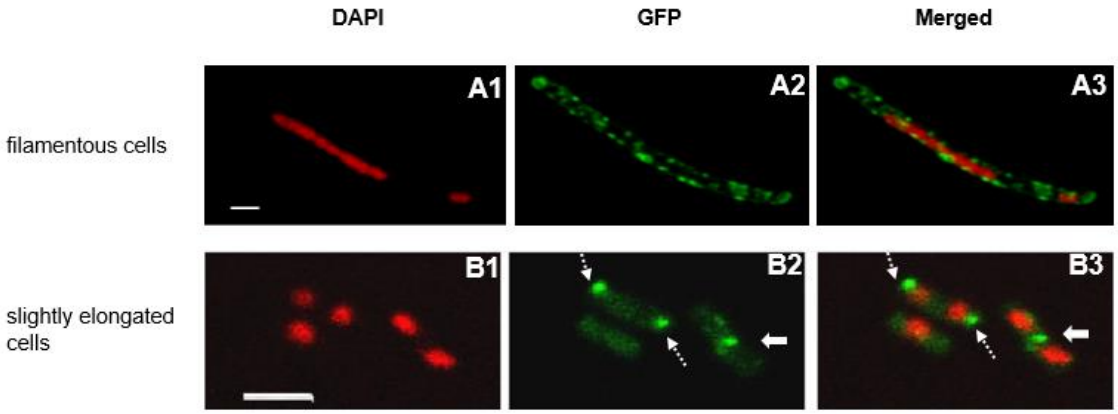

Fig. 3.2. Representative images of GFP-FtsA$_{Ng}$ localization in *E. coli* PB103. In filamentous cells containing GFP-FtsA$_{Ng}$, the chromosome was not properly segregated (A1) as shown by DAPI staining. GFP-FtsA$_{Ng}$ showed a punctate localization pattern along the membrane (A2 and A3). In slightly elongated cells which have a properly segregated chromosome (B1), GFP-FtsA$_{Ng}$ was either noted at the mid-cell area (solid arrows) or the cell poles (dashed arrows) (B2) and was away from the chromosome (B3). Scale bar represents 2 µm.

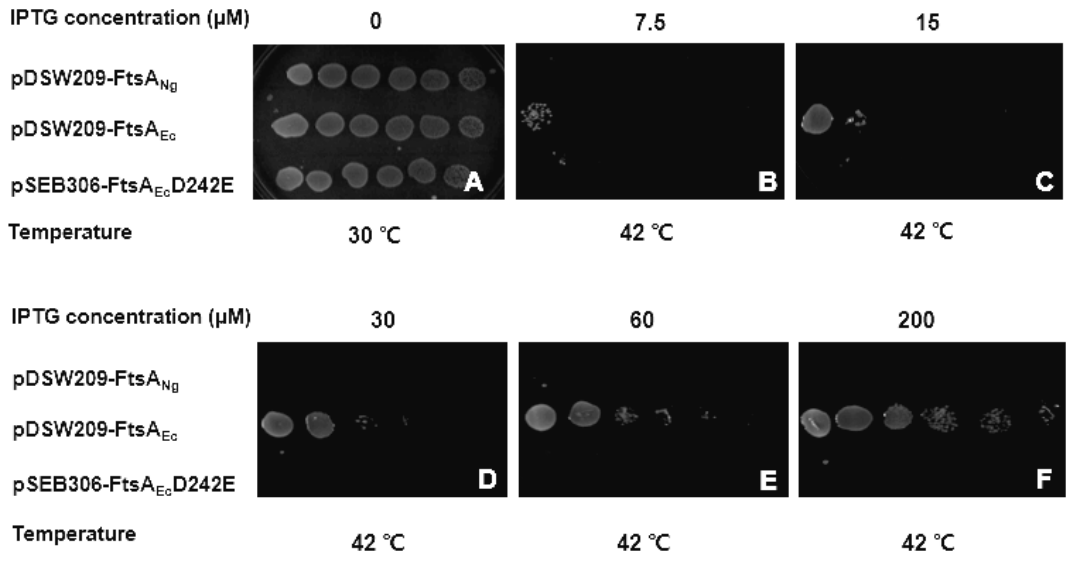

Fig. 3.3 Complementation of the *E. coli* P163 *ftsA* temperature sensitive strain by $FtsA_{Ng}$. *E. coli* P163 containing plasmids harboring the indicated FtsA were tested for colony growth after incubation at 30°C (permissive temperature) or 42°C (non-permissive temperature). IPTG was present in LB agar at 0, 7.5, 15, 30, 60 or 200 μM, as shown at the top of each image. 20 μl of serial 10-fold dilutions was spotted onto LB agar at each concentration of IPTG.

Table 3.3. (A) Interactions between gonococcal FtsA and *E. coli* cell division proteins as determined by B2H assays. (B) Interactions between gonococcal FtsA and its truncations and *E. coli* cell division proteins FtsN as determined by B2H assays.

(A)

	***E. coli* cell division proteins**					
	$FtsN_{Ec}$	**$FtsZ_{Ec}$**	**$FtsA_{Ec}$**	**$FtsQ_{Ec}$**	**$FtsI_{Ec}$**	**$FtsZ_{Ng}$**
Residual β-galactosidase activity (%) with $FtsA_{Ng}$	**<u>25</u>**	**100**	**73**	**88**	**89**	**<u>40</u>**

(B)

	$FtsA_{Ng}$ truncations					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Residual β-galactosidase activity (%) with $FtsN_{Ec}$	**53**	**57**	**<u>47</u>**	**<u>37</u>**	**<u>38</u>**	**71**

By comparing to positive controls (*E. coli* R721 without plasmids), interactions that have less than 50% of residual ß-galactosidase activity (underlined) were considered as positive interactions, $FtsA_{Ng}$-$FtsZ_{Ng}$ interaction was used as a positive control

3.3A). However, $FtsA_{Ng}$ did interact with $FtsN_{Ec}$ (residual β-galactosidase activity 25%; Table 3.3A), suggesting $FtsN_{Ec}$ was the only *E. coli* cell division protein that $FtsA_{Ng}$ interacted with. Notably $FtsA_{Ng}$ did interact with $FtsZ_{Ng}$ in positive control (residual β-galactosidase activity 40%; Table 3.3A). This result was confirmed by GST pull-down assay in which His-$FtsN_{Ec}$ was pulled down by GST-$FtsA_{Ng}$ protein, but not GST protein (Fig. 3.4). This confirmed the result obtained from B2H assays.

3.4.5. *N. gonorrhoeae* FtsA interacts with *E. coli* FtsN via its 2A and 2B subdomains

Our previous research using a B2H assay indicated that $FtsA_{Ng}$ interacted with $FtsN_{Ng}$ through its 2A and 2B subdomains (Zou *et al.*, 2017a). To ascertain whether these domains were also implicated in the $FtsA_{Ng}$ interaction with $FtsN_{Ec}$, B2H assays were performed using $FtsA_{Ng}$ truncations. Interactions of $FtsN_{Ec}$ with $FtsA_{Ng}$ truncations T3, T4 and T5 (corresponding to 2A and 2B sub-domains) were positive (47%, 37% and 38% of residual β-galactosidase activities) (Table 3.3B). These data were confirmed by GST pull-down assay in which His tagged $FtsA_{Ng}$ truncation His-FtsAT3, His-FtsAT4 and His-FtsAT5 were pulled down by GST-$FtsN_{Ec}$ but not GST protein (Fig. 3.5). While His-FtsAT2 and His-FtsAT6 were not pulled down by either GST-$FtsN_{Ec}$ or GST itself (Fig. 3.5).

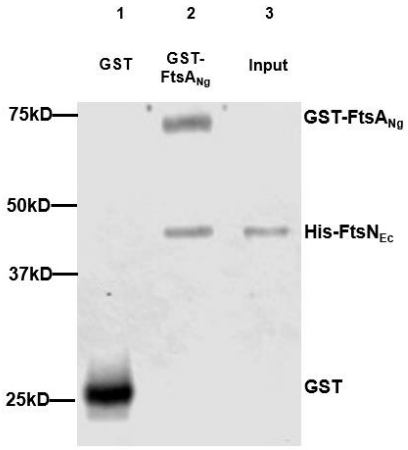

Fig. 3.4. GST pull-downs between $FtsN_{Ec}$ and $FtsA_{Ng}$. Lane 1: GST and His-$FtsN_{Ec}$ mixture; Lane 2: GST-$FtsA_{Ng}$ and His-$FtsN_{Ec}$ mixture; Lane 3: His-$FtsN_{Ec}$ input;

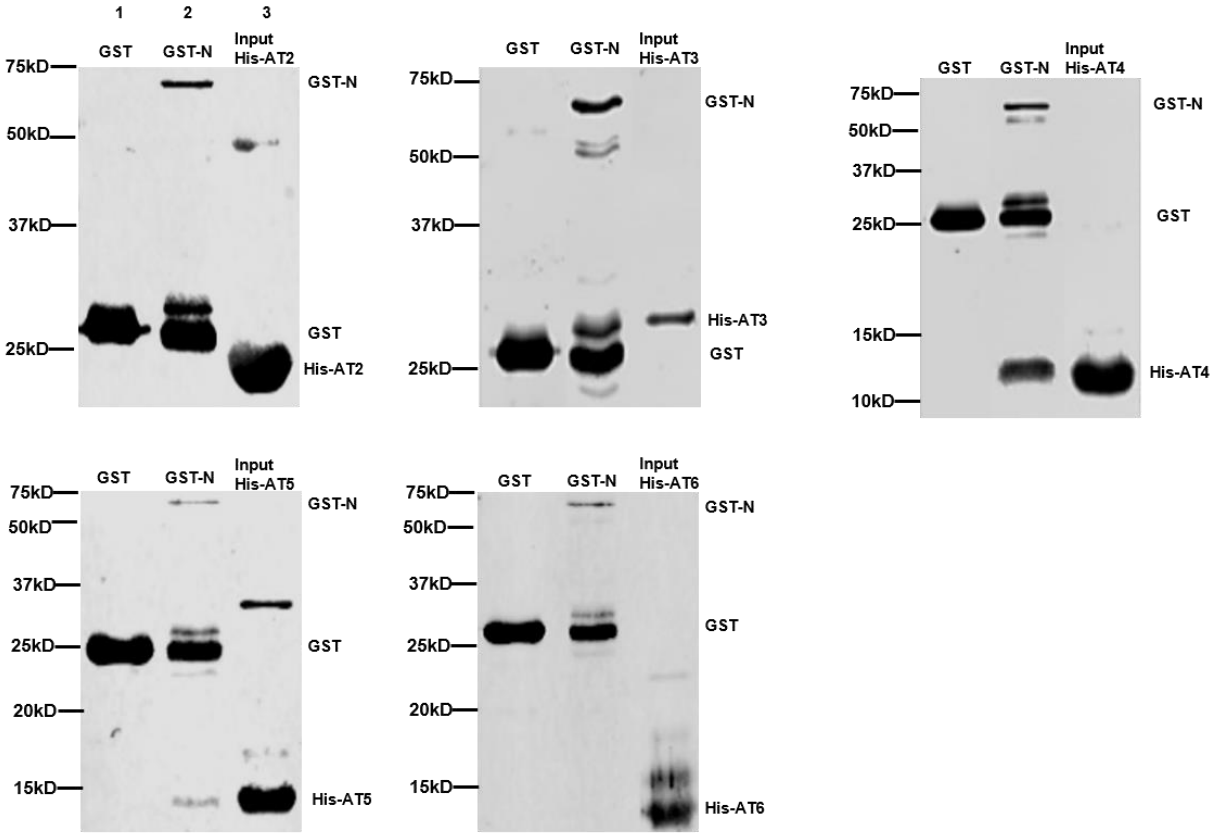

Fig. 3.5. GST pull-downs between $FtsN_{Ec}$ and $FtsA_{Ng}$ truncations. Lane 1: GST and His-$FtsA_{Ng}$ mixture; Lane 2: GST-$FtsN_{Ec}$ and His-$FtsA_{Ng}$ truncation mixture, GST-$FtsN_{Ec}$ was loaded with co-purified GST and GST-$FtsN_{Ec}$ degradation products; Lane 3: His-$FtsA_{Ng}$ truncation input; His-$FtsN_{Ec}$ was visualized using anti-6×His antibody; GST and GST-$FtsA_{Ng}$ was visualized using anti-GST antibody.

3.5. Discussion

Previous studies demonstrated that *E. coli* is a useful model organism to analyse the function and localization of heterologous cell division proteins (Ouellette et al., 2015; Moll and Thanbichler, 2009; Wang and Lutkenhaus, 1996; Beall et al., 1988; Honrubia *et al.*, 1998). Furthermore, the *E. coli* expression model can uncover functions of cell division proteins from species where genetic tools for such studies are limited (Gaiwala Sharma *et al.*, 2016; Honrubia et al., 1998). Complementation of mutants in *E. coli* is a common method to investigate functions of heterologous cell division proteins. We determined $FtsA_{Ng}$ could not complement an *E. coli* temperature sensitive strain, suggesting that this protein did not recognize the specific FtsA-targeting sites for proper cell division in *E. coli*. Similarly, *Chlamydia* FtsQ and *B. subtilis* FtsA were incapable of complementing mutations in their corresponding *E. coli* homologs (Ouellette et al., 2015; Beall et al., 1988), suggesting that functions of cell division proteins from these species differed from their homologs in *E. coli*.

The overexpression of $ftsA_{Ec}$ in *E. coli* has been previously shown to produce a filamentous cell phenotype (Wang and Gayda, 1990). We have also observed a significant number (36%, data not shown) of filamentous cells when $ftsA_{Ng}$ was overexpressed from a high copy number plasmid (pUC18) in *E. coli*. Since protein overexpression can cause stoichiometry issues which can lead to an abnormal cell morphology (Sopko et al., 2006), we cloned $ftsA_{Ng}$ into a medium copy number plasmid (pDSW209) with a weakened *trc* promoter to achieve physiologically appropriate levels of expression. Expression of *gfp-*$ftsA_{Ng}$ from $pDSW209A_{Ng}$ still increased the average cell length in *E. coli*, but with fewer

filamentous cells. This suggested that although $FtsA_{Ng}$ could inhibit *E. coli* cell division, it is not a strong inhibitor.

B2H results showed that $FtsA_{Ng}$ did not interact with either $FtsA_{Ec}$ or $FtsZ_{Ec}$. Further, while $FtsA_{Ec}$ interacts with $FtsQ_{Ec}$, $FtsI_{Ec}$ and $FtsN_{Ec}$ (Di Lallo et al., 2003; Karimova et al., 2005), $FtsA_{Ng}$ only interacted with $FtsN_{Ec}$. This showed that $FtsA_{Ng}$ does not interact with the same *E. coli* cell division proteins as $FtsA_{Ec}$. The 1C subdomain, which is 86 residues, of $FtsA_{Ec}$ interacts with the cytoplasmic domain of $FtsN_{Ec}$ (Busiek et al., 2012). We showed that the 2A and 2B domains (108 residues) of $FtsA_{Ng}$ interact with $FtsN_{Ec}$ and the only region available for this interaction is the cytoplasmic domain of $FtsN_{Ec}$. The cytoplasmic region of $FtsN_{Ec}$ shares four common residues (KXSXSXK) with $FtsN_{Ng}$ and the first serine is necessary for the $FtsA_{Ec}$-$FtsN_{Ec}$ interaction (Busiek et al., 2012). This most probably explains the reason for the $FtsA_{Ng}$-$FtsN_{Ec}$ interaction. The association of $FtsA_{Ng}$ with $FtsN_{Ec}$ would likely block any association by $FtsA_{Ec.}$ Since they are both mediated by the same region of $FtsN_{Ec}$, these interactions would be mutually exclusive. Thus, the reduced availability of $FtsN_{Ec}$ postpones full $FtsN_{Ec}$ recruitment to the divisome, delaying the cell division process and resulting in a slightly elongated phenotype.

FtsN depletion can cause abnormal nucleoid segregation by affecting the stability of $ZipA_{Ec}$ at the division site (Rico et al., 2010) which further disrupts $FtsK_{Ec}$ recruitment (Hale and de Boer, 2002). The $FtsA_{Ng}$-$FtsN_{Ec}$ interaction likely reduced the amount of FtsN available for cell division in *E. coli* cells. Insufficient FtsN may further lead to a disruption in native FtsK recruitment and nucleoid segregation resulting in long filamentous cell phenotypes.

Yeast-two-hybrid (Y2H) assays are sometimes used as an alternative method to verify bacterial cell division protein interactions obtained from B2H assays since no bacterial cell division genes are present in yeast (Daniel et al., 2006). Our previous research showed that Y2H assays could not detect a number of gonococcal cell division protein interactions due to high background (unpublished data, Greco-Stewart et al., 2007; Ramirez-Arcos et al., 2004). Therefore, GST pull-down assays were used as a confirmatory method for the interactions observed in this study.

The diverse functionality of FtsA from different species has been reported by others. In organisms such as *S. aureus* (Fujita et al., 2014) and *Deinococcus radiodurans* (Modi and Misra, 2014), FtsA could destabilize their native FtsZ filaments by enhancing the GTPase activity of FtsZ. However, FtsA from *D. radiodurans* reduced the GTPase activity of *E. coli* FtsZ (Modi and Misra, 2014). In addition, while all FtsAs from different species exhibit ATP binding capacity (Lara et al., 2005), their abilities to hydrolyse ATP varies (Lara et al., 2005; Beuria et al., 2009; Feucht et al., 2001). Our results show that FtsA from *E. coli* and *N. gonorrhoeae* are not interchangeable functionally. This has also been shown for FtsA from *B. subtilis* (Beall et al., 1988). Further, we determined $FtsA_{Ng}$ did not share the same *E. coli* divisome interacting proteins as $FtsA_{Ec}$. In some cases, however, the interacting proteins of heterologous proteins in *E. coli* can be identical to the native protein. For example, *Chlamydia trachomatis* FtsQ interacted with the same cell division proteins in *E. coli* as $FtsQ_{Ec}$ (Ouellette et al., 2015; Karimova et al., 2005).

In summary, we demonstrated that expression of $ftsA_{Ng}$ in *E. coli* could disrupt host cell division, but was not able to complement loss of $FtsA_{Ec}$ function. We also identified that $FtsA_{Ng}$ localization in *E. coli* was dependent on $FtsN_{Ec}$. This protein interaction was

mediated via the 2A and 2B subdomains of $FtsA_{Ng}$, which is different from its *E. coli* homolog. Therefore, we concluded that $FtsA_{Ng}$ has species-specific functionality in cell division.

Chapter 4 Three conserved residues at the N-terminal of *Neisseria gonorrhoeae* FtsI are crucial for interaction with FtsW and penicillin binding

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Keywords: *N. gonorrhoeae*, FtsI, PBP2, cell division, penicillin binding

Abbreviations: PBP, penicillin-binding protein; Ng, *Neisseria gonorrhoeae*; HMW, high molecular weight; LMW, low molecular weight; TPase, transpeptidase; B2H, bacterial two-hybrid; CD, circular dichroism; Tm, melting temperature.

4.1. Abstract

Neisseria gonorrhoeae (Ng) FtsI, also known as penicillin-binding protein 2 (PBP 2), is the primary target of β-lactam antibiotics, such as penicillin. The C-terminal transpeptidase domain contains an active site formed by three conserved sequence motifs for antibiotic binding. Mutations in this domain lead to chromosomally-mediated penicillin resistance. The function of the N-terminal domain of $FtsI_{Ng}$ has not been characterized. We identified three conserved residues in the N-terminal periplasmic region, Arg75(R75), Arg167(R167) and Glu193(E193), which form a conserved motif located in the linker region that connects the C-terminal and N-terminal domains. Mutations of these residues disrupted the interaction of $FtsI_{Ng}$ with $FtsW_{Ng}$, a cell division protein. Alterations of Arg75 and Arg167 also impaired penicillin binding capacity, whereas a mutation at Glu193 had no effect. Circular dichroism analysis indicated that the E193G mutant altered the secondary structure and protein stability of $FtsI_{Ng}$, while both R75G and R167G mutants had no significant impact on conformation. Generation of a gonococcal chromosomal *ftsIR167G* mutant was not successful since the insertional mutagenesis of chromosomal *ftsI* led to a hetero-diploid genotype. Our study suggests that the conserved residues in the N-terminal periplasmic region of $FtsI_{Ng}$ are necessary for bacterial cell division protein interaction and penicillin binding.

4.2. Introduction

Penicillin-binding proteins (PBPs) are involved in peptidoglycan synthesis and antimicrobial resistance in bacteria. Based on their molecular mass, PBPs can be divided into two categories: high- (HMW) and low-molecular-weight (LMW) PBPs. According to the function, PBPs can be separated into three classes: class A with N-terminal transglycosylase and transpeptidase (TPase) activities, class B with only TPase activity and class C with carboxypeptidase activity and sometimes with endopeptidase activity (Ghuysen, 1991; Sauvage *et al.*, 2008).

Four PBPs have been identified in *Neisseria gonorrhoeae* (Ng): one HMW class A protein, PBP1; one HMW class B, protein PBP2; and two LMW class C proteins, PBP3 and PBP4 (Barbour, 1981). Previous studies have shown that *N. gonorrhoeae* PBP3 and PBP4 are not essential for cell viability (Stefanova et al., 2003), while deletion of PBP1 or PBP2 are lethal (Ropp and Nicholas, 1997; Barbour, 1981). In addition, PBP2 is the primary killing target of penicillin since the minimum inhibitory concentration (MIC) of PBP2 is 10-fold lower than that of PBP1 (Barbour, 1981).

PBP2, also known as FtsI, is a class B, HMW PBP which is involved in the final stage of peptidoglycan synthesis (Ghuysen, 1991). The crystal structure of *N. gonorrhoeae* FtsI shows that it contains two domains: an N-terminal domain and a C-terminal transpeptidase (TPase) domain (Powell et al., 2009). The N-terminal domain can be further divided into three regions: an N-terminal cytoplasmic tail, a transmembrane region, and a non-catalytic periplasmic region with unknown function (Powell et al., 2009). The C-terminal TPase domain of FtsI catalyzes cross-linking of adjacent peptide side chains of

peptidoglycan strands. Furthermore, the TPase domain is also recognized as a lethal target of β-lactam antibiotics, such as penicillin and third generation cephalosporins (Barbour, 1981). Mutations in the TPase domain of $FtsI_{Ng}$ confer chromosomally mediated resistance against β-lactam antibiotics (Powell et al., 2009).

Although the function of the N-terminal domain of *N. gonorrhoeae* FtsI is not clear yet, studies on its homolog in *Escherichia coli* (Ec) can provide insight in this respect. The $FtsI_{Ng}$ homolog in *E. coli*, PBP3, localizes at the division site via its N-terminal domain (Piette *et al.*, 2004). The M1-to-E56 region of *E. coli* FtsI acts as a membrane anchor-containing module and the G57-to-E258 non-catalytic region is essential for correct protein folding (Goffin *et al.*, 1996). An N-terminal linker region formed by three degenerated motifs was identified in *E. coli*, *S. pneumoniae* and *S. aureus* (Sauvage et al., 2014; Marrec-Fairley *et al.*, 2000). The non-catalytic region is also essential for protein-protein interactions (Goffin et al., 1996). The *E. coli* cell division interactome established by Di Lallo *et al.* (Di Lallo et al., 2003) and Karimova *et al.* (Karimova et al., 2005) suggests that $FtsI_{Ec}$ interacts with several cell division proteins including $FtsA_{Ec}$, $FtsQ_{Ec}$, $FtsN_{Ec}$, $FtsW_{Ec}$, $FtsK_{Ec}$, $FtsB_{Ec}$, $FtsL_{Ec}$, and $FtsI_{Ec}$ itself. The *E. coli* FtsW, a lipid II flippase, is required for $FtsI_{Ec}$ localization to the division site by forming a protein complex (Mercer and Weiss, 2002; Fraipont et al., 2011). Interestingly, two adjacent arginine residues, R166 and R167, on the N-terminal region of *E. coli* $FtsI_{Ec}$, are required to maintain the penicillin binding capacity since alterations at these sites result in the loss of penicillin binding ability (Marrec-Fairley et al., 2000).

Previously, we determined that *N. gonorrhoeae* FtsI only interacts with $FtsW_{Ng}$ (Zou et al., 2017a), indicating that the localization of $FtsI_{Ng}$ potentially depends on its

interaction with $FtsW_{Ng}$. Since the C-terminal TPase domain of $FtsI_{Ng}$ has been extensively investigated for antimicrobial resistance, and no research has explored the role of the β-terminal domain of $FtsI_{Ng}$ in cell division. We hypothesized that the N-terminal region of *N. gonorrhoeae* FtsI may mediate key cell division protein-protein interactions and serve as the conformational support for the proper function of the C-terminal domain regarding penicillin binding. In this study, we identified highly conserved residues at the N-terminal periplasmic region of $FtsI_{Ng}$ and investigated the role of these residues on cell division and antimicrobial resistance using a combination of protein-protein interaction assays and penicillin binding assays.

4.3. Materials and Methods

4.3.1. Strains and growth conditions

Bacterial strains used in this study are shown in Table 4.1. *E. coli* DH5α was used as a host for cloning. *E. coli* BL21 (DE3) was used as the expression host for protein purification. *E. coli* R721 was used in B2H assays (Di Lallo et al., 2001). *E. coli* DH5α and BL21(DE3) were grown in Luria-Bertani (LB) medium (BD Difco™, Sparks, MD), for 16–18 hours, at 37°C. *E. coli* R721 was grown in the same medium and incubated at 34°C, as described previously (Di Lallo et al., 2003).

N. gonorrhoeae FA1090 was grown on GC medium base agar (GCMB, Oakville, ON), supplemented with Kellogg's defined supplement (GCMBK, 40 g D-glucose, 1 g glutamine, 10 ml of 0.5% ferric nitrate and 1 ml of 20% cocarboxylase), at 35°C, in a humid environment, with 5% CO_2, for 18 to 24 h (Kellogg et al., 1963).

When required, the following concentrations of antibiotics were added to LB medium: 50 μg/ml kanamycin for protein purification. For B2H assays, 34 μg/ml chloramphenicol, 30 μg/ml kanamycin, and 50μg/ml ampicillin were added to LB medium. For *N. gonorrhoeae* transformation, 2 μg/ml erythromycin or 100 μg/ml streptomycin was added to GCMB medium supplemented with Kellogg's supplement.

4.3.2. Plasmid construction

For bacterial two-hybrid (B2H) assays, *ftsI* mutants with a single amino acid substitution were generated using site-directed mutagenesis described by Ho *et al* (1989). The R75G *ftsI* mutant was generated as follows: two DNA segments were amplified using primer pairs P1/P4 and P2/P3 from *N. gonorrhoeae* FA1090, covering the upstream and

Table 4.1 Bacterial strains used in this study

Stain	Relevant characteristics	Source/reference
E. coli DH5α	*supE44 ΔlacU169* (*80lacZΔM15*) *hsdR17 endA1 gyrA96 thi-1 relA1*	Gibco
E. coli BL21(DE3)	F^-*, dcm Δ, ompT, hsdS* (r^-_B m^-_B)*, gal, λ(DE3)*	Stratagene
E. coli R721	*supE thy D*(*lac-proAB*) F' [$proAB^+$ $lacI^q$ *lac*ZDM15] *glpT*::O-$_{P434/P22}$*lacZ*	(Di Lallo et al., 2001)
N. gonorrhoeae FA1090	A/ S/ P: proline/IB-2/2.6	(West and Clark, 1989)

Table 4.2 Primers used in this study

Primer name		**Sequences (5'-3')**
P1	fwSalI-ftsI	GCGCGTCGACCATGTTGATTAAAAGCGAATATAAGCC
P2	reBamHI-ftsI	CGCGGGATCCTTAAGACGGTGTTTTGACGGCTGC
P3	fwftsIR75G	CGGCTACAgGCGGTACGGT
P4	reftsIR75G	GAAACCGTACCGCcTGTAGCC
P5	fwftsIR167G	AAAAGAATTAAAAgGCCATTACCCGAT
P6	reftsIR167G	CCCATCGGGTAATGGCcTTTTAATTCT
P7	fwftsIE193G	GGAAGGTTTGGAgCTTTCGCTTGA
P8	reftsIE193G	TCAAGCGAAAGcTCCAAACCTTCC
P9	fwEcoRI-ftsIF	CGGAATTCTGCGACGCGCAC
P10	reBamHI-ftsI	CGCGGGATCCTTAAGACGGTGTTTTGACGG
P11	fwEcoRI-It	CGCGAATTCCAGACGGTAACGTATAACTTTTTG
P12	reXhoI-ftsI	GCGCTCGAGTTAAGACGGTGTTTTGACGG
P13	fwftsIF	TCCTGCATCAGGATAATAATAACG

P14	reftsIF	AAACAATCTCGTTGATACTCGGATTAAG

downstream sequences of the R75G mutation site (Table 4.2). Both segments had 20–30 bp overlapping sequences that contained a single base pair substitution that resulted in substitution from arginine to glycine. These two PCR amplicons, generated in the first PCR, were fused in a subsequent primer extension reaction using the primer pair P1/P2. The resulting *ftsI* amplicon, containing the desired mutation, was digested with SalI and BamHI and ligated into pre-digested B2H vectors pcI_{p22} and pcI_{434}, yielding pcI_{p22}-IR75G and pcI_{434}-IR75G constructs, respectively (Table 4.3). R167G and E193G *ftsI* mutants were generated following the same protocol for R75G using primer pairs P5/P6 and P7/P8 (Table 4.2), introducing pcI_{p22}-IR167G, pcI_{434}-IR167G, pcI_{p22}-IE193G and pcI_{434}-IE193G (Table 4.3).

To generate *ftsI* insertional mutants, wildtype *ftsI* and 350 base pair (bp) of its upstream sequence were amplified from *N. gonorrhoeae* FA1090 using a primer set P9/P10 (Table 4.2). The PCR amplicon was double-digested with EcoRI and BamHI followed by ligation into a pre-digested pKH6, yielding pKIF construct (Table 4.3). The *ermC/rpsL* cassette was isolated from the pKC1 using NheI and HindIII restriction enzymes and treated with T4 DNA polymerase (M0203S, New England Biolabs) to form blunt ends. The pKIF construct was digested with MscI to create the blunt end close to the R167G mutation site followed by dephosphorylation using calf-intestinal alkaline phosphatase (CIP, cat# M0290S, New England Biolabs). The *ermC/rpsL* cassette DNA segment was ligated into pre-digested pKIF, yielding the pKIFrpsL/ermC construct (Table 4.3). For the second transformation, two DNA fragments, containing regions from 350 bp upstream sequence of FtsI to the R167G mutation site and from the downstream sequence of the mutation site to the stop codon, were amplified using primer sets P9/P4 and P5/P10

Table 4.3 Plasmids used in this study

Plasmid	Relevant genotype	Source/Reference
pcI_{p22}	pC132 derivative carrying N-terminal end of P22 repressor	(Di Lallo et al., 2001)
pcI_{434}	pACYC177 derivative carrying N-terminal end of 434 repressor	(Di Lallo et al., 2001)
pcI_{p22}-IR75G	$pcIp_{22}$ derivative carrying the $ftsI_{Ng}$ containing C-G mutation at position 223	This study
pcI_{434}-IR75G	pcI_{434} derivative carrying the $ftsI_{Ng}$ containing C-G mutation at position 223	This study
pcI_{p22}-IR167G	$pcIp_{22}$ derivative carrying the $ftsI_{Ng}$ containing C-G mutation at position 499	This study
pcI_{434}-IR167G	pcI_{434} derivative carrying the $ftsI_{Ng}$ containing C-G mutation at position 499	This study
pcI_{p22}-IE193G	$pcIp_{22}$ derivative carrying the $ftsI_{Ng}$ containing A-G mutation at position 579	This study
pcI_{434}-IE193G	pcI_{434} derivative carrying the $ftsI_{Ng}$ containing A-G mutation at position 579	This study
pcI_{p22}-I	$pcIp_{22}$ derivative carrying the $ftsI_{Ng}$ gene	(Zou et al., 2017a)
pcI_{434}-I	pcI_{434} derivative carrying the $ftsI_{Ng}$ gene	(Zou et al., 2017a)
pcI_{p22}-W	$pcIp_{22}$ derivative carrying the $ftsW_{Ng}$ gene	(Zou et al., 2017a)

pcI_{434}-W	pcI_{434} derivative carrying the *ftsW*$_{Ng}$ gene	(Zou et al., 2017a)
pKH6	pUP1 derivative carrying one copy of 12bp extended DUS and one copy of 10bp DUS	(Dillard, 2011)
pKC1	pIDN1 with *N. gonorrhoeae* F62 *rpsL* inserted at XhoI and KpnI sites (Erm^r Str^s)	(Cloud and Dillard, 2002)
pKIF	pKH6 derivative carrying wildtype *ftsI* and its 350bp upstream sequences	This study
pKIFrpsL/ermC	pKIF derivative carrying the *ermC/rpsL* cassette inserted at MscI site	This study
pKIFR167G	pKIF derivative carrying the *ftsIR75G* gene	This study
pET30a	Kan^R P_{T7}::6Xhis	EMD Millipore, Billerica, MA
pETIt	pET30a derivative carrying the *ftsI* gene without first 48 residues	This study
pETItR75G	pETIt derivative carrying the R75G mutation	This study
pETItR167G	pETIt derivative carrying the R167G mutation	This study
pETItE193G	pETIt derivative carrying the E193G mutation	This study

followed by a primer extension reaction using primer pair P9/P10. The PCR amplicon containing the R167G mutation was digested with EcoRI and BamHI followed by ligation into a pre-digested pKH6, yielding the pKIFR167G construct (Table 4.3).

For circular dichroism and penicillin binding assays, wildtype *ftsI* without the first 48 residues containing the N-terminal tail and transmembrane helices was amplified from *N. gonorrhoeae* FA1090 using primer pair P11/P12 (Table 4.2). The PCR amplicon was digested with EcoRI and XhoI and ligated into pre-digested pET30a vector, introducing pETIt (Table 4.3). R75G, R167G and E193G *ftsI* mutants were created as described above using primer sets P3/P4, P5/P6 and P7/P8, yielding pETItR75G, pETItR167G and pETItE193G, respectively.

4.3.3. Protein purification

Plasmids pETIt, pETItR75G, pETItR167G and pETItE193G (Table 4.3) were transformed into *E. coli* BL21(DE3). Overexpression of His-tagged FtsI was induced by 400 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for two hours. Cells were centrifuged and resuspended in Binding Buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole; pH 7.4) followed by sonic disruption and centrifugation at 11 000 *g* at 4 ºC. Supernatant containing His-tagged fusion proteins were purified using His60 Ni Superflow Resin (Clontech) including an elution step with 300 mM of imidazole. Purified proteins were dialyzed into PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.9) and stored at -80 ºC. Integrity and purity of proteins were checked by SDS–PAGE and quantified as described by Greenfield (2006).

4.3.4. Bacterial two-hybrid assay

B2H assays were performed as previously described (Zou et al., 2017a). Briefly, pcI_{p22} or pcI_{434} derived recombinant constructs containing *ftsI* mutants (R75G, R167G and E193G) were transformed into *E. coli* R721 in combination with pcI_{434}-W or pcI_{p22}-W containing wildtype *ftsW*. *N. gonorrhoeae* FtsI-FtsW interaction was used as a positive control. R721 without plasmids was used as a negative control. The β-galactosidase activity of each combination was compared to that of R721 which had a full β-galactosidase activity of around 2504±34 Miller units. A positive interaction was defined as a protein pair with less than 50% (<1250 Miller Units) of residual β-galactosidase activity, while a negative interaction was considered that with a value more than 50% (>1250 Miller Units)(Di Lallo et al., 2003). Standard deviations were calculated for the average Miller units from three independent experiments. Statistical analyses were performed using the unpaired Student t-test.

4.3.5. Transformation

Transformation of *N. gonorrhoeae* was performed following the liquid transformation protocol described by Dillard (2011). Breifly, 10 μg of linearized plamid DNA was added into 200 μl of prewarmed GCBL medium (1.5% proteose peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl; pH 7.2) containing 5 mM Mg_2SO_4. An overnight culture of piliated *N. gonorrhoeae* was collected and re-suspended into 200 μl of prewarmed GCBL medium containing 5 mM Mg_2SO_4 followed by dilution into an optical density of 0.18 (at 560 nm). 20 μl of cell suspension was transferred into 180 μl of DNA solution and incubated at 35°C for 20 minutes. The cell-DNA mixture was inoculated onto GC base agar plates supplemented with Kellogg's supplement and incubated at 35°C for six hours. Enriched culture was then collected and suspended into 1 ml of pre-warmed

GCBL medium following by plating 200 µl of the cell suspension onto GC base agar plates supplemented with Kellogg's supplement and appropriate anitbiotics. These plates were incubated at 35°C with 5% CO_2 until colonies appeared.

4.3.6. Circular dichroism

Circular dichroism experiments were performed using a Pistar-180 CD spectrometer (Applied Photophysics, Leatherhead, UK) with a 1 mm path length quartz cell. Far UV spectra (185–260 nm) were collected at 25°C for both wildtype FtsI and the mutants at a concentration range of 0.7 to 1.0 mg/ml in PBS buffer. Data points were recorded every 0.5 nm. All measurements were performed in triplicate. All spectra were standardized against the spectrum obtained from the buffer only. The CD spectra acquired were deconvoluted using CDNN v2.1 (Böhm *et al.*, 1992).

The melting curves for both wildtype FtsI and the mutants were recorded at 218 nm within a temperature range from 25 to 60°C. Residual ellipticity of each protein was measured four times and averaged using Pistar software. All spectra were standardized against the spectrum obtained from the PBS buffer only. The melting temperature for each protein was calculated as the point at which there was a maximum change in the slope of the melting curve.

4.3.7. Penicillin binding assay

The penicillin binding assay was performed using BOCILLIN™ FL Penicillin (Thermo Fisher Scientific; Lenexa, KS). For each reaction, 100 µM of BOCILLIN™ FL was added into 8 µM of purified wildtype FtsI/mutant in PBS buffer to a final volume of 20 µl. Reactions were protected from light exposure and incubated for 3 minutes at 25°C.

Reactions were quenched by the addition of 5X Laemmli buffer, and the mixtures were boiled at 95°C for five minutes. The FtsI-penicillin complex and unbound penicillin were separated using 10% SDS-PAGE. Visualization of the FtsI-penicillin complex was achieved using the Molecular Imager® VersaDoc™ MP 4000 System (Bio-Rad Laboratories).

4.3.8. Production of anti-FtsI antibodes

To produce rabbit polyclonal antiserum to $FtsI_{Ng}$, Freund's complete (Sigma) was used as an adjuvant for the first injection and Freund's incomplete as an adjuvant for the following boosters. 150 µl adjuvant was added to 150 µl purified wildtype His-$FtsI_{Ng}$ for injection of rabbits. The resultant mixtures were injected into female New Zealand White rabbits. The first booster was administered three weeks after the initial injection (300 µl for each rabbit). Blood was tested for the level of antibody ten days after the first booster and a second booster was administered (Animal Care Unit, Vaccine and Infectious Disease Organization - International Vaccine Centre, University of Saskatchewan). Serum was collected from rabbit blood ten days after the last booster using established procedures (Sambrook and Russel, 2001). The antibody was then purified using Protein G Sepharose® (Sigma).

4.4. Results

4.4.1. The N-terminus of $FtsI_{Ng}$ contains three conserved residues

To investigate the function of the N-terminal region of $FtsI_{Ng}$, we applied multisequence alignment using FtsI homologous amino acid sequences from 30 different Gram-negative bacterial species to identify conserved residues (Fig. 4.1). These alignments showed three highly conserved residues, Arg75 (R75), Arg167 (R167) and Glu193 (E193), in the N-terminal periplasmic region of the 30 different species (Fig. 4.1). R75 and R167 residues were located at the β1n and β3n strands in the periplasmic N-terminal region, respectively, while the E193 residue was located at the α3n helix (Fig. 4.2) (Powell et al., 2009). Although R75, R167, and E193 were not close to each other in the primary sequence, localization of these residues in the three-dimensional (3D) structure of $FtsI_{Ng}$ showed that they were all located in the linker region that connects the C-terminal and N-terminal domains (Fig. 4.2). A similar structure was also identified in FtsI from *E. coli*, *S. pneumoniae* and *S. aureus* (Contreras-Martel *et al.*, 2009).

4.4.2. Alteration of each conserved residue affected FtsI-FtsW interaction

Previously, we reported that *N. gonorrhoeae* FtsI only interacts with $FtsW_{Ng}$. The homologs of these two proteins in *E. coli* form a protein complex, suggesting the importance of the FtsI-FtsW interaction in both species for cell division (Zou et al., 2017a; Fraipont et al., 2011). To examine the effect of the conserved residues at the N-terminal region on cell division, we created three $FtsI_{Ng}$ mutants, R75G, R167G and E193G, and tested the capacity of each of these mutants to interact with wildtype $FtsW_{Ng}$ by B2H assay. The residual β-galactosidase activity of each mutant was 56% (R75G), 76% (R167G) and

60% (E193G), whereas the wildtype $FtsI_{Ng}$-$FtsW_{Ng}$ interaction had 35% of residual β-galactosidase activity (Fig. 4.3). These data suggested that alterations of these conserved residues blocked the interaction between FtsI and FtsW in *N. gonorrhoeae*. In addition, we tested the self-interaction capacity of $FtsI_{Ng}$. B2H assay revealed 58% of residual β-galactosidase activity, indicating the lack of self-interaction in $FtsI_{Ng}$.


N_gonorrhoeae
N_meningitidis
N_lactamica
B_bronchiseptica
E_coli
P_ananatis
M_succiniciproducens
H_influenzae
H_parasuis
S_oneidensis
P_aeruginosa
X_axonopodis
L_pneumophila
M_catarrhalis
P_arcticus
A_ferrooxidans
G_metallireducens
G_bemidjiensis
P_carbinolicus
D_magneticus
T_thermophilus
O_carboxidovorans
G_xylinus
R_capsulatus
S_japonicum
C_Pelagibacter
D_desulfuricans
W_chondrophila
H_pylori
P_americana

Fig. 4.1 Multi-sequence alignment of N-terminal region of FtsI from 30 different Gram-negative species. Star indicates highly conserved residues.

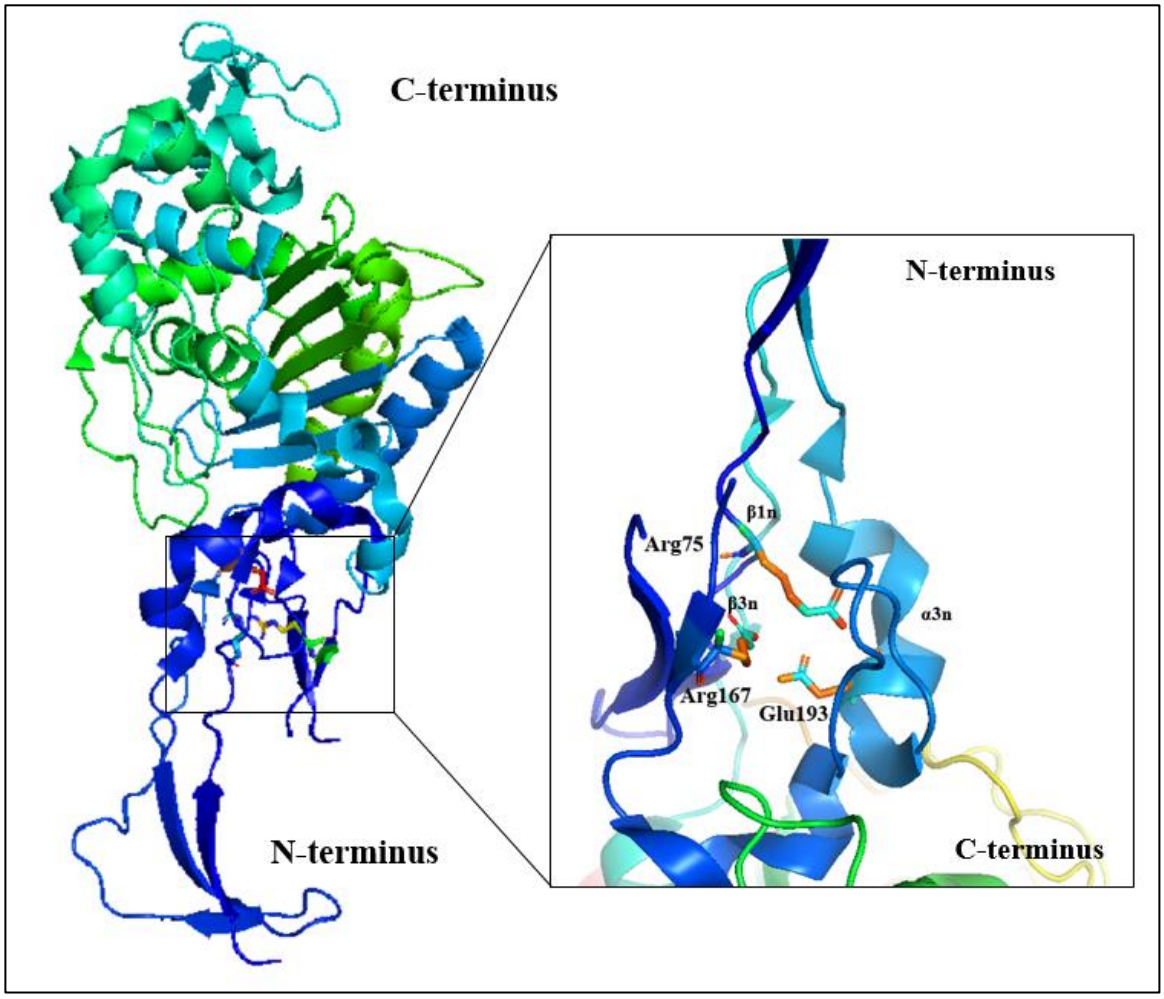

Fig. 4.2 Localization of conserved residues at the N-terminal domain of FtsI in *N. gonorrhoeae* (PDB: 3EQU). Arg75, Arg167 and Glu193 are shown in sticks. Arg75 was located at the β1n strand, Arg167 was located at the β3n strand and Glu193 was α3n helix.

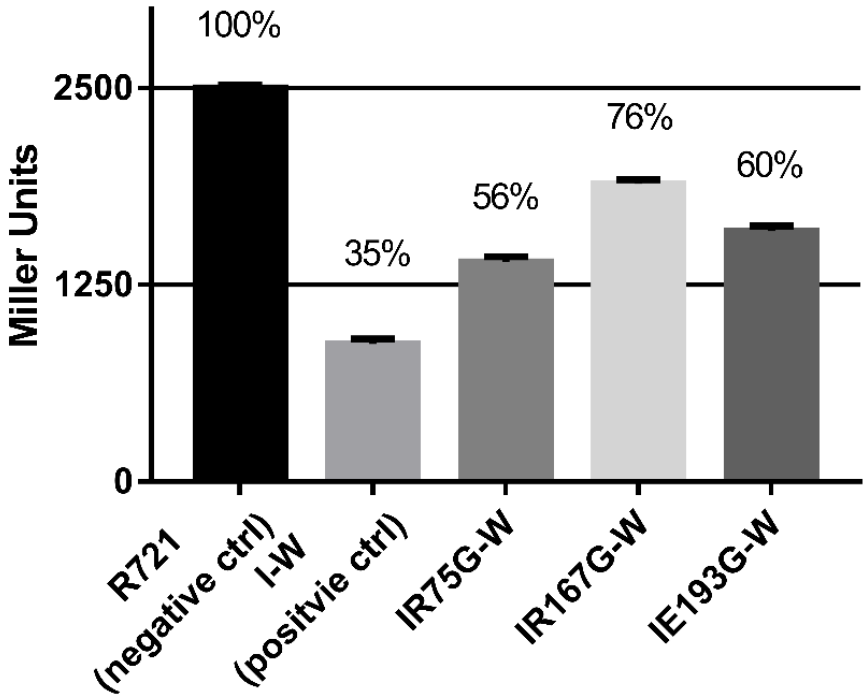

Fig. 4.3 Residual β-galactosidase activities between FtsW (W) and FtsI (I) mutants (R75G, R167G, and E193G). R721 without plasmids was used as a negative control which had a β-galactosidase activity of 2504±34 Miller units. Wild type FtsI-FtsW interaction was used as a positive control. Values of less than 50% (<1250 Miller Unites) indicate a positive interaction between two proteins, while values of more than 50% (>1250 Miller Unites) indicate a negative interaction.

4.4.3. E193G mutation affected secondary structure and protein stability

To determine whether the inability of $FtsI_{Ng}$ to interact with $FtsW_{Ng}$ was because of the conformational changes caused by single amino acid substitution, circular dichroism was used to ascertain the secondary structure and stability in both wildtype FtsI and its mutants (Fig. 4.4). All three mutants had lower Tm values compared to wildtype FtsI. Melting temperature (Tm) values, calculated based on the melting curves, showed that the Tm of each protein is 43.58°C (wildtype), 40.55°C (R75G), 40.97°C (R167G) and 38.82°C (E193G). However, the reduction of Tm values in R75G and R167G mutants were less than 3°C, whereas the difference of Tm values between E193G and wildtype was close to 5°C.

The Far-UV spectrum obtained using circular dichroism showed that wildtype $FtsI_{Ng}$ and its mutants contained α-helices, with negative bands at 208 and 222 nm and a positive band at 193 nm, and antiparallel β-sheet structures with negative bands at 218 nm and positive bands at 195 nm (Fig. 4.5A) (Holzwarth and Doty, 1965; Greenfield and Fasman, 1969). Deconvolution analysis using CDNN software showed that wildtype $FtsI_{Ng}$ comprised 25.6% of α-helical structure and 17% of β-sheet structure which was consistant with the data for $FtsI_{Ng}$ (26% of α-helices and 18% of β-sheet) provided by the Protein Data Bank (PDB ID: 3EQU). Compared to wildtype, R75G and R167G did not have significant differences in secondary structures, whereas the α-helical content in E193G was 7.5% lower than wildtype and the proportion of antiparallel β-sheet increased by 19.1% (Fig. 4.5B).

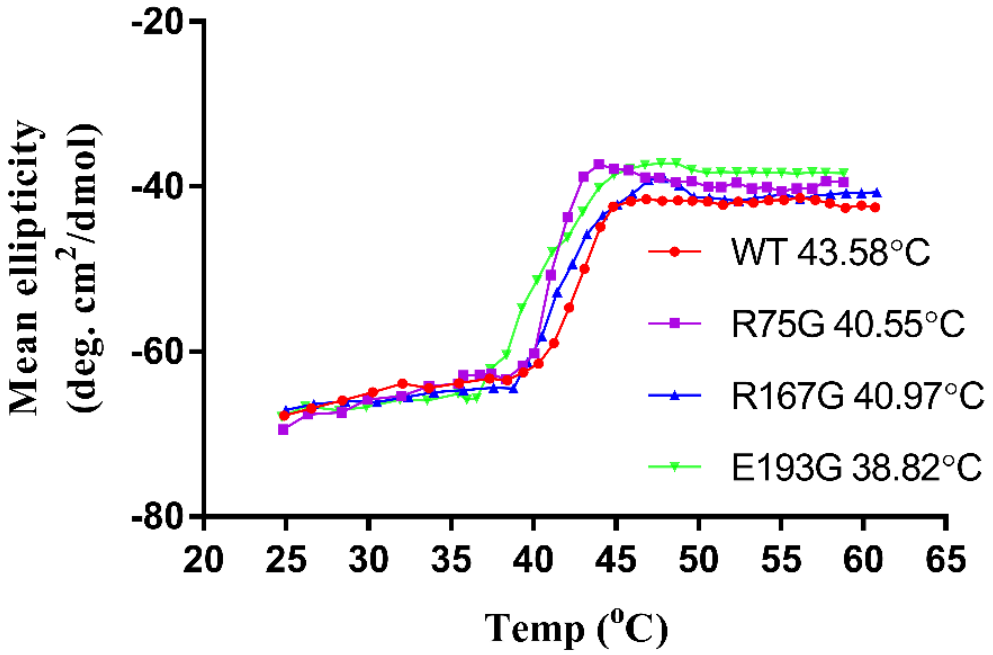

Fig. 4.4 Thermal stabilities of wild-type FtsI and its mutant. Melting curves were determined using circular dichroism by measuring the mean ellipticity at 222 nm in 1 °C intervals. WT: wild-type FtsI; R75G: FtsIR75G mutant; R167G: FtsIR167G mutant; E193G: FtsIE193G mutant.

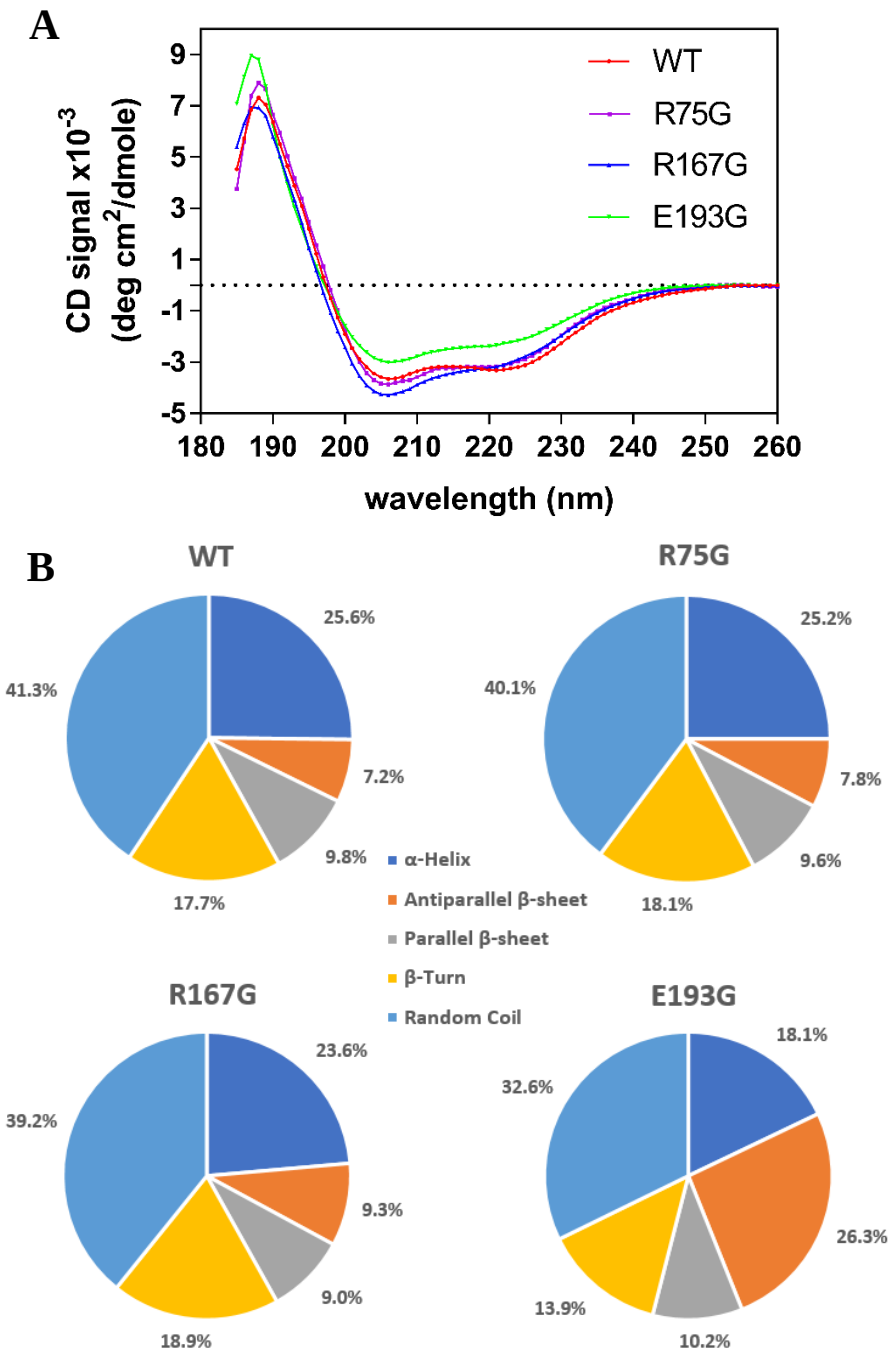

Fig. 4.5 Secondary structure analysis of wildtype FtsI and its mutants (R75G, R167G and E193G) using circular dichroism. (A) Far-UV spectra of wildtype FtsI and its mutants at the wavelength range of 185-260 nm. WT: wild-type FtsI; R75G: FtsIR75G mutant; R167G: FtsIR167G mutant; E193G: FtsIE193G mutant. (B) The secondary structure composition of wildtype FtsI and its mutants deconvoluted by CDNN software.

4.4.4. Generation of *ftsI* knock-out strain resulted in an *ftsI/ftsI*::*ermC/rpsL* hetero-diploid genotype

To investigate the effect of N-terminal mutations of $FtsI_{Ng}$ in cell division , we introduced an unmarked R167G mutation in chromosomal $ftsI_{Ng}$ using a two-step transformation protocol established by Dillard (2011). We used this mutation because it had the strongest inhibitory effect on $FtsI_{Ng}$-$FtsW_{Ng}$ interaction. The first step of the protocol involves insertion of the *ermC/rpsL* cassette at the MscI site (836 bp) of chromosomal $ftsI_{Ng}$ by homologous recombination, resulting in an $ftsI_{Ng}$::*ermC/rpsL* genotype. Transformants from this step were selected by erythromycin. At the second step, the $ftsI_{Ng}$::*ermC/rpsL* was replaced by *ftsIR167G* through homologous recombination. Colonies from the second transformation that contained the R167G mutation were selected using streptomycin (Dillard, 2011).

After the first transformation, the erythromycin-resistant transformants appeared within 48 hours at a frequency of 1.03×10^{-6}. Colonies were sub-cultured onto fresh GCMBK plates supplemented with erythromycin followed by PCR screening for a $ftsI_{Ng}$::*ermC/rpsL* genotype using a primer pair P13/P14 that flanks the chromosomal $ftsI_{Ng}$. Agarose gel electrophoresis showed that two amplicons corresponding to the estimated size of insertional mutated (3429 bp) and wildtype (1749 bp) $ftsI_{Ng}$ were amplified (Fig. 4.6A). The presence of *ftsI* insertional mutant suggested that the *ermC/rpsL* cassette was inserted into chromosomal $ftsI_{Ng}$ by homologous recombination, resulting in *ftsI*::*ermC/rpsL*. However, the presence of wildtype $ftsI_{Ng}$ suggested that the transformation resulted in a hetero-diploid genotype which both wild type *ftsI* and the mutant were present on the chromosome.

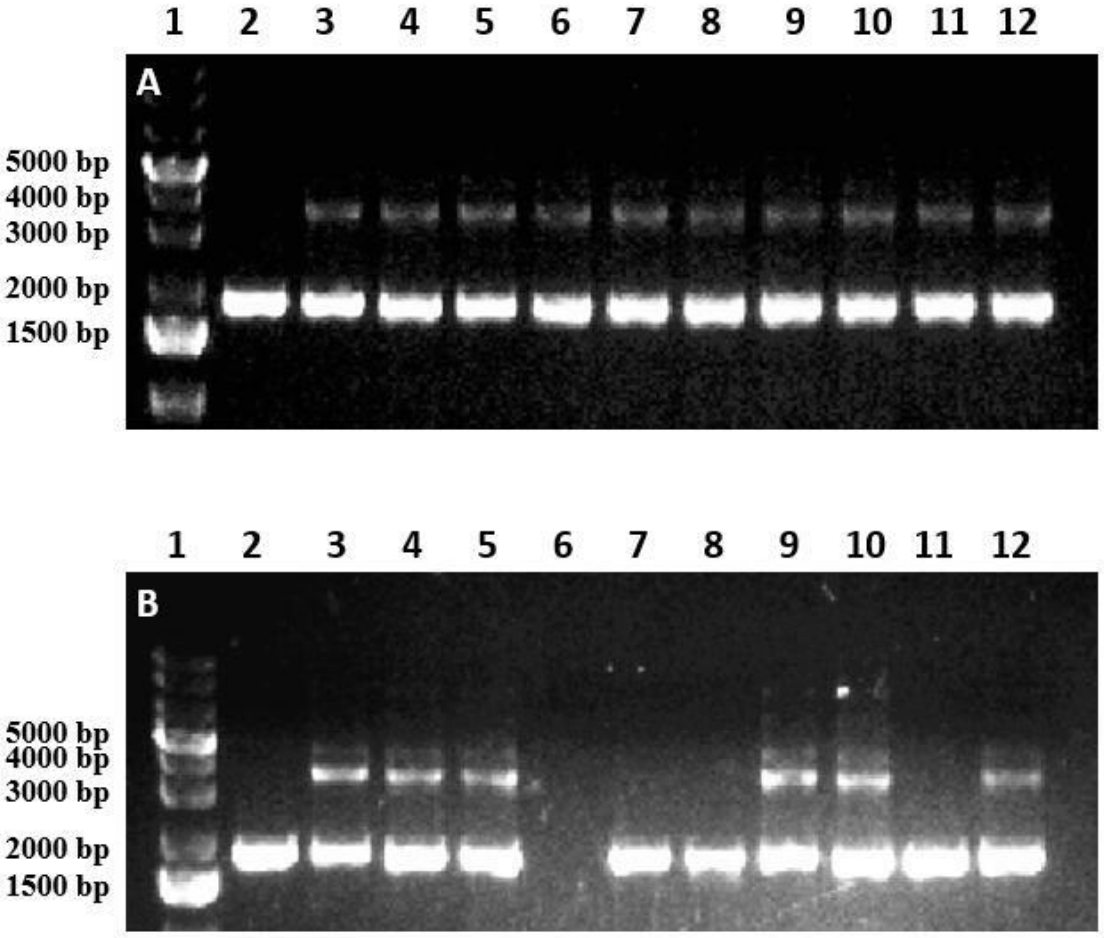

Fig. 4.6 Agarose (1%) electrophoresis of PCR amplified products using the primer pair P13/P14. (A) PCR products amplified from colonies grown on GCMBK plates containing erythromycin. (B) amplicons generated from colonies grown on GCMBK plates without antibiotics. Wild type *N. gonorrhoeae* FA1090 genomic DNA was used as a positive control. Lane 1: DNA marker; Lane 2: PCR products amplified from *gonorrhoeae* FA1090; Lane 2-12: PCR products amplified from individual colonies;

Interestingly, when colonies were sub-cultured onto fresh GCMBK plates without antibiotics, colony PCR results showed that the *ftsI*::*ermC/rpsL* genotype was absent in six out of 18 colonies (Fig. 4.6B, Lane 7, 8, and 11), indicating that the *ftsI*::*ermC/rpsL* genotype is less stable and favorable than the wildtype.

4.4.5. R75G and R167G led to reduction in penicillin binding capacity

Although there is no evidence to support the involvement of the N-terminal region of $FtsI_{Ng}$ in penicillin binding, previous studies with *E. coli* FtsI suggested that mutations in the N-terminal permiplasmic region of $FtsI_{Ec}$ could destabilize the protein structure and lead to the loss of penicillin binding capacity (Marrec-Fairley et al., 2000; Wissel and Weiss, 2003). Hence, we ascertained the effect of R75G, R167G and E193G mutations on penicillin binding capacity for penicillin V (BOCILLIN FL penicillin) (Zhao *et al.*, 1999). An equal amount of wildtype $FtsI_{Ng}$ and its mutants were used for penicillin binding assays as determined by immunoblotting using polyclonal anti-$FtsI_{Ng}$ antibodies (Fig. 4.7A). As described by the BOCILLIN FL fluorescence measurement in SDS-PAGE gels, the BOCILLIN fluorescence intensity of both R75G and R167G was much lower compared to wildtype $FtsI_{Ng}$ (Fig. 4.7B), indicating weakened penicillin binding capacities in both mutants. There was no significant difference of fluorescence intensity between wildtype $FtsI_{Ng}$ and the E193G mutant of $FtsI_{Ng}$ (Fig. 6B), suggesting that the mutation at E193 did not influence penicillin binding.

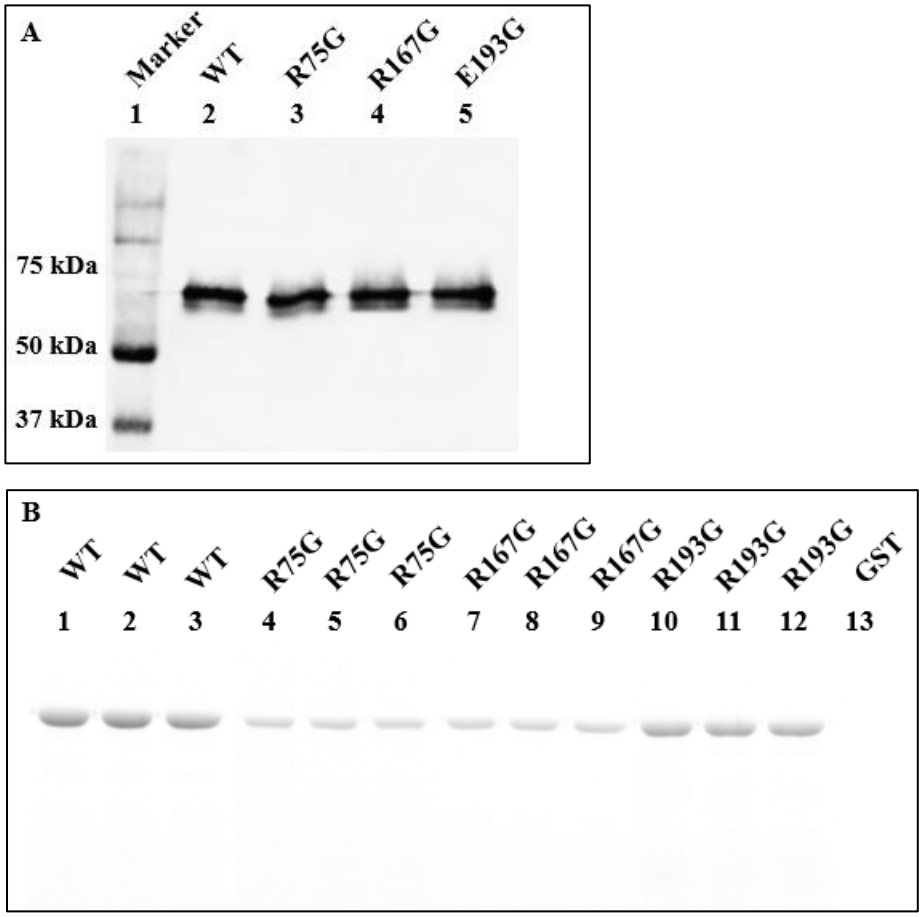

Fig. 4.7 Immunoblotting and penicillin binding assays of purified wildtype FtsI and its mutants. (A) Immunoblotting result of purified FtsI and its mutants using polyclonal anti-FtsI antibody. Lane 1: protein marker; Lane 2: purified wildtype FtsI without transmembrane region; Lane 3: purified FtsIR75G mutant; Lane 4: purified FtsIR167G mutant; Lane 5: purified FtsIE193G mutant; (B) Penicillin binding assays using BOCILLIN FL penicillin. Wildtype FtsI was used as a positive control and purified GST protein was used as a negative control. Lane 1-3: purified wildtype FtsI; Lane 4-6: purified FtsI R75G mutants; Lane 7-9: purified FtsI R167G mutants; Lane 10-12: purified FtsI E193G mutants; Lane 13: purified GST protein.

4.5. Discussion

Using multisequence alignment, we identified three highly conserved amino acids, R75, R167 and E193, lying at the N-terminal permiplasmic noncatalytic region of $FtsI_{Ng}$ (PDB ID: 3EQU)(Powell et al., 2009). 3D structure analysis of $FtsI_{Ng}$ revealed that these three residues were located at the linker region that connects the C-terminal TPase domain and N-terminus (Fig. 1) (Powell et al., 2009; Sauvage et al., 2014). Similar structures comprising two arginines and one glutamic acid were also observed in FtsIs of *Streptococcus pneumoniae* and *Staphylococcus aureus* which were proposed to stabilize the protein structure by direct interactions among these residues (Contreras-Martel et al., 2009). Interestingly, this conserved linker structure is only present in Class B PBPs, indicating that it may be involved in the novel cell division function of PBPs from this class (Zapun *et al.*, 2008; den Blaauwen *et al.*, 2008). Comparison of FtsIs structures from *E. coli* (PDB ID: 4BJP), *S. aureus* (PBD ID: 1VQQ) and *S. pneumoniae* (PDB ID: 2WAD) revealed that the C-terminal TPase domains could be superposed onto each other, reflecting the highly conserved function of this region (Sauvage et al., 2014). In contrast, the N-terminal region lay at different positions, implying that the linker region that connects both domains may be flexible (Sauvage et al., 2014; Contreras-Martel et al., 2009; Lim and Strynadka, 2002).

A previous study conducted by Powell *et al* (Powell et al., 2009) proposed that the N-terminal region of *N. gonorrhoeae* FtsI may serve as a pedestal for proper protein functions. Recently, we reported that $FtsI_{Ng}$ interacted with the cell division protein $FtsW_{Ng}$, which is a common interaction present in other species (Zou et al., 2017a; Fraipont et al., 2011; Mistry et al., 2008; Datta et al., 2006). In this study, we found that alterations

of the N-terminal highly conserved residues could disrupt the interaction between $FtsI_{Ng}$ and $FtsW_{Ng}$. In *E. coli*, the localization of $FtsI_{Ec}$ is dependent on its interaction with $FtsW_{Ec}$ (Mercer and Weiss, 2002). Using B2H assays, the transmembrane segment and a short N-terminal periplasmic region (G40-E56) of *E. coli* FtsI were recognized as a structural determinant for the interaction with $FtsW_{Ec}$ and its localization to the septal region, whereas the N-terminal noncatalytic region was not necessary for its positioning to the division site (Piette et al., 2004; Fraipont et al., 2011). A single amino acid change at positions 167 and 193 of *E. coli* FtsI, corresponding to the R167 and E193 residues in *N. gonorrhoeae* FtsI, caused cell filamentation which was an indication of impaired cell division (Marrec-Fairley et al., 2000; Goffin et al., 1996). Apart from the R167 and E193 residues, we found that the R75 residue of *N. gonorrhoeae* FtsI was also necessary for the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction. Thus, our B2H results suggest that the conserved Arg-Arg-Glu motif at the linker region of *N. gonorrhoeae* $FtsI_{Ng}$ is required for proper protein interactions in cell division. In addition to the E193G mutation, we also determined that the interaction of $FtsI_{Ng}$-$FtsW_{Ng}$ could be maintained when the glutamic acid at position 193 was substituted with aspartic acid (Fig 4.3). Since both residues are physicochemically similar to each other, our B2H result suggested that E193 provided structural support for the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction. Since $FtsW_{Ng}$ was identified as the only cell division protein that interacted with $FtsI_{Ng}$ (Zou et al., 2017a), we propose that the Arg-Arg-Glu structure is vital for its localization to the septum during the gonococcal cell division process.

Due to the inability of $FtsI_{Ng}$ to interact with $FtsW_{Ng}$ caused by alterations of conserved residues at the linker region, we further examined the potential effect on gonococcal cell division by introducing an R167G mutation in chromosomal *ftsI*$_{Ng}$ using a

two-step transformation protocol (Dillard, 2011). However, we were unable to isolate a single *ftsI*::*ermC/rpsL* mutant. Instead, an *ftsI/ftsI*::*ermC/rpsL* hetero-diploid genotype was observed by PCR screening. This was possibly caused by illegitimate recombination, a process in which two non-homologous DNA regions are joined together. A similar observation was reported by Taha *et al* (1988) in an attempt to generate an insertional mutation in *pilA*. Since *pilA* is essential for *N. gonorrhoeae*, insertional mutagenesis resulted in a *pilA/pilA*::*mTn3Cm-3a* hetero-diploid mutant, as determined by Southern blot analysis. In our study, the *ftsI/ftsI*::*ermC/rpsL* hetero-diploid mutant was well maintained until erythromycin was removed from the medium, suggesting that *ftsI* is necessary for gonococcal viability.

Previous studies on *E. coli* PBP3 showed that the G188-D197 motif, corresponding to the G188-E197 region of $FtsI_{Ng}$, is highly conserved among class B PBPs (Goffin and Ghuysen, 1998). Mutation of E193 in this motif resulted in a highly unstable $FtsI_{Ec}$ mutant, suggesting that this motif might serve as a structural determinant for proper folding (Marrec-Fairley et al., 2000; Goffin et al., 1996). In our study, conformational analysis using circular dichroism demonstrated that the E193G mutant of $FtsI_{Ng}$ had a lower percentage of α-helical structure and reduced melting temperature than wildtype. The negative effect of this E193G mutation on protein stability and protein-protein interaction (i.e. $FtsI_{Ng}$-$FtsW_{Ng}$ interaction) suggests that the E193 residue plays a crucial role for the proper folding of the protein.

An earlier study on *E. coli* FtsI showed that both *E. coli* FtsI R71 and R167 residues, counterparts of *N. gonorrhoeae* FtsI R75 and R167, were located at two conserved motifs R71–G79 and R167–G172 (Goffin et al., 1996). Alterations of residues in motif R71–G79

led to a less stable form of $FtsI_{Ec}$ that had decreased penicillin binding capacity, whereas its role in cell division was not affected since it could still complement an *ftsI*$_{Ec}$ temperature-sensitive mutant at non-permissive temperature (Marrec-Fairley et al., 2000; Goffin et al., 1996). Double mutations of R167Q and its adjacent arginine R166Q, located in motif R167–G172 of *E. coli* FtsI, could completely abolish penicillin binding capacity and disrupt cell division, suggesting that this motif plays a crucial role in cell division and antimicrobial resistance (Marrec-Fairley et al., 2000). In our study, circular dichroism analysis illustrated that neither the secondary structure nor the stability of *N. gonorrhoeae* FtsI was significantly affected by the R75G or R167G mutation, whereas the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction was abolished. Similar results were observed when *E. coli* counterparts of *N. gonorrhoeae* FtsI R75 and R167 were mutated (Goffin et al., 1996; Marrec-Fairley et al., 2000). Our interpretation is that a mutation at R75 or R167 residues decreased the amphiphilicity of the peptide segments containing these residues, which resulted in a loss of function of the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction.

Although the Arg-Arg-Glu motif is conserved among difference species, there is no direct evidence suggesting that it is involved in antimicrobial resistance. Structural analysis showed that the linker region containing the Arg-Arg-Glu residues was far away from the active site of $FtsI_{Ng}$ which is located at the groove of an α-subdomain and an α/β-subdomain (Powell et al., 2009). However, data from *E. coli* FtsI clearly showed that mutations at the N-terminal permiplasmic region affected the affinity of penicillin binding. It was believed that the alteration of penicillin binding capacity was due to conformational changes caused by mutations in the N-terminal permiplasmic region of *E. coli* FtsI (Goffin et al., 1996). Using fluorescently labelled BOCILLIN FL, we determined that the R75G

and R167G $FtsI_{Ng}$ mutants had decreased affinity with penicillin, while the E193G mutation had a similar binding level compared to wildtype. Since mutations at R75 and R167 did not cause significant structural changes, how these mutations impose an influence on penicillin binding capacity is still unclear. Surprisingly, E193G did not affect the C-terminal TPase function, although it caused protein destabilization and failure of the FtsI-FtsW interaction. In total, the penicillin binding results suggest that the Arg-Arg-Glu motif lying in the N-terminal linker region of *N. gonorrhoeae* FtsI is necessary to maintain the function of the C-terminal domain.

The B2H results showed that there was no self-interaction of $FtsI_{Ng}$, indicating that it does not form a dimer. Previous studies revealed that FtsIs from both *E. coli* and *S. pneumoniae* self-interacted as determined by B2H assays (Di Lallo et al., 2003; Maggi et al., 2008), although the role of FtsI self-interaction in both species is still unidentified. The distinction in self-interaction between $FtsI_{Ng}$ and its homologs from *E. coli* and *S. pneumoniae* may implicate a difference in protein functions.

In summary, we identified three conserved residues located at the N-terminal linker region of *N. gonorrhoeae* FtsI, which form a conserved structure among different species. Our B2H results suggest that all these residues are necessary for the interaction between $FtsI_{Ng}$ and $FtsW_{Ng}$. Penicillin binding assays indicated that two of these residues, R75 and R167, are required for penicillin binding. Structural analysis using circular dichroism showed that R75 and R167 residues have a minor effect on protein conformation and, therefore, do not affect the stability of $FtsI_{Ng}$. On the other hand, a mutation at E193 destabilized the protein by affecting the secondary structure. The introduction of a single amino acid substitution at the R167 position was not successful since insertional

mutagenesis on *ftsI*$_{Ng}$ led to a hetero-diploid genotype, suggesting that FtsI$_{Ng}$ is essential for gonococcal cell viability. Therefore, we conclude that the conserved residues at the N-terminal region of *N. gonorrhoeae* FtsI$_{Ng}$ are essential for both cell division protein interaction and penicillin binding capacity.

Chapter 5 General conclusion and future considerations

Infections with multidrug-resistant bacteria have become a threat to public health worldwide (Unemo and Nicholas, 2012; Unemo and Shafer, 2011). Most antibiotics used to treat bacterial infections target key metabolism pathways including DNA, RNA, protein and cell wall synthesis. Due to the increasing numbers of bacterial infections with reduced susceptibility to current clinical antibiotics, it is urgent to find new antibacterial drugs targeting novel mechanisms. Recently, bacterial cell division has received more attention for new drug development due to its necessity for bacterial reproduction (Sass and Brotz-Oesterhelt, 2013; Misra et al., 2018). Understanding of bacterial cell division is heavily dependent on model organisms, such as Gram-negative *E. coli* and Gram-positive *B. subtilis*, due to the great availability of genetic tools. Knowledge of cell division in non-model organisms, such as *N. gonorrhoeae*, is still insufficient. My study of *N. gonorrhoeae* cell division in Dr. Dillon's laboratory was focused on *N. gonorrhoeae* cell division protein-protein interactions and functions of two proteins, $FtsA_{Ng}$ and $FtsI_{Ng}$, in cell division and antimicrobial resistance.

During my Ph.D. study in Dr. Dillon's laboratory, I have ascertained the protein-protein interactions among eight gonococcal cell division proteins and established a cell division interactome of *N. gonorrhoeae*. This is the third interactome describing the cell division protein-protein interaction network in bacteria. As a non-model organism, the *N. gonorrhoeae* cell division interactome unveiled some unique features of gonococcal cell division protein-protein interactions which were different from the knowledge obtained from the model organism (i.e., *E. coli*) and other non-model species (i.e., *S. pneumoniae*). My second study on the function of *N. gonorrhoeae* FtsA in cell division using an *E. coli*

system has clearly shown that gonococcal FtsA had a different function and interaction pattern compared to its well-studied *E. coli* homolog, indicating a species-specific function of FtsA. Finally, the third study on *N. gonorrhoeae* FtsI showed that the linker structure located in the N-terminal domain played a vital role in the interaction between $FtsI_{Ng}$ and $FtsW_{Ng}$ and, surprisingly, in penicillin binding. These finding extended our understanding of $FtsI_{Ng}$ in *N. gonorrhoeae* cell division as well as in antimicrobial resistance and provided fundamental knowledge for future studies.

5.1. *N. gonorrhoeae* possesses a distinct cell division interactome

Previously, our laboratory identified most of the gonococcal cell division genes in the *dcw* cluster (Francis et al., 2000). In this study, nine pairs of protein-protein interactions among eight *N. gonorrhoeae* cell division proteins were ascertained using a combination of B2H, GST pull-down and surface plasmon resonance assays and established a *N. gonorrhoeae* cell division interactome.

Despite of the presence of two common interactions which are $FtsA_{Ng}$-$FtsZ_{Ng}$ and $FtsZ_{Ng}$-$FtsK_{Ng}$, *N. gonorrhoeae* possesses two novel interactions, the $FtsA_{Ng}$-$FtsW_{Ng}$ and $FtsN_{Ng}$-$FtsK_{Ng}$ interactions. The $FtsA_{Ng}$-$FtsW_{Ng}$ interaction is unique to *N. gonorrhoeae*. FtsW is known as an integral membrane protein that forms a protein complex with FtsI, a cell division protein associated with cell wall synthesis, in *E. coli*, *S. coelicolor* and *M. tuberculosis* (Fraipont et al., 2011; Mistry et al., 2008; Datta et al., 2006). Several studies have indicated that FtsW is required for FtsI localization (Mercer and Weiss, 2002; Wang *et al.*, 1998; Gamba et al., 2016). We found that the *N. gonorrhoeae* FtsI only interacted with $FtsW_{Ng}$. Therefore, we propose that the localization of $FtsI_{Ng}$ is dependent on $FtsW_{Ng}$

in *N. gonorrhoeae*. In addition to $FtsI_{Ng}$, $FtsW_{Ng}$ interacted with two early divisome proteins, $FtsZ_{Ng}$ and $FtsA_{Ng}$, suggesting that recruitment of $FtsW_{Ng}$ is dependent on either $FtsA_{Ng}$ or $FtsZ_{Ng}$ in *N. gonorrhoeae*. In *E. coli*, localization dependency of cell division proteins is assessed using fluorescence microscopy (Goehring et al., 2006). A previous study conducted in our laboratory showed that expression of $FtsZ_{Ng}$-GFP from a shuttle vector, pFPHS, in *N. gonorrhoeae* fluoresced entire cells and displayed no specific localization pattern due to the small size of the cells compared to those of *E. coli* (Salimnia et al., 2000). Future work needs to focus on improving the specificity of fluorescent signals from GFP fusion in *N. gonorrhoeae* by replacing the *lac* promoter in pFPHS with a weakened *trc* promoter from pDSW209 (Weiss et al., 1999).

The role of the $FtsN_{Ng}$-$FtsK_{Ng}$ interaction in *N. gonorrhoeae* cell division is unknown. Hints from a study conducted in *E. coli* showed that overexpression of $FtsN_{Ec}$ in a *ftsK*-depletion strain could restore the localization of some FtsK-dependant proteins, including $FtsQ_{Ec}$, $FtsL_{Ec,}$ and $FtsI_{Ec}$, indicating that these two proteins are interconnected (Goehring et al., 2007). What is the role the $FtsN_{Ng}$-$FtsK_{Ng}$ interaction in *N. gonorrhoeae* cell division? To answer this question, future work needs to focus on revealing the region of $FtsN_{Ng}$ involoved in the interaction with $FtsK_{Ng}$ using truncated $FtsN_{Ng}$ by B2H assays. Based on the B2H result, mutations that blocks the FtsN-FtsK interaction will be introduced onto chromosomal $ftsN_{Ng}$ as described by Dillard (2011). The loss of functional $FtsN_{Ng}$ can be complemented by inserting wild-type $ftsN_{Ng}$ in the region between *iga* and *trpB* in *N. gonorrhoeae* with a controllable promoter (i.e. tetracycline-inducible promoter) as described by Ramsey *et al* (Ramsey *et al.*, 2012). The effect of the FtsN-FtsK interaction

on *N. gonorrhoeae* cell division can be determined by examining the morphological changes when the inducer is present and absent in the growth medium.

The biggest surprise from the cell division interactome study in *N. gonorrhoeae* was that $ZipA_{Ng}$ did not interact with any other cell division protein. A previous study has shown that similarities existed in gene location and predicted protein structures between $ZipA_{Ng}$ and its *E. coli* homolog (Du and Arvidson, 2003). Complementation assays showed that $ZipA_{Ng}$ could partially complement an *E. coli zipA* temperature-sensitive mutant at the non-permissive temperature. In *E. coli*, $ZipA_{Ec}$ localizes $FtsZ_{Ec}$ to the inner membrane together with $FtsA_{Ec}$. Both $ZipA_{Ec}$ and $FtsA_{Ec}$ are required for the recruitment of downstream proteins to the division site (Hale and de Boer, 2002; Pichoff and Lutkenhaus, 2002). Since $ZipA_{Ng}$ did not interact with any other cell division protein in *N. gonorrhoeae*, we propose that the $FtsZ_{Ng}$ localization and recruitment of downstream proteins are dependant on $FtsA_{Ng}$. I have shown that localization of gonococcal $FtsA_{Ng}$ can be ascertained in an *E. coli* system using confocal microscopy in (Chapter 3). Future studies on $ZipA_{Ng}$ may focus on determining the localization of $ZipA_{Ng}$ in an *E. coli zipA* temperature sensitive mutant, such as the *E. coli* CH3 (Hale and de Boer, 1997), at the non-permissive temperature.

Although FtsN is present in many Gram-negative bacterial species, such as *E. coli*, *C. crescentus*, and *Vibrio cholerae* (Moll and Thanbichler, 2009; Möll *et al.*, 2014; Dai et al., 1993), An FtsA-FtsN interaction was only identified in *E. coli* (Di Lallo et al., 2003; Busiek et al., 2012). Recent studies indicated the importance of the $FtsA_{Ec}$ in its interaction with $FtsN_{Ec}$ in *E. coli* with respect to $FtsN_{Ec}$ recruitment and signal transmission for cell wall synthesis and constriction (Busiek and Margolin, 2014; Liu et al., 2015; Pichoff et al.,

2015). Our data from B2H, GST pull-down, and SPR assays clearly shown that an $FtsA_{Ng}$-$FtsN_{Ng}$ interaction is also present in *N. gonorrhoeae*. Since the 2A and 2B subdomains of $FtsA_{Ng}$ are necessary for the interaction with $FtsN_{Ng}$, the role of this interaction in *N. gonorrhoeae* cell division may be evaluated by introducing mutations in 2A and/or 2B subdmains of $FtsA_{Ng}$ that disrupt the $FtsA_{Ng}$-$FtsN_{Ng}$ interaction and examining its potential effect on cell morphology of *N. gonorrhoeae*.

5.2. *N. gonorrhoeae* FtsA has a species-specific function in cell division

Since FtsA is crucial for recruitment of many cell division proteins in *E. coli* and interactions between $FtsA_{Ng}$ and other gonococcal cell division proteins were observed in this study (i.e. $FtsA_{Ng}$-$FtsZ_{Ng}$, $FtsA_{Ng}$-$FtsQ_{Ng}$, $FtsA_{Ng}$-$FtsN_{Ng}$, and $FtsA_{Ng}$-$FtsW_{Ng}$), I further evaluated the function of $FtsA_{Ng}$. Due to limited availability of genetic tools in *N. gonorrhoeae*, the function study of $FtsA_{Ng}$ was conducted in an *E. coli* system.

A previous report in *E. coli* showed that overexpression of $ftsA_{Ec}$ led to a filamentous cell phenotype (Wang and Gayda, 1990). Similar cell morphology (36%) was observed in my study when $ftsA_{Ng}$ was overexpressed from a high copy number plasmid pUC18 in *E. coli* (data not shown). Since the abnormal cell morphology could result from overexpression-induced stoichiometry issues (Sopko et al., 2006), I further examined the *E. coli* morphology changes by expressing $ftsA_{Ng}$ from a medium copy number plasmid with a weakened P_{trc} promoter, ensuring that the expression of $ftsA_{Ng}$ was at an appropriate physiological level. Microscopic results showed that a low percentage (2.5%) of the cells still exhibited a filamentous phenotype, indicating that $FtsA_{Ng}$ was an inhibitor for *E. coli* cell division, but the inhibitory effect was not strong.

Since the FtsZ-FtsA interaction is common in most bacterial species, an initial hypothesis for the disrupted *E. coli* cell division caused by $FtsA_{Ng}$ could be that the presence of $FtsA_{Ng}$ altered the ratio of $FtsA_{Ec}$: $FtsZ_{Ec}$ by a direct interaction with either $FtsA_{Ec}$ or $FtsZ_{Ec}$. However, further analysis, using the B2H assay, showed that $FtsA_{Ng}$ interacted with neither $FtsA_{Ec}$ nor $FtsZ_{Ec}$. Instead, $FtsA_{Ng}$ only interacted with $FtsN_{Ec}$ which was further confirmed by GST pull-down assay. B2H assays showed that the 2A and 2B subdomains of $FtsA_{Ng}$ interacted with $FtsN_{Ec}$, the same subdomains that interact with several gonococcal cell division proteins including $FtsZ_{Ng}$, $FtsN_{Ng}$, $FtsQ_{Ng,}$ and $FtsW_{Ng}$ (Zou et al., 2017a). In *E. coli*, $FtsA_{Ec}$ mediates its interaction with early and late divisome proteins via different subdomains (i.e., 2B subdomain for the $FtsA_{Ec}$-$FtsZ_{Ec}$ interaction and 1C subdomain for self-interaction and interactions with late divisome proteins $FtsQ_{Ec}$ and $FtsN_{Ec}$). The difference in domain usage for protein-protein interactions between $FtsA_{Ng}$ and $FtsA_{Ec}$ suggests that FtsA has a species-specific function in cell division.

Complementation of $FtsA_{Ng}$ in an *E. coli ftsA*-depleted strain showed that $FtsA_{Ng}$ could not compensate for the loss of native $FtsA_{Ec}$ at the non-permissive temperature, indicating that the functions of these two homologs were different. Although both proteins share 41% of identity in their primary sequences, our results suggest that the functions of these proteins do not overlap. Proteins from other species also demonstrate species-specific functions in cell division, including *Mycoplasma pulmonis* FtsZ (Wang and Lutkenhaus, 1996), *B. subtilis* FtsZ (Beall et al., 1988), *C. trachomatis* FtsQ (Ouellette et al., 2015), and *C. crescentus* FtsN (Moll and Thanbichler, 2009). Expression of all these proteins in *E. coli* failed to complement mutations in their corresponding *E. coli* homologs, suggesting a different role in cell division in their native cells (Wang and Lutkenhaus, 1996; Beall et al.,

1988; Ouellette et al., 2015; Moll and Thanbichler, 2009). Nevertheless, these studies, in addition to my $FtsA_{Ng}$ research, proved that *E. coli* is a useful model to determine functions of heterologous cell division proteins where availability of genetic tools are poor in their native environment (Honrubia et al., 1998; Gaiwala Sharma et al., 2016).

5.3. The Arg-Arg-Glu motif in the N-terminal domain of *N. gonorrhoeae* is associated with cell division and penicillin binding

The C-terminal TPase domain of *N. gonorrhoeae* FtsI has been extensively investigated with respect to antimicrobial resistance because it is the primary target of β-lactam antibiotic. However, the role of its N-terminal domain is still unclear. To uncover the function of this region, I identified three conserved residues, Arg75, Arg167, and Glu193, in the N-terminus of $FtsI_{Ng}$ by aligning amino acid sequences of FtsI homologs from 30 Gram-negative bacterial species. Structural analysis indicated that these residues formed a Arg-Arg-Glu motif located in the linker region that connects the N-terminal and C-terminal TPase domains. Superimposition of FtsIs from *E. coli*, *S. aureus*, and *S. pneumoniae* suggested that the flexibility of these linker regions determined the position of their N-terminal domains (Sauvage et al., 2014). Interestingly, the Arg-Arg-Glu motif has only been observed in class B PBPs, implying that the motif may be associated with the novel function of class B PBPs (Zapun et al., 2008; den Blaauwen et al., 2008). B2H assays showed that mutations at each conserved residue that I identified blocked the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction in *N. gonorrhoeae*, suggesting that the Arg-Arg-Glu motif is crucial for cell division protein-protein interactions.

I further analyzed the potential structure and stability changes caused by these alterations using circular dichroism. The CD results showed that the R75G and R167G mutations did not cause a significant change in the secondary structure of $FtsI_{Ng}$ compared to the wildtype, whereas the E193G mutant had a much reduced α-helical content and an increase in β-sheet structure. The calculated melting temperature of each mutant suggested that the E193G mutation imposed a greater impact on the thermal stability of $FtsI_{Ng}$ compared to the other two mutations. My interpretation is that the E193G mutation affected the protein stability by direct disruption on the secondary structure, whereas the R75G and R167G mutations decreased the amphiphilicity of the peptide segments containing these residues, which resulted in a loss of function of the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction. Aside from the R75G, R167G, and E193G mutations, I also created an E193D mutation and tested its effect on the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction. The B2H result showed that the wildtype $FtsW_{Ng}$ could still interact with the E193D mutant possibly because of the physicochemical similarity between glutamic and aspartic acids. Thus, my conclusion is that the Arg-Arg-Glu motif in *N. gonorrhoeae* FtsI acts as the structural determinant for the interaction with $FtsW_{Ng}$.

Previous studies in *E. coli* showed that several motifs in the N-terminal domain of $FtsI_{Ec}$ containing corresponding residues of R75, R167, and E193 of $FtsI_{Ng}$ were important for cell division and penicillin binding (Goffin et al., 1996; Marrec-Fairley et al., 2000). Structural analysis of $FtsI_{Ng}$ showed that the linker region containing the Arg-Arg-Glu motif in the N-terminus was far away from the active site for penicillin binding (Powell et al., 2009). In my research, penicillin binding assays showed that the R75G and R167G mutants of $FtsI_{Ng}$ had decreased capacity for penicillin binding. Since alterations at both

arginine residues did not affect the secondary structure, it is unclear how mutations in both arginine residues affected the penicillin binding activity of the C-terminal domain. Surprisingly, the E193G mutant did not affect the penicillin binding capacity, although the mutation destabilized the secondary structure of $FtsI_{Ng}$. In total, the results obtained from B2H, circular dichroism and penicillin binding assays suggested that the three key residues that formed the Arg-Arg-Glu motif were required for the $FtsI_{Ng}$-$FtsW_{Ng}$ interaction and penicillin binding.

The attempt to create a gonococcal chromosomal *ftsIR167G* mutant was not successful because of the appearance of a hetero-diploid genotype containing both a wildtype $ftsI_{Ng}$ and a $ftsI_{Ng}$ insertional mutant. Similar genotype was reported by Taha et al (1988) when they tried to generate a *pilA* insertional mutant. The presence of wildtype $ftsI_{Ng}$ in the hetero-diploid mutant indicated that $ftsI_{Ng}$ was an essential gene for cell viability. Using the method described by Ramsey *et al* (2012), the lethal effect of the *ftsIR167G* mutation can be complemented by introducing an extra copy of $ftsI_{Ng}$ on gonococcal genome that is expressed under a tetracycline-inducible promoter. The *N. gonorrhoeae ftsIR167G* mutant will be maintained with the presence of anhydrotetracycline as the inducer, and the effect of *ftsIR167G* mutation on cell division can be ascertained by removing the inducer from the growth medium.

5.4. Limitations

The current model of *N. gonorrhoeae* cell division interactome does not include a putative $FtsL_{Ng}$ for protein interaction analysis because it has low similarity with other homologs (Francis et al., 2000; Snyder et al., 2001). In *E. coli*, $FtsL_{Ec}$ interacts with $FtsK_{Ec}$,

$FtsQ_{Ec}$, $FtsB_{Ec}$, $FtsI_{Ec}$, and $FtsN_{Ec}$ (Di Lallo et al., 2003; Karimova et al., 2005) and forms the FtsQBL complex with FtsQ and FtsB, which is considered as a signal transmitter for cell wall synthesis and constriction (Tsang and Bernhardt, 2015). Thus, it is necessary to assess the involvement of $FtsL_{Ng}$ in *N. gonorrhoeae* cell division. Future studies may focus on ascertaining the role of the putative $FtsL_{Ng}$ for cell division by generating an *N. gonorrhoeae ftsL*-knockout mutant following the method described by Ramsey et al (2012). The potential effect of the *ftsL*-knockout mutation on gonococcal cell division can be determined by removing inducer from the growth medium and examining morphological changes using electron microscopy. Potential interactions of the *N. gonorrhoeae* FtsL with other cell division proteins may be examined using B2H and other *in vitro* assays including GST pull-down and surface plasmon resonance.

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Appendix

Appendix A. Generation of chromosomal *ftsIR167G* mutant with kanamycin resistance gene upstream

Generation of *N. gonorrhoeae* chromosomal *ftsIR167G* mutant was not successful due to the presence of a *ftsI/ftsI*::*ermC/rpsL* hetero-diploid genotype, indicating the necessity of *ftsI* for cell viability (Chapter 4). To examine the effect of R167G mutation of $FtsI_{Ng}$ on *N. gonorrhoeae* cell morphology, I tried to create a chromosomal *ftsIR167G* mutant with a *kan* gene upstream of the start codon of *ftsI*, which confers kanamycin resistance for transformant selection.

Material and methods

Plasmid construction for *N. gonorrhoeae* transformation

The R167G *ftsI* mutant was generated as follows: two DNA segments were amplified using primer pairs AP1/AP4 and AP2/AP3 from *N. gonorrhoeae* FA1090, covering the upstream and downstream sequences of the R167G mutation site (Table S2). Both segments had 20–30 bp overlapping sequences that contained a single base pair substitution that resulted in substitution from arginine to glycine. These two PCR amplicons, generated in the first PCR, were fused in a subsequent primer extension reaction using the primer pair AP1/AP2. The resulting *ftsI* amplicon, containing the desired mutation, was digested with BamHI and EcoRV and ligated into a pre-digested pKH6 and treated with T4 DNA polymerase (M0203S, New England Biolabs) to form blunt ends, yielding pKHIF500 (Table A2). The *kan* gene was isolated from the pET30a using a primer set AP5/AP6 and treated with T4 DNA polymerase (M0203S, New England Biolabs) to

form blunt ends. The pKHIF500 was digested with EcoNI to create the blunt end close to the start codon of $ftsI_{Ng}$ followed by dephosphorylation using calf-intestinal alkaline phosphatase. The *kan* segment was ligated into pre-digested pKHIF500, yielding the pKHIF500kan (Table A2).

Transformation

Transformation of *N. gonorrhoeae* was performed following the liquid transformation protocol described by Dillard (2011). Breifly, 10 µg of linearized plamid DNA was added into 200 µl of prewarmed GCBL medium (1.5% proteose peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl; pH 7.2) containing 5 mM Mg_2SO_4. An overnight culture of piliated *N. gonorrhoeae* was collected and re-suspended into 200 µl of prewarmed GCBL medium containing 5 mM Mg_2SO_4 followed by dilution into an optical density of 0.18 (at 560 nm). 20 µl of cell suspension was transferred into 180 µl of DNA solution and incubated at 35°C for 20 minutes. The cell-DNA mixture was inoculated onto GC base agar plates supplemented with Kellogg's supplement and incubated at 35°C for six hours. Enriched culture was then collected and suspended into 1 ml of pre-warmed GCBL medium following by plating 200 µl of the cell suspension onto GC base agar plates supplemented with Kellogg's supplement and kanamycin. These plates were incubated at 35°C with 5% CO_2 until colonies appeared.

Table A1. Primers used in this study

Primer name		**Sequences (5'-3')**
AP1	fwBamHIFtsIF500	CGGGATCCTCCAGGCAATCCGCATCTTTA
AP2	reEcoRVFtsI500	GCGATACTCTTTGCCGTTTTGCG
AP3	fwftsIR167G	AAAAGAATTAAAAgGCCATTACCCGAT
AP4	reftsIR167G	CCCATCGGGTAATGGCcTTTTAATTCT
AP5	fwKanR	ATGAGCCATATTCAACGGGAAACG
AP6	reKanR	TTAGAAAAACTCATCGAGCATCAAATGAAAC
AP7	fwKan-veri	CTGTGTGCCGGAATCG
AP8	reKan-veri	TTACTTCTCGTTATTATTATCCTGATG
AP9	fwEcoRI-FtsI-pDSW209	CGCGGGAATTCATGTTGATTAAAAGCGAATATAAG
AP10	reBamHI-FtsI-pDSW209	CCCGGGGATCCTTAAGACGGTGTTTTGACG

Table A2. Plasmids used in this study

Plasmid	Relevant genotype	Source/Reference
pKH6	pUP1 derivative carrying one copy of 12bp extended DUS and one copy of 10bp DUS	(Dillard, 2011)
pKHIF500	pKH6 derivative carrying wildtype *ftsI* containing C-G mutation at position 499and its 500bp upstream sequences	This study
pET30a	Kan^R P_{T7}::6Xhis	EMD Millipore, Billerica, MA
pKHIF500kan	pKHIF500 derivative carrying *kan* gene upstream of the start codon of $ftsI_{Ng}$	This study
pDSW209	pGFPmut2 derivative carrying the *gfp* gene	(Weiss et al., 1999)
pDSW209-I_{Ng}	$ftsI_{Ng}$ in pDSW209	This study

Results and discussion

Transformants appeared after 24 hours incubation at 35°C with 5% CO_2. Colonies were sub-cultured onto fresch GCMBK plates containing kanamycin. Overnight cultures were collected and screened for the presence of *kan* gene by PCR using a primer set AP7/AP8 flanking the *kan* gene in pKHIF500kan. Gel electrophorasis showed that all transformants (n=40) contained the *kan* gene inserted upstream of chromosomal

$ftsI_{Ng}$ (Fig. A1). Subsequently, $ftsI_{Ng}$ was PCR amplified using the primer pair AP1/AP2 from these colonies. PCR amplicons were sequenced to confirm the presence of R167G in $ftsI_{Ng}$. The sequencing results showed that none of these transformants contained the R167G mutation in chromosomal $ftsI_{Ng}$. For homologous recombination event in *N. gonorrhoeae*, a flanking DNA sequence with a length between 500-1000 bp is usually required (Dillard, 2011; Hamilton *et al.*, 2001). The R167G mutation site is 530 bp downstream of the insertion site of *kan* gene in this study. Homologous recombination should introduce both *kan* gene and the R167G by the double cross-over event. The lack the R167G in kanamycin-resistant colonies may the lethal effect of the R167G mutation to gonococcal cells.

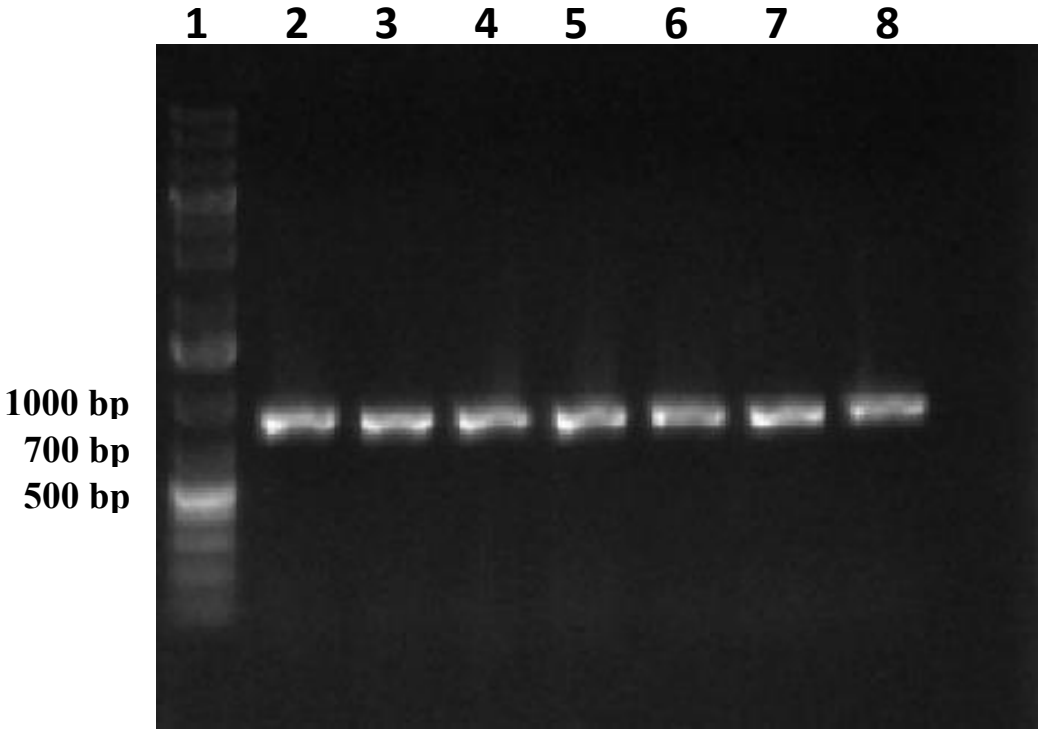

Fig. A1. Agarose (1%) electrophoresis of PCR amplified products using the primer pair AP7/AP8. Lane 1: DNA marker; Lane 2-8: PCR products amplified from individual colonies

Appendix B. $FtsI_{Ng}$ localization in *E. coli*

Since I have successful use an *E. coli* system to study the localization pattern of *N. gonorrhoeae* FtsA, I further created a construct containing wildtype $ftsI_{Ng}$ and expressed it in *E. coli* to ascertain the localization of gonococcal FtsI in *E. coli*.

Material and methods

Plasmid construction for *N. gonorrhoeae* FtsI localization study

Wildtype $ftsI_{Ng}$ was PCR amplified from *N. gonorrhoeae* FA1090 with primers AP9/AP10 (Table A1), PCR amplicon was digested with EcoRI and BamHI and ligated into EcoRI/BamHI-digested pDSW209 [which contained GFP derived from pGFPmut2 (Cormack et al., 1996)], creating pDSW209-I_{Ng} (Table A2).

Results and discussion

Sequencing results confirmed the presence of wildtype $ftsI_{Ng}$ in pDSW209-I_{Ng}. The construct has been transformed into *E. coli* PB103 for localization study. Immuno-blotting analysis needs to be performed to examine the expression level of *gfp-*$ftsI_{Ng}$ in *E. coli* PB103.

Curriculum Vitae

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Academic Credentials

2012-present **Ph.D. in Department of Microbiology & Immunology**

- University of Saskatchewan (Canada)
- Thesis: "*Neisseria gonorrhoeae* cell division interactome and roles of FtsA and N-terminus of FtsI in cell division and antimicrobial resistance"
- Supervisor: Professor Jo-Anne R. Dillon
- Expected date of completion: July 2018

2010-2012 **Master of Science, Department of Biology**

- Saint Mary's University (Canada)
- Thesis: "Effect of H_2 on soil bacterial community structure and gene expression"
- Supervisor: Professor Zhongmin Dong

2006-2010 **Bachelor in Biotechnology, College of Zhongbei**

- Nanjing Normal University (China)
- Thesis: "Anatomical structure of the sexual gland of *Takifugu obscures*"
- Supervisor: Shuyu Gu

Honours And Grants

2012 - 2017	Research Scholarship, Department of Microbiology and Immunology, University of Saskatchewan
2010 - 2012	Research Fellowship, Saint Mary's University Faculty of Graduate Studies and Research Graduate Award, Saint Mary's University
2008 - 2009	Secondary Feng Ruer Scholarship Excellent Student Cadre Excellent Youth League Member
2007 - 2008	'Outstanding Individual in Social Practice' Award Social Work Scholarship Excellent Student Cadre Triple A Student Award

Working Experience

2014-2017	**Teaching assistant** (20 hours per year) • Department of Microbiology & Immunology, University of Saskatchewan (Canada) • Lab course(s): MCIM 390 Experimental Microbiology&Immunology
2010-2012	**Teaching assistant** (100 hours per semester) • Department of Biology, Saint Mary's University (Canada) • Lab course(s): BIOL 1201 Molecular and Cell Biology

	▪ BIOL 2307 Genetics
2007	**Volunteer** • College of Forest Resources and Environment, Nanjing Forestry University • **Project:** Genetic transformation of poplar trees by use of multiple genes and techniques in culturing poplar tissues

Research Experience

2012-present

Location: Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada

Project: *Neisseria gonorrhoeae* cell division interactome

Skills used in project:

- ✓ **Microbiology:** aseptic techniques; Gram stain; bacteria culture
- ✓ **Molecular Biology:** primer design; gene cloning and construction of recombinant expression plasmid, transformation; polymerase chain reaction; DNA gel electrophoresis; site-directed mutagenesis
- ✓ **Protein expression and analysis:** expression; isolation and purification of heterologous protein; sodium dodecyl sulfate polyacrylamide gel electrophoresis; polyclonal antibody preparation; western blots; Bacterial two-hybrid assay; glutathione S-transferase pull-down assay; surface plasmon resonance; circular dichroism; dynamic light scattering
- ✓ **Microscopy:** transmission electron microscopy
- ✓ **Bioinformatics:** Graphpad Prism; Clone Manager; ClustalX; GenBank; PyMOL

2010-2012

Location: Department of Biology, Saint Mary's University, Halifax, Canada

Project: Rhizobacteria of legume crops and mechanisms of rotation benefit

Skills used in project:

- ✓ **Microbiology:** see skills above

- ✓ **Molecular Biology:** see skills above plus total RNA extraction from soil sample; Real-time PCR; terminal restriction fragment length polymorphism
- ✓ **Bioinformatics:** see skills above

2008-2010 **Location:** College of Zhongbei, Nanjing Normal University, Nanjing, Canada

Project: Anatomical structure of the sexual gland of *Takifugu obscures*

Skill used in project:

- ✓ **Histology:** tissue slices
- ✓ **Microscopy:** see skills above

Publications

Peer-reviewed:

Zou, Y., Li, Y., & Dillon, JR. (2017). The distinctive cell division interactome of *Neisseria gonorrhoeae*. *BMC Microbiology*, *17*, 232. http://doi.org/10.1186/s12866-017-1140-1

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In preparation:

Zou, Y., & Dillon, JR. The effect of three conserved amino acids in *Neisseria gonorrhoeae* penicillin binding protein 2 on cell division and antimicrobial resistance. (in progress).

Zou Y., & Dong, Z. Meta-transcriptome Analysis of Soil Microbial Structure and Function Changes Induced by Hydrogen Treatment (in progress).

Conferences:

Zou, Y., Li, Y & Dillon, J.R. (2016). Unique features of the cell division interactome of *Neisseria gonorrhoeae*. 2016 International Pathogenic Neisseria Conference. Manchester, Britain. Abstract 103. Abstract book 20th International Pathogenic Neisseria Conference; 4th–9th September 2016; Manchester, United Kingdom. P.193. http://www.ipnc2016.org/IPNC2016AbstractBook.pdf

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Zou, Y., and Dong, Z. (2012). The Effect of H_2 Treatment on Soil Bacterial Community Structure and Gene Expression. 8th Canadian Plant Biotechnology conference. Guelph, Canada. Abstract IV-3

Zou, Y., and Dong, Z. (2011). Plant-microbe interaction and rotation benefit. Plant Canada

2011 conference. Halifax, Canada. Abstract CSA-S3

Zou, Y., and Dong, Z. (2011). Rhizobacteria of legume crops and mechanisms of rotation benefit. Green Crop Network forum. Montreal, Canada.

Seminars:

Zou, Y. (2017). A distinct cell division interactome in *Neisseria gonorrhoeae*. Department of Microbiology & Immunology, College of Medicine, University of Saskatchewan

Zou, Y. (2016). An *Escherichia coli* expression model reveals the species-specific function of FtsA from *Neisseria gonorrhoeae* in cell division. Vaccine and Infectious Disease Organization - International Vaccine Centre

Zou, Y. (2016). *Neisseria gonorrhoeae* FtsA affects *E. coli* cell division through its interaction with FtsN. Department of Microbiology & Immunology, College of Medicine, University of Saskatchewan

Zou, Y. (2015). The *Neisseria gonorrhoeae* cell division interactome and function of N-domain of gonococcal FtsI protein. Department of Microbiology & Immunology, College of Medicine, University of Saskatchewan.

Zou, Y. (2014). Exploring the cell division interactome in *Neisseria gonorrhoeae*. Department of Microbiology & Immunology, College of Medicine, University of Saskatchewan.

Zou, Y. (2013). Cell division in Gram-negative bacteria. Vaccine and Infectious Disease Organization - International Vaccine Centre

Supervision and training

I have mentored three undergraduate students and supervised the work of a lab technician in Dr. Jo-Anne Dillon's laboratory.

Hobbies

I enjoy playing piano and listening to piano music.

I am a big fan of team sports, such as soccer. I like to watch/play soccer with friends.

I enjoy reading novels in leisure time.

I really enjoy cooking for family and friends.