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Splitting Heirs: A Study of the Cell Division Site Determinant MinD from the Coccus Neisseria gonorrhoeae

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Splitting heirs: A study of the cell division site determinant MinD from the coccus *Neisseria gonorrhoeae*

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Department of Biochemistry, Microbiology, and Immunology

Faculty of Medicine

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ABSTRACT

Proper cell division site placement in the extensively studied rod *Escherichia coli* (Ec) is determined by the Min proteins. MinD<sub>Ec</sub> recruits MinC<sub>Ec</sub> to the membrane to inhibit cell division. MinE<sub>Ec</sub> restricts MinC<sub>Ec</sub>-MinD<sub>Ec</sub> activity to cell pole regions by inducing MinD<sub>Ec</sub> ATPase activity and causing it to dynamically oscillate from end-to-end in *E. coli*. The Dillon laboratory has identified *min* gene homologues in the coccus *Neisseria gonorrhoeae* (Ng) which, unlike *E. coli*, divides along two dimensions. This study investigates the role and identifies functional domains of gonococcal MinD (MinD<sub>Ng</sub>).

MinD is implicated in *Neisseria gonorrhoeae* cell division. Inactivation of *minD<sub>Ng</sub>* dramatically disrupted *N. gonorrhoeae* division patterns and reduced cell viability. Overexpression of MinD<sub>Ng</sub> and MinC<sub>Ng</sub> together in the gonococcus inhibited cell division, producing grossly enlarged cells. MinD<sub>Ng</sub> could complement an *E. coli* minD mutant and induced cell division arrest in this organism, provided endogenous *E. coli* MinC was present. GFP-MinD<sub>Ng</sub> fusions also displayed MinE<sub>Ng</sub>-dependent oscillations within a coiled array in rod-shaped and round mutant *E. coli*.

The self-interaction of MinD<sub>Ng</sub>, as well as MinD<sub>Ec</sub>, was detected using yeast two-hybrid assays, the first report of such interactions. Furthermore, size-exclusion chromatography and analytical ultracentrifugation showed purified MinD<sub>Ng</sub> to be dimeric. MinD<sub>Ng</sub> also interacted with both MinE<sub>Ng</sub> and MinC by yeast two-hybrid experiments.

Structural and sequence alignments implicated a polar region, containing amino acids 92-94, in MinD dimerization. However, mutations to these residues did not eliminate interaction of MinD<sub>Ng</sub> with itself, MinC, or MinE. Interestingly, this region may have a role in permitting bacterial MinD to respond to MinE stimulation, since mutant GFP-MinD fusions failed to oscillate from pole-to-pole in *E. coli*, despite being distributed uniformly within a coiled array. This was supported by *in vitro* assays, where MinE<sub>Ng</sub> was unable to stimulate the ATPase activity of mutated MinD<sub>Ng</sub>.
The conserved extreme N-terminus of MinD_{Ng} was also studied and shown to have a role in affecting MinD_{Ng} ATPase activity and localization dynamics. Successive truncation or mutation of this region affected MinD_{Ng} interaction with other Min proteins. Significantly, these alterations resulted in faster GFP-MinD_{Ng} oscillation cycles and increased tendencies to remain in the cytoplasm. Mutant MinD_{Ng} displaying this behaviour possessed hyper-ATPase activities independent of MinE_{Ng}.

This is the first study to characterize MinD from a naturally occurring coccus and demonstrates the protein acts as a cell division site determinant in *N. gonorrhoeae* and in *E. coli*. Results from this study were used to generate a model for *N. gonorrhoeae* cell division.
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Cheers,

Jason
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>Af</td>
<td>Archaeoglobus fulgidus</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Bs</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>CT</td>
<td>C-terminal truncation</td>
</tr>
<tr>
<td>dcw</td>
<td>division cell wall cluster</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>Ec</td>
<td>Escherichia coli</td>
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<td>kilobase</td>
</tr>
<tr>
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<td>kilodalton</td>
</tr>
<tr>
<td>MTS</td>
<td>membrane targeting sequence</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>Ng</td>
<td>Neisseria gonorrhoeae</td>
</tr>
<tr>
<td>NT</td>
<td>N-terminal truncation [removal of a designated number of amino acids (or codons) from the N-terminus, and replaced with a start codon]</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pf</td>
<td>Pyrococcus furiosus</td>
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<tr>
<td>s</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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CHAPTER 1

INTRODUCTION, HYPOTHESIS, AND OBJECTIVES
INTRODUCTION

Cell division, or cytokinesis, is an essential process that permits proper growth, development, and reproduction of all living organisms. In bacteria, this process generally results in the formation of equivalent viable daughter cells that each possess a full genetic complement. In order to achieve this result, proper spatial and temporal coordination must occur between chromosome replication, chromosome segregation, and ultimate septation (Donachie, 1993).

Detailed studies of the mechanisms of bacterial cell division are relatively new in comparison to that of eukaryotic cells. Since the discovery that a crucial bacterial cell division protein, FtsZ, formed a ring-like structure at midcell in *Escherichia coli* (Bi and Lutkenhaus, 1991), molecular studies have substantially advanced the identification of many of the components required for successful bacterial cytokinesis. Almost all of our present knowledge of this event has been acquired from studying rod-shaped bacteria (bacilli), such as *E. coli* and *Bacillus subtilis*, which, under normal conditions, will divide at the middle of the cell to form two equally sized progeny.

Prior to the actual division event, the proper cytokinetic site at midcell must be chosen in order to ensure the production of equivalent daughter cells. It is now recognized that bacteria can possess quite elaborate mechanisms to specify the placement of septation sites. In *E. coli*, the Min system relies on the dynamic localization and interaction of MinC, MinD, and MinE proteins in order to achieve proper midcell division. However, many species of bacteria are not rod-shaped, and exhibit round, spiral, and asymmetric morphologies instead. Whether the strategies used to address cytokinesis in bacilli can be applied to these other organisms is not known. The present study is the first to characterize the cell division site-determinant MinD from a naturally occurring round bacterium, or coccus, using *Neisseria gonorrhoeae* as a model organism.

In the first section of the Introduction, an overview of bacterial cell division and the key components of the cytokinetic and chromosome segregation machinery will be described using the two most commonly studied organisms, *E. coli* and *B. subtilis*, as examples. In particular, the cell
division components of *E. coli* will be reviewed more extensively, as it is a Gram-negative organism like *N. gonorrhoeae*. The second section will discuss how the placement of cell division sites is regulated in rod-shaped bacteria by the Min proteins. The final section of the Introduction will describe the coccus *N. gonorrhoeae* and the current state of knowledge in gonococcal cell division.
1.1. Bacterial cell division

1.1.1. The division cell wall (dcw) cluster of genes

Bacterial cell division has been most extensively studied in the Gram-negative rod *E. coli*. In this organism, a cluster of 16 genes directly involved in cell division or cell wall biosynthesis has been identified: *mraZ, mraW, ftsL, ftsI (php3), murE, murF, murD, ftsW, murG, murC, ddl, ftsQ, ftsA, ftsZ*, and *envA*. The genes within this division cell wall (dcw) cluster are organized as an operon with transcription proceeding in the same direction (Ayala et al., 1994). Several promoters have been identified within the *E. coli* dcw cluster; however, the main promoter seems to be the one located upstream of *mraZ* (Mengin-Lecreulx et al., 1998) which drives co-transcription of all dcw genes, ending at the single transcriptional terminator identified downstream of *envA* (Lutkenhaus and Addinall, 1996). The remaining promoters are likely involved in regulating different expression levels of individual genes (Lutkenhaus and Addinall, 1996; Vicente et al., 1998).

Such dcw clusters have also been identified in many other bacteria, including *B. subtilis* (Henriques et al., 1992; Daniel et al., 1996), *Haemophilus influenzae* (Fleischmann et al., 1995), *Staphylococcus aureus* (Pucci et al., 1997), *Streptococcus* species (Massidda et al., 1998), and *N. gonorrhoeae* (Dillon laboratory; Francis et al., 2000). While not all bacteria possess the same dcw gene homologues, the order of gene arrangement is generally conserved (Vicente et al., 1998).

The following sections review some of the key proteins encoded by the dcw cluster which are directly involved in the formation of the bacterial division apparatus. Other proteins, not encoded in the dcw cluster, but which are also recruited to the cytokinetic apparatus, are described as well.

1.1.2. The essential cell division protein FtsZ

The actual physical process of bacterial cell division can be divided into several key steps: a) assembly of the cytokinetic apparatus at a specified division site; b) anchoring of the division machinery to the membrane; and c) synthesis of cell wall and cell envelope material to ultimately divide a cell (Errington et al., 2003). Probably the most important bacterial cell division protein is the
highly conserved GTPase FtsZ, which is found in almost all eubacteria (with Chlamydia trachomatis being an exception; Stephens et al., 1998), in Archaea, in chloroplasts, and in mitochondria (Addinall and Holland, 2002).

Green fluorescent protein (GFP) fusions to FtsZ, as well as immunostaining with FtsZ antibodies, have shown that the protein localizes as a distinct ring-like structure (Z-ring) associated with the normal midcell division plane in E. coli (Figure 1.1A) (Ma et al., 1996; Addinall and Holland, 2002). FtsZ proteins also exhibit cross-species functionality, and it has observed that expression of a GFP fusion to N. gonorrhoeae FtsZ localized to the E. coli midcell as well (Figure 1.1B) (Salimmia et al., 2000). Z-ring cytoskeleton formation appears to originate at a single nucleation point and progresses bidirectionally until a closed ring is formed (Sun and Margolin, 1998). This process is completed within one minute of initiation in E. coli cells (Addinall et al., 1997). As cell division proceeds, the Z-ring contracts while remaining at the leading edge of the developing septum between two daughter cells (Bi and Lutkenhaus, 1991). This suggests that the Z-ring is, or is part of, a contractile motor that helps drive septation (Lutkenhaus and Addinall, 1997).

While reduced FtsZ levels result in fewer division events, two distinct E. coli phenotypes are observed upon its overexpression. A two- to seven-fold increase in FtsZ produces a ‘minicell’ phenotype in E. coli characterized by septation at the cell ends (cell poles), resulting in round minicells, in addition to septation at the normal midcell region. At higher expression levels, a block in cell division occurs, giving rise to long filamentous cells (Ward and Lutkenhaus, 1985). It is estimated that there are approximately 15,000 FtsZ molecules in an E. coli cell (Lu et al., 1998), sufficient to form a cytoskeleton that encircles the cell midpoint several times over (Bramhill, 1997). However, recent fluorescence recovery after photobleaching (FRAP) studies in E. coli have indicated that only about 30 % of total FtsZ is found in the Z-ring at any particular time, with the remaining protein engaged in rapid dynamic exchange between the ring and the cytoplasm (Stricker et al., 2002).
Figure 1.1. Cell division in the rod *E. coli*. (A) *E. coli* stained with anti-FtsZ antisera show FtsZ localization at the middle of each cell [reprinted with permission from Elsevier (Addinall and Holland, 2002)]. (B) Expression of GFP-fusion to *N. gonorrhoeae* FtsZ protein in *E. coli* also results in midcell localization of fluorescent fusion protein (Dillon laboratory). (C) Assembly of cytokinetic proteins at the *E. coli* cell division site. Numbers indicate the general order to which proteins are recruited to the division site. FtsZ (Z) initiates polymerization at the division site and may be stabilized by FtsA (A) and ZipA (ZA). ZipA may act as a membrane anchor for FtsZ. Proteins subsequently recruited to the septal FtsZ-ring include FtsK (K), FtsQ (Q), FtsL (L) and YgbQ (Y), FtsW (W), FtsI (I), and FtsN (N) [reprinted with permission from the American Society for Microbiology (Errington et al., 2003)]. (D) MinD is proposed to direct MinC to its target, FtsZ, at impending septation sites along the inner cell membrane. The N-terminus of *E. coli* MinC inhibits FtsZ polymerization, while the C-terminus is involved in self-association and interaction with MinD (Hu and Lutkenhaus, 2000; Szeto et al., 2001b) (Figure obtained from Errington et al., 2003).
Crystallographic studies of FtsZ from the Archaea Methanococcus jannaschii have shown that it bears a remarkable structural resemblance to eukaryotic tubulin, despite overall sequence dissimilarities (Löwe and Amos, 1998). Like tubulin, FtsZ is able to polymerize in a GTP-dependent manner. In vitro studies have shown that FtsZ-GTP is able to assemble into several structures, including protofilaments and sheets (Löwe and Amos, 1999), while the hydrolysis of GTP or addition of GDP promotes FtsZ disassembly and/or formation of curved protofilaments and tubes (Mukherjee and Lutkenhaus, 1998; Lu et al., 2000).

The actual arrangement of FtsZ subunits within the Z-ring and the mechanism of its constriction in vivo are currently not known. In one model, the Z-ring is proposed to be formed from short, overlapping filaments of FtsZ that slide along each other by means of an unidentified motor protein, leading to Z-ring constriction (Bramhill, 1997). Another model suggests that Z-ring constriction occurs by depolymerization of FtsZ subunits from one, or both, end(s) of an FtsZ polymer (Bramhill, 1997). A third model proposes that bending or curving of FtsZ filaments due to GTP hydrolysis may result in Z-ring constriction (Lu et al., 2000). FtsZ does not possess any defined membrane spanning sequence (Errington et al. 2003), hence each model requires that the FtsZ cytoskeleton be anchored to the membrane by another partner in order to exert its constrictive effects (Bramhill, 1997); however, such an anchor has yet to clearly identified.

Aside from its proposed ‘contractile’ role during septation, the FtsZ ring is believed to serve as a scaffold to which other cell division proteins are recruited. Thus far, all other cell division proteins seem to require the presence of the Z-ring for their successful recruitment to the division site (Errington et al., 2003). In addition, there is a specific sequence in which downstream cell division proteins appear. Each protein depends on the previous protein in order to be properly localized to the E. coli cytokinetic ring in the following order: FtsA and ZipA, FtsK, FtsQ, FtsI and YgbQ, FtsW, FtsI, and FtsN (Errington et al., 2003) (Figure 1.1 C).
1.1.3. FtsA, ZipA and Z-ring stabilization

The next proteins to associate with the cytokinetic Z-ring are FtsA and ZipA. Z-rings are capable of forming in the presence of either FtsA or ZipA, but not when both proteins are absent (Pichoff and Lutkenhaus, 2002). In turn, both proteins require FtsZ for their localization to the division plane, however they do so independently of each other (Hale and de Boer, 1997; Hale and de Boer, 1999; Pichoff and Lutkenhaus, 2002).

FtsA is an ATP-binding protein that belongs to the actin/hsp70/sugar kinase superfamily (Sánchez et al., 1994; Feucht et al., 2001) and the crystal structure of FtsA from the thermophilic bacterium Thermotoga maritima shows that it is structurally homologous to actin (van den Ent and Löwe, 2000). FtsA co-localizes as a ring with FtsZ at division sites (Ma et al., 1996). In E. coli, overexpression of FtsA will arrest cell division (Wang and Gayda, 1990); however, increasing FtsZ levels can counteract this effect (Dai and Lutkenhaus, 1992). This suggests that the ratio of FtsZ to FtsA (normally ~100:1) is required for proper cell division to occur (Dai and Lutkenhaus, 1992). No defined role for FtsA in cytokinesis has been established; however, it may play a role in cross-linking and stabilizing Z-ring polymers and/or recruiting other cell division proteins (Errington et al., 2003).

ZipA (Z-interacting protein A) is an inner membrane protein containing an N-terminal membrane domain, and a C-terminal cytoplasmic domain (Ohashi et al., 2002). Currently, ZipA is the leading candidate for the FtsZ membrane anchor (Erickson, 2001). ZipA is recruited to the division site by FtsZ (Hale et al., 1999; Liu et al., 1999), with both proteins interacting at their C-termini (Hale et al., 1997; Hale et al., 2000; Haney et al., 2001). In vitro experiments have shown that ZipA stabilizes (RayChaudhuri, 1999) and induces bundling of FtsZ polymers (Hale et al., 2000).

FtsZ polymerization is also prevented by certain proteins. The general cell division inhibitor MinC, part of the Min system in E. coli (de Boer et al., 1989), has been shown to interact with and disassemble FtsZ polymers in vitro (Hu and Lutkenhaus, 2000). Furthermore, overexpression of MinC in E. coli will lead to a filamentous phenotype, indicative of cell division arrest (de Boer et al., 1992). Hence, it is proposed that MinC can regulate cell division events at the membrane in vivo by
inhibiting FtsZ-ring formation, and thus subsequent recruitment of other downstream cytokinetic proteins (Bi and Lutkenhaus, 1993; Hu et al., 1999) (Figure 1.1 D). More detailed description of MinC and the Min system can be found in Section 1.3.2. of the Introduction.

There are several other proteins, not necessarily found in E. coli, which may also contribute to Z-ring stability. In the Gram-positive rod B. subtilis, two proteins, EzrA and ZapA, also seem to be involved in maintaining Z-ring integrity. The recently described ZapA (Z-ring associated protein A) is distributed more widely than ZipA, and orthologs are found in B. subtilis and in E. coli (Gueiros-Filho and Losick, 2002). Like ZipA, ZapA localizes to the B. subtilis Z-ring at an early stage in cell division and this localization is dependent upon FtsZ. In vitro, ZapA can also bind FtsZ and stimulate FtsZ-GTP protofilament bundling into branched networks; therefore, ZapA likely plays a role in Z-ring stability (Gueiros-Filho and Losick, 2002).

In B. subtilis, deletion of erzA lowers the critical concentration of FtsZ needed to form Z-rings and results in the formation of multiple Z-rings distributed along the length of the cylindrical cell (Levin et al., 1999). These results suggest that erzA may have the opposite function of ZipA from E. coli, and acts to promote Z-ring depolymerization. Hence, bacterial cell division proteins such as FtsA, ZipA, ZapA, and EzrA, appear to be involved in FtsZ polymer stabilization/destabilization. The actions of these proteins may also account for the dynamic remodeling of the Z-ring observed with the continuous exchange of FtsZ subunits between Z-polymers and the cytoplasmic pool (Gueiros-Filho and Losick, 2002; Stricker et al., 2002; Margolin, 2003).

1.1.4. FtsN, FtsL, YgbQ, and FtsQ

Comparatively little is known about FtsN, FtsL, YgbQ, and FtsQ transmembrane proteins relative to the other members of the cytokinetic ring. The ftsQ gene is commonly found directly upstream of ftsA and ftsZ in the deo gene cluster in many bacteria (Errington et al., 2003). FtsQ is a low copy number protein in E. coli (~20 copies/cell; Carson et al., 1991) and contains a short N-terminal cytoplasmic domain, a transmembrane region, and a periplasmic domain; however, only the
periplasmic domain is required for localizing to the septum (Chen et al., 1999). Although essential for cell division, the role of FtsQ in bacterial cytokinesis is not known (Chen et al., 1999; Errington et al., 2003).

FtsL is present in low abundance in *E. coli* (20-40 copies/cell) and contains a coiled-coil motif in its periplasmic domain implicated in homodimerization and localization to the division site (Guzman et al., 1992; Ghigo et al., 2000). Since FtsL has no apparent catalytic domains, it is proposed to have a structural role in cell division (Ghigo et al., 2000). Another protein, YgQ, has recently been shown to localize to the division site with FtsL. Both proteins required one another for this localization, and it is proposed that they interact through a coiled-coil structure (Buddelmeijer et al., 2002).

FtsN carries a short N-terminal cytoplasmic domain, a single transmembrane region, and a large periplasmic domain that is responsible for targeting to the septal ring (Addinall et al., 1997). FtsN has some similarity to cell wall amidases and may play a role in degrading cell wall material required during the constriction process of cytokinesis (Errington et al., 2003).

1.1.5. **Cell wall synthesis at the division site: FtsI and FtsW**

In order to physically separate two future daughter cells, new peptidoglycan (murein) must be incorporated at the division site. The gene *ftsI* encodes penicillin-binding protein 3 (PBP-3 or FtsI), a transpeptidase that catalyzes the addition of new cell wall material, in the form of glycan strands, to septation sites (Bramhill, 1997). A related protein, PBP-2, is involved in the addition of peptidoglycan strands to the lateral cell wall, and hence for cell elongation and growth (Errington et al., 2003).

FtsI contains an N-terminal transmembrane region and a larger C-terminal domain containing the catalytic motifs common to other PBPs (Goffin and Ghuysen, 1998). This protein is proposed to form part of a murein-synthesizing complex that adds glycan strands in such a way that a constriction forms at the site of division. A “three-for-one” model for glycan strand insertion has been proposed to
account for the sharp inward growth of murein observed during cell division (Höltje, 1998). In
general, the model suggests that three crosslinked strands of new glycan are brought to an existing
murein ‘docking’ strand at a designated cell division site. The FtsI-containing complex incorporates
these crosslinked strands underneath the single docking strand so that the latter is ‘straddled’ by the
new strands. The murein-synthesizing complex also bears lytic transglycosylase and endopeptidase
activity, and can degrade the old docking strand (Bramhill, 1997). In essence, the three new glycan
strands (murein triplets) underneath the docking strand will form the basis for murein constriction.
The addition of more murein triplets to the apex of the constriction, coupled with the inward pull of
the membrane, driven by the anchored Z-ring, is proposed to result in cell constriction and the
formation of two cells (Höltje, 1998).

FtsW contains 10 membrane spanning regions (Lara and Ayala, 2002), and its presence is
always correlated with that of FtsI, suggesting a link in their functions (Errington et al., 2003).
Interestingly, FtsW has high homology to RodA, a shape-determinant in E. coli (Bramhill, 1997).
RodA is one of several proteins implicated in maintaining the rod-shape typically found in E. coli,
and abrogation of this gene results in round E. coli cells (Begg et al., 1986). Studies investigating
murein synthesis in temperature sensitive rodA E. coli cells have shown that murein synthesis
directed towards cell elongation is affected upon rodA impairment (de Pedro et al., 2001). Due to
their similarities, it has been proposed that FtsW and RodA act as translocation proteins that shuttle
murein precursors to their corresponding PBPs, namely PBP-3 (cell division) and PBP-2 (cell
elongation), respectively (Höltje, 1998).

1.1.6. FtsK

FtsK is a multi-role protein and has two distinct functions mediated by its N-terminal
membrane domain and its C-terminal cytoplasmic domain. The first domain is involved in cell
division, as cells encoding only the N-terminal domain of FtsK are still able to divide (Liu et al.,
1998). In addition, while a deletion of ftsK inhibits E. coli cell division, a corresponding
overexpression of the FtsK N-terminus can reverse cell division arrest (Draper et al., 1998). The formation of smooth filaments (without any signs of constriction) in ftsK mutants (Wang and Lutkenhaus, 1998) and the dependence on FtsK for FtsQ, FtsL, and FtsI localization (Chen and Beckwith, 2001) supports the notion that the protein is required at an early stage in cytokinesis.

FtsK is a member of the SpoIIE family of DNA translocases and its C-terminal domain contains an ATP-binding site that functions in chromosome partitioning, or the movement of replicated bacterial chromosomes through the closing septum (see below for an overview of chromosome segregation). The protein has been shown to use an ATP-dependent mechanism to actively push DNA through the septum (Aussel et al., 2002). *E. coli* lacking the C-terminus of FtsK exhibit defective chromosome segregation (Liu et al., 1998). Hence, FtsK also plays an essential role late in the cytokinetic process, and provides an intimate link between cell division and chromosome segregation.

Cell division must ensure that progeny cells each receive complete chromosomal material; however, chromosome replication can often lead to the formation of circular dimers of daughter chromosomes formed by odd numbers of recombination events (Draper and Gober, 2002). FtsK functions to resolve such dimers prior to their segregation into daughter cells. Resolution is achieved by a recombination event mediated through a 28 base pair sequence, termed dif, that is found in each daughter chromosome near the replication terminus (Kuempel et al., 1991). The C-terminus of FtsK is believed to activate the XerC and XerD DNA resolvases that will catalyze dimer resolution at dif sites and produce separate, uncatenated chromosomes (Aussel et al., 2002). Since FtsK is localized to the septum, the resolution of chromosome dimers is ensured prior to full septum closure (Draper and Gober, 2002).
1.2. Bacterial chromosome segregation

In most cases, the bacterial chromosome is circular and DNA replication is initiated at a single origin of replication \((\text{ori}C)\) and proceeds bidirectionally towards a terminus located approximately opposite of \(\text{ori}C\) (Draper and Gober, 2002). Successful DNA replication, followed by the complete distribution of chromosomes to each daughter cell during cytokinesis, is paramount to the production of viable cells. One model of chromosome segregation proposes that newly replicated chromosomes are attached to the inner membrane at midcell and that cell elongation occurring prior to cell division would provide the motive force needed to equally distribute chromosomal material to each offspring cell (Jacob et al., 1963). A more contemporary ‘transversion-mediated’ segregation model has been proposed, whereby newly replicated DNA is linked to the membrane by virtue of coupled transcription, translation, and translocation of membrane proteins, creating bidirectionality in chromosome segregation in the predivisional cell (Woldringh, 2002).

1.2.1. Intracellular migration of the chromosomal origin of replication

Recent advances in intracellular localization and biochemical techniques have challenged the above proposals. By introducing \(\text{lac}\) operator sites near \(\text{ori}C\), the position of the replication origin could be tracked in \(E.\ coli\) and in \(B.\ subtilis\) cells that encoded GFP-fusions to the \(\text{lac}\) repressor (LacI-GFP) (Gordon et al., 1997; Webb et al., 1997; Webb et al., 1998). These and other studies have shown that \(\text{ori}C\)s exhibit dynamic movement throughout the cell cycle.

In cells undergoing rapid growth and DNA replication, two copies of GFP-labeled \(\text{ori}C\) were observed positioned at opposite cell poles. These labeled \(\text{ori}C\)s migrated towards the cell center prior to re-initiating DNA replication. After replication, four origins were observed in a single cell, with one of the origins from each chromosome moving to the cell pole while the other remained near midcell. Hence, two chromosomal DNA masses (nucleoids) were situated at the one- and three-quarter positions of the predivisional cell (Hiraga et al., 1990). Cell division and septum formation
then proceeds, resulting in daughter cells that each have oriC regions at their cell poles (Webb et al., 1998; Draper and Gober; 2002).

Since the oriC regions could move irrespective of cell elongation, it was unlikely that simple lateral cell envelope growth was responsible for their migration (Sharpe and Errington, 1998; Webb et al., 1998; Draper and Gober; 2002). Furthermore, since the measured rate of oriC movement in B. subtilis (0.17 mm/min) was greater than that of cell elongation (0.011-0.025 mm/min), it argued against the chromosome segregation model based on simple nucleoid/membrane association (Webb et al., 1998).

1.2.2. The extrusion-capture model of chromosome partitioning

What drives the migration of bulk chromosomal material towards cell pole regions in the predivisional cell? Recently, it was shown that the DNA replication complex, or replisome, is located at a relatively fixed site at midcell in B. subtilis (Lemon and Grossman, 1998). Experiments using [³H] thymidine to localize newly duplicated DNA in E. coli have also indicated that DNA replication occurs at a midcell site (Koppes et al, 1999). Thus, it is proposed that a spooling effect caused by the movement of replicating DNA through a stationary replisome may provide some of the necessary motive force needed for chromosome segregation (Lemon and Grossman, 1998; Lemon and Grossman, 2000; Lemon and Grossman, 2001).

The ‘push’ imparted on nascent chromosomes by the replisome may also be aided by corresponding ‘pulls’ caused by DNA condensation proteins, such as MukB (from E. coli) or Smc (structural maintenance of chromosomes) in B. subtilis (Draper and Gober, 2002). Both proteins belong to a large SMC family of proteins involved in DNA repair, condensation, and segregation (Draper and Gober, 2002). MukB is an ATP-binding protein that exhibits ATPase activity in the presence of DNA (Niki et al., 1991; Niki et al., 1992). Both MukB and Smc are large proteins (176 kDa and 135 kDa, respectively) and electron microscopy has shown each to exist in homodimeric
arrangements characterized by two globular domains linked by a flexible coiled-coil domain that allows significant changes in conformation (Niki et al., 1992; Melby et al., 1998).

Mutations in *E. coli mukB* produced a significant proportion of cells bearing improperly segregated chromosomes, including DNA masses of varying size and location (Niki et al., 1991). Null mutations in *B. subtilis* Smc also affected chromosome segregation (Britton et al., 1998; Moriya et al., 1998).

While MukB localizes to the nucleoid, Smc localizes to both nucleoid and adjacent polar regions (Britton et al., 1998; Graumann et al., 1998). It is proposed that these DNA condensation proteins may disassemble from DNA prior to its replication and reassemble onto the nascent chromosomes to provide the additional tension required to direct chromosome movement away from midcell (Lemon and Grossman, 2001).

As replication is completed, two interlinked chromosomal DNA circles result, which are decatenated by topoisomerase IV. Any chromosome dimers that may have also formed, due to odd numbers of recombination events, are resolved by FtsK (already recruited to the constricting midcell division ring) and the XerCD recombinases (Lemon and Grossman, 2001; Donachie 2001). Remaining DNA is finally translocated by FtsK to their respective daughter cell compartments before septum closure (Aussel et al., 2002). Hence, the combined motive force provided by a stationary replisome and DNA organizing molecules have led to this current ‘extrusion-capture’ model of chromosome partitioning (Lemon and Grossman, 2001; Draper and Gober, 2002). However, it is still possible that co-transcription/translation of proteins coupled to the membrane may also play some role in active chromosome partitioning (Lemon and Grossman, 2001).

1.2.3. Par proteins and their involvement in chromosome partitioning

Eukaryotic sister chromosomes are separated by the actions of a mitotic spindle apparatus originating from each end of the dividing cell. The spindle apparatus is attached to chromosomal centromere regions through protein structures termed kinetochores (Nanninga, 2001). The possibility
that bacteria possess a functionally equivalent apparatus has been raised with several nucleoid associated proteins, including members of the Par protein family, which may help direct the movement of nascent chromosomal origins towards the cell poles (Draper and Gober, 2002).

Par proteins were first studied in the *E. coli* low-copy number plasmids P1 and F, where they are involved in efficient plasmid partitioning between daughter cells (Gerdes *et al.*, 2000). Plasmid P1 encodes ParA and ParB proteins. ParB binds co-operatively to a *parS* DNA site on the plasmid, and also interacts with the ATPase ParA (Gerdes *et al.*, 2000). ParA itself is membrane associated and may tether the ParB-*parS* complex to specific cellular locations (Lin and Mallavia, 1998).

Many bacteria possess chromosomal homologues of these plasmid-borne *par* genes, including *B. subtilis* [Soj, ParA homologue; SpoOJ, ParB homologue] and *Caulobacter crescentus* (Draper and Gober, 2002); however *E. coli* lacks any such genes (Lemon and Grossman, 2001). Like plasmid P1, the *B. subtilis* chromosome also possesses several *parS* sites near its *oriC* and localization experiments have revealed that SpoOJ binds to these sites and localizes to the cell poles (Lewis and Errington, 1997). In contrast, Soj undergoes dynamic SpoOJ-dependent movement that has been described as ‘internucleoid jumping’, involving its apparent assembly into a single, large nucleoid-associated patch, followed by dynamic movement of the Soj patch onto an adjacent nucleoid. Such movement occurred on a timescale of minutes (Marston and Errington, 1999a). Others have observed that Soj movement is more of a pole-to-pole pattern, rather than nucleoid-to-nucleoid (Quisel *et al.*, 1999). The intracellular jumping of Soj, and the binding of SpoOJ to *parS* regions near *oriC*, has led to the proposal that Par systems may act to direct *oriC* to cell pole regions during chromosome partitioning, possibly comparable to the eukaryotic centromeric system (Gordon and Wright, 2000; Bignell and Thomas, 2001; Draper and Gober, 2002).

Another possible candidate for restricting nascent chromosomes to cell polar regions is the recently described *B. subtilis* RacA protein (Ben-Yehuda *et al.*, 2003). Analysis of RacA revealed a putative helix-turn-helix DNA binding motif and RacA-GFP fusions were colocalized with the nucleoid, particularly around the origin region. However, the protein was also strongly associated
with the cell poles, prompting the proposal that RacA may form a chromosomal anchor (Ben-Yehuda et al., 2003). RacA may be tethered to the cell poles by DivIVA, another protein normally found *B. subtilis* cell poles. Hence, RacA may be a kinetochore-like protein that is attached to both cell poles and that binds to a centromere-like region in newly replicated *B. subtilis* chromosomes which anchors them to the cell poles (Ben-Yehuda et al., 2003).
1.3. Spatial regulation of the cell division site

In general, rod-shaped cells divide at midcell to produce viable bacterial cells that each contain a full genetic complement. However, before cell division is even initiated, the correct site for cell division must be determined. Currently, there are two proposed mechanisms of toporegulating bacterial division sites: nucleoid occlusion and the Min protein system. Little is known about the molecular details responsible of the first mechanism in comparison to the second; hence, a brief discussion of nucleoid occlusion will be followed by a more detailed review of Min proteins.

1.3.1. Nucleoid occlusion model

The nucleoid occlusion model states that cell division generally occurs in regions devoid of nucleoid masses, such as the space between newly separated chromosomes (Mulder et al., 1989; Woldringh et al., 1990) (Figure 1.2). In this way, the predivisional cell ensures that its progeny will each receive a complete chromosome. This model was developed through the observation that visible cytokinesis in *E. coli* does not occur over regions occupied by a nucleoid mass (Woldringh et al., 1991). This was further supported by FtsZ localization studies showing that Z-rings usually do not form over nucleoids (Yu and Margolin, 1999; Sun and Margolin, 2001). In addition, mutant cells with non-replicating nucleoids still display preferential Z-ring formation over nucleoid free regions (Gullbrand and Nordstrom, 2000; Sun and Margolin, 2001).

The compactness of the nucleoid itself may play a role in preventing Z-ring assembly. In wild-type *E. coli*, Z-ring formation coincides with the termination of DNA replication, a time when most of the chromosomal mass has been cleared away from midcell (Den Blaauwen et al., 1999). In *E. coli* that cannot resolve chromosome dimers (due to mutations in *dif, xerC*, and *xerD*), cell division can occur on top of unresolved dimer chromosomes, resulting in a guillotining effect on the DNA. In this case, it is proposed that the remaining unresolved DNA at the midcell is ineffective at imparting nucleoid occlusion, thus permitting complete cell division (Hendricks et al., 2000). Moreover, inactivation of the DNA condensation protein MukB in *E. coli* produces decondensed chromosomes
Figure 1.2. Nucleoid occlusion and the Min protein system act to toporegulate cell division in E. coli. (A-B) In the predivisional cell, the nucleoid mass (black oval) prevents FtsZ ring formation throughout the central region of the cell (red bar). The Min system acts to prevent FtsZ ring formation at cell pole regions (black bars). (C) As DNA replication terminates, a nucleoid-free zone between each daughter chromosome is formed. The lack of nucleoid occlusion in this central region allows FtsZ polymerization to proceed (dotted line). The Min system continues to inhibit cell division at cell pole regions. (D) Cell division is completed and nucleoid occlusion and the Min system are re-established in each daughter cell.
over which FtsZ ring assembly can occur, further suggesting that the compactness of the nucleoid plays a role in regulating Z-ring formation (Yu et al., 2001).

Although a defined mechanism of nucleoid occlusion has not been elucidated, one recent proposal attempts to link the transsertion-mediated model of chromosome segregation with nucleoid occlusion (Woldringh, 2002). It is proposed that the coupled transcription, translation, and translocation of membrane proteins from nascent DNA creates a crowded condition around the nucleoid that prevents FtsZ assembly. This inhibition may be suppressed towards the end of DNA replication by virtue of a decreased number of expressed genes in the chromosomal terminus region. Since the terminus is localized to the midcell at a late stage in the DNA replication cycle, Z-ring assembly at this ‘spatially-free’ site can proceed (Woldringh, 2002; Zaritsky and Woldringh, 2003). Interestingly, in outgrowing B. subtilis spores grown under thyminineless conditions that prevented DNA replication, it was found that Z-ring assembly could still occur over the still compact nucleoid mass, which argues against the idea of nucleoid occlusion, at least in this gram-positive rod (Regamey et al., 2000).

1.3.2. The Min system of cell division site determination

1.3.3. The min genes

Although the mechanism of nucleoid occlusion remains unclear, cell division toporegulation by Min proteins in bacilli is significantly better elucidated. The min genes were first identified by characterizing E. coli cells that displayed a ‘minicell’ phenotype due to mutations at a locus termed the minB (minicell) operon, containing minC, minD, and minE genes (Davie et al., 1984; de Boer et al., 1988; de Boer et al., 1989). Such mutants displayed cell division at their cell poles, in addition to cytokinesis at the normal midcell site, resulting in a population consisting of round chromosomeless minicells, normal sized cells, and filamentous cells of varying lengths (Adler et al., 1967; de Boer et al., 1988; de Boer et al., 1989). Although minicells are not viable, normal midcell division occurs in sufficient numbers of min mutant cells to maintain the population (Margolin, 2001a).
Cytokinesis in cells lacking the min genes will still occur between nucleoid masses, indicating that nucleoid occlusion functions independently of the Min proteins (Yu and Margolin, 1999; Margolin, 2001a). In cells lacking both normal chromosome segregation and Min proteins, clusters of closely spaced FtsZ rings are observed within the nucleoid free regions of these filamentous cells, suggesting roles for both the nucleoid and Min proteins in Z-ring localization. In addition, these studies revealed the potential for Z-ring formation at all positions along the length of the *E. coli* rod (Yu and Margolin, 1999). Hence, while nucleoid occlusion seems to prevent untimely Z-ring formation over unsegregated nucleoids, the Min system appears to act by inhibiting cell division at the cell poles (Figure 1.2). The overall result is the restriction of cell division to the proper midcell site upon completion of chromosome replication (Margolin, 2001a).

1.3.4. MinC

*E. coli* MinC (MinC Ec; ~25 kDa) acts as a general cell division inhibitor. Overexpression of MinC Ec in wild-type cells leads to the formation of long filaments, a hallmark of cell division inhibition, while the loss of MinC Ec produces a minicell phenotype (de Boer et al., 1989). In *E. coli* lacking all its chromosomal min genes, the overexpression of both MinC Ec and MinD Ec in the absence of MinE Ec could induce total cell division arrest, causing a long filamentous morphology. This suggested that MinC Ec and MinD Ec form a division inhibitor that acts throughout the length of the cell (de Boer et al., 1989). Further studies showed that a >25-fold increase in the levels of MinC Ec alone will inhibit *E. coli* cytokinesis (de Boer et al., 1992). The requirement for such a large increase in MinC to arrest cell division suggested that MinD may function to augment MinC activity (de Boer et al., 1992).

MinC Ec [231 amino acids (aa) long] consists of two distinct domains, with the N-terminus comprising amino acids 1-99 and the C-terminus formed by residues 125-231, both joined by a linker region (Hu and Lutkenhaus, 2000). Size exclusion chromatography has shown MinC Ec to be a dimer (Hu and Lutkenhaus, 2000; Szeto et al., 2001b), while yeast two-hybrid studies have detected a strong
interaction between MinC_{Ec} and MinD_{Ec} (Huang et al., 1996). Using far-UV circular dichroism the MinC_{Ec} C-terminus has been shown to contain predominantly β-sheet secondary structure (Szeto et al., 2001b). Furthermore, the MinC_{Ec} C-terminus has been implicated in the self-association of the protein and in its interaction with MinD_{Ec} (Hu and Lutkenhaus, 2000; Szeto et al., 2001b).

The crystal structure of MinC from the thermophilic bacterium *Thermotoga maritima* (Tm) has been elucidated, showing the protein existing as four MinC_{Tm} molecules arranged as two dimers (Cordell et al., 2001). Similar to *E. coli* MinC, each MinC_{Tm} monomer contains two domains. The N-terminus is composed of two α-helices and five β-strands, while the C-terminus consists of a right-handed β-helix that forms a triangular barrel-like structure with three surfaces, designated A, B, and, C (Cordell et al., 2001). The dimerization interface of MinC_{Tm} appears to lie on the hydrophobic A surface (Cordell et al., 2001).

Using biosensor technology, a purified MalE-MinC_{Ec} fusion was shown to interact with FtsZ (Hu et al., 1999). Furthermore, FtsZ polymers are disassembled in the presence of MalE-MinC_{Ec}; however, MinC_{Ec} disassembles FtsZ polymers by a mechanism that does not involve modifying FtsZ GTPase activity, in contrast to another characterized FtsZ inhibitor, SulA (Hu et al., 1999). *In vitro*, the N-terminus of MinC_{Ec} has been shown to be sufficient to inhibit FtsZ polymerization, and overexpression of a MalE-MinC_{Ec} fusion encoding only the first 115 residues of MinC in *E. coli* can inhibit cell division (Hu and Lutkenhaus, 2000).

**1.3.5. MinD**

Of the three Min proteins, MinD is the most ubiquitously distributed (Ramirez-Arcos et al., 2001a) and is the most conserved bacterial protein involved in cell division, aside from FtsZ (Sakai et al., 2001). This protein has a conserved ATP-binding site and is part of the ParA subfamily of proteins, which includes proteins involved in DNA segregation (de Boer et al., 1991; Lutkenhaus and Sundaramoorthy, 2003).

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In *E. coli* (Ec), the presence of MinD_{Ec} (270 residues; ~30 kDa) greatly reduces the amount of MinC_{Ec} required to induce cell filamentation; hence, MinD_{Ec} has been termed an activator of MinC_{Ec} (de Boer *et al.*, 1992). In contrast, overexpression of MinD_{Ec} does not block *E. coli* cell division in the absence of MinC_{Ec} (de Boer *et al.*, 1992). *E. coli* that lack functional MinD_{Ec} exhibit a classic minicell phenotype and experience cell division at polar sites, in addition to the cell center (de Boer *et al.*, 1988). Immunoelectron microscopy localized MinD to the inner cell membrane of *E. coli* (de Boer *et al.*, 1991), consistent with initial proposals that the protein may act at the cell envelope (with MinC_{Ec}) to block cell division (de Boer *et al.*, 1989).

MinD proteins contain what is classified as a ‘deviant’ Walker A ATP-binding motif (P-loop) at its N-terminus (aa 10-17 in *E. coli* MinD) (de Boer *et al.*, 1991; Lutkenhaus and Sundaramoorthy, 2003) (Figure 1.3 A, B; green and blue residues). Classic Walker A motifs have a GXXGXGK[T/S] sequence (where X is any amino acid), while the deviant consensus sequence is XKGGXXK[T/S] and includes a signature lysine (bold) found in MinD proteins (Koonin, 1993; Lutkenhaus and Sundaramoorthy, 2003) (Figure 1.3 A, B; black arrow). Purified MinD_{Ec} can bind ATP (de Boer *et al.*, 1991) and dimerizes in the presence of this nucleotide (Hu *et al.*, 2003). By itself, MinD_{Ec} has relatively weak ATPase activity, and point mutations within the Walker A motif (e.g. K16Q) abolished the ability of the protein to activate MinC_{Ec}-mediated division inhibition in *E. coli*, thus indicating a role for ATP in this process (de Boer *et al.*, 1991) (K16 residue of a MinD homologue from *Archaeoglobus fulgidus* is shown as blue residue in Figure 1.3 A, B).

The crystal structures of several MinD homologues have been elucidated from the Archaeal organisms *A. fulgidus* (Af), *Pyrococcus furiosus* (Pf), and *P. horikoshii* (Ph). While all structures consisted of monomeric protein, they were obtained bound to ADP (MinD_{Af} and MinD_{Pf}) (Hayashi *et al.*, 2001; Sakai *et al.*, 2001); to an ATP analogue, AMPPCP (MinD_{Af}) (Hayashi *et al.*, 2001); or in the absence of nucleotide (MinD_{N}) (Cordell and Löwe, 2001). All proteins consisted of a curved β-sheet formed of seven parallel, and one antiparallel, β-strands (Figure 1.3 A, B; orange residues). In
Figure 1.3. Crystal structure of MinD from *Archaeoglobus fulgidus* (Cordell and Löwe, 2001). (A, B) MinD consists a curved β-sheet formed of seven parallel and one antiparallel β-strands (orange residues) surrounded by a network of 11 α-helices (red residues). Residues forming the Walker A ATP-binding motif are shown in green and blue. Blue residue represents residue K16. Signature lysine in this ATP-binding motif is indicated with a black arrow. Purple and cyan residues indicate position of switch I and switch II sites, respectively. The MinD α-7 helix is indicated with a green arrow. (B) depicts a 90° forward rotation of (A).
turn, this centralized β-sheet is surrounded by a network of 11 α-helices (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001) (Figure 1.3 A, B; red residues). Structurally, the solved MinD proteins most closely resemble the monomeric subunits that form dimeric nitrogenase iron proteins (NIPs) (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001), such as NifH that are involved in nitrogen fixation (Schindelin et al., 1997).

The NifH dimer interface includes the ATP binding sites of each monomer, such that a nucleotide sandwich is formed (Schindelin et al., 1997). NifH also contains a deviant Walker A motif, and its signature lysine has been shown to contact the nucleotide that is bound by the opposite monomer. This is proposed to stabilize the negative charges of ATP, permitting efficient ATP hydrolysis (Schindelin et al., 1997). Like NifH, MinD proteins also contain switch I and switch II sites (Cordell and Löwe, 2001; Sakai et al., 2001) (Figure 1.3 A, B; purple residues (switch I) and cyan residues (switch II); see also Figure 3.1], which have been implicated in nucleotide dependent conformational changes in other proteins (Vale, 1996). Using dimeric NifH as a template, a model for dimeric Archaec MinD has been generated (Hu and Lutkenhaus, 2003). It was shown that the modeled MinD dimer could also have intermonomer contacts between the signature lysine of one monomer and the ATP molecule of the opposite monomer, suggesting that dimerization may be important for aiding MinD ATPase activity, similar to NifH (Lutkenhaus and Sundaramoorthy, 2003).

### 1.3.6. MinE

MinE is the smallest of the three Min proteins (88 residues, ~10 kDa). In contrast to MinC Ec and MinD Ec, the overexpression of MinE Ec in wild-type E. coli cells resulted in a minicell phenotype, not filamentous cells. Furthermore, minicells are also formed when all three Min proteins are overexpressed simultaneously, showing the epistatic effects of MinE Ec over MinC Ec and MinD Ec (de Boer et al., 1989). Using the yeast two-hybrid system, an interaction between MinE Ec and MinD Ec was detected, as well as the ability of MinE Ec to disrupt MinC Ec-MinD Ec association (Huang et al., 1996). The ability of MinE to suppress the MinCD division block at polar and midcell sites suggested
two roles for the protein: 1) to impart topological specificity to the MinCD complex and, 2) to inhibit MinCD activity (de Boer et al., 1989; Pichoff et al., 1995, Zhao et al., 1995).

Separate domains in MinE E. coli seem to be responsible for these functions. Deletion analyses have shown that the N-terminal amino acids 1-22 of MinE E. coli contain an 'anti-MinCD' domain that interacts with MinD E. coli (Zhao et al., 1995), while the remaining residues (aa 23-88) contain the topological specificity domain (TSD) that is responsible for restricting MinCD division inhibition to the cell pole regions (Pichoff et al., 1995; Zhao et al., 1995; King et al., 1999). Nuclear magnetic resonance (NMR) analysis of the N-terminal anti-MinCD domain of MinE E. coli revealed it to be a peptide region that can rapidly convert between randomly coiled and α-helical conformations, with the latter being a possible interacting surface with MinD E. coli (King et al., 1999). The N-terminal domain of MinE E. coli (aa 6-35) is predicted to form an α-helix, with one face containing residues that are involved in interaction with MinD E. coli, as determined by yeast two-hybrid studies (King et al., 1999; Ma et al., 2003). The coiled-coil nature of this region has led to the proposal that the N-terminus of MinE E. coli interacts with a complementary helix in MinD E. coli (Ma et al., 2003).

Using yeast two hybrid studies and gel-filtration analyses, MinE E. coli has also been shown to be dimeric, with residues 36-62 of the TSD likely involved in protein self-association (Pichoff et al., 1995). Analytical ultracentrifugation of a peptide comprised of MinE E. coli residues 31-88 also showed it was dimeric (King et al., 1999). NMR studies have further confirmed the self-association of the MinE E. coli TSD (King et al., 2000). Each TSD contains a long α-helix and dimerization seems to occur by extensive hydrophobic interactions between the α-helices of each TSD monomer, forming an antiparallel coiled-coil. Two β-strands from each TSD also form a four stranded beta-sheet against which the coiled-coil packs to form a hydrophobic sandwich (King et al., 2000).
1.3.7. Min protein localization in *Escherichia coli*

The detailed localization of GFP fusions to *E. coli* Min proteins have greatly advanced the study of Min protein function in this organism. Using a GFP-fusion to the N-terminus of MinD<sub>Ec</sub> (GFP-MinD<sub>Ec</sub>), several groups have demonstrated that the protein exhibits a rapid, MinE<sub>Ec</sub>-dependent, pole-to-pole oscillation in rod-shaped *E. coli* (Figure 1.4 A) (Raskin and de Boer, 1999a; Rowland *et al.*, 2000). This dynamic localization is characterized by the progressive assembly of GFP-MinD<sub>Nz</sub> along the inner membrane, from the pole towards midcell, to form a tube-like structure that dwells for a short period of time (~10 seconds) at one cell half before disassembling and reforming at the opposite cell pole (Figure 1.4 A) (Raskin and de Boer, 1999a; Rowland *et al.*, 2000). The period of oscillation of GFP-MinD<sub>Ec</sub> (defined by the time required for fluorescent signal to leave one cell pole, migrate to the other and back) was approximately 50 seconds and was dependent only upon MinE<sub>Ec</sub> and not MinC<sub>Ec</sub> (Raskin and de Boer, 1999a). In the absence of MinE<sub>Ec</sub>, GFP-MinD<sub>Ec</sub> localized along the entire cell periphery without evidence of pole-to-pole movement (Raskin and de Boer, 1999a; Rowland *et al.*, 2000).

Recently, quantitative immunoblotting indicated that there are approximately 1400 and 2000 molecules of MinE<sub>Ec</sub> and MinD<sub>Ec</sub> in an *E. coli* cell (Shih *et al.*, 2002). Increasing the ratio of GFP-MinD<sub>Ec</sub>/MinE<sub>Ec</sub> appears to increase the dwell time within each cell pole, and hence the oscillation period, of the fusion protein (Raskin and de Boer, 1999a). This increased period was accompanied by minicell formation, suggesting that a certain minimum oscillation frequency, mediated by a proper MinD<sub>Ec</sub>/MinE<sub>Ec</sub> ratio, is required to maintain correct midcell division in rod-shaped *E. coli* (Raskin and de Boer, 1999a).

Recently, using deconvolution and three-dimensional imaging of *E. coli*, Shih *et al.* (2003) observed that the GFP-MinD<sub>Ec</sub> 'tube' is actually organized into one or two helical, or coiled, structures that wind around the inner membrane of the cylindrical cell, suggesting the existence of a possible Min cytoskeletal apparatus (Figure 1.4 B). The appearance of these coils is MinE<sub>Ec</sub>-dependent and they are not restricted to polar regions. Interestingly, a faint coiled structure remains in
Figure 1.4. Localization of GFP-MinD_{Ec} in *E. coli*. (A) GFP-MinD_{Ec} exhibits pole-to-pole movement in rod-shaped *E. coli*. Timepoints are indicated in seconds in each panel (image from Raskin and de Boer, 1999a). (B) Image deconvolution shows GFP-MinD_{Ec} localizing as a coiled-structure in *E. coli*. Note the majority of fluorescent signal at one half of the cell and a residual coiled array at the other. Image 1 is raw image, image 2 shows deconvolution of image 1 [Reprinted with permission from the National Academy of Sciences, USA (Shih *et al.*, 2003)].
one cell pole after the majority of GFP-MinD_{Ec} has localized as a coiled array in the opposite cell half (Figure 1.4 B) (Shih et al., 2003). Hence, it would appear that this helical array(s) is extended throughout the length of the cell. The apparent oscillatory movement of GFP-MinD_{Ec} is attributed to a dynamic shift in fusion protein concentration along the coiled array from one cell pole to the other (Shih et al., 2003).

Similar to GFP-MinD_{Ec}, a GFP-MinC_{Ec} fusion also displayed periodic membrane-associated pole-to-pole movement in *E. coli* (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999b). This oscillation pattern was dependent upon both MinD_{Ec} and MinE_{Ec}, and the period of oscillation was ~40-50 seconds, comparable to that observed with GFP-MinD_{Ec} (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999b). In the absence of MinE_{Ec}, GFP-MinC_{Ec} would be localized along the entire inner cell periphery, provided that MinD_{Ec} was present (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999b). Similar to GFP-MinD_{Ec}, refined imaging of GFP-MinC_{Ec} expressing *E. coli* showed it also localized as a membrane-associated coiled structure (Shih et al., 2003). While the majority of GFP-MinC_{Ec} was assembled into spirals in one cell half, a residual array remains at the opposite cell pole, as observed with GFP-MinD_{Ec} (Shih et al., 2003).

Interestingly, using an N-terminal truncation to MinC_{Ec} that does not inhibit Z-ring assembly, it was demonstrated that MinD_{Ec} is able to direct the GFP-MinC_{Ec} mutant to septal rings (Johnson et al., 2002). Presumably, this was not observed previously since functional GFP-MinC_{Ec} would immediately disassemble Z-rings and concurrently eliminate evidence of GFP-MinC_{Ec} localization to these regions as well (Johnson et al., 2002). Hence, the MinCD_{Ec} complex not only accumulates within a coil along one half of the cell, but likely also actively targets impending division septa.

Although initially reported to localize as a ring at midcell (Raskin and de Boer, 1997), further studies with MinE_{Ec}-GFP revealed that this protein also displayed MinD_{Ec}-dependent intracellular oscillatory movement (Fu et al., 2001; Hale et al., 2001). In live cells, the majority of MinE_{Ec}-GFP signal was assembled in a ring-like structure near midcell; however, a significant amount of fluorescent signal was also seen along the membrane on one just side of the MinE_{Ec}-ring and was
termed the peripheral extra-annular (PEA) signal (Fu et al., 2001; Hale et al., 2001). Hence, MinE<sub>Ec</sub>-GFP was proposed to localize as a tube-like structure with a defined ‘rim’ composed of the MinE<sub>Ec</sub>-ring (Hale et al., 2001).

Time-lapse imaging has revealed that the centrally located MinE-ring moved towards the cell pole containing the PEA signal such that the latter would decrease in size. Once the MinE-ring signal reached the cell pole, it would dissolve and reform at or near the middle of the cell. At the same time, a new PEA signal would form at the other cell half and the MinE-ring would subsequently move towards this other cell pole (Hale et al., 2001; Fu et al., 2001). The average time required for a complete cycle of MinE-ring movement, dissolution, and reformation at midcell was very similar to that required for a cycle of MinD oscillation (Hale et al., 2001).

Recent studies by Shih et al. (2003) now suggest that MinE<sub>Ec</sub> actually localizes in a MinD<sub>Ec</sub>-dependent coiled array within the cell. The MinE-ring did not appear to be closed, and seemed to be composed of 1-2 tight loops of the coil. These midcell MinE<sub>Ec</sub>-GFP coils also appeared to be continuous with those that extended into the polar zone. Again, while the fluorescent intensity of the MinE<sub>Ec</sub>-GFP coil was greater at midcell and along one half of the cell, a faint MinE array could still be observed in the other cell half (Shih et al., 2003).

1.3.8. Min proteins and the bacterial membrane

Studies with GFP fusions to each Min<sub>Ec</sub> protein have shown that the recruitment of both MinC<sub>Ec</sub> and MinE<sub>Ec</sub> to the inner cell membrane, and their characteristic intracellular oscillations, are dependent upon MinD<sub>Ec</sub>. In addition, the oscillation of MinD<sub>Ec</sub> is dependent upon MinE<sub>Ec</sub> (Raskin and de Boer, 1999a; Raskin and de Boer, 1999b; Hu and Lutkenhaus, 1999; Shih et al., 2003). What are the molecular mechanism(s) that drive this behaviour?

Recently, Hu et al. (2002) demonstrated that, in the presence of ATP, MinD could bind to phospholipid vesicles in vitro, forming an ordered helical array than converts round vesicles into extended tubes. The binding of phospholipid vesicles by MinD is also cooperative, such that
increasing the concentration of protein stimulates an increased fraction of MinD to become membrane-associated (Lackner et al., 2003). In contrast, incubation of MinD with ADP, or the use of a MinD mutant bearing a K16Q substitution in its Walker A ATP-binding motif, did not result in protein binding to phospholipid vesicles (Hu and Lutkenhaus, 2002). Another group has also shown that, in the absence of phospholipid vesicles, MinD can rapidly assemble into filaments in an ATP-dependent manner (Suefuji et al., 2002). The addition of E. coli phospholipid vesicles promoted lengthening and bundling of these MinD filaments (Suefuji et al., 2002).

Interestingly, it was shown that the ATPase activity of MinDEc could be stimulated almost 10-fold in the presence of MinEEc and phospholipid vesicles (Hu and Lutkenhaus, 2001). Significantly, this stimulation of MinD Ec ATPase activity was accompanied by the release of MinD from the vesicles (Hu et al., 2002). Hence, it was proposed that ATP-binding may induce conformational change(s) in MinD that give it higher affinity for phospholipids, and that subsequent hydrolysis of ATP, induced by MinE, will result in the dissociation of MinD from the membrane (Hu et al., 2002). In vivo, MinEEc proteins containing mutations within their N-terminal anti-MinCD domain were less able to stimulate MinD ATPase activity. This was correlated with a loss of, or an increased oscillation period of, GFP-MinDEc (Hu and Lutkenhaus, 2001).

Surprisingly, while wild-type MinEEc could promote the disassembly of MinDEc filaments (Hu et al., 2002; Suefuji et al., 2002), it could also induce additional bundling of MinDEc polymers in the presence of lipid vesicles when both proteins were at equimolar amounts (Suefuji et al., 2002). This dual role of MinEEc may reflect the requirement of this protein for both MinDEc oscillation and MinDEc assembly into higher order structures (Suefuji et al., 2002).

MinDEc-ATP was also able to recruit MinCEc onto phospholipid vesicles (Hu et al., 2003; Lackner et al., 2003), in support of in vivo studies where the association of GFP-MinCEc with the E. coli cell poles was dependent upon MinDEc (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a). Purified MinDEc and a MalE-MinCEc fusion have been shown to interact using size-exclusion chromatography in an ATP-dependent manner (Hu et al., 2003). Yeast two-hybrid studies have
indicated that residues within the \( \alpha-7 \) helix of MinD<sub>Ec</sub> (E146 and D152) may be involved in binding to MinC<sub>Ec</sub> (\( \alpha-7 \) helix of \textit{A. fulgidus} MinD is indicated in Figure 1.3 B, green arrow). Recent studies have also indicated that residues within the switch I and II sites of MinD<sub>Ec</sub> are involved in interaction with MinC<sub>Ec</sub>. While these mutations (including G42A, R44G, and I125E) did not affect MinD membrane association, they impaired MinC<sub>Ec</sub> binding, as assessed by yeast two-hybrid methods (Zhou and Lutkenhaus, 2004).

\textit{In vitro}, both MinC<sub>Ec</sub> and MinD<sub>Ec</sub> proteins can be removed from lipid vesicles by the addition of MinE<sub>Ec</sub> (Lackner \textit{et al.}, 2003; Hu \textit{et al.}, 2003). It has been suggested that a MinE<sub>Ec</sub>-induced conformational change in membrane-bound MinD<sub>Ec</sub> may be responsible for displacing MinC<sub>Ec</sub> (Lackner \textit{et al.}, 2003; Hu \textit{et al.}, 2003). However, MinE<sub>Ec</sub>-induced dissociation of MinC<sub>Ec</sub> and MinD<sub>Ec</sub> are likely distinct events, since MinE<sub>Ec</sub> could displace MinC<sub>Ec</sub> from vesicles even in the absence of ATP hydrolysis by MinD<sub>Ec</sub> (Lackner \textit{et al.}, 2003; Hu \textit{et al.}, 2003). MinC<sub>Ec</sub> and MinE<sub>Ec</sub> also do not seem to compete for the same MinD<sub>Ec</sub> binding site, since the former could not displace MinD<sub>Ec</sub>-MinE<sub>Ec</sub> complexes from vesicles, nor interfere with MinE<sub>Ec</sub> stimulation of MinD<sub>Ec</sub> (Hu \textit{et al.}, 2003). However, it is also possible that both MinC<sub>Ec</sub> and MinE<sub>Ec</sub> share similar or overlapping binding sites on MinD<sub>Ec</sub>, and that MinE<sub>Ec</sub> has a greater affinity for this MinD<sub>Ec</sub> region than MinC<sub>Ec</sub> does (Dr. Glenn King, University of Connecticut Health Center).

A short C-terminal membrane targeting sequence (MTS) in MinD has been identified as the region responsible for membrane localization (Szeto \textit{et al.}, 2002; Hu and Lutkenhaus, 2003). This extreme C-terminal region likely consists of a highly conserved 8-12 residue amphipathic helix (aa 261-268; KGFLKRLFL in \textit{E. coli} MinD as an example). In general, one face of the helix is polar (lysine and arginine residues in MinD<sub>Ec</sub>), while the other face contains hydrophobic residues (leucine, phenylalanine, and methionine in MinD<sub>Ec</sub>) (Szeto \textit{et al.}, 2002). Removal of this predicted amphipathic helix from both \textit{E. coli} MinD and \textit{B. subtilis} MinD resulted in GFP-MinD fusions that were unable to localize to the membrane in \textit{E. coli}, remaining in the cytosol instead (Szeto \textit{et al.},
Despite retaining interaction with MinC<sub>Ec</sub>, these MinD<sub>Ec</sub> deletion variants were unable to activate MinC-dependent cell division arrest (Hu and Lutkenhaus, 2003). Mutations that disrupted either the helicity or amphipathicity of the MinD<sub>Ec</sub> MTS also abrogated membrane localization of the protein (Szeto et al., 2002).

Interestingly, the short MinD MTS can be fused to other proteins that are normally cytosolic and target them to the inner membrane of E. coli. However, while a single B. subtilis MinD MTS fused to proteins such as GFP or MinC could recruit them to the E. coli inner membrane, a bivalent arrangement of E. coli MinD MTSs was required to do the same. In B. subtilis, MinD<sub>Bs</sub> does not undergo the dynamic oscillation observed with MinD<sub>Ec</sub>; therefore, it was suggested that the higher membrane affinity of the MinD<sub>Bs</sub> MTS may be responsible for this lack of intracellular mobility (Szeto et al., 2003).

How does the C-terminus of MinD associate with the cell membrane? Although there are several MinD crystal structures available from Archaeal organisms, the proteins either do not naturally contain MTS regions or the corresponding region is structurally disordered (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001). Using far-UV circular dichroism, it was shown that the E. coli and B. subtilis MTSs do not exhibit any stable secondary structure in the absence of lipids. However, in the presence artificial phospholipid vesicles, these MTS peptides experienced a random coil-to-helix transition and adopted an α-helical conformation (Szeto et al., 2003). The resulting amphipathic helix is predicted to generate a positively charged polar face; hence, it was predicted that the MinD MTS preferentially interacts with negatively-charged (anionic) phospholipids (Szeto et al., 2003). In fact, MinD MTSs do display a preference for anionic phospholipids (Mileykovskaya et al., 2003). While the MinD<sub>Bs</sub> MTS has distinct preference for phosphatidylglycerol (PG) over cardiolipin (CL), the MinD<sub>Ec</sub> MTS associates equally well with both of these anionic phospholipids (Szeto et al., 2003). Hence, it is proposed that each MinD MTS is adapted to interact with specific phospholipids present in the inner membrane of their respective organisms (Szeto et al., 2003).
The orientation of the MinD MTS helix is predicted to be parallel to the membrane surface, such that the hydrophobic residues on one face of the amphipathic helix are buried and interact with lipid acyl chains, while the cationic residues on the opposite face interact with the headgroups of anionic phospholipids (Szeto et al., 2002; Mileykovskaya et al., 2003). Experimental support for this was obtained by Zhou and Lutkenhaus (2003), who substituted the MTS hydrophobic residues with the hydrophobic residue tryptophan, and used fluorescence spectroscopy to reveal that tryptophan did insert into the hydrophobic interior of a lipid bilayer, likely reflecting what might occur with the wild-type MinD MTS (Zhou and Lutkenhaus, 2003).

Since dimerization and membrane association of MinD$_{66}$ are ATP-dependent (Hu et al., 2003), a ‘dimer trigger’ model has been proposed in which MinD$_{66}$ dimerizes upon binding ATP, leading to conformational changes that activate, or expose, the MinD$_{66}$ MTS sequence for membrane binding (Hu and Lutkenhaus, 2003). This dimerization would also ensure that two MTSs are available to increase the membrane affinity of the MinD$_{66}$-ATP complex for the *E. coli* membrane (Hu and Lutkenhaus, 2003). It has also been recently proposed that the MTS itself may mask the MinD dimerization interface, and that the presence of ATP and phospholipid may promote MinD self-interaction by shifting its MTS to expose the dimeric interface (Zhou et al., 2004).

A second model has also been proposed to explain how MinD may associate with membranes. In the ‘zipper’ model, ATP-binding will promote the cooperative assembly of a MinD polymer on the membrane (Szeto et al., 2003). This model does not necessarily require MinD-ATP dimers, only the recruitment of MinD-ATP subunits that continually increase the valency of MTSs that result in enhanced membrane affinity (Szeto et al., 2003). The growing MinD-ATP polymer(s) can thus be compared to a zipper that gains stability on the membrane as more subunits, and hence more MTSs, are recruited.
1.3.9. Model for Min system function in rod-shaped *E. coli*

The accumulation of data obtained from studying the three MinEc proteins has led to a gradual development of a model for Min-dependent cell division site selection in *E. coli* (Figure 1.5) (Hu and Lutkenhaus, 2003; Hu *et al.*, 2003; Lackner *et al.*, 2003; Shih *et al.*, 2003). In the cytosol, monomeric MinD is proposed to bind ATP, resulting in a MinD dimer with activated MTSs that confer membrane affinity. Alternatively, individual MinD-ATP subunits may be recruited in a cooperativemanner to the cell membrane as proposed by the ‘zipper’ model (Szeto *et al.*, 2003). Regardless, the majority of MinD-ATP becomes preferentially accumulated at the membrane and/or polymerized lattice (collectively referred to as a MinD*ec* ‘zone’) at one half of the cell (Figure 1.5 A, B). This directed accumulation may be the result of a local self-enhancing reaction (Meinhardt and de Boer, 2001), and is supported by the cooperative nature of MinD binding to membranes in *vitro* (Lackner *et al.*, 2003). Based on the recent observation of MinC, MinD, and MinE spiral arrays in *E. coli* (Shih *et al.*, 2003), this lattice may actually consist of a permanent coiled array of Min proteins that extends across the entire cell length to be used as a template, or nucleation point, for the incorporation of more Min proteins to either cell half (Shih *et al.*, 2003) (Figures 1.4 B and 1.5).

MinD*ec* is able to recruit the cell division inhibitor MinC*ec* to the inner membrane/Min helical array at one end of the cell as well. Whether MinD interacts with MinC prior to associating with the membrane is presently unclear. On one hand, size-exclusion chromatography suggests a weak, ATP-dependent, interaction between the two purified proteins in solution; however, the recruitment of MinC by MinD is more easily observed using a sedimentation assay once phospholipids vesicles are also included (Hu *et al.*, 2003). Recent evidence also showed MinD*ec* mutants that fail to bind membranes can still interact with MinC*Ec* (Zhou and Lutkenhaus, 2004), suggesting a cytosolic interaction between the two proteins may exist. As MinD*Ec* has been demonstrated to recruit GFP-MinC*Ec* to septal targets (Johnson *et al.*, 2002), any FtsZ*Ec* ring assembly, and cell division, would be transiently inhibited along one half of the cell where MinC*Ec*-MinD*Ec* have accumulated (Figure 1.5 B).
Figure 1.5. Model for MinE<sub>E</sub>-dependent cell division regulation in cylindrical E. coli. This model is adapted from Shih et al. (2003) and incorporates cumulative data obtained from studying the three MinE<sub>E</sub> proteins. (A) In the cytosol, MinD<sub>E</sub> (open circles) is proposed to bind ATP (MinD<sub>E</sub>-ATP; green circles), dimerize, and gain affinity for the membrane and/or a permanent MinCDE<sub>E</sub> scaffold base (coils). Alternatively, individual MinD<sub>E</sub>-ATP subunits may be recruited in a cooperative manner according to the ‘zipper model’ (Szeto et al., 2003). MinD<sub>E</sub> also recruits the division inhibitor MinC<sub>E</sub> (squares). MinD<sub>E</sub>-ATP and MinC<sub>E</sub> preferentially accumulate at one half of the cell (MinD<sub>E</sub> ‘zone’). (B) MinE<sub>E</sub> (triangles) attracted by MinD<sub>E</sub>-ATP, localizes near the cell center as 1-2 loops of the coiled structure. MinE<sub>E</sub> induces the release of MinC<sub>E</sub> and stimulates the ATPase activity of MinD<sub>E</sub>-ATP at the edge of the accumulated MinD<sub>E</sub> zone. MinD<sub>E</sub>-ADP dissociates from the membrane/MinCDE<sub>E</sub> coil. MinE<sub>E</sub> travels along the Min helical array towards one cell pole and gradually induces the disassembly of the MinD<sub>E</sub> zone. (C) Cytoplasmic MinD<sub>E</sub> can diffuse towards the other cell pole, during which it binds ATP again, and associates with the membrane/Min lattice at the opposite cell pole. (D) The continuous MinE<sub>E</sub>-dependent movement of MinD<sub>E</sub>, and associated MinC<sub>E</sub>, between each cell half will result in the inhibition of Z-ring assembly in these regions. Furthermore, this oscillation pattern keeps the time-averaged concentration of MinC<sub>E</sub> lowest at midcell, permitting stable FtsZ assembly at that point (thunderbolts).
MinE<sub>Ec</sub>, attracted by MinD<sub>Ec</sub>-ATP, forms a ‘ring-like’ structure near the cell center which actually consists of 1-2 loops of a coiled structure (Figure 1.5 B) (Shih et al., 2003). The interaction of MinE<sub>Ec</sub> ‘ring’ molecules with MinD<sub>Ec</sub>-ATP found at the edge of the accumulated MinD<sub>Ec</sub>-ATP zone at one cell half would induce the release of MinC<sub>Ec</sub> from the membrane. Furthermore, MinE<sub>Ec</sub> will stimulate the ATPase activity of MinD<sub>Ec</sub> and cause the latter protein to dissociate from the membrane and into the cytoplasm (Figure 1.5 C). A constant ‘wave’ of MinE travelling along the Min helical array would gradually induce the disassembly of MinD<sub>Ec</sub> from the cell half in which it resides (or from its coiled array) (Margolin, 2001b) (Figure 1.5 C). Presumably, nucleoid occlusion will also prevent untimely cell division at the midcell (Figure 1.2).

The MinE<sub>Ec</sub>-ring has also been proposed to act as a ‘stop-growth’ mechanism that prevents the MinC-MinD division complex from extending beyond the midcell division site. Studies have shown that a MinE<sub>Ec</sub> mutant that fails to form a MinE-ring (when tagged to GFP) will permit the extension of the MinD<sub>Ec</sub> polar zone well past the midcell (Shih et al., 2002).

Cytoplasmic MinD<sub>Ec</sub> can subsequently diffuse to the other cell pole, during which it binds ATP again, and associates with the membrane/Min lattice at the opposite cell pole (Figure 1.5 C, D). It is proposed that any nascent cytosolic MinD<sub>Ec</sub>-ATP will have diffused away sufficiently, such that it avoids being reincorporated into any remaining MinD<sub>Ec</sub> lattice at the original cell pole (Lackner et al., 2003). In addition, a new MinD<sub>Ec</sub> zone can assemble at the opposite end of the cell, since the majority of MinE<sub>Ec</sub> is still involved in disassembling MinD<sub>Ec</sub> at the original cell half. Once the MinE<sub>Ec</sub>-ring has completely disassembled the original MinD zone, it dissolves and reforms at midcell to act on MinD<sub>Ec</sub> in the other cell half (Lackner et al., 2003).

The continuous movement of MinD<sub>Ec</sub> and associated MinC<sub>Ec</sub>, between each cell half will result in the inhibition of Z-ring assembly in these regions (Raskin and de Boer, 1999a; Lackner et al., 2003). This oscillation pattern will keep the time-averaged concentration of MinC<sub>Ec</sub> lowest at midcell, permitting stable FtsZ assembly at that point (Raskin and de Boer, 1999b) (Figure 1.5 D). The observed oscillations of each Min protein is proposed to be a result of the continuous
redistribution of MinC<sub>Ee</sub>, MinD<sub>Ee</sub>, and MinE<sub>Ee</sub> subunits across a permanent Min scaffold (Shih et al., 2003); however, whether this scaffold requires another underlying cytoskeletal frame is not known.

Interestingly, several groups have used computer simulation models to show that Min protein oscillations can occur in a closed system, and that no other additional components other than MinD and MinE are required (Howard et al., 2001, Meinhardt and de Boer, 2001; Kruse, 2002). However, since the discovery that Min proteins assemble as intracellular coils (Shih et al., 2003), it is unclear whether these in silico models remain applicable.

1.3.10. Min proteins in the Gram-positive rod *Bacillus subtilis*

The only other organism in which Min proteins have been studied in significance is the Gram-positive rod *B. subtilis* (Bs). *B. subtilis* is the most widely studied model for Gram-positive cell division and clearly contains minC and minD homologues, while lacking minE (Levin et al., 1992; Varley and Stewart, 1992).

Overexpression of both MinC<sub>Bs</sub> and MinD<sub>Bs</sub> will inhibit *B. subtilis* cell division and cause filamentation (Marston and Errington, 1999b), while inactivation of either gene results in a minicell-like phenotype (Varley and Stewart, 1992). Localization studies have shown that MinD<sub>Bs</sub> is found at both cell poles (forming an arc that follows the curvature of the cell membrane) and at the midcell between separated sister nucleoids. This suggests that the protein has affinity for impeding and former division sites (Marston et al., 1998). Presently it is not known whether MinD<sub>Bs</sub> can localize as higher ordered structures, such as coils. MinD<sub>Bs</sub> also interacts with MinC<sub>Bs</sub>, as determined by yeast two-hybrid studies (Marston and Errington, 1999b), and MinC<sub>Bs</sub> has been shown to target both cell poles and the impending midcell division site in a MinD<sub>Bs</sub>-dependent manner (Marston and Errington, 1999b).

Unlike *E. coli* MinC and MinD, the *B. subtilis* proteins do not display any periodic intracellular oscillations. In place of MinE, an unrelated protein termed DivIVA (~19 kDa) is involved in the topological regulation of the *B. subtilis* MinCD division inhibition complex (Cha and
Stewart, 1997; Edwards and Errington, 1997). Similar to MinD<sub>bs</sub>, studies using DivIVA-GFP show that the protein localizes as discrete foci at the cell poles (old division sites) and as bands near the cell center (new division sites) just prior to the completion of septation (Edwards and Errington, 1997; Marston et al., 1998). In the absence of DivIVA, long <i>B. subtilis</i> filaments are formed (Edwards and Errington, 1997) and MinD<sub>bs</sub> localizes throughout the cell in diffuse patches along the membrane; hence, proper MinD<sub>bs</sub> localization is dependent upon DivIVA (Marston et al., 1998).

How might DivIVA regulate the topological specificity of the MinCD<sub>bs</sub>-cell division inhibitor complex in <i>B. subtilis</i>? In the ‘polar piloting’ model, a cell would have DivIVA localized at both poles, sequestering MinCD<sub>bs</sub> to these regions as well (Figure 1.6 A) (Marston et al., 1998). Once DNA replication nears completion, Z-ring assembly and recruitment of other cell division proteins is free to occur at a midcell location. Recent evidence using germinating <i>B. subtilis</i> spores has indicated that DivIVA and MinD<sub>bs</sub> (and presumably MinC<sub>bs</sub>) can assemble at midcell division sites before Z-ring constriction begins (Figure 1.6 B) (Harry and Lewis, 2003). Presumably, the Z-ring has assembled to a point where it will be resistant to the effects of MinCD<sub>bs</sub>. Upon completion of cell division, each daughter cell has an old cell pole and a new one formed by the act of division. Since the DivIVA-MinCD<sub>bs</sub> complex remains associated with nascent and old cell poles in each daughter cell, both progeny will have MinCD<sub>bs</sub> retained at each of their cell ends to control future cytokinetic events (Figure 1.6 C) (Marston et al., 1998).

<i>B. subtilis</i> is a spore-forming bacterium. Sporulation involves the use of septation sites at cell pole regions, as opposed to midcell division sites, in order to divide the cell into mother and prespore compartments (Errington, 2001). Whether Min proteins have a role in this event is unclear, since min mutants are still able to sporulate quite efficiently (Lee and Price, 1993). However, one study showed that MinC<sub>bs</sub> and MinD<sub>bs</sub> may be involved in regulating the proper transfer of DNA from the mother cell into the forespore during sporulation (Sharp and Pogliano, 2002b). This function is fulfilled by the DNA translocase SpoIIIIE (homologous to <i>E. coli</i> FtsK) and is normally found in both mother cell and forespore; however, SpoIIIIE in the mother cell localizes specifically to the septum that separates

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Figure 1.6. Model for MinCD<sub>Bs</sub>-DivIVA dependent cell division regulation in *B. subtilis* (adapted from Marston *et al.*, 1998). (A) In the ‘polar piloting’ model, *B. subtilis* cells have DivIVA localized at both poles, which sequesters MinCD<sub>Bs</sub> to these regions as well, leading to division inhibition at the cell ends. (B) Once DNA replication nears completion, Z-ring assembly and recruitment of other cell division proteins is free to occur at a midcell location. DivIVA, MinD<sub>as</sub>, and MinC<sub>as</sub> can assemble at midcell division sites before Z-ring constriction begins. Presumably, the Z-ring has assembled to a point where it will be resistant to the effects of MinCD<sub>Bs</sub>. (C) Upon completion of cell division, the DivIVA-MinCD<sub>Bs</sub> complex remains associated with nascent and old cell poles in each daughter cell; thus, both cells have MinCD<sub>Bs</sub> retained at either ends to control future cytokinetic events.
the two compartments to ensure that the direction of DNA translocation proceeds towards the future spore (Sharp and Pogliano, 2002a). Interestingly, removal of MinCD<sub>Bs</sub> resulted in the assembly of forespore-expressed SpoIIE to the septum, resulting in a reversal of DNA translocation. Therefore, MinCD<sub>Bs</sub> may play a role in inhibiting SpoIIE assembly/activity within the forespore to ensure proper DNA translocation in sporulating <i>B. subtilis</i> (Sharp and Pogliano, 2002b). MinD<sub>Bs</sub> may also play a role in chromosome partitioning in <i>B. subtilis</i>. Interestingly, Soj internucleoid ‘jumping’ (reviewed above) has been shown to be dependent upon MinD<sub>Bs</sub> (Autret and Errington, 2003). In the absence of MinD, Soj patches were observed further away from cell poles, suggesting that MinD may be a target for Soj (Autret and Errington, 2003).

Hence, while having MinC and MinD in common, Gram-negative <i>E. coli</i> and Gram-positive <i>B. subtilis</i> rods appear to have significantly different mechanisms to control their subcellular localization and action. Interestingly, other bacilli such as <i>Caulobacter crescentus</i> and <i>Haemophilus influenzae</i> do not encode any Min proteins (Margolin, 2001a). In general, Gram-positive organisms lack <i>minE</i>, with the exception of Clostridia bacilli, which seem to encode MinC, MinD, MinE, and DivIVA in their genomes (Errington <i>et al.</i>, 2003). Interestingly, Gram-positive cocci, including <i>Staphylococcus aureus</i>, <i>Streptococcus pneumoniae</i>, and <i>Enterococcus faecalis</i>, do not have any Min proteins, despite retaining <i>divIVA</i> homologues (Margolin, 2001a; Massidda <i>et al.</i>, 1998; Ramirez-Arcos <i>et al.</i>, manuscript in preparation). Therefore, it is likely that several other mechanisms for regulating cell division site selection exist in different groups of bacteria.

In contrast to Gram-positive cocci, the Dillon laboratory has identified homologues to all three <i>min</i> genes in the Gram-negative coccus <i>Neisseria gonorrhoeae</i> (Ramirez-Arcos <i>et al.</i>, 2001a). The presence of these genes in a round bacterium was intriguing, particularly since Min protein functions have been well documented in rod-shaped bacteria, which have obvious cell poles and midcells. In addition, gonococcal cells divide along two perpendicular dimensions, in stark contrast to
the single division plane inherent to bacilli. Hence, *N. gonorrhoeae* was selected as a model organism to study the role of Min proteins in coccal systems.
1.4. Current state of knowledge of cell division in the coccus *Neisseria gonorrhoeae*

1.4.1. The Gram-negative organism *N. gonorrhoeae*

*Neisseria gonorrhoeae* (Ng) is a Gram-negative coccus that is the causative agent of the sexually transmitted disease gonorrhea, perhaps the most reported communicable disease worldwide. The World Health Organization (1995) estimated that there are approximately 62 million reported cases of gonorrhea annually worldwide, although the actual number of infections is likely much higher due to asymptomatic infections. *N. gonorrhoeae* is an obligate human pathogen. The primary site of infection in males is the urethra, characterized by a painful urethral discharge. Gram-staining of this discharge invariably reveals the presence of Gram-negative diplococci found within polymorphonuclear neutrophils (Howard, 1994). Women typically experience endocervical infections; however, asymptomatic carriage of the disease is more common in females, making infection seem less obvious. If left untreated, the disease can ascend into the uterus and fallopian tubes and cause pelvic inflammatory disease (Howard, 1994). Persistent pain in the abdomen results and damage to the fallopian tubes may lead to ectopic pregnancy and infertility. Newborns can also acquire gonorrhea in their eyes as they pass through an infected birth canal. Rare cases of systemic infection have also been reported, leading to arthritis, endocarditis, and meningitis (Howard, 1994). Gonococcal infection, as well as infection with other bacterial sexually transmitted diseases (e.g. chlamydia), has also been linked to an increase in HIV transmission (Wasserheit, 1992). It is believed that genitial inflammation and lesions caused by STDs may accommodate HIV transmission from person to person (Holmes, 1994; Sparling *et al.*, 1994).

*N. gonorrhoeae* infects by adhering to and invading mucosal epithelial surfaces (Merz and So, 2000). The binding of gonococcal type IV pili and other adhesins can affect host cell signalling and stimulate bacterial ingestion. This is followed by transcytosis of bacteria to the basolateral surface, where they are released to the underlying tissue (Dehio *et al.*, 2000; Merz and So, 2000). To avoid the host immune system, the surface antigens of *N. gonorrhoeae*, including pilin subunits, opacity (Opa) proteins, and lipooligosaccharide (LOS) are extremely variable (Merz and So, 2000).
Such antigenic variation can be achieved through several mechanisms, including genetic recombination between multiple gene copies and slipped-strand mispairing (Kline et al., 2003). To date, gonococcal surface variability has confounded the development of any successful vaccine.

1.4.2. Morphological analyses of dividing N. gonorrhoeae

The coccus *N. gonorrhoeae* exhibits cell division along two planes, resulting in the formation of a tetrad of daughter cells (Westling-Häggström et al., 1977) (see also Figure 1.7 A, D). This differs from rod-shaped *E. coli* where cell division occurs along a single plane, resulting in two progeny cells (Nanninga, 1998). Analysis of gonococcal cell cultures invariably reveals the presence of single cocci, diplococci, and cells arranged in tetrads (Figure 1.7 A). By observing gonococci on agar slides, others have found that they grew in at least two dimensions, first expanding perpendicular to the initial division plane followed by growth along a second dimension that is parallel to the existing septum (Westling-Häggström et al., 1977).

Using transmission electron microscopy, the earliest visible event of gonococcal cell division appeared to be a constriction that is more pronounced on one side of the coccus than the other (Fitz-James, 1964) (see also Figure 1.7 B). At the tip, or apex, of this constriction, extension of the inner membrane could be visualized forming ahead of the growing cell wall septum (Fitz-James, 1964; Westling-Häggström et al., 1977); hence, the initial constriction serves as a marker for the plane of impending division (Fitz-James, 1964). Furthermore, as division proceeds, the growing constrictions did not appear to have any close association with chromosomal material (Fitz-James, 1964).

Although inner membrane constriction results in two separated gonococcal cell compartments, the completion of cell wall synthesis is delayed; hence, daughter cells remained attached to one another, resulting in a high proportion of diplococci (Fitz-James, 1964). Cell division along a second, perpendicular plane follows and is also characterized by an ingrowth of cytoplasmic membrane enclosing a peptidoglycan fold. This invagination occurs at the diplococcal cell junction.
Figure 1.7. Cell division patterns in wild-type *N. gonorrhoeae*. (A) Differential interference contrast (DIC) image of wild-type *N. gonorrhoeae* CH811 cells showing single, diplococcal, and tetrad arrangements of cells. Bar indicates 2 μm length. (B) Transmission electron micrograph of *N. gonorrhoeae* CH811 cell at the start of cell division, showing initial invagination at one side of the coccus. (C) Transmission electron micrograph of secondary division planes (green arrows) initiating perpendicular to the original plane (black arrow) (figure obtained from Westling-Häggström et al., 1977). Note the new constrictions occur at the diplococcal junction. (D) Schematic showing the perpendicularly alternating division pattern characteristic of *N. gonorrhoeae*. 
(Figure 1.7 C, green arrows) and at a right angle to the first septum (Figure 1.7 C, black arrow; Westling-Häggström et al., 1977), such that four cells result in a two-by-two (tetrad) arrangement (Figure 1.7 A, D).

The physical separation of gonococcal cells from each other may be partly mediated by the product of the tpc (tetrapac) gene (Fussenegger et al., 1996). Disruption of tpc results in >95% of cells exhibiting a tetrad arrangement, instead of the usual diplococcal and single cell morphologies. Electron microscopic analysis of these mutants revealed that they shared continuous outer membrane, with the two diplococci interconnected by a double murein layer; hence, it is believed that tpc encodes a murein hydrolase that acts after septation to facilitate cell separation (Fussenegger et al., 1996).

1.4.3. Identification and characterization of the dcw cluster of N. gonorrhoeae

Using raw data from the N. gonorrhoeae strain FA1090 genome project (Roe et al., 2001), the dcw cluster of N. gonorrhoeae was identified and assembled (Francis et al., 2000). This gene cluster contained 17 genes in the following 5’ to 3’ order: mraZ, mraW, ftsI (penA), murE, hyp1, murF, mraY, hyp2, murD, ftsW, murG, murC, ddl, ftsQ, ftsA, ftsZ, and hyp3 (Francis et al., 2000). Overall, the organization of the gonococcal dcw genes was similar to that found in the E. coli dcw cluster.

In E. coli, ftsL is located between mraW and ftsI; however, the putative protein encoded in this same region in N. gonorrhoeae had low similarity (22% at the amino acid level) to E. coli FtsL, hence ftsL was not included in this analysis of the gonococcal dcw cluster (Francis et al., 2000). A subsequent Neisserial dcw cluster analysis by another group did include ftsL as part of this gene cluster (Snyder et al., 2001). Three hypothetical genes, hyp1, hyp2, and hyp3 were also identified between murE-murF, mraY-murD, and following ftsZ, respectively (Francis et al., 2000). The hyp1 gene, also named dca (division cluster associated), likely encodes an inner membrane protein that is
essential for DNA uptake by *N. gonorrhoeae* (Snyder et al., 2001); however, the functions of the remaining hyp genes are currently unknown (Francis et al., 2000).

Co-transcription of all the gonococcal dcw genes does not occur, since four transcriptional terminators were identified in the gene cluster and shown to be functional by RT-PCR strategies (Francis et al., 2000). Three of these terminators consisted of inverted repeats of the gonococcal uptake sequence (5'-'GCCGTCTGAA-3'), previously shown to be required for gonococcal DNA transformation and to act as transcriptional terminator sequences (Goodman and Scocea, 1988; Francis et al., 2000). These uptake sequence repeats were located at the junctions between mraY-hyp2, murD-fisW, and murG-murC genes. A novel transcriptional terminator consisting of a Correia element, a repetitive sequence commonly found in *Neisseria* species, was also found to act as a transcriptional terminator between murF and mraY (Francis et al., 2000). However, there is conflicting evidence for the role of this Correia element, as others studying the dcw clusters of *N. gonorrhoeae*, and the closely related *N. meningitidis*, have shown that the Correia element does not act to terminate transcription within the dcw cluster (Snyder et al., 2003).

1.4.4. Gonococcal FtsZ

The gonococcal ftsZ gene was previously identified using raw data from the *N. gonorrhoeae* FA1090 genome project (Salimnia et al., 2000). Gonococcal FtsZ (FtsZ<sub>Ng</sub>, 392 aa long, 41.5 kDa) shares high identity (95-98%) with FtsZ from *N. meningitidis*. The protein also has 67% and 65% similarity with FtsZ from *E. coli* and *B. subtilis*, respectively. All attempts to generate a *N. gonorrhoeae* ftsZ knockout were unsuccessful, indicating that FtsZ was likely essential for gonococcal survival (Salimnia et al., 2000). FtsZ<sub>Ng</sub> is active across species and could inhibit cell division when overexpressed in wild-type *E. coli*. Furthermore, a GFP-FtsZ<sub>Ng</sub> fusion could localize to the midcell in *E. coli*, similar to *E. coli* FtsZ (Salimnia et al., 2000). Overexpression of GFP-FtsZ<sub>Ng</sub> in *N. gonorrhoeae* from a shuttle vector resulted in the formation of aberrant cells that had multiple and atypically positioned division sites that separated the cell into small compartments. However, most of
the GFP fusion protein was insoluble and subcellular localization of FtsZ within the gonococcus was not possible (Salimnia et al., 2000).

There are at least six promoters that may control the expression of \( ftsZ_{N_g} \) (Francis et al., 2000). Under conditions of anaerobiosis, the \( ftsZ_{N_g} \) promoter region had a significantly higher expression level than under aerobic conditions. In particular, of the three promoters that are directly upstream of \( ftsZ_{N_g} \), the most proximal, \( P_{Z1} \), seems to be the most active under anaerobic conditions (Francis et al., 2000; Ramirez-Arcos et al., 2001b). In addition, the presence of urea led to a decrease in \( ftsZ_{N_g} \) promoter region activity (Ramirez-Arcos et al., 2001b). Hence, it is possible that \( ftsZ \) expression (as well as other gonococcal cell division genes) is regulated, in part, by environmental conditions that mimic the host genitourinary tract (Ramirez-Arcos et al., 2001b).

1.4.5. Identification of \textit{min} genes in \textit{N. gonorrhoeae}.

The Dillon laboratory is the first group to identify and to investigate Min proteins from a naturally occurring coccus. Analysis of the raw data from the \textit{N. gonorrhoeae} strain FA1090 genome project (Roe et al., 2001), revealed the presence of \textit{minC}, \textit{minD}, and \textit{minE} homologues (Ramirez-Arcos et al., 2001a). The three gonococcal \textit{min} genes (\textit{minC}_{N_g}, \textit{minD}_{N_g}, and \textit{minE}_{N_g}) are part of a large 17 kilobase (kb) gene cluster (Figure 1.8) (GenBank accession number AF345908; Ramirez-Arcos et al., 2001a). In contrast, the three \textit{min} genes in \textit{E. coli} are within a 1.7 kb gene cluster (Figure 1.8), while \textit{minC} and \textit{minD} of \textit{B. subtilis} are contained in a 5 kb gene cluster (Figure 1.8). Included in the \textit{N. gonorrhoeae min} cluster are genes for \textit{rpoA} (\( \alpha \) subunit of RNA polymerase), \textit{secY} (secretion protein Y), IF1 (initiation factor 1) and 20 structural ribosomal genes upstream of \textit{minCDE}, with \textit{oxyR} (oxidative stress transcriptional regulator) situated downstream (Figure 1.8). All genes are transcribed in the same direction and are flanked by Neisserial uptake sequences (Figure 1.8) (Ramirez-Arcos et al., 2001a). In addition, the closely related \textit{N. meningitidis} also contains a \textit{minCDE} cluster, with the \textit{min} cluster from \textit{N. meningitidis} Z2491/serotype A sharing 97% identity with that of the gonococcus (Ramirez-Arcos et al., 2001a).
Figure 1.8. Comparison of min gene clusters from *N. gonorrhoeae*, *E. coli*, and *B. subtilis*. Gonococcal minC, minD, and minE homologues are part of a 17 kb gene cluster flanked by two neisserial uptake sequences (US) that can act as transcriptional terminators (T1). *E. coli* min genes are found in a smaller 1.7 kb cluster, with rho-independent transcription terminator sequences (T2). *B. subtilis* contains minC and minD genes, but no minE. Arrows indicate direction of transcription (Figure from Ramirez-Arcos et al. 2001).
Within the *N. gonorrhoeae* minCDE cluster, minC$_{Ng}$ and minD$_{Ng}$ are separated by 28 bp, while minD$_{Ng}$ and minE$_{Ng}$ have only a 3 bp intergenic region (based on the distance between the stop codon of an upstream gene and the start codon of its downstream gene) (Ramirez-Arcos et al., 2001a). Promoter regions for minC$_{Ng}$, minD$_{Ng}$, and minE$_{Ng}$ were also identified between *rplQ* and minC$_{Ng}$ at the 3’ end of minC$_{Ng}$, and within the 3’ half of minD$_{Ng}$, respectively (Ramirez-Arcos et al., 2001b). Similar to the fisZ$_{Ng}$ promoter region, minC$_{Ng}$ and minD$_{Ng}$ promoter regions are significantly more active under anaerobic conditions (Ramirez-Arcos et al., 2001b).

1.4.6. Gonococcal MinC

Gonococcal MinC (MinC$_{Ng}$) is 237 aa in length (26.3 kDa) and is 36% and 22% identical to MinC from *E. coli* and *B. subtilis*, respectively. Deletion of minC in *N. gonorrhoeae* led to severe aberrations in cell morphology and cell division. Mutant cells appeared to have several planes of division resulting in the formation of multi-lobed compartments. In addition, there was significant evidence of cell lysis and cells devoid of intracellular contents (Ramirez-Arcos et al., 2001a). Furthermore, the gonococcal minC mutant had significantly decreased cell viability (Ramirez-Arcos et al., 2001a). MinC$_{Ng}$ is also active across species and inhibited wild-type *E. coli* cell division when overexpressed, causing the formation of long filamentous cells. The protein could also complement an *E. coli* minC mutant by restoring its ability to divide at midcell, further evidence of its cross species functionality (Ramirez-Arcos et al., 2001a).

Based on sequence alignment of MinC proteins from various species, four completely conserved C-terminal glycine residues were identified (Ramirez-Arcos et al., in press). Mutation of each of these residues in MinC$_{Ng}$ and in MinC$_{Ec}$ resulted in inactive proteins which were unable to arrest cell division in *E. coli* backgrounds. From yeast two-hybrid analyses, it was determined that glycines corresponding to MinC$_{Ec}$ G135, G154, and G171 are involved in MinC-MinD interaction, and that G161 was implicated in MinC dimerization. Each mutation was subsequently mapped onto the triangular β-helix surfaces (A, B, C) that form the C-terminus of the solved *T. maritima* MinC
crystal structure. This revealed that glycines at, or near, the B/C surface junction of MinC are implicated in MinD binding, while the single glycine within the A surface is involved in MinC dimerization (Ramirez-Arcos et al., in press). Using molecular modeling, it was proposed that the exposed conserved glycines within the B/C surfaces junction of MinC may interact with exposed residues of the α-7 helix in MinD (Ramirez-Arcos et al., in press) (α-7 helix of A. fulgidus MinD is indicated in Figure 1.3 B, arrow).

In addition, N-terminal truncation studies with MinC_{Ng} revealed that the thirteenth amino acid is required for the ability of the protein to inhibit cell division. While the extreme N-terminus of MinC is not conserved across species, there may be conserved structural motifs that are important for protein function (Greco, 2004).

1.4.7. Gonococcal MinE

Studies with N. gonorrhoeae MinE (MinE_{Ng}) have also been initiated. Expression studies in E. coli indicate that this protein can disrupt cell division site, resulting in minicell formation (Eng and Dillon, unpublished results), similar to overexpression of E. coli MinE (de Boer et al., 1989). Analytical ultracentrifugation of MinE_{Ng} showed the protein could self-associate, existing in a monomer-dimer-tetramer equilibrium (Dillon laboratory, unpublished results), similar to E. coli MinE (Zhang et al., 1998). Yeast two-hybrid assays also detected a MinD_{Ng}-MinE_{Ng} interaction (see Chapter 5, this study); hence truncations to both the N- and C-termini of MinE_{Ng} were constructed to delineate the minimum MinD_{Ng}-binding region of the protein (see Appendix, this study).

MinE_{Ng} is currently being studied by another doctoral student in the Dillon laboratory. Sequence alignment of MinE proteins from various organisms revealed several highly conserved residues which were mutated to assess their functionality. These studies have indicated that residues within the N-terminus of MinE_{Ng}, specifically A18D and L22D, are required for interaction with MinD_{Ng}. As a result, these MinE_{Ng} mutant proteins are unable to affect E. coli cell division (Eng and Dillon, unpublished results).
1.5. Rationale and Hypothesis of this study

Cytokinesis is a crucial event common to all lifeforms, including bacteria. From detailed studies in rod-shaped bacteria, it is evident that bacteria possess elaborate mechanisms to specify the localization of cell division sites. Bacteria exist in a number of morphologies, including rods, spirals, and round cells. While it is clearly established that Min proteins have a role in regulating proper septum placement in both Gram-negative and Gram-positive rod-shaped bacteria, comparatively little is known about how this is achieved in cells with other morphologies.

Since cell division is such a crucial event, components of the bacterial cell division machinery and their associated proteins can also provide promising targets for the development of new antimicrobials. This is especially important since bacteria are constantly acquiring resistance to our limited antibiotic arsenal. *N. gonorrhoeae* is a prime example of such developed resistance.

While gonorrhea is treatable with antibiotic therapy, *N. gonorrhoeae* has a well documented history of developing antibiotic resistance. The first treatment of gonorrhea used sulfonamide drugs to which gonococcal resistance developed quickly, prompting the use of penicillin as the primary therapy. By the 1970s and 1980s, resistance to penicillin developed through chromosomally-mediated mechanisms and the acquisition of penicillinase-producing plasmids which effectively terminated the use of this antibiotic to treat gonorrhea (Dillon and Yeung, 1989). High levels of resistance to tetracycline have also developed, mediated by the *tetM* determinant on a conjugative plasmid (Morse *et al*., 1986; Roberts, 1988). Multidrug resistant strains of *N. gonorrhoeae* also contain the *mtr* (multiple transferable resistance) locus, which encodes components for an energy-dependent drug efflux pump (Hagman *et al*., 1995) and confers resistance to a range of antimicrobials including erythromycin, tetracycline, chloramphenicol, rifampicin, and penicillin (Maness and Sparling, 1973; Hagman *et al*., 1995; Shafer *et al*., 1995; Veal *et al*., 1998). Currently, gonococcal infections are treated with third generation cephalosporins (e.g. ceftriaxone) and fluoroquinolones (e.g. ciprofloxacin). Unfortunately, the adaptive nature of *N. gonorrhoeae* constantly challenges antibiotic therapy, such that resistance to current antimicrobials is now reported (Fox and Knapp, 1999).
The presence of these minC, minD, and minE genes in the gonococcus is intriguing, since these genes are already established to encode cell division site-determinants in rod-shaped bacteria. Since all three min genes are found in *N. gonorrhoeae*, it is possible that a functional Min system exists to direct septum placement in this round bacterium. Studies with MinC<sub>Ng</sub> have demonstrated that it is likely involved in determining proper cell division sites in *N. gonorrhoeae* (Ramirez-Arcos *et al.*, 2001). The high conservation of MinD across bacterial species also suggests that this protein is required to maintain the proper cell division pattern in a variety of bacteria, including round cells. It is hypothesized that MinD<sub>Ng</sub> is involved in regulating the characteristic division pattern of the coccus *N. gonorrhoeae* and that the protein achieves this through interactions with the other Min proteins.

These studies will help gain insight into the mechanisms that bacteria, other than rods, use to achieve proper cell division. In addition, due to the ever-increasing prevalence of antibiotic resistant bacteria, what can be learned from studying cell division processes in these organisms can ultimately be applied to the development of novel antimicrobials and therapies for treating bacterial infections.
1.6. Objectives

The present study will determine whether *N. gonorrhoeae* MinD is involved in cell division site selection in the gonococcus. In addition, functional regions of MinD<sub>Ng</sub> will be identified and characterized to better elucidate its role. Collectively, these studies will be used to generate a model for topological regulation of cell division site placement in *N. gonorrhoeae*. In order to achieve this, the following objectives are proposed:

a) To establish whether MinD is involved in maintaining the characteristic division pattern of the gonococcus by using gene disruption, mutation, and overexpression studies in *N. gonorrhoeae*.

b) To investigate the use of *E. coli* as an indicator system for MinD<sub>Ng</sub> functionality to further elucidate the role of this protein. This will be achieved by heterologous expression studies of MinD<sub>Ng</sub> in rod-shaped and round mutant *E. coli* strains, coupled with examination of cell morphology and protein localization studies using GFP-fusions.

c) To characterize functional regions within MinD<sub>Ng</sub> using various genetic and biochemical strategies, including yeast two-hybrid analyses, site-directed mutagenesis, gene truncations, and protein techniques.

d) To use the culmulative data from these studies to develop a model for Min function in coccal cell division, using *N. gonorrhoeae* as a model organism.
CHAPTER 2

GENERAL MATERIALS AND METHODS
The following protocols outline methods that were common to most, or all, of the studies in this project. In addition, Tables describing strains and DNA primers used in this project are also included in this chapter. More specific Materials and Methods, as well as plasmids for each particular study, are described in their respective chapters.

2.1. General protocols and methods

**Strains and growth conditions.** The strains used in this study are presented in Table 2.1. *E. coli* strains DH5α and XLI-Blue were used as hosts for DNA cloning. *N. gonorrhoeae* CH811 was used to generate gonococcal minDNg mutant strains CJSD1 and SCD-1, and to provide chromosomal DNA template for PCR amplifications. *N. gonorrhoeae* F62 was used for minNg gene overexpression studies in the gonococcus. *N. gonorrhoeae* CH811 and F62 were grown on GC medium base (Difco) supplemented with Kellogg’s defined supplement (GCMBK) for 18 to 24 hours at 35°C in a humid, 5% CO₂ environment (Kellogg et al., 1963; Pagotto et al., 2000). Liquid cultures of *N. gonorrhoeae* were grown in GCMBK containing 0.04% NaHCO₃, incubated at 35°C with 5% CO₂ and shaking at 200 rpm.

*E. coli* strains PB103, DR105 (minCEc), PB104 (minDEc), WM1032 (∆minCDEEc), PB114 (∆minCDEEc), and KJB24 (rodA) (Table 2.1) were used in MinDNg expression and localization studies. *E. coli* PB103 was also used to provide chromosomal DNA for PCR amplification of *E. coli* minD (minDEc) and minC (minC Ec). *E. coli* GM48 (dam) was used to provide unmethylated plasmid DNA necessary for construction of the *N. gonorrhoeae* minDNg insertional mutant CJSD1 (Table 2.1). *E. coli* M15 and *E. coli* BL21 (DE3) were used as expression strains for the purification of N-terminal His-tagged MinCNg (His-MinCNg) and His-MinDNg, respectively. *E. coli* C41 (DE3) (Table 2.1) was used to overexpress C-terminal His-tagged MinDNg (MinDNg-His) proteins. *E. coli* C43 (DE3) (Table 2.1) was also used to overexpress N-terminal His-tagged MinCNg. *E. coli* strains WM1032 and PB114 were used for localization of GFP-MinDNg fusions (Table 2.1). *E. coli* strains were grown at 37°C in
<table>
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<th>Strains</th>
<th>Relevant genotype</th>
<th>Source/Reference</th>
</tr>
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<td><em>N. gonorrhoeae</em> CH811 Str’ <em>minD</em>:Cm’</td>
<td>This study</td>
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<td><em>N. gonorrhoeae</em> SCD-1</td>
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<td>This study</td>
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<td>P. de Boer, Case Western Reserve University</td>
</tr>
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<td>Organism</td>
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<td>Source</td>
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<td><em>S. cerevisiae</em> SFY526</td>
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**Plasmids**

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</table>
Luria-Bertani (LB) medium (Difco), except for *E. coli* strains WM1032 and PB114, which were grown at 30°C. Frozen stocks of all bacterial strains were stored at -70°C in brain heart infusion broth (Difco) supplemented with 20% glycerol.

When required, antibiotics were added to media in the following concentrations: 100 μg ampicillin (Amp)/ml, 25 μg chloramphenicol (Cm)/ml, and 100 μg kanamycin (Kan)/ml for *E. coli*; and 1 mg streptomycin (Str)/ml, 5 μg Cm/ml, and 75 μg (Kan)/ml for *N. gonorrhoeae*.

*Saccharomyces cerevisiae* SFY526 (Table 2.1) was used in yeast two-hybrid assays to study protein-protein interactions. Yeast were grown at 30°C on YPAD media, or on the appropriate synthetic dropout (SD) media, as described by the Clontech Yeast Two-Hybrid Protocols Manual (Clontech). Frozen stocks of yeast were stored at -70°C in YPAD media (Clontech Yeast Protocols Handbook) supplemented with 20% glycerol.

**Oligonucleotide primers and PCR.** Oligonucleotide primers used for PCR amplification (Table 2.2) were designed using Primer Designer Software (Scientific and Education Software). All primers were synthesized at the Core DNA Sequencing and Synthesis Facility, University of Ottawa. Whole cell suspensions of *N. gonorrhoeae* CH811 or *E. coli* PB103 (Table 2.1) were used to provide chromosomal DNA template for PCR by diluting cells in deionized distilled water (ddH2O). Cell concentrations were adjusted to 0.5 McFarland Equivalence Turbidity Standard (Remel). Where required, plasmid DNA to be used as PCR template was adjusted to 0.01 μg/ml.

PCR reactions were carried out in a Perkin Elmer Gene Amp PCR System 9600 Thermocycler (Perkin Elmer Corp.) as follows: 3 minutes at 94°C; 30 cycles of 15 s at 94°C, annealing for 15 s at temperatures dependent upon the primer pair used, extension at 72°C for times dependent on expected product size, and a final 5 minutes extension at 72°C. Reactions were carried out in a final volume of 100 μl containing: 1X PCR buffer with 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2
## Table 2.2. Primers used in this study

Primer sequences are listed in alphabetical order. Each primer sequence is encoded for a specific mutation or truncation, and the restriction sites are underlined.

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Note: The above sequences are from the Golliver system and are used in the construction of lambda minicircles. The exact functions and implications of these sequences are detailed in the referenced experimental study.
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<sup>1</sup> Denotes primer annealing to complementary strand.

<sup>2</sup> Gonococcal uptake sequence indicated in italics.

<sup>3</sup> 'CT' denotes truncation from the C-terminus.

<sup>4</sup> 'NT' denotes deletion of the designated number of amino acids (or codons) from the N-terminus, which are replaced with a start codon.
µg of each primer and 2.5 U Taq DNA polymerase (Boehringer Mannheim or Fermentas). In PCR reactions where Vent or Pfu DNA polymerase was used, the reactions conditions were modified as follows: 0.2 U Vent (New England BioLabs) or 2.5 U Pfu (Fermentas) polymerase and 1X PCR buffer (Vent or Pfu-specific buffer provided by manufacturers) in 100 µl final reaction volumes. When required to screen for minD_{Nt} mutations encoded on plasmids carried by E. coli, colony PCR was carried out as follows: individual E. coli colonies were selected with a sterile toothpick and resuspended in 25 µl ddH₂O. 1.25 µl of this suspension was used to provide plasmid DNA template for a 25 µl final volume PCR reaction, with volumes of other reagents adjusted accordingly.

**Agarose gel electrophoresis, plasmid isolation, and PCR amplicon purification.** DNA was separated on 1-2 % agarose gels using Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA) run at 6 volts/cm. The gels were stained in 1 mg/L ethidium bromide solution and the DNA visualized under a UV illuminator and digital imaging system (Alpha Innotech). Plasmids were isolated from E. coli and N. gonorrhoeae using the Qiagen Miniprep Kit according to the manufacturer’s instructions. PCR amplicons to be used in restriction digestion and cloning were purified using the Qiagen PCR Purification kit in order to remove residual salts and nucleotides.

**E. coli transformation** Plasmids were transformed into E. coli according to standard CaCl₂ methods outlined in Sambrook *et al.* (1989).

**Protein sequence alignment of MinD homologues.** The minD gene sequences from various bacteria were obtained at the WIT web site (formerly http://wit.mcs.anl.gov/WIT2 and now http://www.genomesonline.org). BLAST searches for minD homologues in other organisms were carried out in their respective sequence databases provided by The Institute for Genomic Research (TIGR) website (http://www.tigr.org). Protein sequences of MinD homologues were also obtained
from completed genome projects available from TIGR. When required, translation of \textit{minD} gene sequences was done using PCGene Software (Intelligenetics Inc.). Protein alignments were performed using ClustalW Version 1.8 software (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and alignments were visualized using BOXSHADE Version 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

**Construction and purification of His-MinD$_{Ng}$ and production of polyclonal anti-MinD$_{Ng}$ antisera.** To purify MinD$_{Ng}$, the coding region of \textit{minD$_{Ng}$} was cloned in frame with an N-terminal 6XHis tag encoded in pET30a (Table 2.1) using primers \textit{minD7pet30} and \textit{minD2} that included \textit{Ncol} and \textit{BamHI} sites, respectively (Table 2.2). The resulting fusion protein (His-MinD$_{Ng}$) would possess an N-terminal tag consisting of six histidine residues followed by a linker region (SSGLVPRGSMKETAAAASKFERQHMDSPDLGTDKKD) fused to the N-terminus of MinD$_{Ng}$. The plasmid was named pJSHD2 and was transformed into \textit{E. coli} BL21 (DE3) for overexpression (Table 2.1).

A 250 ml log-phase culture of \textit{E. coli} BL21 (DE3) carrying pJSHD2 was induced with 1 mM IPTG for 4 hours at 37°C and shaking at 250 rpm. Cells were harvested by centrifugation (5,000 X g for 10 minutes) (Sorvall RC 50) and stored at -20°C. Cells were resuspended in 12 ml binding buffer (prepared according to Novagen His-Bind protocols) containing 5 mM imidazole. Cells were redistributed to three tubes and each sample was sonicated eight times, for 30 seconds each time, with 1 minute intervals on ice (Fisher Scientific 60 Sonic Dismembrator). Lysed cells were then centrifuged at 100,000 X g for 30 minutes at 4°C (Beckman L8-55M ultracentrifuge). Soluble protein extract was applied to a column with 3 ml of His-Bind resin (Novagen) and protein purification was carried out as described by the manufacturer using the following solutions (with the indicated changes in imidazole concentration): 30 ml of binding buffer (5 mM imidazole), 21 ml wash buffer 1 (20 mM imidazole), 18 ml wash buffer 2 (30 mM imidazole), 9 ml wash buffer 3 (60 mM imidazole), and 12 ml elution buffer (250 mM imidazole). Eluted protein was dialyzed overnight at 4°C against Buffer A.
(20 mM Tris-HCl pH 7.5, 2 mM EDTA, 200 mM NaCl, 10% glycerol; final pH adjusted to pH 7.2). Protein identity was confirmed by Dr. P. Thibault, Institute of Biological Sciences, National Research Council of Canada, using MALDI-TOF mass spectrometry.

To prepare His-MinD<sub>Ng</sub> for rabbit immunization, the protein was eluted from SDS-PAGE gel fragments using the Model 422 Electroeluter (Bio-RAD) and concentrated using Centricon YM-10 (10,000 molecular weight cutoff) centrifuge devices. This protein was used to immunize female New Zealand white rabbits. Each immunization consisted of 80 µg of His-MinD<sub>Ng</sub> mixed with Gerbu adjuvant according to the manufacturer’s instructions (GERBU Biotechnik). Two additional boosters were administered over three week intervals. Whole serum was obtained from rabbit blood as outlined in established protocols (Sambrook <i>et al.</i>, 1989). MinD<sub>Ng</sub> specific antisera was affinity-purified using immobilized His-MinD<sub>Ng</sub> on nitrocellulose membranes, as outlined previously (Koelle, 1998).

**Construction and purification of His-MinC<sub>Ng</sub>, and production of polyclonal anti-MinC<sub>Ng</sub> antisera.** Using the primer pair JSC1/JSC2 (Table 2.2), incorporating BamHI and PstI restriction sites, respectively, minC<sub>Ng</sub> was PCR amplified and cloned into pQE30 to produce pJSHC (Table 2.1), encoding a 6XHis tag fusion to the N-terminus of MinC<sub>Ng</sub> (His-MinC<sub>Ng</sub>).

Plasmid pJSHC was transformed into <i>E. coli</i> M15 (Table 2.1) for expression of His-MinC<sub>Ng</sub>. One litre of LB broth supplemented with 100 µg/ml Amp and 25 µg/ml Kan was inoculated with 20 ml of overnight culture of <i>E. coli</i> M15 (pJSHC). Cells were grown at 250 rpm for 1 hour at 37°C prior to the addition of IPTG to 1 mM concentration, followed by an additional 3 hours of growth. 750 ml of culture was pelleted and resuspended in 15 ml of 8 M urea lysis buffer (pH 8.0), as outlined in the QIAexpressionist Protocols booklet for protein purification (Qiagen), in preparation for purifying His-MinC<sub>Ng</sub> from inclusion bodies. After incubation for 20 minutes at room temperature, cells were centrifuged at 10,000 X g for 25 minutes (Sorvall RC 50) and the supernatant was mixed gently with
1 ml of Ni-NTA resin (Qiagen) for 1 hour at room temperature. Resin and supernatant were then loaded into a 5 ml Qiagen polypropylene column. The resin bed was washed with 8 ml pH 6.3 buffer, 1 ml pH 5.9 buffer, 1.5 ml pH 5.0 buffer, and denatured His-MinC₉₈ was eluted with 1.5 ml of pH 4.5 buffer according to the manufacturer’s instructions (Qiagen). Purified protein was used to immunize female New Zealand white rabbits for the production of polyclonal anti-MinC₉₈ sera. Each immunization consisted of 20 μg of His-MinC₉₈ mixed with Gerbu adjuvant according to the manufacturer’s instructions (GERBU Biotechnik). Three additional boosters, similar to the initial immunization, were administered over three week intervals. Antisera were fractionated with 70% NH₄SO₄ and concentrated 10 times to obtain antiserum rich in IgG (Hebert et al., 1973). The antiserum was subsequently affinity purified by binding to immobilized His-MinC₉₈ on nitrocellulose membranes, as outlined previously (Koelle, 1998).

**Construction and purification of MinE₉₈-His, and production of polyclonal anti-MinE₉₈ antisera.** To obtain purified MinE₉₈, minE₉₈ was PCR amplified from gonococcal cell suspensions using primers ESminE1 and ESminE2 (Table 2.2), and cloned into the NdeI and XhoI restriction sites of pET30a above to form plasmid pEC1 (Table 2.1). E. coli C41 (DE3) was transformed with pEC1 and 0.4 mM IPTG was used to induce a 350 ml log phase culture for 2-3 hours at 250 rpm and 37°C. Cells were harvested and resuspended in four 8 ml volumes of 5 mM imidazole binding buffer, prepared according to Novagen His-Bind resin protein purification protocols. Resuspended cells were lysed by sonication (45 second bursts, repeated four times on ice) (Fisher Scientific 60 Sonic Dismembrator). Soluble cell supernatant was applied to a 3 ml His-Bind resin column which was washed with buffers containing increasing concentrations of imidazole (5 mM to 100 mM; Novagen protocols), and MinE₉₈-His was eluted with 250 mM imidazole buffer. Purified protein was dialyzed in Buffer B (50 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 7.4). MinE₉₈-His was concentrated using Biomax-5 centrifugal filter columns with a 5000 Dalton molecular weight cutoff (Millipore). Purified MinE₉₈ protein was provided to Dr. John Webb, Department of Biochemistry, Microbiology, and
Immunology, University of Ottawa, to immunize mice for raising polyclonal anti-MINNg antisera according to his established protocols.

**Protein analysis and Western blots.** In general, whole cell extracts from *N. gonorrhoeae* and *E. coli* were prepared by resuspending cells in appropriate volumes of PBS (pH 7.4) (depending on the size of initial harvested pellets), followed by the addition of 5X SDS-PAGE loading buffer (15% β-mercaptoethanol, 15% SDS, 1.5% bromophenol blue, 50% glycerol). Cell suspensions were boiled for 10 minutes and then centrifuged for 10 minutes at 10,000 X g (Sorvall MC 12V Microfuge). The supernatant fractions were recovered and separated by SDS-PAGE using 12% polyacrylamide gels (15% polyacrylamide gels when studying MINNg) prepared as outlined in Sambrook *et al.* (1989). SDS-PAGE was carried out using the Mini-PROTEAN II electrophoresis system (Bio-Rad). Prior to Western blotting, protein concentrations were standardized for each sample using densitometric analysis of resolved cell extracts. In general, after Coomassie blue staining of resolved cell extracts (Sambrook *et al.*, 1989), the intensities of several common protein bands in each sample were compared using densitometric analysis available on Alpha imager 1220 v5.04 software (AlphaEase™ version 5.00, Alpha Innotech Corp.). These comparisons were then used to adjust, and equalize, the amount of each protein sample that was to be resolved in a subsequent polyacrylamide gel to be used in Western blotting. The Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was used for Western transfer onto Immobilon-P membranes (Millipore Corporation). Immobilon-P SQ membranes were used when MINNg was to be detected by Western blotting. After protein transfer, membranes were blocked with 3% skim milk for one hour at room temperature.

Blots were probed for one hour at room temperature (anti-MINNg sera) or overnight at 4°C (anti-MINC Ng, anti-MIND Ng, or anti-MINNg sera). Anti-gonococcal MinD antisera was diluted 1:800, while anti-MINC Ng and anti-MINNg antisera were used at 1:100 in TTBS (50 mM Tris, 1.25 M NaCl, 0.05% Tween-20, pH 7.5). Blots were then washed 3 X 10 minutes in TTBS, followed by incubation
with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Bio-Rad, 1:3000 dilution) when anti-MinC$_{N_8}$ or anti-MinD$_{N_8}$ was used as a primary antibody. Biotinylated goat anti-mouse antisera was used as a secondary (1:40,000; 2 hours room temperature; Sigma) for blots probed with anti-MinE$_{N_8}$ sera, followed by 2 hour incubation with Extravidin (1:150,000; Sigma). All blots were then washed with TTBS (3 X 10 minutes) and developed using the AttoPhos Plus kit (JBL Scientific INC). Where required, densitometric analysis was performed using the Alpha imager 1220 v5.04 (AlphaEase™ version 5.00, Alpha Innotech Corp.) to compare signal intensity levels.

**Phase contrast microscopy.** *N. gonorrhoeae* and *E. coli* cells to be examined by phase contrast microscopy were fixed with 0.2% glutaraldehyde and 6% formaldehyde, prior to adhesion for 30 minutes onto coverslips precoated with 0.01% polylysine. Coverslips were then washed gently with PBS buffer (pH 7.4) and placed onto a slide containing a drop of 50% glycerol and sealed. Samples were analyzed by phase contrast microscopy using a Zeiss Axioskop microscope under a 100X oil immersion lens. Images were collected using Northern Eclipse (Version 5.0) software. When required, differential interference contrast (DIC) imaging was performed using Olympus BX60 or BX61 microscopes equipped with DIC prisms.

**Yeast-two hybrid assays.** Prior to testing for protein-protein protein interactions, each yeast plasmid was transformed singly using the lithium acetate method (Clontech Yeast Two-Hybrid Manual) into the yeast reporter strain *S. cerevisiae* SFY526 (Table 2.1) to ensure that the GAL4 fusions could not activate the β-galactosidase reporter gene by themselves. Transformants carrying pGBT9-derived vectors were plated on SD –Trp media, while those carrying pGAD424-derived plasmids were grown on SD –Leu. When testing for interacting proteins, double transformants were plated on SD -Leu/-Trp plates. All media were prepared as described in the Clontech Yeast two-hybrid manual (Clontech). β-galactosidase activities were initially assessed using colony-lift assays and X-gal substrate, as
described by Clontech. The relative strength of each interaction was determined by β-galactosidase activity assays using o-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate, as described by Clontech. These liquid assays were performed at least twice, in duplicate, for each interaction.
CHAPTER 3

MinD and *Neisseria gonorrhoeae* cell division

Portions of this chapter were published in:

3.1 PURPOSE OF THIS STUDY

Virtually all of our present knowledge of bacterial cell division has been obtained from studies with rod-shaped organisms, such as *E. coli* and *B. subtilis* (Errington et al., 2003). Normally, cytokinesis in these bacteria occurs at a midcell site that results in two equally sized daughter cells. In both rods, the Min proteins have been shown to be involved in determining the proper placement of the cell division site (de Boer et al., 1989, Levin et al., 1992, Errington et al., 2003).

Homologues to minC, minD, and minE have been identified in the coccus *N. gonorrhoeae* (Ramirez-Arcos et al., 2001a). Unlike rod-shaped bacteria, *N. gonorrhoeae* divides along two dimensions (Fitz-James, 1964; Westling-Häggström et al., 1977). We have previously shown that deletion of minC will disrupt normal cell division in *N. gonorrhoeae* (Ramirez-Arcos et al., 2001a). Studies in this chapter were performed to determine whether MinD$_{Ng}$ is also involved in maintaining the characteristic cell division pattern in the gonococcus. Here, it is shown that minD$_{Ng}$ disruption or mutation in *N. gonorrhoeae* leads to aberrant cell division and morphology, and reduces cell viability. Furthermore, overexpression of MinD$_{Ng}$ and MinC$_{Ng}$ together from a *N. gonorrhoeae*-*E. coli* shuttle vector, modified from a previous vector constructed in our laboratory (Pagotto et al., 2000), could induce cell division arrest in *N. gonorrhoeae*. MinD$_{Ng}$ was found to reside in both cytosolic and membrane fractions of the gonococcus. The localization of the essential cell division protein FtsZ in *N. gonorrhoeae* was also carried out using immunoelectron microscopy. Overall, these studies demonstrate that MinD$_{Ng}$ is required to maintain proper cell division site placement in the coccus *N. gonorrhoeae*.
3.2. MATERIALS AND METHODS

Identification of the gonococcal min gene cluster. Our laboratory previously identified and cloned the *N. gonorrhoeae* minCDE cluster (Ramirez-Arcos *et al.*, 2001a) after analysis of the raw DNA sequence data from the *N. gonorrhoeae* FA1090 genome project (Roe *et al.*, 2001). The minCNg, minDNg, and minENg genes from *N. gonorrhoeae* strain CH811 (Table 2.1) were PCR amplified, cloned into plasmid pSR1 (Table 3.1), and sequenced (GenBank accession number AF345908) (Ramirez-Arcos *et al.*, 2001a).

Insertional knockout of chromosomal minDNg in *N. gonorrhoeae* CH811. A chloramphenicol acetyltransferase (cat) cassette was inserted into the chromosomal minDNg gene of *N. gonorrhoeae* strain CH811 by allelic exchange in order to disrupt minDNg expression. Plasmid pSR1 (Table 3.1) was transformed into *E. coli* GM48 (dam) in order to obtain unmethylated plasmid DNA. This plasmid was digested at its unique BclI site near the 5' end of minDNg, 179 bp downstream from the translational start site of minDNg. The cat cassette from pACYC184 (Table 3.1) was PCR amplified using primers HMcat1 and HMcat2 (Table 2.2). Both the cat cassette and BclI-digested pSR1 were blunt-ended with T4 DNA polymerase (New England Biolabs) and ligated to form pJSDcat (Table 3.1). The presence and orientation of the cat insert in minDNg was confirmed by PCR analysis and by DNA sequencing at the Core DNA Sequencing and Synthesis Facility, University of Ottawa. *N. gonorrhoeae* CH811 cells (Table 2.1) were transformed with pJSDcat to allow for insertional inactivation of minDNg by homologous recombination as described previously (Janik *et al.*, 1976). The insertion of cat into *N. gonorrhoeae* CH811 chromosomal minDNg was confirmed by PCR analysis. The resulting gonococcal minDNg knockout was named *N. gonorrhoeae* strain CJSD1 (Table 2.1).

Growth studies. The effect of minDNg disruption in the gonococcus was monitored by growth and viability studies. To ensure that equivalent numbers of *N. gonorrhoeae* CH811 and CJSD1 cells
### Table 3.1 Plasmids used in this study

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<tr>
<td>pJATP1</td>
<td>pSR1; ( \text{P}<em>{\text{lac}}:: \text{minC}</em>{\text{Ng}}, \text{minD}<em>{\text{Ng}-\text{K16Q}}, \text{minE}</em>{\text{Ng}}, \text{oxyR} ) (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pJATP1CAT</td>
<td>pSR1; ( \text{P}<em>{\text{lac}}:: \text{minC}</em>{\text{Ng}}, \text{minD}<em>{\text{Ng}-\text{K16Q}}, \text{minE}</em>{\text{Ng}}, \text{cat}, \text{oxyR} ) (Amp&lt;sup&gt;R&lt;/sup&gt;Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td><strong>N. gonorrhoeae shuttle vectors</strong></td>
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<tr>
<td>pFP10</td>
<td>Gonococcal shuttle vector containing ( \text{ori} ) from ( \beta )-lactamase producing plasmid pID5 (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Pagotto <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>pFP20</td>
<td>pFP10 with ( \text{cat} ) removed, replaced with ( \text{aph(3')} ) (Kan&lt;sup&gt;R&lt;/sup&gt; gene from pET30a)</td>
<td>This study</td>
</tr>
<tr>
<td>pFP21</td>
<td>pFP20 + ( \text{minC}<em>{\text{Ng}}, \text{minD}</em>{\text{Ng}}, \text{minE}_{\text{Ng}} ) (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pFP22</td>
<td>pFP20 + ( \text{minC}_{\text{Ng}} ) (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pFP23</td>
<td>pFP20 + ( \text{minD}_{\text{Ng}} ) (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pFP24</td>
<td>pFP20 + ( \text{minE}_{\text{Ng}} ) (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pFP25</td>
<td>pFP20 + ( \text{minC}<em>{\text{Ng}}, \text{minD}</em>{\text{Ng}} ) (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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</table>
would be used in growth studies, the inocula were standardized as follows. First, to determine the
number of colony-forming units (cfu)/ml of wild-type and mutant bacteria in overnight cultures, each
strain was diluted 25-fold from overnight liquid GCMBK cultures to obtain an OD_{550} of 0.05. Ten-
fold serial dilutions were performed to determine the numbers of cfu/ml that were present in each
culture.

For growth studies, equivalent numbers of cells (1 × 10^6 cfu) from overnight cultures of *N.
gonorrhoeae* CH811 and CJSD1 were used to inoculate 25 ml of liquid GCMBK, and cells were
grown as outlined in Chapter 2 for a 13 hour time period. 1 ml of culture was removed at fixed times
to measure absorbance at OD_{550} and was serially diluted in liquid GCMBK. 100 µl from selected
dilutions were plated on GCMBK agar media supplemented with Str for *N. gonorrhoeae* CH811, or
with Str and Cm for *N. gonorrhoeae* CJSD1. The plates were incubated for 24 hours prior to
enumerating colonies, or for an additional 24 hours if the colonies were too small to be effectively
counted. Growth studies were performed twice for each strain.

**Creation of a chromosomal minD_Ng (K16Q) point mutation in *N. gonorrhoeae*.** Allelic exchange
was used to replace the wild-type chromosomal minD_Ng gene of *N. gonorrhoeae* with a minD_Ng gene
encoding a K16Q mutation in the Walker A ATP-binding motif of the protein. The minD_Ng gene in
plasmid pSR1 (Table 3.1) was mutated using an inverse PCR (IPCR) strategy. Primers JSDATP1,
encoding a K16Q mutation and a Hpy188IX restriction site, and JSDATP5 (Table 2.2) were designed
to anneal adjacent to each other and amplify in opposite directions using *Pfu* DNA polymerase
(Fermentas). The resulting blunt-ended amplicon was phosphorylated using T4 polynucleotide kinase
(New England BioLabs), re-circularized with T4 DNA ligase (Gibco), and transformed into *E. coli*
DH5α. Colony PCR was used to amplify plasmid-encoded minD_Ng genes that could be screened for
the K16Q mutation by virtue of their ability to be cleaved by *Hpy*188IX. The resulting plasmid
(containing minC_Ng, minD_Ng-K16Q, minE_Ng, and oxyR) was named pJATP1 (Table 3.1).
A cat cassette was inserted between minE_Ng and oxyR in pJATP1 prior to transformation into N. gonorrhoeae CH811 (Table 2.1). This site for cat insertion was selected to avoid disrupting the min cluster, and would allow for selection of successful chromosomal recombinants. To do this, IPCR was carried out using primers min40 and min40rev (Table 2.2), annealing adjacent to each other at the intergenic region between minE_Ng and oxyR, and amplifying in opposite directions. The resulting amplicon was blunt-ended using T4 DNA polymerase (New England BioLabs) and T4 polynucleotide kinase (PNK) (New England BioLabs). A cat cassette, PCR amplified from pACYC184 (Table 3.1) using primers min21 and min22 (Table 2.2), was also blunt-ended as above and ligated into the min40/min40rev amplicon, to produce plasmid pJATP1CAT (Table 3.1). PCR methods were used to determine that the orientation of the cat cassette was in the same direction as minCDE transcription.

Plasmid pJATP1CAT was transformed into N. gonorrhoeae CH811 as outlined previously (Janik et al., 1976). PCR using various combinations of primer pairs that annealed both outside and inside of the recombination region was performed on selected recombinants to confirm that a double crossover had occurred. DNA sequencing confirmed the presence of the chromosomal mutation. The resulting gonococcal strain, containing chromosomal minD_Ng-K16Q was named N. gonorrhoeae SCD-1 (Table 2.1).

Overexpression studies of minNg genes in N. gonorrhoeae. The Dillon laboratory has previously constructed a cloning vector, pFP10 (Table 3.1), capable of replicating within several hosts, including N. gonorrhoeae and E. coli (Pagotto et al., 2000). This N. gonorrhoeae-E. coli shuttle vector was modified by replacing its resident cat cassette with a kanamycin resistance cassette and by the incorporation of additional restriction sites as follows. The kanamycin resistance cassette from pET30a (Table 3.1) was PCR amplified using the 5’ primer CJ1 and the 3’ primer DJ26 (Table 2.2). Each primer also contained a gonococcal uptake sequence (US) (Goodman and Scocca, 1988) to better facilitate the transformation of N. gonorrhoeae cells. CJ1 also incorporated a HindIII site, while DJ26 contained restriction sites for Psil, Kpnl, NcoI, EcoRI, and Ndel (Table 2.2). The amplicon was
digested with *PstI* and *HindIII* prior to ligation. pFP10 was also digested with *PstI* and *HindIII* to eliminate the *cat* cassette, and the desired vector fragment was isolated from 1% agarose gels using the QIAquick gel extraction kit (Qiagen). The resulting pFP10 vector fragment and kanamycin resistance cassette were subsequently ligated to generate pFP20 (Table 3.1).

Gonococcal min genes and their upstream regions shown previously to have promoter activity (Ramirez-Arcos et al., 2001b) were PCR amplified from *N. gonorrhoeae* cell suspensions and cloned separately, or in combination, into pFP20. Gonococcal minC and its 503 bp upstream region shown to possess promoter activity (Ramirez-Arcos et al., 2001b) were PCR amplified using the primer pair CJ3/JSC2 (Table 2.2) which incorporated *EcoRI* and *PstI* sites, respectively. The resulting amplicon was digested with *EcoRI* and *PstI* and ligated into similarly digested pFP20, forming plasmid pFP22 (Table 3.1). The other min genes were also amplified with their respective upstream regions as follows. Gonococcal minD was PCR amplified with primers min12 and JSD2 (Table 2.2). This amplicon included minD and an upstream region consisting of the coding region of minC, shown to have promoter activity (Ramirez-Arcos et al., 2001b). The minE gene and a 720 base pair upstream sequence, also shown to possess promoter activity (Ramirez-Arcos et al. 2001b), was amplified with primers minD5 and JSE2 (Table 2.2). Both minC and minD were amplified together using primers CJ3 and JSD2 (Table 2.2). The entire minCDE cluster was also amplified with primers CJ3 and JSE2. All amplicons were digested with *EcoRI* and *PstI* and individually ligated into pFP20 as above to form pFP21 (minC, minD, minE), pFP23 (minD), pFP24 (minE), and pFP25 (minC, minD).

Each plasmid was transformed into *N. gonorrhoeae* F62 using a protocol modified from Seifert and So (1991). Frozen stocks of *N. gonorrhoeae* F62 were restreaked onto GCMBK agar, grown for 18 hours, and examined for T2 colonies (Juni and Heym, 1977). T2 colonies were resuspended to a 0.5 MacFarland Standard, in GCMBK broth supplemented with 10 mM MgSO4. 20 μl of the bacterial suspension was diluted in 200 μl of the same broth and 0.5-1.0 μg of plasmid DNA was added. The cells were mixed gently and incubated at 35°C and 5% CO2 for 30 minutes without
shaking, then transferred to a 15 ml Falcon tube containing 2 ml of GCMBK supplemented with 10 mM MgSO₄. Cells were incubated for an additional 5-6 hours with shaking (150 rpm) at 35°C and 5% CO₂. Afterwards, 100 µl of cells were plated onto kanamycin selective GCMBK agar (100 µg Kan/ml) and incubated at 35°C and 5% CO₂. The stability of each plasmid within the transformants was verified by plasmid isolation and DNA gel electrophoresis.

**Transmission electron microscopy and immunogold localization studies.** Transmission electron microscopy was performed on *N. gonorrhoeae* strains CH811 and CJSD1 (Table 2.1) in collaboration with Dr. T. Beveridge, University of Guelph, using general protocols as previously described (Beveridge *et al.*, 1994). *N. gonorrhoeae* F62 (Table 2.1) transformed with pFP20 (negative control vector) and pFP25 (*minC₅₉, minD₅₉*) (Table 3.1), and *N. gonorrhoeae* SCD-1 (Table 2.1) were fixed in 1.6% glutaraldehyde, sectioned, and examined by transmission electron microscopy according to protocols established at the Laboratory Pathology facilities, Ottawa Hospital, Civic Campus, Ottawa Hospital, Ottawa, ON.

The diameters of forty *N. gonorrhoeae* F62 cells transformed with pFP20 or pFP25 (Table 3.1) were measured from electron micrographs taken at the same magnification and average diameters at their widest points were calculated for both samples. Statistical analysis using the unpaired Student's *t*-test was applied to the averages, and differences were considered significant for *p* values <0.001.

Immunogold labelling of *N. gonorrhoeae* CH811 to localize MinD₅₉ or FtsZ₅₉ protein was also carried out according to protocols established at the Laboratory Pathology facilities, Ottawa Hospital, using affinity purified anti-MinD₅₉ antisera (this study) or affinity purified anti-FtsZ₅₉ antisera prepared previously in our laboratory (Salimnia *et al.*, 2000).

**Separation of soluble and insoluble gonococcal cell fractions.** *N. gonorrhoeae* CH811 cells were collected from solid GCMBK media and resuspended in 2 ml of PBS (pH 7.4). Cells were lysed by
sonication (Fisher Scientific 60 Sonic Dismembrator) using 3 cycles of four 5 second bursts, with a 1 minute time interval on ice between cycles. The cell extract was centrifuged at 100,000 x g for 1 hour at 4°C (Beckman TL-100 ultracentrifuge) and the soluble supernatant was retained. The insoluble pellet was washed twice with 2 ml PBS and resuspended in 2 ml PBS. Protein concentrations for both soluble and insoluble fractions were determined using the Bio-Rad Protein assay kit and similar amounts of protein from each fraction were resolved by SDS-PAGE and used for Western blotting to detect MinD_{Ng}. 
3.3. RESULTS

MinD proteins are highly conserved. The minD gene is the most ubiquitously distributed of the three min genes, with homologues being found in Gram-negative and Gram-positive bacteria, in chloroplasts, and in Archaea (Margolin, 2001). MinD from *N. gonorrhoeae* (MinD<sub>Ng</sub>; 271 aa) is 73% identical and 85% similar to *E. coli* MinD (MinD<sub>E</sub>), and is 42% identical and 66% similar to *B. subtilis* MinD (MinD<sub>bs</sub>).

Alignment of *N. gonorrhoeae* MinD from strain FA1090, used in the genome project (Roe *et al.*, 2001), with the deduced amino acid sequences of 23 other MinD homologues from various organisms shows regions of extensive conservation distributed throughout the protein, particularly the N-terminal half of the protein (corresponding to amino acids 1-166 of MinD<sub>Ng</sub>) (Figure 3.1). All MinD proteins analyzed also have a conserved Walker A ATP-binding motif, corresponding to residues 10-17 of MinD<sub>Ng</sub> (GKGGVGKT) (Figure 3.1; residues underneath red line). The overall consensus sequence of this motif is GKGG[T/V]GK[T/S]. The MinD Walker A motif can be classified as a part of the ‘deviant’ subgroup of such motifs by virtue of the signature lysine (shown in bold) found at the N-terminus of the sequence (Koonin, 1993). Both *N. gonorrhoeae* and *N. meningitidis* MinD also contain a sequence at their extreme C-termini (KSFFKRLF) (Figure 3.1, residues underneath green line), which is nearly identical to the membrane targeting sequence (MTS) identified in *E. coli* MinD (KGFFKRLF) (Szeto *et al.*, 2002; Hu and Lutkenhaus, 2003). In addition, bacterial MinD proteins contain switch I and switch II sites, implicated in nucleotide-induced conformational changes (Vale, 1996), which have also been identified in Archaeal MinD structures (Cordell and Löwe, 2001; Sakai *et al.*, 2001) (Figure 3.1, residues underneath black lines).

There are only two nucleotide differences between minD from *N. gonorrhoeae* strain FA1090 (used in the genome project), and from strain CH811 (used in this study). In strain CH811, base number 479 is a cytosine, while in strain FA1090 the nucleotide is an adenine. In either case, the respective GGC and GGA codons still encode glycine at amino acid residue 160. Strain CH811 minD<sub>Ng</sub> also has a guanine nucleotide at base number 702, resulting in a
Figure 3.1. Sequence alignment of MinD proteins from various species. Abbreviations used: *Methanococcus jannaschii* (Mj), *Archaeoglobus fulgidus* (Af), *Pyrococcus furiosus* (Pf), *P. horikoshii* (Ph), *Neisseria gonorrhoeae* (Ng) strain FA1090, *N. meningitidis* (Nm), *Escherichia coli* (Ec), *Salmonella enterica* serovar Typhimurium (St), *Yersinia pestis* (Yp), *Vibrio cholerae* (Vc), *Pseudomonas aeruginosa* (Pa), *Brucella melitens* (Bm), *B. suis* (Bs), *Agrobacterium tumefaciens* (At), *Helicobacter pylori* (Hp), *Aquifex aeolicus* (Aa), *Thermotoga maritima* (Tm), *Bacillus subtilis* (Bs), *Listeria monocytogenes* (Lm), *Clostridium perfringens* (Cp), *Synechocystis sp.* (Sy), *Guillardia theta* (Gt), *Deinococcus radiodurans* (Dr), and *Chlamydia trachomatis* (Ct). Black boxes indicate amino acid residues that are identical to the consensus. Gray boxes indicate amino acids that are similar to the consensus. Single red bar indicates position of the deviant Walker A ATP-binding motif. Black bars indicate positions of switch I and II sites. Single green bar indicates MinD membrane targeting sequence (MTS) (Szeto et al., 2002; Hu and Lutkenhaus, 2003). Short coloured bars adjacent to organism names indicate their general classification: green = Archaea, red = Gram-negatives, orange = Thermophilic bacteria, blue = Gram-positives, black = cyanobacteria, violet = chloroplast, grey = Chlamydiae. (*) indicates residue 235 (threonine) of *N. gonorrhoeae* MinD FA1090, which is appears as an alanine residue in *N. gonorrhoeae* CH811 MinD.
GCG codon encoding alanine at residue 235 of its MinD\textsubscript{Ng}. In this unconserved gene region, strain FA1090 contains an ACG codon encoding threonine at residue 235 (Figure 3.1, asterisk), instead of an alanine.

**Disruption of minD\textsubscript{Ng} results in aberrant gonococcal cell division and morphology and decreases cell viability.** To determine whether minD\textsubscript{Ng} is involved in maintaining proper cell division patterns in the gonococcus, the gene was disrupted using a homologous recombination strategy to insert a cat cassette near the 5' end of chromosomal minD\textsubscript{Ng} in *N. gonorrhoeae* CH811 (Table 2.1). PCR analysis and DNA sequencing showed that the cat insert was in the opposite orientation of transcription of minD\textsubscript{Ng}. Using affinity purified anti-MinD\textsubscript{Ng} polyclonal antisera, Western blot analysis of cell extracts from wild-type *N. gonorrhoeae* CH811 (Figure 3.2 F, lane 1) and the minD knockout strain, CJS1D1, (lane 2) showed no MinD\textsubscript{Ng} expression in the latter strain.

Under phase-contrast microscopy, *N. gonorrhoeae* CJS1D1 exhibited cells of varying sizes which were often clustered together and showed signs of lysis (Figure 3.2 B), while wild-type *N. gonorrhoeae* CH811 cells displayed uniform size and shape (Figure 3.2 A). Closer inspection of *N. gonorrhoeae* CJS1D1 by electron microscopy revealed grossly abnormal cell morphologies, characterized by irregularly shaped cells and the presence of lysed cells devoid of intracellular contents (Figure 3.2 D, E). Furthermore, septum formation resulted in unequally sized cells. These observations contrast the typical, uniform diplococcal morphology of wild-type *N. gonorrhoeae* CH811 (Figure 3.2 C).

The growth of *N. gonorrhoeae* CJS1D1 was also compared to its parental wild-type strain in liquid cultures. The number of colony forming units (cfu)/ml of *N. gonorrhoeae* CJS1D1 was higher than that of wild-type *N. gonorrhoeae* CH811 for approximately the first 6 hours, but subsequently became significantly less than that of wild-type for the following 7 hours (Figure 3.3 A). In addition, the mutant strain appeared to reach stationary phase after approximately 6 hours, which was 4-5 hours sooner than wild-type, as assessed by viable counts (Figure 3.3 A). Wild-type colonies were easily
Figure 3.2. Effects of insertional inactivation of minD on the cell morphology of *N. gonorrhoeae* CH811. Phase contrast micrographs of wild-type *N. gonorrhoeae* CH811 (A) and *N. gonorrhoeae* minD knockout mutant, CJSD1 (B). Both (A) and (B) are at the same magnification and the scale bar in (A) represents 5 μm. (C) Transmission electron microscopy shows the typical diplococcal morphology of *N. gonorrhoeae* CH811. (D and E) Electron micrographs of *N. gonorrhoeae* CJSD1 showing aberrant cell morphologies, including cells of varying sizes and cells devoid of intracellular contents. Scale bars in (C, D and, E) represent 0.5 μm. (F) Western blot analysis of cell extracts using affinity-purified anti-MinD<sub>Ng</sub> antisera show the expression of MinD<sub>Ng</sub> in *N. gonorrhoeae* CH811 (lane 1), but not in *N. gonorrhoeae* CJSD1 (lane 2).
Figure 3.3. Cell viability of wild-type *N. gonorrhoeae* strain CH811 and *minD*<sub>Ng</sub> knockout strain CJSD1. (A) Comparison of the viability of wild-type *N. gonorrhoeae* CH811 (circles) and *minD* knockout CJSD1 (triangles) based on colony forming units (cfu/ml) counts of each strain grown in GCMBK broth over 13 hours. (B) Wild-type *N. gonorrhoeae* CH811 colonies appeared 18-24 hours after plating from broth to solid media and displayed uniform size, while CJSD1 colonies (C) were not readily visible until 42-48 hours, and appeared smaller. Both (B) and (C) display colonies at the same magnification.
visualized and counted after the normal incubation time of 18-24 hours; however, *N. gonorrhoeae* CJSD1 colonies did not appear until after 42-48 hours. Furthermore, once these colonies became visible, they were always variable in size, especially with the appearance of many tiny colonies (Figure 3.3 C). In contrast, *N. gonorrhoeae* CH811 colonies were much larger after only a single day of incubation, and were of uniform size (Figure 3.3B).

In addition to the insertional *minD*<sub>Ng</sub> knockout mutant, a second gonococcal mutant was generated to express MinD<sub>Ng</sub> bearing a glutamine substitution at the extremely conserved lysine-16 residue of the Walker A-ATP binding motif. The same mutation in *E. coli* MinD was shown to render the protein unable to induce cell division arrest in those cells (de Boer et al., 1991). *N. gonorrhoeae* SCD-1, encoding *minD*<sub>Ng</sub>-K16Q in place of wild-type *minD*<sub>Ng</sub> (Table 2.1), showed aberrant morphology similar to strain CJSD1, including cells that possessed multiple invaginations (Figure 3.4 B, arrows), suggestive of multiple cell division events, as well as cells that had varying shapes and sizes (Figure 3.4 C, D). These morphologies were in contrast to the round diplococci typically seen in wild-type *N. gonorrhoeae* (Figure 3.4 A). Western blotting verified the expression of wild-type MinD<sub>Ng</sub> in *N. gonorrhoeae* CH811 (Figure 3.4 E, lane 1) and MinD<sub>Ng</sub>-K16Q in *N. gonorrhoeae* SCD-1 (Figure 3.4 E, lane 2).

Hence, the effects of abrogating MinD<sub>Ng</sub> expression, or replacing wild-type *minD*<sub>Ng</sub> with a gene containing a mutated Walker A ATP-binding motif, suggests that the protein is involved in maintaining proper cell division, cell morphology, and viability of the gonococcus.

**Overexpression of MinD<sub>Ng</sub> and MinC<sub>Ng</sub> together in *N. gonorrhoeae* causes cell enlargement.** Our laboratory has previously constructed a unique shuttle vector capable of replicating in *N. gonorrhoeae, E. coli,* and *Haemophilus* species (Pagotto et al., 2000). To determine the effects of overexpressing gonococcal Min proteins in their native background, the shuttle vector pFP10 (Table 3.1) (Pagotto et al., 2000) was first modified to include more restriction sites for cloning purposes, additional gonococcal uptake sequences to aid transformation, and a kanamycin resistance cassette.
Figure 3.4. Transmission electron micrographs of wild-type *N. gonorrhoeae* CH811 and mutant SCD-1 encoding MinD_{NGK16G}. (A) *N. gonorrhoeae* CH811 shows typical, round gonococcal morphology. (B, C, and D) Aberrant morphology is displayed by strain SCD-1, including multiple invaginations (arrows). Scale bar represents 1 μm in all figures. (E) Wild-type (lane 1) and mutant (lane 2) MinD_{NG} protein expression is confirmed in both strains by Western blotting with anti-MinD_{NG} antisera.
Each minNg gene was cloned individually, or in combination with the other minNg genes, into the new shuttle vector, pFP20 (Table 3.1).

Attempts to transform the shuttle expression vectors into the plasmid-free strain N. gonorrhoeae CH1811 (Table 2.1) were unsuccessful. Interestingly, N. gonorrhoeae F62 (Table 2.1), which naturally carries a 4.2 kb cryptic plasmid found in many gonococcal strains (Roberts et al., 1979), could be transformed with each of the shuttle vectors including pFP20 (negative control vector), pFP21 (minCNg, minDNg, minENg), pFP22 (minCNg), pFP23 (minDNg), pFP24 (minENg), and pFP25 (minCNg, minDNg) (Table 3.1). The cell morphologies of each transformant were examined by phase contrast microscopy. Gonococci transformed with the unmodified pFP20 vector exhibited a typical, uniform, round shape and cell diameters of ~1 μm or less (Figure 3.5 A). Cells transformed with pFP21, pFP22, pFP23, and pFP24 did not exhibit any noticeable morphological changes from the control, as assessed by phase contrast microscopy (Figure 3.5 B-E). However, transformants carrying pFP25, encoding both minCNg and minDNg, exhibited obvious cell enlargement, with some cells having over twice the diameter of control cells (Figure 3.5 F-G, arrows).

Western blotting using affinity purified anti-MinCNg antisera showed that gonococcal cells transformed with pFP21 (minCNg, minDNg, minENg), pFP22 (minCNg), and pFP25 (minCNg, minDNg) had 4, 2, and 2 times, respectively, the level of MinCNg (Figure 3.5 I; lanes 2, 3 and 6) found in negative control cells transformed with pFP20 (lane 1). MinCNg levels in pFP23 (minDNg) and pFP24 (minENg) transformants (lanes 4 and 5) resembled that in the control (lane 1).

When cell extracts were probed with affinity purified anti-MinDNg antisera, cells transformed with pFP21 (minCNg, minDNg, minENg) (Figure 3.5 J, lane 2) had approximately twice the amount of MinDNg detected in negative control cells (lane 1). N. gonorrhoeae transformed with pFP25 (minCNg, minDNg) also had increased MinDNg levels to ~1.2 times the control (Figure 3.5 J, lane 6). All other transformants did not have increased levels of MinDNg (Figure 3.5 J, lanes 3, 4, and 5).

The ability of N. gonorrhoeae F62 to maintain the shuttle vectors was confirmed by plasmid isolation followed by gel electrophoresis of the isolated DNA. In addition to the cryptic plasmid (4.2
Figure 3.5. Phase contrast micrographs to determine the effects of transforming *N. gonorrhoeae* F62 with shuttle vector pFP20 encoding individual, and combinations of, *min* genes. (A) *N. gonorrhoeae* F62 transformed with control shuttle vector pFP20 exhibit characteristic uniform coccal morphology (diameter <1μm). *N. gonorrhoeae* F62 transformed with (B) pFP21 (minC<Ng> minD<Ng> minE<Ng>), (C) pFP22 (minC<Ng>), (D) pFP23 (minD<Ng>), and (E) pFP24 (minE<Ng>), do not show obvious altered morphologies by phase-contrast microscopy. (F-H) *N. gonorrhoeae* F62 transformed with pFP25 (minC<Ng> minD<Ng>) exhibit noticeable cell enlargement, including cells up to twice the normal diameter. Panels A-H are all at the same magnification, and the scale bar in (A) represents 5 μm. Western blots to determine MinC<Ng> (I) and MinD<Ng> (J) expression levels in *N. gonorrhoeae* F62 transformants was carried out. Cell extracts of *N. gonorrhoeae* F62 transformed with: Lane 1, control pFP20; lane 2, pFP21 (minC<Ng> minD<Ng> minE<Ng>); lane 3, pFP22 (minC<Ng>); lane 4, pFP23 (minD<Ng>); lane 5, pFP24 (minE<Ng>); and lane 6, pFP25 (minC<Ng> minD<Ng>) were probed with affinity-purified anti-MinC<Ng> (I) or anti-MinD<Ng> (J) antisera.
kb) of *N. gonorrhoeae* F62, transformants also maintained their respective shuttle vectors [Figure 3.6: lane 2, pFP20 (5.2 kb); lane 3, pFP21 (7.5 kb); lane 4, pFP22 (6.4 kb); lane 5, pFP23 (6.8 kb); lane 6, pFP24 (6.2 kb); and lane 7, pFP25 (7.2 kb)].

Electron microscopy was used to further examine the morphology of the enlarged *N. gonorrhoeae* F62 carrying pFP25 (*minC*$_{Ng}$, *minD*$_{Ng}$) (Figure 3.7 C-F). These cells were larger than those transformed with the negative control vector pFP20 (Figure 3.7 A, B), and still retained diplococcal morphology. Even individual cocci of enlarged pFP25 transformants were often as large as diplococci of control cells. Out of 40 randomly measured cells containing pFP25 (*minC*$_{Ng}$, *minD*$_{Ng}$), 38 had diameters ranging from ~0.95 μm to 1.50 μm, which were greater than the average diameter of the control transformants (~0.90 μm). The average diameter of pFP25 transformants was calculated to be ~28% greater than that of pFP20 transformants and statistical analysis confirmed that this difference was significant (*p*<0.001). Hence, gonococcal cell enlargement correlated with increased expression of both MinC$_{Ng}$ and MinD$_{Ng}$ together. Overall, these results indicated that MinD$_{Ng}$ acts in conjunction with MinC$_{Ng}$ to inhibit cell division in gonococcal cells.

**MinD is located in cytosolic and membrane fractions of gonococcal cell extracts.** Soluble and insoluble wild-type *N. gonorrhoeae* CH811 cell fractions were separated by high speed centrifugation and probed with affinity purified anti-MinD$_{Ng}$ antisera. Densitometric analysis revealed that approximately 40% of total MinD$_{Ng}$ was associated with the insoluble membrane fraction (Figure 3.8 A, lane 1), while 60% of the protein was detected in the cytosol (Figure 3.8 A, lane 2). Immunogold labeling of fixed *N. gonorrhoeae* CH811 cells to localize MinD$_{Ng}$ also showed that the protein could be found in the cytoplasm (Figure 3.8 B, D; black arrows) and along the inner cell membrane (Figure 3.8 B-D, red arrows). However, there was insufficient labeling in the cell sections to accurately determine any MinD$_{Ng}$ localization pattern in these samples.
Figure 3.6. *N. gonorrhoeae* F62 are able to maintain shuttle vectors carrying gonococcal *min* genes. Plasmids isolated from gonococci transformed with pFP20 (negative control vector), pFP21 (minC<sub>N</sub>g, minD<sub>N</sub>g, minE<sub>N</sub>g), pFP22 (minC<sub>N</sub>g), pFP23 (minD<sub>N</sub>g), pFP24 (minE<sub>N</sub>g), and pFP25 (minC<sub>N</sub>g, minD<sub>N</sub>g) were resolved by agarose gel electrophoresis to verify their presence and general integrity. Black arrows indicate supercoiled DNA ladder bands that were used to estimate plasmid size (lane 1). White arrows indicate resolved gonococcal plasmid bands used to estimate shuttle vector sizes. Lane 2 = pFP20 (5.2 kb); lane 3 = pFP21 (7.5 kb); lane 4 = pFP22 (6.4 kb); lane 5 = pFP23 (6.8 kb); lane 6 = pFP24 (6.2 kb); and lane 7 = pFP25 (7.2 kb). Plasmid band at ~4.2 kb represents gonococcal cryptic plasmid inherent to *N. gonorrhoeae* F62.
Figure 3.7. Electron micrographs of *N. gonorrhoeae* F62 transformed with pFP20 control shuttle vector and pFP25 encoding \(\text{minC}_{Ng} \text{ and minD}_{Ng}^*\) (A and B) Control transformants display normal gonococcal morphology. (C-F) *N. gonorrhoeae* F62 transformed with pFP25 (\(\text{minC}_{Ng} \text{ and minD}_{Ng}^*\)) are noticeably enlarged. All panels are at the same magnification, and scale bars represent 1 \(\mu\text{m}\).
Figure 3.8. Detection of MinD$_{Ng}$ in gonococcal cell fractions. (A) Western blot using anti-MinD$_{Ng}$ antisera was used to probe insoluble membrane fraction (lane 1) and soluble cytosolic fraction (lane 2) of N. gonorrhoeae CH811 cell extracts. (B-D) Immunogold labeling and transmission electron microscopy was used to localize MinD$_{Ng}$ in N. gonorrhoeae CH811. Black arrows indicate cytosolic MinD$_{Ng}$ and red arrows show inner membrane associated MinD$_{Ng}$. 
Localization of FtsZ in dividing *N. gonorrhoeae* cells. Gonococci characteristically divide along two alternating, perpendicular planes (Fitz-James, 1964; Westling-Häggström *et al*., 1977). As part of the effort to generate a refined model of cell division in *N. gonorrhoeae*, localization of FtsZ in dividing gonococcal cells was conducted using immunoelectron microscopy. Previous studies in our laboratory have shown that *N. gonorrhoeae* possesses *ftsZ* (*ftsZNg*) within the division cell wall (*dcw*) cluster of genes (Salimnia *et al*., 2000).

Immunogold labeling using anti-*FtsZNg* antiserum (Salimnia *et al*., 2000) was performed on sections of fixed *N. gonorrhoeae*. In single coci, transmission electron micrographs revealed a distinct accumulation of FtsZ along one side of the cell, within a single invagination point that presumably indicated the initiation of a cell division event along one plane (Figure 3.9 A, B; arrows). Further along in the division process, a constrictive septum was observed along a midline of the cell, with *FtsZNg* signal found predominantly at the two leading edges of the constriction (Figure 3.9 C, arrows). As division proceeded, a single band of *FtsZNg* signal is observed between two daughter cells (Figure 3.9 D), prior to the formation of a diplococcus.

In order to produce the typical 2 x 2 tetrad formation of daughter cells, the next *FtsZNg* assembly point must be repositioned perpendicularly to its initial point prior to cell division along the second plane. This was supported by observing *FtsZNg* signal distinctly localizing at single invagination points at the junction between each cell of a diplococcus (Figure 3.9 E, arrows), such that septum formation along this second, perpendicular plane should result in a tetrad of daughter cells.
Figure 3.9. Immunogold localization of *N. gonorrhoeae* FtsZ (*FtsZ\(_{Ng}\)*) in cross sections of dividing gonococcal cells. (A and B) In individual cocci, cell division is initiated at a single invagination point (arrows), where distinct accumulation of *FtsZ\(_{Ng}\) is observed (black dots). (C) Further along in the division process, a constrictive septum is observed along a midline of the cell, with *FtsZ\(_{Ng}\) signal found predominantly at the two leading edges of the constriction (arrows). (D) As division proceeds, a single band of *FtsZ\(_{Ng}\) signal is observed between two daughter cells, prior to the formation of a diplococcus. (E) Septum formation along a second, perpendicular plane is initiated by *FtsZ\(_{Ng}\) localizing at single invagination points at the junction between each cell of a diplococcus (arrows).
3.4. DISCUSSION

Most of our present knowledge of bacterial cell division has arisen from extensive studies of cell division in rod-shaped bacteria. Since cocci lack obvious cell poles, it has been suggested that they would not contain any min genes (Weaver, 2000). Our laboratory has pioneered the study of cell division proteins from round bacteria, and we have shown that the Gram-negative coccus N. gonorrhoeae contains a min operon with minC, minD, and minE genes (Ramirez-Arco et al., 2001a). This is the first study to characterize MinD from a naturally occurring coccus.

MinD homologues are found in a variety of organisms, ranging from Gram-negative and Gram-positive bacteria to Archaea and chloroplasts (Margolin, 2001a). Alignment of MinD proteins showed that they are well conserved, particularly at the N-terminus, corresponding to the first 166 amino acids of MinDNe. From the crystal structure of P. furiosus MinD, the residues directly involved in binding ATP are mostly found distributed throughout the N-terminal half of the protein, in support of the overall conserved nature of this region (Hayashi et al., 2001).

Their wide distribution across genera and species suggests MinD proteins have an important role for cellular function(s). It is noted that the presence of MinD in an organism does not necessarily indicate that MinC and/or MinE are present as well. For example, Archaeal organisms possess MinD homologues (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001), yet analysis of Archaeal genome projects show they lack MinC and MinE. In addition, no min genes have been identified in any Gram-positive cocci to date, with the exception of a minD homologue in Deinococcus radiodurans. However, like Archaea, the D. radiodurans genome project does not identify any minC or minE genes. Hence, MinD may serve another function in Archaea and in D. radiodurans, other than regulating cell division.

Since N. gonorrhoeae and N. meningitidis contain all three min genes, they provide exceptional cases for investigating the possible role of a Min system regulating the placement of cytokinetic machinery in naturally occurring cocci. Although these organisms do not have obvious cell poles, the disruption of cell division site machinery should elicit some morphological changes.
This was demonstrated by the *N. gonorrhoeae minD* knockout strain, CJSD1, and by strain SCD-1, bearing a minD_{Ng-K16Q} chromosomal mutation. These strains exhibited grossly aberrant morphology characterized by daughter cells of differing sizes and shapes, and had multiple, improperly placed division planes initiated. A similar phenotype has also been observed in a *N. gonorrhoeae minC* insertional mutant, constructed in our laboratory, which displayed atypical division as well (Ramirez-Arcos *et al.*, 2001a). Hence, these studies indicate that MinD_{Ng} is involved in maintaining proper cell division and morphology in the gonococcus.

Mutation of the conserved K16 Walker A ATP-binding motif of *E. coli* MinD has been shown to render the protein incapable of regulating proper cell division arrest and unable to associate with phospholipids (de Boer *et al.*, 1991; Hu *et al.*, 2002). It is proposed that the K16Q substitution may prevent MinD_{Ee} from undergoing necessary conformational changes that permit membrane association (Hu *et al.*, 2002). Hence, the inability of MinD_{Ng-K16Q} to properly bind to the inner membrane in *N. gonorrhoeae* SCD-1 would conceivably allow improper initiation of FtsZ constriction events. Similarly, the loss of MinD_{Ng} in *N. gonorrhoeae* CJSD1 would presumably result in an inability for the division inhibitor MinC_{Ng} to properly localize to the inner membrane and exert its effects.

To date, *N. gonorrhoeae* SCD-1 is the only described gonococcal strain, and perhaps natural coccus, that contains a constructed point mutation in one of its min genes. In *E. coli*, removal of MinD or MinC produces a minicell phenotype characterized by small anucleate minicells and rods of varying lengths (de Boer *et al.*, 1989); therefore, it is tempting to speculate the phenotypes observed with *N. gonorrhoeae* CJSD1 and SCD-1 represent the equivalent of a ‘minicell’ phenotype in cocci.

Although Western blotting confirmed that MinD_{Ng-K16Q} was still expressed, it was present at a lower level compared to wild-type MinD_{Ng} in control cells. It could be argued that MinD_{Ng-K16Q} remains active, and that the aberrant phenotype of *N. gonorrhoeae* strain SCD-1 was due to lowered levels of MinD_{Ng-K16Q}, and not specifically to the K16Q mutation itself. However, further work with MinD_{Ng-K16Q} in this study provides more evidence for its impaired activity (see following chapters). In
addition, the fact that the depletion of MinD<sub>NgK16Q</sub> itself (whether still functional or not) results in aberrant cell division is significant evidence in itself that MinD protein is required for proper gonococcal cell division.

Disruption of minD<sub>Ng</sub> may have affected gonococcal cell wall integrity as well. Evidence of cell lysis in <i>N. gonorrhoeae</i> CJSD1 was observed by phase contrast and electron microscopy, and a similar marked increase in lysis was observed in a <i>N. gonorrhoeae minC</i> insertional mutant constructed in our laboratory (Ramirez-Arcos <i>et al.</i>, 2001a). It is possible that the enlarged cells, produced as a result of asymmetric cell division in <i>N. gonorrhoeae</i> CJSD1 are more sensitive to lysis. From the surface stress theory of Koch (1985), where T = Pr/2 in cocci (T, surface tension; P, hydrostatic pressure; and r, radius), an increase in cell size (r) results in an increase in cell wall tension. Significant increases in cell size can result in sufficient tension on peptidoglycan bonds that make them more susceptible to breaking. Alternatively, peptidoglycan bonds may be more susceptible to hydrolysis since the activity of autolytic enzymes increases under stress conditions (Koch, 1985).

Since cocci need only half the hydrostatic pressure required in rods to increase their surface tension by similar amounts (Koch, 1985), gonococci are likely more sensitive to the effects brought by changes in cell size, including those caused by disrupting minD. It is also possible that lysis resulted from a compromise in cell wall integrity due to cell wall components/precursors being diverted to multiple division sites forming in the gonococcal minD mutant.

The lack of MinD<sub>Ng</sub> also led to decreased cell viability of <i>N. gonorrhoeae</i> CJSD1 during the latter half of the growth curve. In rod-shaped bacteria, polar divisions due to min mutations produce non-viable, anucleate minicells (de Boer <i>et al.</i>, 1988; de Boer <i>et al.</i>, 1989). It is possible that improper cell division in strain CJSD1 would also result in 'daughter' cells with decreased and/or lost viability, which was observed. Increased cell lysis likely further contributed to the lower viability of the gonococcal minD mutant. The production of non-viable cells and/or lysed cells during each
successive division event would also account for the delayed appearance and smaller size of *N. gonorrhoeae* CJS31 colonies on solid media.

Curiously, this mutant displayed a higher viability compared to wild-type cells early in the growth experiments (<6 hours). It is possible that mutant cells may have become multinucleated at the onset of growth, resulting in a higher number of viable nucleated cells than the control. As more undesirable cell division events occur, a corresponding increase in anucleate and non-viable mutant cells may account for the decreased viability of strain CJS31 at later growth stages. Unlike *E. coli*, all attempts by our laboratory to generate a *N. gonorrhoeae* mutant lacking all three *min* genes have been unsuccessful, suggesting that the gonococcus may be inherently more sensitive to the effects of *min* gene abrogation than *E. coli*. Hence, differences in the ability of Min proteins to affect cell division in rods and cocci may reflect differences in the sensitivity of each model system to these proteins.

In *E. coli*, MinD appears to dynamically shuttle between the cytoplasm and membrane target sites as it recruits MinC from pole-to-pole (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a; Rowland et al., 2000; Hu et al., 2003). The detection of MinD$_{Ng}$ in both soluble and insoluble gonococcal fractions also suggests that the protein may exhibit such dynamic movement in *N. gonorrhoeae*. Furthermore, immunoelectron microscopy showed evidence that MinD$_{Ng}$ was found in both the cytosol and along the inner membrane. In support of this, examination of the MinD$_{Ng}$ sequence shows it contains a C-terminal motif that closely resembles the *E. coli* MinD membrane targeting sequence identified by others (Szeto et al., 2002, Hu and Lutkenhaus, 2003). Experiments to attempt localization of GFP-MinD$_{Ng}$ fusions in live *N. gonorrhoeae* should prove interesting. Due to the small size and fastidious nature of this organism, such studies should prove challenging.

Cloning the gonococcal *min* genes into the modified shuttle vector pFP20 allowed the first study to examine the effects of Min protein overexpression in natural cocci. Such studies in *N. gonorrhoeae* would have previously been more difficult, if not impossible, due to a lack of suitable genetic tools for this organism. Overexpression studies provided further evidence that MinD$_{Ng}$ is involved in gonococcal cell division regulation. *N. gonorrhoeae* F62 cells became noticeably
enlarged when both MinC<sub>Ng</sub> and MinD<sub>Ng</sub> were overexpressed from pFP25 (minC<sub>Ng</sub>, minD<sub>Ng</sub>), indicative of a relationship between the two proteins to inhibit cytokinesis. Similarly, the *E. coli* and *B. subtilis* MinC-MinD complexes act together to inhibit cell division, with MinD recruiting MinC to the cell membrane (Huang et al., 1996; Hu and Lutkenhaus, 1999; Marston and Errington, 1999b; Hu et al., 2003; Lackner et al., 2003). Other studies have shown that inhibiting cell division in cocci results in enlarged cells. For example, penicillin G induces cell enlargement in *N. gonorrhoeae* (Westling-Häggström et al., 1977), and round roda mutants of *Salmonella typhimurium* grown under conditions that prevent cell division become significantly larger in size (Costa and Antôn, 1999).

Gonococci transformed with pFP21 (encoding minC<sub>Ng</sub>, minD<sub>Ng</sub>, and minE<sub>Ng</sub>) also had increased levels of MinC<sub>Ng</sub> and MinD<sub>Ng</sub>; however, there was no obvious cell enlargement. It has been shown that *E. coli* transformed with a plasmid containing all three *E. coli* min genes exhibit a minicell phenotype, similar to overexpressing minE<sub>E</sub> alone. This suggests an increase in MinE<sub>E</sub> can overcome any cell division arrest that may arise from a concurrent increase in MinC<sub>E</sub>-MinD<sub>E</sub> levels (de Boer et al., 1989). Therefore, it is possible that, despite increased MinC<sub>Ng</sub> and MinD<sub>Ng</sub> levels in gonococcal pFP21 transformants, there may have been sufficient MinE<sub>Ng</sub> expression from this vector to maintain a proper stoichiometric ratio between the three Min<sub>Ng</sub> proteins that countered any effects on cell morphology. Unfortunately, any increase in MinE<sub>Ng</sub> levels could not be ascertained in gonococcal transformants since our current polyclonal anti-MinE<sub>Ng</sub> antisera only has sufficient sensitivity to detect MinE<sub>Ng</sub> expressed from high copy number vectors in *E. coli* backgrounds.

Although MinC<sub>Ng</sub> levels were also increased in gonococci transformed with pFP22 (minC<sub>Ng</sub>), there was no increase in MinD<sub>Ng</sub> levels in cells carrying pFP23 (minD<sub>Ng</sub>). Studies in our laboratory indicated the region upstream of minD<sub>Ng</sub> (included in pFP23) possesses promoter activity (Ramirez-Arcos et al., 2001b). However, it is possible that flanking chromosomal DNA sequences that normally influence minD<sub>Ng</sub> promoter activity are disrupted upon cloning minD<sub>Ng</sub> into the shuttle vector. Hence, whether the overexpression of MinC<sub>Ng</sub> and MinD<sub>Ng</sub> together is an absolute requirement for gonococcal cell enlargement is not known. In wild-type *E. coli*, the overexpression of
either MinC\textsubscript{Ec} or MinD\textsubscript{Ec} can inhibit cell division (de Boer \textit{et al.}, 1989). Therefore, it is possible that overexpression of either MinC\textsubscript{Ng} or MinD\textsubscript{Ng} to levels \textit{higher} than that achieved in the present study may affect normal cell division of \textit{N. gonorrhoeae}. To further study the effects of overexpressing each \textit{min}\textsubscript{Ng} gene in the gonococcus, each gene could be introduced into the shuttle vector under the control of a more active, or inducible, promoter system, rather than their natural upstream promoter regions. While the consequences of overexpressing MinD\textsubscript{Ng} alone in \textit{N. gonorrhoeae} could not be determined in the present study, it is predicted that sufficiently increased MinD\textsubscript{Ng} levels should lead to cell enlargement, similar to the elongation of wild-type \textit{E. coli} cells that overexpress MinD\textsubscript{Ec} (de Boer \textit{et al.}, 1989).

Interestingly, the plasmid-free \textit{N. gonorrhoeae} strain CH811 used to generate the two gonococcal \textit{minD} mutants in this study could not be transformed with the shuttle vector pFP20. In contrast, \textit{N. gonorrhoeae} strain F62, which harbours a 4.2 kb cryptic plasmid (Roberts \textit{et al.}, 1979), could successfully maintain pFP20 and its derivatives. It is noted that \textit{N. gonorrhoeae} strain FA1090, which also carries the cryptic plasmid, can be transformed with the shuttle vector as well (data not shown). Although most \textit{N. gonorrhoeae} clinical isolates bear the cryptic plasmid, it has, to date, no known function (Roberts \textit{et al.}, 1979). Studies thus far have identified up to 10 open reading frames (ORFs) encoded by this plasmid (Korch \textit{et al.}, 1985), including a possible Mob protein gene, which acts as a mobilization factor in other plasmid systems (Sarandopoulos and Davies, 1993). It is possible that the cryptic plasmid may encode other factors that permit the maintenance of other plasmids, including our shuttle vector. It is also possible that \textit{N. gonorrhoeae} strain CH811 lacks certain chromosomally encoded determinants that are required for general plasmid upkeep.

Since Min proteins are implicated in the ultimate positioning of the Z-ring in bacteria (Margolin, 2001b; Errington \textit{et al.}, 2003), FtsZ was localized in dividing \textit{N. gonorrhoeae} in order to provide spatial information that will be used to generate a model for gonococcal cell division. Using immunoelectron microscopy, FtsZ was localized at the leading edge of constrictions, which defined cell division along one dimension. This is consistent with previous observations that gonococcal cell
division is initiated by a single invagination point along one side of the cell (Fitz-James, 1964). In addition, the localization of FtsZ at invaginations between two daughter cells is consistent with observations that a second cytokinetic event occurs along a plane perpendicular to the first, which is initiated at the junction between two cells (Fitz-James, 1964; Westling-Häggström et al., 1977).

These are the first studies of MinD function in coccal bacteria. By disrupting minDNg, it was demonstrated that MinDNg is involved in maintaining the proper cell division pattern, morphology, and cell viability of the coccus N. gonorrhoeae. Using a stable shuttle vector, it was also demonstrated that MinDNg likely acts with MinCNg to inhibit cell division in the gonococcus. This, and other studies in our laboratory (Ramirez-Arcos et al., 2001a), offer further evidence that a functional Min protein system exists in N. gonorrhoeae to regulate proper cytokinesis.
CHAPTER 4

Functionality of Neisseria gonorrhoeae MinD in E. coli backgrounds

Portions of this chapter were published in:


and

4.1. PURPOSE OF THIS STUDY

*N. gonorrhoeae* is a fastidious organism, with only a few genetic tools, such as the gonococcal shuttle vectors prepared in our laboratory, available for studying molecular mechanisms in this bacterium (Pagotto et al., 2000; this study). In contrast, *E. coli* is much easier to culture and to genetically manipulate. Furthermore, there are many expression vectors that are suitable for protein studies in *E. coli*. Since both *N. gonorrhoeae* and *E. coli* are Gram-negative organisms that contain all three *min* genes, the present study was conducted to determine whether gonococcal MinD is functional in *E. coli* backgrounds.

The overexpression of *E. coli* MinD will induce MinC$_{Ec}$-dependent cell division arrest in wild-type cells (de Boer et al., 1989). GFP-MinD$_{6c}$ also oscillates *in vivo* in the presence of MinE$_{Ec}$ (Raskin and de Boer, 1999a; Rowland et al., 2000), and does so by dynamically localizing within intracellular coiled arrays (Shih et al., 2003). Hence, the ability of gonococcal MinD to perform the above activities was assessed to determine its functionality in *E. coli*. I show that MinD$_{Ng}$ can be expressed heterologously in *E. coli*, and can induce cell division arrest in these cells provided that resident *E. coli* MinC is available. In addition, MinD$_{Ng}$ is able to partially complement an *E. coli* minD mutant. Furthermore, GFP-MinD$_{Ng}$ exhibits dynamic intracellular movement in *E. coli* where it localizes, in a MinE$_{Ng}$-dependent manner, within coiled arrays, similar to studies with GFP-fusions to *E. coli* MinD (Raskin and de Boer, 1999a; Rowland et al., 2000; Shih et al., 2003). Hence, intracellular dynamism is not exclusive to *E. coli* MinD. These studies demonstrate that *E. coli* can be used as an effective indicator organism for MinD$_{Ng}$ functionality.
4.2. MATERIALS AND METHODS

Cloning minD<sub>Ng</sub> for expression studies in E. coli. Wild-type minD<sub>Ng</sub> was PCR amplified from gonococcal cell suspensions using the primers minD1 and minD2 (Table 2.2), incorporating EcoRI and BamHI restriction sites to the 5’ and 3’ ends of the gene. The amplicon was ligated into EcoRI/BamHI digested pUC18 (Amersham) to form pSR3 (Table 4.1). Plasmid pSR3 was transformed into E. coli strains PB103 (wild-type), DR105 (minC<sub>Ec</sub> mutant), PB104 (minD<sub>Ec</sub> mutant), PB114 (∆minCDE<sub>Ec</sub>), and KJB24 (rodA mutant) (Table 2.1) to assess the effects of MinD<sub>Ng</sub> expression in E. coli backgrounds using phase contrast microscopy. As a negative control, minD<sub>Ng</sub><br> K16Q was PCR amplified from pJATP1 (Chapter 3, Table 3.1) using primers minD1 and minD2 (Table 2.2), and cloned into the EcoRI and BamHI sites of pUC18 to form pMJ3 (Table 2.1).

Construction of GFP fusions to wild-type MinD<sub>Ng</sub>. A fusion of green-fluorescent protein (GFP) to the N-terminus of wild-type MinD<sub>Ng</sub> was constructed for localization experiments in E. coli backgrounds. Primers minD1 and minE2 (Table 2.2) were used to PCR amplify both minD<sub>Ng</sub> and minE<sub>Ng</sub> from N. gonorrhoeae chromosomal DNA and the resulting amplicon was digested with EcoRI and BamHI and ligated into similarly digested pDSW209 (encoding GFP) (Weiss et al., 1999), forming plasmid pSR15 (Table 4.1). This plasmid encoded GFP fused to the N-terminus of wild-type MinD<sub>Ng</sub> and encoded MinE<sub>Ng</sub> immediately downstream.

A vector encoding GFP-MinD<sub>Ng</sub> alone (without MinE<sub>Ng</sub>), was also constructed by amplifying minD<sub>Ng</sub> from N. gonorrhoeae CH811 chromosomal DNA using primers minD1 and minD2 (Table 2.2), digesting the amplicon with EcoRI and BamHI, and ligating into pDSW209 as above. This plasmid was named pJS10 (Table 4.1). As a negative control, primers minD1 and minE2 were also used to amplify minD<sub>Ng</sub><br> K16Q and minE<sub>Ng</sub> from plasmid pJATP1 (Chapter 3, Table 3.1) for ligation into pDSW209 as above to create plasmid pJDE1 (gfp-minD<sub>Ng</sub><br> K16Q, minE<sub>Ng</sub>) (Table 4.1).
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<tr>
<th>Plasmids</th>
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<td><strong>Plasmids for expression studies</strong></td>
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<td>$P_{lac}$ (Amp$^R$)</td>
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<td>pJDE1</td>
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<td>pWM1255</td>
<td>pDSW209; $P_{trc}$::gfp-$minD_{Ec}$, $minE_{Ec}$ (Amp$^R$)</td>
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Plasmid pWM1255 encoded a GFP-fusion to *E. coli* MinD (GFP-MinD<sub>Ec</sub>), as well as MinE<sub>Ec</sub> downstream (Table 4.1) (Corbin *et al.*, 2002), and was used to compare oscillation cycles with GFP-MinD<sub>Ng</sub>.

**GFP-MinD<sub>Ng</sub> localization studies in *E. coli*.** Protein localization studies were performed using two separate fluorescent microscope systems. Initial studies were done in collaboration with Dr. William Margolin, University of Texas Medical School, using an Olympus BX60 fluorescence microscope and a 100X oil immersion objective. Images were captured with a Photometric CoolSnap fx CCD camera and QED software. Approximately 3 μl of log-phase *E. coli* WM1032 (ΔaminCDE) (Table 2.1) transformed with pSR15 (Table 4.1) were grown for 4-5 hours under 40 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and 30°C incubation. 3 μl of cells were immobilized by mixing with 3 μl of 2% low-melting-point agarose in LB and immediately applied to a microscope slide. Localization of GFP-MinD<sub>Ng</sub> in round *E. coli* KJB24 (rodA) cells (Table 2.1) containing pSR15 was also examined. *E. coli* KJB24 were grown overnight at room temperature without any agitation or IPTG, and supplemented with 50 μg/ml thymine. Time-lapse fluorescence images were taken every 10-15 seconds, with exposure times ranging from 0.5-1.2 sec. Images were saved and compiled with Adobe Photoshop software.

Subsequent GFP-MinD<sub>Ng</sub> localization studies were conducted in our laboratory, after acquiring an Olympus BX61 fluorescence microscope system equipped with oil-immersion 60X and 100X objectives, a Photometrics CoolSnap ES camera, and Image Pro (Version 4.5.1) software. Time lapse images were taken every 6 seconds, with exposure times of 0.250-0.500 seconds. Localization studies GFP-MinD<sub>Ng</sub> in *E. coli* PB114 (ΔaminCDE) transformed with pSR15, pJDE1, or pJS10 were conducted (Table 4.1). In addition, GFP-MinD<sub>Ng</sub> localization in round *E. coli* KJB24 cells (Table 2.1) transformed with pSR15 (Table 4.1) was also investigated. GFP-MinD<sub>Ng</sub> oscillation cycles were defined as the time required for fusion protein signal to travel from one cell pole to the other, and
back again. The unpaired Student’s $t$-test was used to determine whether average oscillation cycles differed from one another, with $p < 0.001$ considered significantly different.
4.3. RESULTS

Expression of MinD\textsubscript{Ng} in \textit{E. coli} backgrounds indicates cross-species functionality. Since gonococcal MinD is 73% identical and 85% similar to \textit{E. coli} MinD, MinD\textsubscript{Ng} was expressed in \textit{E. coli} backgrounds to determine whether it could affect cell division in another organism. Because \textit{E. coli} is easier to genetically manipulate and is less fastidious than \textit{N. gonorrhoeae}, these studies will determine the possibility of using \textit{E. coli} as an indicator strain for MinD\textsubscript{Ng} functionality.

Rod-shaped wild-type \textit{E. coli} PB103 cells (Table 2.1) were transformed with plasmid pSR3, encoding minD\textsubscript{Ng} (Table 4.1). While cells transformed with the negative control pUC18 vector exhibited normal short rod morphology (Figure 4.1 A), the same strain transformed with pSR3 exhibited a filamentous phenotype, indicative of cell division arrest (Figure 4.1 B). As another negative control, \textit{E. coli} PB103 was also transformed with pMJ3, encoding MinD\textsubscript{Ng,K16Q} (Table 4.1). Cells expressing MinD\textsubscript{Ng,K16Q} could still divide normally and appeared as short rods (Figure 4.1 C), similar to pUC18 transformants. Western blot analysis confirmed that MinD\textsubscript{Ng} was overexpressed in \textit{E. coli} cells transformed with pSR3 (Figure 4.1 D, lane2). The anti-MinD\textsubscript{Ng} antisera could also detect the resident MinD\textsubscript{Ec} found in wild-type \textit{E. coli}, which appeared as a faint band in pUC18 transformed cell extracts (Figure 4.1 D, lane 1). Western blotting also confirmed that wild-type MinD\textsubscript{Ng} (Figure 4.1 E, lane 1) and MinD\textsubscript{Ng,K16Q} (Figure 4.1 E, lane 2) were expressed at similar levels in \textit{E. coli}.

Plasmid pSR3 was also transformed into \textit{E. coli} PB104 (Table 2.1) to determine whether minD\textsubscript{Ng} could complement the minD\textsubscript{Ec} mutation in this strain. \textit{E. coli} PB104 encodes a MinD\textsubscript{Ec} protein with a G262A substitution that results in an inactive protein (de Boer \textit{et al.}, 1988), likely due to an abrogated C-terminal membrane targeting sequence (Szeto \textit{et al.}, 2002). As a result, these cells display a classic minicell phenotype characterized by rods of varying lengths (Figure 4.2 A, black arrows) and minicells (Figure 4.2 A, white arrows). However, when transformed with pSR3, the majority of \textit{E. coli} PB104 cells exhibited a short rod morphology, with very few filaments and minicells (Figure 4.2 B). This indicated that a partial complementation of the minD\textsubscript{Ec} mutation was achieved. Western blot analysis shows the overexpression of gonococcal MinD\textsubscript{Ng} in the
Figure 4.1. Effects of overexpressing \textit{MinD}_{N\text{g}} in wild-type \textit{E. coli} PB103. (A) \textit{E. coli} PB103 transformed with pUC18 negative control vector have typical wild-type short rod morphology. (B) \textit{E. coli} PB103 transformed with pSR3 (\textit{minD}_{N\text{g}}) exhibit a severe filamentous phenotype. Scale bar in (A) is 5 \textmu m and all figures are at the same magnification. (C) \textit{E. coli} PB103 transformed with pMJ3 (\textit{minD}_{N\text{g}-K16Q}) exhibit a short rod morphology. (D) Western blot using anti-\textit{MinD}_{N\text{g}} antisera to probe cell extracts of \textit{E. coli} PB103 pUC18 transformants (lane 1) and pSR3 (\textit{minD}_{N\text{g}}) transformants (lane 2). (E) Western blot using anti-\textit{MinD}_{N\text{g}} antisera to probe cell extracts of \textit{E. coli} PB103 pSR3 (\textit{minD}_{N\text{g}}) transformants (lane 1) and pMJ3 (\textit{minD}_{N\text{g}-K16Q}) transformants (lane 2). Note: (D) and (E) are separate blots. Protein concentrations for samples within each respective blot were equalized.
Figure 4.2. Complementation of minD mutation in E. coli PB104 expressing MinD<sub>Nr</sub>. (A) E. coli PB104 transformed with pUC18 displays a typical minicell phenotype characterized by rods of varying lengths (black arrows), and minicells (white arrows) (B) The majority of E. coli PB104 transformed with pSR3 (minD<sub>Nr</sub>) have restored short rod morphology, with few longer filaments and minicells. Scale bar in (A) is 5 µm and both figures are at the same magnification. (C) Western blot using anti-MinD<sub>Nr</sub> antisera to probe cell extracts of E. coli PB104 (pUC18) (lane 1) and E. coli PB104 (pSR3) (lane 2).
complemented cells (Figure 4.2 C, lane 2), compared to background expression of inactive MinD<sub>Ec</sub> (Figure 4.2 C, lane 1). Overall, these studies show that MinD<sub>Ng</sub> is active in <em>E. coli</em> and can influence cell division in these cylindrical cells.

To determine whether MinD<sub>Ng</sub> acted in a MinC-dependent manner in <em>E. coli</em>, MinD<sub>Ng</sub> expression studies were carried out in strains deficient in minC or all three min genes. When pSR3 (<em>minD<sub>Ng</sub></em>) was transformed into <em>E. coli</em> PB114 (<em>ΔminCDE<sub>Ec</sub></em>) (Table 2.1), the cells retained their characteristic minicell phenotype (Figure 4.3 B), similar to the negative control pUC18 transformants (Figure 4.3 A), despite the overexpression of MinD<sub>Ng</sub> shown by Western blotting (Figure 4.3 C, lane 2). Furthermore, transformation of pSR3 into the <em>E. coli minC</em> mutant strain DR105 (Table 2.1) did not affect the minicell phenotype of this mutant (Figure 4.3 E), such that the cells were similar to pUC18 transformants (Figure 4.3 D). Western blotting confirmed the overexpression of MinD<sub>Ng</sub> in <em>E. coli</em> DR105 transformants (Figure 4.3 F, lane 2). Hence, these results indicate MinD<sub>Ng</sub> requires resident MinC to affect cell division in <em>E. coli</em> cells.

Round <em>E. coli</em> KJB24 cells (Table 2.1) were also transformed with pSR3 to determine the effects of MinD<sub>Ng</sub> overexpression in a coccal cell background. These cells lack the shape-determinant gene <em>rodA</em> and present a round cell morphology (Begg and Donachie, 1998). In addition, these cells divide along two planes, as <em>N. gonorrhoeae</em> does, to produce a tetrad of daughter cells (Begg and Donachie, 1998); hence, they may present a suitable model to study cell division in coccal Gram-negative organisms. <em>E. coli</em> cells KJB24 cells transformed with pUC18 appeared uniform in size and shape (Figure 4.4 A), while those transformed with pSR3 exhibited gross enlargement (Figure 4.4 B), with cell diameters up to 3-4 times that of control cells. Immunoblotting confirmed the overexpression of MinD<sub>Ng</sub> in the enlarged round cells (Figure 4.4 C, lane 2) compared to the background level of native <em>E. coli</em> MinD in negative control cells (Figure 4.4 C, lane 1).

Collectively, these heterologous expression studies indicate MinD<sub>Ng</sub> is able to induce cell division arrest in <em>E. coli</em> backgrounds, provided that functional <em>E. coli</em> MinC is present. In addition,
Figure 4.3. Expression of MinD<sub>N</sub><sub>g</sub> in *E. coli* PB114 (Δ<em>minCDE</em>) and *E. coli* DR105 (<em>minC</em> mutant). (A) *E. coli* PB114 (Δ<em>minCDE</em>) lacks all <em>min</em> genes and typically displays a minicell phenotype, as observed with pUC18 transformants. (B) *E. coli* PB114 transformed with pSR3 (min<sub>D<sub>N</sub><sub>g</sub></sub>) retain the minicell phenotype. (C) Western blotting confirmed the overexpression of MinD<sub>N</sub><sub>g</sub> in *E. coli* PB114 pSR3 transformants (lane 2) compared to pUC18 transformants (lane 1). (D) *E. coli* DR105, a <em>minC</em> mutant, also exhibits a minicell phenotype, as observed with pUC18 transformants. (E) Transformation of *E. coli* DR105 with pSR3 (min<sub>D<sub>N</sub><sub>g</sub></sub>) did not change its minicell phenotype. (F) Western blotting confirmed the overexpression of MinD<sub>N</sub><sub>g</sub> in *E. coli* DR105 pSR3 transformants (lane 2) compared to pUC18 transformants (lane 1). Scale bar in (A) is 5 μm and all figures are at the same magnification.
Figure 4.4. Overexpression of MinD<sub>N</sub> in round *E. coli* KJB24 (*rodA*) cells. (A) *E. coli* KJB24 transformed with pUC18 control display their characteristic coccoid morphology, with all cells presenting a similar size. (B) Round *E. coli* transformed with pSR3 (*minD<sub>N</sub>*) exhibit gross cell enlargement. The scale bar in (A) is 5 µm and both figures are at the same magnification. (C) Western blot using anti-MinD<sub>N</sub> antisera confirmed the overexpression of MinD<sub>N</sub> in cells transformed with pSR3 (lane 2), compared to pUC18 transformants (lane 1).
the functionality of MinD<sub>Ng</sub> in <i>E. coli</i> backgrounds showed that <i>E. coli</i> can be used as an effective reporter strain for gonococcal MinD function.

**A GFP-MinD<sub>Ng</sub> fusion exhibits MinE<sub>Ng</sub>-dependent intracellular movement in <i>E. coli</i> and localizes within coiled arrays.** Since gonococcal MinD could affect <i>E. coli</i> cell division, a GFP-MinD<sub>Ng</sub> fusion was constructed to determine whether the protein would also oscillate intracellularly in <i>E. coli</i>. Since it is recognized that dynamic movement of <i>E. coli</i> GFP-MinD requires MinE<sub>Ec</sub> (Raskin and de Boer, 1999a; Rowland <i>et al.</i>, 2000), gonococcal minE<sub>Ng</sub> was provided downstream of gfp-minD<sub>Ng</sub> in pSR15 (Table 4.1). These initial localization studies were conducted in collaboration with Dr. W. Margolin, University of Texas Medical School. <i>E. coli</i> WM1032 (Table 2.1) was used in these studies since this strain lacks its own native min genes, thus eliminating any possible interference from endogeneous <i>E. coli</i> Min proteins.

Fluorescence microscopy of live cells revealed dynamic fusion protein behaviour characterized by intracellular shifting of fluorescent signals (Figure 4.5 A). Closer inspection of cells, particularly shorter rods (~3-4 μm in length), revealed GFP-MinD<sub>Ng</sub> moving from pole-to-pole (Figure 4.5 A), similar to reports for <i>E. coli</i> GFP-MinD (Raskin and de Boer, 1999a; Rowland <i>et al.</i>, 2000). Where pole-to-pole oscillation could be readily observed, the average oscillation cycle of GFP-MinD<sub>Ng</sub> (movement from one pole to the other, and back) in 14 cells examined took 70 ± 25 seconds. The average oscillation cycle of GFP-MinD<sub>Ng</sub> in <i>E. coli</i> WM1032 was significantly slower than GFP-MinD<sub>Ec</sub> (encoded on pWM1255; Table 4.1), which exhibited an average oscillatory cycle of 37 ± 12 seconds, measured in 10 cells (<i>p</i> < 0.001). Western blotting confirmed the expression of GFP-MinD<sub>Ng</sub> (Figure 4.5 B) and MinE<sub>Ng</sub> (Figure 4.5 C) in <i>E. coli</i> WM1032 cells containing pSR15. Hence, these preliminary studies showed that GFP-MinD<sub>Ng</sub> exhibits dynamic localization in an <i>E. coli</i> background. Furthermore, these studies indicated that intracellular oscillatory movement is not exclusive to <i>E. coli</i> MinD.
Figure 4.5. GFP-MinD<sub>N<sub>G</sub></sub> displays intracellular movement within rod-shaped *E. coli* WM1032 (ΔminCDE<sub>EC</sub>) cells. (A) Plasmid pSR15, encoding GFP-MinD<sub>N<sub>G</sub></sub> and untagged MinE<sub>N<sub>G</sub></sub> was transformed into *E. coli* WM1032 (ΔminCDE<sub>EC</sub>) and fusion protein localization was followed over time, as indicated in seconds. Arrows indicate pole-to-pole shifting of GFP-MinD<sub>N<sub>G</sub></sub> signal in three different cells. DIC = differential interference contrast image. Bar in first panel represents 5 μm. Western blotting using anti-MinD<sub>N<sub>G</sub></sub> antisera and anti-MinE<sub>N<sub>G</sub></sub> antisera detects GFP-MinD<sub>N<sub>G</sub></sub> (B), and MinE<sub>N<sub>G</sub></sub> (C) in cell extracts of *E. coli* WM1032 transformed with pSR15.
Upon acquiring a microscope system with improved magnification, resolution, and image analysis software, more refined GFP-MinD_Ng localization studies were conducted. *E. coli* strain PB114 (ΔminCDE_Ec) was used for these studies (Table 2.1). Similar to *E. coli* WM1032, wild-type GFP-MinD_Ng expressed from pSR15 (Table 4.1) displayed oscillatory movement in *E. coli* PB114 (Figure 4.6). Since *E. coli* ΔminCDE strains exhibit a minicell phenotype that results in cells of varying lengths, pole-to-pole oscillatory cycles were monitored in rods of similar length (2.0-2.5 μm) to maintain consistency. In raw images, wild-type GFP-MinD_Ng displayed periodic oscillations and moved from the membrane at one cell pole region to the other, forming a tube-shaped fluorescent signal that transiently lined each cell half (Figure 4.6 A, B). The average oscillation cycle (from one pole to the other and back) of GFP-MinD_Ng measured in twenty short rods was 29.1 ± 7.3 seconds.

Studies with *E. coli* GFP-MinD_Ec have shown that its dynamic localization is dependent upon MinE_Ec. To determine whether MinE_Ng is responsible for the oscillatory movement of GFP-MinD_Ng, *E. coli* PB114 was transformed with pJS10 (Table 4.1), encoding GFP-MinD_Ng but no MinE_Ng. In this case, the fusion protein appeared along the entire inner cell periphery, presenting a smooth and unbroken fluorescent signal without any evidence of pole-to-pole movement (Figure 4.6 C). Therefore, GFP-MinD_Ng oscillates intracellularly in a MinE_Ng-dependent manner.

Since MinD_Ng-K16Q could not induce cell division arrest in wild-type *E. coli*, it was fused to GFP as well to determine whether its inactivity was due to an inability to localize to the membrane, as reported with an *E. coli* MinD_K16Q GFP-fusion (Hu et al., 2002). As expected, expression of GFP-MinD_Ng-K16Q from pJDE1 resulted in the fusion protein localizing throughout the cytoplasm in *E. coli*, despite the presence of minE_Ng supplied on the same plasmid (Figure 4.6 D). Thus, due to its cytosolic localization, GFP-MinD_Ng-K16Q can act as a suitable negative control for further in vivo membrane association studies.

Western blotting using affinity purified anti-MinD_Ng antisera confirmed the expression of each GFP-MinD_Ng protein in *E. coli* PB114 transformed with pSR15 (gfp-minD_Ng, minE_Ng) (Figure
Figure 4.6. GFP-MinD$_{N_1}$ localization in *E. coli* PB114 (ΔminCDE$_{E_c}$). (A and B) *E. coli* transformed with pSR15 (gfp-minD$_{N_1}$, minE$_{N_1}$) exhibit periodic GFP-MinD$_{N_1}$ movement from pole-to-pole. Time lapse between (A) and (B) is 18 seconds. Note the ‘U’-shaped fluorescent pattern that transiently lines each cell pole. (C) In the absence of MinE$_{N_1}$, GFP-MinD$_{N_1}$ localizes along the inner cell periphery, presenting a smooth, unbroken signal. (D) GFP-MinD$_{N_1,K16Q}$ does not oscillate and remains distributed throughout the cytosol. (E and F) In longer *E. coli* cells (>5 µm) containing pSR15, a GFP-MinD$_{N_1}$ banding pattern is observed. These bands display dynamic localization, and shift positions rapidly (compare arrows in E and F; 12 seconds between each image). (G) Image enhancement of (E) shows fluorescent bands are composed of GFP-MinD$_{N_1}$ signal accumulating within segments of a coiled array. White arrows indicate regions of high GFP-MinD$_{N_1}$ accumulation. Red arrows show weaker GFP-MinD$_{N_1}$ signal indicating extension of the coiled array throughout the cell. Bar in (A) indicates 1 µm and (A-G) are all at the same magnification. (H) Magnified segment of *E. coli* cell showing GFP-MinD$_{N_1}$ coiled array. Note the different diagonal orientations of the GFP-MinD$_{N_1}$-containing coiled segments (white arrow compared to green arrow). (I) Western blotting using anti-MinD$_{N_1}$ antisera detects expression of GFP-MinD$_{N_1}$ (lanes 1, 4) and GFP-MinD$_{N_1,K16Q}$ (lane 2), in *E. coli* transformed with pSR15 (gfp-minD$_{N_1}$, minE$_{N_1}$), pJDE1 (gfp-minD$_{N_1,K16Q}$, minE$_{N_1}$), and pJS10 (gfp-minD$_{N_1}$ alone), respectively, compared to untransformed *E. coli* cell extract (lane 3). (J) Western blotting using anti-MinE$_{N_1}$ antisera detects expression of MinE$_{N_1}$ in cells transformed with pSR15 (lane 1) and pJDE1 (lane 3), but not in untransformed *E. coli* cell extract (lane 2).
4.6 I, lane 1), pJDE1 (gfp-minD_{Ng,K16Q}, minE_{Ng}) (lane 2), and pJS10 (gfp-minD_{Ng} alone) (lane 4), in comparison to untransformed negative control cells (lane 3). Western blotting also showed the expression of MinE_{Ng} in E. coli PB114 transformed with pSR15 (Figure 4.6 J, lane 1) and pJDE1 (lane 3). No MinE_{Ng} was detected in untransformed E. coli PB114 cell extracts (Figure 4.6 J, lane 2).

Raw fluorescent images also provided evidence that GFP-MinD_{Ng} likely formed coil-like structures that wound around the inner cell periphery of E. coli (Figure 4.6 E, F). These coiled structures were more readily visible in longer cells, since fluorescent signal concentration at the poles of shorter cells tended to obscure details of the helical array. In elongated cells, GFP-MinD_{Ng} signal appeared as regularly spaced bands that shifted position along the cell length over a matter of seconds (Figure 4.6 E, F; arrows indicate shifted fluorescent signals), similar to previous reports with GFP-MinD_{Ec} (Raskin and de Boer, 1999a). This suggested that GFP-MinD_{Ng} subunits move by undergoing dynamic assembly/disassembly along a helical array. Image enhancement revealed that these dynamic bands were actually composed of accumulated GFP-MinD_{Ng} signal within at least two turns of a coil (Figure 4.6 G, white arrows). Weaker coil signals were also seen in areas with less fusion protein accumulation, suggesting the array extended the length of the cell (Figure 4.6 G, red arrows), as similarly reported with GFP-MinD_{Ec} by Shih et al. (2003). In addition, some images showed fluorescent coil strands along different diagonal orientations (Figure 4.6 H, magnified section of filamentous cell transformed with pSR15; compare diagonal coil segment indicated by green arrow with diagonal coil segment indicated by white arrow). This suggested that the GFP-MinD_{Ng} coiled array may consist of at least two coils, possibly arranged in a double-helix conformation (Figure 4.6 H), as noted with GFP-MinD_{Ec} (Shih et al., 2003).

Overall, these results show that wild-type GFP-MinD_{Ng} exhibits MinE_{Ng}-dependent intracellular movement and localization within a coiled array in rod-shaped E. coli. Furthermore, these results indicate dynamic localization along a coiled array is not exclusive to E. coli Min proteins.
**GFP-MinD_{Ng} oscillates in round E. coli cells.** In an effort to elucidate the intracellular localization of GFP-MinD_{Ng} in cocci, *E. coli* KJB24 (*rodA*) (Table 2.1) was selected as a model organism, since it presents a round morphology, and divides in alternating perpendicular planes as *N. gonorrhoeae* does (Begg and Donachie, 1998; Westling-Häggström, 1977).

Upon expressing GFP-MinD_{Ng} from pSR15 (Table 4.1), two cell populations of *E. coli* KJB24 were observed. Approximately 20% were normal-sized cells (~1 μm diameter), while the majority were enlarged cells (> 1 μm). In larger cells, the direction of GFP-MinD_{Ng} movement appeared to be random, with fluorescent signals moving from one point to another without any obvious pattern (Figure 4.7 A). However, in smaller cells, the oscillation of GFP-MinD_{Ng} presented a discernible pattern. In diplococci with apparently completed septa, fusion protein movement was characterized by a back and forth motion in parallel to the septum, with 15 second time lapses from one end of the cell to the other (Figure 4.7 B; arrows indicate fusion protein movement in right cell, arrowheads indicate fusion protein movement in left cell). Hence, GFP-MinD_{Ng} is also able to move within coccal shaped *E. coli* along a direction that is parallel to nascent septa, provided that the cells are not grossly enlarged.

Using an improved microscopy system, round *E. coli* cells containing pSR15 also showed evidence of a GFP-MinD_{Ng}-containing coiled array as well. Due to their small size, these coils were much more difficult to visualize in round *E. coli* than in rod-shaped cells. *E. coli* KJB24 cells are not perfectly spherical and the coils appeared to wind around the inner cell membrane in a direction that was parallel to the longest available cell axis (Figure 4.7 C). The longitudinal axis also seemed to dictate the direction of GFP-MinD_{Ng} oscillation, which moved along the same axis. The coiled array was most visible near the center of round cells, where cell diameter was greatest and where fluorescence would not be too concentrated to visualize the array (Figure 4.7 C, middle panel; red arrows indicate two segments of a possible coiled array). As GFP-MinD_{Ng} moved, fluorescent coil signal would diminish at the cell center, and fluorescence lining one cell half would appear (Figure

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Figure 4.7. GFP-MinD<sub>N<sub>g</sub> localization in round *E. coli* cells. Time-lapse fluorescent images of round *E. coli* KJB24 (*rodA*) cells transformed with pSR15, encoding GFP-MinD<sub>N<sub>g</sub> and untagged MinE<sub>N<sub>g</sub> (A) GFP-MinD<sub>N<sub>g</sub> movement in enlarged cells shows apparently uncoordinated movement (arrows indicate GFP-MinD<sub>N<sub>g</sub> movement in the bottom cell, arrowheads indicate GFP-MinD<sub>N<sub>g</sub> movement in top cell). (B) GFP-MinD<sub>N<sub>g</sub> movement in normal sized cells shows an oscillation pattern parallel to the septum that separates two cells. Arrows indicate GFP-MinD<sub>N<sub>g</sub> movement in rightmost cell, arrowheads indicate GFP-MinD<sub>N<sub>g</sub> movement in leftmost cell. Times are indicated in seconds. Bar in (A, B) represents 1 µm. DIC = differential interference contrast image. (C) GFP-MinD<sub>N<sub>g</sub> oscillates in the same direction as the longest available axis in round *E. coli* cells. White arrows in left and right panels indicate GFP-MinD<sub>N<sub>g</sub> movement along this axis. Center panel shows evidence of GFP-MinD<sub>N<sub>g</sub> localizing within a coiled array (coil segments indicated with red arrows). Bar represents 1 µm.
4.7 C, left and right panels). This suggested that the movement of fluorescent signal was due to GFP-
MinD_{Ne} subunits undergoing dynamic assembly/disassembly within a helical array, similar to GFP-
MinD_{Ne} movement in rod-shaped *E. coli* PB114. Overall, these results show that GFP-MinD_{Ne}
exhibits dynamic localization in round *E. coli* cells and can assemble into an intracellular helical array
cooled in the same direction as the longest cell axis, which corresponds to the axis of GFP-MinD_{Ne}
oscillation.
4.4. DISCUSSION

This study showed that MinD$_{Ng}$ is active across species of differing morphologies and can function with non-gonococcal Min systems. The high identity (73%) between MinD from *N. gonorrhoeae* and *E. coli* likely permitted the functionality of the protein in *E. coli* backgrounds.

The overexpression of MinD$_{Ng}$ in wild-type *E. coli* PB103 led to a filamentous phenotype, indicative of cell division arrest at all potential division sites along the length of the rod, similar to the effects of overexpressing *E. coli* MinD (de Boer *et al.*, 1989). This ability to arrest *E. coli* cell division indicates MinD$_{Ng}$ is able to recruit the resident *E. coli* MinC cell division inhibitor to inner cell membrane targets. This is consistent with the ability of both MinD$_{Ng}$ and MinC$_{Ng}$ to inhibit gonococcal cell division when overexpressed together (Chapter 3).

In the absence of MinC$_{Ec}$, the overexpression of MinD$_{Ng}$ did not alter the phenotype of *E. coli* strains DR105 (minC$_{Ec}$ mutant) and PB114 (ΔminCDE$_{Ec}$), further evidence that MinD$_{Ng}$ requires MinC protein, either from *N. gonorrhoeae* (Chapter 3) or from *E. coli*, to influence cell division. While unable to overexpress MinD$_{Ng}$ by itself from a shuttle vector in a *N. gonorrhoeae* background (Chapter 3), the overexpression of plasmid-encoded MinD$_{Ng}$ in a round *E. coli* rodA mutant provides evidence that increased levels of MinD$_{Ng}$ alone can induce cell division arrest in coccal backgrounds and lead to gross cell enlargement.

Expression of gonococcal MinD in *E. coli* PB104 (minD$_{Ec}$ mutant) could also partially correct the minicell phenotype of this strain. Since interactions between MinC$_{Ec}$, MinD$_{Ec}$, and MinE$_{Ec}$ are required for proper *E. coli* cell division site selection (Huang *et al.*, 1996; Raskin and de Boer, 1999a; Hu and Lutkenhaus, 1999; Hu and Lutkenhaus, 2000; Hale *et al.*, 2001; Lackner *et al.*, 2003; Hu *et al.*, 2003), MinD$_{Ng}$ likely interacted with MinE$_{Ec}$ in *E. coli* as well. In support of this, our laboratory has demonstrated the ability of *E. coli* MinE to induce GFP-MinD$_{Ng}$ oscillation in *E. coli* (Ramirez-Arcos *et al.*, 2002). Excessive production of gonococcal MinD may have prevented the full complementation of *E. coli* PB104. An increased MinD/MinE ratio is proposed to extend the oscillation cycle of MinD in *E. coli*, which would permit FtsZ ring formation at cell pole regions,
leading to some to minicell formation (Raskin and de Boer, 1999a), which was observed in the partially complemented *E. coli* PB104 cells of the present study.

Further evidence of MinD<sub>Ng</sub> cross species functionality was provided by dynamic localization patterns of GFP-MinD<sub>Ng</sub> expressed in *E. coli* backgrounds. These studies demonstrate, for the first time, that intracellular movement is not exclusive to *E. coli* MinD. In addition, these results indicate that MinD<sub>Ng</sub> can oscillate in *E. coli* independently of its native gonococcal environment. The fusion protein was able to move, in a MinE<sub>Ng</sub>-dependent manner, from the membrane at one cell pole to the other in rod-shaped *E. coli*, similar to previous reports with GFP-MinD<sub>Ec</sub> (Raskin and de Boer, 1999a; Rowland et al., 2000). In *E. coli*, MinE<sub>Ec</sub> is proposed to induce the ATPase activity of MinD<sub>Ec</sub>, which stimulates dynamic MinD<sub>Ec</sub> movement *in vivo* (Hu et al., 2002; Lackner et al., 2003). Hence, it is highly likely that MinE<sub>Ng</sub> can interact with MinD<sub>Ng</sub> to stimulate the enzymatic activity, and subsequent movement, of the latter as well. In accordance with this, when expressed without any MinE<sub>Ng</sub>, GFP-MinD<sub>Ng</sub> was localized all along the inner cell periphery of *E. coli* and did not show any evidence of movement. Similar peripheral localization has been observed with GFP-MinD<sub>Ec</sub> when MinE<sub>Ec</sub> is not present (Raskin and de Boer, 1999a, Shih et al., 2003).

In contrast to wild-type GFP-MinD<sub>Ng</sub>, GFP-MinD<sub>Ng,K16Q</sub> did not localize to the membrane, but remained distributed in the cytosol. A similar observation has been made with *E. coli* MinD<sub>K16Q</sub> fused to GFP, and it is proposed that such mutants may not respond to the effects of ATP, despite retaining the ability to bind this nucleotide (Hu et al., 2002). The cytosolic distribution of GFP-MinD<sub>Ng,K16Q</sub> supports the aberrant phenotype of the *N. gonorrhoeae minD<sub>Ng,K16Q</sub>* encoding strain, SCD-1 (Chapter 3). By remaining in the cytoplasm, MinD<sub>Ng,K16Q</sub> would not be able to recruit MinC<sub>Ng</sub> to the inner membrane in order to regulate proper cell division in *N. gonorrhoeae* SCD-1.

The present study also shows the ability to assemble into higher ordered structures is not restricted to *E. coli* MinD. Moreover, GFP-MinD<sub>Ng</sub> required MinE<sub>Ng</sub> to localize within a helical array in *E. coli* backgrounds, suggesting the two proteins participated in the formation of this coiled structure. This is in support of observations by Shih et al. (2003), who also demonstrated a MinE<sub>Ec</sub>-
dependent ability of GFP-MinD<sub>Ec</sub> to localize as a coiled array. Interestingly, it has recently been demonstrated that MinE<sub>Ec</sub> can promote the bundling of MinD<sub>Ec</sub> filaments, in addition to inducing filament disassembly; therefore, these two apparently opposing roles may explain the requirement for MinE in both MinD coil assembly and MinD oscillation (Suefuji et al., 2002).

GFP-MinD<sub>Ng</sub> movement was also observed in round *E. coli* cells. Interestingly, these cells display an alternating division plane pattern similar to *N. gonorrhoeae* (Begg and Donachie, 1998). In stark contrast to its oscillation in rod-shaped *E. coli*, GFP-MinD<sub>Ng</sub> moved parallel to the nascent septum between two cells, consistent with restricting a subsequent division event to a plane that is perpendicular to the first. A similar localization pattern has been observed in round *E. coli* cells expressing GFP-MinD<sub>Ec</sub> (Corbin et al., 2002).

Bacterial geometry may play a role in determining the direction of Min protein oscillation (Corbin et al., 2002). While coccal in shape, *E. coli* rodA cells are not necessarily perfectly round, and a GFP-MinD<sub>Ec</sub> fusion has been shown to almost invariably oscillate parallel to the slight long axis that is characteristic of these cells (Corbin et al., 2002). After one cell division event, a diplococcus is formed and the long axis of each newly formed round *E. coli* is parallel to the nascent septum. GFP-MinD<sub>Ec</sub> oscillates along this new plane (Corbin et al., 2002), similar to GFP-MinD<sub>Ng</sub> observed in this study.

This distinct movement of MinD proteins in round *E. coli* cells suggested that a Min protein coiled array should exist in cocci as well, and would be arranged parallel to the longest available axis. Indeed, this was supported with evidence of GFP-MinD<sub>Ng</sub> decorated coils wound along the longest axis of round *E. coli* cells. This is the first observation of such helical structures in round bacteria. Hence, the ability of MinD<sub>Ng</sub> to localize within helical arrays in both rod-shaped and round bacteria suggests that this may represent the standard assembled conformation of the protein *in vivo*. Furthermore, it appears that the direction Min protein array coiling specifies the direction of GFP-MinD<sub>Ng</sub> movement. Whether the ability to localize as an extended intracellular coil is limited to MinD proteins from Gram-negative organisms is currently unknown. In the Gram-positive rod *B. subtilis*,

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GFP-MinD_{Es} localizes distinctly at the cell poles (Marston et al., 1998); however, the conformation of any higher ordered structures at the poles, if they exist, is unclear. Furthermore, it would be interesting to determine whether MinD_{Es} can be induced to oscillate by MinE in a Gram-negative background.

Although difficult to observe GFP-MinD_{Ng} helical arrays in round cells, many enlarged coccal *E. coli* cells did display curiously uncoordinated movement of GFP-MinD_{Ng}, similar to a report with GFP-MinD_{Es} (Corbin et al., 2002). Cells likely became enlarged due to functional GFP-MinD_{Ng} acting with resident MinC_{Es} to inhibit cell division. It has been proposed that such enlarged cells are more ‘round’ and may not have obvious long axes, resulting in apparently disordered MinD protein movement (Corbin et al., 2002). It is also possible that cell enlargement, combined with a dosage effect due to endogenous MinD_{Es}, might disrupt the proper coiling specification of the Min protein array upon overexpression of GFP-MinD_{Ng} in round *E. coli*. This could conceivably lead to the apparent uncoordinated movement of GFP-MinD_{Ng} within enlarged cocci. Such disruption of intracellular cytoskeletal structures upon overexpression of their subunits has been observed with FtsZ protein. FtsZ-GFP normally localizes as a ring at the *E. coli* midcell (Ma et al., 1996); however, severe overexpression of FtsZ-GFP fusion protein can lead to the formation of tubules that localize improperly and extend throughout the length of the cell (Ma et al., 1996; Salimnia et al., 2000).

The average oscillation cycle of GFP-MinD_{Ng} was slower than that of GFP-MinD_{Es} in *E. coli* WM1032 cells. This may be due to differences in the membrane/phospholipid targets that each MinD protein normally associates with. Although MinD_{Ng} and MinD_{Es} share nearly identical membrane targeting sequences (MTSs), it is possible that the single amino acid difference between each MTS (S263 in MinD_{Ng} and G262 in MinD_{Es}) represents species specific adaptations that optimize in vivo interactions with phospholipids in their respective inner membranes.

The orientation of the MinD MTS is predicted to be parallel to the membrane surface (Mileykovskaya et al., 2003). This would permit the hydrophobic residues on one face of the amphipathic helix to be buried within lipid acyl chains, and allow the charged residues on the opposite face to interact with phospholipids headgroups, preferably those with anionic charge (Szeto
et al., 2002; Szeto et al., 2003; Mileykovskaya et al., 2003). In comparison to the helicity of the *E. coli* MinD MTS (Szeto et al., 2002), the S263 residue of the MinD₉₆ MTS is predicted to lie on the polar face of the helix. Hence, it is possible that this residue plays a role in modulating protein-membrane association. Similarly, apparent *in vitro* differences in membrane affinity between the MTSs of *E. coli* and *B. subtilis* may be due to inherent amino acid differences (Szeto et al., 2003).

The main constituents of the *E. coli* cell membrane are phosphatidylethanolamine (PE; ~75%), phosphatidylglycerol (PG; ~20%) and cardiolipin (CL; ~5%) (Raetz, 1978). Several groups have also characterized the phospholipids in *N. gonorrhoeae* membranes and have shown that the major classes include PE (65-77%) and PG (14-24%); however, there are conflicting results for the proportion of CL, with reports ranging from trace levels up to ~20% of total phospholipids (Senff et al., 1976; Sud and Feingold, 1975; Rahman et al., 2000). Since it is likely that MinD₉₆ and MinDₑₑ would each be adapted to optimally bind membranes from their own respective organisms, it is possible that the difference in the oscillation frequency of each protein in *E. coli* is a reflection of this evolution. Differences in oscillation periods could also account for the inability of MinD₉₆ to fully complement the *E. coli minD* mutant.

In addition, it is currently unknown whether MinD helical arrays associate with another, as yet unidentified, cytoskeletal scaffold (Shih et al., 2003). Should this be the case, then differences in interaction with this latter scaffold may also account for the differences in the oscillation period of heterologously expressed MinD₉₆ in *E. coli*.

It is noted that the average oscillation cycle of GFP-MinD₉₆ was significantly faster in *E. coli* PB114 cells than in *E. coli* WM1032 cells. It is possible that interstrain differences in inner membrane phospholipid composition may be responsible for this observation. GFP-MinD₉₆ localization studies in *E. coli* WM1032 were also carried in a collaborating laboratory using a less advanced microscope system, which did not provide the accurate, automated time-lapse capabilities of the microscope system used to study GFP-MinD₉₆ movement in *E. coli* PB114. Furthermore, strict
cell length limitations were used in GFP-MinD_{Ng} localization studies in *E. coli* PB114, which may have also contributed to the difference in fusion protein oscillation cycles in *E. coli* WM1032.

Due to their small size (less than or equal to 1 μm) and fastidious nature, it would be difficult to localize Min proteins in live gonococci; hence, round *E. coli* rodA cells were used to gain insight on how MinD_{Ng} might behave in a coccus. However, from the studies in various *E. coli* backgrounds, it is highly likely that MinD_{Ng}, as well as the other gonococcal Min proteins, undergo dynamic localization and assembly into higher ordered structures in their native organism, *N. gonorrhoeae*. Indirect evidence of dynamic behaviour is provided by the detection of MinD_{Ng} in both soluble and insoluble fractions of gonococcal cell extracts, suggesting an exchange of protein between the cytosol and membrane (Chapter 3). Furthermore, there is evidence from immunogold localization that MinD_{Ng} is found in the cytoplasm and along the inner cell membrane (Chapter 3). In addition, electron micrographs show that gonococci exhibit growth along one dimension before dividing, and can therefore possess a slight long axis, which is parallel to the nascent septum in diplococci (Westling-Häggström et al., 1977). Hence, it is likely that a Min protein array forms along this axis in *N. gonorrhoeae* as well, similar to the observation with GFP-MinD_{Ng} in round *E. coli* cells used in this study. Future considerations should be directed towards localizing MinD_{Ng} in live *N. gonorrhoeae* to ultimately verify dynamic MinD_{Ng} protein localization in this organism.

Overall, these studies show that MinD_{Ng} is fully active in *E. coli* backgrounds. The gonococcal protein is able to induce cell division arrest in the presence of MinC_{Ec} and can complement an *E. coli* minD mutant. Furthermore, GFP-MinD_{Ng} can oscillate intracellularly and localize within helical arrays *independent* of its native gonococcal environment. These studies also indicate that dynamic behaviour and coiled array formation is not exclusive to *E. coli* MinD. Therefore, it is demonstrated that *E. coli* can act as a suitable indicator strain for gonococcal MinD function.
CHAPTER 5

Identification of MinD<sub>Nr</sub> protein-protein interactions

Portions of this chapter were published in:


and

5.1. PURPOSE OF THIS STUDY

In *E. coli*, the three Min proteins are dependent upon each other to properly regulate cell division site placement (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a; Rowland *et al.*, 2000; Fu *et al.*, 2001; Hu *et al.*, 2003; Lackner *et al.*, 2003). MinD_{ec} recruits MinC_{ec} to the membrane where it can act to inhibit cell division (Hu and Lutkenhaus, 1999; Hu *et al.*, 2003). MinE_{ec} acts with MinD_{ec} to localize within intracellular helical arrays and can stimulate the ATPase activity of MinD_{ec} to induce its dynamic protein movement (Hu and Lutkenhaus, 2001; Shih *et al.*, 2003). Having demonstrated that gonococcal MinD requires the presence of MinC to induce cell division arrest (Chapters 3 and 4) and can localize dynamically, in a MinE-dependent manner, within coiled arrays in *E. coli* (Chapter 4), yeast two-hybrid assays were used to determine what protein interactions MinD_{Ng} may participate in.

Using this system, I was the first to demonstrate that MinD proteins can self-associate. MinD_{Ng}, as well as MinD_{ec}, displayed self-interaction. Purified MinD_{Ng} was also shown to exist as a dimer using size-exclusion chromatography and sedimentation equilibrium analysis. In addition, an interaction between MinD_{Ng} and MinE_{Ng} was detected using the yeast two-hybrid system. Although yeast two-hybrid assays did not detect MinD_{Ng}-MinC_{Ng} binding, likely due to MinC_{Ng} instability in the yeast reporter strain, a strong interaction between MinD_{Ng} and *E. coli* MinC was observed, in support of MinD_{Ng} functionality in *E. coli* backgrounds. The ability of each gonococcal Min protein to interact with other cell division proteins, including FtsZ, FtsA, PenA (FtsI), and FtsY, was also investigated.

These studies indicate that interactions do exist between MinD_{Ng} and other Min proteins. The self-interaction of MinD proteins from cocci and rods was the first evidence that this property of MinD may be important for controlling cell division site selection across species, even those with morphological differences.
5.2. MATERIALS AND METHODS

Cloning min genes into yeast two-hybrid vectors. The yeast two-hybrid system was used to determine interactions between the gonococcal Min proteins. The genes minC<sub>Nc</sub>, minD<sub>Nc</sub>, and minE<sub>Nc</sub> were individually PCR amplified from gonococcal chromosomal DNA using the primer pairs min12/min29, minD1/minD2, and minE1/minE2, respectively (Table 2.2). Each amplicon was digested at their ends with EcoRI and BamHI and ligated in frame to both the GAL4-activation domain (AD) of pGAD424 and the GAL4 DNA-binding domain (BD) of pGBT9 (Clontech; Table 5.1). The plasmids obtained were pGADminC (GAL4-AD-MinC<sub>Nc</sub>), pGBT9minC (GAL4-DNA-BD-MinC<sub>Nc</sub>), pGADminD (GAL4-AD-MinD<sub>Nc</sub>), pGBT9minD (GAL4-DNA-BD-MinD<sub>Nc</sub>), pGADminE (GAL4-AD-MinE<sub>Nc</sub>), and pGBT9minE (GAL4-DNA-BD-MinE<sub>Nc</sub>) (Table 5.1). E. coli minC and minD were also amplified using the primer pairs EcMinC-up/EcMinC-down and EcMinD-up/EcMinD-down (Table 2.2) and were ligated into the EcoRI/BamHI sites of pGAD424 and pGBT9 as above, generating plasmids pSRAD-C (GAL4-AD-MinC<sub>Ec</sub>), pSRBD-C (GAL4-DNA-BD-MinC<sub>Ec</sub>), pSRAD-D (GAL4-AD-MinD<sub>Ec</sub>) and pSRBD-D (GAL4-DNA-BD-MinD<sub>Ec</sub>) (Table 5.1).

Cloning ftsZ, ftsA, ftsY, and penA into yeast two-hybrid vectors. Similar to each min<sub>Nc</sub> gene, gonococcal ftsZ, ftsA, penA (ftsI), and ftsY were PCR amplified from gonococcal chromosomal DNA using primer pairs Z1/Z2, A1/A2, penA1/penA2, and Y1/Y2, respectively (Table 2.2). Each gene was cloned into pGBT9 and pGAD424 as above. Plasmids encoding FtsZ, FtsA, PenA(FtsI), and FtsY fused to the GAL4-DNA-AD were: pGADftsZ, pGADftsA, pGADpenA, and pGADftsY (Table 5.1). Plasmids encoding FtsZ, FtsA, PenA(FtsI), and FtsY fused to the GAL4-AD were: pGBT9ftsZ, pGBT9ftsA, pGBT9penA, and pGBT9ftsY (Table 5.1).

Yeast-two hybrid assays. Yeast two-hybrid plasmids were transformed into yeast as outlined in Chapter 2. None of the GAL4-fusions could activate the reporter gene when expressed by themselves,
Table 5.1. Plasmids used in this study

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**Plasmids for protein purification**

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<td>This study</td>
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</table>

$^1$ GAL4-activation domain
$^2$ GAL4-DNA binding domain
with the exception of GAL4-BD-FtsZ (encoded on pGBT9ftsZ; Table 5.1). β-galactosidase activities were assessed by colony-lift and liquid assays using X-gal and ONPG substrates, as outlined in Chapter 2.

**Purification of His-MinD₉₉.** N-terminal His-tagged MinD₉₉ (His-MinD₉₉) was purified from *E. coli* BL21 (DE3) transformed with pJSHD2 (Tables 2.1) as detailed in Chapter 2. The His-minD₉₉ gene was also excised from pJSHD2 using *Xba*I and *Sal*I and cloned into similarly digest pUC18 (Table 2.1) to produce plasmid pMJ1 (Table 5.1) for use in *E. coli* morphology studies.

**Gel-filtration chromatography of His-MinD₉₉.** His-MinD₉₉ protein was dialyzed overnight at 4°C against PBS (pH 7.4) with 0.2% Tween-20. The protein was applied to a 25 ml Sephadex 200 column (Amersham Pharmacia Biotech) equilibrated with PBS (pH 7.4) and a flow rate of 0.5 ml/min for size-exclusion chromatography. Protein from the column was monitored by UV absorbance at 280 nm and 0.5 ml fractions were collected and analyzed by SDS-PAGE. The elution pattern of His-MinD₉₉ was compared to that of standard proteins provided in the Bio-Rad Gel-Filtration Standard.

**Sedimentation equilibrium analysis.** Sedimentation equilibrium analyses on purified His-MinD₉₉ was done in collaboration with Dr. Cyril Kay and Leslie Hicks, University of Alberta. Prior to analytical ultracentrifugation analysis, purified His-MinD₉₉ was dialyzed overnight at 4°C against Buffer A (50 mM Tris, 20 mM NaCl, 3 mM β-mercaptoethanol, 1 mM EDTA; pH 7.5). Sedimentation equilibrium experiments were carried out at 20°C in a Beckman XLI analytical ultracentrifuge using absorbance optics following the procedures previously described (Laue and Stafford, 1999). 110 μl aliquots of sample solution were loaded into 6-sector CFE sample cells, allowing 3 concentrations of sample to be run simultaneously. Runs were performed at a minimum of
2 different speeds and each speed was maintained until there was no significant difference in \( r^2/2 \) versus absorbance scans taken 2 hours apart to ensure that equilibrium was achieved.

The sedimentation equilibrium data was evaluated using the NONLIN program, which incorporates a nonlinear least-squares curve-fitting algorithm (Johnson et al., 1981). This program allows the analysis of both single and multiple data files. Data can be fit to either a single ideal species model or models containing up to four associating species, depending on which parameters are permitted to vary during the fitting routine. The partial specific volume of the protein and the buffer solution density were estimated using the SEDNTERP program, which incorporates calculations detailed previously (Laue et al., 1991).

**Purification of soluble MinC_{Ng}** To obtain soluble MinC_{Ng}, gonococcal minC was PCR amplified using primers min15 and HLC1 (Table 2.2), which incorporated BamHI and HindIII sites, respectively. BamHI/HindIII-digested amplicon was digested and ligated into similarly treated pET30a to give plasmid pHLCN, encoding His-MinC_{Ng} (Table 5.1). This plasmid was transformed into the expression strain *E. coli* C43 (Table 2.1). A one litre log-phase culture of these cells was induced with 1 mM IPTG for 4 hours at 37°C and shaking at 250 rpm. Cells were harvested by centrifugation (5,000 X g for 10 minutes) (Sorvall RC 5C) and soluble protein purification using 3 ml His-Bind resin (Novagen) was carried out as described by the manufacturer using the following solutions (with the indicated changes in imidazole concentration): 30 ml of binding buffer (5mM imidazole), 18 ml wash buffer 1 (20 mM imidazole), 18 ml wash buffer 2 (30 mM imidazole), 18 ml wash buffer 3 (60 mM imidazole), and 18 ml elution buffer (250 mM imidazole). Construction of pHLCN and purification of His-MinC_{Ng} was carried out by Hui Li, University of Ottawa.

**Dot blot assay to detect His-MinC_{Ng} and His-MinD_{Ng} interaction.** A dot blot assay was used to determine whether purified His-MinC_{Ng} and His-MinD_{Ng} could interact together. Immobilin-P membrane (Millipore) was washed in methanol for 15 seconds, in ddH_{2}O for 2 minutes, and in TBS
buffer (50 mM Tris, 1.25 M NaCl, pH 7.5) for 5 minutes. The membrane was then placed over 3 MM filter paper (Whatman) soaked in TBS. Membrane and filter were assembled into a Bio-Dot vacuum dot blot apparatus (Bio-Rad). Low vacuum pressure was applied to the assembled apparatus to draw out excess buffer.

Two-fold serial dilutions of His-MinD₇g were made in TBS buffer, such that 1.2 µg, 0.6 µg, 0.3 µg, 0.15 µg and 0.075 µg of protein in 20 µl volumes could be loaded into the dot blot chambers under light vacuum pressure. 10 µl of TBS was added to each well and vacuum was applied again. The membrane was then removed and blocked with 1% skim milk overnight at 4°C. Purified His-MinC₇g (final concentration of 1.5 µg protein/ml) diluted in TTBS (TTBS = TBS with 0.05% Tween) was subsequently used as a probe to detect His-MinD₇g on the dot blot membrane. After 2 hours exposure at room temperature to His-MinC₇g, the membrane was washed 3 times in TTBS (10 minutes per wash) and probed with anti-MinC₇g sera (1:170 in TTBS) for 1.5 hours. Following three additional TTBS washes, the membrane was probed with goat anti-rabbit sera (Bio-Rad, 1:3000 in TTBS) for 1 hour, washed again, and developed using Attphos substrate (JBL Scientific INC.). As a negative control, serial dilutions of lysozyme protein (Sigma) were also prepared similarly, transferred onto Immobilon-P membrane, and probed with His-MinC₇g as above.
5.3. RESULTS

Interactions between Min proteins detected by yeast two-hybrid assays. To determine what interactions might exist between the gonococcal Min proteins, fusions of MinC<sub>Ng</sub>, MinD<sub>Ng</sub>, and MinE<sub>Ng</sub> were made to GAL4-DNA-binding (BD) and GAL4-activation (AD) domains for yeast two-hybrid protein-protein interaction assays.

Using this system, a novel gonococcal MinD self-interaction was detected (Table 5.2). Yeast colonies transformed with pGBT9minD and pGADminD (Table 5.1), encoding fusions of MinD<sub>Ng</sub> to GAL4-BD and GAL4-AD, respectively, appeared blue in colour. This was the first report of MinD self-association. Yeast transformed singly with either one of the plasmids appeared white, indicating that the fusion proteins themselves were incapable of activating the yeast lacZ reporter gene. Liquid β-galactosidase assays performed on the yeast transformants also verified the MinD<sub>Ng</sub>-MinD<sub>Ng</sub> interaction, giving values of 12.3 ± 7.3 Miller units relative to the 27.2 ± 6.0 Miller units recorded for the positive control interaction of SV40 large T-antigen and p53 (Table 5.2). For the first time, the self-association of <i>E. coli</i> MinD was also detected, although the strength of interaction was not as strong as that observed for gonococcal MinD, based on β-galactosidase activity (Table 5.2). Interestingly, MinD<sub>Ng</sub> and MinD<sub>Ec</sub> could interact with each other in either orientation tested (Table 5.2). The self-association of MinE<sub>Ng</sub> or of MinC<sub>Ng</sub> was not observed (Table 5.2).

MinD<sub>Ng</sub> was also able to interact with MinE<sub>Ng</sub> and produced a β-galactosidase activity of 2.8 ± 0.4 Miller units (Table 5.2). This interaction was only detected when MinE<sub>Ng</sub> was fused to the GAL4-DNA-BD (Table 5.2). An interaction between MinE<sub>Ng</sub> and MinC<sub>Ng</sub> was not detected in this assay (Table 5.2).

Yeast two-hybrid assays have revealed strong interactions between MinC and MinD from <i>E. coli</i> (Huang <i>et al.</i>, 1996) and from <i>B. subtilis</i> (Marston and Errington, 1999b). However, interaction between gonocoecal MinC and MinD was not detected using the yeast two-hybrid system in this
<table>
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<tr>
<th>Fusion to GAL4-AD&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Fusion to GAL4-DNA-BD&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Intensity of blue colony colour&lt;sup&gt;3&lt;/sup&gt;</th>
<th>β-galactosidase Activity (Miller Units)&lt;sup&gt;4&lt;/sup&gt;</th>
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<sup>1</sup> GAL4-activation domain  
<sup>2</sup> GAL4-DNA binding domain  
<sup>3</sup> Yeast colony colour indicating strength of interaction: (++) = dark blue; (+) = blue; (+) = faint blue; (-) = white colonies  
<sup>4</sup> One unit equals the amount of β-galactosidase which hydrolyzes 1 μmol of ONPG per minute per cell  
<sup>5</sup> *N. gonorrhoeae* MinD  
<sup>6</sup> *E. coli* MinD  
<sup>7</sup> ND<sup>7</sup> indicates β-galactosidase activity Not Determined  
<sup>8</sup> *E. coli* MinC
study (Table 5.2). It is noted that gonococci become enlarged when MinC<sub>Ng</sub> and MinD<sub>Ng</sub> are overexpressed simultaneously (Chapter 3), suggesting an interaction between the two proteins. From heterologous expression studies of MinD<sub>Ng</sub> in *E. coli* backgrounds, it is clear that gonococcal MinD can act with *E. coli* MinC to inhibit cell division (Chapter 4). MinC<sub>E</sub> has 36% identity to *N. gonorrhoeae* MinC. Therefore, the ability of MinC<sub>E</sub> to interact with MinD<sub>Ng</sub> was assessed. Using the yeast two-hybrid system, MinD<sub>Ng</sub> and MinC<sub>E</sub> interaction was observed in both orientations tested, with the fusion of GAL4-AD-MinD<sub>Ng</sub> interacting particularly well with the GAL4-BD-MinC<sub>E</sub> fusion to produce a β-galactosidase activity of 131.5 ± 84.6 units (Table 5.2). In addition, the strong interaction between *E. coli* MinC and *E. coli* MinD (Huang et al., 1996) was also confirmed (Table 5.2).

Overall, these studies demonstrate that interactions between the gonococcal Min proteins exist. Significantly, the detected self-associations of MinD<sub>Ng</sub> and of MinD<sub>E</sub> were novel findings. The self-association of MinD from species with differing morphologies suggests that this characteristic is important for MinD function in general. Furthermore, MinD<sub>Ng</sub> interacts with MinE and MinC, in support of previous yeast two-hybrid studies with *E. coli* Min proteins (Huang et al., 1996).

**Testing gonococcal Min protein interactions with cell division proteins.** Each gonococcal Min protein was also assayed for interaction with other *N. gonorrhoeae* cell division and cell growth proteins, including FtsZ, FtsA, and PenA (FtsI) from the *dcw* cluster (Francis et al., 2000; Salimnia et al., 2000) (see Introduction for a review of these proteins). FtsZ and FtsA were selected since they are recruited early in the cell division process, when Min proteins appear to act (Hu et al., 1999; Errington et al., 2003; Johnson et al., 2002). FtsI acts at a later stage in cell division to help form the cell wall between daughter cells (Bramhill, 1997). FtsY, an inner membrane protein, was also included since it was originally classified as a cell division protein (Gill and Salmond, 1987). This protein has since been characterized as a receptor for the signal recognition particle (SRP) involved in
targeting integral membrane proteins to the inner membrane (Luirink et al., 1994). In *N. gonorrhoeae*,
the FtsY homologue is also known as PilA (Arvidson et al., 1999).

Using the yeast two-hybrid system, no interactions between any of the Min proteins and FtsZ,
FtsA, FtsI or FtsY were detected (Table 5.3). In addition, no interactions between FtsZ, FtsA, PenA,
and FtsY were detected. When fused to the GAL4-BD, gonococcal FtsZ was able to activate the yeast
lacZ reporter gene by itself, similar to a report of *E. coli* FtsZ (Huang et al., 1996); therefore, only
FtsZ-GAL4-AD was used in these experiments (Table 5.3).

**His-MinD<sub>Nε</sub> exhibits self-association.** To confirm that MinD<sub>Nε</sub> can self-associate, N-terminal Histagged MinD<sub>Nε</sub> (His-MinD<sub>Nε</sub>) was purified for gel-filtration and sedimentation equilibrium analyses.
*E. coli* BL21(DE3) expressing the fusion protein were filamentous, indicating cell division arrest
(Figure 5.1 B). This was in contrast to the short rod morphology of *E. coli* BL21 (DE3) cells
transformed with the control pET30a vector (Figure 5.1 A). SDS-PAGE analysis of IPTG induced
cell extracts from pET30a (Figure 5.1C, lane 2) and pJSHD2 (lane 3) transformants show high
overexpression of His-MinD<sub>Nε</sub> in the latter cells (lane 3, arrow).

The functionality of the fusion protein was further verified by expressing His-MinD<sub>Nε</sub> from
the pUC18-derived plasmid pMJ1 (Table 5.1) in *E. coli minC* and *minD* mutants, specifically strains
DR105 and PB104, respectively (Table 2.1). *E. coli* DR105 expressing His-MinD<sub>Nε</sub> from pMJ1
retained its minicell phenotype (compare Figures 5.1 D, E), while *E. coli* PB104 became filamentous
(compare Figures 5.1 G with F), indicative of cell division arrest. Overexpression of the fusion
protein in both strains was verified by Western blotting [Figure 5.1 H; lane 1, *E. coli* DR105 (pMJ1);
lane 2, *E. coli* PB104 (pMJ1)]. Together these observations show that His-MinD<sub>Nε</sub> is active and
requires the presence of functional MinC, found in strain PB104, but not in DR105, to inhibit cell
division.

His-MinD<sub>Nε</sub> was purified from the *E. coli* BL21 (DE3) expression strain (Figure 5.2) and
subsequently applied to a 25 mL Superdex 200 size exclusion column (Figure 5.3 A, B). At the time

138
Table 5.3. Yeast two-hybrid assays to assess gonococcal Min protein interactions with selected cell division and cell growth proteins

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<sup>1</sup> A fusion of the GAL4-DNA binding domain to FtsZ<sub>Ng</sub> could activate the β-galactosidase reporter gene in yeast; therefore, GAL4-DNA-BD-FtsZ<sub>Ng</sub> fusion was not tested for interaction with other proteins.
Figure 5.1. Functionality of N-terminal His-tagged MinD<sub>N</sub> (His-MinD<sub>N</sub>). (A) *E. coli* BL21 (DE3) cells transformed with pET30a control vector display short rod morphology after induction with 1 mM IPTG, while (B) induced cells transformed with pJSHD2, encoding His-MinD<sub>N</sub>, have a filamentous phenotype. (C) Induced cell extract from *E. coli* BL21 pET30a transformants was resolved by SDS-PAGE (lane 2) and compared to induced pJSHD2 transformant cell extracts (lane 3; arrow indicates overexpressed His-MinD<sub>N</sub>). Lane 1 contains broad range protein marker. (D) *E. coli* DR105 (minC mutant) transformed with pUC18 display a typical minicell phenotype, which is also retained upon transformation with pMJ1 (E), a pUC18 derived plasmid encoding His-MinD<sub>N</sub>. (F) *E. coli* PB104 (minD mutant) transformed with pUC18 also exhibits a minicell phenotype; however, cells transformed with pMJ1 (G) become very filamentous. Scale bar in (A) is 5 µm and all figures are at the same magnification. (H) Western blotting using affinity-purified anti-MinD<sub>N</sub> antisera detects His-MinD<sub>N</sub> expression in *E. coli* DR105 (pMJ1) and *E. coli* PB104 (pMJ1).
Figure 5.2. Purification of His-MinD$_{\text{Ng}}$ from *E. coli* BL21 (DE3). Nickel affinity chromatography was used to purify His-MinD$_{\text{Ng}}$ from induced *E. coli* BL21 (DE3) transformed with pJSHD2. Lane 1, induced whole cell extract; lane 2, 5 mM imidazole buffer wash; lane 3, 20 mM imidazole buffer wash; lane 4, 30 mM imidazole buffer wash; lane 5, 60 mM imidazole buffer wash; lane 6, 250 mM imidazole elution.
Figure 5.3. **Size exclusion chromatography of purified His-MinD$_{Ng}$**. Purified protein was applied to a Superdex 200 column equilibrated with PBS (pH 7.4) at a flow rate of 0.5 ml/min. (A) Eluted fractions were analyzed by SDS-PAGE. (B) Elution of His-MinD$_{Ng}$ relative to protein standards (1) thyroglobulin (∼669 kDa), (2) goat anti-human IgG (∼150 kDa), (3) bovine prothrombin (72 kDa), and (4) carbonic anhydrase (31 kDa). His-MinD$_{Ng}$ eluted between 11 and 12.5 ml (horizontal line), with the majority eluting at ∼12 ml (red circle).
of these particular studies, the implications of ATP for MinD self-association were not considered; hence, experiments with His-MinD_{N_{6}} in this chapter were conducted in the absence of ATP. In comparison to the elution of control proteins thyroglobulin (~669 kDa), goat IgG (~150 kDa), bovine prothrombin (~72 kDa), and carbonic anhydrase (31 kDa), the majority of His-MinD_{N_{6}} eluted from the size exclusion column at an apparent molecular weight of at least twice its predicted monomeric molecular weight of 34 kDa (Figure 5.3 A, B). This indicated that the protein passed through the column at least as a dimer, in support of the yeast two-hybrid data. The elution of proteins from size-exclusion columns is dependent upon protein mass and shape. Since the crystal structure of several MinD proteins show it is a globular protein (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001), the elution profile of purified MinD_{N_{6}} in this study likely correlates well with apparent state of protein association.

The self-interaction of His-MinD_{N_{6}} was also confirmed by sedimentation equilibrium analysis. The data points from three different loading concentrations of His-MinD_{N_{6}}, depicting both the concentration and migration of the protein matched well with the theoretical fit lines of a dimer (Figure 5.4 A-C, lower panels). Further analysis showed that these data points had little deviation from their respective theoretical fit lines (Figure 5.4 A-C, upper panels). The protein was shown to have an average apparent molecular weight of 67,955, supporting a single species model of a dimer, which would have an expected molecular weight of 69,364. At a rotor speed of 16,000 rpm, there was also some evidence of decreasing apparent molecular weight with decreasing loading concentrations, suggesting that the protein could exist in a monomer-dimer equilibrium model (Table 5.4). Degradation and/or dissociation of the protein sample was observed over time when sedimentation analyses were run at rotor speeds greater than 16,000 rpm (Table 5.4).

Collectively, yeast two-hybrid, size-exclusion chromatography, and sedimentation equilibrium analyses show for the first time that bacterial MinD proteins are capable of dimer formation, suggesting that self-association may be important for MinD function across species.
Figure 5.4. Analytical ultracentrifugation analyses of His-MinD_{NG}. Plots illustrating sedimentation equilibrium data from 3 loading concentrations of His-MinD_{NG} fit globally to a single species model indicating a dimeric protein. A: 0.39 mg/ml. B: 0.27 mg/ml. C: 0.18 mg/ml. The run was performed at 20°C and 16,000 rpm. Lower panels display radial distance2/2 versus absorbance plots. Symbols represent measured data points and solid lines represent theoretical fit lines. Upper panels display the random deviation of the data points from the fit lines, indicating a good fit to the dimeric model.
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<th>Ultracentrifugation speed (rpm)</th>
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<sup>1</sup> Predicted molecular weight of His-MinD dimer is 69,364 Daltons
MinD_{Ng} interaction with MinC_{Ng} detected using a dot blot assay. Since the yeast two-hybrid system did not detect MinD_{Ng}-MinC_{Ng} interaction, a dot blot assay was developed to determine whether the two gonococcal proteins could bind to one another (Figure 5.5).

His-MinC_{Ng} was incubated over an Immobilon-P membrane previously spotted with varying concentrations of His-MinD_{Ng}. His-MinC_{Ng} could bind to the immobilized His-MinD_{Ng}, as detected by polyclonal anti-MinC_{Ng} (Figure 5.5, rows 1-4). This ability to interact was also dependent upon the amount of His-MinD_{Ng} present, as evidenced by the decreased His-MinC_{Ng} signal as levels of His-MinD_{Ng} dropped from 1.2 μg to 0.075 μg spotted per well (Figure 5.5, rows 1-4). As a negative control, lysozyme, an unrelated protein, was also spotted onto the membrane and probed for interaction with His-MinC_{Ng}. As expected, no significant binding of His-MinC_{Ng} to lysozyme was detected (Figure 5.5, row 5). In addition, His-MinC_{Ng} was not included as a probe in some experiments to determine whether the anti-MinC_{Ng} sera itself would detect His-MinD_{Ng} on the membrane and produce a false positive signal. It was found that the anti-MinC_{Ng} antibodies presented only minimal detection of His-MinD_{Ng} (Figure 5.5, rows 6-7). This minor cross-reaction of anti-MinC_{Ng} antisera can likely be attributed to the common N-terminal peptide sequence found, in addition to the 6XHis tag, within both His-MinC_{Ng} and His-MinD_{Ng} (see description of pJSHD2 construction, Chapter 2).

Hence, a direct interaction was observed between purified MinD_{Ng} and MinC_{Ng} proteins, in support of overexpression studies in *N. gonorrhoeae* (Chapter 3), as well as yeast two-hybrid results using MinD_{Ng} and *E. coli* MinC.
Figure 5.5. Dot blot assay to detect MinD<sub>Ng</sub> interaction with MinC<sub>Ng</sub>. The ability of purified His-MinC<sub>Ng</sub> to bind membrane-immobilized His-MinD<sub>Ng</sub> was determined. Two-fold serial dilutions of purified His-MinD<sub>Ng</sub> were applied to an Immobilin-P membrane. (Rows 1-2 and 3-4): His-MinD<sub>Ng</sub> from two separate purification preparations. (Row 5): Lysozyme was used as a negative control and was applied to the same blot. Following blocking, the membrane was probed with purified His-MinC<sub>Ng</sub> and bound His-MinC<sub>Ng</sub> was detected by incubating the membrane with affinity-purified anti-MinC<sub>Ng</sub> antisera. (Rows 6-7): A parallel blot was also spotted with His-MinD<sub>Ng</sub> and probed with anti-MinC<sub>Ng</sub> antisera, without prior incubation with His-MinC<sub>Ng</sub> itself, to determine the degree of cross-reactivity with His-MinD<sub>Ng</sub>.
5.4. DISCUSSION

This study is the first to identify the ability of bacterial MinD proteins to self-associate. Using the yeast two-hybrid system, both MinD<sub>N</sub> and MinD<sub>E</sub> displayed self-association, and could interact as heterodimers as well. These interaction studies offered the first physical evidence for the possibility that bacterial MinD might self-assemble. The self-association of MinD from either cocci or rods suggests that this interaction may be important for controlling cell division site selection in both cell types despite their morphological differences. In support of this, GFP-MinD<sub>N</sub> (Chapter 4) and GFP-MinD<sub>E</sub> (Shih et al., 2003) are shown to assemble along higher ordered coil structures in vivo.

What is the role for dimeric MinD? MinD<sub>E</sub> has recently been shown to dimerize in the presence of ATP, which is proposed to activate its membrane targeting sequence to promote membrane affinity, as proposed by the ‘dimer trigger’ model (Hu and Lutkenhaus, 2003; Hu et al., 2003). MinD<sub>E</sub> can also polymerize in vitro, and these polymers appear to consist of paired protofilaments with a combined diameter of ~ 6 nm, consistent with a pair of MinD monomers when measured across their narrowest profile (~3 nm) (Hayashi et al., 2001; Suefuji et al., 2002). Hence, dimeric MinD may be the basic building block, and perhaps nucleating seed, of MinD polymers (D. RayChaudhuri, Tufts University, personal communication). Dimerization may also be important for optimizing the ATPase activity of MinD proteins once at the membrane. Examination of a modeled MinD dimer suggested that the signature lysine (corresponding to K11 in MinD<sub>N</sub> and MinD<sub>E</sub>) within the deviant Walker A motif of each MinD subunit may stabilize ATP that is bound by the opposite subunit, thus aiding nucleotide hydrolysis (Lutkenhaus and Sundaramoorthy, 2003).

Size-exclusion chromatography and sedimentation equilibrium analyses also indicated that purified N-terminal His-tagged MinD<sub>N</sub> was dimeric, in support of yeast two-hybrid studies. Although these in vitro studies were performed prior to indications that ATP may play a role in MinD dimerization (Hu et al., 2003), purified His-MinD<sub>N</sub> was observed to be dimeric, despite the absence
of ATP. In the following chapter, evidence that C-terminal His-tagged MinD_{Ng} can be induced to dimerize in the presence of ATP is presented (Chapter 6).

What might account for the ATP-independent dimerization of N-terminal His-tagged MinD_{Ng}? It is possible that ATP remained bound to His-MinD_{Ng} during the purification process, causing it to remain dimeric during its gel-filtration and sedimentation equilibrium assays. The relatively long peptide sequence that is fused to the N-terminus of His-MinD_{Ng}, in addition to the His-tag, may have also affected the ability of the protein to hydrolyze ATP, thus maintaining its self-association. However, it is clear that this long N-terminal peptide did not abrogate the ability of His-MinD_{Ng} to induce MinC-mediated cell division, as evidenced by the functional expression studies in various *E. coli* backgrounds. Interestingly, others have also observed that MinD_{lg} can exist as a dimer in the absence of ATP (D. RayChaudhuri, Tufts University, personal communication).

Furthermore, it is possible that MinD proteins can exist in ATP-independent monomer-dimer equilibria which, in the case of His-MinD_{Ng}, was shifted in favour of dimers under its assay conditions. It is noted that the concentrations of His-MinD_{Ng} (0.18-0.39 mg/ml) used in sedimentation equilibrium analyses were higher than *in vivo* estimates in *E. coli* (~1-2 μM or 0.03-0.06 mg/ml) (Mileykovskaya *et al.*, 2003), and likely in *N. gonorrhoeae*. Thus, it is possible that these higher protein concentrations may have driven His-MinD_{Ng} more favourably towards dimerization, even in the absence of ATP. Interestingly, sedimentation equilibrium analyses of His-MinD_{Ng} provided some evidence of decreasing apparent molecular weight with decreasing loading concentrations, suggesting the protein might exist in a monomer-dimer equilibrium model (Leslie Hicks, University of Alberta, personal communication), however, further testing would need to be done to verify this. There are many examples of protein monomer-dimer equilibria being influenced by environmental factors. *E. coli* SecA and *P. furiosus* ferredoxin can both experience different monomer-dimer equilibrium reactions depending upon ionic strength and/or temperature (Hasan *et al.*, 2002; Woodbury *et al.*, 2002). In addition, the *B. subtilis* ATP-binding protein OpuAA also exhibits increased dimerization
upon increasing protein concentration, despite the absence of its nucleotide substrate (Horn et al., 2003).

In *E. coli*, MinE interacts with MinD to promote the ATPase activity, oscillation, and coiled-array localization of MinD (Huang et al., 1996; Raskin and de Boer, 1999a; Rowland et al., 2000; Hu and Lutkenhaus, 2001; Hu et al., 2002; Shih et al., 2003). This study also detected an interaction between MinD$_{Ng}$ and MinE$_{Ng}$, further supporting the *in vivo* dynamism and helical assembly of GFP-MinD$_{Ng}$ in the presence of MinE$_{Ng}$ (Chapter 4). The association of MinD$_{Ng}$ with MinE$_{Ng}$ indicates that the latter likely stimulates the ATPase activity of MinD$_{Ng}$ to drive its intracellular movement, both in *E. coli* and, most probably, in *N. gonorrhoeae*.

Although evidence from overexpression studies suggests MinC$_{Ng}$ and MinD$_{Ng}$ are both required to inhibit gonococcal cell division (Chapter 3), a MinC$_{Ng}$-MinD$_{Ng}$ interaction was not detected using the yeast two-hybrid system. This is in contrast to reports for MinC and MinD homologues from *E. coli* or *B. subtilis*, which do interact (Huang et al., 1996, Marston and Errington, 1999b). However, the yeast two-hybrid system did detect a strong interaction between gonococcal MinD and *E. coli* MinC, in support of its functionality in *E. coli* backgrounds (Chapter 4).

It is noted that different yeast two-hybrid systems may detect different Min protein interactions. For example, the dimerization of *E. coli* MinE was detected by one group using yeast two-hybrid methods (Pichoff et al., 1995), but not by others using a different yeast two-hybrid system (Huang et al., 1996; this study). These differences may be due to steric considerations, protein expression, and protein stability within various yeast systems. Hence, it is possible that the GAL4-MinC$_{Ng}$ fusions were unstable or poorly expressed in the yeast reporter strain, such that no interaction between MinC$_{Ng}$ and MinD$_{Ng}$ was detected. Studies have indicated that the C-terminus of *E. coli* MinC is responsible for interaction with its MinD partner (Hu and Lutkenhaus, 2000). From this, our laboratory has constructed a chimeric protein consisting of the N-terminus of *E. coli* MinC and the C-terminus of *N. gonorrhoeae* MinC. Using the yeast two-hybrid system, we were able to observe a strong interaction between the chimeric MinC and MinD$_{Ng}$ (Ramirez-Arcos et al., in press).
Therefore, this chimeric MinC protein is likely more stable in our yeast reporter strain than MinC_{Ng}, and can be used to study MinC_{Ng} and MinD_{Ng} interactions in future experiments.

No interactions between the three Min proteins and the cell division/cell development proteins FtsZ, FtsA, PenA (FtsI), and FtsY were detected. An interaction between MinC_{Ng} and FtsZ would be expected, since purified *E. coli* MinC has been demonstrated to interact with FtsZ (Hu *et al.*, 1999). However, yeast two-hybrid assays also do not detect any association between *E. coli* MinC and FtsZ (Huang *et al.*, 1996; Hu *et al.*, 1999), suggesting this system may not be the optimal for studying this interaction.

Studies with *E. coli* proteins have shown that the ATP-bound form of MinD_{Ec} is required to interact with MinC_{Ec} *in vitro* (Hu *et al.*, 2003), suggesting dimeric MinD is the active MinC partner. However, in the present study, an interaction between purified His-MinC_{Ng} and His-MinD_{Ng} was detected in the absence of additional ATP. Hence, it is possible that His-MinD_{Ng} could bind His-MinC_{Ng} under these conditions since His-MinD_{Ng} was already dimerized, as discussed above.

Could there be a biological function for ATP-free MinD dimers? In order to oscillate within the cell, membrane-associated MinD must be released into the cytoplasm upon hydrolysis of its bound ATP molecule (Hu *et al.*, 2002; Lackner *et al.*, 2003). It has been proposed that simple diffusion in the cytosol may be sufficient to ensure that ATP-free MinD proteins do not re-associate with existing MinD assemblies (Lackner *et al.*, 2003). However, it is possible that rapid ATP-binding may drive the protein back to its original membrane (or MinCDE array) location, and thus negate proper MinD movement. It is tempting to speculate that, *in vivo*, nucleotide-free MinD dimers may form from nascent ATP-free MinD monomers that are released from the membrane. This could conceivably delay ATP-binding and serve as an additional safeguard that prevents MinD from re-associating at the same membrane location.

This study demonstrates that interactions exist between MinD_{Ng} and the other Min proteins. The MinD_{Ng}-MinE_{Ng} interaction offers support for the ability of GFP-MinD_{Ng} to undergo MinE_{Ng}-dependent movement and localization within a helical array in *E. coli* backgrounds. The MinD_{Ng}-
MinC interaction is consistent with the ability of MinD_{Ng} to induce MinC-mediated cell division arrest in *N. gonorrhoeae* and *E. coli* backgrounds. Significantly, a novel self-association of bacterial MinD proteins was observed. Both MinD_{Ng} and MinD_{Ec} were able to interact with themselves, suggesting the self-interaction of MinD may be a conserved characteristic that is important for MinD function in different species of varying morphologies. This self-association also supports the ability of MinD proteins to localize as higher ordered structures *in vivo* (Chapter 4; Raskin and de Boer, 1999a; Rowland *et al.*, 2000; Shih *et al.*, 2003).
CHAPTER 6

Structural homology modeling strategy identifies a region in MinD_{Ng} that sensitizes the protein to MinE stimulation

Portions of this chapter are found in the following submitted manuscript:

[Szeto, J., Eng, N. F., Acharya, S., Rigden, M. D., and Dillon, J. R. (2004). A distinct region in the cell division site-determinant MinD is implicated in sensitizing the protein to MinE stimulation Submitted for publication to Research in Microbiology.]
6.1. PURPOSE OF THIS STUDY

Is is now established that bacterial MinD proteins dimerize (this study; Hu et al., 2003). The self-association of MinD is proposed to be important for its ability to localize to the membrane and to hydrolyze ATP (Hu and Lutkenhaus, 2001; Hu et al., 2003). The crystal structures of several MinD homologues from Archaeal organisms show that the protein is monomeric. These crystal structures observed were probably monomeric because the proteins were bound to ADP, AMPPCP, or to no nucleotide (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001). Interestingly, the Archaeal MinD proteins most closely resemble the monomeric subunits that form dimeric nitrogenase iron proteins (NIPs) (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001), such as NifH from Azotobacter vinelandii (Schindelin et al., 1997).

In order to identify regions in MinDNg that may be involved in homodimerization, a model of dimeric MinD was generated by superimposition of solved Archaeal MinD monomers onto NifH. Structural modeling and sequence alignment of 24 MinD proteins revealed a polar region in residues 92-94 of bacterial MinD. To assess the importance of this region in MinD functionality, mutant proteins were generated and subjected to several functional assays. These MinDNg mutants inhibited E. coli cytokinesis and retained interaction with all Min proteins, including MinENg, GFP-MinDNg and GFP-MinDEc, both mutated at MinD residues 92-94, localized as coiled arrays in E. coli, similar to wild-type GFP-MinD. However, unlike wild-type fusions, mutant proteins were distributed uniformly throughout the array and presented no obvious oscillation patterns, despite the presence of MinE. Although the MinDNg mutant dimerized in the presence of ATP, its ATPase activity was not stimulated by MinENg, unlike wild-type MinDNg. Hence, despite localizing to the membrane as a helical array and interacting with MinENg, the mutant MinD proteins in this study have lost the ability to be efficiently stimulated by MinENg, resulting in a loss of distinct pole-to-pole oscillation.
6.2. MATERIALS AND METHODS

**Structural modeling of dimeric MinD.** NifH from the nitrogenase complex of *Azotobacter vinelandii* (PDB# 1N2C; Schindelin *et al.*, 1997) is a structural homologue of MinD proteins (Cordell and Löwe, 2001; Hayashi *et al.*, 2001; Sakai *et al.*, 2001), and was used as a template to generate a model for dimeric MinD. Using SWISS-PDB Viewer (Version 3.7 b2), the solved structure for monomeric *P. furiosus* MinD bound to ADP (PDB# 1G3Q; Hayashi *et al.*, 2001) was superimposed manually onto each monomeric subunit of NifH that comprises the solved NifH dimer. Ribbon diagrams were generated with RasMol Molecular Graphics Visualization Tool, (Version 2.7.2.1) [http://www.bernstein-plus-sons.com/software/RasMol_2.7.2.1/doc/rasmol.html].

**Construction of minD<sub>Ng</sub> genes encoding point mutations to amino acids 92-94.** Plasmid pSR3 (Table 6.1 and Chapters 4) encoding wild-type minD<sub>Ng</sub> was used as a template to generate the simultaneous substitutions R92L, D93L, K94I (collectively referred to as ‘loop’ mutations) using IPCR and Vent DNA polymerase.

  Primers SJ1 (encoding all three amino acid mutations) and SJ2 (Table 2.2) were designed to anneal within minD<sub>Ng</sub> at the region of interest and to initiate amplification in opposite directions. The resulting blunt-ended amplicon was phosphorylated using T4 polynucleotide kinase (New England BioLabs), recircularized with T4 DNA ligase (Gibco BRL), and transformed into *E. coli* DH5α. Plasmid-encoded mutant genes were screened by performing colony PCR on candidate clones to amplify an internal segment of minD<sub>Ng</sub> and digesting the amplicons with *KspI*, since this restriction site would have been incorporated by primer SJ1 (Table 2.2). The resulting plasmid encoding MinD<sub>Ng-R92L,D93L,K94I</sub> (referred to as MinD<sub>Ng-loop</sub>), was named pJS7 (Table 6.1).

  Similar IPCR strategies were employed to generate minD<sub>Ng</sub> genes encoding K94A and E103I mutations. K94A mutations were made using the mutant primer DJ1 (which also incorporated an *NruI* restriction site for screening purposes) and primer SJ2 (Table 2.2) on pSR15 template to form plasmid pJS32, encoding GFP-MinD<sub>Ng-K94A</sub> (Table 6.1). The minD<sub>Ng-K94A</sub> gene was subsequently PCR
Table 6.1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Relevant genotype</th>
<th>Source/reference</th>
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<td>de Boer et al.(1988)</td>
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<td>P_{uc}::gfp (Amp^{R})</td>
<td>Weiss et al. (1999)</td>
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<td>Plasmid</td>
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<td>pDSW209;( \text{P}<em>{\text{irc}}::\text{gfp-minD}</em>{\text{Ng-E103I}}, \text{minE}_{\text{Ng}}(\text{Amp}^R) )</td>
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<td>( \text{Amp}^R ) ( \text{P}<em>{\text{lac}}::\text{gfp-minD}</em>{\text{Ec}}, \text{minE}_{\text{Ec}}(\text{Amp}^R) )</td>
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<td>pDR122;( \text{P}<em>{\text{lac}}::\text{gfp-minD}</em>{\text{Ec-loop}}(\text{R92L, D93L, K94I}), \text{minE}_{\text{Ec}}(\text{Amp}^R) )</td>
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**Yeast-two hybrid plasmids**

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$^a$ GAL4 activation domain

$^b$ GAL4 DNA binding domain
amplified from pJS32 using primers minD1/minD2 (Table 2.2) and ligated into pUC18 to give plasmid pJS31 (Table 6.1).

An E103I substitution in MinD_Ng was made using primer SA2 and the mutant primer SA3 (Table 2.2) on pSR3 template (Table 6.1). Clones were screened by digestion of minD_Ng colony PCR amplicons with BclI (incorporated by primer SA3), and the resulting plasmid encoding MinD_Ng-E103I was named pSIA5 (Table 6.1). Gene integrity and the presence of the each mutation was verified by DNA sequencing performed at the Core DNA Sequencing and Synthesis Facility, University of Ottawa. Plasmids pSR3, pJS7, pJS31, and pSIA5 (Table 6.1) were transformed into *E. coli* PB103 (Table 2.1) for morphology studies.

IPCR methods using the relevant primers listed above were also used on pSR15 template (Chapter 4; encoding wild-type GFP-MinD_Ng and MinE_Ng) (Table 6.1) to generate GFP-fusions to MinD_Ng-loop (pJS9), MinD_Ng-K94A (pJS32), and MinD_Ng-E103I (pJS17) for localization studies. Plasmid pJS10, encoding GFP-MinD_Ng alone (without downstream minE_Ng), as well as pJDE1, encoding GFP-MinD_Ng-K16Q, were constructed previously in Chapter 4. In addition, R92I, D93L, and K94I substitutions were also simultaneously introduced into *E. coli* MinD (MinDEc) by using IPCR on pDR122 (*gfp-minD_Ec, minE_Ec*) (Raskin and de Boer, 1999a) with primers DJ2 (mutant primer, incorporating VspI site as well for screening) and DJ3 (Table 2.2). The resulting plasmid, encoding GFP-MinD_Ec-R92I,D93L,K94I (referred to as GFP-MinD_Ec-loop), was named pJS35 (Table 6.1). The integrity of all min genes was confirmed by DNA sequencing performed at the Core DNA Sequencing and Synthesis Facility, University of Ottawa.

**Yeast two-hybrid plasmids.** Fusions of MinD_Ng-loop, and MinD_Ng-E103I to GAL4-DNA-BD and to GAL-4AD were constructed. Each gene was PCR amplified using primers minD1 and minD2 (Table 2.2) from pJS7 and pSIA5, respectively, digested with *Eco*RI and *Bam*HI, and cloned into pGBT9 and pGAD424. Fusions of MinD_Ng-loop, and MinD_Ng-E103I to GAL4-DNA-BD were encoded on
plasmids pJminD19 and pSIA7 (Table 6.1). Fusions of MinD<sub>Ng-loop</sub> and MinD<sub>Ng-E103I</sub> to GAL4-AD were similarly constructed, and encoded on plasmids pJminD18 and pSIA8 (Table 6.1). Fusions of GAL4-AD (pJminD16) and GAL4-DNA-BD (pJminD17) to MinD<sub>Ng-K16Q</sub> were also constructed similarly (Table 6.1), using pJATP1 (Table 3.1) as a PCR template with primers minD1 and minD2 (Table 2.2). Yeast two-hybrid assays were conducted as outlined in Chapter 2.

**GFP-MinD fusion localization.** For protein localization studies, *E. coli* PB114 cells (Table 2.1) transformed with plasmids encoding various GFP-MinD<sub>Ng</sub> fusions were induced with 40 μM IPTG, grown, and immobilized on coverslips as previously described (Chapter 4). *E. coli* PB114 cells transformed with plasmids encoding GFP-MinD<sub>Ng</sub> were induced with 27 μM IPTG (Raskin and de Boer, 1999a) and also grown and immobilized, as described before (Chapter 4). Fluorescence microscopy was performed with an Olympus BX61 microscope equipped with a Photometrics CoolSnap ES camera and Image Pro (Version 4.5.1) software. To maintain consistency, the oscillation cycle (movement from one pole to the opposite, and back again) of each GFP-MinD<sub>Ng</sub> fusion was measured in 20 cells of near equivalent length (~2.0-2.5 μm). Time lapse images were taken every 10 seconds. In each cell observed, at least two complete oscillation cycles were used to verify oscillation periods. Statistical analyses using unpaired Student’s t-tests were performed to determine whether differences in average oscillation times between GFP-MinD<sub>Ng</sub> fusions were significant, with \( p<0.001 \) considered significant. When required, enhancement of raw images was done using standard options available on Image Pro software: Contrast enhancement was used to increase contrast and to decrease gamma. Gauss filtering using a 3 X 3 kernel size with 4 passes at strength 10 was also used to help visualize intracellular substructures.

**Protein purification.** PCR was carried out using primers SJ3 and SJ4 (Table 2.2) on four templates: pSR3, pJATP1, and pJS7 (Table 6.1) in order to amplify wild type minD<sub>Ng</sub>, minD<sub>Ng-K16Q</sub>, and minD<sub>Ng</sub>.
loop, respectively. Each amplicon was digested with NdeI and XhoI and ligated into similarly digested pET30a (Table 6.1) to generate plasmids encoding C-terminal His-tagged MinE<sub>N</sub> (pSC9), MinE<sub>N</sub>-K<sub>16Q</sub> (pSC10), and MinE<sub>N</sub>-loop (pJS8) (Table 6.1) for purification using nickel affinity chromatography.

Each plasmid was transformed into *E. coli* C41 (DE3) (Table 2.1) for overexpression. 50 ml log phase cultures of cells were induced with 1mM IPTG for 4 hours at 37°C and shaking at 250 rpm. Induced cells were pelleted for 10 minutes at 5000 X g resuspended in 2 ml binding buffer (5mM imidazole, 1M NaCl, 20mM Tris-HCl pH 7.9) and incubated on ice for 30 minutes. The cells were lysed by sonication (Fisher Scientific 60 Sonic Dismembrator) using 4 cycles, each consisting of two six-second bursts, with at least a 1 minute interval on ice between cycles. Cell extracts were centrifuged at 100,000 X g for 20 minutes at 4°C (L8-M Ultracentrifuge, Beckman) and the soluble supernatant fraction was retained.

Protein purification was performed with a 1ml bed volume of His-Bind resin (Novagen) for each protein using the following solutions, described by the manufacturer, for column washing: 10 ml binding buffer (5 mM imidazole), 5 ml wash solution (60 mM imidazole), and 1 ml of elution buffer (1 M imidazole). Eluted protein was dialyzed overnight at 4 °C against Buffer A (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 200 mM NaCl, 10% glycerol; final pH adjusted to pH 7.2) in glassware previously washed with 7X detergent (ICN Biomedicals Inc.), distilled/deionized water (ddH<sub>2</sub>O), 1 M HCl, and a final ddH<sub>2</sub>O rinse to eliminate possible contaminating inorganic phosphates.

To obtain purified MinE<sub>N</sub>, minE<sub>N</sub> was PCR amplified from gonococcal cell suspensions using primers ESmE1 and ESmE2 (Table 2.2), and cloned into pET30a as above to form plasmid pEC1 (Table 6.1). *E. coli* C41 (DE3) was transformed with pEC1 and 0.4 mM IPTG was used to induce a 350 ml log phase culture for 2-3 hours at 250 rpm and 37°C. Soluble cell supernatant was applied to 3 ml of His-Bind resin and the column was washed with buffer containing increasing concentrations of imidazole buffer (5 mM to 100 mM; Novagen) and MinE<sub>N</sub>-His was eluted with 250 mM imidazole. Purified protein was dialyzed in Buffer B, consisting of 50 mM Tris, 20 mM
NaCl, 1 mM EDTA, pH 7.4. MinE_Ng-His was concentrated using Biomax-5 centrifugal filter columns with a 5000 Dalton molecular weight cutoff (Millipore).

**Circular dichroism spectroscopy.** Circular dichroism (CD) spectroscopy was performed on purified C-terminal His-tagged wild-type MinD_Ng, MinD_NgK16Q, and MinD_NgNgoop using an AVIV Model 62DS CD spectrometer (AVIV Instruments, Inc., Lakewood NJ), with a 1 mm path length quartz cell at 20°C. The temperature of the sample was computer controlled to within 0.2 °C. For the wild-type protein and the mutant, far-UV CD spectra were recorded at a protein concentration of ~0.1 mg/ml in Buffer B. All CD experiments were repeated three times to ensure reproducibility. Following the baseline subtraction, the CD data were normalized to the protein concentration measured by Bradford method.

**MinD_Ng ATPase stimulation assays.** A rapid protocol for the formation of vesicles composed of the anionic phospholipid 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol) (phosphatidylglycerol; PG) (Avanti Polar Lipids, Inc.) was developed. 0.1 ml of PG (10 mg/ml in chloroform) was dried under a stream of filtered air and resuspended with reaction buffer (25 mM Tris-Cl, 50 mM KCl, pH 7.5) to give a stock PG vesicle solution of 5 mg/ml. PG vesicles were visualized by phase contrast microscopy to verify structural integrity and stored at -20°C. PG vesicles appeared to remain stable for at least four months, as assessed by phase contrast microscopy.

Prior to each experiment, the concentrations of MinD_Ng and MinE_Ng proteins were determined using the Bio-Rad protein assay and adjusted as required by diluting in their respective dialysis buffers. In a typical 100 μl reaction, the following reagents were added (final concentrations in brackets): reaction buffer (volume added dependant on other reagents to be included), MinD_Ng protein (0.012 mg/ml), ATP (1 mM), PG vesicles (0.5 mg/ml), and MgCl₂ (1 mM). Reactions were incubated at room temperature for five minutes and, if required, MinE_Ng-His (0.012 mg/ml) was added followed
by an additional five minutes incubation. Storage buffers for MinD_{Ng} or MinE_{Ng} were used to adjust reaction volumes to 100 μl when protein was not used.

At specified timepoints, 30 μl of the each reaction was removed, centrifuged at high speed for 1 minute, and 25 μl of supernatant was removed and added to 50 μl of malachite green solution in a microtitre dish, similar to the procedure described by Harder et al. (1994). Colour development was allowed to proceed for 20 minutes at room temperature and the absorbance of the reaction at 620 nm was determined using a TECAN Spectra Shell microplate reader (TECAN U.S. Inc.) and compared to a blank containing all the reagents in a standard reaction, except storage buffers were used in place of each protein.

All reactions were carried out in duplicate and colour development was compared to a blank containing ATP, PG, and MgCl₂, as described above, with the remaining volume consisting of Buffers A and B. The amount of inorganic phosphate released was determined by comparing absorbance readings with those obtained from inorganic phosphate standards prepared from dilutions of KH₂PO₄ in the blank buffer.

**Size exclusion chromatography.** Purified MinD_{Ng}, MinD_{Ng-K16Q}, and MinD_{Ng-loop} (0.35-0.45 mg/ml) were preincubated with, or without, 1 mM ATP-γ-S and 1 mM MgCl₂ for 40 minutes at room temperature and 450 μl reaction volumes were each applied to a 100 ml column of Superdex 200 (Amersham Pharmacia) equilibrated with PBS (pH 7.4). Flow rate was set at 0.7 ml/min. Eluted protein from 0.5 ml fractions was collected and detected in a microtitre plate using the Bio-Rad protein assay. Protein elution profiles were compared to those of Amersham Gel-filtration Standards.
6.3. RESULTS

Generating a proposed model for dimeric MinD

To identify regions in MinD that may be implicated in homodimerization and/or function, structural homology alignment was used to generate a possible model for dimeric MinD (Figure 6.1 A). Structurally, the Archaeal MinD proteins are very similar to the monomeric subunits of dimeric nitrogenase iron proteins (Cordell and Löwe, 2001; Hayashi et al., 2001; Sakai et al., 2001), such as NifH from Azotobacter vinelandii (Schindelin et al., 1997). Therefore, solved monomeric structures of a representative Archaeal MinD from Pyrococcus furiosus (MinD_p) (Hayashi et al., 2001) were superimposed onto the structure of dimeric NifH (Schindelin et al., 1997).

Structural alignment of the predicted MinD_p dimer (Figure 6.1 B) and the solved A. vinelandii NifH dimer (Figure 6.1 A) revealed a common exposed region in both proteins. In each MinD_p monomer, this region appears as a short α-helix containing residues 87-93 (Figure 6.1 B, green residues; Figure 6.2, residues of MinD_p below the bar indicated ‘2’). In each NifH monomer, the corresponding region is in the form of a loop comprising residues 92-98 (Figure 6.1 A green residues; Figure 6.2, residues of NifH above the bar indicated ‘4’) (Schindelin et al., 1997; Schlessman et al., 1998). Previous structural analyses of NifH indicated that amino acids within this region (particularly Glu92, Pro93, Val95, Ala98) are involved in mediating monomer-monomer contact through polar interactions, such as hydrogen bonding and salt-bridge formation (Schlessman et al., 1998).

Sequence alignment of twenty-four MinD proteins showed the presence of several consecutive polar amino acids in a corresponding region in bacteria (Figure 6.2; as a reference, residues 86-97 of MinD_Nb are aligned below the bar indicated ‘3’). These residues are highly conserved, particularly among Gram-negative organisms, which have a consensus sequence of L[P/A]ASQ[T/S][R/K]DK[D/N][A/T/N/I]L (polar amino acids are underlined). Since this region also comprises the NifH loop, it is reasoned that it may be implicated in protein interactions and/or function; hence, three conserved polar residues in MinD_Nb were selected for simultaneous substitution.
Figure 6.1. Model of dimerized MinD based on structural alignment with *Azotobacter vinelandii* NifH dimer. (A) The crystal structure of NifH from *A. vinelandii* shows a dimeric protein (Schindelin et al., 1997). Position of each NifH monomer is indicated by brackets. Green residues correspond to a loop region (aa 92-98) in each monomer that is implicated in mediating monomer-monomer contact (Schlessman et al., 1998). (B) Model of dimeric MinD generated by superimposition of *P. furiosus* MinD (MinD$_{pf}$) (Hayashi et al., 2001) monomers onto each subunit of the *A. vinelandii* NifH dimer. The green residues (aa 87-93) of MinD$_{pf}$ represent a region that corresponds with the loop region in each NifH monomer [green residues in (A)]. Cyan residues indicate MinD$_{pf}$ residues involved in binding ATP (Hayashi et al., 2001).
Figure 6.2. Alignment of MinD sequences corresponding to residues 82-104 of A. vinelandii NifH. Abbreviations used: Methanococcus janaschii (Mj), Archaeoglobus fulgidus (Af), Pyrococcus furiosus (Pf), P. horikoshii (Ph), Neisseria gonorrhoeae (Ng), N. meningitidis (Nm), Escherichia coli (Ec), Salmonella enterica serovar Typhimurium (St), Yersinia pestis (Yp), Vibrio cholerae (Vc), Pseudomonas aeruginosa (Pa), Brucella melitensis (Bm), B. suis (Bsui), Agrobacterium tumefaciens (At), Helicobacter pylori (Hp), Aquifex aeolicus (Aa), Thermotoga maritima (Tm), Bacillus subtilis (Bs), Listeria monocytogenes (Lm), Clostridium perfringens (Cp), Synechocystis sp. (Sy), Guillardia theta (Gt), Deinococcus radiodurans (Dr), Chlamydia trachomatis (Ct). Residues R92, D93, K94 of MinDNg and MinDNe are aligned below the bar designated ‘1’. MinDPr residues 87-93 are aligned below the bar designated ‘2’. Residues 86-97 of MinDNg are aligned aligned below the bar designated ‘3’. Residues in NifH aligned above the bar designated ‘4’ are part of one region involved in NifH dimerization through polar interactions (Schlessman et al., 1998). Arrow shows position of E103 in MinDNg.
with hydrophobic residues (R92L, D93L, and K94I) (Figure 6.2, residues of MinD_Ng below the bar indicated ‘1’). For convenience, the resulting mutant will be referred to as MinD_{Ng-loop}, although the exact structure of this region in bacterial MinD is currently not known.

Furthermore, an E103I mutant was also generated at this non-conserved residue position (Figure 6.2, arrow), in close proximity to MinD_{Ng} residues 92-94. This mutation is predicted not to adversely affect MinD_{Ng} function and was constructed to evaluate any functional significance of mutations in non-conserved residues in this region. In addition, MinD_{Ng-K16Q} was selected as a negative control since it is unable to induce cell division arrest in *N. gonorrhoeae* (Chapter 3) and *E. coli* backgrounds (Chapter 4), and localizes to the cytosol *in vivo* (Chapter 4).

**MinD_{Ng} with mutations at residues 92-94 can still induce cell division arrest in *E. coli***

MinD_{Ng} is active in *E. coli* and works with *E. coli* MinC to arrest cell division in this background. To initially assess the functionality of the various MinD_{Ng} mutants, the ability of each protein to induce cell division arrest in wild-type *E. coli* was determined. Wild-type *E. coli* PB103 (Table 2.1) was transformed with plasmids encoding wild-type MinD_{Ng}, MinD_{Ng-loop}, MinD_{Ng-E103I}, and MinD_{Ng-K16Q}. Overexpression of wild-type MinD_{Ng} or MinD_{Ng-E103I} could induce cell division arrest in these cells, leading to cell filamentation (Figure 6.3 B, E). Similarly, *E. coli* cells overexpressing MinD_{Ng-loop} displayed filamentation as well (Figure 6.3 C). Residue K94, the most conserved of the polar residues at positions 92-94 in MinD_{Ng}, was also substituted with an alanine (K94A) to determine the effects of mutating only a single residue in this region. *E. coli* overexpressing MinD_{Ng-K94A} were also filamentous (Figure 6.3 D). As negative controls, *E. coli* cells were transformed with the pUC18 plasmid or with a derivative plasmid encoding MinD_{Ng-K16Q}. Both transformants presented normal short rod morphology (Figure 6.3 A, pUC18 transformed cells shown).

Western blotting confirmed the overexpression of wild-type (Figure 6.3 F, lane 5) and mutant MinD_{Ng} (MinD_{Ng-loop}, lane 3; MinD_{Ng-K94A}, lane 2; MinD_{Ng-E103I}, lane 1) in *E. coli* PB103 relative to
Figure 6.3. Effects of overexpressing MinD<sub>Ng</sub> proteins bearing mutations in the 'loop' region in wild-type E. coli PB103. (A) Cells transformed with pUC18 show normal short rod morphology. Cells transformed with (B) pSR3 (<i>minD<sub>Ng</sub></i>), (C) pJS7 (<i>minD<sub>Ng-loop</sub></i>), (D) pJS31 (<i>minD<sub>Ng-K94A</sub></i>), and (E) pSIA5 (<i>minD<sub>Ng-E103I</sub></i>) displayed filamentous phenotypes. Bar shown in (A) is 10 μm, and all images are at the same magnification. (F) Western blot analysis using anti-MinD<sub>Ng</sub> antisera shows the overexpression of each MinD<sub>Ng</sub> protein in E. coli PB103: Lane 1, MinD<sub>Ng-E103I</sub>; lane 2, MinD<sub>Ng-K94A</sub>; lane 3, MinD<sub>Ng-loop</sub>; lane 4, pUC18 transformed E. coli cell extract; lane 5, wild-type MinD<sub>Ng</sub>.
background *E. coli* MinD signal (lane 4). These results show that radical mutations to residues R92, D93 and K94 do not affect the ability of MinD<sub>Ng</sub> to induce cell division arrest in wild-type *E. coli*.

**MinD<sub>Ng</sub>-loop retains self-association and interaction with all Min proteins**

To determine whether the identified polar region in MinD<sub>Ng</sub> is involved in protein dimerization, as predicted by structural homology modeling with the NifH dimer, the yeast two-hybrid system was used to test protein-protein interactions. As expected, wild-type MinD<sub>Ng</sub> could self-associate, in addition to the MinD<sub>Ng</sub>-E103I mutant (Table 6.2). Interestingly, MinD<sub>Ng</sub>-loop retained the ability to self-associate as well. Both MinD<sub>Ng</sub>-loop and MinD<sub>Ng</sub>-E103I self-interactions were stronger than the wild-type MinD<sub>Ng</sub> control. In contrast, the negative control mutant MinD<sub>Ng</sub>-K16Q displayed a significantly decreased ability to dimerize (Table 6.2). MinD<sub>Ng</sub>-loop and MinD<sub>Ng</sub>-E103I also retained interactions with MinE<sub>Ng</sub> which were either stronger than, or comparable with, wild-type MinD<sub>Ng</sub>, respectively. Conversely, MinD<sub>Ng</sub>-K16Q did not display interaction with MinE<sub>Ng</sub> (Table 6.2).

MinD<sub>Ng</sub>-loop also retained an ability to interact with MinC<sub>Ec</sub>, as did MinD<sub>Ng</sub>-E103I. Both interactions were weaker relative to the wild-type control, based on β-galactosidase activities (Table 6.2). *E. coli* MinC was used in these assays since MinC<sub>Ng</sub> is likely unstable in the yeast reporter strain (Chapter 5, Ramirez-Arcos et al., in press). Despite diminished interactions observed by the yeast two-hybrid system, the abilities of MinD<sub>Ng</sub>-loop and MinD<sub>Ng</sub>-E103I to effectively inhibit cell division in wild-type *E. coli* (Figure 6.3 C, E) shows that sufficient functional interaction is retained between each mutant MinD and MinC<sub>Ec</sub> (Table 2). In contrast to the other MinD<sub>Ng</sub> proteins tested, MinD<sub>Ng</sub>-K16Q did not display interaction with MinC<sub>Ec</sub>. Overall, these results show that mutations within, or in proximity to, the predicted ‘loop’ region of MinD<sub>Ng</sub> do not abrogate interactions with other Min proteins or MinD<sub>Ng</sub> homodimerization.
Table 6.2. Yeast two-hybrid analysis of the interactions of wild-type and mutant MinD$_{Ng}$ with the Min system

<table>
<thead>
<tr>
<th>Fusion to GAL4-BD$^a$</th>
<th>Fusion to GAL4-AD$^b$</th>
<th>Intensity of colony$^c$</th>
<th>$\beta$-galactosidase activity$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MinD$_{Ng}$</td>
<td>MinD$_{Ng}$</td>
<td>+++</td>
<td>8.81 ± 0.20</td>
</tr>
<tr>
<td>MinD$_{Ng}$-loop$^f$</td>
<td>MinD$_{Ng}$-loop</td>
<td>+++</td>
<td>21.79 ± 0.64</td>
</tr>
<tr>
<td>MinD$_{Ng}$-E103I</td>
<td>MinD$_{Ng}$-E103I</td>
<td>+++</td>
<td>15.18 ± 0.08</td>
</tr>
<tr>
<td>MinD$_{Ng}$-K16Q</td>
<td>MinD$_{Ng}$-K16Q</td>
<td>+</td>
<td>1.68 ± 0.09</td>
</tr>
<tr>
<td>MinE$_{Ng}$</td>
<td>MinD$_{Ng}$</td>
<td>+++</td>
<td>8.31 ± 0.40</td>
</tr>
<tr>
<td>MinE$_{Ng}$</td>
<td>MinD$_{Ng}$-loop</td>
<td>+++</td>
<td>31.24 ± 0.22</td>
</tr>
<tr>
<td>MinE$_{Ng}$</td>
<td>MinD$_{Ng}$-E103I</td>
<td>+++</td>
<td>7.60 ± 0.42</td>
</tr>
<tr>
<td>MinE$_{Ng}$</td>
<td>MinD$_{Ng}$-K16Q</td>
<td>.</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td>MinC$_{Ec}$</td>
<td>MinD$_{Ng}$</td>
<td>+++</td>
<td>935.43 ± 19.90</td>
</tr>
<tr>
<td>MinC$_{Ec}$</td>
<td>MinD$_{Ng}$-loop</td>
<td>+++</td>
<td>75.76 ± 0.96</td>
</tr>
<tr>
<td>MinC$_{Ec}$</td>
<td>MinD$_{Ng}$-E103I</td>
<td>+++</td>
<td>149.71 ± 2.71</td>
</tr>
<tr>
<td>MinC$_{Ec}$</td>
<td>MinD$_{Ng}$-K16Q</td>
<td>.</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

$^a$GAL4-DNA binding domain

$^b$GAL4-activation domain

$^c$Colour intensity relative to the respective positive control interactions: (+++) dark blue; (+++) blue; (+) faint blue; (-) white colonies

$^d$Activity in Miller Units. One unit equals the amount of $\beta$-galactosidase which hydrolyzes 1 $\mu$mol of ONPG per minute per cell

$^e$N. gonorrhoeae MinD

$^f$MinD$_{Ng}$-loop contains R92L, D93L, K94I substitutions

$^g$E. coli MinC
MinD$_{Ng}$-loop can still dimerize in the presence of ATP

It was recently shown that *E. coli* MinD dimerizes in the presence of ATP (Hu *et al.*, 2003). To confirm that MinD$_{Ng}$-loop could still self-interact in an ATP-dependent manner, the dimerization potential of purified MinD$_{Ng}$-loop in the presence or absence of nucleotide was compared to that of purified wild-type MinD$_{Ng}$ and MinD$_{Ng,K16Q}$ using size-exclusion chromatography. The short C-terminal His-tag of both MinD$_{Ng}$ and MinD$_{Ng}$-loop did not affect the function of the proteins as they both caused a filamentous phenotype upon overexpression in *E. coli* C41 (DE3) (Figure 6.4 A), similar to the untagged proteins in wild-type *E. coli* PB103 (Figure 6.3 B, C). Overexpression of the negative control, His-tagged MinD$_{Ng,K16Q}$, in *E. coli* C41 (DE3) did not inhibit cell division (Figure 6.4 E), and cells appeared as short rods, similar to cells carrying the negative control vector pET30a (Figure 6.4 G). His-tagged MinE$_{Ng}$ was also active, causing a minicell phenotype in *E. coli* (Figure 6.4 H, arrows indicate minicells), similar to overexpression of untagged *E. coli* MinE (de Boer *et al.*, 1989).

Each MinD protein was readily purified using nickel-affinity chromatography (Figure 6.4 B-F). Far-UV circular dichroism analyses conducted on each MinD$_{Ng}$ protein confirmed they all had similar secondary structure; hence, the mutant proteins did not have significant structural perturbations compared to the wild-type (Figure 6.5 A, B).

Purified MinD has been shown to bind to the non-hydrolyzable ATP derivative, ATP-$\gamma$-S (Hu *et al.*, 2002; Lackner *et al.*, 2003). ATP-$\gamma$-S was used in these experiments to prevent any nucleotide hydrolysis that may occur during gel-filtration. In the absence of nucleotide, the majority of wild-type MinD$_{Ng}$ (Figure 6.6 A), MinD$_{Ng,K16Q}$ (Figure 6.6 C), and MinD$_{Ng}$-loop (Figure 6.6 E) eluted from the column at an apparent molecular weight consistent with monomeric protein, as seen by a large single absorbance peak corresponding to elution volumes from 71-75 ml. In each case, a small fraction of protein was dimeric, as indicated by a minor absorbance peak that appeared in the 63-68 ml fractions.
Figure 6.4. Expression and purification of C-terminal His-tagged MinD<sub>N<sub>g</sub></sub> proteins in *E. coli* C41 (DE3). A) Cells transformed with pSC9 (minD<sub>N<sub>g</sub></sub>-His) display a filamentous phenotype. B) Purification of MinD<sub>N<sub>g</sub></sub>-His from induced *E. coli* cultures. Lane 1, 5 mM imidazole wash buffer eluate; lane 2, 60 mM imidazole wash buffer eluate; lane 3, 1 M imidazole elution. (C) Cells transformed with pJS8 (minD<sub>N<sub>g</sub>-loop</sub>-His) display a filamentous phenotype. (D) Purification of MinD<sub>N<sub>g</sub>-loop</sub>-His from induced *E. coli* cultures. Lane 1, 5 mM imidazole wash buffer eluate; lane 2, 60 mM imidazole wash buffer eluate; lane 3, 1 M imidazole elution. E) Cells transformed with pSC10 (minD<sub>N<sub>g</sub>-K16Q</sub>-His) have a short rod morphology. (F) Purification of MinD<sub>N<sub>g</sub>-K16Q</sub>-His from induced *E. coli* cultures. Lane 1, 5 mM imidazole wash buffer eluate; lane 2, 60 mM imidazole wash buffer eluate; lane 3, 1 M imidazole elution. G) Cells transformed with the negative control vector pET30a have a short rod morphology. (H) Cells transformed with pEC1 (minE<sub>N<sub>g</sub></sub>-His) have a minicell phenotype. Arrows indicate minicells. Scale bar in (A) represents 5 μm and all figures are at the same magnification.
Figure 6.5. Circular dichroism spectroscopy analysis of purified C-terminal His-tagged MinD$_{Ng}$, MinD$_{Ng}$-K16Q, and MinD$_{Ng}$-loop. Far-UV Circular dichroism (CD) spectroscopy was performed at 20°C and at protein concentrations of ~0.1 mg/ml in Buffer A. All CD experiments were repeated three times to ensure reproducibility. (A) Far-UV spectra of wild-type (WT) MinD$_{Ng}$ and MinD$_{Ng}$-K16Q. (B) Far-UV spectra of wild-type MinD$_{Ng}$ and MinD$_{Ng}$-loop.
Figure 6.6. Gel-filtration of purified wild-type MinD_Ng, MinD_Ng-loop, and MinD_Ng-K16Q (A, C, E) Elution profiles of (A) wild-type MinD_Ng, (C) MinD_Ng-K16Q, and (E) MinD_Ng-loop applied individually to a Superdex 200 column equilibrated with PBS (pH 7.4). (B, D, F) Elution profiles of (B) wild-type MinD_Ng, (D) MinD_Ng-K16Q, and (F) MinD_Ng-loop preincubated with 1 mM ATP-γ-S and 1 mM MgCl₂, and applied individually to the same column equilibrated with PBS (pH 7.4) supplemented with 0.5 mM ATP and 1 mM MgCl₂. Flow rate was set at 0.7 ml/min. 0.5 ml fractions were collected and analyzed for protein content in order to generate elution profiles. The association state of MinD_Ng proteins, based on elution profiles of gel-filtration standards, is also shown above the elution profiles.
Upon addition of ATP-γ-S, the elution profile of wild-type protein changed, with more protein shifting in favour of a dimeric association (Figure 6.6 B). MinD_{Ng-loop} also had an increase in eluted dimeric protein, with a greater proportion of dimers formed relative to monomers compared to wild-type (Figure 6.6 F). This result is consistent with the stronger self-interaction of MinD_{Ng-loop} compared to wild-type MinD_{Ng}, observed using the yeast two-hybrid system. No significant increases in the absorbance peak corresponding to dimeric MinD_{Ng,K16Q} was observed when this protein was preincubated with ATP-γ-S (Figure 6.6 D) compared to protein in the absence of nucleotide. Overall, these results confirm the self-association of MinD_{Ng-loop} observed by the yeast two-hybrid system and shows the mutant protein dimerizes in an ATP-dependent manner.

ATPase activity of purified MinD_{Ng-loop} is not stimulated by MinE_{Ng} and phospholipids

Since MinD_{Ng-loop} could still dimerize in the presence of ATP, the ATPase activity of this mutant was investigated to determine whether mutations to residues 92-94 affect MinD enzymatic activity. *E. coli* MinD has been shown to preferentially bind anionic phospholipids (Mileykovskaya, 2003; Szeto *et al.*, 2003); hence, artificial phosphatidylycerol (PG) vesicles were made and used for MinD_{Ng} ATPase stimulation assays with MinE_{Ng}. In the absence of MinE_{Ng}, wild-type MinD_{Ng} exhibited a basal level of ATPase activity when combined with phospholipid vesicles (Figure 6.7). However, the addition of MinE_{Ng} resulted in a significantly increased release of inorganic phosphate, up to threefold the basal level after 90 minutes (Figure 6.7).

Without MinE_{Ng}, the ATPase activities of MinD_{Ng-loop} and MinD_{Ng,K16Q} were low, similar to wild-type protein (Figure 6.7). Interestingly, the addition of MinE_{Ng} did not significantly raise the levels of inorganic phosphate released from MinD_{Ng-loop}, similar to MinD_{Ng,K16Q} (Figure 6.7). Therefore, despite retaining homodimerization and strong interaction with MinE, as shown by yeast two-hybrid assays, and the ability to form cell-spanning intracellular coils *in vivo*, the ATPase activity of MinD_{Ng-loop} could not be stimulated in the presence of MinE_{Ng} and lipid vesicles.
Figure 6.7. MinD ATPase stimulation assays using wild-type MinD_{Ng}, MinD_{Ng-loop}, and MinD_{Ng-K16Q}. Equal amounts of purified MinD_{Ng}, MinD_{Ng-loop}, and MinD_{Ng-K16Q} were incubated with PG vesicles and 1 mM ATP. The ATPase activities of each were tested in the presence or absence of MinE_{Ng} over a 90 minute period. Inorganic phosphate released due to ATP hydrolysis was monitored using a malaichite green based method. Buffer A is MinD_{Ng} storage buffer, Buffer B is MinE_{Ng} storage buffer.
GFP-fusions to MinDN_{Ng} and MinDE_{Ec} proteins containing substitutions within residues 92-94 do not display distinct pole-to-pole oscillations in *E. coli*

While mutations at residues 92-94 did not disrupt the ability of MinDN_{Ng-loop} to inhibit cytokinesis in wild-type *E. coli*, the inability of MinDN_{Ng-loop} to be stimulated by MinEN_{Ng} suggested the dynamic localization of the mutant might be affected. GFP-MinDN_{Ng} exhibits distinct, MinEN_{Ng}-dependent, pole-to-pole oscillation in *E. coli* rods (Chapter 4). Hence, plasmid-encoded GFP fusions to mutant MinDN_{Ng} were constructed for dynamic localization studies in *E. coli* PB114 (ΔminCDE) (Table 2.1). The minEN_{Ng} gene was included downstream of each fusion gene to provide the stimulation needed to induce MinDN_{Ng} oscillation. Since *E. coli* PB114 has a minicell phenotype consisting of small anucleate cells and rods of varying lengths, oscillatory movement was measured in cells of similar length (2.0-2.5 μm) to maintain consistency.

As expected, wild-type GFP-MinDN_{Ng} displayed periodic oscillations and clearly moved from the membrane at one polar region to the other (Figure 6.8 A, B). The average oscillation cycle of GFP-MinDN_{Ng} (from one pole to the other, and back again) measured in twenty cells was 29.1 ± 7.3 seconds. GFP-MinDN_{Ng-E103I} also displayed a similar pole-to-pole oscillation in *E. coli* PB114 rods as the wild-type fusion (Figure 6.8 E, F), with an average cycle, calculated from twenty cells, of 18.6 ± 2.0 seconds. In the absence of MinEN_{Ng}, GFP-MinDN_{Ng} appeared along the entire inner cell periphery, presenting a smooth, unbroken fluorescent signal without evidence of oscillatory movement (Figure 6.8 K), similar to a report with GFP-MinDE_{Ec} (Raskin and de Boer, 1999a).

Despite the presence of MinEN_{Ng}, raw images of GFP-MinDN_{Ng-loop} localization in *E. coli* PB114 showed that the fusion protein displayed no obvious pole-to-pole oscillations and was distributed along the entire inner periphery of the cell (Figure 6.8 H). This resulted in GFP-MinDN_{Ng-loop} localizing not only along cell pole regions, but across the membrane at midcell as well, similar to wild-type GFP-MinDN_{Ng} in the absence of MinEN_{Ng} (Figure 6.8 K). Western blotting confirmed similar expression
levels of all GFP-MinD<sub>Ng</sub> fusions (Figure 6.8 L) and the expression of MinE<sub>Ng</sub> (Figure 6.8 M) in each
_E. coli_ transformant used in localization studies.

Hence, these experiments indicate that mutations within amino acid residues 92-94 of
MinD<sub>Ng</sub> and MinD<sub>Ex</sub> disrupt the topological specificity of MinD in _E. coli_, despite the presence of
MinE. As a result, the mutant proteins no longer undergo the distinct pole-to-pole movement that
normally sequesters the protein away from the midcell.

**Uniform distribution of GFP-MinD<sub>loop</sub> proteins throughout an intracellular coiled array**

Closer examination of GFP-MinD<sub>Ng-loop</sub> localization revealed that its membrane association
appeared discontinuous, with punctate points of higher fluorescence along the membrane (Figure 6.8
H). Since this localization of GFP-MinD<sub>Ng-loop</sub> might represent contact sites between the membrane
and an intracellular GFP-MinD<sub>Ng-loop</sub>-containing array, image enhancement was used to determine
whether such a higher ordered structure could be visualized _in vivo._

As observed previously (Chapter 4), raw fluorescent images of wild-type GFP-MinD<sub>Ng</sub>,
provided evidence of the fusion dynamically localizing within coil-like structures extending along the
long axis of cells that also encoded MinE<sub>Ng</sub> (Figure 6.8 C), similar to a recent report of wild-type
GFP-MinD<sub>Es</sub> (Shih _et al._, 2003). Again, these coiled structures were readily visible in longer cells (≥
5 μm), since fluorescent signal concentration at the poles of shorter cells tended to obscure details of
the helical array. Image enhancement revealed that these dynamic bands were composed of
accumulated GFP-MinD<sub>Ng</sub> signal that localized in at least two adjacent turns of a coil (Figure 6.8 D,
arrows).

It was also revealed that GFP-MinD<sub>Ng-E110T</sub> assembled dynamically within coiled structures
similar to wild-type GFP-MinD<sub>Ng</sub>, with brighter regions defined by accumulated fluorescent signal
along at least two turns of the coil (Figure 6.8 G, arrows). Although localized along the entire inner
cell membrane, there was no evidence of any GFP-MinD<sub>Ng</sub> decorated coils when the fusion was
expressed in the absence of MinE_{Ng} (Figure 6.8 K). The negative control GFP-MinD_{Ng-K16Q} appeared distributed in the cytoplasm, despite the presence of MinE_{Ng} (see Figure 4.6 D).

Interestingly, enhancement of raw images revealed that GFP-MinD_{Ng-loop} still localized as coiled structures along the length of the cell. However, unlike wild-type protein, coordinated fluorescent signal movement could not be distinguished at all. While there seemed to be some movement of protein, it appeared highly disordered along the coil, such that any distinct pole-to-pole motion or fusion protein banding was not observed. As a result, fluorescent signal was distributed throughout the entire helical array extending the length of the cell (Figure 6.8 I). Due to this uniform localization of GFP-MinD_{Ng-loop}, it was also possible to discern that MinD coiled arrays likely consisted of at least two coils arranged in a double-helix conformation (Figure 6.8 I), similar to observations with MinD_{Ec} (Shih et al., 2003). Hence, while still able to localize as a membrane-associated coil, GFP-MinD_{Ng-loop} has lost its topological specificity, since the protein did not preferably localize to any particular region of the inner membrane or the coiled array. Similar uniform distribution of fluorescent signal along a coiled array was also observed with GFP-MinD_{Ng-K94A} (Figure 6.8 J), again in stark contrast to the obviously distinct banding seen with wild-type GFP-MinD_{Ng} (Figure 6.8 D).

To determine whether these attributes were characteristic of other MinD proteins bearing mutations within the ‘loop’ region, a GFP fusion to an _E. coli_ MinD mutant containing R92L, D93L, and K94I substitutions (GFP-MinD_{Ec-loop}) was created. This mutant also did not display obvious oscillation patterns. Image enhancement revealed that the fusion protein still localized uniformly along a coiled array throughout the cell, including at the midcell (Figure 6.9 D), similar to its gonococcal counterpart. In contrast, wild-type GFP-MinD_{Ec} exhibited pole-to-pole oscillation (Figure 6.9 A, B). Furthermore, unlike GFP-MinD_{Ec-loop}, evidence of wild-type GFP-MinD_{Ec} localizing within a coiled array could only be clearly visualized in specific regions (e.g. the cell poles) at any particular time (Figure 6.9 C), consistent with the coordinated dynamic movement of GFP-MinD_{Ec} subunits over the array.
Figure 6.9. Localization of GFP-fusions to wild-type *E. coli* MinD (MinD<sub>Ec</sub>) and a 'loop' region MinD<sub>Ec</sub> mutant in the presence of minE<sub>Ec</sub> within *E. coli* PB114. (A, B) Pole-to-pole oscillation of wild-type GFP-MinD<sub>Ec</sub>. Time between images is 30 seconds. (C) Enhanced image showing localization of wild-type GFP-MinD<sub>Ec</sub> as a coil at one cell pole. (D) Enhanced image of GFP-MinD<sub>Ec-loop</sub> localizing throughout a coiled structure extending the length of the cell. Bar in (A) indicates 1 μm and all images are at the same magnification.
Hence, GFP-MinD mutants bearing hydrophobic substitutions in the polar region encompassing residues 92-94 of MinD can still localize as intracellular coils. However, they do not display obvious intracellular oscillation patterns. As a result, they appear to have lost normal topological specificity by accumulating at all potential division sites, including the midcell, by virtue of their uniform distribution along a membrane-associated helical array.
6.4 DISCUSSION

Using both structural and protein sequence alignments, coupled with a number of functional assays, a region in bacterial MinD has been identified to be implicated in rendering the protein responsive to MinE stimulation. Originally, this study was directed towards characterizing potential MinD homodimerization interfaces; however, unlike its structural counterpart NifH, the polar residues at positions 92-94 of MinDNg are not required for MinDNg dimerization. Furthermore, mutation of these residues did not disrupt the ability of MinDNg to induce cell division arrest or to abrogate its interaction with MinC and MinE.

The relatively uniform distribution of GFP-MinD ‘loop’ mutants within coiled arrays was a striking contrast to wild-type GFP-MinD coiled arrays, which displayed clearly defined regions of dynamic protein accumulation. GFP-MinDEc has been shown to localize within coils in a MinEc-dependent manner (Shih et al., 2003). It is likely that GFP-MinDNg-loop was still able to form coiled arrays because MinDNg-loop retained interaction with MinENg, as shown by our yeast two-hybrid assays. Since GFP-fusions to either MinDNgK94A or MinDEc-loop also localized as helical arrays in vivo, they likely interact with MinENg as well.

This study indicates that the inability of MinENg to effectively stimulate the ATPase activity of MinDNg-loop might account for the absence of distinct, coordinated oscillations of GFP-MinDNg-loop in vivo. It is also likely that MinDNgK94A and MinDEc-loop are not responsive to MinE stimulation, leading to their near uniform distribution along intracellular coiled arrays. In support of this, E. coli MinE mutants that are deficient in stimulating the ATPase activity of wild-type GFP-MinDEc cannot induce the intracellular oscillation of the latter, which remains distributed along the entire inner cell membrane (Hu and Lutkenhaus, 2001); however, whether GFP-MinDEc can still localize within coiled arrays in this case is not known. Moreover, the diminished interaction of MinDNg-loop with MinC could not possibly have contributed to the loss of coordinated MinDNg-loop oscillation, since MinC was not present in the E. coli strain used in our localization studies. Furthermore, it is established that MinC is not required for MinD oscillation (Raskin and de Boer, 1999a).
The lack of distinct pole-to-pole movement in *N. gonorrhoeae* and *E. coli* GFP-MinD ‘loop’ mutants effectively led to their localization along coiled arrays across the midcell, in addition to the remainder of the cell. This indicated a loss of topological specificity normally defined by clear oscillations that direct MinD away from the midcell site (de Boer *et al.*, 1989; Raskin and de Boer, 1999a; Hu and Lutkenhaus, 2001). This disrupted topological specificity differed fundamentally from that of wild-type GFP-MinD<sub>Ng</sub> expressed in the absence of MinE. While the lack of MinE<sub>Ng</sub> resulted in a uniform distribution of GFP-MinD<sub>Ng</sub> along the inner cell membrane, the fusion protein did not localize within any coiled arrays, in stark contrast to our mutant GFP-MinD proteins. It is noted that GFP-MinD ‘loop’ mutants were not totally devoid of dynamism, and did exhibit what appeared to be random disordered shifting throughout their coiled structures. The residual basal ATPase activity remaining in these mutants may have been sufficient to allow this disorganized protein movement that clearly did not present any preference for specific coil and/or membrane regions. Hence, the MinD<sub>Ng-loop</sub> mutant, and likely MinD<sub>Ng-K94A</sub> and MinD<sub>Ec-loop</sub> of the present study represent the first MinD proteins characterized to be non-responsive to MinE-induced stimulation and topological specificity, despite the ability to interact with all other Min proteins and to localize within a membrane-associated coiled array.

MinE<sub>Ec</sub> has also been shown to promote the bundling of MinD<sub>Ec</sub> filaments under certain *in vitro* conditions, leading to the suggestion that MinE may have a secondary role of cross-linking polymerized MinD (Suefuji *et al.*, 2002). Since an increased interaction of MinD<sub>Ng-loop</sub> with MinE<sub>Ng</sub> was detected using the yeast two-hybrid system, it is tempting to speculate that, in addition to its inability to be stimulated by MinE<sub>Ng</sub>, increased bundling of GFP-MinD<sub>Ng-loop</sub> by MinE<sub>Ng</sub> may have prevented the normal dissociation of GFP-MinD<sub>Ng</sub> subunits from the membrane-associated Min protein coil. It is possible that the residues within the defined ‘loop’ region of MinD are involved in providing optimal binding affinity to MinE to correctly regulate MinD bundling and/or ATPase activity. Proper protein bundling has also been shown to be important in FtsZ function, as it was recently shown that a mutant FtsZ with altered bundling properties could not initiate cell division,
despite its proper localization at midcell (Koppelman et al., 2004). In addition, the increased affinity between MinD_{Ng-loop} and MinE_{Ng}, as observed by yeast two-hybrid assays, may prevent proper conformational changes from occurring in each protein that are essential prerequisites for MinD ATPase induction.

Possibly the stronger self-association of MinD_{Ng-loop} compared to wild-type MinD_{Ng} (determined by the yeast two-hybrid system) may have contributed to the inefficient disassembly of MinD_{Ng-loop} subunits from their intracellular helical array. However, yeast two-hybrid assays also detected a stronger self-association of MinD_{Ng-E103I} in comparison to wild-type MinD_{Ng}. Since GFP-MinD_{Ng-E103I} could still clearly oscillate in vivo, it argues against the idea that increased MinD self-association affinity would lead to a loss of pole-to-pole oscillation along the coiled-array.

The MinD_{Ng-K16Q} mutant did not dimerize as efficiently as the other MinD_{Ng} proteins, although there was slight shift of the MinD_{Ng-K16Q} ‘monomer’ peak towards shorter retention times suggesting weak self-association. An E. coli MinD_{K16Q} mutant was reported to bind ATP as strongly as wild-type protein (Hu and Lutkenhaus, 2001); hence, the diminished self-association of MinD_{Ng-K16Q} in the present study suggests that such mutants in general may be deficient in a conformational change that normally allows efficient protein dimerization following nucleotide binding. As a result of disrupted homodimerization, this mutant protein would not bind membrane targets, in accordance with the ‘dimer trigger’ model of MinD membrane association (Hu and Lutkenhaus, 2003), and would not be stimulated by MinE. Since MinD_{Ng-K16Q} lost interaction with MinC and MinE, it also raises the possibility that dimerization of MinD may have a role in its interaction with the other two Min proteins. The lack of interaction detected between MinD_{Ng-K16Q} and MinE_{Ng} would also likely render this MinD mutant unable to form any helical array, further contributing to its uniform cytoplasmic distribution. It is also noted that, given the high MinD_{Ng} concentrations and excess ATP-γ-S used in the gel-filtration studies, the proportion of wild-type MinD_{Ng} or MinD_{Ng-loop} that dimerized was still quite low. This might signify a low dimerization constant for MinD_{Ng} proteins in general.
Alternatively, it has been shown that MinD has less affinity for ATP-γ-S than ATP (Lackner et al., 2003), which may have contributed to less dimerization than expected.

Whether the affinity of MinD ‘loop’ mutants for ATP is altered remains to be determined. The R92, D93, and K94 residues of MinD_{Nz} and MinD_{Es} do not correspond to ATP-binding residues, based on Archaeal MinD structures (Figure 6.1B, cyan residues) (Hayashi et al., 2001; Sakai et al., 2001). However, should an increased affinity for ATP exist, it is conceivable that these MinD mutants may have increased propensities to remain on, or rapidly return to, their membrane-associated coiled array, such that normal dynamic oscillation is lost. The loss of topological specificity of MinD aa 92-94 mutants in this study was also not a result of mutating residues directly implicated in hydrolyzing ATP (e.g. D40 and A121, as indicated by the P. furiosus MinD structure) (Hayashi et al., 2001) or those found in the switch I and switch II sites of MinD (Cordell et al., 2001; Sakai et al., 2001). Switch I and II sites in various ATPases normally undergo conformational changes during nucleotide hydrolysis (Vale, 1996). A recent report showed that MinD_{Es} switch I and II mutants lose interaction with MinC_{Es}, but not their MinE-induced ATPase activity (Zhou and Lutkenhaus, 2004), in contrast to MinD_{Nz}-loop in this study. Hence, the activity of the switch I and II sites in our MinD_{Nz}-loop mutant was likely not affected.

What function might the polar MinD ‘loop’ region have in allowing the protein to be topologically regulated? Based on our model of dimeric MinD, the residues of interest correspond to a short α-helix in each P. furiosus MinD monomer (Figure 6.10 A, green residues) located directly opposite the C-terminal membrane targeting sequence (MTS). While the actual crystal structure of the MTS of Archaeal MinD has not been obtained due to structural disorder in this region (Cordell and Löwe, 2001; Hayashi et al., 2001), it will be assumed that the MTS is in proximity to the C-terminus indicated in Figure 6.10 A (cyan residues). In vitro experiments have shown that MinD polymer bundles may possess polarity (Suefuji et al., 2002), similar to other proteins such as tubulin; hence, a possible arrangement for MinD polymers at the membrane surface is shown in Figure 6.10 C’. Upon
Fig. 6.10. A proposed model for MinD ‘loop’ region function. (A) The predicted MinD dimer generated by superimposition of \textit{P. furiosus} \textit{MinD} (MinD\textsubscript{Pr}) (Hayashi et al., 2001) monomers onto each subunit of the \textit{A. vinelandii} NiFH dimer. The green residues (aa 87-93) of MinD\textsubscript{Pr} represent a region that corresponded with the loop region in each NiFH monomer. Note: the structure and exact location of the C-terminal membrane targeting sequence (MTS) of MinD has not been determined yet. The cyan residues indicate the C-terminal residues in the solved structure that are likely in proximity to the MTS. Dotted line indicates proposed MinD dimerization interface. (B) A schematic representation of a MinD monomer. Here, the membrane targeting sequence (MTS), is deployed in this representative molecule, which has bound ATP (red circle) at the dimerization interface (partner MinD not shown). (B) Conserved polar residues R92, D93, and K94 of MinD\textsubscript{Ng} and MinD\textsubscript{Ec} are predicted to be in proximity to the MinD dimerization interface and exposed to the cytosol. The ‘loop’ region, containing R92, D93, and K94 residues is shown opposite of the MTS. (C) Dimeric, ATP-bound MinD associates with the inner cell membrane and/or Min protein coil through the activated MTS. The division inhibitor MinC (not shown) would also be recruited to the membrane by MinD. MinE (triangles) initiates binding to MinD-ATP. MinD will also polymerize along the membrane to form polymeric structures. (C') Possible arrangement of a MinD protofilament at the membrane. Note, the membrane is not shown to simplify the schematic. (+) and (-) symbols designate the possibility of polarity in the MinD filaments (Szeto et al., 2003), as suggested by \textit{in vitro} polymerization studies (Suefuji et al., 2002). (D) Residues R92, D93, and K94 are not directly involved in MinE binding; however, the effects of MinE binding may be transmitted by these MinD residues (blue ovals) to other parts of the protein, particularly sites involved in ATPase activity at the MinD dimer interface. As a result, MinD ATPase activity is stimulated and ATP is hydrolyzed to form ADP (white circles). (E) ATP hydrolysis terminates MinD dimerization and its association at the membrane and with the remaining MinD polymer (not shown). Cytosolic MinD subunits are free to move to other membrane/Min coil segments.
membrane association, it is possible that R92, D93, K94, and surrounding residues of bacterial MinD proteins, will remain exposed and oriented towards the cytoplasm, opposite the MTS (Figure 6.10 B, C). These residues would be situated in close proximity to, but not within, the MinD dimerization interface (Figure 6.10 A and B). This interface includes the ATP binding sites of each MinD monomer (Figure 6.1 B, cyan residues), based on structural similarities of MinD to NifH (Lutkenhaus and Sundaramoorthy, 2003; Cordell and Löwe, 2001; Sakai et al., 2001; this study), such that the dimerized form of MinD is primed for ATP hydrolysis (Lutkenhaus and Sundaramoorthy, 2003).

Although not essential for interaction with MinE, the residues in the ‘loop’ region may serve to sensitize and/or transmit the effects of MinE binding to the MinD-MinD interface through conformational change, thus inducing ATPase activity and MinD oscillation (Figure 6.10 D, E). The B-factor profile of residues corresponding to the ‘loop’ region in at least one Archaeal MinD homologue (from *Archaeoglobus fulgidus*) (Cordell and Löwe, 2001) shows this region possesses some flexibility, which might also be found in bacterial MinD homologues to allow for conformational change. By mutating this region, the ability of MinD to properly respond to MinE binding would be disrupted and its ATPase activity and coordinated pole-to-pole oscillation would not be induced.

The MinD_{Ng-loop} mutant, and likely MinD_{Ng-K94A} and MinD_{Ec-loops} constructed in the present study represent the first MinD proteins characterized to have retained interaction with all components of the Min protein system, and yet have truly lost topological specificity while at the membrane. Without an intact C-terminal membrane targeting sequence, mutants of *E. coli* MinD also do not oscillate (Szeto et al., 2002; Hu and Lutkenhaus, 2003), and it could be argued that they have lost topological specificity. However, like MinD_{K16Q} mutants, this ‘loss’ of topological specificity is simply due the inability of these C-terminal MinD mutants to localize to the membrane. Topological specificity mutants of *B. subtilis* MinD (MinD_{Bs}) have been described that localize peripherally along the membrane (El Karoui and Errington, 2001). However, unlike the MinD ‘loop’ mutants in the present study, the MinD_{Bs} mutants no longer activated MinC cell division arrest, or had lost
interaction with their Gram-positive specific topological specificity determinant DivIVA (El Karoui and Errington, 2001). Interestingly, while residues 86-97 of the predicted 'loop' region of MinD<sub>N</sub> are extremely conserved in Gram-negative organisms, there is less conservation in the corresponding region of MinD from Gram-positive bacteria. It is possible that this amino acid degeneracy reflects the absence of MinE homologues in Gram-positive bacteria (Margolin, 2001a).

This study employed a homologous structure-based approach to identify a polar region in bacterial MinD that, when mutated, clearly disrupts MinD pole-to-pole oscillation, but not its ability to localize as intracellular coils. Hence, these mutations disrupted the ability of MinD to have topological specificity imparted upon it. It is proposed that amino acid residues 92-94, and possibly the spatially proximal residues as well, may play a role in transmitting the effects of MinE-binding to the ATPase regions of MinD dimers. Significantly, these studies show the ability of MinD to bind to MinE is not sufficient for the latter to stimulate MinD ATPase activity and to coordinate its dynamism. While MinD proteins bearing mutations in residues 92-94 can still interact with MinE, the latter protein cannot efficiently stimulate MinD ATPase activity and/or induce significant MinD movement along the Min protein membrane lattice. Clearly, structural studies should provide additional insight into the role that specific residues selected in this study may have in modulating effective MinD-MinE interaction.
CHAPTER 7

Mutational analysis of the N-terminus of MinD_{Ng} indicates this region contains determinants that affect MinD_{Ng} ATPase activity and dynamic localization

Portions of this chapter were published in:


and also included in the following submitted manuscript:

7.1. PURPOSE OF THIS STUDY

Sequence alignment of MinD proteins show they are highly conserved. In general, the N-terminus of MinD appears to have greater sequence conservation than the C-terminal half (See Figure 3.1). While there is increasing information regarding the membrane targeting role of the extreme C-terminus of MinD (Szeto et al., 2002; Szeto et al., 2003; Hu and Lutkenhaus, 2003; Zhou and Lutkenhaus, 2003), little is known about its N-terminus. Perhaps the most characterized N-terminal region of MinD is its Walker A ATP-binding motif [amino acids (aa) 10-17 in MinD_{E.coli} and MinD_{Ng}] (Figure 3.1). In particular, mutation of the conserved K16 residue disrupted MinD_{Ng} ATPase activity, dimerization, and membrane localization (Chapters 5 and 6).

This study was conducted to determine whether the conserved residues at the extreme N-terminus of MinD_{Ng} are involved in protein functionality. To characterize this region, sequential deletions or point mutations were generated to residues at the N-terminus of MinD_{Ng} and mutant proteins were evaluated using several functional assays. These truncations or mutations disrupted MinD_{Ng} interaction with other Min proteins, particularly with itself and MinE. The majority of GFP-MinD_{Ng} mutants could still oscillate from pole-to-pole in E. coli. However, as truncations or mutations were successively generated from the N-terminus of MinD_{Ng}, GFP-MinD_{Ng} oscillation cycles became significantly faster, accompanied by an increased tendency for GFP-MinD_{Ng} fusions to remain distributed in the cytoplasm. Interestingly, in vitro ATPase assays indicated that MinD_{Ng} proteins lacking the first three residues (replaced with a start codon), or having an I5E substitution, possessed hyper-ATPase activities that were independent of MinE_{Ng}. From these studies, it is proposed that determinants found within the extreme N-terminus of MinD_{Ng} are involved in regulating the enzymatic activity of the protein, which in turn affects its dynamic localization.
7.2. MATERIALS AND METHODS

Generation of deletions derivatives and N-terminal mutants of \( \text{minD}_{\text{Ng}} \) for \( E. \text{coli} \) expression studies. N-terminal truncated \( \text{minD}_{\text{Ng}} \) genes were cloned into pUC18 (Table 7.1) for morphology studies in \( E. \text{coli} \) PB103 (Table 2.1). In order to preserve protein translation, the primers used to generate N-terminal deletions were designed to place the natural GTG start codon of \( \text{minD}_{\text{Ng}} \) directly upstream of codons encoding residues 3, 4, 5, and 20, to generate N-terminal deletions to \( \text{MinD}_{\text{Ng}} \). These primers included MinD-N2, MinD-N3, minD11B, and minD12B, and were used in conjunction with primer minD2 (Table 2.2) to PCR amplify truncated \( \text{minD}_{\text{Ng}} \) from gonococcal DNA. As a result, \( \text{minD}_{\text{Ng}} \) genes where generated where the first 2, 3, 4, and 19 codons (and hence, amino acids) were deleted and replaced with a start codon. The resulting amplicons were digested with \( \text{EcoRI} \) and \( \text{BamHI} \) (incorporated by each mutant primer and minD2, respectively) and digested with similarly digested pUC18 to produce plasmids pSIA10 (\( \text{minD}_{\text{Ng}}\text{-3aaNT} \)), pSIA11 (\( \text{minD}_{\text{Ng}}\text{-4aaNT} \)), pJS4B (\( \text{minD}_{\text{Ng}}\text{-4aaNT} \)), and pJS5B (\( \text{minD}_{\text{Ng}}\text{-19aaNT} \)), where ‘NT’ designates the number of N-terminal amino acids replaced with a start codon (Table 7.1).

To create mutant \( \text{minD}_{\text{Ng}} \) genes containing specific N-terminal substitutions, site-directed mutagenesis was employed. Primers incorporating the specific mutation of interest were used with primer minD2 (Table 2.2) to amplify \( \text{minD}_{\text{Ng}} \) from pSR3 (Table 7.1) or from gonococcal cell suspensions. Mutant primers included (with respective substitutions in parentheses): SA5 (K3E), SA6 (K3I), SA7 (K3A), SA13 (K3F), SA8 (I4Q), SA10 (I5E), and SA11 (I5A) (Table 2.2). Each amplicon was digested with \( \text{EcoRI} \) and \( \text{BamHI} \) and ligated into similarly digested pUC18 to generate plasmids pSIA13, pSIA22, pSIA23, pJS16, pSIA24, pJS13 and pJS14 (Table 7.1), respectively.

Construction of yeast two-hybrid vectors encoding GAL4 fusions to N-terminal mutants of \( \text{MinD}_{\text{Ng}} \). Amplicons used to generate the N-terminal deletion and mutant forms of \( \text{minD}_{\text{Ng}} \) were also cloned into \( \text{EcoRI} \) and \( \text{BamHI} \) digested yeast two-hybrid vectors pGAD424 and pGBT9 (Table 7.1).
### Table 7.1. Plasmids used in this study

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<th>Plasmids</th>
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<td><strong>Yeast two-hybrid plasmids</strong></td>
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<td>pGBT9; P&lt;sub&gt;ADH1&lt;/sub&gt;::gal4(BD)-minD&lt;sub&gt;Ng&lt;/sub&gt;-15E (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
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pJS21  pGBT9; PAHII::gal4(BD)-minDNg15A (Amp^R)  This study
pSIA3  pGAD424; PAHII::gal4(AD)-minDNg2aNT (Amp^R)  This study
pSIA4  pGAD424; PAHII::gal4(AD)-minDNg3aNT (Amp^R)  This study
pJminD12 pGAD424; PAHII::gal4(AD)-minDNg4aNT (Amp^R)  This study
pJminD13 pGAD424; PAHII::gal4(AD)-minDNg19aNT (Amp^R)  This study
pJminD3 pGAD424; PAHII::gal4(AD)-minDNg31aNT (Amp^R)  This study
pJminD4 pGAD424; PAHII::gal4(AD)-minDNg183aNT (Amp^R)  This study
pSIA14 pGAD424; PAHII::gal4(AD)-minDNgK3E (Amp^R)  This study
pJS25 pGAD424; PAHII::gal4(AD)-minDNgK3H (Amp^R)  This study
pSIA27 pGAD424; PAHII::gal4(AD)-minDNgK3A (Amp^R)  This study
pSIA28 pGAD424; PAHII::gal4(AD)-minDNg14Q (Amp^R)  This study
pJS11 pGAD424; PAHII::gal4(AD)-minDNg15E (Amp^R)  This study
pJS20 pGAD424; PAHII::gal4(AD)-minDNg15A (Amp^R)  This study

GFP-fusion plasmids

pDSW209  pDSW209; Prc::gfp (Amp^R)  Weiss et al., 1999
pSR15  pDSW209; Prc::gfp-minDNg, minENg (Amp^R)  Chapter 4, this study
pJDE1  pDSW209; Prc::gfp-minDNgK16Q, minENg (Amp^R)  Chapter 4, this study
pSIA16  pDSW209; Prc::gfp-minDNg2aNT, minENg (Amp^R)  This study
pSIA17  pDSW209; Prc::gfp-minDNg3aNT, minENg (Amp^R)  This study
pJDE2  pDSW209; Prc^d::gfp-minDNg4aNT, minENg (Amp^R)  This study
pJDE3  pDSW209; Prc^d::gfp-minDNg19aNT, minENg (Amp^R)  This study
pSIA18  pDSW209; Prc::gfp-minDNgK3E, minENg (Amp^R)  This study
pJS30  pDSW209; Prc::gfp-minDNgK3I, minENg (Amp^R)  This study
pJS18  pDSW209; Prc::gfp-minDNg14Q, minENg (Amp^R)  This study
pJS29  pDSW209; Prc::gfp-minDNg15E, minENg (Amp^R)  This study
pJS19  pDSW209; Prc::gfp-minDNg15A, minENg (Amp^R)  This study

Plasmids for protein purification

pET30a  Pr7:: 6XHis (Kan^R)  Novagen
pSC9  pET30a; Pr7:: minDNg-6XHis (Kan^R)  Chapter 6, this study
pJS33  pET30a; Pr7:: minDNg15E-6XHis (Kan^R)  This study
pJS34  pET30a; Pr7:: minDNg3aNT-6XHis (Kan^R)  This study
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<th>pSC10</th>
<th>pET30a; P&lt;sub&gt;T7&lt;/sub&gt;: minD&lt;sub&gt;Ne,K16Q&lt;/sub&gt;-6XHis (Kan&lt;sup&gt;R&lt;/sup&gt;)</th>
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</tr>
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</table>

* NT denotes N-terminal truncation [e.g. 2aaNT = deletion of first 2 amino acids (codons) from N-terminus]. In place of the removed codons is a start codon.

b Encodes GAL4 activation domain (AD)

c Encodes GAL4 DNA-binding domain (BD)
GAL4-DNA-BD fusions to MinD_{Ng-2aaNT}, MinD_{Ng-3aaNT}, MinD_{Ng-4aaNT}, MinD_{Ng-19aaNT} were encoded on pSIA1, pSIA2, pJminD8, and pJminD9, respectively (Table 7.1). GAL4-AD fusions to MinD_{Ng-2aaNT}, MinD_{Ng-3aaNT}, MinD_{Ng-4aaNT}, and MinD_{Ng-19aaNT} were encoded pSIA3, pSIA4, pJminD12, and pJminD13, respectively (Table 7.1). Additional N-terminal deletions, MinD_{Ng-31aaNT} and MinD_{Ng-183aaNT}, were also made using primers minD5 or minD6 paired with minD2 (Table 2.2), which were ligated in-frame with the GAL4-AD in pGAD424 to produce pJminD3 and pJminD4, respectively (Table 7.1). PCR amplicons encoding minD_{Ng} with N-terminal substitutions K3E, K3I, K3A, I4Q, I5E, and I5A (see above) were also cloned into EcoRI/BamHI digested yeast two-hybrid vectors. Plasmids encoding fusions to the GAL4-DNA-BD include, pSIA15 (MinD_{Ng-K3E}), pJS26 (MinD_{Ng-K3I}), pSIA29 (MinD_{Ng-K3A}), pJS22 (MinD_{Ng-I4Q}), pJS24 (MinD_{Ng-I5E}), and pJS21 (MinD_{Ng-I5A}) (Table 7.1). Vectors encoding fusions to the GAL-4-AD include pSIA14 (MinD_{Ng-K3E}), pJS25 (MinD_{Ng-K3I}), pSIA27 (MinD_{Ng-K3A}), pSIA28 (MinD_{Ng-I4Q}), pJS11 (MinD_{Ng-I5E}), and pJS20 (MinD_{Ng-I5A}) (Table 7.1).

Plasmids encoding GAL4 fusions to wild-type MinD_{Ng}, MinE_{Ng}, and MinC_{Es} (Table 7.1) were constructed previously (Chapter 5). Yeast two-hybrid studies were carried out as described in Chapter 2.

**Nucleoid localization in E. coli using DAPI staining.** To determine the location of nucleoids in *E. coli*, DAPI stain (4’-6-diamidino-2-phenylindole-2HCl) (Molecular Probes) was employed. Cells were fixed and adhered to polylysine coated coverslips as outlined in Chapter 2. Five μl of DAPI (0.2 μg/ml stock) was placed on a small sheet of Parafilm and coverslips containing attached bacteria were gently pressed onto the DNA stain. After 2 minutes incubation, coverslips were removed and gently washed with 10 ml PBS (pH 7.4) dispensed dropwise from a pipette. Coverslips were then placed over a 5 μl drop of 50% glycerol on a coverslip and sealed. Fluorescence microscopy was performed
on an Olympus BX61 microscope equipped with a Photometrics CoolSnap ES camera and Image Pro Version 5.0 software.

**Construction of GFP-fusions to MinD<sub>Ng</sub> mutants.** For localization studies, GFP-fusions to mutant MinD<sub>Ng</sub> were constructed. Primer MinE2 was used in combination with primers MinD-N2, MinD-N3, minD7, minD8, SA5, SA6, SA8, SA10, or SA11 (Table 2.2) to amplify specific truncated/mutated minD<sub>Ng</sub> genes together with minE<sub>Ng</sub> from gonococcal chromosomal DNA. The PCR amplicons, as well as plasmid pDSW209, encoding GFP (Weiss et al., 1999) (Table 7.1), were digested with EcoRI and BamHI and ligated. The resulting plasmids encoded GFP-MinD<sub>Ng-2anNT</sub> (pSIA16), GFP-MinD<sub>Ng-3anNT</sub> (pSIA17), GFP-MinD<sub>Ng-4anNT</sub> (pJDE2), GFP-MinD<sub>Ng-19anNT</sub> (pJDE3), GFP-MinD<sub>Ng-K3E</sub> (pSIA18), GFP-MinD<sub>Ng-K3I</sub> (pJS30), GFP-MinD<sub>Ng-14Q</sub> (pJS18), GFP-MinD<sub>Ng-15E</sub> (pJS29), and GFP-MinD<sub>Ng-15A</sub> (pJS19) (Table 7.1). Plasmids encoding wild-type GFP-MinD<sub>Ng</sub> (pSR15) and GFP-MinD<sub>Ng-K16Q</sub> (pJDE1) (Table 7.1) were constructed previously (Chapter 4). All plasmids also encoded MinE<sub>Ng</sub> downstream of each *gfp-minD<sub>Ng</sub>* variant.

**Localization of GFP-MinD<sub>Ng</sub> fusions.** *E. coli* WM1032 (Table 2.1) transformed with pSR15 (encoding wild-type GFP-MinD<sub>Ng</sub>) pJDE1 (GFP-MinD<sub>Ng-K16Q</sub>), pJDE2 (GFP-MinD<sub>Ng-4anNT</sub>), and pJDE3 (GFP-MinD<sub>Ng-19anNT</sub>) (Table 7.1) were examined using an Olympus BX60 fluorescence microscope, in collaboration with Dr. W. Margolin, University of Texas Medical School. Localization studies were carried out as essentially described in Chapter 4 Materials and Methods.

Subsequent studies involving wild-type GFP-MinD<sub>Ng</sub> (pSR15), GFP-MinD<sub>Ng-K16Q</sub> (pJDE1), GFP-MinD<sub>Ng-2anNT</sub> (pSIA16), GFP-MinD<sub>Ng-3anNT</sub> (pSIA17), GFP-MinD<sub>Ng-K3E</sub> (pSIA18), GFP-MinD<sub>Ng-K3I</sub> (pJS30), GFP-MinD<sub>Ng-14Q</sub> (pJS18), GFP-MinD<sub>Ng-15E</sub> (pJS29), and GFP-MinD<sub>Ng-15A</sub> (pJS19) (Table 7.1) were conducted using an Olympus BX61 microscope system equipped with a Photometrics CoolSnap ES camera and Image Pro (Version 5.0) software. To measure oscillation cycle periods,
each GFP-MinD<sub>Ng</sub> fusion was observed in 30 cells of near equivalent length (~2.0-2.5 µm) and time-
lapse images were taken every 5 seconds. In each cell observed, at least two complete oscillation
cycles were used to verify oscillation periods. Statistical analyses using unpaired Student’s t-tests
were performed to determine whether differences in average oscillation periods between GFP-
MinD<sub>Ng</sub> fusions were significant, with p<0.001 considered significant. When required, enhancement
of raw images was done using standard options available on Image Pro software. Contrast
enhancement was used to increase contrast and to decrease gamma. Gauss filtering using a 3 X 3
kernel size with 4 passes at strength 10 was performed to help visualize intracellular substructures.

**Purification of MinD<sub>Ng</sub> N-terminal mutants and ATPase stimulation assays.** Plasmids encoding
C-terminal His-tagged MinD<sub>Ng</sub>-15A and MinD<sub>Ng</sub>-3aaNT were obtained using an inverse PCR strategy with
pSC9 (encoding C-terminal His-tagged wild-type MinD<sub>Ng</sub>) (Chapter 6 and Table 7.1) as a template.
Primer SA19 and a mutant primer (SA17 or SA18; incorporating I5E or 3aaNT mutations,
respectively) (Table 2.2) were designed to anneal adjacent to each other and amplify in opposite
directions using Pfu DNA polymerase (Fermentas). The resulting blunt-ended linear DNA product
was subsequently religated to give pJS33 and pJS34 (Table 7.1). Plasmids pSC9 and pSC10,
encoding C-terminal His-tagged wild-type MinD<sub>Ng</sub> and MinD<sub>Ng</sub>-K16Q (Chapter 6 and Table 7.1), served
as a sources for positive and negative control proteins, respectively. Protein purification was carried
out as described in Chapter 6. Following dialysis in Buffer A, purified MinD<sub>Ng</sub>-15A and MinD<sub>Ng</sub>-3aaNT
were concentrated using Microcon YM-3 centrifuge devices (3,000 molecular weight cutoff)
(Amicon). MinD<sub>Ng</sub> ATPase stimulation assays with PG vesicles and MinE<sub>Ng</sub> were also conducted as
outlined in Chapter 6 Materials and Methods.
7.3. RESULTS

Deletion and point mutation studies of the MinDNg N-terminus indicate this region contains residues that are important for inducing cell division arrest. The high conservation of MinD makes it difficult to select any one particular amino acid for functional studies. Sequence alignment of MinD proteins revealed that the extreme N-terminus is well conserved (Figure 7.1), suggesting this region is important for protein function. In particular, Gram negative organisms feature a consensus sequence of M[A/G][K/R][I/V][I/V] for the first five residues, with the exception of *Vibrio cholerae* MinD, which contains a serine at position two; *Helicobacter pylori* MinD, which contains isoleucine at position three; and *Pseudomonas aeruginosa* MinD, containing a leucine at position five (Figure 7.1). Of particular note is that this region is identical in MinD from either *N. gonorrhoeae* or *E. coli*, except that MinDnc contains an arginine residue instead of a lysine residue at position three. Thus, structure/function analyses of the gonococcal protein can serve as a paradigm for other MinD proteins, especially those from Gram negative organisms including *E. coli*, since it is evident that MinDNg behaves in a similar manner as MinDnc (Chapter 4).

To elucidate residues at the extreme N-terminus of MinDNg that may be involved in protein function, deletion derivatives of MinDNg were constructed that removed the first 2, 3, 4 or 19 amino acids of the protein. As a result, each gene contained the 3rd, 4th, 5th, and 20th codon of minDNg immediately downstream of a start codon. The resulting truncated MinDNg proteins will be referred to as MinDNg-2aaNT, MinDNg-3aaNT, MinDNg-4aaNT, and MinDNg-19aaNT (NT indicating ‘N-terminal deletion’ of the specified number of residues, which are replaced by a single start codon). While the first three mutant proteins are comprised of successive deletions, MinDNg-19aaNT would contain a deletion of the entire Walker A ATP-binding motif (aa 10-17) (Figure 7.1, residues underneath black bar).

To determine whether these truncations affect the ability of MinDNg to induce cell division arrest in wild-type *E. coli* PB103 (Table 2.1), plasmids encoding each truncated protein were transformed into the indicator strain. While cells transformed with pUC18 negative control vector exhibited normal short rod morphologies (Figure 7.2 A), transformant expressing wild-type MinDNg
Figure 7.1. Sequence alignment of the N-terminus of MinD proteins. Abbreviations used: Methanococcus janaaschii (Mj), Archaeoglobus fulgidus (Af), Pyrococcus furiosus (Pf), P. horikoshii (Ph), Neisseria gonorrhoeae (Ng), N. meningitidis (Nm), Escherichia coli (Ec), Salmonella enterica serovar Typhimurium (St), Yersinia pestis (Yp), Vibrio cholerae (Vc), Pseudomonas aeruginosa (Pa), Brucella melitensis (Bm), B. suis (Bsui), Agrobacterium tumefaciens (At), Helicobacter pylori (Hp), Aquifex aeolicus (AA), Thermotoga maritima (Tm), Bacillus subtilis (Bs), Listeria monocytogenes (Lm), Clostridium perfringens (Cp), Synechocystis sp. (Sy), Gluillardia theta (Gt), Deinococcus radiodurans (Dr), and Chlamydia trachomatis (Ct). Residues of the Walker A ATP-binding motif are aligned below the black bar.
from pSR3 (Table 7.1) exhibited a filamentous phenotype, indicative of cell division arrest (Figure 7.2 B). *E. coli* expressing MinD_{Ng-2aaNT} were filamentous as well (Figure 7.2 C). In contrast, cells expressing MinD_{Ng-3aaNT} or MinD_{Ng-4aaNT} presented a minicell phenotype, characterized by rods of varying lengths and round minicells (Figure 7.2 D, E; arrows indicate minicells). Cells transformed with pJS58 (minD_{Ng-19aaNT}) (Table 7.1) presented a short rod morphology (Figure 7.2 F), similar to the negative control cells containing pUC18 (Figure 7.2 A). Western blotting showed the overexpression of wild-type MinD_{Ng} (Figure 7.2 G, lanes 2, 6), MinD_{Ng-2aaNT} (lane 3), MinD_{Ng-3aaNT} (lane 4), and MinD_{Ng-4aaNT} (lane 7), in comparison to background levels of resident *E. coli* MinD (lanes 1 and 5). The expression of MinD_{Ng-19aaNT} could not be detected in *E. coli*, and only background MinD_{Ec} was observed by Western blotting (lane 8). Overall, these studies suggested that removal of only 3 amino acids from the N-terminus of MinD_{Ng} will affect its ability to induce total cell division arrest.

Amino acid substitutions were also made to alter the charge and/or side chain bulkiness of selected N-terminal residues. These mutations included K3E, K3I, K3A, K3F, I4Q, I5A and I5E. Expression of most MinD_{Ng} N-terminal point mutants, including MinD_{Ng-K3E}, MinD_{Ng-K3I}, MinD_{Ng-K3A}, MinD_{Ng-K3F}, and MinD_{Ng-I4Q}, also produced filamentous *E. coli* (Figure 7.3 C-G), similar to cells expressing wild-type MinD_{Ng} (Figure 7.3 B). In contrast, expression of MinD_{Ng-I5E} or MinD_{Ng-I5A} did not lead to *E. coli* filamentation, but produced a minicell phenotype instead (Figure 7.3 H, I; arrows show minicells). The levels of each MinD_{Ng} point mutant in *E. coli* PB103 were similar or slightly greater than wild-type MinD_{Ng} (Fig. 2I), as determined by Western blotting. In addition, DAPI staining confirmed the absence of DNA in minicells of *E. coli* expressing MinD_{Ng-3aaNT} (Figure 7.4 A), MinD_{Ng-I5E} (Figure 7.4 B), and MinD_{Ng-I5A} (Figure 7.4 C).

**N-terminal deletions and point mutations to MinD_{Ng} affect protein-protein interactions.** Since interactions between the Min proteins are important for proper functioning of the Min system (Huang *et al.*, 1996; Hu and Lutkenhaus, 2003; Lackner *et al.*, 2003; Chapters 4, 5 and 6, this study), yeast
Figure 7.2. Expression of wild-type and N-terminal deletion derivatives of MinD_Ng in wild-type *E. coli* PB103. (A) *E. coli* PB103 transformed with pUC18 exhibit a typical short rod morphology, while (B) cells transformed with pSR3 (minD_Ng) display a filamentous phenotype. (C) pSIA10 (minD_Ng-2aaNT) transformants also display filamentation. D) pSIA11 (minD_Ng-3aaNT) transformants exhibit a minicell morphology, as do (E) cells containing pJS4B (minD_Ng-4aaNT). (F) pJS5B (minD_Ng-19aaNT) transformants have a short rod morphologies. Scale bar in (A) represents 5 μm and all figures are at the same magnification. Arrows indicate minicells. (G) Western blotting using anti-MinD_Ng antisera to probe *E. coli* cell extracts transformed with: lanes 1 and 5, pUC18; lanes 2 and 6, pSR3 (minD_Ng); lane 3, pSIA10 (minD_Ng-2aaNT); lane 4, pSIA11 (minD_Ng-3aaNT); lane 7 pJS4B (minD_Ng-4aaNT); and lane 8, pJS5B (minD_Ng-19aaNT). The antiserum also detects background expression of native *E. coli* MinD in strain PB103 (lane 1). Each panel in (G) was a separate Western blot experiment, with equalized loading concentrations in their respective wells.
Figure 7.3. Expression of wild-type MinD\textsubscript{Np} and N-terminal MinD\textsubscript{Np} mutants in wild-type \textit{E. coli} PB103. (A) Cells transformed with pUC18 negative control have a normal short rod morphology. (B) pSR3 (\textit{minD}\textsubscript{Np}) transformants display a filamentous phenotype. Cells transformed with (C) pSIA13 (\textit{minD}\textsubscript{Np-K3E}), (D) pSIA22 (\textit{minD}\textsubscript{Np-K3I}), (E) pSIA23 (\textit{minD}\textsubscript{Np-K3A}), (F) pJS16 (\textit{minD}\textsubscript{Np-K3F}) or (G) pSIA24 (\textit{minD}\textsubscript{Np-K3}) all exhibited filamentation. Cells transformed with (H) pJS13 (\textit{minD}\textsubscript{Np-K3E}) or (I) pJS14 (\textit{minD}\textsubscript{Np-K3A}) displayed minicell phenotypes. Scale bar in (A) represents 10 \(\mu\)m and all figures are at the same magnification. (J) Western blotting using anti-MinD\textsubscript{Np} antisera confirms expression of each MinD\textsubscript{Np} protein in \textit{E. coli}: lane 1, negative control pUC18 transformants; lane 2, wild-type MinD\textsubscript{Np}; lane 3, MinD\textsubscript{Np-K3E}; lane 4, MinD\textsubscript{Np-K3I}; lane 5, MinD\textsubscript{Np-K3A}; lane 6, wild-type MinD\textsubscript{Np}; lane 7, MinD\textsubscript{Np-K3F}; lane 8, MinD\textsubscript{Np-K3E}; lane 9, MinD\textsubscript{Np-K3A}; and lane 10, MinD\textsubscript{Np-K3F}. Each panel in (J) was a separate Western blot experiment.
Figure 7.4. DAPI staining verifies absence of DNA in minicells resulting from expression of N-terminal MinD$_{Np}$ truncation and mutation derivatives in wild-type *E. coli* PB103. In all cases, the left panel represents DIC image, while the right panel shows the corresponding DAPI fluorescence image. Black arrows indicate minicells. Note these minicells are devoid of DNA in all cases. (A) *E. coli* PB103 transformed with pSIA11 (minD$_{Np-3aaNT}$). (B) *E. coli* transformed with pJS13 (minD$_{Np-151}$). (C) *E. coli* transformed with pJS14 (minD$_{Np-15A}$).
two-hybrid assays were employed to determine how mutations to the N-terminus of MinD<sub>Ng</sub> may affect interactions with other Min proteins. Since interactions between wild-type MinD<sub>Ng</sub> and MinE<sub>Ng</sub> or MinC<sub>Ec</sub> are easily detected when the latter two proteins are fused to the GAL4-BD (Chapter 5), each N-terminal MinD<sub>Ng</sub> truncation was tested for interaction with other Min proteins fused to this particular GAL4 domain. As in Chapters 5 and 6, <i>E. coli</i> MinC<sub>Ec</sub> was used in these studies since it could interact well with wild-type MinD<sub>Ng</sub>.

Removal of the first four residues of MinD<sub>Ng</sub> drastically reduced the strength of MinD<sub>Ng-4aaNT</sub> interaction with MinC<sub>Ec</sub>, based on β-galactosidase activities (3.18 ± 0.16 Miller units for the mutant and 141.07 ± 4.43 units for wild-type (Table 7.2). MinD<sub>Ng-4aaNT</sub> lost interaction with itself and with MinE<sub>Ng</sub>; however, it still maintained weak interaction with wild-type MinD<sub>Ng</sub> (Table 7.2). More drastic deletions from the N-terminus of MinD<sub>Ng</sub>, including MinD<sub>Ng-19aaNT</sub>, MinD<sub>Ng-31aaNT</sub> and MinD<sub>Ng-183aaNT</sub> derivatives, resulted in lost interaction with MinC<sub>Ec</sub>, MinE<sub>Ng</sub>, and MinD<sub>Ng</sub> (Table 7.2). Similar to wild-type MinD<sub>Ng</sub> (Szeto <i>et al.</i>, 2001), MinD<sub>Ng-2aaNT</sub> could interact with itself and with wild-type MinD<sub>Ng</sub> (Table 7.2). Furthermore, MinD<sub>Ng-2aaNT</sub> retained strong interaction with MinE<sub>Ng</sub> and MinC<sub>Ec</sub>, relative to the respective wild-type control interactions (Table 7.2). In contrast, yeast two-hybrid assays showed that MinD<sub>Ng-3aaNT</sub> did not interact with itself, and had severely diminished interactions with wild-type MinD<sub>Ng</sub> and MinE<sub>Ng</sub> (Table 7.2). However, MinD<sub>Ng-3aaNT</sub> retained interaction with MinC<sub>Ec</sub> to produce dark blue yeast colonies, but this interaction was weaker (57.45 ± 9.64 Miller units) relative to wild-type control (141.07 ± 4.43 Miller units), based on β-galactosidase activity (Table 7.2). Since MinD<sub>Ng-3aaNT</sub> had disrupted interaction with itself and with MinE<sub>Ng</sub>, but retained interaction with MinC<sub>Ec</sub>, this suggests that the truncated protein is likely to be folded correctly, and can be stably expressed in the yeast reporter strain.

Hence, in support of its preserved functionality, MinD<sub>Ng-2aaNT</sub> interacted with all other Min proteins as did wild-type MinD<sub>Ng</sub>. However, removal of only the first three residues of the MinD<sub>Ng</sub>,
<table>
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<th>Fusion to GAL4-DNA-BD</th>
<th>Intensity of blue colony colour</th>
<th>$\beta$-galactosidase Activity (Miller Units)</th>
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</tr>
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<td>MinD$_{Ng}$-183aaNT</td>
<td>MinC$_{Ec}$</td>
<td>-</td>
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1 GAL4-activation domain
2 GAL4-DNA binding domain
3 Yeast colony colour intensity indicating strength of interaction, ranging from dark blue (+++++) to faint blue (+); (-) = white colonies
4 One unit equals the amount of $\beta$-galactosidase which hydrolyzes 1 $\mu$mol of ONPG per minute per cell
5 N. gonorrhoeae MinD
6 E. coli MinC
7 NT denotes N-terminal truncation [e.g. 2aaNT = deletion of first 2 amino acids (codons) from N-terminus]. In place of the removed codons is a start codon.
was sufficient to disrupt or reduce interaction with all other Min proteins, particularly with itself and MinE<sub>N</sub>.

MinD<sub>N</sub> bearing N-terminal point mutations were also examined for Min protein interactions. Each of the MinD<sub>N</sub> mutants, with the exception of MinD<sub>N</sub>-I5E and possibly MinD<sub>N</sub>-K3A, retained the ability to self-associate as determined by the appearance of blue yeast colonies (Table 7.3). Overall, the strengths of mutant MinD<sub>N</sub> self-interaction, as assessed by β-galactosidase activities, were less than that of wild-type MinD<sub>N</sub> (8.75 ± 0.29 Miller units) (Table 7.3). In addition, all the proteins that retained self-interaction could interact with wild-type MinD<sub>N</sub> (Table 7.3).

Most of the MinD<sub>N</sub> point mutants had noticeably less interaction with MinE<sub>N</sub>, as indicated by faint blue and/or white yeast colonies (Table 7.3). This was confirmed by β-galactosidase assays showing all mutants, with the exception of MinD<sub>N</sub>-K3E and MinD<sub>N</sub>-I4Q, had clearly diminished interaction with MinE<sub>N</sub> (Table 7.3).

In general, almost all MinD<sub>N</sub> mutants retained interaction with MinC<sub>E</sub>, to produce dark blue colonies when assayed with the yeast two-hybrid system (Table 7.3). Only the MinD<sub>N</sub>-I5E–MinC<sub>E</sub> interaction produced faint blue colonies (Table 7.3). β-galactosidase assays showed that, with the exception of MinD<sub>N</sub>-K3E, the interaction between each N-terminal MinD<sub>N</sub> mutant and MinC<sub>E</sub> was markedly decreased compared to the wild-type MinD<sub>N</sub>–MinC<sub>E</sub> interaction. However, it is noted that, although mutations at the third or fourth residues of MinD<sub>N</sub> diminished MinD<sub>N</sub>–MinC<sub>E</sub> interactions, it is apparent that these mutant proteins (K3E, K3I, K3A, and I4Q mutants) could still recruit sufficient MinC<sub>E</sub> to the E. coli membrane to arrest cell division in wild-type E. coli (Figure 7.3 C, D, E, and G).

It is also noted that all but one (MinD<sub>N</sub>-I5E) of the six MinD<sub>N</sub> N-terminal mutants retained interaction with at least one of MinC<sub>E</sub>, wild-type MinD<sub>N</sub>, MinE<sub>N</sub>, or with itself. This indicates that these MinD<sub>N</sub> mutants were likely folded properly, without gross structural perturbations. MinD<sub>N</sub>-I5E did not have detectable interactions with most of the other Min proteins. Ideally, Western blotting
Table 7.3. Yeast two-hybrid assays of the interactions between Min proteins and N-terminal mutation derivatives of MinD<sub>Ng</sub>

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</table>

<sup>1</sup> GAL4-activation domain  
<sup>2</sup> GAL4-DNA binding domain  
<sup>3</sup> Yeast colony color intensity indicating strength of interaction, ranging from dark blue (++++) to faint blue (+); (-) = white colonies; (+/−) = indicates a combination of faint blue and white colonies  
<sup>4</sup> One unit equals the amount of β-galactosidase which hydrolyzes 1 μmol of ONPG per minute per cell  
<sup>5</sup> <i>N. gonorrhoeae</i> MinD  
<sup>6</sup> <i>E. coli</i> MinC
should be used to detect whether GAL4-MinD<sub>Ng·15E</sub> fusions are expressed in the yeast; however, it is recognized that the yeast two-hybrid system used in this study has inherently low expression levels of GAL-4 fusion genes (Clontech). Furthermore, the Dillon laboratory has not been able to detect GAL4-fusions in the yeast reporter strain SFY526 (Ramirez-Arcos et al., in press). However, since MinD<sub>Ng·15E</sub> did have a weak interaction with MinC<sub>E</sub>, it suggests that the mutant MinD<sub>Ng</sub> was still expressed stably to some degree in the yeast reporter strain. Overall, these results suggest residues at the extreme N-terminus of MinD<sub>Ng</sub> are involved in MinD<sub>Ng</sub> interaction with all other Min proteins.

**Deletion of MinD<sub>Ng</sub>, N-terminal residues affects dynamic localization of GFP-MinD<sub>Ng</sub> fusions.**

GFP-fusions to N-terminal deletion derivatives of MinD<sub>Ng</sub> were constructed to determine whether this region of MinD<sub>Ng</sub> is implicated in dynamic localization of the protein. Initial studies of GFP-MinD<sub>Ng·4aaNT</sub> and GFP-MinD<sub>Ng·19aaNT</sub> were conducted in *E. coli* WM1032 (ΔminCDE<sub>Ec</sub>) (Table 2.1), in collaboration with Dr. W. Margolin, University of Texas Medical School. GFP-MinD<sub>Ng·K16Q</sub> was used as a negative control. In all cases, the *minE<sub>Ng</sub>* gene was also provided immediately downstream of each plasmid-encoded mutant *gfp-minD<sub>Ng</sub>* gene to stimulate fusion protein movement.

*E. coli* WM1032 transformed with pJDE2 (GFP-MinD<sub>Ng·4aaNT</sub>) and pJDE3 (GFP-MinD<sub>Ng·19aaNT</sub>) (Figure 7.5 A, B) did not display any discernible intracellular fusion protein movement seen with wild-type GFP-MinD<sub>Ng</sub> (See Figure 4.5 A), and presented cytosolic fusion protein localization instead. This localization was similar to that observed with the negative control GFP-MinD<sub>Ng·K16Q</sub> (Figure 7.5 C).

Western blotting indicated the expression of GFP-MinD<sub>Ng·K16Q</sub> was similar to that of wild-type GFP-MinD<sub>Ng</sub> (Figure 7.5 D, lanes 1 and 2). GFP-MinD<sub>Ng·4aaNT</sub> and GFP-MinD<sub>Ng·19aaNT</sub> levels were slightly less, at 86% and 70% of the wild-type fusion protein level, respectively (Figure 7.5 D, lanes 3 and 4). Furthermore, immunoblotting showed that the levels of MinE<sub>Ng</sub> in all transformants were similar (Figure 7.5 E). These localization results suggested that deletion of only the first four N-
Figure 7.5. Localization of GFP-fusions to N-terminal MinDNg deletion derivatives in *E. coli* WM1032. Examination of cells expressing (A) GFP-MinDNg-4aaNT+, (B) GFP-MinDNg-19aaNT+, and (C) GFP-MinDNg-K16Q+. In all cases, no evidence of intracellular fusion protein movement is seen and fluorescent signal appears distributed throughout the cytoplasm. Bar in (A) indicates 5 μm and all images are at the same magnification. (D and E) Western blotting verifies expression of GFP-MinDNg and MinENg in *E. coli* WM1032 (ΔminCDENg). (D) Anti-MinDNg antisera detects wild-type GFP-MinDNg (lane 1), GFP-MinDNg-K16Q (lane 2), GFP-MinDNg-4aaNT (lane 3), and GFP-MinDNg-19aaNT (lane 4) in *E. coli* WM1032 cells transformed with pSR15 (gfp-minDNg, minENg), pJDE1 (gfp-minDNg-K16Q, minENg), pJDE2 (gfp-minDNg-4aaNT, minENg), and pJDE3 (gfp-minDNg-19aaNT, minENg), respectively. (E) Anti-MinENg antisera detects MinENg in cell extracts of *E. coli* WM1032 transformed with pSR15 (lane 1), pJDE1 (lane 2), pJDE2 (lane 3), and pJDE3 (lane 4).
terminal amino acid residues of MinD_{Ng} is sufficient to disrupt the intracellular movement and membrane association of GFP-MinD_{Ng}.

More sophisticated localization studies were conducted with GFP-MinD_{Ng-2anNT} and GFP-MinD_{Ng-3anNT} fusions using an advanced microscope system acquired by our laboratory. These experiments were conducted in *E. coli* PB114, which also lacks its native minCDE gene cluster, similar to *E. coli* WM1032 (Table 2.1). MinE_{Ng} was encoded on the same plasmid as each GFP-MinD_{Ng} fusion to induce *in vivo* movement.

Positive control cells expressing wild-type GFP-MinD_{Ng} displayed dynamic intracellular fusion protein movement (Figure 7.6 A). Wild-type GFP-MinD_{Ng} oscillation required an average of 33.2 ± 5.8 seconds (Table 7.4). GFP-MinD_{Ng-2anNT} displayed similar pole-to-pole movement as wild-type GFP-MinD_{Ng} (Figure 7.6 C), with an average oscillation cycle of 32.5 ± 5.2 seconds that was not significantly different from the positive control (Table 7.4).

At first glance, GFP-MinD_{Ng-3anNT} did not appear to move in *E. coli* PB114 cells, with much of the fluorescent signal distributed throughout the cytoplasm. However, closer inspection revealed faint oscillatory signals shifting from one end of the cell to the other (Figure 7.6 E, arrows), amidst the increased cytosolic fluorescence relative to wild-type GFP-MinD_{Ng} and GFP-MinD_{Ng-2anNT}. The increased tendency to remain distributed in the cytoplasm made visualizing clear pole-to-pole movement of GFP-MinD_{Ng-3anNT} more difficult (Figure 7.6 E). Interestingly, the average oscillation time of this GFP-MinD_{Ng-3anNT} (19.8 ± 3.8 seconds) was significantly faster than GFP fusions to both wild-type and MinD_{Ng-2anNT} (p<0.001) (Table 7.4).

*E. coli* GFP-MinD has been shown to localize in a MinE-dependent manner within a membrane-associated coiled array (Shih *et al.*, 2003). GFP-MinD_{Ng} localizes similarly in a MinE_{Ng}-dependent manner within an *E. coli* background (Chapter 4, Chapter 6). As with wild-type GFP-MinD_{Ng} (Figure 7.6 B), GFP-MinD_{Ng-2anNT} was localized in what appeared to be a coiled array (Figure 7.6 D), with increased fluorescence within specific segments of the array that contributed to the
Figure 7.6. Localization of wild-type and N-terminal deletion derivatives of MinD_Ng in *E. coli* PB114. Oscillation cycles (from one pole to the other, and back) of GFP-MinD_Ng fusions were measured in *E. coli* rods. (A) Distinct pole-to-pole movement of wild-type GFP-MinD_Ng in *E. coli*. Leftmost panel shows DIC image. Remaining panels show GFP-MinD_Ng localization. One cycle of fusion protein movement required 30 seconds in this cell. Note the U-shaped fluorescent signal of the fusion protein alternately lining each cell pole region (arrows). Scale bar in (A) indicates 5 μm and all other images are at similar magnification. (B) GFP-MinD_Ng localizes in longer *E. coli* cells as regularly spaced bands. Each band contains fusion protein arranged within a coil (arrows). (C) GFP-MinD_Ng_2aaNT undergoes pole-to-pole oscillation. The time required for the fusion protein to complete one oscillatory cycle is 30 seconds (arrows). (D) GFP-MinD_Ng_2aaNT can localize within segments of a coiled array (arrows). (E) GFP-MinD_Ng_3aaNT can also undergo pole-to-pole oscillation (arrows), with one cycle requiring 15 seconds in the cell shown. Note that much of the fusion protein signal is found distributed throughout the cytosol. (F) Raw image of *E. coli* PB114 expressing GFP-MinD_Ng_3aaNT. Note the near uniform cytosolic localization of the protein. (F') Image enhancement of previous figure reveals presence of GFP-MinD_Ng_3aaNT localizing within coil-like structure (arrows). (G) Western blot using anti-MinD_Ng antisera to detect GFP-MinD_Ng proteins in *E. coli* PB114: Lane 1, cell extract from untransformed *E. coli* PB114; lane 2, wild-type GFP-MinD_Ng (pSR15); lane 3, GFP-MinD_Ng_2aaNT (pSIA16); and lane 4, GFP-MinD_Ng_3aaNT (pSIA17). (H) Western blot using anti-MinE_Ng antisera to detect MinE_Ng in *E. coli* PB114 transformed with: Lane 1, no plasmid; lane 2, pSR15; lane 3, pSIA16; and lane 4, pSIA17.
Table 7.4. Dynamic localization characteristics of GFP-fusions to MinD<sub>N</sub><sup>N</sup>-terminal mutants

<table>
<thead>
<tr>
<th>GFP-fusion</th>
<th>Dynamic movement&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average oscillation cycle (seconds)</th>
<th>Ability to localize in coiled array&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Propensity to remain in cytoplasm&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>Wild-type MinD&lt;sub&gt;N&lt;/sub&gt;</td>
<td>Yes</td>
<td>33.2 ± 5.8 (n=30)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>MinD&lt;sub&gt;N&lt;/sub&gt;-2aaNT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Yes</td>
<td>32.5 ± 5.2 (n=30)</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>MinD&lt;sub&gt;N&lt;/sub&gt;-3aaNT</td>
<td>Yes</td>
<td>19.8 ± 3.8 (n=30)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>MinD&lt;sub&gt;N&lt;/sub&gt;-K3E</td>
<td>Yes</td>
<td>25.8 ± 5.7 (n=30)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>MinD&lt;sub&gt;N&lt;/sub&gt;-K3I</td>
<td>Yes</td>
<td>20.5 ± 3.9 (n=30)&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>++</td>
</tr>
<tr>
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<td>Yes</td>
<td>19.9 ± 3.1 (n=30)&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>+++</td>
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<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>None</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
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<td>No</td>
<td>None</td>
<td>-</td>
<td>+++</td>
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<sup>a</sup> Dynamic movement of GFP-fusions in <i>E. coli</i> PB114 as determined by pole-to-pole oscillation in 2.0-2.5 μm long cells, or discernable fluorescent signal movement in longer cells.

<sup>b</sup> As determined by the ability of fusion protein to localize distinctly to intracellular coiled arrays, easily distinguishable from cytosolic fluorescent signals. All observations compared to wild-type MinD<sub>N</sub>, set at (+++). An inability to localize within a coiled array is denoted by (-).

<sup>c</sup> In comparison to negative control GFP-MinD<sub>N</sub>-K16Q, which remains wholly cytoplasmic and is set at (++++) value. In contrast, wild-type GFP-MinD<sub>N</sub> is recruited to the membrane at cell poles and to a coiled array; hence its propensity to remain cytosolic is set at (+).

<sup>d</sup> n = number of cells observed to obtain average oscillation cycle

<sup>e</sup> NT denotes N-terminal truncation [e.g. 2aaNT = deletion of first 2 amino acids (codons) from N-terminus]. In place of the removed codons is a start codon.

<sup>f</sup> Average oscillation cycle calculated to be significantly faster than wild-type, as determined by the unpaired Student’s t-test (p<0.001).

<sup>g</sup> ND, not determined. Due to excessive background fluorescent signal from GFP-MinD<sub>N</sub>-15A localized throughout the cytoplasm, clearly distinguishable pole-to-pole oscillation could not be determined; however, oscillatory patterns were observed in longer cells in these samples.
dynamic ‘zebra-stripe’ banding patterns typical of GFP-MinD proteins in elongated *E. coli* cells (See Figure 4.6 E, F). As before (Chapters 4 and 6), GFP-MinD<sub>N<sub>Ng</sub></sub>-decorated coiled arrays were easier to visualize in *E. coli* cells that were ≥ 5 μm in length.

Despite the increased cytosolic localization of GFP-MinD<sub>N<sub>Ng</sub>-3aaNT</sub> (Figure 7.6 F), image enhancement revealed that a small proportion of fusion protein could also localize in what appeared to be segments of a helical array (Figure 7.6 F’, arrows). Hence, despite a tendency to localize mainly in the cytosol, GFP-MinD<sub>N<sub>Ng</sub>-3aaNT</sub> is still able to localize within a coil, likely permitting the faint oscillation patterns observed. As expected, the negative control GFP-MinD<sub>N<sub>Ng</sub>-K16Q</sub> was distributed uniformly throughout the cytoplasm, without any evidence of localizing within a spiral array (see Figure 4.6 D).

Western blotting using anti-MinD<sub>N<sub>Ng</sub></sub> antisera (Figure 7.6 G) verified the overexpression of wild-type GFP-MinD<sub>N<sub>Ng</sub></sub> (lane 2), GFP-MinD<sub>N<sub>Ng</sub>-2aaNT</sub> (lane 3) and GFP-MinD<sub>N<sub>Ng</sub>-3aaNT</sub> (lane 4) in *E. coli* PB114 transformants, relative to untransformed cells (lane 1). Densitometric analysis indicated that GFP-MinD<sub>N<sub>Ng</sub>-2aaNT</sub> and GFP-MinD<sub>N<sub>Ng</sub>-3aaNT</sub> levels were at ~92% and ~86% of wild-type GFP-MinD<sub>N<sub>Ng</sub></sub>, respectively. Western blotting detected similar levels of MinE<sub>N<sub>Ng</sub></sub> in all *E. coli* PB114 transformants used in these studies (Figure 7.6 H; lanes 2, 3, and 4), in comparison to the untransformed cell extract (lane 1).

These studies indicate that removal of the first three amino acids of MinD<sub>N<sub>Ng</sub></sub> affects the dynamic localization of the protein by producing a faster oscillation rate and an increased propensity to remain in the cytosol, in comparison to wild-type protein.

**Mutation of MinD<sub>N<sub>Ng</sub></sub> N-terminal residues affects dynamic localization of GFP-MinD<sub>N<sub>Ng</sub></sub> fusions.**

Similar to GFP-fusions to N-terminal deletion derivatives of MinD<sub>N<sub>Ng</sub></sub>, localization of GFP-fusions to MinD<sub>N<sub>Ng</sub></sub> bearing K3E, K3I, I4Q, I5A, or I5E mutations was examined in *E. coli* PB114 (Table 2.1). From yeast two-hybrid assays, MinD<sub>N<sub>Ng</sub>-K3E</sub> interacted with the other Min proteins in a
similar manner as wild-type MinD_{Ne}. As expected, GFP-MinD_{Ne,K3E} displayed clear pole-to-pole movement in *E. coli* PB114 rods, characterized by its distinct localization along the inner cell periphery at either cell pole as the fusion oscillated (Figure 7.7 A), similar to wild-type GFP-MinD_{Ne} (Figure 7.6 A). However, the average oscillation cycle of GFP-MinD_{Ne,K3E} required only 25.8 ± 5.7 seconds, significantly faster than the wild-type control (*p*<0.001) (Table 7.4). Similar to wild-type GFP-MinD_{Ne}, GFP-MinD_{Ne,K3E} also localized as a coiled array, with dynamic fluorescent bands resulting from fusion protein accumulation along evenly spaced segments of the coil (Figure 7.7 B, arrows).

GFP-MinD_{Ne,K31} also displayed pole-to-pole movement (Figure 7.7 C). However, like GFP-MinD_{Ne,K3E}, the fusion had a significantly faster oscillation cycle compared to the wild-type fusion, with an average oscillation cycle of only 20.5 ± 3.9 seconds (*p*<0.001) (Table 7.4). Furthermore, while it was clear that pole-to-pole oscillation was occurring, a slightly greater fraction of GFP-MinD_{Ne,K31} appeared to be distributed in the cytosol at any particular time (Figure 7.7 C, D), in comparison to wild-type GFP-MinD_{Ne} (Figure 7.6 A, B) and to GFP-MinD_{Ne,K3E} (Figure 7.7 A, B). This increased cytosolic fluorescence also made it difficult to visualize any intracellular GFP-MinD_{Ne,K31} within coiled arrays; however, fluorescent banding patterns within cells suggested the presence of this array (Figure 7.7 D). Image enhancement confirmed that GFP-MinD_{Ne,K31} could still localize within a membrane-associated coiled array (Figure 7.7 D'), despite an apparently increased propensity to remain cytosolic (Table 7.4).

The localization of GFP-MinD_{Ne,K44Q} proved to be even more preferentially distributed in the cytosol than GFP-MinD_{Ne,K31}. From fluorescent images, it was apparent that a significant portion of GFP-MinD_{Ne,K44Q} did not localize to membrane sites at the cell poles, but remained distributed in the cytoplasm instead (Figure 7.7 E). While pole-to-pole movement of GFP-MinD_{Ne,K44Q} could still be discerned (Figure 7.7 E), it required an average oscillation period of 19.9 ± 3.1 seconds, significantly faster the wild-type control (*p*<0.001) (Table 7.4). In addition, the average oscillation cycle of GFP-
Figure 7.7. Localization of N-terminal mutants of MinD<sub>Ng</sub> fused to GFP in <i>E. coli</i> PB114. (A) Distinct pole-to-pole movement of GFP-MinD<sub>Ng,K31</sub> in <i>E. coli</i>. Here, the fusion protein required 25 seconds to complete one cycle of oscillation. Note the U-shaped fluorescent signal of the fusion protein alternately lining each cell pole region. Bar in (A) represents 5 µm and all figures are at the same magnification. (B) GFP-MinD<sub>Ng,K31</sub> localizes in longer <i>E. coli</i> cells as regularly spaced bands. Each band consists of fusion protein arranged within a coiled segment (arrows). (C) GFP-MinD<sub>Ng,K31</sub> also undergoes pole-to-pole oscillation (arrows); however, more of the fusion protein signal is also found distributed throughout the cytosol. The time required for the fusion protein to complete one oscillatory cycle in the cell shown is 20 seconds. (D) Raw image of <i>E. coli</i> PB114 expressing GFP-MinD<sub>Ng,K31</sub>. Note the increased cytosolic localization of GFP-MinD<sub>Ng,K31</sub> relative to GFP-MinD<sub>Ng,K31</sub> (B). (D') Image enhancement of (D) reveals GFP-MinD<sub>Ng,K31</sub> localizing within segments of a coiled array (arrows). (E) GFP-MinD<sub>Ng,14Q</sub> can move from pole-to-pole (arrows); however, much of the fusion protein is found distributed throughout the cytoplasm. The time required for GFP-MinD<sub>Ng,14Q</sub> to complete one oscillatory cycle in the indicated cell is 15 seconds (arrows). (F) Raw image of elongated <i>E. coli</i> PB114 expressing GFP-MinD<sub>Ng,14Q</sub>. Note the near uniform cytosolic localization of the protein. (F') Image enhancement of previous image shows evidence of GFP-MinD<sub>Ng,14Q</sub> still localizing along a coiled array (arrows). (G) GFP-MinD<sub>Ng,15A</sub> is mostly localized to the cytosol. (G') Image enhancement of previous image shows GFP-MinD<sub>Ng,15A</sub> retains the ability to localize as coils. (H) GFP-MinD<sub>Ng,15E</sub> is uniformly distributed throughout the cytoplasm. (I) Western blot using anti-MinD<sub>Ng</sub> antiserum to detect GFP-MinD<sub>Ng</sub> fusions in <i>E. coli</i> PB114: Lane 1, wild-type GFP-MinD<sub>Ng</sub> (pSR15); lane 2, GFP-MinD<sub>Ng,K31</sub> (pSIA18); lane 3, MinD<sub>Ng,K31</sub> (pJS30); lane 4, GFP-MinD<sub>Ng,14Q</sub> (pJS18); lane 5, GFP-MinD<sub>Ng,15E</sub> (pJS29); lane 6, GFP-MinD<sub>Ng,15A</sub> (pJS19); and lane 7, untransformed <i>E. coli</i> cell extract. (J) Western blotting using anti-MinEng antiserum to detect MinEng in <i>E. coli</i> transformed with: Lane 1, no plasmid; lane 2, pSR15; lane 3, pSIA18; lane 4, pJS30; lane 5, pJS18; lane 6, pJS29; lane 7, pJS19.
MinD$_{Ng}$-14Q was significantly faster than that of GFP-MinD$_{Ng}$-K3E ($p<0.001$), but not of GFP-MinD$_{Ng}$-K31 (Table 7.4). Image enhancement revealed that GFP-MinD$_{Ng}$-14Q could also localize within a helical array (Figure 7.7 F and F'; arrows), despite its increased distribution in the cytosol.

GFP-MinD$_{Ng}$-15A tended to remain almost entirely in the cytoplasm. While faint evidence of dynamic fusion protein movement in bands could be discerned in longer filamentous *E. coli* PB114 cells (Table 7.4), it was not possible to clearly measure pole-to-pole movement in *E. coli* rods due to the increased cytosolic fluorescent signal. However, evidence of GFP-MinD$_{Ng}$-15A localizing as a coiled array (Figure 7.7 G') was obtained from enhancement of raw images of filamentous cells (Figure 7.7 G). The final mutant, GFP-MinD$_{Ng}$-15E, appeared to be wholly distributed in the cytosol, presenting uniform fluorescence throughout the cell with no evidence of dynamic movement or helical array formation (Figure 7.7 H) (Table 7.4).

Western blotting confirmed that similar levels of GFP-MinD$_{Ng}$ fusions were found in each *E. coli* PB114 transformant used in these studies (Figure 7.7 I). In addition, immunoblotting showed similar levels of MinE$_{Ng}$ expressed from each plasmid used in the localization studies (Figure 7.7 J).

Hence, as residues from the third to the fifth position of MinD$_{Ng}$ are sequentially targeted for mutation, there is a decrease in the time required for GFP-MinD$_{Ng}$ to oscillate from pole-to-pole in *E. coli*. As a result, oscillation periods were as follows: wild-type GFP-MinD$_{Ng}$ > GFP-MinD$_{Ng}$-K3E > GFP-MinD$_{Ng}$-K31 = GFP-MinD$_{Ng}$-14Q. Interestingly, this is accompanied by an increased tendency of each successive GFP-MinD$_{Ng}$ mutant to remain in the cytosol, such that 15A and 15E mutations conferred near-complete and complete tendencies to localize in the cytoplasm, respectively.

**N-terminal mutation derivatives of MinD$_{Ng}$ exhibit increased basal ATPase activity that is not affected by MinE$_{Ng}$**

MinE-induced stimulation of MinD ATPase activity is believed to be a key factor regulating the dynamic localization of Min proteins (Hu et al., 2001; Hu et al., 2002; Lackner et al., 2003). To elucidate a possible biochemical explanation for the altered *in vivo* dynamism of some of the N-
terminal MinD<sub>Ng</sub> mutants, ATPase stimulation assays were conducted on purified MinD<sub>Ng-3aaNT</sub> and MinD<sub>Ng-15E</sub>. These mutants were selected to gain insight into why N-terminal mutations to MinD appear to confer increased cytosolic distribution (MinD<sub>Ng-3aaNT</sub> and MinD<sub>Ng-15E</sub>) or faster oscillation rates (MinD<sub>Ng-3aaNT</sub>). MinD<sub>Ng-K16Q</sub> served as a negative control in these experiments, since this mutant exhibited little ATPase activity, even in the presence of MinE<sub>Ng</sub> and phospholipids (Chapter 6).

Functionality of each C-terminal His-tagged MinD<sub>Ng</sub> protein was first monitored in the E. coli expression strain C41 (DE3) (Table 2.1). As shown previously (Chapter 6), the short C-terminal 6XHis-tag did not affect the ability of wild-type MinD<sub>Ng</sub> to inhibit cell division in the E. coli expression strain (Figure 7.8 A). As expected, overexpression of the negative control protein, C-terminal His-tagged MinD<sub>Ng-K16Q</sub>, did not alter the typical short rod morphology of this E. coli strain (See Figure 6.4 E). Finally, cells expressing C-terminal His-tagged MinD<sub>Ng-3aaNT</sub> and MinD<sub>Ng-15E</sub> presented minicell phenotypes (Figure 7.8 C and D), similar to the expression of corresponding untagged proteins in E. coli PB103 (Figures 7.2 D and 7.3 H). Therefore, the cell morphologies induced by each His-tagged MinD<sub>Ng</sub> protein were consistent with those caused by equivalent untagged proteins. While MinD<sub>Ng-3aaNT</sub> could be readily purified from induced cultures (Figure 7.8 E), a large fraction of MinD<sub>Ng-15E</sub> was found in insoluble E. coli fractions (Figure 7.8 F, lane 1). However, there remained sufficient MinD<sub>Ng-15E</sub> in soluble fractions (lane 2) to be purified by nickel affinity chromatography (lane 3, arrow).

As in Chapter 6, artificial phosphatidylglycerol (PG) vesicles were made and used for the ATPase assays. Purified wild-type MinD<sub>Ng</sub>, MinD<sub>Ng-3aaNT</sub>, MinD<sub>Ng-15E</sub>, MinD<sub>Ng-K16Q</sub>, and MinE<sub>Ngd</sub> were incubated in the presence of ATP and PG vesicles to measure the release of inorganic phosphate (P<sub>i</sub>) from hydrolyzed ATP. In the absence of MinE<sub>Npg</sub>, wild-type MinD<sub>Ng</sub> incubated with phospholipid vesicles and ATP displayed a low level of basal enzymatic activity (Figure 7.9). Addition of MinE<sub>Npg</sub> increased the amount of P<sub>i</sub> released from wild-type MinD<sub>Npg</sub> up to three times the basal level after 90
Figure 7.8. Effects of expressing C-terminal His-tagged MinDNg proteins in E. coli C41 (DE3). (A) Cells transformed with the negative control vector pET30a have a short rod morphology. (B) Cells transformed with pSC9 (minDNg-His) display a filamentous phenotype. (C and D) Cells transformed with pJS33 (minDNg-15E-His) or pJS34 (minDNg-3aaNT-His) show a minicell phenotype. Scale bar in (A) represents 10 μm and all figures are at the same magnification. (E) Purification of MinDNg-3aaNT-His from induced E. coli cultures. Lane 1, 5 mM wash buffer eluate; lane 2, 60 mM imidazole wash buffer eluate; lane 3, 1 M imidazole elution. (F) Lane 1: insoluble fraction of E. coli C41 (DE3) expressing MinDNg-15A-His (pJS33). Lane 2: soluble fraction of pJS33 transformants. Lane 3: Eluted MinDNg-15A-His from nickel affinity chromatography (arrow).
minutes (Figure 7.9). In contrast, the MinD$_{Ng-K16Q}$ negative control exhibited little ATPase activity irrespective of the presence or absence of MinE$_{Ng}$ (Figure 7.9).

Interestingly, in the absence of MinE$_{Ng}$, both MinD$_{Ng-3aaNT}$ and MinD$_{Ng-15E}$ incubated with PG vesicles and ATP exhibited markedly greater ATPase activities than the basal wild-type MinD$_{Ng}$ reaction (Figure 7.9). After 45 minutes, wild-type MinD$_{Ng}$ had not released any measurable amount of P$_1$ in the absence of MinE$_{Ng}$. However, at the same timepoint, both MinD$_{Ng-3aaNT}$ and MinD$_{Ng-15E}$ had already released significantly more P$_1$ (>350 pmol), despite the lack of MinE$_{Ng}$ (Figure 7.9). By the end of the assay, both mutants released approximately twice as much P$_1$ as wild-type (Figure 7.9). Strikingly, the addition of MinE$_{Ng}$ did not result in any significant increase in the ATPase activities of MinD$_{Ng-3aaNT}$ and MinD$_{Ng-15E}$ over the period of the assay (Figure 7.9).

Therefore, mutations to the N-terminus of MinD$_{Ng}$ that decreased dynamic oscillatory periods and/or promoted cytosolic localization in vivo also conferred MinE$_{Ng}$-independent hyper-ATPase activity to MinD$_{Ng}$ in the presence of phospholipids, which was not further stimulated by the addition of MinE$_{Ng}$. These studies indicate there are determinants within the extreme N-terminus of MinD$_{Ng}$ involved in regulating the dynamic localization and intrinsic enzymatic activity of the protein.
Figure 7.9. MinD$_{Np}$ ATPase stimulation assays using MinD$_{Np}$, MinD$_{Np}$-15E$^3$, MinD$_{Np}$-3aaNT$^3$, and MinD$_{Np}$-K16Q$^*$. Equal amounts of purified MinD$_{Np}$, MinD$_{Np}$-15E$^3$, MinD$_{Np}$-3aaNT$^3$, and MinD$_{Np}$-K16Q$^*$ were incubated with PG vesicles and 1 mM ATP. The ATPase activities of each were tested in the presence or absence of MinE$_{Np}$ over a 90 minute period. Inorganic phosphate released due to ATP hydrolysis was monitored using a malachite green based method. Buffer A is MinD$_{Np}$ storage buffer, Buffer B is MinE$_{Np}$ storage buffer.
7.4. DISCUSSION

The coordinated intracellular movement of MinD is essential for maintaining proper septum placement in bacteria (Raskin and de Boer, 1999a; Rowland et al., 2000). To achieve this, MinD must bind reversibly to specific membrane and/or membrane-associated targets (Hu et al., 2002; Lackner et al., 2003; Szeto et al., 2003, Mileykovskaya et al., 2003, Shih et al., 2003). Several studies have now highlighted the role of the extreme C-terminus of MinD in membrane targeting (Szeto et al., 2002; Hu et al., 2003; Szeto et al., 2003; Zhou and Lutkenhaus, 2003). However, little is known about the function(s) of the N-terminus of MinD which, based on MinD sequence alignments, exhibits higher overall conservation than the C-terminus (Szeto et al., 2001). Interestingly, this study showed that mutation or deletion of residues at the extreme N-terminus of MinD affected protein dynamism and interactions with other Min proteins.

To date, the only factors shown to affect MinD oscillation frequency in vivo are mutations within MinE or changes in intracellular MinD/MinE ratios (Raskin and de Boer, 1999a; Hu and Lutkenhaus, 2001). Inadequate stimulation of E. coli MinD ATPase activity by MinE_Ec mutants has been shown to extend the oscillation period of GFP-MinD_Ec (Hu and Lutkenhaus, 2001). In support of this, MinE_Ng mutants with impaired MinD_Ng-MinE_Ng interaction will induce slower oscillation cycles of GFP-MinD_Ng (N. F. Eng and J. R. Dillon, unpublished data). In contrast, the mutant GFP-MinD_Ng proteins in the present study had faster oscillation rates, despite the presence of wild-type MinE_Ng. Furthermore, yeast two-hybrid studies showed MinD_Ng bearing 3aaNT, K3A, and K3I mutations all had significantly diminished abilities to interact with wild-type MinE_Ng. Hence, the rapid oscillation cycles of these GFP-MinD_Ng variants were likely due to determinants within MinD_Ng itself.
The oscillation frequency of MinD is also inversely related to intracellular MinD/MinE ratios (Raskin and de Boer, 1999a). An increased ratio results in extended oscillation cycles while a decreased ratio is proposed to have the opposite effect (Raskin and de Boer, 1999a). In this study, the intracellular levels of all GFP-MinD_{Ng} variants (except for GFP-MinD_{Ng-3aaNT}), and of their co-expressed MinE_{Ng}, were the same. Despite this, the average oscillation cycles of several N-terminal MinD_{Ng} mutants were significantly faster than wild-type. In addition, oscillation cycle times between some mutants were also different (e.g. GFP-MinD_{Ng-14Q} had a significantly faster oscillation cycle than GFP-MinD_{Ng-K3E}). Therefore, it appears that the decreased oscillation periods of the MinD_{Ng} mutants were not due to gross changes in MinD/MinE ratios, but rather due to changes intrinsic to MinD_{Ng}, and independent of MinE_{Ng}. Since MinD_{Ng-3aaNT} had significantly diminished interaction with MinE_{Ng}, it remains possible that the effects of this deletion on GFP-MinD_{Ng-3aaNT} were MinE_{Ng}-independent as well.

The observed changes in the dynamism of N-terminal MinD_{Ng} mutants were not likely due to diminished interaction with MinE_{Ng} for several reasons. Firstly, MinD_{Ng} mutants that retained similar interaction with MinE_{Ng} as wild-type MinD_{Ng} (e.g. K3E and I4Q mutants) still had faster oscillation rates than the control, indicating the phenotypes observed were independent of MinE_{Ng}. Secondly, it has been demonstrated that diminished and/or abrogated interaction with mutant MinE will cause GFP-MinD_{Ec} (Ma et al., 2003) or GFP-MinD_{Ng} (N.F. Eng and J.R. Dillon, unpublished results) to oscillate slower and remain for an extended period at one cell pole, presumably due to inefficient stimulation of MinD ATPase. However, it is clear from our studies that the MinD_{Ng} mutants with decreased interaction with MinE_{Ng} had significantly faster oscillation than wild-type and decreased
propensities to remain associated with the membrane. Thirdly, the increased ATPase activity of two of the mutants, MinD_{Ng-3aaNT} and MinD_{Ng-I5A}, was not affected by the presence MinE_{Ng}. Finally, studies by others have revealed evidence that the MinE binding site on MinD is within the $\alpha$-7 helix of MinD, which is not in proximity to the MinD N-terminus (G. King, University of Connecticut Health Centre, personal communication). Hence, the phenotypes of the mutant MinD_{Ng} proteins were likely MinE-independent.

While several N-terminal deletion and point mutation mutants of MinD_{Ng} had significantly diminished interaction with MinC, this could not possibly have contributed to the faster oscillation and increased cytosolic localization of these GFP-MinD_{Ng} variants. Firstly, all localization studies were conducted in an *E. coli* system devoid of any MinC. Secondly, it is well established that GFP-MinD localization is independent of MinC (Raskin and de Boer, 1999a). These studies do indicate that the extreme N-terminus of MinD_{Ng} contains determinants that are involved in, but not entirely responsible for, interaction with the MinC division inhibitor. In support of this, previous yeast two-hybrid studies have indicated that residues within the $\alpha$-7 helix of MinD_{Ec} (e.g. E146 and D152), which are not in proximity to the extreme N-terminus, may play a more direct role in binding MinC (Hayashi *et al.*, 2001). Despite interacting as strongly with MinC_{Ec} as some of the other MinD_{Ng} mutants, the greater tendencies of MinD_{Ng-3aaNT} and MinD_{Ng-I5A} to remain and/or return to the cytosol, in comparison to the other mutants, may have prevented them from recruiting sufficient MinC_{Ec} to the membrane *in vivo*. As a result, expression of these two mutant MinD_{Ng} proteins did not induce total cell division arrest in wild-type *E. coli*, unlike the other mutant MinD_{Ng} proteins.
This study demonstrated that, despite the absence of MinE_{Ng}, MinD_{Ng-3aaNT} and MinD_{Ng-1SE} each had significantly greater MinE_{Ng}-independent ATPase activities than wild-type protein. Such hyper-ATPase activity in these, and the remaining N-terminal mutants, could account for their faster oscillation cycles and/or increased cytosolic distribution relative to wild-type MinD_{Ng}. Interestingly, both GFP-MinD_{Ng-1SE} and the negative control GFP-MinD_{Ng-K16Q} were found entirely in the cytosol. However, since MinD_{Ng-1SE} had increased ATPase activity, in contrast to the low activity of MinD_{Ng-K16Q}, it suggests that their common intracellular localization pattern was due to separate factors. While a K16Q mutation to MinD is proposed to render the protein insensitive to the effects of ATP-binding (Hu et al., 2002), it is conceivable that increased intrinsic ATPase activity of MinD_{Ng-1SE} would prevent sufficient stable MinD-ATP to localize effectively along the membrane in vivo.

Under normal circumstances, the MinE-induced ATPase activity of MinD has been proposed to be the rate-limiting step for GFP-MinD oscillation (Hu and Lutkenhaus, 2001). However, as a result of increased intrinsic ATPase activity at the membrane, it is possible that GFP-MinD_{Ng-3aaNT} and GFP-MinD_{Ng-1SE} were more prone to dissociate from their membrane targets regardless of MinE_{Ng}. In effect, GFP-fusions to N-terminal MinD_{Ng} mutants would be released sooner into the cytosol than wild-type GFP-MinD_{Ng}, allowing them to migrate towards the opposite cell pole and to produce faster oscillation cycles, which was observed. This would account for the positive correlation observed between the relative amount of each GFP-MinD_{Ng} residing in the cytoplasm and its rate of protein oscillation. In contrast, wild-type MinD_{Ng} would require sufficient MinE_{Ng} recruitment and stimulation prior to commencing a cycle of oscillation in vivo. This delay would result in a more stable
MinD<sub>Ng</sub> membrane association and longer oscillation cycles than the N-terminal mutants. The diminished and lost interactions of MinE<sub>Ng</sub> with MinD<sub>Ng-3aaNT</sub> or MinD<sub>Ng-1SE</sub> respectively, would have also contributed to the inability of MinE<sub>Ng</sub> to further stimulate their \textit{in vitro} ATPase activities.

MinD oscillation and ATPase activity are intimately linked and require MinD self-association (Hu and Lutkenhaus, 2001; Hu \textit{et al}., 2003; Lutkenhaus and Sundaramoorthy, 2003). Hence, the faster oscillation cycles and increased ATPase activities of the MinD<sub>Ng</sub> mutants in this study indicate they still possess significant functional self-association, despite their decreased affinities to homodimerize, as detected by the yeast two-hybrid system. Results from the \textit{in vitro} ATPase assays also provide an explanation for this decreased self-affinity. While ATP-binding induces MinD dimerization, the hydrolysis of ATP should lead to the dissociation of MinD dimers as well (Hu \textit{et al}., 2002; Hu \textit{et al}., 2003). Hence, while still able to interact in the yeast reporter strain, hyper-ATPase activity of N-terminal MinD<sub>Ng</sub> mutants within yeast may have destabilized the self-association of these proteins, resulting in the diminished strengths of interaction observed.

Interestingly, each of the MinD<sub>Ng</sub> mutants displaying tendencies to localize cytoplasmically (3aaNT, K3I, I4Q, I5A, or ISE mutations) had less than half the strength of self-interaction of wild-type protein, based on β-galactosidase activities. In contrast, MinD<sub>Ng-2aaNT</sub> and MinD<sub>Ng-K3E</sub> each retained at least half of the strength of self-interaction of wild-type MinD<sub>Ng</sub>, and were localized clearly along the membrane. Therefore, it is possible that a certain threshold of self-binding affinity must be achieved prior to the commitment of MinD to bind membrane targets.
The changes in the dynamic localization characteristics of the N-terminal MinD_{Ng} mutants were not likely due to inefficiencies in ATP-binding, but rather due to the effects of MinE-independent hyper-ATPase activities. In support of this, examination of Archaeal MinD crystal structures show that the extreme N-terminus of MinD does not contain residues that directly participate in nucleotide binding (Hayashi et al., 2001; Sakai et al., 2001). In addition, the N-terminal MinD_{Ng} mutants which exhibited faster intracellular oscillation, as well as increased ATPase activities, would first require ATP-binding. Moreover, MinC-MinD interaction appears to require ATP (Hu et al., 2003; Lackner et al., 2003), and almost all of the MinD_{Ng} N-terminal mutants could still interact with MinC and could induce total cell division arrest in *E. coli*.

These studies also support the notion that a basic, MinD-containing, helical scaffold is required for any degree of intracellular MinD protein oscillation. It should be noted that each GFP-MinD_{Ng} mutant that displayed oscillation, no matter how faint, could still localize within coil-like arrays. The ability of each MinD_{Ng} variant to form these arrays corresponded with its ability to interact with MinE_{Ng}, even if this interaction was severely diminished (e.g. MinD_{Ng-S5A}). This is in support of a proposal that MinE may serve as a cross-linker for MinD filaments, in addition to its role in inducing MinD disassembly from the membrane (Suefuji et al., 2002). Hence, these studies indicate that some (even minimal) interaction with MinE, must be maintained in order to form MinD-containing coiled arrays that direct protein oscillation *in vivo*.

What role might the N-terminus of MinD_{Ng} play in the functionality of the protein? It is possible that the N-terminus of MinD_{Ng} may be involved in controlling intrinsic ATPase activity. Using the solved structure of MinD from *Archaeoglobus fulgidus* as a representative structure (Figure 7.10), residues of the extreme N-terminus (yellow residues, aa 1-5) form one end of a buried β-strand
Figure 7.10. Structure of *Archaeoglobus fulgidus* MinD highlighting the N-terminus residues (Cordell and Löwe, 2001). Yellow residues indicate the extreme N-terminus amino acids at positions 1-5. Red residues (aa 6-9), in conjunction with yellow residues (1-5) form a $\beta$-strand that connects to the P-loop (Walker A ATP-binding motif, green residues). The general position of the ATP-binding face of MinD is indicated. Ribbon diagram generated with RasMol Molecular Graphics Visualization Tool (Version 2.7.2.1).
(yellow and red residues) that connects directly to the P-loop (Walker A ATP-binding motif, green residues) which is found at the opposite side of the protein (Cordell and Löwe, 2001). Hence, it is possible that some of the effects of ATP-binding are transmitted from the ATP-binding face (Figure 7.10) through the N-terminus residues (yellow and red residues) to other regions of the protein in preparation for membrane association and/or ATP hydrolysis. Mutation of this N-terminus region may therefore render MinD more sensitive to the effects of ATP-binding, in contrast to the desensitization proposed with a K16Q mutation (Hu et al., 2002), and result in increased intrinsic enzymatic activity.

There are several examples of ATPases in which the N-terminus is directly involved in regulating functionality. Sequential truncations to the first five residues of the chloroplast ATP synthase epsilon subunit resulted in significant increases in the ATPase activity of the holoenzyme (Shi et al., 2001). Furthermore, deletion of the N-terminal ‘A’ domain of the transcriptional regulator XylR has been shown to lock the regulator in a fully active form (Fernandez et al., 1995), which displays a strongly stimulated ATPase activity in the presence of DNA activation sequences (Perez-Martin and de Lorenzo, 1996). In addition, the N-terminal domain of the DmpR transcriptional activator has been shown to act as a repressor of its ATPase activity (Shingler and Pavel, 1995). Normally, DmpR ATPase activity is induced by binding of aromatic compounds (Shingler and Pavel, 1995), which draws an interesting comparison to the effects of MinE binding to MinD.

This study demonstrates that mutations or truncations within the first five residues of the conserved N-terminus of MinD_{N} affect the movement and localization properties of the protein. Residues 6-9 of MinD are even more conserved than the first five, and it would be interesting to study their functional roles as well. Since the extreme N-terminus of MinD is highly conserved, its function is likely shared across MinD proteins from various organisms, particularly Gram-negative bacteria. Evidence is provided that the altered dynamism of these MinD_{N} mutants may result from increased intrinsic basal ATPase activities. Further work, including in vitro membrane binding assays using
vesicles that closely approximate bacterial cell membranes, as well as combined structural and enzymatic studies, should prove useful in further elucidating the function of the MinD N-terminus. Since correct cell division site-selection is such an important event in the bacterial cell cycle, there should conceivably be several mechanisms and/or factors that regulate dynamic Min protein movement, including determinants within MinD itself that ensure its proper ATPase activity.
CHAPTER 8

General discussion, future considerations, and concluding statements
The study of Min proteins from rod-shaped organisms is well advanced. However, the
discovery of min gene homologues in the coccus *N. gonorrhoeae* was intriguing (Ramirez-Arcos *et al.*, 2001a) and prompted our laboratory to initiate investigations into the function of Min proteins
from coccal organisms. Using a variety of methods and assays, it was shown that MinD_{Ng} functions as
a cell division site determinant in the gonococcus, and is active across species as well. Despite
differences in morphology and cell division patterns, both *N. gonorrhoeae* and the rod *E. coli* share
similar proteins to achieve successful cell division. The tripartite Min system (MinC, MinD, and
MinE), with its highly conserved MinD protein, therefore appears to be a relatively common cell
division regulator in Gram-negative organisms. However, in contrast to the rod *E. coli*, it would
appear that the Min system in gonococcal cells must function in two dimensions to maintain the
perpendicularly alternating division planes characteristic of *N. gonorrhoeae*.

Overall, these studies suggest a universality of MinD function, at least in Gram-negative
organisms. Based on *in vivo* studies in *E. coli*, it is apparent that MinD_{Ng} is active in this background,
since it displayed several characteristics that have also been reported with *E. coli* MinD, including the
induction of MinC-activated cell division arrest and MinE-dependent pole-to-pole oscillation along a
coiled scaffold. These observations suggest that *N. gonorrhoeae* and *E. coli* MinD have more
functional similarities than differences. The ability of GFP-MinD_{Ng} to localize as coils in *E. coli*
backgrounds suggests that the formation of such MinD helical arrays may be common in all Gram-
negative organisms bearing a Min system, regardless of shape. For MinD_{Ng}, this ability was not
entirely unexpected, since it shares significant identity (73%) with MinD_{Ec}, and comes from an
organism that also contains MinC and MinE proteins. Of course, more subtle differences between
each protein, such as slight differences in the C-terminal MTS region for example (KSFFKRLF in
MinD_{Ng} and KGFFKRLF in MinD_{Ec}), likely reflect evolutionary changes that accommodate
 corresponding changes in their respective host targets, including membrane, MinC, and MinE
partners.
Interestingly, there is one species of *Neisseria, N. elongata*, which presents a rod-shaped morphology (Bovre and Hotten, 1970). While there is no available genome project for this organism, it should, more than likely, contain MinD, MinC, and MinE homologues, which would predictably behave as Min proteins in other rods, such as *E. coli*. It is also conceivable that MinD from the rod-shaped *N. elongata* would have an even higher identity to MinD$_{N_{g}}$ than to MinD$_{E_c}$, further blurring the line of distinction, if any, between 'coccal'- and 'rod'-derived MinD. However, further investigation of a potential min system in *N. elongata* must be done to confirm this.

From accumulated studies in *E. coli*, several models of cell division site selection have been generated and progressively modified (Hu and Lutkenhaus, 1999; Hu and Lutkenhaus, 2001; Margolin, 2001a; Lackner et al., 2003; Shih et al., 2003). By combining observations from the present study with *E. coli* Min protein models, a model for division site selection in Gram-negative round bacteria such as *N. gonorrhoeae* is proposed (Figure 8.1). Since GFP-MinD$_{N_{g}}$ was able to oscillate and to assemble/localize within coiled structures in rod-shaped and round *E. coli*, it is likely that MinD$_{N_{g}}$ undergoes similar behaviour within gonococcal cells. Nucleoid occlusion also influences cell division site placement (Woldringh, 1990; Yu and Margolin, 1999); therefore, the effect of bulk chromosomal DNA is also considered in this model, based on preliminary data obtained from nucleoid localization studies conducted in dividing gonococcal cells (see Appendix, Part D).

The model of cell division toporegulation in *N. gonorrhoeae* is similar to that of *E. coli*, as both require the localization of Min proteins along a helical scaffold. In *N. gonorrhoeae*, this basic Min protein array, which includes MinD$_{N_{g}}$, is proposed to coil along the longest available axis of the cell (Figure 8.1 A, red arrowheads), similar to that observed in both round and rod-shaped *E. coli* (Chapter 4). In fact, *N. gonorrhoeae* cells have been shown to elongate during their growth cycle, resulting in a slight longitudinal axis (Westling-Häggström et al., 1977); hence, it could be argued that gonococcal cells resemble extremely short rods prior to cytokinesis. Transmission electron microscopy in the present study also verified that gonococcal cells are not perfectly round, and can present a long axis (Figures 3.4 A and 3.9 E). Interestingly, the Min protein scaffold in *E. coli* does
not appear to be associated with other helical cytoskeletal structures, such as MreB arrays, which are involved in maintaining bacterial shape (Shih et al., 2003). Hence, it is tempting to speculate that the coiling orientation of Min protein arrays is dependent upon cell shape which, in turn, is dictated by cell growth.

Upon binding ATP, MinD<sub>N</sub> dimerizes and gains membrane affinity. Accumulation of MinD<sub>N</sub>-ATP subunits (green circles) along the helical array at one half of the cell commences, forming a ‘MinD<sub>N</sub> zone’, which recruits the MinC<sub>N</sub> division inhibitor (red squares) to that region as well (Figure 8.1A). MinE<sub>N</sub> would also be required for the localization of MinD<sub>N</sub> within a coil, possibly acting as a crosslinker (Suefuji et al., 2003). The extent of the MinD<sub>N</sub> zone is restricted to one cell half, as accumulated MinE<sub>N</sub> near the middle of the cell (MinE<sub>N</sub> ring-like structure; triangles) induces the dissociation of MinC<sub>N</sub> from MinD<sub>N</sub>, and stimulates the ATPase activity and release of MinD<sub>N</sub> from the membrane/Min protein lattice (Figure 8.1 B). Cytoplasmic MinD<sub>N</sub>·ADP (white circles) can diffuse towards the opposite end of the cell, where it re-initiates localization within a coil upon binding ATP (Figure 8.1 B, C). Hence, the continued oscillation of the MinCD<sub>N</sub> complex between opposite cell hemispheres should allow FtsZ assembly to commence at a midcell site (thunderbolts), which is relatively free of division inhibition (Figure 8.1 B-E).

Immunoelectron microscopy revealed that FtsZ initially accumulates specifically at a single invagination point in dividing gonococci. What role might the chromosome play in this process? Studies in <i>E. coli</i> and <i>B. subtilis</i> have shown that chromosomal DNA replication machinery (DNA replisome) is localized at midcell, suggesting that replicating chromosomes are threaded through a stationary replisome (Lemon and Grossman, 1998; Koppes et al., 1999). Therefore, it is possible that such a fixed DNA replisome is also present in growing <i>N. gonorrhoeae</i> cells. In Figure 8.1 D, this replisome is depicted at the ‘bottom’ of the cell (orange box). Hence, the periodic oscillation of the MinCD<sub>N</sub> complex from ‘left to right’ hemispheres, coupled with the presence of a replicating chromosomal mass at the ‘bottom’ of the cell would result in cytokinetic inhibition at all sites, except at the location opposite of the replisome (i.e. the ‘top’ of the cell in Figure 8.1 E), where FtsZ
polymerization can initiate. Once DNA replication and chromosome segregation events approach termination, the effects of nucleoid occlusion at midcell are diminished and full septation can occur.

Upon completing the first division event, each cell in the resulting diplococcus experiences growth along a second dimension that is perpendicular to the first, producing a new longitudinal axis (Westling-Häggström et al., 1977) (Figure 8.1 F, red arrowheads). A corresponding re-orientation of the Min protein coil would also occur, in order to direct Min protein subunit oscillations parallel to the nascent septum. This pattern of oscillation was observed in diplococcal E. coli cells expressing GFP-MinD\textsubscript{Ng} (this study) and GFP-MinD\textsubscript{Ec} (Corbin et al., 2002). In addition, it is possible that the DNA replisome in each daughter cell attaches to a new intracellular site, directly opposite the nascent septum (Figure 8.1 G; also Appendix, Figure A.6 B, C), which would further restrict FtsZ polymerization to the junction point between two cells (Figure 8.1 H). Hence, the redirection of Min protein oscillation and the effects of bulk chromosomal DNA will result in cell division along a plane that is perpendicular to the first (Figure 8.1 H, thunderbolts).

Therefore, based on their septation patterns, the major difference between the N. gonorrhoeae and E. coli Min systems would seem to be the reorientation of Min protein oscillation in the coccal system. From the present study, it appears that the direction of MinD oscillation corresponds to the longitudinal direction of its winding coil. It is possible that the ‘permanent’ MinCDE coiled array proposed to exist in rod-shaped bacteria (Shih et al., 2003) may not be as ‘fixed’ in coccal backgrounds, thus allowing the disassembly and/or reorientation of the array along a second dimension in cells such as N. gonorrhoeae. Interestingly, E. coli GFP-MinD also undergoes a perpendicular shift in oscillation direction in dividing round cells (Corbin et al., 2002); hence, the reorientation of GFP-MinD\textsubscript{Ng} in round cells was not due to MinD\textsubscript{Ng} regions that are specifically adapted for coccal cell systems. Again, it is interesting to propose that the coiling of the Min protein array is under the influence of the longest available cell axis created by cell growth.

There is evidence that lipid domains within the bacterial membrane can also influence the assembly of MinD \textit{in vivo}. In \textit{E. coli} lacking the zwitterionic phospholipid phosphatidylethanolamine,
and containing only anionic phospholipids cardiolipin and phosphatidylglycerol, GFP-MinD_{Ec} assembled into dynamic foci rather than its characteristic bands, despite the presence of MinE (Mileykovskaya et al., 2003). Therefore, since lipid composition seems to affect Min protein assembly in vivo, it is possible that rearrangements in lipid domains within actively dividing coccal bacteria can also contribute to reorienting Min protein oscillation.

Future studies to investigate the formation of Min protein coiled arrays in dividing cocci should prove very interesting, particularly during the transition from oscillating along one dimension to the other. These studies should shed light onto whether the MinCDE helical arrays are disassembled and re-assembled along new axes, or whether the coiled array is maintained, but rotated to accommodate the second oscillation direction. Such investigations could be achieved by live cell imaging of dividing round E. coli cells expressing GFP-fusions to MinD_{Ng} or to MinD_{Ec}. In addition, colocalization studies of FtsZ and nucleoids should increase our knowledge of the effects of nucleoid occlusion on multi-septating bacteria such as N. gonorrhoeae. Furthermore, studies to address the mechanisms that induce the direction of cell growth in relation to nascent septa should be of particular interest, especially when combined with Min coil localization.

Several questions still need to be addressed in the model of N. gonorrhoeae cell division. The challenges of genetically manipulating and localizing proteins in N. gonorrhoeae limited the MinD_{Ng} studies that could be performed in its native organism. While it is highly likely that the Min proteins exhibit dynamic localization and assembly in N. gonorrhoeae, in vivo localization studies using GFP-fusions should be performed to verify this. Cloning of these fusions into the gonococcal shuttle vector, or their integration into the gonococcal chromosome, should facilitate these studies. However, gonococci are prone to cell autolysis and are extremely fastidious; hence, in vivo localization studies may prove to be very difficult.

MinD is a complex protein which must perform a variety of activities in order to properly influence cell division site placement. These include binding ATP, dimerization and polymerization,
interaction with membrane and/or coiled array targets, recruitment of MinC, binding to MinE and having its ATPase activity stimulated, and dissociation from the membrane (Raskin and de Boer, 1999a; Hu and Lutkenhaus, 2001; Hu et al., 2002; Hu and Lutkenhaus, 2003; Hu et al., 2003, Lackner et al., 2003, Szeto et al., 2003; this study). With all these different functions, it is not surprising that bacterial MinD studies show that regions involved in mediating these different functions are distributed throughout the protein. By mapping these regions onto the solved crystal structure of Archaeal MinD (Figure 8.2; A. fulgidus MinD; Cordell and Löwe, 2001) the distribution of functional sites is more evident. While the actual structure and position of the C-terminal MTS of MinD is not known, it is predicted to form an amphipathic helix (Szeto et al., 2002); hence, an α-helix was manually inserted in Figure 8.2 A to complete the protein structure.

Although each MinD region shown in Figure 8.2 has been assigned a 'main' role, it remains difficult to designate any clear-cut regions of function, except perhaps the MTS. For example, while residues within the α-7 helix are implicated in MinC interaction (Hayashi et al., 2002) (Figure 8.2; purple residues in yellow helix), their effects on MinE-binding, dimerization, ATPase activity, and protein dynamism are unexplored. In addition, while the switch I and II sites may play a role in mediating MinC interaction (Figure 8.2, blue and green residues), they also affect MinE-induced ATPase activity of MinD, and their role in affecting MinD dynamic behaviour is not known (Zhou and Lutkenhaus, 2004). Furthermore, the K16Q mutation is of key importance in almost every facet of MinD function (Hu et al., 2002; this study). Hence, it appears that many aspects of MinD function are intimately linked with one another, and that assigning certain regions with a single function should be carefully considered.

The results from the present study further identify new functional regions in bacterial MinD (Figure 8.2). Each region was characterized by a battery of assays in order to better address their specific, as well as global, roles in MinD function. Interestingly, the 'loop' and extreme N-terminal regions identified in this study have almost opposite effects. In terms of MinD dynamics, loop region
8.2. Functional regions identified in MinD proteins from Gram-negative bacteria mapped onto the crystal structure of MinD from the Archaea *A. fulgidus* (MinDzf). (A) Residues and regions identified to have functional significance in MinDzf and MinDzc are indicated by arrows pointing to corresponding sites in the MinDzf crystal structure (Cordell and Löwe, 2001). Unless indicated, amino acid designations correspond to the MinDzf protein. Red helix represents the C-terminal membrane targeting sequence; however, its exact position and structure are currently not known and was therefore manually inserted into the figure to present a complete protein. Yellow helix, and the purple residues within, shows the α-7 helix, containing residues involved in binding to MinC, based on yeast two hybrid studies (Hayashi *et al.*, 2001) and as proposed by molecular modeling by our laboratory (Ramirez-Arcos *et al.*, in press). Single red residue represents K16 of the Walker A-ATP binding motif found. Blue and green residues indicate location of switch I and II sites, respectively, that appear to be required for interaction with MinC (Zhou and Lutkenhaus, 2004). ‘Loop’ region, identified in the present study, is shown in cyan and is implicated in conferring to MinD the ability to respond to MinE binding. N-terminal residues (orange) are involved in affecting the dynamics of MinD association with the membrane and/or Min coiled array, as well as interactions with other Min proteins (this study). The proposed dimerization interface is shown along the protein face indicated by the bracket. (B) represents a 90° downward rotation of (A). The proposed C-terminal MTS is not shown in (B).
Generalized proposed dimerization interface

Switch I region:
(aa 40-46; aa 40-46 in MinD_{E_{c}} and MinD_{E_{c},This study});
Interaction with MinC (Zhou and Lutkenhaus, 2004)

Switch II region:
(aa 117-123; aa 120-126 in MinD_{E_{c}} and 122-128 in MinD_{E_{c},This study});
Interaction with MinC (Zhou and Lutkenhaus, 2004)

K16: Interaction with MinD, MinC, MinE, membrane localization
(This study; Hu et al., 2002; Zhou and Lutkenhaus, 2004)

D149
(comm in MinD_{E_{c}} and E154 in MinD_{E_{c},This study});
Interaction with MinC
(Hayashi et al., 2001)

E143
(comm in MinD_{E_{c}} and E148 in MinD_{E_{c},This study});
Interaction with MinC
(Hayashi et al., 2001)

α-7 helix: Interaction with MinC
(Hayashi et al., 2001; Ramirez-Arcos et al., in press)

Extreme N-terminal region (aa 1-5 in MinD_{E_{c}}): Involved in controlling ATPase activity and dynamic localization, affects interaction with MinC, MinE_{E_{c}} and self-interaction (This study)

C-terminal membrane targeting sequence (MTS); (Szeto et al., 2002; Hu and Lutkenhaus, 2003) (Note: exact positioning/structure of MTS is currently unknown. C-terminus is predicted to form a helix, which was added to this crystal structure to complete the protein)

90° downward rotation of (A)
mutants lost the ability to oscillate clearly from pole-to-pole, and localized throughout a membrane-associated coil. In contrast, N-terminal MinD mutants experienced faster oscillation cycles, accompanied by increased tendencies to localize in the cytosol. Furthermore, while loop region mutations did not eliminate MinD<sub>N</sub> interaction with any other Min proteins, N-terminal mutations did diminish or abolish such interactions. Finally, while MinD<sub>N</sub>-loop exhibited low ATPase activity, even in the presence of MinE,<sub>N</sub> N-terminal MinD<sub>N</sub> mutants displayed MinE-independent ATPase activity. Hence, it is interesting to propose that these two separate regions may be involved in fine-tuning the dynamic properties of MinD through opposing effects. As such, MinD proteins would contain determinants within themselves that help autoregulate their activity, since correct cell division site placement is so vital for proper cell development.

In terms of MinD<sub>N</sub> itself, there are many interesting avenues of research that may be further pursued. Optimization of MinD<sub>N</sub> membrane binding assays are underway (Appendix) and should provide an additional useful functional assay for studying MinD<sub>N</sub> mutant activity. Furthermore, our laboratory has initiated NMR structural studies of MinD<sub>N</sub>. While there are several solved structures of Archaeal MinD proteins, there is, to date, no evidence that any of them are involved in cell division regulation, particularly since these organisms lack the other min genes. Hence, structural information from a bacterial MinD protein should be extremely useful to those studying in this field.

Aside from additional standard mutational analyses of other regions in MinD, another attractive project would be to extend MinD<sub>N</sub> studies (and Min proteins in general) to examine their role in bacterial pathogenicity. Such studies would be unique to the Min protein field. Both <i>N. gonorrhoeae</i> minC<sub>N</sub> (Ramirez-Arcos et al., 2001a) and minD<sub>N</sub> (this study) mutants exhibit decreased cell viability, and preliminary in vitro studies with collaborators at the University of Iowa have shown that these mutants are severely attenuated in adhesion and invasion of host epithelial cells (Dillon et al., unpublished results). Future studies combining molecular and cellular biology may even lead to unique Min protein target sites for the development of novel antimicrobials.
It is noted that a BLAST search of the human genome reveals two human proteins that have some identity to MinDNg. One protein, E2F transcription protein 3, has 27% identity and 54% similarity to MinDNg over a region corresponding to residues 49-120 of the bacterial protein. Interestingly, this region does not include the switch I and II sites of MinD, which one might expect to be conserved among various proteins in different organisms. Instead, the similarity encompasses a region between both switch sites, which contains the identified MinD ‘loop’ region in this study. Another human protein, identified as a sperm protein, has 20% identity and 43% similarity to MinDNg residues 103-228, which contains the switch II site and α-7 helix. Since these human proteins share homologous regions with MinD, the bacterial protein may not be suitable for antimicrobial targeting. However, by focusing on truly MinD-specific residues/regions which are not shared with human proteins, this issue could be circumvented.

Environmental factors from the host may also contribute to Min expression in N. gonorrhoeae and could also be an interesting focus for research. The min genes of N. gonorrhoeae are part of a large gene cluster containing several other genes that seem to be unrelated to the act of cell division, including those involved in transcriptional regulation (e.g. rpoA and oxyR) and translation (e.g. structural ribosomal genes) (Ramirez Arcos et al., 2001a). This is in contrast to the three E. coli min genes which seem to form an independent transcriptional unit themselves (Ramirez-Arcos et al., 2001a). Our laboratory has previously shown that the activity of promoter regions identified upstream of each minNg gene is influenced by factors that resemble the host genitourinary environment, including anaerobic conditions and the presence of urea (Ramirez-Arcos et al., 2001b). It is possible that the remaining genes residing in the 17 kb cluster with minCDENg are also involved, in part, in regulating gonococcal min gene expression under different host environmental conditions. For example, oxyR, located downstream of minCDENg, encodes a transcriptional regulator that acts as a repressor of catalase expression. In the presence of hydrogen peroxide, catalase production is induced through OxyR-mediated derepression (Tseng et al., 2003). Hence, it would be interesting to determine whether the expression of minCDENg genes is affected by OxyR and/or hydrogen peroxide.
This is the first study of MinD from a coecal bacterium. The construction of \( \text{min}D_{N_g} \) knockout and K16Q point mutation strains, as well as MinC_{N_g}-MinD_{N_g} overexpression studies, demonstrated that MinD_{N_g} is required to maintain the characteristic division pattern of \( N.\ gonorrhoeae \). In addition, MinD_{N_g} was shown to be active in \( E.\ coli \) backgrounds, being able to influence cell division and to exhibit intracellular dynamism and coil assembly in this organism. The use of \( E.\ coli \) as an indicator for MinD_{N_g} function greatly facilitated this study, since genetic manipulation, protein expression studies, and GFP-MinD_{N_g} localization could be readily conducted. Using various genetic and biochemical techniques, interactions between MinD_{N_g} and the other Min proteins were identified. Significantly, this study was the first to observe the self-association of bacterial MinD, now considered a key property of the protein that allows for its dynamic assembly. Finally, the use of structural homology modeling and protein sequence alignments, coupled with mutational analyses, identified several regions in MinD_{N_g} that are involved in maintaining function, including an exposed polar region implicated in sensitizing MinD_{N_g} to MinE binding/stimulation, and the extreme N-terminus of the protein that affects intrinsic MinD_{N_g} ATPase activity and general dynamism.

While found in many bacteria, Min protein-containing systems are not universal. With the numerous morphologies and septation patterns encountered in bacteria, it is not surprising that several other mechanisms have likely evolved to correctly localize septation planes in other microorganisms. For example, some bacteria, including Gram-positive cocci, seem to be devoid of any \( \text{min} \) genes, and their mechanisms of regulating cytokinesis are entirely unknown. Hence, while the Min proteins provide one solution for proper cell division site selection, the rich diversity in bacteria should ensure no shortage in studies that address alternative mechanisms for splitting heirs.
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CONTRIBUTION OF COLLABORATORS

The author would like to thank past and present members of the Dillon laboratory for their technical help in various aspects of this project. Dr. Sandra Ramirez-Arcos constructed plasmids pSR15, pSR3, pSRBD-C and pSRAD-C and provided much useful insight throughout this study. In addition, Dr. Ramirez-Arcos initiated GFP-localization studies. Nelson Eng provided purified MinE_{Ng} and assisted with ATPase assays and GFP-localization studies. Sudeep Acharya constructed many of the plasmids (designated with pSIA-) used in the studies of the MinD_{Ng} N-terminus while he was under my direct supervision, and contributed to both MinD ‘loop’ region and N-terminus experiments. Sheila Costford generated the *N. gonorrhoeae* mutant strain SCD-1 and constructed pSC9 and pSC10 plasmids as an Honour’s student under my supervision. Claude Raymond constructed the modified gonococcal shuttle vector and all its derivatives, while Mylène Theriault constructed plasmids designated with the pMJ- prefix, with both students under my direct supervision. Hui Li constructed pHLCN and purified soluble MinC_{Ng}. Dr. P. Anderson, University of Ottawa, provided access to size-exclusion chromatography equipment and Marc Rigden provided technical assistance with standardizing gel-filtration conditions. Polyclonal mouse anti-MinE_{Ng} antiserum was prepared by Dr. J. Webb, University of Ottawa. Peter Rippstein, Ottawa Hospital, Civic Campus, performed immunolocalization studies of MinD_{Ng}. He also immunogold labeled FtsZ_{Ng} in gonococcal cells and provided me with access to the electron microscope at the Laboratory Pathology facilities, Ottawa Hospital, to visualize FtsZ_{Ng} localization, as well as to examine *N. gonorrhoeae* SCD-1 morphology. Dr. P. Thibault, Institute of Biological Sciences, National Research Council of Canada, provided MALDI-TOF mass spectrometry analysis of His-MinD_{Ng}.

The author would also like to thank collaborators from other universities. Dr. T. Beveridge and Diane Moyles, University of Guelph, performed electron microscopy on *N. gonorrhoeae* strain CJS1D1 and control cells. Dr. C. Kay and Leslie Hicks, University of Alberta, performed analytical ultracentrifugation analyses on purified MinD_{Ng}. Initial GFP-MinD_{Ng} localization studies were done
in collaboration with Dr. W. Margolin, University of Texas Medical School. Far-UV circular dichroism was conducted by Dr. J. Wang and D. Fan, Southern Illinois University.
APPENDICES: Additional experiments
The following chapter details some studies performed in addition to the main body of work. Each study contains a brief description on its purpose and why they were not investigated further in this project. Results and Discussion are combined for each respective section. Table A.1 describes all plasmids used in the Appendix.

A. INVESTIGATION OF THE MinD$_{Ng}$ C-TERMINUS

Similar to the N-terminal studies performed on MinD$_{Ng}$ (Chapter 7), the C-terminus was also subjected to truncation to determine the effects on protein function and interactions. However, as it was reported that the C-terminus of MinD proteins contains a membrane targeting sequence (Szeto et al., 2002; Hu and Lutkenhaus, 2003), these studies were not pursued further, and experiments were re-directed towards investigating the MinD$_{Ng}$ N-terminus.

MATERIALS AND MATERIALS

Construction of C-terminal truncations to minD$_{Ng}$. C-terminal truncations (CT) to minD$_{Ng}$ were made to remove the last 21 and 38 amino acids from MinD$_{Ng}$ (MinD$_{Ng}$-21aaCT and MinD$_{Ng}$-38aaCT) to determine the effects on protein functionality. Primers minD10B or minD9B, each encoding a stop codon, were used with primer minD1 (Table 2.2) to amplify truncated minD$_{Ng}$ from N. gonorrhoeae CH811 (Table 2.1) chromosomal DNA. PCR amplicons were digested with EcoRI and BamHI and ligated into similarly digested pUC18 as above to form pMJ4 (minD$_{Ng}$-21aaCT), and pMJ2 (minD$_{Ng}$-38aaCT) (Table A.1). These plasmids, as well as pUC18 (negative control) and pSR3 (positive control; previously constructed in Chapter 4) (Table A.1) were transformed in wild-type E. coli PB103 for morphology studies as outlined in Chapters 2 and 4.

Construction of yeast two-hybrid plasmids encoding GAL4 fusions to C-terminal truncated minD$_{Ng}$. C-terminal truncated minD$_{Ng}$ genes, encoding MinD$_{Ng}$-21aaCT, MinD$_{Ng}$-38aaCT, MinD$_{Ng}$-83aaCT,
Table A.1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>$\text{P}_{\text{lac}}$ (Amp$^R$)</td>
<td>Amersham</td>
</tr>
<tr>
<td>pSR3</td>
<td>pUC18; $\text{P}<em>{\text{lac}}$::$\text{minD}</em>{\text{Ng}}$ (Amp$^R$)</td>
<td>Chapter 4, this study</td>
</tr>
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<td>pMJ4</td>
<td>pUC18; $\text{P}<em>{\text{lac}}$::$\text{minD}</em>{\text{Ng-21aaCT}}$ (Amp$^R$)</td>
<td>This study</td>
</tr>
<tr>
<td>pMJ2</td>
<td>pUC18; $\text{P}<em>{\text{lac}}$::$\text{minD}</em>{\text{Ng-38aaCT}}$ (Amp$^R$)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Yeast two-hybrid plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGAD424</td>
<td>$\text{P}_{\text{ADH1}}$::$\text{gal4(AD)}^1$ (Amp$^R$)</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGBT9</td>
<td>$\text{P}_{\text{ADH1}}$::$\text{gal4(BD)}^2$ (Amp$^R$)</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGADminD</td>
<td>pGAD424; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(AD)-minD}</em>{\text{Ng}}$ (Amp$^R$)</td>
<td>Chapter 5, this study</td>
</tr>
<tr>
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<td>pGBT9; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(BD)-minD}</em>{\text{Ng}}$ (Amp$^R$)</td>
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<td>pGBT9minE</td>
<td>pGBT9; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(BD)-minE}</em>{\text{Ng}}$ (Amp$^R$)</td>
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<td>pSRBD-C</td>
<td>pGBT9; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(BD)-minC}</em>{\text{Ec}}$ (Amp$^R$)</td>
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<td>pGAD424; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(AD)-minD}</em>{\text{Ng-21aaCT}}^3$ (Amp$^R$)</td>
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<td>pJminD11</td>
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<td>This study</td>
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<td>pJminD6</td>
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<td>This study</td>
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<td>pJminE7</td>
<td>pGBT9; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(BD)-minE}</em>{\text{Ng-4aaCT}}$ (Amp$^R$)</td>
<td>This study</td>
</tr>
<tr>
<td>pJminE8</td>
<td>pGBT9; $\text{P}<em>{\text{ADH1}}$::$\text{gal4(BD)-minE}</em>{\text{Ng-15aaCT}}$ (Amp$^R$)</td>
<td>This study</td>
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<td>pJminE2</td>
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<td>pJminE1</td>
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<td>This study</td>
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</table>

$^1$ GAL4-activation domain  
$^2$ GAL4 DNA-binding domain  
$^3$ CT denotes C-terminal truncation (e.g. 21aaCT = deletion of the 21 amino acids from the C-terminus)
and MinD_{Ng-237aaCT} were also generated using primer pairs minD1/minD9, minD1/minD10, minD1/minD4, and minD1/minD3 (Table 2.2). Each of these were cloned into the EcoRI and BamHI sites of pGAD424 to produce fusions with GAL4-AD encoded on pJminD10 (MinD_{Ng-21aaCT}), pJminD11 (MinD_{Ng-38aaCT}), pJminD2 (MinD_{Ng-83aaCT}), and pJminD1 (MinD_{Ng-237aaCT}) (Table A.1). Similarly, plasmids pJminD6 (MinD_{Ng-21aaCT}) and pJminD7 (MinD_{Ng-38aaCT}) were also constructed, where truncated MinD_{Ng} was in-frame with the GAL4-DNA BD encoded on pGBT9 (Table A.1). Yeast two-hybrid studies were carried out as described in Chapter 2.

RESULTS AND DISCUSSION

To determine whether the C-terminus of MinD_{Ng} is involved in inducing cell division arrest, deletion derivatives of minD_{Ng} were constructed to encode MinD_{Ng} bearing 21 and 38 aa C-terminal deletions (CT). Wild-type E. coli PB103 transformed with pMJ4 (minD_{Ng-21aaCT}) and pMJ2 (minD_{Ng-38aaCT}) (Table A.1) retained the characteristic short rod morphology (Figure A.1 C, D) seen with pUC18 negative control transformants (Figure A.1 A). As expected, cells transformed with pSR3, encoding wild-type MinD_{Ng} (Table A.1), were filamentous (Figure A.1 B), indicative of cell division arrest. Western blotting of cell extracts showed the overexpression of wild-type MinD_{Ng} (Figure A.1 E, lane 2) and MinD_{Ng-21aaCT} (lane 3) in comparison to the background levels of native E. coli PB103 MinD (lane 1). Overexpression of MinD_{Ng-38aaCT} was not observed (Figure A.1 E, lane 4), such that only native E. coli MinD was detected in the pMJ2 (minD_{Ng-38aaCT}) transformants.

The yeast two-hybrid system was also employed to determine how deletions from the C-terminus of MinD_{Ng} may affect interactions with other Min proteins. While unable to induce cell division inhibition in E. coli, MinD_{Ng-21aaCT} could still interact with MinC_{Ec} and with MinE_{Ng}; however, the apparent strength of interaction with MinC_{Ec} (34.73 ± 3.77 Miller units) was considerably less than wild-type MinD_{Ng} (141.01 ± 4.43 Miller units) (Table A.2). The interaction between MinD_{Ng-21aaCT} and wild-type MinD_{Ng} was abrogated (Table A.2). Further C-terminal deletions to MinD_{Ng}, including MinD_{Ng-38aaCT}, and the more drastic MinD_{Ng-83aaCT} and MinD_{Ng-238aaCT}, resulted in
Figure A.1. Expression of C-terminal truncated MinD<sub>Np</sub> derivatives in wild-type E. coli PB103. (A) Cells containing pUC18 display a short rod morphology. (B) Cells transformed with pSR3 wild-type minD<sub>Np</sub> are filamentous. Cells transformed with (C) pMJ4 (minD<sub>Np-21aaCT</sub>) and (D) pMJ2 (minD<sub>Np-38aaCT</sub>) retain short rod morphologies. (E) Western blotting using anti-MinD<sub>Np</sub> antisera shows overexpression of wild-type MinD<sub>Np</sub> (lane 2) and MinD<sub>Np-21aaCT</sub> (lane 3) in comparison to the background levels of native E. coli PB103 MinD (lane 1). Overexpression of MinD<sub>Np-38aaCT</sub> was not observed, and only native E. coli MinD was detected in pMJ2 (minD<sub>Np-38aaCT</sub>) transformants (lane 4). Scale bar in (A) indicates 10 μm, and all images are at the same magnification.
Table A.2. Yeast two-hybrid assays to determine Min protein interactions with C-terminal deletion derivatives of MinD_{N}\text{g}

<table>
<thead>
<tr>
<th>Fusion to GAL4- AD(^1)</th>
<th>Fusion to GAL4-DNA-BD(^2)</th>
<th>Intensity of blue colony colour(^3)</th>
<th>β-galactosidase Activity (Miller Units)(^4)</th>
</tr>
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<tbody>
<tr>
<td>MinD_{N}\text{g} (^5)</td>
<td>MinC_{Ec} (^6)</td>
<td>+++</td>
<td>141.07 ± 4.43</td>
</tr>
<tr>
<td>MinD_{N}\text{g}</td>
<td>MinE_{N}\text{g}</td>
<td>++</td>
<td>8.70 ± 0.05</td>
</tr>
<tr>
<td>MinD_{N}\text{g}</td>
<td>MinD_{N}\text{g}</td>
<td>++</td>
<td>23.14 ± 7.33</td>
</tr>
<tr>
<td>MinD_{N}\text{g}-21aaCT</td>
<td>MinC_{Ec}</td>
<td>++</td>
<td>34.73 ± 3.77</td>
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<tr>
<td>MinD_{N}\text{g}-21aaCT</td>
<td>MinE_{N}\text{g}</td>
<td>++</td>
<td>12.21 ± 0.29</td>
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<tr>
<td>MinD_{N}\text{g}-38aaCT</td>
<td>MinC_{Ec}</td>
<td>-</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>MinD_{N}\text{g}-38aaCT</td>
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<td>0.02 ± 0.01</td>
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<td>ND</td>
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<tr>
<td>MinD_{N}\text{g}-83aaCT</td>
<td>MinC_{Ec}</td>
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<td>MinD_{N}\text{g}-238aaCT</td>
<td>MinD_{N}\text{g}</td>
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<td>ND</td>
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</tbody>
</table>

\(^1\) GAL4-activation domain
\(^2\) GAL4-DNA binding domain
\(^3\) Yeast colony colour intensity corresponding to strength of interaction: (+++) = dark blue; (++) = blue; (+) = faint blue; (-) = white colonies
\(^4\) One unit equals the amount of β-galactosidase which hydrolyzes 1 µmol of ONPG per minute per cell
\(^5\) N. gonorrhoeae MinD
\(^6\) E. coli MinC
\(^7\) 'CT' indicates C-terminal truncation (e.g. 21aaCT = 21 amino acid truncation from the C-terminus of MinD_{N}\text{g})
\(^8\) 'ND' indicates β-galactosidase activity Not Determined
the loss of interaction with MinC_{Ec}, MinE_{Ng}, and MinD_{Ng} (Table A.2). These results suggest the last 21 residues from the C-terminus of MinD_{Ng} may play a role in its self-association; however, this region does not appear to be absolutely required for interaction with MinC and MinE.

These studies also indicate that removal of the last 21 residues from MinD_{Ng} abrogates its ability to induce cell division arrest. It has now been demonstrated that the C-terminus of bacterial MinD proteins contains a relatively conserved membrane targeting sequence (MTS) (Szeto et al., 2002; Hu and Lutkenhaus, 2003). Hence, deletion of the last 21 amino acids of MinD_{Ng} will remove its MTS (KSFKKRLF; Figure 3.1, green bar), resulting in its inability to cause cell division arrest upon overexpression in wild-type *E. coli* by virtue of its disrupted membrane association.

Similarly, a 20 amino acid C-terminal deletion to *E. coli* MinD disrupted the ability of MinD_{Ec} to activate MinC-dependent cell division arrest (Hu and Lutkenhaus, 2003). However, this MinD_{Ec} truncation also lost interaction with MinC, and was therefore proposed to be misfolded (Hu and Lutkenhaus, 2003). In contrast, the MinD_{Ng-21aaCT} derivative used in the present study retained interaction with both MinC_{Ec} and MinE_{Ng}, suggesting its inactivity in wild-type *E. coli* was not due to misfolding, but rather to a direct result of MTS removal. Furthermore, since MinD_{Ng-21aaCT} no longer interacted with wild-type MinD_{Ng}, as detected by yeast two-hybrid assays, it suggests that the regions responsible for self-interaction and for interaction with MinC/MinE do not have significant overlap. In addition, the loss of MinD_{Ng-21aaCT} interaction with wild-type protein suggests that determinants within the C-terminus of MinD_{Ng} may have a role in homodimerization; however, further studies, including a yeast two hybrid assay of MinD_{Ng-21aaCT}, MinD_{Ng-21aaCT} should be initiated to address this issue.
B. **IN VITRO MinD<sub>NM</sub> LIPID VESICLE BINDING ASSAYS**

Several groups have employed *in vitro* studies to investigate MinD membrane association (Hu *et al.*, 2002; Hu *et al.*, 2003; Lackner *et al.*, 2003; Mileyskovskaya *et al.*, 2003). In general, purified MinD proteins are incubated with artificial vesicles composed of phospholipids such as phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), or total *E. coli* membrane phospholipids, with the binding of protein to vesicles monitored by sedimentation assays (Hu *et al.*, 2002; Lackner *et al.*, 2003; Mileyskovskaya, 2003). It has been shown that *E. coli* MinD-ATP binds preferentially to anionic phospholipids such as PG and CL (Mileyskovskaya *et al.*, 2003), and can be induced to dissociate from lipid vesicles by MinE<sub>Ec</sub> (Hu *et al.*, 2002).

The following study was initiated to develop an additional *in vitro* functional assay for MinD<sub>NM</sub>, which would complement the MinD-ATPase stimulation assay developed in Chapter 6. While MinD<sub>NM</sub> bound preferentially to PG over PE vesicles, and could be induced to dissociate from PG vesicles by MinE<sub>NM</sub>, there was also evidence of some non-specific binding. As further work must be done to standardize reaction conditions, these results are presented in the Appendix for future considerations.

**MATERIALS AND METHODS**

**MinD<sub>NM</sub> lipid vesicle binding assays.** The ability of MinD<sub>NM</sub> to bind to phospholipid vesicles was examined using a sedimentation based assay, modified from previous protocols (Hu *et al.*, 2002; Lackner *et al.*, 2003) Purified N- and C-terminal His-tagged MinD<sub>NM</sub> proteins, as well as MinE<sub>NM</sub>, were obtained as outlined in Chapters 5 and 6, respectively.

Zwitterionic *E. coli* L-α-phosphatidylethanolamine (PE) and anionic 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG) (Avanti Polar Lipids, Inc.) vesicles were prepared for MinD<sub>NM</sub>-His vesicle binding studies. For PE vesicle formation, the method of Lackner *et al.* (2003) was modified...
as follows. 0.5 ml of stock PE (supplied at 25 mg/ml in chloroform) was dispensed into a borosilicate glass tube and allowed to dry under a gentle stream of filtered air. Dried PE was resuspended in 1.25 ml of reaction buffer (RB; 25 mM Tris-Cl, 50 mM KCl, pH 7.5) to give a final lipid concentration of 10 mg/ml. The solution was incubated at 65ºC with frequent vortexing and pipetting using a fine microtip. PE vesicles were prepared fresh prior to each experiment. Anionic PG vesicles were prepared according to the protocol detailed in Chapter 6 Materials and Methods.

Purified His-MinDN_{Eg}, MinDN_{Eg}-His, and MinDN_{Eg,K16Q}-His were incubated with PG or PE vesicles, ATP, and MinE_{Ng} to determine their associative properties with lipids. Prior to each experiment, protein concentrations were determined using the Bio-Rad protein assay kit and equalized as required by diluting in their respective dialysis buffers. In a typical 50 µl reaction, the following reagents were added in this order (final concentrations in brackets): reaction buffer (RB: reaction buffer, 25 mM Tris-Cl, 50 mM KCl, pH 7.5; volume added depending on other reagents to be included), MinDN_{Eg} protein (0.2-0.24 mg/ml), ATP (1mM), lipid vesicles (0.5 mg/ml), and MgCl₂ (1mM). Reactions were incubated at room temperature for five minutes and, if required, MinE_{Ng} (0.5 mg/ml final concentration) was then added for an additional five minutes. The reactions were then centrifuged at high speed for one minute to pellet vesicles and any associated proteins. The supernatant was removed and the pellet was resuspended in 50 µl RB. Proteins within the supernatant and pellet fractions were analyzed by SDS-PAGE.
RESULTS AND DISCUSSION

In the presence of ATP, *E. coli* MinD has been shown to associate with artificial vesicles composed of PG, PE, CL, total *E. coli* membrane phospholipids, and various combinations of these components as well (Hu *et al.*, 2002; Lackner *et al.*, 2003; Mileykovskaya *et al.*, 2003). To determine membrane binding capabilities of MinD<sub>N</sub>, purified N-terminal His-tagged MinD<sub>N</sub> (His-MinD<sub>N</sub>) was incubated in the presence or absence of ATP and artificial vesicles composed of either the zwitterionic phospholipid PE, or the anionic phospholipid PG. Protein-vesicle complexes were subsequently isolated by sedimentation and analyzed by SDS-PAGE, similar to assays performed previously (Lackner *et al.*, 2003). Any MinD<sub>N</sub> protein remaining in the supernatant is presumed not associated with any lipid vesicles.

Prior to their use, the integrity of PE (Figure A.2 A) and PG (Figure A.2 B) vesicles was determined using phase contrast microscopy. Vesicle diameters ranged from ~3-10 µm (Figure A.2 A, B). When incubated with ATP and PE vesicles, the majority of His-MinD<sub>N</sub> was found in the supernatant fraction, indicating little association between MinD<sub>N</sub> and zwitterionic vesicles (Figure A.2 C; lanes 1, 2). However, incubation of His-MinD<sub>N</sub> with PG vesicles and ATP resulted in the majority of protein associated with lipids in the pellet fraction (Figure A.2 D; lanes 1, 2). This indicates that MinD<sub>N</sub> preferentially binds anionic phospholipid vesicles over zwitterionic ones. Even in the absence of ATP, a greater proportion of His-MinD<sub>N</sub> was still associated with PG in the pellet fraction (Figure A.2 D; lanes 5) compared to the supernatant (lane 6). In the absence of vesicles, His-MinD<sub>N</sub> was found almost exclusively in the supernatant fraction (Figure A.2 D; lanes 3, 4).

His-MinD<sub>N</sub> could also be induced to dissociate from PG vesicles by MinE<sub>N</sub>. Addition of MinE<sub>N</sub> to a reaction containing His-MinD<sub>N</sub> and PG resulted in a greater proportion of His-MinD<sub>N</sub> appearing in the supernatant fraction (Figure A.2 D; lanes 7, 8) as compared to a similar reaction without MinE<sub>N</sub> (Figure A.2 D; lanes 1, 2).

C-terminal His-tagged MinD<sub>N</sub> (MinD<sub>N</sub>-His) was also assayed for vesicle binding. When incubated with PE vesicles and ATP, almost all MinD<sub>N</sub>-His was found in the supernatant fraction.
Figure A.2. Vesicle binding studies using N-terminal His-tagged MinDNg (His-MinDNg). Pellet (P) and supernatant (S) fractions are denoted. (A) Zwitterionic PE vesicles observed under phase-contrast microscopy. All panels are at the same magnification. (B) Anionic PG vesicles observed under phase-contrast microscopy. All panels are at the same magnification. (C) Vesicle sedimentation assay conducted with His-MinDNg, ATP, and PE vesicles. (D) Vesicle sedimentation studies conducted with His-MinDNg and PG vesicles. Lanes 1,2: ATP and PG included. Lanes 3,4: only ATP included. Lanes 5,6: only PG included. Lanes 7,8: ATP, PG, and MinENg included. All reactions carried out in presence of 1 mM MgCl₂.
(Figure A.3; lanes 7, 8), indicating a low affinity for zwitterionic phospholipid vesicles. In contrast, MinD_{Ng}-His was found in the pellet fraction when incubated with PG vesicles and ATP (Figure A.3; lanes 5, 6). When ATP was omitted, the majority of MinD_{Ng}-His was still associated with PG vesicles (Figure A.3; lanes 3, 4), behaving similarly as His-MinD_{Ng} in the absence of ATP (Figure A.2 D; lanes 5, 6). In the absence of PG vesicles, MinD_{Ng}-His was found almost entirely in the supernatant fraction (Figure A.3; lanes 1, 2).

As with N-terminal His-tagged MinD_{Ng}, MinD_{Ng}-His could also be induced by MinE_{Ng} to dissociate from PG vesicles (Figure A.4). This ability was concentration dependent, as increasing MinE_{Ng} concentration resulted in greater amounts of MinD_{Ng}-His dislodged from the PG vesicles. As a result, increased MinD_{Ng}-His was observed in supernatant fractions (Figure A.4; lanes 2, 4, 6, 8), with corresponding decreases in MinD_{Ng}-His levels in pellet fractions (Figure A.4; lanes 1, 3, 5, 7). In contrast, MinE_{Ng} storage buffer could not induce the dissociation of MinD_{Ng}-His from PG vesicles. Hence, the ability to remove of MinD_{Ng}-His from lipid vesicles is specific to MinE_{Ng}.

Surprisingly, the negative control MinD_{Ng,K16Q}-His protein could also associate with PG vesicles in the presence or absence of ATP (Figure A.5; lanes 1, 2 and 5, 6). This result was unexpected, since GFP-MinD_{Ng,K16Q} was observed to localize to the cytoplasm when expressed in E. coli (Chapter 4). Furthermore, studies using purified E. coli MinD_{K16Q} and total E. coli phospholipid vesicles have indicated a lack of association between the two (Hu et al., 2002). Interestingly, in parallel experiments, the addition of MinE_{Ng} could induce a greater dissociation of MinD_{Ng,K16Q}-His from PG vesicles (Figure A.5; lanes 7, 8) than wild-type MinD_{Ng}-His (Figure A.5; lanes 9, 10). While 57% and 43% of wild-type MinD_{Ng}-His was distributed in the pellet and supernatant fractions, respectively, MinD_{Ng,K16Q}-His was distributed almost equally in both fractions (49% and 51%).

Overall, these studies show that purified MinD_{Ng} proteins display a preference to bind anionic vesicles over zwitterionic ones. This supports observations using purified E. coli MinD, as well as E. coli and B. subtilis MinD MTS peptides (Szeto et al., 2003; Mileykovskaya et al., 2003). The MTS of MinD is proposed to form an amphipathic helix that lies parallel to the inner cell membrane surface.
Figure A.3. Vesicle binding studies using C-terminal His-tagged MinD<sub>Ne</sub> (MinD<sub>Ne</sub>-His). Pellet fractions denoted by 'P' and supernatant fractions denoted by 'S'. Sedimentation studies conducted with MinD<sub>Ne</sub>-His and: ATP (lanes 1, 2); PG (lanes 3, 4); ATP and PG (lanes 5, 6); and ATP with PE (lanes 7, 8). All reactions carried out in presence of 1 mM MgCl<sub>2</sub>. 

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**Figure A.4. MinE<sub>Ng</sub>-induced dissociation of MinD<sub>Ng</sub>-His from PG vesicles.** Lanes 1-8: MinD<sub>Ng</sub> bound to PG vesicles was incubated with increasing quantities of MinE<sub>Ng</sub>. Note the corresponding decrease in the amount of MinD<sub>Ng</sub> found in the pellet fraction (P; lanes 1, 3, 5, and 7) and the increase in MinD<sub>Ng</sub>-His appearing in the supernatant (S; lanes 2, 4, 6, 8). Lanes 9 and 10 show that MinE<sub>Ng</sub> storage buffer is unable to induce dissociation of MinD<sub>Ng</sub> from PG vesicles. All reactions carried out in presence of 1 mM MgCl<sub>2</sub>. 

**Table:**

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<tr>
<td>PG</td>
<td>+</td>
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<tr>
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<tr>
<td>[Lysozyme] mg/ml</td>
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Figure A.5. Vesicle binding studies using MinD$_{Nega}$-His and wild-type MinD$_{Nega}$-His. Pellet fractions denoted by ‘P’ and supernatant fractions denoted by ‘S’. Sedimentation studies conducted with MinD$_{Nega}$-His and: ATP and PG (lanes 1, 2); ATP only (lanes 3, 4); PG only (lanes 5, 6); and ATP, PG, and MinE$_{Nega}$ (lanes 7, 8). Lanes 9 and 10 are equivalent to lanes 7 and 8, except wild-type MinD$_{Nega}$ was used. All reactions carried out in presence of 1 mM MgCl$_2$. 

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In doing so, the hydrophobic face of the helix interacts with lipid acyl chains, while the positively charged face interacts with polar lipid headgroups (Szeto et al., 2002). Examination of the MinD_{Ng} MTS (KSFFKRLF) shows that it is very similar to the MTS of MinD_{E} (KGFLKRLF) (Figure 3.1, green bar), with both containing positively charged residues that, when arranged in a helix, would be predicted to preferentially interact with negatively charged lipid headgroups (Szeto et al., 2002), such as those found in PG vesicles used in this study.

Why MinD_{Ng-K16Q}-His associates with PG vesicles is unclear. It is possible that the C-terminal His-tag common to both MinD_{Ng-K16Q}-His and MinD_{Ng}-His contributed to non-specific binding with anionic phospholipids, resulting in the ATP-independent association of each protein to the vesicles. However, it is noted that His-MinD_{Ng}, bearing a tag-free C-terminus, also associated specifically with PG vesicles in the absence of ATP. Therefore, it is unlikely that a C-terminal 6XHis tag is responsible for ATP-independent MinD_{Ng} membrane binding.

Gonococcal and E. coli cell membranes are not naturally composed of pure PG (each containing approximately 20% PG) (Raetz, 1978; Senff et al., 1976; Sud and Feingold, 1976; Rahman et al., 2000). Hence, it is possible that the excessive concentration of anionic phospholipid may have accounted for some ATP-independent binding of purified MinD_{Ng} and MinD_{Ng-K16Q} to PG vesicles used in this study. In support of this, studies using vesicles composed of increasing concentrations of another anionic phospholipid, cardiolipin, have also demonstrated a corresponding increase in ATP-independent association of E. coli MinD with the vesicles (Mileykovskaya et al., 2003). Further studies using vesicles that better represent the gonococcal cell membrane should be investigated to in order to optimize conditions for MinD_{Ng} vesicle binding assays.

Despite this, it is clear that the PG vesicles made in this study can be used successfully for MinE_{Ng}-induced ATPase stimulation assays of MinD_{Ng} (Chapter 6). Wild-type MinD_{Ng} ATPase is stimulated in the presence of these vesicles and MinE_{Ng}, similar to E. coli MinD (Hu et al., 2002), while the negative control MinD_{Ng-K16Q} was not (Chapter 6).
C. PRELIMINARY STUDIES ELUCIDATING REGIONS IN MinE<sub>Ng</sub> RESPONSIBLE FOR INTERACTION WITH MinD<sub>Ng</sub>

MinE<sub>Ng</sub> has been shown to interact with MinD<sub>Ng</sub> in this study using the yeast two-hybrid system (Chapter 5) and is required to stimulate GFP-MinD<sub>Ng</sub> oscillation and its localization within coiled arrays (Chapter 4). A recent study using *E. coli* MinE has implicated specific residues within the N-terminus (aa 1-31) of the protein to be involved in binding to MinD<sub>Ec</sub> (Ma et al., 2003). These residues are all located along the same face of an α-helix, and are proposed to interact with a corresponding helical region in MinD (King et al., 1999). Mutation of residues from positions 15 to 30 along this particular α-helix face were shown to disrupt MinE<sub>Ec</sub> interaction with MinD<sub>Ec</sub> (Ma et al., 2003). To determine what region of MinE<sub>Ng</sub> binds to MinD<sub>Ng</sub>, N- and C-terminal truncations to MinE<sub>Ng</sub> were constructed for use in yeast two-hybrid assays.

MATERIALS AND METHODS

**Construction of GAL4-BD fusions to MinE<sub>Ng</sub> N- and C-terminal deletion derivatives.**

Truncations to *minE*<sub>Ng</sub> were made and cloned into the yeast two-hybrid vector pGBT9 (Table A.1) to determine MinE<sub>Ng</sub> regions (full length of 87 aa) that interact with MinD<sub>Ng</sub>. Deletions to the 5’ end of *minE*<sub>Ng</sub> were made using primer pairs JSminE7/minE2, JSminE8/minE2, minE5/minE2, and minE6/minE2 (Table 2.2). PCR amplicons were digested with *EcoR*I and *BamH*I and ligated into similarly digested pGBT9 produced plasmids pJminE5 (MinE<sub>Ng-5aaNT</sub>), pJminE6 (MinE<sub>Ng-11aaNT</sub>), pJminE3 (MinE<sub>Ng-26aaNT</sub>), and pJminE4 (MinE<sub>Ng-54aaNT</sub>), respectively ('NT' denotes N-terminal truncation) (Table A.1). Deletions to the 3’ end of *minE*<sub>Ng</sub> were also made using primer pairs minE1/JSmE9, minE1/JSmE10, minE1/minE3, and minE1/minE4 (Table 2.2), and ligated into pGBT9 as above. The resulting plasmids were pJminE7 (MinE<sub>Ng-4aaCT</sub>), pJminE8 (MinE<sub>Ng-15aaCT</sub>), pJminE2 (MinE<sub>Ng-30aaCT</sub>), and pJminE1 (MinE<sub>Ng-59aaCT</sub>), respectively ('CT' denotes C-terminal truncation) (Table A.1). The integrity of all GAL4 fusions was verified by DNA sequencing.
RESULTS AND DISCUSSION

MinE interacts with MinD to stimulate MinD ATPase activity and intracellular movement (Chapter 4, 5, and 6, this study; Huang et al., 1996; Raskin and de Boer, 1999a; Rowland et al., 2000; Hu and Lutkenhaus, 2001). Preliminary yeast two-hybrid studies were initiated using N- and C-terminal deletion derivatives of MinE_Ng in order to begin elucidating potential interacting regions with MinD_Ng. Severe truncation derivatives to either the N- or the C-terminus of MinE_Ng were constructed, and included MinE_Ng-26aaNT, MinE_Ng-54aaNT, MinE_Ng-30aaCT, and MinE_Ng-59aaCT. All of these deletions abrogated MinE_Ng interaction with MinD_Ng (Table A.3). However, less drastic deletions, including removal of the first 5 or 11 N-terminal residues (MinE_Ng-5aaNT and MinE_Ng-11aaNT), or the last 4 or 15 residues (MinE_Ng-4aaCT and MinE_Ng-15aaCT) of MinE_Ng did not eliminate its interaction with MinD_Ng (Table A.3).

These studies suggest that residues found at the extreme N- and C-termini of MinE_Ng are not implicated in its interaction with MinD_Ng, and that region(s) involved in binding to MinD_Ng are located within residues 12-72 of MinE_Ng. The N-terminus of E. coli MinE (aa 6-35) is predicted to form an α-helix that interacts with MinD_Ec (Ma et al., 2003). Since the N-terminus of MinE_Ng (aa 1-35) has 31% identity and 57% similarity to that of MinE_Ec, it is possible that gonococcal MinE also contains a similar N-terminal α-helix. Mutations within residues 15-30 that are proposed to lie along the same face of this helix can disrupt MinE_Ec binding to MinD_Ec. Hence, deletion of the first 11 residues in MinE_Ng-11aaNT would not likely disturb MinD_Ng binding, while more severe N-terminal truncations would, as seen in the present study. Comprehensive studies with MinE_Ng are currently being conducted by another doctoral student in the Dillon laboratory.
Table A.3. Yeast two-hybrid analysis of the interactions of MinE<sub>Ng</sub> deletion derivatives with wild-type MinD<sub>Ng</sub>

<table>
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<th>Fusion to GAL4-BD&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Fusion to GAL4-DNA-AD&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Intensity of blue colony colour&lt;sup&gt;3&lt;/sup&gt;</th>
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<tr>
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<td>MinD&lt;sub&gt;Ng&lt;/sub&gt;&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>MinD&lt;sub&gt;Ng&lt;/sub&gt;</td>
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<sup>1</sup> GAL4-DNA binding domain  
<sup>2</sup> GAL4-activation domain  
<sup>3</sup> Colour intensity corresponding to strength of interaction: (+) denotes blue; (+) denotes light blue; (-) denotes white colonies  
<sup>5</sup> N. gonorrhoeae MinE  
<sup>6</sup> N. gonorrhoeae MinD
D. NUCLEOID LOCALIZATION IN DIVIDING *N. gonorrhoeae* CELLS

The nucleoid appears to have a role in toporegulating cell division site placement (Woldringh, 1990). To determine what information can be obtained about the role that the nucleoid may have specifying the alternating division planes in gonococci, DAPI staining was employed to visualize chromosomes in dividing *N. gonorrhoeae*. These experiments are preliminary, but they do indicate that the nucleoids in dividing gonococci are arranged such that they would accommodate the typical division pattern of these cells. However, due to the small size of *N. gonorrhoeae*, microscopy and image collection techniques should be standardized to better elucidate the effects of nucleoid occlusion on gonococcal cell division. Hence, results are presented in the Appendix to provide considerations for future study.

MATERIALS AND METHODS

**Nucleoid localization in *N. gonorrhoeae* cells using DAPI staining.** To determine the position of the bulk chromosomal mass in dividing *N. gonorrhoeae* CH811 cells (Table 2.1), DAPI (4', 6-diamidino-2-phenylindole-2HCl) (Molecular Probes) staining was used. A log phase culture of *N. gonorrhoeae* CH811 was fixed, adhered to polylysine coated coverslips, and stained with DAPI as outlined in Chapter 7 Materials and Methods. Fluorescence microscopy was performed on an Olympus BX50 fluorescent microscope (Dr. Douglas Franks, Department of Pathology, University of Ottawa) equipped with a CCD Monichron camera and Northern Eclipse software.
RESULTS AND DISCUSSION

In addition to the Min system, the nucleoid itself is proposed to have a role in determining the positioning of bacterial division sites, supposedly by interfering with FtsZ polymerization through nucleoid ‘occlusion’ (Woldringh, 1990). In *N. gonorrhoeae*, the first round of cell division is initiated by a single invagination point at one side of the cell (Figure 1.7 B), while a second cell division event begins at the junction between two connected daughter cells (Figure 1.7 C). Both of these events involve the localization of FtsZ to the leading edge of cell envelope constrictions (Figure 3.9 A, B, and E).

To determine whether the nucleoid influences the positioning of cytokinetic constrictions in dividing *N. gonorrhoeae*, DAPI staining was employed to localize bulk chromosomal material in these cells. While difficult to draw conclusions regarding the influence of the nucleoid, if any, on gonococcal cell division, fluorescence microscopy did reveal that the general localization of chromosomal material was consistent with the cell division pattern observed in *N. gonorrhoeae*. While individual cocci each presented a single circular DAPI signal (Figure A.6 A), each cell comprising a *diplococcal* arrangement typically exhibited a curved ‘C’-shaped chromosomal mass (Figure A.6 B, C). These curved chromosomal masses were distributed such that the junction between two cells was devoid of any DNA material (Figure A.6 C, arrow). This is consistent with leaving the junction free for FtsZ accumulation (as observed in Figure 3.9 E), in preparation for division along a plane that is perpendicular to the first. Some diplococci also displayed evidence of four nucleoid bodies, with each cell containing two DAPI stained chromosomes (Figure A.6 D, arrows). In these instances, the nucleoids were consistently arranged such that they would NOT block the second plane of division (Figure A.6 D’, dotted line). Cytokinesis along this second dimension would then result in four cells, each having its own nucleoid.

Overall, these studies show that the nucleoid in *N. gonorrhoeae* is arranged in patterns that are consistent with accommodating the nature of cytokinesis in this organism. Future studies to localize
Figure A.6. Localization of DAPI stained nucleoids in wild-type *N. gonorrhoeae* CH811 cells. In all images, fluorescent nucleoids are presented in the left panel, and the corresponding phase contrast image is shown on the right. (A) Individual coci, each containing a single, circular DAPI fluorescent signal. (B, C) Diplococci contain nucleoids that localize in a crescent shaped arrangement in each cell. Arrow in (C) indicates DNA-free zone in the middle of the junction point between each cell comprising a diplococcus. (D) Diplococcus showing four DAPI-stained chromosomal masses (arrows). Each cell contains two distinct DAPI signals. (D’) The arrangement of the four nucleoids in (D) is consistent with allowing cell division to proceed along a plane (dotted line) that is perpendicular to the first. Scale bar in (A) indicates 5 μm, and all images are at the same magnification.
both nucleoid and FtsZ should further enlighten details regarding alternating cell division planes that are characteristic of *N. gonorrhoeae*.
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Thesis title- Splitting heirs: A study of the cell division site determinant
MinD from the coccus Neisseria gonorrhoeae

Department of Biology
University of Ottawa, Ottawa, Ontario
Thesis title- The role of integration host factor binding sites in gonococcal
β-lactamase producing plasmid replication

Honours and Distinctions

Canadian Association of Gastroenterology (CAG)/Canadian Institutes of Health Research (CIHR)/Solvay Fellowship (2004-2005)

Canadian Institutes of Health Research (CIHR) Doctoral Research Award (2002-2005)

Winner at the 2001 Canadian Society of Microbiologists Student Award Symposium Presentations

Natural Sciences and Engineering Research Council (NSERC) PGS B Award (2000-2002)

University of Ottawa Strategic Areas of Development Award (1999-2000, 2000-2001)

Natural Sciences and Engineering Research Council (NSERC) PGS A Award (1998-2000)

University of Ottawa Undergraduate Summer Research Award (1997)


Canada Scholarship (1994-1998)

University of Ottawa Admission Scholarship (1994)
**Professional associations**

Canadian Society of Microbiologists  
American Society of Microbiology  
Canadian Association of Gastroenterology

**University activities**

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<th>Date</th>
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<tr>
<td>09/1999-12/1999</td>
<td>Teaching assistant for General Microbiology course</td>
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<tr>
<td>09/2000-12/2000</td>
<td>Department of Biology, University of Ottawa</td>
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<tr>
<td>09/2001-12/2001</td>
<td>Teaching assistant for Medical Microbiology Laboratory</td>
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<td>10/1998-11/1998</td>
<td>Faculty of Medicine, University of Ottawa</td>
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<tr>
<td>05/1998-08/1998</td>
<td>Research assistant for Dr. Jo-Anne R. Dillon</td>
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<tr>
<td></td>
<td>Department of Biochemistry, Microbiology, and Immunology, University of Ottawa</td>
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<tr>
<td>01/1998-04/1998</td>
<td>Teaching assistant for undergraduate Molecular Biology course,</td>
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<tr>
<td>05/1997-08/1997</td>
<td>Research assistant for Dr. Linda Bonen</td>
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<td>Department of Biology, University of Ottawa</td>
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**Supervision of undergraduate students**

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<th>Date</th>
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<tr>
<td>09/2002-04/2003</td>
<td>Sudeep Acharya, 4th year undergraduate Honours student</td>
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<tr>
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<td>Biopharmaceuticals Program, University of Ottawa</td>
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<td>09/2001-04/2002</td>
<td>Sheila Costford, 4th year undergraduate Honours student</td>
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<tr>
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<td>05/2001-08/2001</td>
<td>Mylène Thériault, undergraduate summer student</td>
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<td>01/2001-04/2001</td>
<td>Yulia Artemenko, undergraduate volunteer student</td>
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<td>Department of Biochemistry, University of Ottawa</td>
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<tr>
<td>05/2000-08/2000</td>
<td>Claude Raymond, undergraduate summer student</td>
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<td>Department of Biology, University of Ottawa</td>
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Publications in refereed journals


Submitted publications


Number of manuscripts reviewed at the request of scientific journals: 1

Patent applications

Provisional patent applied for: "Blocking cell-division as a therapeutic treatment of pathogenic bacteria".

Joint inventors: Dr. Jo-Anne R. Dillon, Dr. Sandra Ramirez-Arcos, Dr. Hossein Salimnia, and Jason Szeto.
Application filed May 26, 2000 in Canada (Ref #08-882835CA) and United States of America (Serial# Ref #08-882757US).
Published conference abstracts


Conference oral presentations