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POSTDOCTORAL STUDIES

Sudeep Acharya

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biochemistry)

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Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

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TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Jo-Anne Dillon

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. Natalie Goto

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Daniel Figeys

Dr. Mary Hefford

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

**BIOCHEMICAL, STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE
CELL DIVISION SITE DETERMINANT MIND_{NG}:
TO DIVIDE OR NOT TO DIVIDE**

Sudeep Acharya

Thesis submitted to
The Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of requirements for the
Master of Science degree in Biochemistry

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
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ABSTRACT

The Min system, constituting MinC, MinD, and MinE proteins plays an essential role in regulating cell division in Gram-negative bacteria. MinD, a peripheral membrane ATPase, functions by recruiting the cell division inhibitor, MinC, to the membrane where it prevents septation. Counteracting this effect is MinE which can bind to MinD, thereby displacing MinC. Formation of the MinE-MinD complex also stimulates the ATPase activity of MinD, and is required for the periodic oscillations of the MinC-MinD complex on and off the membrane required for normal cell division. Our laboratory has shown that MinD self-associates, and this ATP-dependent dimerization is essential for protein function. Recently I and others in the laboratory demonstrated that, unlike wild type MinD, a 3 amino acid (MinD_{Ng-3aaNT}) deletion from the N-terminus of MinD is sufficient to abrogate MinD_{Ng-3aaNT} self interaction, its interaction with wild type MinD_{Ng}, and MinE_{Ng} (Szeto *et al.*, 2004). In addition, a polar loop on the surface of MinD implicated in its dynamic oscillation and topological specificity (Szeto *et al.*, 2005) was identified. Despite the presence of MinE, hydrophobic mutations at these polar residues (R92, D93, and K94) abolished MinD oscillations. While crystal structures are available for monomeric MinD it is not yet known how dimerization occurs or how interactions with MinE can stimulate MinD ATPase activity. In order to address these questions I developed protocols for the production of MinD samples suitable for analysis by solution NMR and to study ATPase and lipid-binding activity of wild type and mutant MinD proteins *in vitro*.

Since the solubility of wild type MinD was found to be limiting for the solution NMR studies, I designed mutants that exhibit increased solubility and can be obtained in higher yields than previously possible. Preliminary results with the most soluble construct indicated that the protein was folded but that higher concentrations will be required for structural studies.

In parallel, functional assays for MinD ATPase and lipid binding activity were also developed. I found that with respect to ATP, MinD displays positive substrate cooperativity both in the presence and absence of MinE_{Ng}. A Hill analysis of these curves suggests that in the absence of MinE there are at least two interdependent binding sites, while in the presence of MinE the cooperativity increases such that at least eight active sites are functionally linked. Since there can be only one active site per MinD monomer, the presence of

cooperativity suggests that MinD must form oligomers that are more active than the monomers. Moreover, the increase in cooperativity in the presence of MinE suggests that MinE promotes the formation of higher order MinD polymers with an extensive network of catalytic sites that positively regulate each other.

New insight were also provided into the role of the N-terminus of MinD by these *in vitro* assays since the mutant MinD_{Ng-3aaNT}, had basal ATPase activity that was ~6-fold higher than wild type and that this activity was not further stimulated by MinE. Thus the N-terminal region of MinD influences ATPase activity and is important for MinE-mediated stimulation of this activity. In contrast I found that a surface exposed loop on MinD would almost completely abrogate ATPase activity if mutated to hydrophobic residues even though it is still able to interact with MinE and phospholipids. Overall, my studies of MinD function, provided insight in which nucleotide binding and hydrolysis control the localization of MinD, and how MinE can promote higher reaction rates in MinD.

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"The greatest accomplishment is not in never falling, but in rising again after you fall."

(Vince Lombardi)

Those that know me see it only fitting that this thesis begin with a sports quote. And who better to quote than the most inspirational and celebrated coach, Vince Lombardi, whose speeches, with their messages of perseverance and dedication, still hold true today. Speaking of great coaches, I have been privileged to learn under the supervision of Dr. Natalie K. Goto and Dr. Jo-Anne Dillon. They were generous enough to supervise and guide me, and their constructive comments enlightened my endeavors with protein chemistry and molecular biology. I would like to thank Dr. Baenziger and Dr. Lorimer; they were members of my thesis committee and have helped in improving it. In addition, I thank Dr. Huber and Dr. Bonen for challenging me, motivating me, and inspiring me to achieve at a higher level.

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Figure 3 – Plot of the amount of inorganic phosphate released as a function of MinD_{Ng-K16Q} and MinD_{Ng-loop} in the presence and absence of MinE_{Ng}

LIST OF ABBREVIATIONS

aa	amino acid
AD	activation domain
Af	<i>Archaeoglobus fulgidus</i>
ATP	adenosine 5'-triphosphate
Amp	ampicillin
BD	binding domain
Bs	<i>Bacillus subtilis</i>
CT	C-terminal truncation
dcw	division and cell wall synthesis gene cluster
ddH ₂ O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
Ec	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
GCMBK	gonococcal medium base with Kellogg's supplement
GFP	green fluorescent protein
GTP	guanosine triphosphate
IPCR	Inverse polymerase chain reaction
IPTG	isopropyl- β -D-thiogalactopyranoside
Kan	kanamycin
kDa	kilodalton
LB	luria bertani
<i>min</i>	minicell gene cluster
ml	milliliter
mM	millimolar
MTS	membrane targeting sequence
Ng	<i>Neisseria gonorrhoeae</i>
NT	N-terminal truncation
OD	optical density

O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pf	<i>Pyrococcus furiosus</i>
Ph	<i>Pyrococcus horikoshii</i>
PG	phosphatidylglycerol
Rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	tris-acetate-EDTA buffer
TBS	tris-buffered saline
TTBS	tris-buffered saline with 0.05% Tween-20
µg	microgram
µl	microlitre
µM	micromolar
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

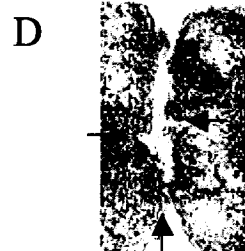
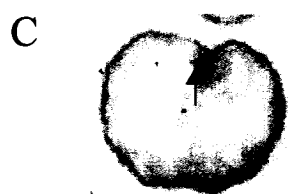
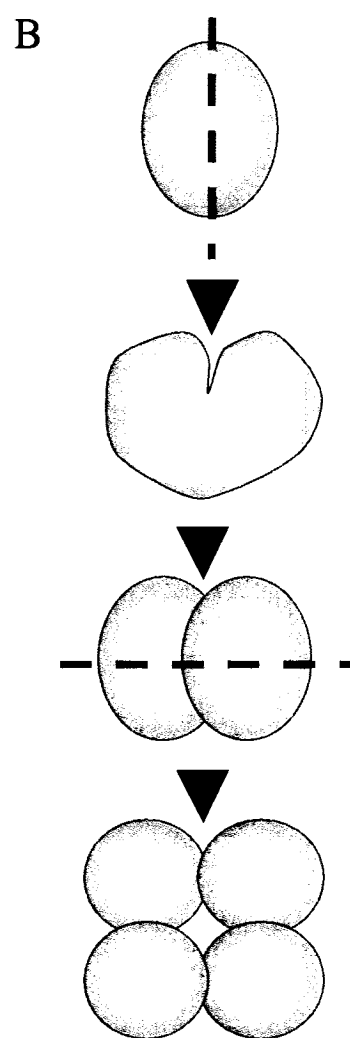
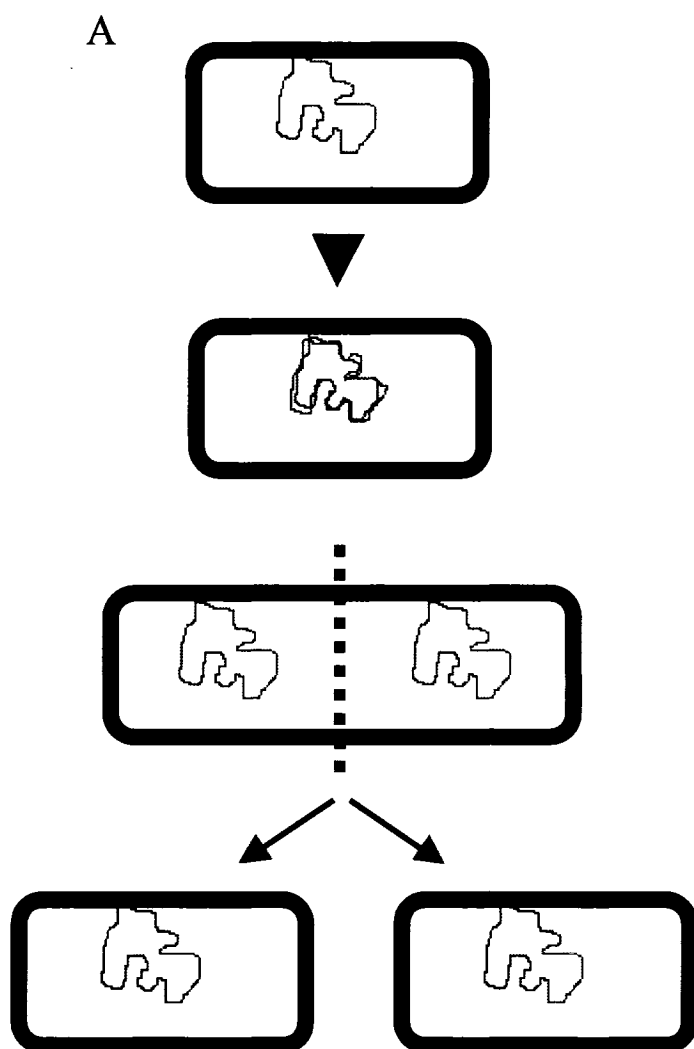
1.1 *Bacterial Cell Division*

In 1858, the German pathologist Rudolph Virchow stated that, “Where a cell arises, there must be a previous cell, just as animals can only arise from animals and plants from plants” (Alberts *et al.*, 1998). This cell doctrine carries with it the fundamental concept that cells are generated from cells, and the only way to make more cells is by the division of those that already exist.

Cell division in its simplest form is the process by which two genetically identical daughter cells are produced, following the equipartitioning of both genetic and cellular components. The process of cell division is essential for the continuity of life. Nevertheless, cell division is a complex process that is still poorly understood, even in relatively simple organisms, including bacteria. Most studies of cell division have been conducted in rod-shaped bacteria where it is easier to envision where the mid-cell site required for normal cell division. However, bacteria have many different shapes and exhibit round, spiral, and asymmetric morphologies with each adding additional complexities to the molecular basis for division site selection.

Bacterial cell division poses some of the most intriguing and fundamental questions in cell biology. Bacterial binary fission – a process that normally occurs symmetrically – causes the cell to constrict along the middle perpendicular to the long axis of the cell, thereby producing two equal-sized daughter cells, following the duplication of DNA and segregation into two daughter nucleoids (Figure 1A) (Buddemeijer and Beckwith, 2002). In contrast, the coccus *N. gonorrhoeae* divides along two perpendicular planes, resulting in the formation of a tetrad of daughter cells (Figure 1B) (Westling-Häggström *et al.*, 1977). Asymmetric

Figure 1. Bacterial cell division. (A) In rod shaped cells the septum forms perpendicular to the long axis of the cell following DNA replication, cell growth, and chromosome segregation producing two equal sized daughter cells. In contrast to rods, (B) the cocci *N. gonorrhoeae* undergoes cell division in alternating, perpendicular planes producing tetrads (Adapted from J. Szeto; Dillon laboratory). Electron micrograph showing the original plane of invagination (C) (blue arrows) and a secondary division plane (D) (black arrows) initiating perpendicular to the original plane.



invagination of the membrane initiates opposite from the nucleoid (Fitz-James, 1964) while the cells grow perpendicular to the initial site of constriction. After the inner membrane constriction yields two separate gonococcal compartments, another division is initiated (Figure 1D, black arrows) in a plane perpendicular to the first septum (Figure 1C, blue arrows) giving rise to a tetrad of gonococcal cells (Westling-Häggström *et al.*, 1977; Szeto *et al.*, submitted for publication).

Regardless of the shape or form, the bacterium must first identify the midcell site where division later initiates (Nanninga, 1998). For proper cell division, numerous cell division proteins must differentiate this site in preparation for cytokinesis and finally form the division septum by the coordinate in growth of the outer membrane of the cell envelope (Rothfield and Justice, 1997). After decades of work, substantial progress has been made in deciphering this complicated phenomenon in both rod-shaped Gram-positive and Gram-negative cells such as *Bacillus subtilis*, and *Escherichia coli* respectively (Margolin, 2000). However, very little is known about the actual process by which even the simplest type of non-rod shaped bacteria, specifically the round bacterium classified as the coccus, such as *Neisseria gonorrhoeae* divide. In order to gain insight into how cocci select a midcell division site our laboratory is using *N. gonorrhoeae* as a model organism.

1.2 Cell division proteins (*dcw* cluster)

Bacterial cell division has been most extensively studied in the Gram-negative rod *E. coli*. In most cases, the division septum is placed with high fidelity at the midpoint, and the placement of this septum at midcell requires that this site be identified and used to support septation in preference to other sites along the cell (Rothfield *et al.*, 1999). This process in

part, is controlled by a division cell wall (*dcw*) cluster of genes coding for enzymes that are directly involved in the synthesis of peptidoglycan precursors and cell division.

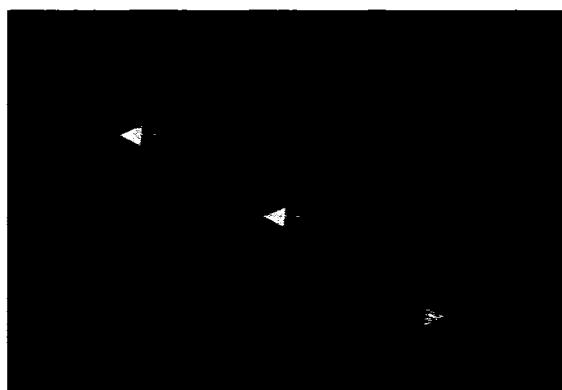
In *E. coli*, the *dcw* cluster consists of 16 tightly packed genes: *mraZ*, *mraW*, *ftsL*, *ftsI*, *murE*, *murF*, *mraY*, *murD*, *ftsW*, *murG*, *murC*, *ddlB*, *ftsQ*, *ftsA*, *ftsZ*, and *envA* which are organized as an operon with transcription proceeding in one direction (Ayala *et al.*, 1994). This tightly packed overlapping cluster contains one initial promoter, *mraZ*, with several internal promoters, and a single transcription terminator downstream of *envA* (Mingorance *et al.*, 2004). While the order of *dcw* genes is mostly conserved across species, our laboratory noted that several differences do exist. Our laboratory showed that in *N. gonorrhoeae* the 17-gene *dcw* cluster is split into four transcriptional units (Francis *et al.*, 2000) as opposed to one transcriptional unit in *E. coli* (Yuan *et al.*, 1992). Perhaps the biggest difference is the presence of six promoters for *ftsZ* alone in the *N. gonorrhoeae* *dcw* cluster, several of which are used under anaerobic conditions (Francis *et al.*, 2000). Despite these differences, sequence homology comparisons of the *dcw* cluster across bacterial genomes revealed that both gene content and gene order are highly conserved in distant bacterial lineages including *B. subtilis* (Daniel *et al.*, 1996), *Haemophilus influenzae* (Fleischmann *et al.*, 1995), *Staphylococcus aureus* (Pucci *et al.*, 1997) and *N. gonorrhoeae* (Francis *et al.*, 2000). In fact, Mingorance and co-workers postulate that the degree of conservation might be directly coupled to cell morphology (2004).

1.3 FtsZ and the Z-ring

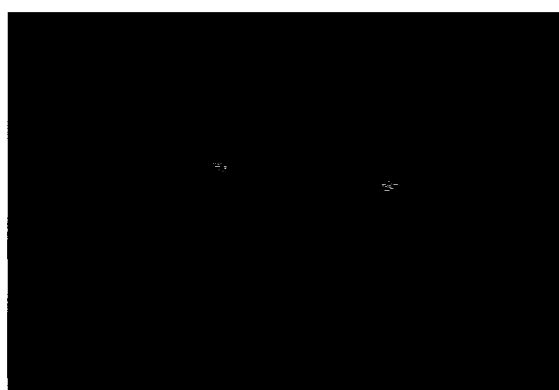
Of the 16 genes involved in cell division, *ftsZ*, a GTPase protein that polymerizes into a constrictive ring like structure, called the FtsZ-ring (Figure 2) (Bi and Lutkenhaus, 1991), is

Figure 2. FtsZ forms rings at midpoint of cells. (A) *E. coli* cells stained with anti FtsZ-antisera show localization of FtsZ to midcell where it forms a ring-like structure called the Z-ring (yellow arrows) [reprinted with permission from Elsevier (Addinall and Holland, 2002)]. (B) GFP fusion to *N. gonorrhoeae* FtsZ protein in *E. coli* also localizes to the midcell (Dillon laboratory).

A



B



by far the most conserved. It is present in most bacteria studied so far as well as the genome of plants where it plays a role in the division of chloroplasts (Osteryoung and Vierling, 1995). Its high conservation is apparent from cross-species functionality studies, where our laboratory showed GFP (green fluorescent protein) fusion to *N. gonorrhoeae* FtsZ localizing to the midcell of *E. coli* cells (Figure 2B) (Salimnia *et al.*, 2000). Interestingly, our GFP images demonstrated the ability of FtsZ to organize into an ordered subcellular architecture, an occurrence not yet observed at that time (Salimnia *et al.*, 2000). Mutational analyses of *ftsZ* suggest its role in the earliest known step in cell division. For instance, mutants of *ftsZ* in *E. coli* fail to divide, producing long filamentous cells that replicate and segregate their chromosome but show no signs of a division septa or cellular constriction.

In 1991, utilizing immunoelectronmicroscopy, Bi and Lutkenhaus showed that FtsZ forms a membrane associated ring-like structure, which extends circumferentially around the cell cylinder at the site of septum formation. The ring-like structure, formed by FtsZ molecules, localizes to the inner surface of the cell membrane. Furthermore, a combination of light microscopy, immunofluorescence and GFP revealed that the Z-ring forms spirals that run along the entire length of the cell and remains associated with the leading edge of the ingrowing septum during septal invagination and disappears once septal formation is complete (Addinall *et al.*, 1996; Levin and Losick, 1996; Ma *et al.*, 1996; Salimnia *et al.*, 2000; Szeto *et al.*, submitted for publication).

FtsZ is a homolog of tubulin, the eukaryotic cytoskeletal protein involved in mitosis (Erickson, 1997). Despite limited primary sequence homology (< than 20%), the solved crystal structures of FtsZ and α , β -tubulin dimers display similar three-dimensional fold (Löwe and Amos, 1998; Nogales *et al.*, 1998A; Nogales *et al.*, 1998) with identical N-

terminal domains and significant structural identity over the core C-terminal domains (Erickson, 1998). In addition, similar to tubulin, FtsZ binds and hydrolyzes GTP and assembles into protofilaments identical to that observed with microtubules (Erickson *et al.*, 1996; Yu and Margolin, 1997). FtsZ protofilaments are dynamic structures, maintaining their polymeric state until GTP is hydrolyzed; after which they rapidly depolymerize (Mukherjee and Lutkenhaus, 1998).

It is obvious that FtsZ and the Z-ring are critical for division since depletion of native FtsZ results in disappearance of the ring and the failure of cells to divide, allowing them to form elongated spaghetti like cells (Addinall *et al.*, 1997). However, the precise function of the Z-ring and the mechanism of its constriction *in vivo* are currently not known. In addition to its proposed motor properties, the FtsZ ring is also believed to act as a scaffold onto which other cell division proteins are recruited (Pogliano *et al.*, 1997). Based on recent studies, a hierarchical order of assembly of the division proteins at midcell has been suggested, where the assembly of an individual protein depends on the presence of one or more other members of the division protein family (Errington *et al.*, 2003). The localization of various proteins to the *E. coli* cytokinetic ring is believed to take place in the following order: ZipA, FtsA, FtsK, FtsQ, FtsL, FtsI, and FtsN (Errington *et al.*, 2003). While formation of the Z-ring and the recruitment of additional cell division proteins are accepted to be the key event in initiating cell division, selection of the correct midcell site for cell division is critical and is achieved through the combination of two separate factors: nucleoid occlusion and the min protein system.

1.4 *Spatial regulation of cell division*

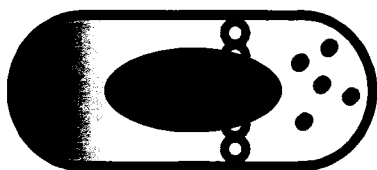
Numerous studies have provided support for two key processes involved in selection of the correct midcell site for cell division. One involves the chromosome itself through a process called nucleoid occlusion (Woldringh *et al.*, 1990; Margolin, 2001). The other process involves the three Min proteins, which are responsible for blocking septation at unwanted potential division sites at the cell poles (Figure 3).

1.4.1 *Nucleoid occlusion model*

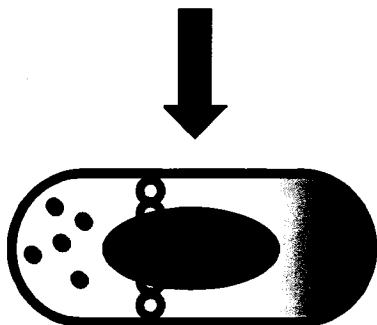
Support for the nucleoid occlusion model stems from observations that the nucleoid acts negatively to inhibit Z-ring formation in its vicinity; thus, Z-rings usually do not form over nucleoids (Figure 3A-E) (Yu and Margolin, 1999). More precisely, recent studies suggest that it is not the presence of DNA per se but the concentration of DNA that is important for this negative regulation (Harry, 2001). This nucleoid veto is the basis of the ‘nucleoid occlusion’ model proposed by Woldringh *et al.*, (1990). The model assumes that all positions along the cell are potential division sites and the nucleoid prevents division from initiating at midcell until the veto is released (Figure 3F). As a consequence, for rod shaped cells about to undergo cell division, there are three potential division sites where DNA is absent or reduced in concentration. One lies at midcell, between the replicated and the segregated chromosomes; the other two lie at the cell poles (Erington *et al.*, 2003). And, it is this division at cell poles that gives rise to small anucleate “minicells”. However, minicell formation is not the norm, and a second factor, the min system, in combination with the nucleoid occlusion model ensures that septum formation initiates at midcell as opposed to cell poles.

Figure 3. Negative regulation of division site placement in rod shaped *E. coli* cells. A combination of nucleoid occlusion and Min protein oscillation determine the precise placement of the Z-ring at midcell. Cells at progressive positions on their cell cycle, before replication to initiation of septum formation, are shown. (A-D) Prior to replication, the cell division inhibitor complex, MinCD, negatively regulates the assembly of the Z-ring, while MinE acts to negatively regulate the action of MinCD. (E-G) Once chromosome replication begins, the veto imparted by the nucleoid is released allowing FtsZ molecules to nucleate such that the Z-ring is formed. Blue ovals represent nucleoids, and red circles near midcell represent the MinE ring. Dark shaded areas at cell poles represent MinCD division inhibitor with the degree of shading corresponding to the concentration. The MinE ring is shown to oscillate at midcell while the division inhibitor oscillates from pole to pole. Purple dots represent FtsZ molecules with potential to polymerize. And, green lightening bolt represents FtsZ molecules that have successfully polymerized into a Z ring. (Modified from Margolin, 2000).

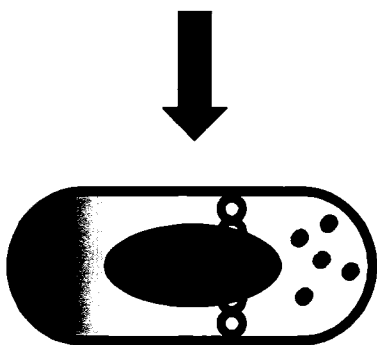
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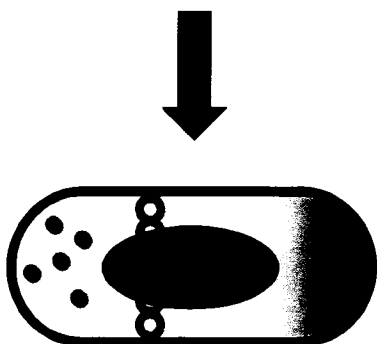
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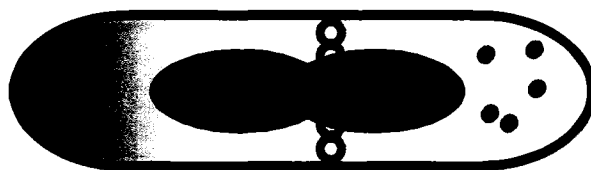
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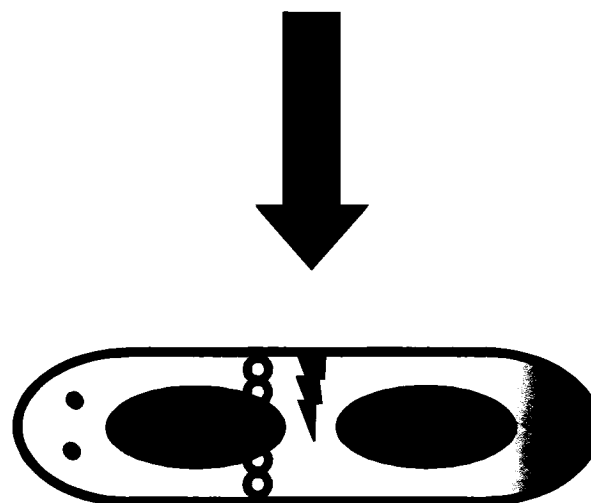
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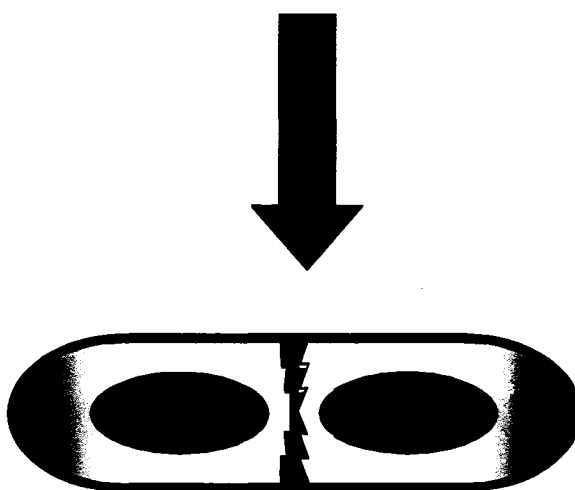
E



F



G



1.4.2 The *min* locus

A second form of regulation on the position of the Z-ring is imparted by the *min* system, comprising MinC, MinD, and MinE. In Gram-negative bacteria, such as *E. coli*, the coordinate expression of the *min* system encoded by the *minB* (minicell) locus is required for the normal division site selection process at midcell (de Boer and Crossley, 1989; Ramirez-Arcos *et al.*, 2001). Mutations to Min proteins in rods normally give rise to small spherical minicells that lack chromosomal DNA due to inappropriate division adjacent to cell poles instead of at midcell (Jaffe *et al.*, 1988). MinC and MinD act in concert to inhibit cell division while MinE acts to negatively regulate the action of MinC and MinD (de Boer and Crossley, 1989).

In contrast, the process is somewhat different in Gram-positive cells, for which the model system is *Bacillus subtilis*. The *divIVB* locus in *B. subtilis* contains homologues of MinC_{Ec} and MinD_{Ec} (Levin *et al.*, 1992; Varley and Stewart, 1992). As in *E. coli*, the function of the MinCD division inhibitor is conserved since similar to Gram-negative organisms MinD_{Bs} mutants generate a minicell phenotype (Levin *et al.*, 1992; Lee and Price, 1993). However, the *B. subtilis* genome lacks a MinE homologue and the topological specificity of the MinCD division inhibitor is mediated by a tropomyosin-like coiled coil protein called DivIVA (Edwards and Errington, 1997; Edwards *et al.*, 2000). Reeve *et al.*, (1973) showed that mutations in DivIVA lead to both minicell formation and long nonseptate filaments. Thus, DivIVA is the MinE-equivalent in Gram-positive organisms since it imparts topological specificity by defining the spatial activity zone of the MinCD division inhibitor.

1.4.3 The Min proteins

The crystal structure of MinC from *Thermotoga maritima* is composed of two distinct domains joined by a flexible linker (Cordell *et al.*, 2001); the N-terminal domain constitutes residues 1 to 99 while the C-terminal domain includes residues 125 to 231 (Hu and Lutkenhaus, 2000). Biochemical studies examining *E. coli* MinC, revealed that the N-terminus interacts with FtsZ (Hu and Lutkenhaus, 2000) while the C-terminal domain is implicated in self interaction (Cordell *et al.*, 2001; Szeto *et al.*, 2001) as well as interaction with MinD (Hu and Lutkenhaus, 2000). Moreover, the two domains are functionally uncoupled since *in vitro*, the N-terminus of MinC_{Ec} was sufficient to inhibit FtsZ polymerization, and overexpression of a MalE-MinC_{Ec} – fusion encoding only the first 115 residues of MinC_{Ec} – inhibited cell division resulting in cellular filamentation (Hu and Lutkenhaus, 2000).

Studies in *E. coli* have shown that the ATPase MinD (270 residues, ~30 kDa) functions by recruiting MinC to polar regions of the cell, thus inhibiting cell division there (Raskin and de Boer, 1999A). This is accomplished through periodic, ATP-dependent, intracellular pole-to-pole oscillations of MinD that constantly shuttle MinC to the membrane from one half of the cell to the other, thereby allowing FtsZ ring formation and cell division to occur exclusively at midcell (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999B). While inactivation of MinC or MinD in *E. coli* produces a minicell phenotype indicative of cell division at polar regions, the overexpression of these proteins induces filamentous cells due to cell division inhibition at all potential division sites (de Boer and Crossley, 1989).

MinE (88 residues, ~10kDa) serves as a topological specificity factor – i.e. it directs the cellular location of the MinCD complex – for the MinCD complex presumably by

stimulating the release of the complex (Lackner *et al.*, 2003) in a site-specific manner that leaves the midcell free of the inhibitory complex. Similar to MinC, this dimeric protein consists of two separate domains: an N-terminal anti-MinCD domain (Zhao *et al.*, 1995; Hu *et al.*, 1999) and a C-terminal topological specificity factor domain (Hu *et al.*, 1999; King *et al.*, 1999). Recent *in vitro* studies have shown that MinE can stimulate MinC dissociation from MinD-membrane complexes through direct interactions via its anti-MinCD domain (Huang *et al.*, 1996; Szeto *et al.*, 2001; Lackner *et al.*, 2003; Hu *et al.*, 2003). In addition, MinE interaction with MinD can increase the ATPase activity of MinD in the presence of phospholipids (Hu and Lutkenhaus, 2001). ATP hydrolysis has been shown to induce MinD to dissociate from the membrane and allow for its subsequent oscillation to the opposite cell half (Hu *et al.*, 2002).

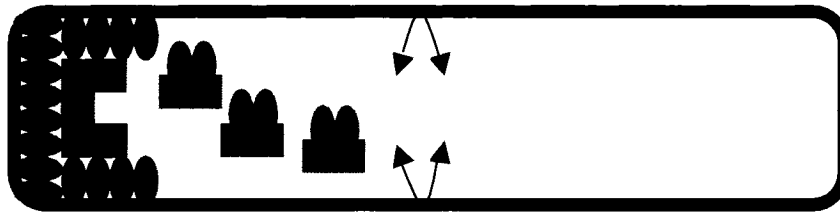
Our laboratory and others have established that MinD can dimerize (Szeto *et al.*, 2001; Hu *et al.*, 2003) and the dimeric ATP-bound form was shown to possess membrane binding affinity (Hu *et al.*, 2003). Recent studies have proposed that the extreme C-terminal region of MinD contains a highly conserved 8-12 residue sequence that forms an amphipathic helix essential for the membrane localization of the protein (Szeto *et al.*, 2002; Hu and Lutkenhaus, 2003). Since ATP binding and dimerization are required for membrane binding, it has been proposed that these events lead to conformational changes that expose specific residues on the amphipathic helix which are required for MinD membrane association (Szeto *et al.*, 2002; Hu and Lutkenhaus, 2003).

1.4.4 Min protein oscillation

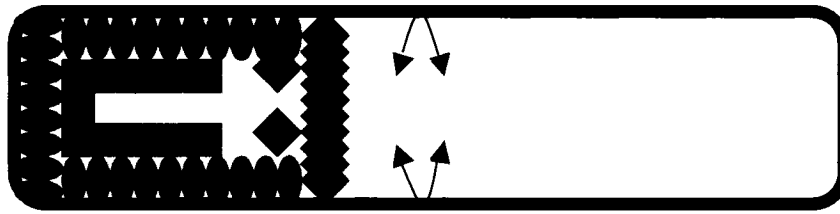
GFP fusions to E. coli Min proteins in combination with the above additional studies have enabled researchers to propose a model (Figure 4), which explains how the Min system

Figure 4. Model of Min protein function in *E. coli* rods. (A) MinD dimerizes after binding to ATP and recruits MinC to polar regions of the cytoplasmic face of the inner membrane. (B) Once the MinCD complex forms a horseshoe-like structure along the periphery of the bilayer, MinE localizes as a ring structure at the edge of the MinCD horseshoe. (C) Membrane bound MinD hydrolyzes ATP in response to MinE, dissociates from the membrane, free to initiate assembly in the opposite cell pole. (D) After the MinE ring reaches the pole, it disassembles and reassembles at the new growing end of the MinD horseshoe. Due to this oscillation, MinC alternately occupies the membrane in polar zones without occupying the midcell. Since the midcell region is free of MinC, Z-ring assembly can proceed, thereby initiating the cytokinetic event that will ultimately result in the formation of two daughter cells. (Modified from Dillon laboratory).

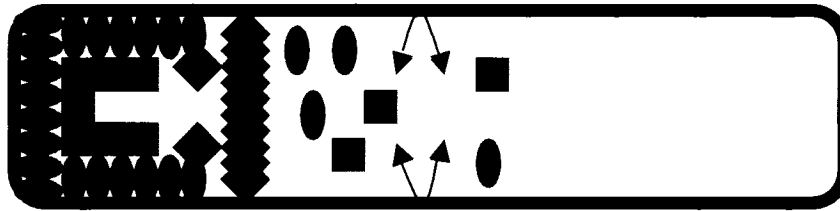
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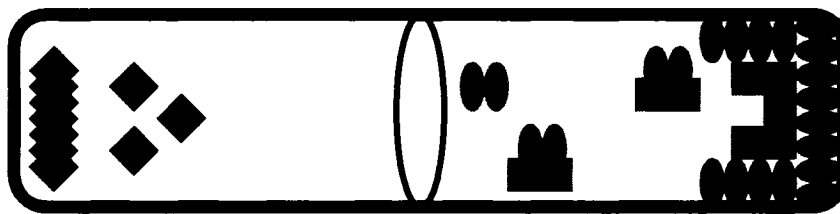
B



C



D



— Phospholipid

■ MinC_{Ec}

● Dimeric MinD-ATP

◆ MinE_{Ec}

● Monomeric MinD

↔ FtsZ-ring

achieves spatial regulation through the oscillatory movement of Min proteins between the two poles of the cell (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999B). In this model, MinD dimerizes in the presence of ATP (Hu and Lutkenhaus, 2003), leading to conformational changes that promote interactions with the phospholipid membrane (Figure 4A) (Hu and Lutkenhaus, 2003). Since MinD also binds the cytoplasmic MinC, the dynamic movement of MinD is directly coupled to MinC. The MinCD inhibitory complex localizes to the membrane where it acts to prevent assembly of the FtsZ ring via MinC (Figure 4A) (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999B). Binding to the membrane causes additional conformational changes that favors both the self-assembly of MinD into protein filaments and makes it more susceptible to MinE-induced ATP hydrolysis (Hu *et al.*, 2002).

Once MinD has bound to the membrane, MinE localizes both as a ring structure (Figure 4B) (E-ring) at the edge of the MinCD-rich polar region as well as the polar zones (Fu *et al.*, 2001; Hale *et al.*, 2001). Subsequent interaction of MinE with the MinCD complex stimulates MinD ATPase activity (Figure 4C), causing rapid shrinkage of the MinCD complex (Hu *et al.*, 2002) at the receding edge of the MinD polar zone. The concomitant release of MinD from the membrane allows these molecules to diffuse and reassemble in the opposite cell pole (Hu *et al.*, 2002). Once the MinE ring reaches the pole after dislodging all MinD molecules, it disassembles so that the liberated MinE can reassemble at the growing end of the new MinD polar zone where the entire process is repeated (Figure 4D) (Hu and Lutkenhaus, 2001). As a result, in the presence of MinE, MinD rapidly oscillates from one cell pole to the other.

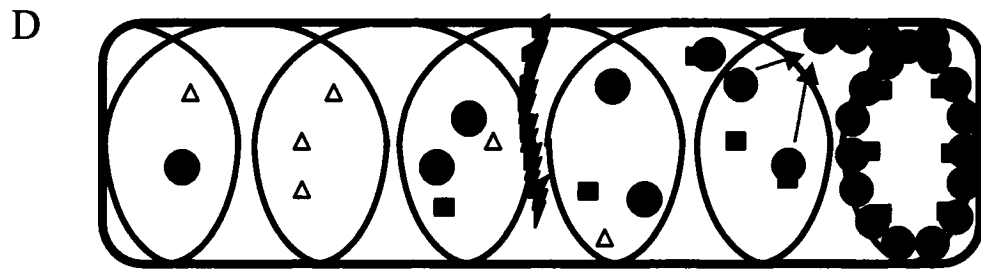
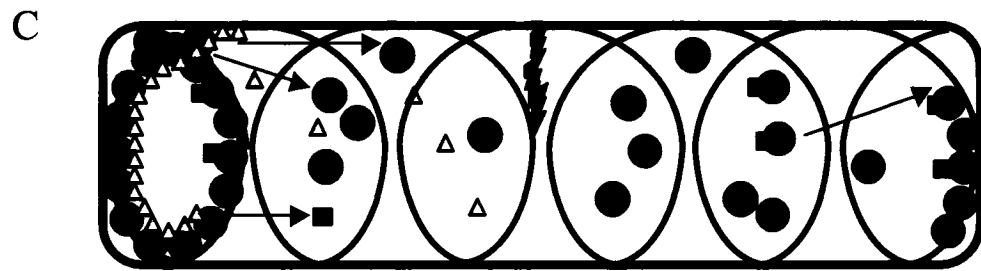
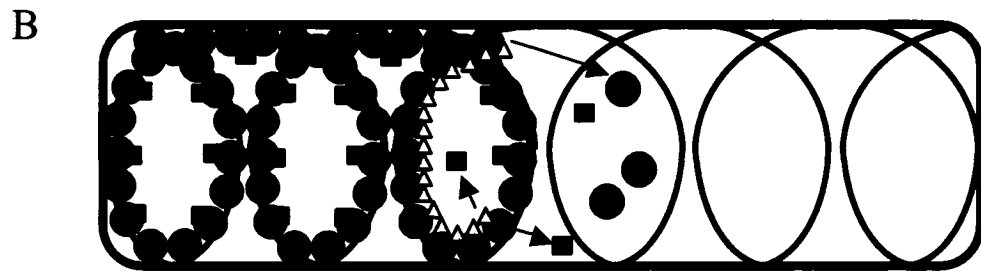
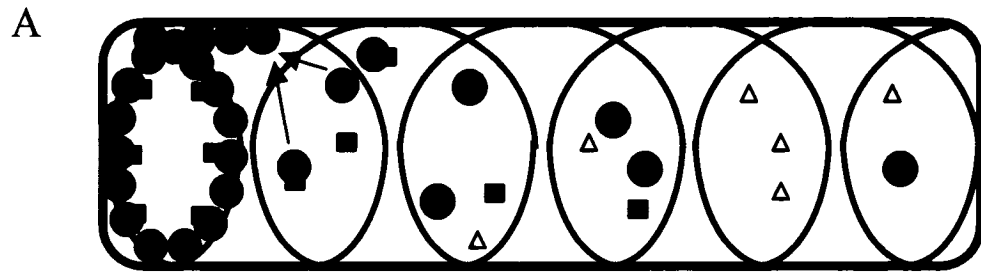
Similarly, MinC is not static. It too oscillates between two halves of the cell assembling and reassembling under the direction of MinE (Hu and Lutkehaus, 1999; Raskin

and de Boer, 1999B). Due to this oscillation, MinC alternately occupies the membrane in polar zones without occupying the membrane at midcell. As a result, the midcell region is free of MinC, allowing Z-ring assembly, thereby, initiating the cytokinetic event that will ultimately result in the formation of two daughter cells (Figure 4D). Recently, additional complexities to the above model were introduced with the discovery that the three Min proteins are organized into membrane-associated helical cytoskeletal substructures, which wind around the cell between the two poles (Shih *et al.*, 2003). Interestingly, heterologous Min proteins make the same structures in *E. coli* (Szeto *et al.*, 2004; Szeto *et al.*, 2005).

Fluorescence microscopy suggests that the MinCDE coiled structures localized at the polar zone do not completely disassemble; the basic MinCDE helical array remains intact as a permanent helical scaffold along the length of the cell. The observed pole-to-pole oscillation of MinC/MinD/MinE appears to occur by the dynamic redistribution of these proteins within this helical framework; and, MinD molecules that are released during disassembly of the old polar zone directly associate with the helical framework that is already in place at the opposite end of the cell to form a new polar zone (Figure 5). Based on these findings it appears that the Min proteins contribute to subcellular architecture in bacteria – a rather revolutionary thought (Shih *et al.*, 2003; Szeto *et al.*, 2004; Szeto *et al.*, 2005).

The validity of this complex Min oscillation has been supported by the construction of mathematical simulations that can accurately reproduce the oscillatory movements observed *in vivo* (Howard *et al.*, 2001; Meinhardt and de Boer, 2001; Kruse, 2002). These simulations are generally based on assumptions arising from observed characteristics of Min protein function. For example, these models assumed that MinD binds to the membrane and recruits MinE, which subsequently causes both to be released. Another reasonable

Figure 5. Model of Min protein function in *E. coli* rods showing the basic MinCDE helical array as a permanent helical scaffold along the length of the cell. (A) Dimeric MinD recruits the cell division inhibitor, MinC, to the helical framework at the polar region of the cell. (B) Following the assembly of the cell division inhibitor complex, MinCD, along the MinCDE scaffold, MinE assembles into a ring-like structure. (C) The MinE ring oscillates along the MinCDE scaffold causing rapid shrinkage of MinCD by stimulating the ATPase activity of MinD. (D) MinD molecules released after disassembly from the old polar zone directly associate with the helical framework that is already in place at the opposite end of the cell to form a new polar zone. Due to this oscillation, the concentration of MinC is lowest at midcell, thereby, allowing Z-ring formation and cytokinesis to occur precisely at midcell. (Adapted from Szeto thesis, 2004).



MinE_{Ec}



$\text{MinD}_{\text{Ec}}\text{-ATP}$



$\text{MinCDE}_{\text{Ec}}$ helical substructure



$\text{MinD}_{\text{Ec}}\text{-ADP}$



FtsZ



MinC_{Ec}

assumption that was important for these models was that the diffusion rate of membrane-bound Min proteins was slower than it would be for the free proteins in the cytoplasm. To obtain the actual oscillation, however, it was necessary to assume that MinD dissociates from the membrane more rapidly than MinE, such that the remaining MinE could prevent MinD from reassociating with the membrane at the same end of the cell (Lutkenhaus and Sundaramoorthy, 2003). Considering that MinE cannot localize to the membrane in the absence of MinD, this particular assumption is difficult to rationalize based on existing data. Also, the recent finding that the Min proteins polymerize into helical filaments is a crucial feature not yet captured by the mathematical simulations. Nonetheless, the ability of simple mathematical models to describe Min protein oscillation provided additional proof that the MinE-MinD interaction, membrane binding and ATP hydrolysis-dependent dissociation could comprise the minimum requirements for Min protein oscillation.

In summary, a combination of negative regulatory signals from both the nucleoid and the Min system appear to be sufficient for the precise localization of the Z-ring. Initially, it can be assumed that all locations on the cell are potential division sites. However, the MinCD inhibitor that oscillates periodically prevents Z-ring assembly at cell poles, while the nucleoid, which occupies the cell center blocks septation at midcell. Once DNA is replicated and segregated the veto imparted by the nucleoid is released allowing Z-ring assembly there (Figure 3F). MinCD would still be able to prevent Z-ring formation at midcell were it not for the presence of MinE, which protects FtsZ from the division inhibitor complex (Figure 3).

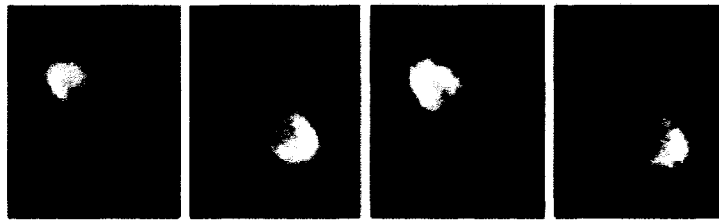
1.5 Min proteins in *N. gonorrhoeae*

Our laboratory previously identified *minC*, *minD*, and *minE* genes in the coccus *N. gonorrhoeae* (Ramirez-Arcos *et al.*, 2001). Unlike rods, round bacteria (cocci) do not possess obvious midcells. Moreover, *N. gonorrhoeae* divides sequentially along two alternating perpendicular planes, resulting in a tetrad of daughter cells (Figure 1B) (Westling-Häggström *et al.*, 1977; Szeto *et al.*, submitted for publication). Of the three Min proteins, MinD is the most ubiquitously distributed and the most conserved across species (Szeto *et al.*, 2001). Disruption of *minD* in *N. gonorrhoeae* (*minD_{Ng}*) and *E. coli* leads to aberrancies in cell division and cell morphology, and reduced cell viability (Szeto *et al.*, 2001). Previously, yeast two-hybrid, size exclusion chromatography, and sedimentation equilibrium studies demonstrated that MinD_{Ng} can self-associate and suggested that this interaction is likely important for MinD function across species (Szeto *et al.*, 2001). Overexpression of both MinD and MinC in *N. gonorrhoeae* leads to gonococcal cell enlargement, revealing a synchronized relationship between the two proteins in controlling cell division (Szeto *et al.*, 2001). MinD_{Ng} is also functional in *E. coli* and can inhibit cell division in this rod-shaped bacterium (Szeto *et al.*, 2001). Furthermore, a green fluorescent protein-MinD_{Ng} fusion (GFP-MinD_{Ng}) has been shown to oscillate in a MinE-dependent manner from pole to pole in *E. coli* backgrounds (Figure 6B) (Ramirez-Arcos *et al.*, 2002), similar to a GFP-fusion to *E. coli* MinD (Figure 6A) (Raskin and de Boer, 1999B).

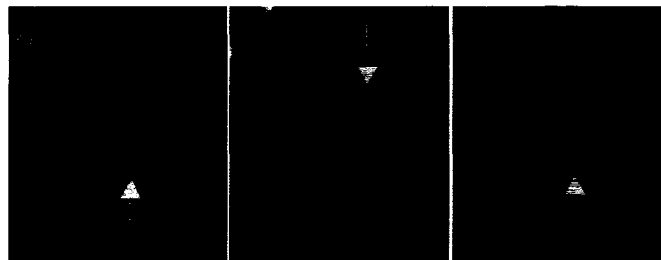
Analogous GFP fusion localization studies of Min proteins in *N. gonorrhoeae* cells are impractical due to the limitations posed by their small size. Nevertheless, our laboratory has overcome this shortcoming by utilizing round *E. coli* KJB24 (*rodA*) since, similar to *N. gonorrhoeae*, this round *E. coli* strain divides in alternating perpendicular planes (Westling-

Figure 6. Localization of GFP-MinD_{Ec} and GFP-MinD_{Ng} in *E. coli*. (A) GFP-MinD_{Ec} exhibits pole-to-pole oscillation in rod-shaped *E. coli* [reprinted with permission from Proceedings of the National Academy of Sciences (image from Raskin and de Boer, 1999B)]. (B) *E. coli* transformed with pSR15 (*gfp-minD_{Ng}, minE_{Ng}*) exhibits periodic GFP-MinD_{Ng} movement from pole-to-pole. Note the localization of GFP-MinD at polar regions of the cell.

A



B



Häggström *et al.*, 1977; Begg and Donachie, 1998). These cells are deficient in the shape determinant gene *rodA*, and thus possess round cell morphology (Begg and Donachie, 1998). Using *E. coli* KJB24 cells as a heterologous host and utilizing GFP localization studies that decipher GFP-MinD_{Ng} oscillatory movements, our laboratory proposed a preliminary model for Min protein function in *N. gonorrhoeae* (Ramirez-Arcos *et al.*, 2002). In 2001, our laboratory also showed that studying gonococcal Min proteins in an *E. coli* background is a viable option since the overexpression of MinD_{Ng} in wild type *E. coli* led to cell filamentation, while overexpression in an *E. coli minD* mutant restored the wild type morphology to majority of the cells (Szeto *et al.*, 2001). Based on these results, and sequence alignments – that demonstrate the high conservation of Min proteins across species – our laboratory showed that gonococcal Min proteins are functional across species (Szeto *et al.*, 2001).

Building on the accumulated studies in *E. coli*, from which several models for Min protein function have been generated, plus our research with *N. gonorrhoeae*, our laboratory has progressively modified the preliminary model of cell division site selection in *N. gonorrhoeae* (Figure 7) (Szeto thesis, 2004). Our laboratory proposes that similar to MinD_{Ec}, dimeric MinD_{Ng}-ATP localizes to one end of the coccus and recruits MinC_{Ng}, resulting in division inhibition at one cell half. As observed for *E. coli* rods (Shih *et al.*, 2003), it is accepted that the gonococcal Min proteins form a helical framework that coils along the longest axis of the cell (Figure 7A) (Szeto thesis, 2004) since studies have demonstrated that *N. gonorrhoeae* cells elongate during their growth cycle, and in the process produce a slightly longitudinal axis (Westling-Häggström *et al.*, 1977). As the MinCD division inhibitor accumulates along the helical array at one end of the cell half, MinE_{Ng} localized

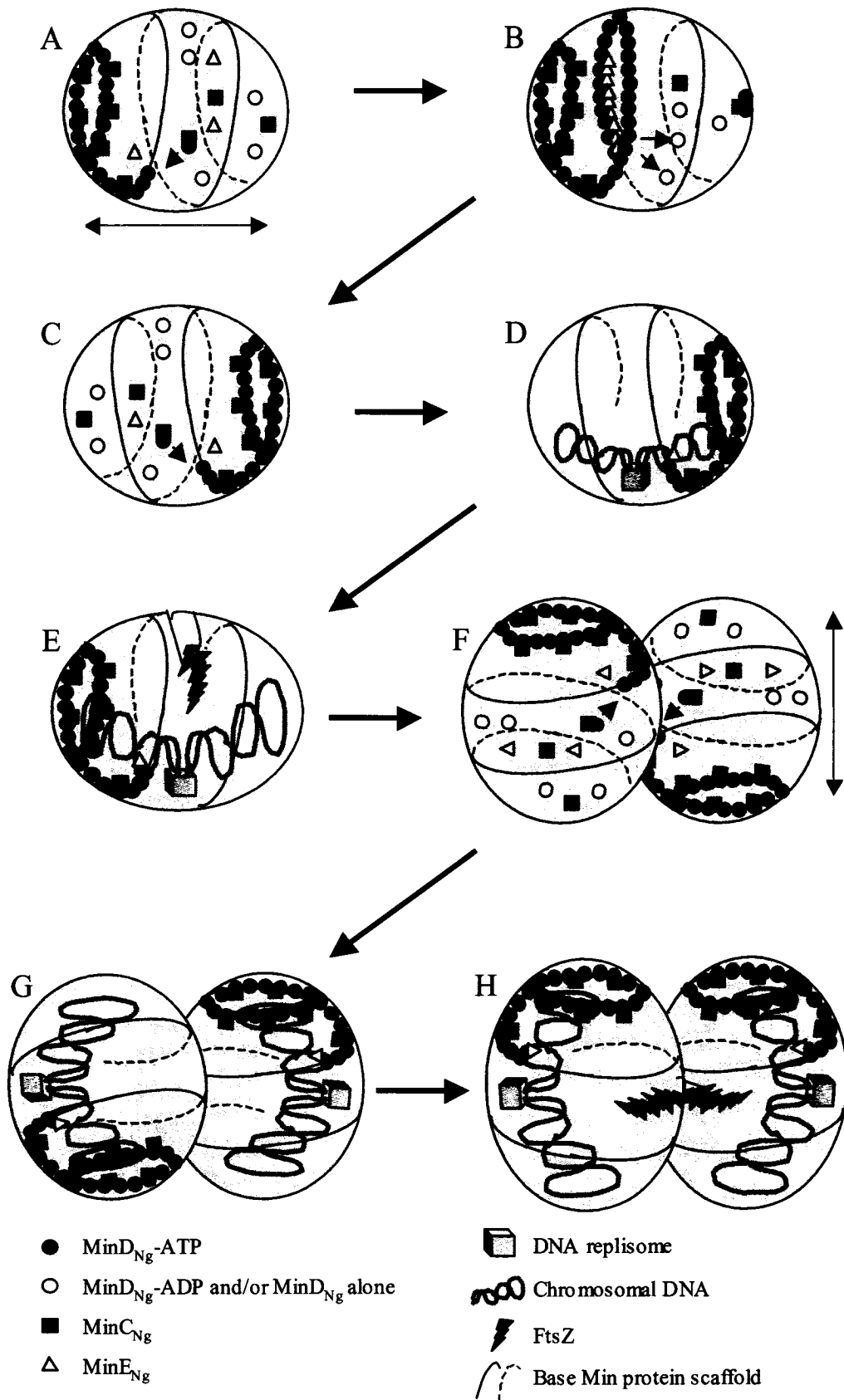
within midcell forms a ring-like structure (Figure 7B). The E-ring induces the dissociation of MinC_{Ng}, and stimulates the ATPase activity of MinD_{Ng} forcing it to dissociate from the membrane. Cytoplasmic MinD_{Ng}-ATP molecules diffuse towards the opposite end of the cell – using the Min protein helical framework as a possible track – bind ATP, and relocalize within the coil/membrane (Figure 7A-D). Due to this continuous oscillation between the opposite cell hemispheres, MinC_{Ng} alternately occupies the membrane in polar zones, thereby, allowing FtsZ assembly to initiate at midcell (Figure 7E). At this stage, the DNA replisome stationed directly opposite the FtsZ polymerization site undergoes replication and segregation allowing full septation (Figure 7E-F) (Szeto thesis, 2004).

Gonococcal cells divide in two alternating perpendicular planes. Hence, after the first division event, each cell in the resulting diplococcus undergoes growth, producing a new longitudinal axis, which is perpendicular to the first (Westling-Häggström *et al.*, 1977; Szeto *et al.*, submitted for publication). The Min protein helical framework re-orientates along the new longitudinal axis such that MinC/D/E oscillate parallel to the nascent septum (Figure 7G) (Szeto thesis, 2004). It is proposed that this redirection of Min protein oscillation in combination with the DNA replisome localized directly opposite the nascent septum restricts FtsZ polymerization to the junction point between two cells resulting in cell division along a plane that is perpendicular to the first (Figure 7H) (Szeto thesis, 2004).

1.6 *MinD_{Ec}: An amphitropic ATPase protein*

Of the three Min proteins, MinD is the most ubiquitous and highly conserved across species (Ramirez-Arcos *et al.*, 2001). For instance, gonococcal MinD (MinD_{Ng}) is 73% identical and 84% similar to *E. coli* MinD (Ramirez-Arcos *et al.*, 2001). The MinD_{Ec} protein consists of

Figure 7. Model for spatial regulation of cell division in *N. gonorrhoeae*. (A) Similar to MinD_{Ec}, dimeric MinD_{Ng}-ATP localizes to one end of the coccus and recruits MinC_{Ng}, to the helical framework that coils along the longest axis of the cell (B) As the MinCD division inhibitor accumulates along the helical array at one end of the cell half, MinE_{Ng} localized within midcell forms a ring-like structure. The E-ring induces the dissociation of MinC_{Ng}, and stimulates the ATPase activity of MinD_{Ng} forcing it to dissociate from the membrane. (C, D) Cytoplasmic MinD_{Ng}-ADP molecules diffuse towards the opposite end of the cell, bind ATP, and relocate within the coil/membrane. (E) Due to this continuous oscillation between the opposite cell hemispheres, MinC_{Ng} alternately occupies the membrane in polar zones, thereby, allowing FtsZ assembly to initiate at midcell. (F) DNA replisome stationed directly opposite the FtsZ polymerization site undergoes replication and segregation allowing full septation. After the first division event, each cell in the resulting diplococcus undergoes growth, producing a new longitudinal axis, which is perpendicular to the first. (G) Min protein helical framework re-orientates along the new longitudinal axis. (H) Redirection of Min protein oscillation combined with the DNA replisome localized directly opposite the nascent septum restricts FtsZ polymerization to the junction point between two cells resulting in cell division along a plane that is perpendicular to the first. Red arrows represent the longitudinal axis of the cell. (Adapted from Szeto *et al.*, submitted for publication).



Szeto *et al.*, submitted for publication

270 residues and is approximately 30 kDa in size. It is an amphitropic peripheral membrane ATPase, and belongs to a large family of ATPases, most of which are involved in plasmid or chromosome partitioning (de Boer *et al.*, 1991).

Recently our laboratory showed that in the presence of phospholipids, MinE_{Ng} stimulates the ATPase activity of MinD_{Ng} (Szeto *et al.*, 2004; Szeto *et al.*, 2005) causing the latter to dissociate from the membrane. This MinE_{Ng} induced stimulation of MinD_{Ng} ATPase activity is the key factor in regulating MinD protein localization. While there is increasing qualitative data regarding Min protein dynamics, little is known about the interaction between MinD, ATP, phospholipids, and MinE.

Recently, several groups have begun to examine the molecular basis for *E. coli* Min protein oscillation by analyzing the interaction of MinD with phospholipids. In 2002 Hu *et al.*, demonstrated that in the presence of ATP, MinD bound phospholipid vesicles and upon binding assembled into a well-ordered helical array. Using ATPase assays it was proven that MinD_{Ec} exhibits positive cooperativity with respect to itself, indicating that self-interaction of MinD facilitated ATP hydrolysis (Hu and Lutkenhaus, 2001). Further proof for polymerization was based on electron microscopy studies which showed that self-assembly into tube-like helical structures was dependent on MinD concentration; a critical concentration of 3.0 μM was required to initiate self-association necessary to form tubes (Hu and Lutkenhaus, 2001). Interestingly, half-maximal activity occurred at 3.7 μM , which is close to both the estimated *in vivo* concentration (3.0 μM) (de Boer *et al.*, 1991), and the critical self-assembly concentration (3.0 μM). In addition, through sedimentation assays examining MinD binding to phospholipid vesicles Hu *et al.*, showed both qualitative and

quantitative data suggesting that ATP regulates MinD_{Ec} binding to phospholipid vesicles and that the binding of MinD_{Ec} to phospholipids is saturable (2002).

In 2003, Lackner *et al.*, extended and clarified the biochemical mechanisms underlying *E. coli* Min protein dynamics by studying the interactions of purified Min proteins with phospholipid vesicles and the role of ATP in these interactions. Through vesicle sedimentation assays in the presence of excess phospholipid and ATP, they demonstrated that increased concentrations of MinD stimulated its binding to the vesicles, suggesting that the cooperativity previously observed in ATPase activity (Hu and Lutkenhaus, 2001) arose from cooperative interactions with the lipid membrane.

1.7 Enzyme Kinetics

MinD is an enzyme that catalyzes the hydrolytic cleavage of a pyrophosphate group in ATP to form ADP and inorganic phosphate (Hu and Lutkenhaus, 2001). Enzymes can generally increase the rate of a chemical reaction through a number of potential mechanisms, including acid-base, electrophilic and/or nucleophilic effects. In addition, by binding to the substrates an enzyme reduces the number of degrees of freedom in the substrates and increases the effective concentration of the reactants. Specific interactions to stabilize the transition state can also occur to provide further specificity and rate acceleration. The result of all of these effects is a lowering of the activation energy required to convert substrates to products. The reaction rate is also influenced by a number of additional factors including: concentration of substrate, temperature, inhibitors, and pH (Copeland, 2000). Enzyme kinetics of wild type and mutant proteins has the potential to reveal functionally important regions of a protein and identify residues that are important for catalysis. In the case of MinD, it should be possible to

compare catalysis in the absence and presence of MinE_{Ng} to gain new insights into the longstanding question of how MinD ATPase activity can be stimulated by MinE_{Ng}.

One of most commonly used approaches in the analysis of enzyme kinetics is to use the equations provided by the Henri-Michaelis-Menten steady state assumption. According to this model a plot of the initial rate versus the substrate concentration should give rise to a rectangular hyperbola where the Michaelis constant, K_m , is the substrate concentration at half V_{max} (Purich, 1983). However, this model requires that the active sites of enzyme molecules behave independently. In fact a large group of enzymes do not display simple Henri-Michaelis-Menten kinetics since they occur as multi-protein complexes with multiple linked active sites. Since MinD is known to dimerize in an ATP-dependent manner, it is possible that the binding of ATP to one active site could confer a structural change that would increase the affinity for ATP at the other active site (Copeland, 2000). This type of effect would be expected to yield a sigmoidal or S shaped curve when initial velocity is plotted as a function of [S]. A sigmoidal curve is characteristic of positive substrate cooperativity; that is, as more substrates bind to the complex the more active the enzyme becomes.

Kinetic studies on MinD_{Ng} have the potential to reveal new insights regarding the molecular mechanisms by which ATP binding promotes MinD dimerization and membrane binding, and how MinE stimulates ATPase activity. Interestingly, our laboratory has identified regions of MinD_{Ng} responsible for regulating its localization and intrinsic enzymatic activity. Of particular interest are the mutants MinD_{Ng-3aaNT} and MinD_{Ng-loop} (R92L, D93L, and K94I). MinD_{Ng-3aaNT} displayed basal ATPase activity that was more than 2-fold greater than wild type basal activity and was not stimulated by MinE (Szeto *et al.*, 2004). In addition, despite retaining interactions with both MinC and MinE, the ATPase

activity of MinD_{Ng-loop} could not be stimulated by MinE (Szeto *et al.*, 2005). Kinetic studies quantitating the reaction rates under varying substrate and protein concentrations should reveal whether the N-terminal region and the solvent-exposed loop affect ATPase activity by changing the affinity of the enzyme for the substrate or by altering the active site environment.

1.8 *MinD* protein structures

Currently, there is no solved structure for bacterial MinD. However, crystal structures of MinD homologues from archaeal organisms have been solved. The three different archaeal structures have a highly homologous fold of seven parallel sheets, and 1 antiparallel strand, surrounded by several helices. Bacterial MinD should have an analogous fold since all these proteins have approximately 30% to 40% sequence identity with the bacterial homologue MinD from *E. coli* and *N. gonorrhoeae*. Undoubtedly, these structures from *Archaeoglobus fulgidus*, *Pyrococcus furiosus*, and *Pyrococcus horikoshi* have provided some insight to the understanding of MinD function. Unfortunately, these archaeal structures are monomeric, and provide only half the story since it is well established that in the presence of ATP MinD dimerizes (Szeto *et al.*, 2001; Hu *et al.*, 2003) and this dimerization is critical for protein function. The observed crystal structures were probably monomeric because they were bound to ADP, AMPPCP, or to no nucleotide (Cordell and Löwe, 2001; Hayashi *et al.*, 2001; Sakai *et al.*, 2001). In addition, it is possible that archaeal MinD proteins are not involved in spatial regulation of cell division, especially given the absence of MinC and MinE in these organisms.

Figure 8. Sequence alignment of MinD proteins from different species. Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>), and alignments were performed using ClustalW Version 1.8 software and visualized using Jalview 2005 Version Release 2.0. Abbreviations used: *Bacillus subtilis* (Bs), *Clostridium perfringens* (Cp), *Listeria monocytogenes* (Lm), *Neisseria gonorrhoeae* (Ng), *Neisseria meningitides* (Nm), *Escherichia coli* (Ec), *Pyrococcus horikoshii* (Ph), *Pyrococcus furiosus* (Pf), and *Archaeoglobus fulgidus* (Af). Dark blue boxes denote amino acid residues that are highly conserved while light blue boxes highlight related amino acids. Red bar indicates position of the 'deviant' Walker A ATP-binding motif. Black bars indicate positions of switch I and II sites, and the grey bar indicates MinD membrane targeting sequence (MTS). Coloured bars adjacent to organism names indicate their general classification: black = Gram-positives, red = Gram-negatives, and blue = Archaea.

Switch I

MinDBs — 57
 MinDCp — 56
 MinDLm — 57
 MinDNg — 57
 MinDNm — 57
 MinDEc — 57
 MinDph — 56
 MinDpf — 56
 MinDaf — 56

Switch II

MinDBs 166 - I PR - - - - VV - - - - HLMN TM DE VQH D - - - - IVAD D IK
 MinDCp 163 - L H - - - - VV - - - - Y MT - - - - S IET K - - - - R ITVT
 MinDLm 165 - I PK - - - - I - - - - TMMN - - - - TTH E - - - - I D I
 MinDNg 168 - - - - - - - - - - I - - - - P - - - - - - - - - - R - - - - S - - - -
 MinDNm 168 - - - - - - - - - - P - - - - - - - - - - R H - - - - S - - - -
 MinDEc 166 - - - - - EPI - - - - PG S - - - - - - - - - - R K - - - - - - - -
 MinDPh 163 - - - - - - - - - - LA F YG SERD PP AAQ - - - - D A - - - - V A EGT
 MinDPf 163 - - - - - - - - - - LA F YG SDRD PP AA - - - - E A - - - - A EGT
 MinDAf 162 - - - - - - - - - - TKV VVV ITTL IEMAKNE A EAK - - - - - - - -

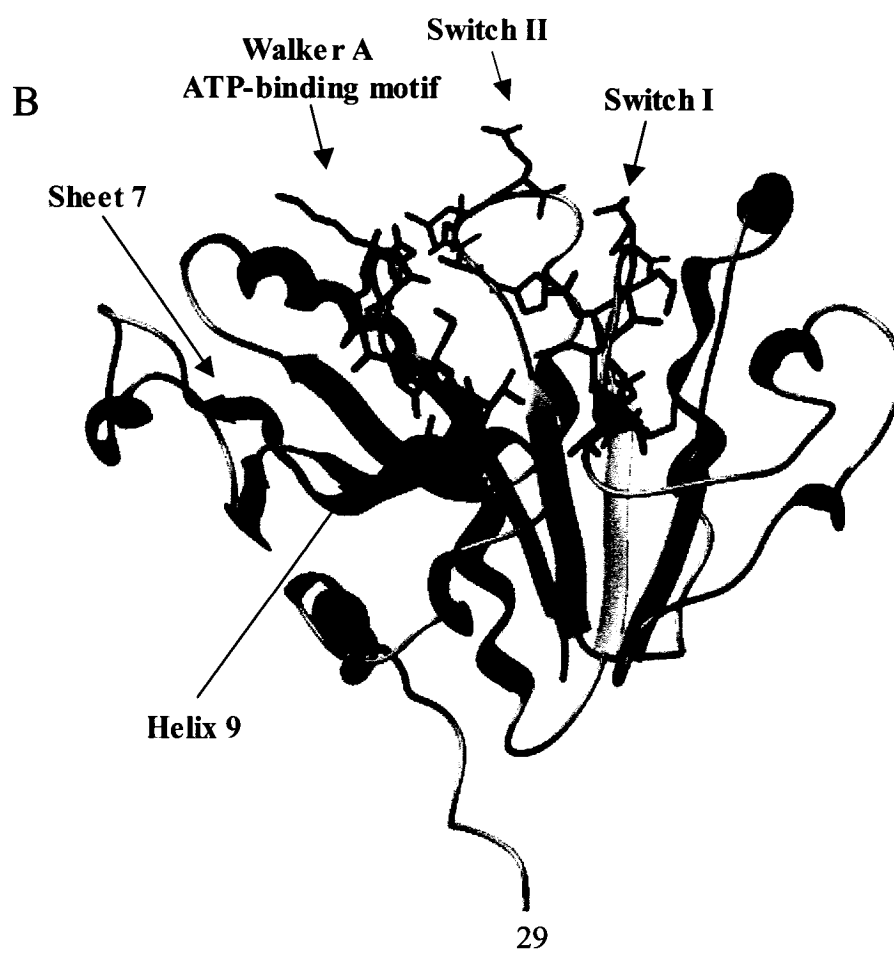
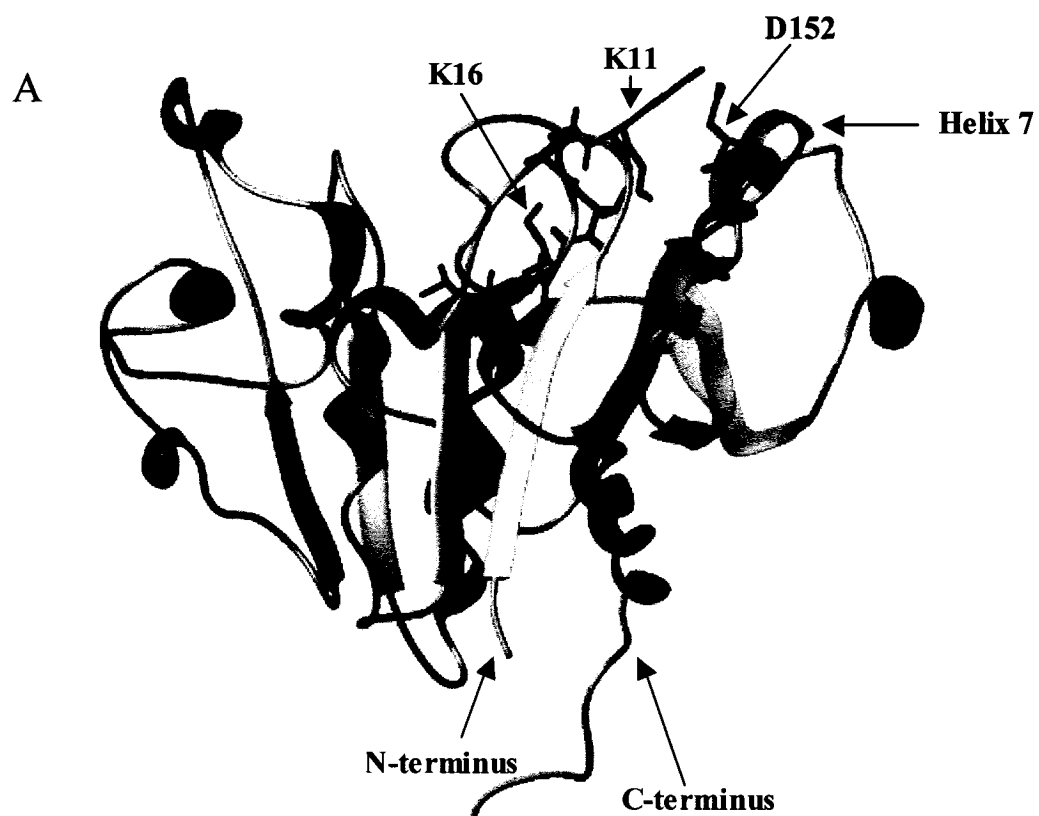
MTS

MinDBs — 215 H I A M - K N I V Q V Q N M M A K S F V R S
 MinDCp — 212 K - - E K A S G G D V M D N H I S S L R R G -
 MinDLm — 214 A M L - - G I M S A A R S K -
 MinDNg — 223 H Q - D S V V I A F E S - - -
 MinDNm — 223 H Q - D S V V I A E E S - - -
 MinDEc — 222 Q - - I A D G A T V E E F L - - -
 MinDPH — 208 L E I A K Y E G I K E D A G A K I M Y - - - -
 MinDPf — 208 L E I A K Y E G I V K E - A - - - -
 MinDaf — 207 A Y K R S P A R I V E N Y A G A K K K V P A V K E K K E G A A K M L R I F R R

Like most ATPases, MinD contains a Walker A motif (Figure 8); a consensus amino acid sequence that makes direct contact with the phosphates of the bound nucleotide (Lutkenhaus and Sundaramoorthy, 2003). More precisely, MinD is classified into the ‘deviant’ Walker A motif subgroup since it has two lysines (Leipe *et al.*, 2002). In the ‘deviant’ consensus sequence (XKGGXX**K**[T/S]) the more common lysine (bold) is situated at the carboxy end of the motif. The side chains of this Lys16 form hydrogen bonds with β - and γ -phosphate oxygens (Hayashi *et al.*, 2001). The second lysine, unique to the ‘deviant’ Walker A motif subgroup, is at the amino terminus. Crystal structures suggest that Lys11 occupies a central position in the network of polar interactions situated close to the nucleotide binding side. The corresponding Lys 11 (Figure 9A) in MinD_{Ec} is hypothesized to interact with a conserved triad comprised of residues E146, S148, and D152 of helix 7 of which were shown to be essential for MinE to stimulate MinD ATPase activity (Zhou *et al.*, 2005). Interestingly, studies mutating K11 or K16 resulted in MinD proteins that lost interaction with the membrane, MinC, MinE, and itself (Hu *et al.*, 2002; Ramirez-Arcos *et al.*, 2002). This provides further evidence for the importance of these residues in dimerization for which ATP binding is a prerequisite.

Coupled with the Walker A motif, two additional conserved regions, switch I and switch II (Figure 9B), form a cavity into which the nucleotide cozily sits (Sakai *et al.*, 2001). In addition to this, recent mutational studies suggest that the switch I and switch II regions may also be implicated in binding MinC (Zhou and Lutkenhaus, 2004). Within the cavity, the adenine base of the nucleotide is stabilized through electrostatic interactions with residues located on sheet 7 and helix 9 (Figure 9B) (Sakai *et al.*, 2001).

Figure 9. Ribbon diagram representation of MinD from *Pyrococcus horikoshii* (PDB# 1ION) (Sakai *et al.*, 2001). (A) Residues that form the Walker A ATP-binding motif (10-18) are highlighted in blue. The two-signature lysine residues (K11, and K16) characteristic of the ‘deviant’ Walker A motif subgroup are labelled. In *E. coli*, Lys 11 forms an electrostatic interacting triad with three residues in helix 7 (cyan), including D152 (black), S148, and E146 (Zhou *et al.*, 2005). (B) Identical structure of (A) depicting the Walker A ATP-binding motif in blue, switch I region (38-46) in black, and switch II region (120-124) in green [Switch I and II sites are normally implicated in protein conformational changes during nucleotide hydrolysis (Vale, 1996)]. Residues on sheet 7 and helix 9 (outlined in royal blue) are proposed to stabilize the adenine base of the nucleotide through electrostatic interactions (Sakai *et al.*, 2001). Ribbon diagrams generated using MOLMOL (version 2K.2) (Koradi *et al.*, 1996).



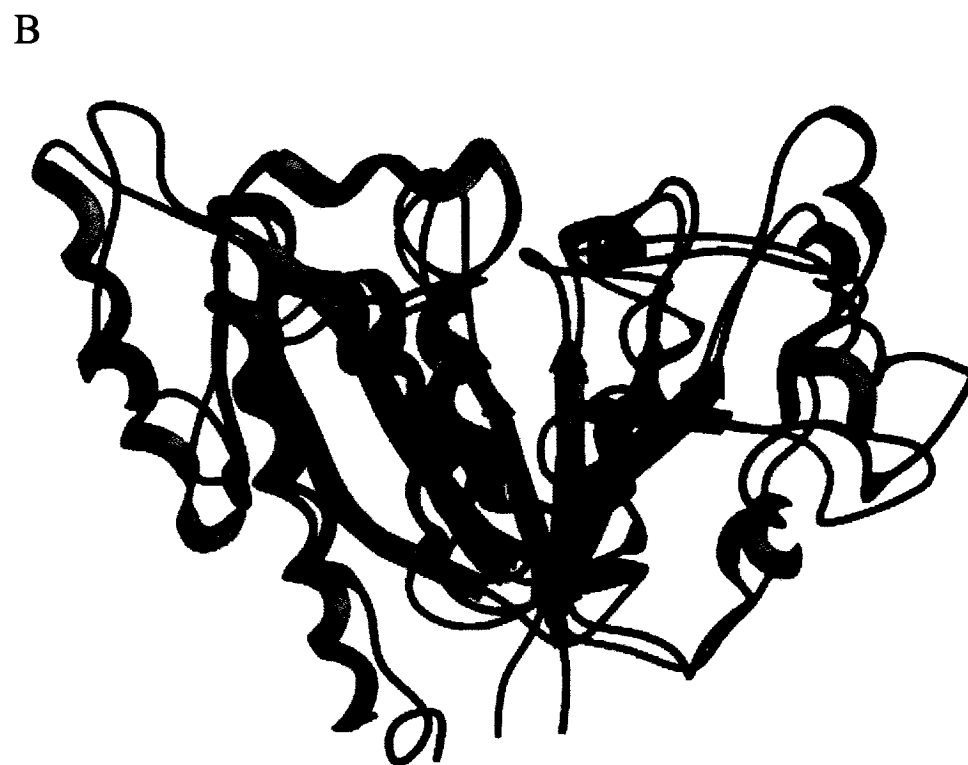
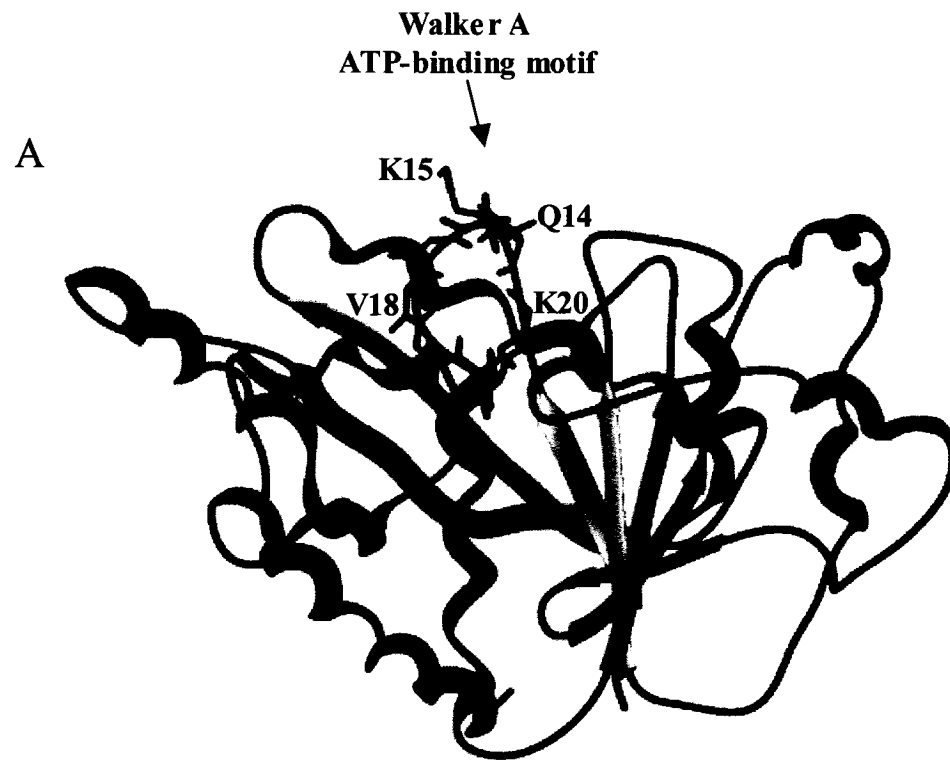
Based on MinD crystal structures and mutagenesis studies the well-conserved helix (a7) was identified as important for both MinC and MinE interactions (Figure 9A) (Hayashi *et al.*, 2001; Ma *et al.*, 2004; Zhou *et al.*, 2005). More specifically in 2004, Ma *et al.*, showed that D152 in MinD_{Ec} (D150 in MinD_{Ph}) was critical for interactions with a 31-residue peptide from the MinE N-terminus (MinE₁₋₃₁) although interactions with full length MinE were preserved (Zhou *et al.*, 2005). Other residues on the same face towards the N-terminal side of helix 7 were also shown to be important for binding this N-terminal MinE peptide and those on the opposite face towards the C-terminal end were important for MinC binding (Ma *et al.*, 2004). Since mutations towards the mid-point of this helix disrupt both MinE N-terminal peptide and MinC interactions with MinD it has been proposed that the two proteins bind to overlapping interaction sites on MinD, providing a reasonable mechanism for MinC displacement by MinE.

The structures of MinD are quite similar to the well-characterized nitrogenase iron protein, NifH, responsible for nitrogen fixation (Cordell and Löwe, 2001; Georgiadis *et al.*, 1992; Szeto *et al.*, 2005). *Azotobacter Vinelandii* NifH has been crystallized with various nucleotides, and the nucleotide dependent conformational changes that form the nucleotide sandwich at the dimer interface have been well documented (Georgiadis *et al.*, 1992; Schindelin *et al.*, 1997). In the NifH, the switch I and switch II regions undergo nucleotide-dependent conformational changes that are responsible for self-association. Comparisons of the NifH dimer in the presence of ADP versus the transition state analogue ADP-AlF₄⁻ revealed that the subunits undergo a ~13 degree rotation towards the nucleotide bound interface which completes the nucleotide sandwich by forming a more compact structure (Schindelin *et al.*, 1997; Lutkenhaus and Sundaramoorthy, 2003). Due to their structural

similarity (Z score 23.0; rmsd 2.9Å for 222 Ca atoms (Sakai *et al.*, 2001)), initially, it was conceivable that MinD monomers could undergo similar nucleotide-dependent conformational changes resulting in dimerization. Surprisingly, no structural changes were observed for the structure of *Pyrococcus furiosus* MinD in the presence of the non-hydrolyzable ATP analogue AMPPCP relative to the ADP-bound structure (Hayashi *et al.*, 2001). However, recently it was demonstrated that AMPPCP may not be a suitable analogue for ATP since it failed to promote MinD membrane localization (Hu and Lutkenhaus, 2003). Thus, the possibility of MinD monomers undergoing similar nucleotide induced conformational changes for dimerization cannot be overlooked.

Recently, Leonard *et al.*, (2005) solved the crystal structure of *Thermus thermophilus* Soj at 1.8 Å resolution in the ATP-bound state (Figure 10A). Soj has been shown to be implicated in chromosome partitioning (Draper and Gober, 2002). Like MinD, Soj is a member of the 'deviant' Walker A ATPases (Leipe *et al.*, 2003). In fact, there are a large number of functional similarities between Soj and MinD. For example, in *B. subtilis* Soj exhibits an oscillatory movement pattern similar to MinD; the MinCD oscillation dependent on MinE is analogous to SpoOJ-dependent oscillation of Soj (Quisel *et al.*, 1999). Moreover, the ATP dependent dimerization and the subsequent localization of MinD to the lipid bilayer is similar to the ATP dependent dimerization of Soj and its ensuing association with the nucleoid. In addition, the MinE induced ATP hydrolysis of MinD and its dissociation from the membrane, in principle, is identical to the SpoOJ induced ATP hydrolysis of Soj and its release from the nucleoid. Interestingly, as observed for MinD, a K16Q mutation in *B. subtilis* Soj also prevents nucleotide binding which has been shown to abolish the *in vivo* oscillatory behavior of Soj, its self association, its association with the nucleoid, and its

Figure 10. Ribbon diagram representation of Soj from hyperthermophile *Thermus thermophilus*. (A) Crystal structure of a Soj monomer at 1.6 Å (2BEJ) (Leonard *et al.*, 2005). Similar to MinD, its overall fold is a twisted arch of stacked β-strands (gold residues) surrounded by α-helices (red residues), with one antiparallel and seven parallel β-strands (Ph, Z score 26.8; rmsd, 2.2Å for 243 equivalent residues). Residues comprising the Walker A ATP-binding motif are outlined in blue. The two-signature lysine residues (K15, and K20), characteristic of the subgroup, and Q14 and V18, residues that partially occlude the active site, are indicated in black. (B) Superposition of the Soj monomer (cyan) on top of the *Pyrococcus horikoshii* MinD structure (red) shows the high similarity between the two structures. Ribbon diagrams generated using MOLMOL (version 2K.2).



response to SpoOJ (Quisel *et al.*, 1999). These functional similarities made it possible to use the Soj structures to gain structural insight into MinD dimerization and to identify residues that might be involved in active site formation.

The structure of the Soj (Figure 10A) monomer is very similar to that of MinD (Figure 10B) (Pf, Z score 26.8; rmsd, 2.2Å for 243 equivalent residues), and NifH. As observed for archaeal MinD, its overall fold is a twisted arch of stacked β -strands surrounded by α -helices, with one antiparallel and seven parallel β -strands (Figure 10A) (Leonard *et al.*, 2005). In the presence of ATP, two Soj monomers form a nucleotide ‘sandwich’ dimer. As was the case for NifH (Georgiadis *et al.*, 1992; Schindelin *et al.*, 1997), the dimerization interface runs along the nucleotide binding surfaces of each monomer, and is stabilized by a network of water mediated H-bonds and H-bonds between residues. The nucleotide binding surface of each monomer contributes hydrophobic residues that form an active site chamber or a cleft that extends down the dimer interface and houses two molecules of ATP. In contrast to the active site of NifH, the ATP molecules in Soj adopt a kinked conformation as opposed to an extended conformation. This could be due to the exclusive conformational changes that the switch II region of NifH undergoes (Leonard *et al.*, 2005).

Interestingly, as hypothesized for MinD, Soj undergoes nucleotide dependent conformational changes. In the Soj apo-protein, the P-loop (Walker A motif) (Figure 10A) adopts an extended conformation between Q14 and V18 (Figure 10A), partially occluding the active site. However, upon ADP binding, the P-loop undergoes a conformational change, making the active site accessible. Similar to the MinD crystal structures (Figure 9B), in the dimer structure, the ‘deviant’ Walker A and switch II motifs of each monomer lie in close proximity (Figure 11). Also, the signature Lys15 (Figure 10A), conserved in all ‘deviant’

Figure 11. Side view of a ribbon diagram representation of hydrolysis deficient Soj D44A dimer (PDB# 2BEK) (Leonard *et al.*, 2005). Similar to MinD, in the dimer structure of Soj D44A, the Walker A ATP-binding motif (residues highlighted in blue) and switch II motifs (residues highlighted in red) of each monomer lie in close proximity. The nucleotide-binding surface of each monomer contributes to the active site, which lies in the interface of the two monomers. Based on the similarity between MinD and Soj monomer structures, it is likely that the MinD dimerization interface is formed by homologous structural elements in MinD. Ribbon diagrams generated using MOLMOL (version 2K.2).

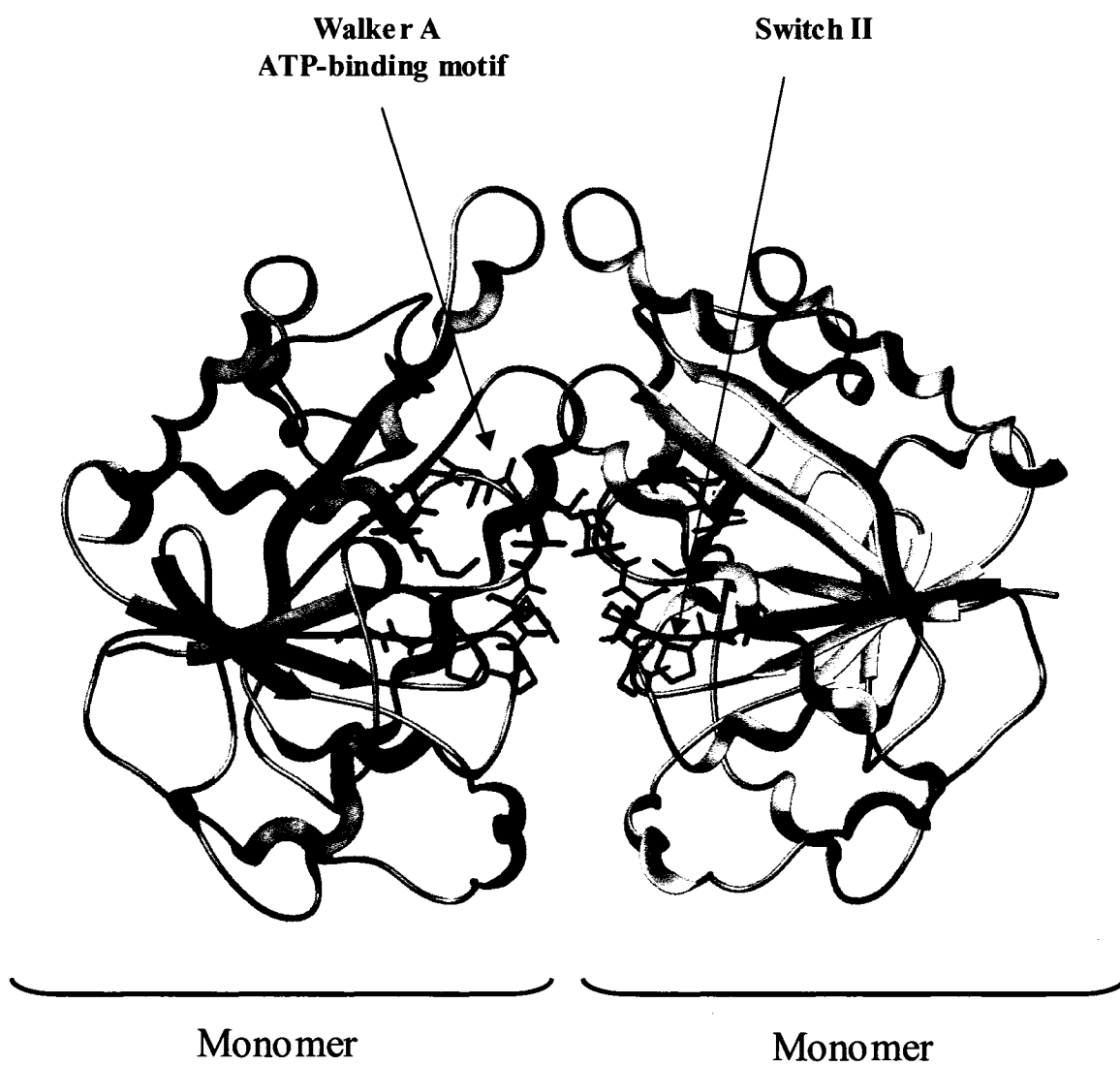
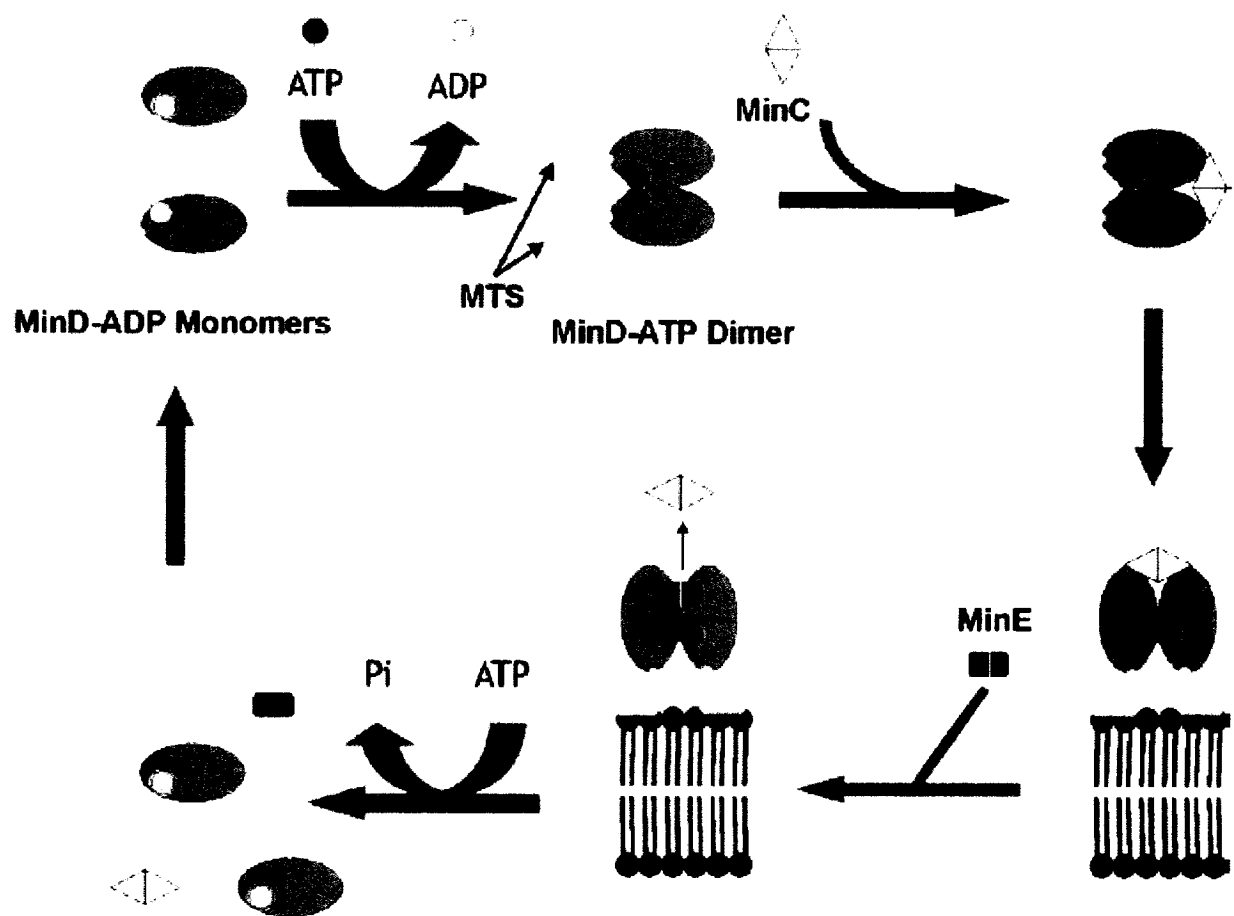


Figure 12. The functional cycle of MinD. During the various steps in its oscillatory cycle, MinD is thought to undergo many conformational changes, adding an additional level of complexity to the mechanism underlying this phenomenon. According to this model, in response to ATP binding, MinD dimerizes and undergoes conformational changes that alter the position of the C-terminal amphipathic helix, thereby allowing MinD to associate with phospholipid membranes. MinD-ATP binds and recruits MinC to polar regions of the cytoplasmic face of the inner membrane. Once membrane bound, MinE displaces MinC while inducing conformational changes that stimulate MinD ATPase activity and force MinD-ADP to dissociate from the membrane.



Walker A ATPases, stabilizes the α and γ phosphates of ATP. Furthermore, the amide nitrogens of G16 and G17 from the P-loops form adjacent chains, which stabilize the γ -phosphate of one ATP moiety. In short, the Soj structures give insight into possible MinD residues involved in active site formation. However, these structures fail to characterize how nucleotide binding could promote MinD dimerization, increase affinity for the membrane leading to its assembly into filaments, and enhance affinity for MinE. Also, it is highly likely that bacterial MinD structures have subtle but critical differences in their three-dimensional structure since the Soj family lacks the MinD-characteristic C-terminal amphipathic helix which mediates membrane association. To reconcile the complex functional cycle of MinD (Figure 12), a structure of a bacterial MinD protein would be of great utility and is therefore a long-term objective for our laboratory.

1.9 Rationale, hypothesis, and objectives

1.9.1 Rationale

Our laboratory is using the Gram-negative coccus *N. gonorrhoeae* (Ng) as a model organism to study the mechanisms of cell division in round bacteria. The presence of *min* homologues in gonococcal cells (Ramirez-Arcos *et al.*, 2001) raised interest regarding the spatial regulation of cell division in cocci, which, unlike bacilli, do not possess obvious midcells. *N. gonorrhoeae* is one of the most clinically significant and prevalent infectious agents. Since 1997, following more than two decades of decline, the reported rate of gonorrhea in Canada has increased by over 60% to 24.3 per 100 000 (7363 cases) in 2003 (Mann *et al.*, 2004). The sexually transmitted infection has a long history of antibiotic resistance (Tapsall, 2002), and a day is envisioned where current antibiotics may no longer be effective. Since cell division

genes represent a potential target for antimicrobial therapy our laboratory is examining how cell division proteins function, with the prospect that this information may be ultimately exploited for the development of new antimicrobial agents. The Min proteins comprise one system that could be targeted for disruption that has yet to be understood at a molecular level. Therefore the focus of this thesis is to study the functional properties of MinD_{Ng}, the enzymatic engine driving Min protein-mediated cell division site placement.

While qualitative experimental studies have shed light into Min protein interactions and oscillation, quantitative experiments examining the molecular mechanism by which ATP binding promotes MinD dimerization and membrane binding have not been performed for MinD_{Ng}. In addition, the mechanism by which MinE stimulates ATPase activity has yet to be elucidated. Therefore, the purpose of this thesis is to gain insight into the mechanism by which ATP binding and hydrolysis control the localization of MinD, and how MinE can promote higher reaction rates in MinD through an *in vitro* analysis of wild type and mutant MinD_{Ng} protein function. In order to facilitate structural studies that would allow the identification of the dimerization interface and the study of conformational changes in MinD, an additional goal of this thesis has been to develop a system for the production of samples suitable for analysis by solution NMR. In the future, the combination of both kinetic and structural studies on MinD should provide new mechanistic insight into motifs responsible for MinD oscillation, dimerization, and ATP hydrolysis.

1.9.2 Hypothesis

- *The conserved N-terminal region of MinD and conserved polar residues (R92, D93 and K94), although far from the active site, influences MinD function.*

- *The rate of ATP hydrolysis is linked to MinD conformational changes that occur in response MinE interactions.*

1.9.3 Objectives

1. *Characterization of function for wild type and mutant MinD ($MinD_{Ng-3aaNT}$, $MinD_{Ng-loop}$) from *N. gonorrhoeae**
 - Vesicle sedimentation studies to study the membrane binding properties of wild type and mutant $MinD_{Ng}$.
 - Develop a micro method based on the procedure described by Harder *et al.*, (1994) for the quantification of inorganic phosphate release.
 - Compare the rate of ATP hydrolysis of wild type $MinD_{Ng}$ to functionally diverse $MinD_{Ng}$ mutants to elucidate the mechanism of MinE stimulation of MinD activity.
2. *Sample development for future NMR Studies of MinD*
 - Design and construct plasmids that can express high levels of $MinD_{Ng}$ that can be easily purified.
 - Optimize expression levels of recombinant $MinD_{Ng}$ and develop a large-scale purification protocol maximizing both protein purity and yield.
 - Determine sample conditions that eliminate or minimize protein aggregation.

2. MATERIALS AND METHODS

2.1 *Strains and growth conditions*

The strains used in this study are presented in Table 1. *E. coli* DH5 α and XL1-Blue were used as hosts to clone both wild type and mutant gonococcal *min* genes. *E. coli* PB103 was used for morphology studies, and *E. coli* PB114 ($\Delta minCDE$) was used to characterize GFP-MinD_{Ng} localization. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (Difco, Detroit, MI), except for *E. coli* PB114, which was grown overnight at 32°C prior to GFP-fusion studies. Induction of *E. coli* PB103 containing recombinant plasmid was done using 100-500 μ M isopropyl-beta-D-thiogalactopyranoside (IPTG). *E. coli* C41 (DE3) was used to overexpress N-terminal or C-terminal His-tagged MinD_{Ng} and His-tagged MinE_{Ng} proteins.

N. gonorrhoeae CH811 was grown on GC Medium Base (GCMB; Difco) supplemented with 1 % modified Kellogg's (GCMBK) defined supplement (40 g glucose, 1 g glutamine, 10 ml of 0.5% ferric nitrate solution, 1 ml of 20% carboxylase), 10 mM MgCl₂, and 0.042% NaHCO₃ for 18 to 24 hours at 35°C in a humid, 5% CO₂ environment (Kellogg *et al.*, 1963; Pagotto *et al.*, 2000).

Saccharomyces cerevisiae strain SFY526 (Clontech, Palo Alto, CA) was used in yeast two-hybrid assays and were grown at 30°C on yeast extract-peptone-adenine-dextrose (YPAD; Clontech) medium or on the appropriate synthetic dropout (SD) media as described in the Clontech Yeast Two-Hybrid Manual. When required, media was supplemented with ampicillin (Amp) at 100 μ g/ml or kanamycin (Km) at 50 μ g/ml (Sigma-Aldrich, St. Louis, MO). Frozen stocks of all bacterial strains were stored at -70°C in Brain Heart Infusion broth (BHI; Difco) supplemented with 20% glycerol.

Table 1. Strains and plasmids used in this study.

Strains/plasmids	Relevant genotype	Source/Reference
<i>E. coli</i> DH5 α	<i>supE44 ΔlacU169 (80lacZΔM15) hsdR17 endA1 gyrA96 thi-1 relA1</i>	Gibco
<i>E. coli</i> XLI-Blue	<i>RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^r ΔM15 Tn10 (Tet^r)</i>]	Stratagene
<i>E. coli</i> PB103	<i>dadR1 trpE61 trpA62 tna-5 purB⁺</i>	de Boer <i>et al.</i> (1988)
<i>E. coli</i> PB114	<i>dadR1 trpE61 trpA62 tna-5 purB⁺ λ^- ΔminCDE aph (Km^r)</i>	de Boer <i>et al.</i> (1988)
<i>E. coli</i> C41(DE3)	<i>F' ompT hsdSB (τ_B^- mτ_B^-) gal dcmD(srl-recA) 306::Tn10 (Tet^r) (DE3)</i>	Miroux and Walker (1996)
<i>N. gonorrhoeae</i> CH811	(P) class: nonrequiring/IB-2/plamid-free, Str ^r	Picard and Dillon (1989)
<i>S. cerevisiae</i> SFY526	<i>trp 1-901 leu2-3 112 can^r gal4-542 gal80-538 URA3::Gal1_{UAS}-GAL1_{TATA}-lacZ</i>	Clontech
<i>Plasmids for expression studies/template DNA</i>		
pUC18	Amp ^R P _{lac}	Amersham
pSIA11	Amp ^R pUC18; Plac::minD _{Ng-3aaNT}	Szeto <i>et al.</i> (2004)*
pSR3	Amp ^R pUC18; P _{lac} ::minD _{Ng}	Szeto <i>et al.</i> (2001)
pJS7	Amp ^R pUC18; P _{lac} ::minD _{Ng-loop} (R92L, D93L, K94I)	Szeto <i>et al.</i> (2005)*
<i>GFP fusion plasmids</i>		
PDSW209	Amp ^R P _{trc} ^d ::gfp	Weiss, <i>et al.</i> (1999)
pSIA17	Amp ^R pDSW209; P _{trc} ^d ::gfp-minD _{Ng} (N-3aa del), minE _{Ng}	Szeto <i>et al.</i> (2004)*
pSR15	Amp ^R pDSW209; P _{trc} ^d ::gfp-minD _{Ng} , minE _{Ng}	Ramirez-Arcos <i>et al.</i> (2002)
pJDE1	Amp ^R pDSW209; P _{trc} ^d ::gfp-minD _{Ng-K16Q} , minE _{Ng}	Szeto <i>et al.</i> (2005)*
pJS9	Amp ^R pDSW209; P _{trc} ^d ::gfp-minD _{Ng-loop} , minE _{Ng}	Szeto <i>et al.</i> (2005)*
<i>Yeast two-hybrid plasmids</i>		
PGAD424	Amp ^R P _{ADHI} ^a ::gal4(AD) ^b	Clontech
PGBT9	Amp ^R P _{ADHI} ::gal4(BD) ^c	Clontech
pSIA4	Amp ^R pGAD424; P _{ADHI} ::gal4(AD)-minD _{Ng} (N-3aa del)	Szeto <i>et al.</i> (2004)*
pSIA2	Amp ^R pGBT9; P _{ADHI} ::gal4(BD)-minD _{Ng} (N-3aa del) ^A	Szeto <i>et al.</i> (2004)*
pGADminD	Amp ^R pGAD424; P _{ADHI} ::gal4(AD)-minD _{Ng}	Szeto <i>et al.</i> (2001)

pGBT9minD	Amp ^R pGBT9; P _{ADH1} :: <i>gal4</i> (BD)- <i>minD</i> _{Ng}	Szeto <i>et al.</i> (2001)
PSRAD-C	Amp ^R pGAD424; P _{ADH1} :: <i>gal4</i> (AD)- <i>minC</i> _{Ng}	Ramirez-Arcos, S. (Dillon Laboratory)
PSRBD-C	Amp ^R pGBT9; P _{ADH1} :: <i>gal4</i> (BD)- <i>minC</i> _{Ng}	Ramirez-Arcos, S. (Dillon Laboratory)
pGBT9minE	Amp ^R pGBT9; P _{ADH1} :: <i>gal4</i> (BD)- <i>minE</i> _{Ng}	Ramirez-Arcos <i>et al.</i> (2002)
PjminD16	Amp ^R pGAD424; P _{ADH1} :: <i>gal4</i> (AD)- <i>minD</i> _{Ng-K16Q}	Ramirez-Arcos <i>et al.</i> (2004)
PjminD17	Amp ^R pGBT9; P _{ADH1} :: <i>gal4</i> (BD)- <i>minD</i> _{Ng-K16Q}	Szeto <i>et al.</i> (2005)*
PjminD18	Amp ^R pGAD424; P _{ADH1} :: <i>gal4</i> (AD)- <i>minD</i> _{Ng-loop}	Szeto <i>et al.</i> (2005)*
PjminD19	Amp ^R pGBT9; P _{ADH1} :: <i>gal4</i> (BD)- <i>minD</i> _{Ng-loop}	Szeto <i>et al.</i> (2005)*
<i>Plasmids for protein purification</i>		
pSC9	Kan ^R pET30a; P _{T7} :: <i>minD</i> _{Ng-6XHis}	Szeto <i>et al.</i> (2004)*
pJS34	Kan ^R pET30a; P _{T7} :: <i>minD</i> _{Ng-3aaNT-6XHis}	Szeto <i>et al.</i> (2004)*
pET30a	Kan ^R P _{T7} ::6XHis	Novagen
pJS8	Kan ^R pET30a; P _{T7} :: <i>minD</i> _{Ng-loop-6XHis}	Szeto <i>et al.</i> (2005)*
pJATP1	Amp ^R pUC18; Plac:: <i>minC</i> _{Ng} <i>minD</i> _{Ng-K16Q} <i>minE</i> _{Ng} , oxyR	Ramirez-Arcos <i>et al.</i> (2001)
pSC10	Kan ^R pET30a; P _{T7} :: <i>minD</i> _{Ng-K16Q-6XHis}	Szeto <i>et al.</i> (2004)*
pSA2	Kan ^R pET30a; P _{T7} :: <i>minD</i> _{Ng-15aaCT^c-6XHis}	This study
PJSHD2	Kan ^R pET30a; P _{T7} ::6XHis-linker- <i>minD</i> _{Ng}	Jason Szeto (Dillon Laboratory)
pSA1	Kan ^R pET30a; P _{T7} ::6XHis- <i>minD</i> _{Ng}	This study
pEC1	Kan ^R pET30a; P _{T7} :: <i>inE</i> _{Ng-6XHis}	Ramirez-Arcos <i>et al.</i> (2002)

^A N-3aa del denotes deletion of first 3 amino acids from N-terminus of MinD_{Ng}

^a P_{ADH1} is a yeast promoter for expression of GAL4 fusion proteins

^b Encodes GAL4 activation domain

^c Encodes GAL4 DNA-binding domain

^d P_{trc} contains mutations at the -10 and -35 region

^e 15aa truncation from the C-terminus

* Publications on which I am co-author

2.2 Polymerase Chain Reaction (PCR) and Inverse PCR (IPCR)

Oligonucleotide primers used for PCR and IPCR amplification were designed using Primer Designer Software (Scientific and Education Software, Durham, NC). PCR reactions were carried out in a Perkin Elmer Gene Amp PCR System 9600 Thermocycler (Perkin Elmer Corporation, Wellesly, MA) as follows: 3 minutes at 94°C; 30 cycles of denaturation for 15 seconds at 94°C, annealing for 15 seconds at temperatures dependent upon the primer pair used, extension at 72°C for times dependent on expected product size (1minute/kb), a final 5 minutes extension at 72°C, and hold at 4°C. All PCR reactions had a final volume of 100 µl, and contained 1X PCR buffer supplemented with 1.5 mM MgCl₂ (Gibco BRL, Gaithersburg, MD), 0.2 mM deoxynucleoside triphosphate (dNTPs) (Roche Diagnostics, Laval, QUE), 2.5U Taq polymerase (Gibco BRL), 0.2 µg of each primer, and 0.01 µg/ml of plasmid template DNA. For IPCR reactions, the reactions conditions were modified as follows: 2.5 U Pfu (Fermentas, Burlington, ON) polymerase and 1X PCR buffer (Pfu-specific buffer provided by manufacturer) in 100 µl final reaction volumes. Pfu DNA polymerase was used instead of Taq polymerase since, in contrast to Taq DNA polymerase, Pfu DNA Polymerase lacks terminal transferase activity and generates blunt-ended PCR products. Where required, a *N. gonorrhoeae* CH811 cell suspension (0.5 McFarland Equivalence Turbidity Standard; Remel, Lenexa, KA) was used as the template DNA source. Concentrations of the plasmid template DNA were determined spectrophotometrically using the GeneQuant RNA/DNA calculator model #80-2103-98 (Amersham Pharmacia, Piscataway, NJ). Primers were synthesized at the University of Ottawa Biotechnology Research Institute. The sequences of the oligonucleotide primers used for PCR and IPCR in this study are listed in Table 2.

Table 2. Oligonucleotide primers used for PCR amplifications.

Primers	Description	Sequence
MinD-N ₃ ^a	generating 3aa N-term truncation	5'- GCGCGAATTCGTGATTATTGTAGTAACTTC- 3'
MinD2 ^b	anneals to 3' terminal of MinD _{Ng}	5'- GCGCGGATCCACCTTATCCTCCGAACA GA- 3'
MinE2 ^b	anneals to 3' terminal of MinE _{Ng}	5'- GCGCGGATCCCATGTCCTATACCTTTTTC- 3'
SA19 [']		5'- TATATCTCCTTCTTAAAG- 3'
SA18	MinD _{Ng} -3aaNT; start codon in bold	5'- CATATGATTATTGTAGTAACTTC- 3'
SJ1 ^c	generating MinD _{Ng} -loop (K92L, K93L, K94I)	5'- GCTTCCCAGACTTTATTAATCGACGCTTTGACAC- 3'
SJ2 [']		5'- CGGCAAAATAAACAGGTTTTC- 3'
MinD1	anneals to 5' terminal of MinD _{Ng}	5'- GCGCGAATTCGTGGCAAAAATTATTGTAG- 3'
SJ3 ^d	MinD _{Ng} -CA15-6XHis; anneals to 5' terminal	5'- GCGCCATATGGCAAAAATTATTGTAG- 3'
SJ4 ^e		5'- GCGCCTCGAGTCCTCCGAACAGACG- 3'
SA20 ^e	MinD _{Ng} -CA15-6XHis; anneals to 3' terminal	5'- GCGCCTCGAGGAAACGCATTTCACG- 3'
SA15	generating 6XHis-MinD _{Ng}	5'- ATGATGATGATGATGGTG- 3'
SA16	generating 6XHis-MinD _{Ng} ; anneals to 5' terminal	5'- GTGGCAAAAATTATTGTA- 3'

a - *Eco*RI restriction site is underlined

b - *Bam*HI restriction site is underlined

c - *Vsp*I restriction site is underlined

d - *Nde*I restriction site is underlined

e - *Xho*I restriction site is underlined

' - Primer annealing to complementary strand

When screening for *minD*_{Ng} mutations encoded on plasmids carried in *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 the plasmid DNA template for a 25 µl final volume PCR reaction, with volumes of other reagents adjusted accordingly. Alternatively, to screen for the correct plasmid size, colonies were picked using a sterile toothpick and resuspended in 25 µl ddH₂O. Following resuspension, the toothpick was used to streak a fresh new LB agar plate with the appropriate antibiotic such that the colony was maintained. A 25 µl cracking solution, composed of 9 parts cracking buffer (11 mM EDTA, pH 8.0, 11% glycerol, 38.5 mM SDS, 0.83 mM bromophenol blue) (Sambrook and Russell, 2001) and 1 part 1 M NaOH, was added to the cell suspension and incubated at room temperature for five minutes to lyse the cells. The reaction mixture was then loaded onto a 1% agarose gel, run for one hour at 100 V, and visualized using the ethidium bromide (EtBr) staining and destaining method outlined below.

2.3 *E. coli* transformations

All transformations into *E. coli* DH5 α , XLI-Blue, PB103, PB114, and C41 (DE3) cells (Table 1), made competent by the CaCl₂ method, were carried out as outlined in Sambrook and Russell (2001). When necessary, plasmid isolation was done using the QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, ON), and plasmid integrity was verified by restriction endonuclease digestion and agarose gel electrophoresis.

2.4 Agarose gel electrophoresis

DNA was characterized by separation on 1% agarose gels in 1X Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA) and stained in 1 mg/ml ethidium bromide followed by visualization under UV light. Gels were photographed in the MultiImage Light Cabinet using the software Alpha Imager 1220 file version 5.04 (Alpha Innotech Corporation, San Leandro, CA). DNA ladders 1Kb plus DNA Ladder, and Supercoiled DNA Ladder (Invitrogen, Burlington, ON) were used to estimate the masses of the PCR amplicon, IPCR amplicon, and plasmid DNA.

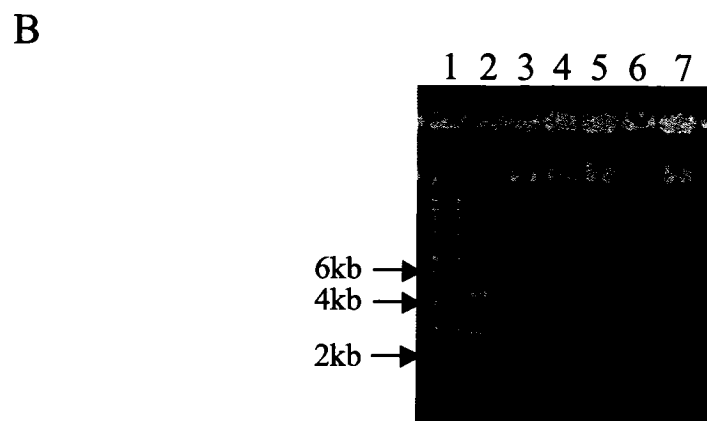
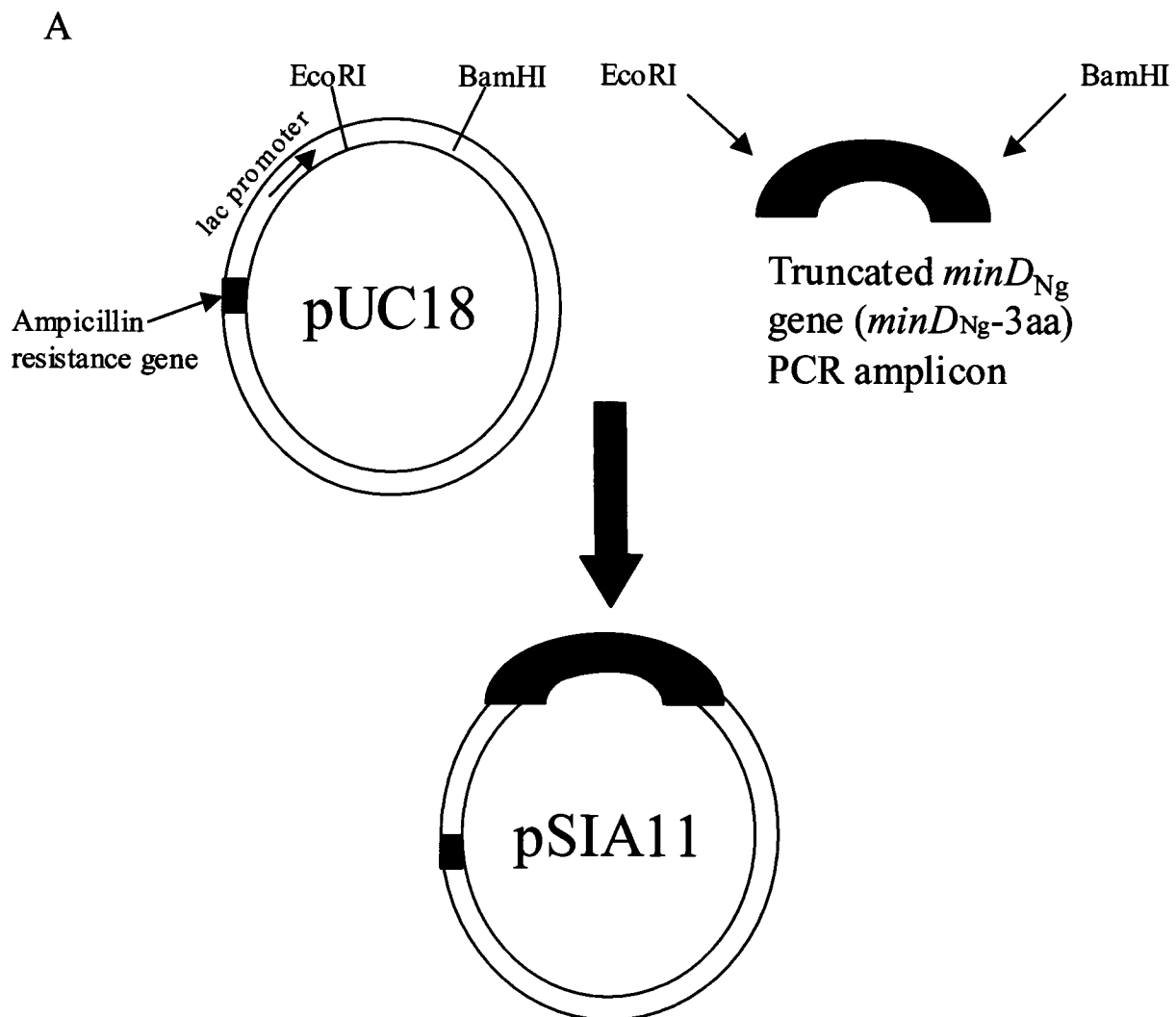
2.5 Plasmids and plasmid constructs

2.5.1 Construction of 3-aa N-terminal deletion derivatives

For expression studies, to create a *minD_{Ng}* gene encoding a 3-aa N-terminal truncation (*minD_{Ng-3aaNT}*), primer MinD-N₃ (Table 2) was used with primer MinD2 (Table 2) to generate the truncated MinD sequence by PCR. The resulting amplicon was digested with both *EcoRI* and *BamHI*, cleaned, and ligated in 20 µl reactions containing 12 µl amplicon, 2 µl of similarly digested pUC18 (Table 1) (Amersham Pharmacia), 2 µl 10X buffer, 2 µl ddH₂O, and 2 µl of DNA ligase buffer (Invitrogen) to produce plasmid pSIA11 (Table 1, Figure 13A). The recombinant plasmid was transformed into *E. coli* DH5a, and XLI-blue cells and potential clones were screened using the aforementioned cracking method (Figure 13B). The generic cloning and screening method outlined in Figure 13 was used for all plasmids constructed in this study.

For yeast two-hybrid studies, PCR amplicons encoding MinD_{Ng-3aaNT} were digested with *EcoRI* and *BamHI* and subsequently cloned in-frame with the GAL4 activation domain

Figure 13. (A) Overview of the procedure used for cloning a PCR amplicon into the plasmid, pUC18 (~2 686 bp). The amplicon (807 bp) (*minD*_{Ng}-3aa) digested with EcoRI and BamHI was ligated into a similarly treated plasmid pUC18 (Table 1) to give rise to a new plasmid, pSIA11 (~3 493 bp) (Table 1). (B) Example of screening for positive clones containing the plasmid, pSIA11 using cracking procedure. Supercoiled DNA ladder (Lane 1); pUC18 control (Lane 2); lanes 3 – 7 correspond to colonies possessing pSIA11 showing decreased mobility relative to pUC18 due to the insertion of the digested PCR amplicon. Similar protocols were followed for all other constructs.



(AD) of pGAD424 and the GAL4 DNA-binding domain (BD) of pGBT9 (Table 1). Plasmids pSIA4 and pSIA2 encode MinD_{Ng-3aaNT} fused to the GAL4-AD and GAL4-BD, respectively (Table 1). Yeast two-hybrid vectors containing the activation domain and binding domain cloned in-frame with one of wild type *minD*_{Ng} (pGADminD, and pGBT9minD), *minC*_{Ng} (pSRAD-C, and pSRBD-C), *minE*_{Ng} (pGBT9minE), or *minD*_{Ng-K16Q} (pJminD16, and pJminD17) were constructed previously (Ramirez-Arcos *et al.*, 2001; Szeto *et al.*, 2001).

Primer MinE2 (annealing to 3' end of *minE*_{Ng}) was used in combination with primer MinD-N₃ (annealing to 5' end of *minD*_{Ng}) (Table 2) to amplify *minD*_{Ng-3aaNT} in combination with *minE*_{Ng} from *N. gonorrhoeae* CH811 chromosomal DNA. The PCR amplicons, as well as plasmid pDSW209, encoding the green fluorescent protein (GFP) (Table 1), were digested with *Eco*RI and *Bam*HI and subsequently ligated. The resulting plasmid, encoding GFP-MinD_{Ng-3aaNT} in combination with MinE_{Ng} was named pSIA17 (Table 1), and used to transform *E. coli* PB114 cells. A fusion of GFP-wild type MinD_{Ng} with MinE (pSR15, Table 1; Ramirez-Arcos *et al.*, 2001) used as a positive control for GFP-MinD_{Ng} localization, and plasmid pJDE1, encoding GFP-MinD_{Ng-K16Q} (Szeto *et al.*, 2005), were constructed previously.

To purify MinD_{Ng-3aaNT}, plasmid encoding C-terminal His-tagged MinD_{Ng-3aaNT} was obtained using an inverse PCR strategy with pSC9 (encoding C-terminal His-tagged wild type MinD_{Ng}) (Table 1) as a template. Primer SA19 and a mutant primer SA18 (incorporating a 3aaNT mutation) (Table 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 pJS34 (MinD_{Ng-3aaNT-6XHis}) (Table 1), and transformed into *E. coli* C41 (DE3) (Table 1) cells for protein overexpression.

2.5.2 Construction of *MinD_{Ng-loop}* (R92L, D93L, K94I) deletion derivatives

To identify whether the highly conserved polar residues R92, D93, and K94 are important for protein function, plasmid pSR3 (Table 1) encoding wild type *minD_{Ng}* was used as a template to generate the triple substitution R92L, D93L, K94I (referred to as ‘loop’ mutation) using IPCR and *Vent* DNA polymerase. Primers SJ1 (encoding all three amino acid mutations) and SJ2 (Table 2) were designed to anneal within *minD_{Ng}* 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, Ipswich, MA), recircularized with T4 DNA ligase (Gibco BRL), and transformed into *E. coli* DH5a. Plasmid-encoded mutant genes were screened by performing colony PCR on candidate clones to amplify an internal segment of *minD_{Ng}* and digesting the amplicons with *VspI*, since this restriction site would have been incorporated by primer SJ1 (Table 2). The resulting plasmid encoding *MinD_{Ng-loop}* (R92L, D93L, K94I) (referred to as *MinD_{Ng-loop}*), was named pJS7 (Table 2).

To create fusions of *MinD_{Ng-loop}* to GAL4-AD and to GAL4-DNA-BD PCR amplicons generated using primers MinD1 and MinD2 (Table 2), and pJS7 (Table 1) as a template, were digested with *EcoRI* and *BamHI*, and cloned into similarly digested pGAD424 and pGBT9. Fusion of *MinD_{Ng-loop}* to GAL4-AD was encoded on plasmid pJminD18 (Table 1). Fusion of *MinD_{Ng-loop}* to GAL4-DNA-BD was similarly constructed, and encoded on plasmid pJminD19 (Table 1).

IPCR methods using the primers SJ1 and SJ2 (Table 2) were used on pSR15 template (encoding wild type GFP-*MinD_{Ng}* and *MinE_{Ng}*) (Table 1) to generate GFP-fusion to *MinD_{Ng-loop}* (pJS9) for localization studies. To purify *MinD_{Ng-loop}*, PCR was carried out using primers SJ3 and SJ4 (Table 2) on template pJS7 (Table 1) in order to amplify *minD_{Ng-loop}*. The

resulting amplicon was digested with *NdeI* and *XhoI* and ligated into similarly digested pET30a (Table 2) to generate a plasmid encoding MinD_{Ng-loop-6XHis} (pJS8).

2.5.3 Construction of plasmids for protein purification

Commercially available plasmid pET30a (Novagen, San Diego, CA) was used to construct 6XHis-*minD*_{Ng} and *minD*_{Ng-6XHis} fusions for protein purification by nickel affinity chromatography. PCR was carried out using primers SJ3 and SJ4 (Table 2) on templates: pSR3, pJATP1, and pJS7 (Table 1). In addition, in an attempt to increase protein solubility, PCR was carried out using primers SJ3 and SA20 (Table 2) on template pSR3 in order to amplify *minD*_{Ng-CA15} (Table 1) (15aa truncation from the C-terminus of wild type MinD_{Ng}). Each amplicon was digested with *NdeI* and *XhoI*, cleaned, and ligated into similarly digested pET30a (Table 1) to generate plasmids encoding C-terminal His-tagged MinD_{Ng} (pSC9), MinD_{Ng-K16Q} (pSC10), and MinD_{Ng-loop} (pJS8), and MinD_{Ng-CA15} (pSA2) (Table 1) for purification (Figure 14). As described in detail above, using IPCR, primers SA18 and SA19 were used in combination with template plasmid pSC9 to generate MinD_{Ng-3aaNT} (pJS34) (Table 1). Note, in the numerous studies that follow, plasmids pSC9 and pSC10, designed previously, encoding C-terminal His-tagged wild type MinD_{Ng-6XHis} and MinD_{Ng-K16Q-6XHis} were utilized as positive and negative controls, respectively.

In contrast to the above C-terminal His tagged MinD proteins, a plasmid encoding N-terminal His-tagged 6XHis-MinD_{Ng} was obtained using an inverse PCR strategy with pJSHD2 (encoding N-terminal His-tagged wild type MinD_{Ng} with a linker region) (Table 1) as a template. Primers SA15 and SA16 (Table 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 produce pSA1 (6XHis-MinD_{Ng}) (Figure 14).

All plasmids used in this study were verified by DNA sequencing by the University of Ottawa Core DNA Sequencing and Synthesis Facility using the Applied Biosystems 373A Sequencer (Perkin Elmer, Wellesley, MA).

2.6 Morphology studies of *E. coli* PB103

MinD_{Ng} is functional when heterologously expressed in an *E. coli* background (Ramirez-Arcos *et al.*, 2002); overexpression of MinD_{Ng} results in cell filamentation because division is inhibited. In contrast, when a MinD_{Ng} mutant is non-functional, filamentation will not occur and wild type morphology will result (Szeto *et al.*, 2001). Thus, to assess the functionality of wild type and mutant MinD_{Ng} proteins, plasmids pSR3 (*minD_{Ng}*), pSIA11 (*minD_{Ng}-3aa_{NT}*), pJS7 (*minD_{Ng}-loop*), pSA1 (6XHis-MinD_{Ng}) and pUC18 (negative control vector) were transformed into *E. coli* PB103 (Table 2) for cell morphology studies. Transformed colonies were suspended in phosphate buffered saline (PBS) (pH 7.4) and centrifuged for 5 minutes at 10,000 X g on a Sorvall MC 12V Microfuge. Pellets were washed three times with PBS, and depending on the size of the pellet, resuspended in 100 µl to 500 µl of PBS to ensure equivalent amounts of cells per slide. Cells were then fixed with 0.2% glutaraldehyde (Fisher Chemical, Nepean, ONT) and 6% formaldehyde (Sigma Chemical, St. Louis, MO). After a 45 minute incubation, fixed cells were allowed to adsorb to coverslips precoated with 0.01% polylysine. The coverslips were then gently washed with PBS and placed on slides containing a drop of 50% glycerol and sealed with transparent nail enamel. Samples were visualized by phase contrast microscopy using a Zeiss Axioskop

Figure 14. Schematic diagram of constructs used to produce wild type and mutant MinD_{Ng} proteins utilized for protein purification, ATPase studies and vesicle sedimentation assays. (A) For wild type MinD_{Ng}, one plasmid encoded a N-terminal six-His tag (pSA1) while the other construct encoded a C-terminal six-His tag (pSC9). (B) For the two C-terminal six-His tagged mutant constructs one plasmid encoded the K16Q mutation (pSC10) while the other construct encoded a 15 amino acid (aa) truncation (pSA2) of the C-terminus of MinD_{Ng}. The wild type and mutant *minD*_{Ng} were cloned into pET30a for protein purification.

A) Wild-type constructs

i) N-terminus tag (pSA1)



ii) C-terminus tag (pSC9)



B) Mutant constructs (pSC10)

i) K16Q mutation



ii) C-terminus truncation (pSA2)



microscope (Carl Zeiss Canada, Toronto, ONT) under a 100X oil immersion lens. Images were captured using a Sony Power HAD 3CCD Color Video Camera (model DX 950), and Northern Eclipse (Version 5.0) software (Northern Eclipse, Mississauga, ONT).

2.7 *Localization and oscillation of GFP-MinD constructs in E. coli PB114*

Previously, our laboratory showed that the ability of GFP-MinD_{Ng} to oscillate in an *E. coli* background is dependent on MinE (Ramirez-Arcos *et al.*, 2002). Thus to determine whether our mutant MinD proteins retain intracellular motility GFP-fusions to mutant MinD_{Ng} were constructed. For localization studies, 50 μ l of an overnight culture of *E. coli* PB114 (Table 1) cells transformed with pSR15, pSIA17, pJS9, or pJDE1 were added to 3 ml of LB in the presence of 40 μ M IPTG. Approximately 5 μ l of *E. coli* PB114 (Δ *minCDE*) cells (Table 1), transformed with each plasmid, was mixed with 5 μ l of 2% low-melting-point agarose in LB and applied immediately to a microscope slide. *E. coli* PB114 (Δ *minCDE*) cells (Table 1), were utilized to eliminate any interference from endogenous *E. coli* Min proteins. Fluorescence microscopy was performed with an Olympus BX61 microscope equipped with a Photometrics CoolSnap ES camera and Image Pro (Version 4.5.1) software (Olympus America, Melville, NY). When the period of oscillation was measured, for consistency, the oscillation cycle of each GFP-MinD_{Ng} fusion was measured in 30 different cells having similar sizes (~2.0 to 3.0 μ m). Time-lapse images were taken every 3-10 seconds for all GFP localization experiments. Enhancement of raw images was done using standard filters of Image Pro software (version 5.0). Contrast enhancement was utilized to increase contrast and to decrease gamma interference. To help visualize intracellular substructures, Gauss filtering using a 3 X 3 kernel size with four passes at strength 10 was used.

2.8 Yeast two-hybrid screening

To determine self-interactions or interactions with other Min proteins the yeast two-hybrid system was utilized. Plasmids were transformed into *S. cerevisiae* SFY526 (Table 1) in various combinations using the lithium acetate method (Clontech Yeast Two-Hybrid Manual). Each yeast vector was first transformed singly into the yeast reporter strain to ensure that their encoded fusion proteins were unable to activate the β -galactosidase reporter gene by themselves. In short, plasmids were transformed singly as a negative control, or in pairs with their corresponding partner to test for protein-protein interactions. Yeast colonies transformed with one or both plasmids were transferred to sterilized filter paper disks, frozen in liquid nitrogen and thawed, and developed overnight in Z-buffer (Clontech) containing the X-gal indicator. Colony lift and liquid β -galactosidase assays, using X-gal and *o*-nitro-phenyl-D-galactopyranoside (ONPG) as substrates, respectively, were used to assess β -galactosidase activity according to the manufacturer's instructions. Transformations and β -galactosidase activity assays were all performed at least twice for each interaction tested and the standard deviation calculated.

2.9 SDS-PAGE and Western Blotting

MinD_{Ng} protein purification was assessed by SDS-PAGE while protein expression levels in transformed *E. coli* was assessed in SDS-PAGE resolved cell extracts by Western blotting using affinity purified polyclonal anti-MinD_{Ng} antisera diluted 1:800 (Szeto *et al.*, 2001). Note that, prior to transfer onto Immobilon-P membranes (Millipore Corporation, Nepean, ONT), protein levels were standardized by densitometry of resolved cell extracts using Alpha imager 1220 v5.04 software (AlphaEaseTM version 5.00; Alpha Innotech Corporation, San

Leandro, CA). Polyclonal mouse antisera against gonococcal MinE was used to detect MinE_{Ng} expressed in *E. coli* PB114 cells as described previously (Ramirez-Arcos *et al.*, 2001).

To assess the purity and yield of wild type and mutant MinD proteins, samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the protocol described by Sambrook *et al.* (1989). Prior to loading, samples diluted with 5X SDS-PAGE loading buffer (15% β -mercaptoethanol, 15% SDS, 1.5% bromophenol blue, 50% glycerol), were boiled for 5 minutes and centrifuged at 10,000 X g (Sorvall MC 12V Microfuge) for 5 minutes. Gels containing two phases – a 5% acrylamide stacking phase, and a 12% acrylamide resolving phase – were loaded with 20 μ l of supernatant and electrophoresed at 130 volts for 80 minutes in 25 mM Tris base, 250 mM glycine, and 0.5% SDS running buffer using the Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). For visualization, gels were stained in Coomassie blue for 60 minutes and destained for 45 minutes in a solution containing 50% ddH₂O, 40% methanol, and 10% acetic acid.

For Western blot analysis (confirm MinD_{Ng} expression), the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was used according to the manufacturer's instructions. An Immobilon-P membrane (Millipore) was washed in methanol for 30 seconds, followed by a 2 minute wash in ddH₂O, and equilibrated in transfer buffer (Sambrook *et al.*, 1989) for 15 minutes. Similarly, after electrophoresis, SDS-PAGE gels were incubated in transfer buffer for 15 minutes to remove any residual salts. The transfer cell was assembled as described by the manufacturer (Bio-Rad) and allowed to proceed for one hour at 100 volts. After protein transfer, the membrane was blocked with 3% skim milk for one hour at room temperature with gentle agitation. The membrane was washed for 3 X

10 minutes in TTBS (50 mM Tris, 1.25 M NaCl, 0.05% Tween-20, pH 7.5), and the blot was then probed with anti-MinD_{Ng} antisera (GERBU Biotechnik, Gailberg, Germany) for one hour at room temperature, and then washed again 3 X 10 minutes in TTBS, followed by incubation with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Bio-Rad, 1:3000 dilution). The blot was once again washed with TTBS (3 X 10 minutes), developed using the AttoPhos Plus kit (JBL Scientific INC), and results were visualized using UV light at 5 minute exposure intervals. The membrane was photographed using Alpha imager 1220 v5.04 software (AlphaEaseTM version 5.00, Alpha Innotech Corporation).

2.10 Protein expression and purification

To prepare samples for NMR analysis, *E. coli* C41 (DE3) cells transformed with pSC9 (MinD_{Ng}-6XHis), pSA1 (6XHis-MinD_{Ng}), or pSA2 (MinD_{Ng}-15aaCT-6XHis) (Table 1) were grown at 37°C in 500 ml of M9 minimal media. For ATPase stimulation assays, *E. coli* C41 (DE3) cells transformed with pSC9 (MinD_{Ng}-6XHis), pJS8 (MinD_{Ng}-loop-6XHis), pJS34 (MinD_{Ng}-3aaNT-6XHis), pSC10 (MinD_{Ng}-K16Q-6XHis), or pEC1 (MinE_{Ng}-6XHis) (Table 1) were grown at 37°C in 250 ml of Luria-Bertani (LB) medium (Difco). Cultures of MinD and MinE cells were induced at A₅₉₅ = 0.6 with 2 mM and 0.4 mM IPTG, respectively, incubated for 6 hours with shaking at 250 rpm, and harvested by centrifugation at 6000 rpm for 10 minutes. The supernatant was discarded, and the 250 ml pellet was resuspended in 12 ml of solution containing 20 mM Tris-HCl (pH 7.9), 0.5 mM NaCl, and 5 mM imidazole (Novagen). The cell suspension was aliquoted into two separate 15 ml falcon tubes and lysed by sonication using a Fisher scientific model 500 Ultrasonic dismembrator (Fisher Scientific, Ottawa, ONT). For lysis – based on SDS-PAGE profiles of cells sonicated at varying amplitude and

repetition – it was empirically determined that cells should be sonicated 12 times each for 12 seconds at 50% amplitude and placed on ice for 1 minute between each repetition. The lysed cell suspension was then centrifuged at 4 °C for 35 minutes at 15 000 rpm. Supernatants were removed and loaded onto a column containing 5 ml of His-bind resin. The following buffers were prepared, as discussed by the manufacturer (Novagen), were used to sequentially wash the column: 40 ml binding buffer (5 mM imidazole), 20 ml wash buffer (30 mM imidazole), 20 ml wash buffer (60 mM imidazole), and 30 ml of elution buffer (1 M imidazole).

For NMR purposes, the purified protein was then dialyzed against Buffer A, (50 mM Tris-HCl, 50 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM EDTA, 2% glycerol, and 10 mM arginine; pH 8.0). After overnight dialysis at 4°C the sample was diluted 1X with ddH₂O and the pH dropped to 5.0 using 200 mM acetate and 200 mM $(\text{NH}_4)_2\text{SO}_4$. The protein solution was then exchanged at 4°C with Buffer B, (20 mM acetate, 50 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM EDTA, 1% glycerol, and 10 mM arginine; pH 5.0). MinD_{Ng} was concentrated using Biomax-5 centrifugal filter columns with a 5 000 Dalton molecular weight cutoff (Millipore). MinE_{Ng} was purified from *E. coli* C41 containing pEC1 (MinE_{Ng}-6XHis), as previously described (Szeto *et al.*, 2004). For ATPase and vesicle sedimentation assays, purification was similar to that described for NMR samples, the only exception being that after elution, proteins were subjected to a single round of dialysis at 4°C against Buffer C, (20 mM Tris-HCl, 0.2 mM EDTA, 200 mM NaCl, and 10% glycerol; pH 7.4).

2.11 Vesicle Sedimentation Assays

To determine the various components required for MinD_{Ng} to bind phospholipid vesicles total *E. coli* phospholipids and anionic 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]

(phosphatidylglycerol [PG]) 25 mg/ml in chloroform were purchased from Avanti Polar Lipids (Avanti, Alabaster, AL). Ultimately, total *E. coli* phospholipids were used over PG vesicles since our studies revealed decreased nonspecific binding of MinD_{Ng} and MinE_{Ng} to total *E. coli* phospholipids relative to PG vesicles (data not shown). A 500 µl aliquot of phospholipid was transferred into a borosilicate glass container with the remaining lipids stored with nitrogen gas in a Teflon coated glass container at -20°C. The 500 µl aliquot was dried using a gentle stream of filtered air and resuspended in 2 500 µl of reaction buffer (25 mM Tris-Cl, and 50 mM KCl; final pH adjusted to 7.5) to obtain a stock 5 mg/ml vesicle solution. Aliquots were stored in microtubes at -20°C and based on phase contrast microscopy appear to be stable for at least 4 months.

After purification and overnight dialysis in their respective buffers, purified MinD_{Ng} (20 mM Tris-Cl, 100 µM EDTA, 200 mM NaCl, and 10% glycerol; final pH adjusted to 7.5) and MinE_{Ng} (50 mM Tris-Cl, 20 mM NaCl, and 1 mM EDTA; final pH adjusted to 7.5) concentrations were quantified and adjusted using the Bio-Rad protein assay. If required, protein samples were concentrated using Biomax-5 centrifugal filter columns with a 5 000 molecular-weight cutoff (Millipore).

Based on the estimated in vivo concentrations of cofactors and interacting proteins (de Boer *et al.*, 1991), a 70 µl reaction mixture carried out in 1.7 ml microtubes consisted of: reaction buffer (25 mM Tris-Cl, and 50 mM KCl; final pH adjusted to 7.5), MinD_{Ng} (3 µM), ATP (1 mM), total *E. coli* phospholipids (300 µg/ml), and MgCl₂ (2 mM). The reaction mixtures were incubated at room temperature (23°C) for 5 minutes after which, depending on the reaction, MinE_{Ng} (3 µM) or MinE buffer was added. This was followed by an additional 30 minute incubation. After incubation, reaction mixtures were centrifuged at 7 000 X g for 2

minutes and the supernatant removed and transferred to a separate microtube while the pellet was resuspended in 70 μ l of reaction buffer. After the addition of 5X SDS-PAGE loading buffer, a 20 μ l aliquot of each sample was electrophoresed on a 12% SDS-PAGE gel and stained using Coomassie blue. The gels were destained in 10% acetic acid, 40% methanol and 50% ddH₂O for one hour such that the amount of protein in the supernatant relative to the pellet could be visualized.

2.12 ATPase Stimulation Assays

Quantitative ATPase activity assays were developed to determine the concentration dependence of wild type MinD ATPase activity. Total *E. coli* phospholipids were prepared as described for vesicle sedimentation assays. In a 150 μ l reaction mixture, the following components were added sequentially: 22 μ l reaction buffer, MinD_{Ng} protein (0.5 – 7.6 μ M), ATP (1 mM), total *E. coli* phospholipids (0.5 mg/ml) and MgCl₂ (10 mM). The reaction mixtures were incubated at room temperature (23°C) for 5 minutes after which depending on the reaction MinE_{Ng} (10 μ M) or buffer with no MinE was added. At specific time points (t = 5 minutes, 15 minutes, 30 minutes, and 45 minutes), 30 μ l aliquots of each reaction mixture were transferred into a new microtube and centrifuged at 16 000 X g for 1 minute. Subsequently, 25 μ l of supernatant was removed and added to 50 μ l of a malachite green solution [3 parts malachite green (0.045% malachite green), 1 part ammonium molybdate (10% ammonium molybdate in 4 N HCl) and 0.01% Tween-20], in a microtiter plate, as described by Harder *et al.*, (1994). For colour development, reaction mixtures were incubated for 15 minutes and the optical density of the reactions at 620 nm was measured using Spectramax Plus 384 (software Softmax PRO) and adjusted for the absorbance of the blank

containing all reagents except the proteins, which were replaced by MinD and MinE storage buffers. The amount of inorganic phosphate released was determined from a standard curve whose values were obtained from serial dilutions of KH_2PO_4 in reaction buffer. Reaction mixtures prepared from at least two different protein preparations were performed and the average inorganic phosphate released determined.

2.13 Protein modeling and alignment

A structural homologue of archaeal MinD proteins, NifH (NIP; Z score = 24.0; Georgiadis *et al.*, 1992) from the nitrogenase complex of *Azotobacter vinelandii* (PDB# 1N2C), was used as a template to generate a homology model of dimeric MinD_{Ng}. Using MOLMOL (Version 2K.2), the solved crystal structure of monomeric *P. furiosus* MinD (PDB# 1G3Q) (Hayashi *et al.*, 2001) was superimposed onto each subunit of the *A. vinelandii* NifH dimer (PDB# 1N2C). In a similar fashion, to demonstrate the structural similarity between the recently solved crystal structure of the Soj protein from *Thermus thermophilus* (PDB# 2BEJ; Leonard *et al.*, 2005) and MinD from *Pyrococcus horikoshii* (PDB# 1ION) (Sakai *et al.*, 2001), the structures were superimposed. For sequence alignment, protein sequences of MinD homologues were obtained from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>). 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 Jalview 2005 Version Release 2.0 (<http://www.jalview.org>).

2.14 NMR spectroscopy

MinD samples were ^{15}N labeled by expression in M9 minimal media containing ^{15}N labeled ammonium chloride (Cambridge Isotope Laboratories Inc.), and purified according to the procedure outlined in section 2.10. After purification and overnight dialysis, concentrations were quantified and modified using the Bio-Rad protein assay. Purity of the samples was assessed by electrophoresis on a 12% SDS-PAGE gel, and protein samples were concentrated using Biomax-5 centrifugal filter columns with a 5,000 molecular-weight cutoff (Millipore). D_2O was added to 10%. Gradient selectivity enhanced $^1\text{H} - ^{15}\text{N}$ HSQC experiments (Kay, LE, Keifer, P, Saainen, T, Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. J. Am. Chem. Soc. 114): 10663-10665) were recorded at 25°C on a 500 MHz Varian Inova spectrometer and data was processed using NMRPipe (Delaglio *et al.*, 1995).

3. RESULTS

3.1 *Characterization of in vivo function for wild type and mutant MinD from N. gonorrhoeae*

3.1.1 *MinD Mutants – Rationale*

Previous studies in our laboratory have demonstrated that a 4-amino acid (MinD_{Ng-4aaNT}) deletion from the N-terminus of MinD is sufficient to prevent its oscillation in *E. coli* PB114 (Szeto *et al.*, 2001). This deletion abrogated MinD self-interaction, and its interaction with MinE (Szeto *et al.*, 2001). In contrast, a 2 amino acid (MinD_{Ng-2aaNT}) truncation was still functional since it could still oscillate in *E. coli* PB114, was able to induce cell filamentation, retained its ability to dimerize, and interact with wild type MinD_{Ng}, MinE_{Ng}, and MinC_{Ng}. This indicated that the N-terminal region of MinD was critical for all aspects of its function and therefore I wanted to further characterize the functional role of amino acid residues in the N-terminus of MinD_{Ng}. An alignment of MinD N-termini from sixteen species revealed that amino acids found directly upstream of the Walker A ATP binding motif are well conserved, particularly among Gram-negative organisms, which share a common MA[K/R]I/VIVVT sequence (Figure 15), corresponding to amino acids 1-8 of MinD_{Ng}. I hypothesized that due to its potential for electrostatic interactions, the conserved positively charged lysine residue in this sequence (K3 in MinD_{Ng}) may be important in MinD function, possibly for interactions with other Min proteins. Thus, to explore the functional role of this residue in MinD_{Ng}, I deleted the first three amino acids including the start codon methionine from the N-terminus and replaced it with a single start codon (MinD_{Ng-3aaNT}) to preserve protein translation, so that its functional characteristics could be compared to the 2 and 4 amino acid truncation mutant properties.

Figure 15. Alignment of N-terminal sequences of MinD from Gram-negative, Gram-positive, and archaeal microorganisms. Alignment was prepared as the aforementioned alignment of MinD proteins from different species. Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>), and alignments were performed using ClustalW Version 1.8 software and visualized using Jalview 2005 Version Release 2.0. Abbreviations used: *Bacillus subtilis* (Bs), *Listeria monocytogenes* (Ls), *Clostridium perfringens* (Cp), *Neisseria gonorrhoeae* (Ng), *Neisseria meningitidis* (Nm), *Escherichia coli* (Ec), *Salmonella enterica* serovar Typhimurium (St), *Yersinia pestis* (Yp), *Vibrio cholera* (Vc), *Brucella melitensis* (Bm), *Brucella suis* (Bsu), *Agrobacterium tumefaciens* (At), *Helicobacter pylori* (Hp), *Pyrococcus furiosus* (Pf), *Pyrococcus horikoshii* (Ph), and *Archeaoglobus fulgidus* (Af). The blue box shows the third amino acid position in Gram-negative MinD proteins. The red bar indicates the position of the 'deviant' Walker A ATP-binding motif. Colored bars adjacent to organism names indicate their general classification: black = Gram-positive, red = Gram-negative, and blue = Archaea. (Adapted from Szeto *et al.*, 2004).

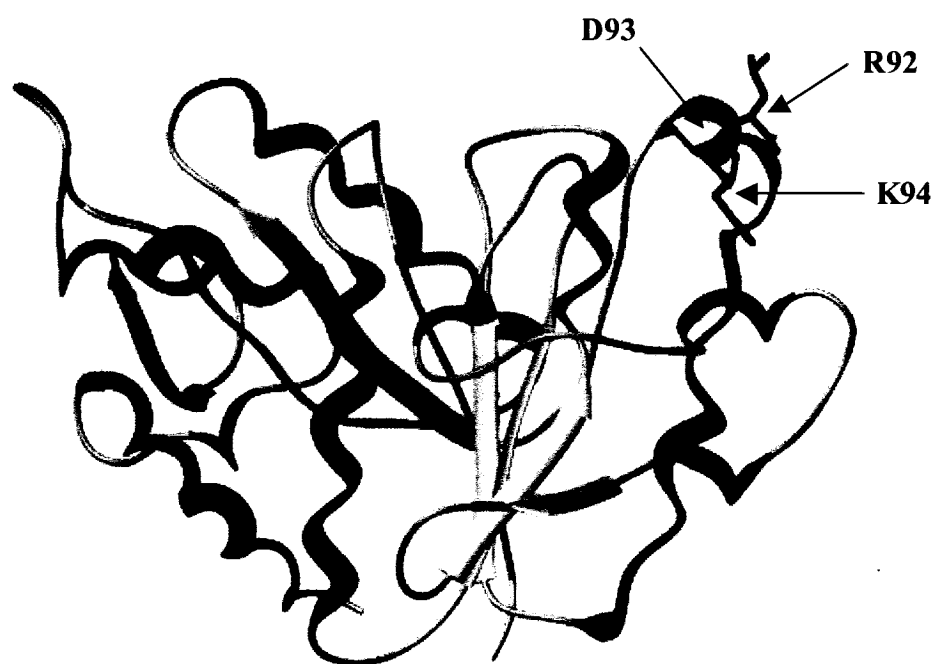
Walker A motif

MinDBs —	1	MGEAIVITSGKGGVGKTTTSANLGTALAILG
MinDLm —	1	MGEAIVITSGKGGVGKTTSTANLGTALALQG
MinDCp —	1	MGVSIIVITSGKGGVGKTTTANIGTALAAQG
MinDNg —	1	MAKIIIVVTSGKGGVGKTTTSASIAATGLALRG
MinDNm —	1	MAKIIIVVTSGKGGVGKTTTSASIAATGLALRG
MinDEc —	1	MARIIVVTSGKGGVGKTTSSAAIATGLAQKG
MinDSt —	1	MARIIVVTSGKGGVGKTTSSAAIATGLAQKG
MinDYp —	1	MARIIVVTSGKGGVGKTTSSAAIATGLAQKG
MinDVc —	1	MSRIIVVTSGKGGVGKTTSSAAIASGLALRG
MinDBm —	1	MOKVIIVVTSGKGGVGKTTSTAALGAALAQRN
MinDBsu —	1	MOKVIIVVTSGKGGVGKTTSTAALGAALAQRN
MinDat —	1	MOKVVVVTSGKGGVGKTTSTAALGAALAQRN
MinDHp —	1	MAIIVVTITSGKGGVGKSTTANLAIGLAESC
MinDPf —	1	MGRITISIVSGKGGTGKTTVTANLSVALGDRG
MinDPh —	1	MTRITISIVSGKGGTGKTTVTANLSVALGEMG
MinDAf —	1	MVRTITIVASGKGGTGKTTITANLGVALAQLG
consensus	1	mgkiivvtSGKGGvGKtTstA lgtala rg

In addition to the studies on the N-terminus of MinD, I was also interested in delineating regions of MinD important for dimerization. Our laboratory has shown that MinD_{Ng} self-associates (Szeto *et al.*, 2001), and this ATP-dependent dimerization is essential for protein function. However, the regions of MinD_{Ng} responsible for this interaction are not known. In order to identify potential regions of MinD_{Ng} that might be involved in dimerization, Szeto (former PhD student; Dillon laboratory) generated a model of the MinD dimer (Figure 16B) (Szeto *et al.*, 2004) using available X-ray crystal structures of homologous proteins. Since *P. furiosus* MinD has low but significant homology to MinD_{Ng} and also exhibits structural similarity with the monomeric subunits forming dimeric nitrogenase iron proteins (Cordell and Lowe, 2001; Hayashi *et al.*, 2001; Sakai *et al.*, 2001), Szeto generated a model of the MinD dimer (Figure 16B) by manual superimposition of representative archaeal MinD monomers from *Pyrococcus furiosus* (Figure 16A) (PDB# 1G3Q) (Hayashi *et al.*, 2001) onto the *A. vinelandii* NifH dimer (Figure 17) (PDB# 1N2C). As shown in Figure 16B, there is a polar loop on the surface of MinD comprising residues R, D, and K – R, D, and K that appear to be in close proximity between monomers in the dimer. Thus, based on the homology structure model and sequence alignment of 24 MinD proteins (Figure 8), Szeto and I focused on the highly conserved residues R92, D93, and K94 (Figure 16A). Interestingly, previous structural studies on NifH had revealed that polar amino acids in the corresponding loop region comprising residues 92-98 (Figure 17) mediate subunit contact via polar interactions (Schlessman *et al.*, 1998). Therefore we hypothesized that, similar to the NifH loop, this conserved region of MinD may be implicated in homodimerization and/or function. Consequently, I simultaneously substituted these polar residues with hydrophobic residues (R92L, D93L, and K94I), to make the mutant which we refer to as MinD_{Ng-loop}.

Figure 16. A homology model of dimeric MinD_{Ng}. (A) Ribbon diagram representation of MinD from *P. furiosus* (1G3Q) (Hayashi *et al.*, 2001) that was used to construct the homology model shown in B (Szeto *et al.*, 2005). The labeled side chains represent those amino acids chosen for simultaneous hydrophobic substitutions (R92L, D93L, and K94I). (B) A homology model of dimeric MinD constructed by superimposition of *P. furiosus* MinD monomers onto each subunit of the *A. vinelandii* NifH dimer. The highly conserved polar region containing the residues R92, D93, and K94 of each monomer corresponding to the loop region in NifH is highlighted. Ribbon diagrams generated using MOLMOL (version 2K.2).

A



B

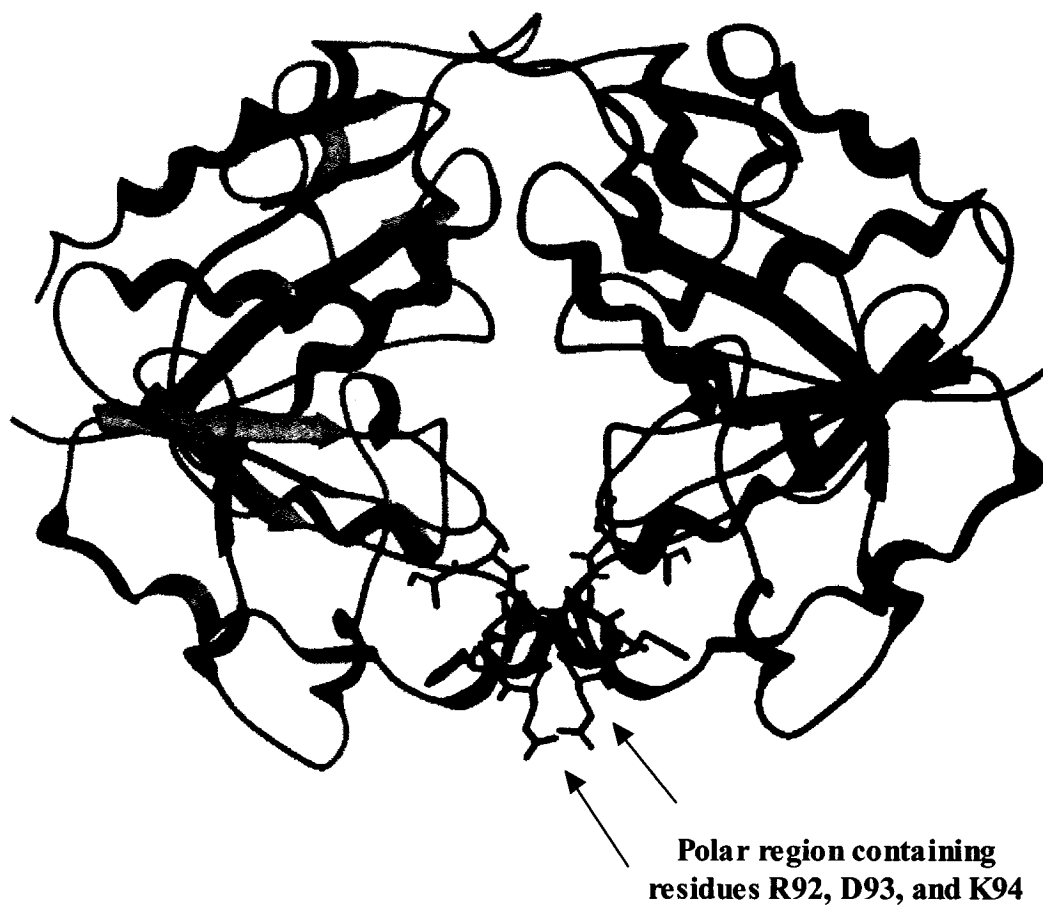
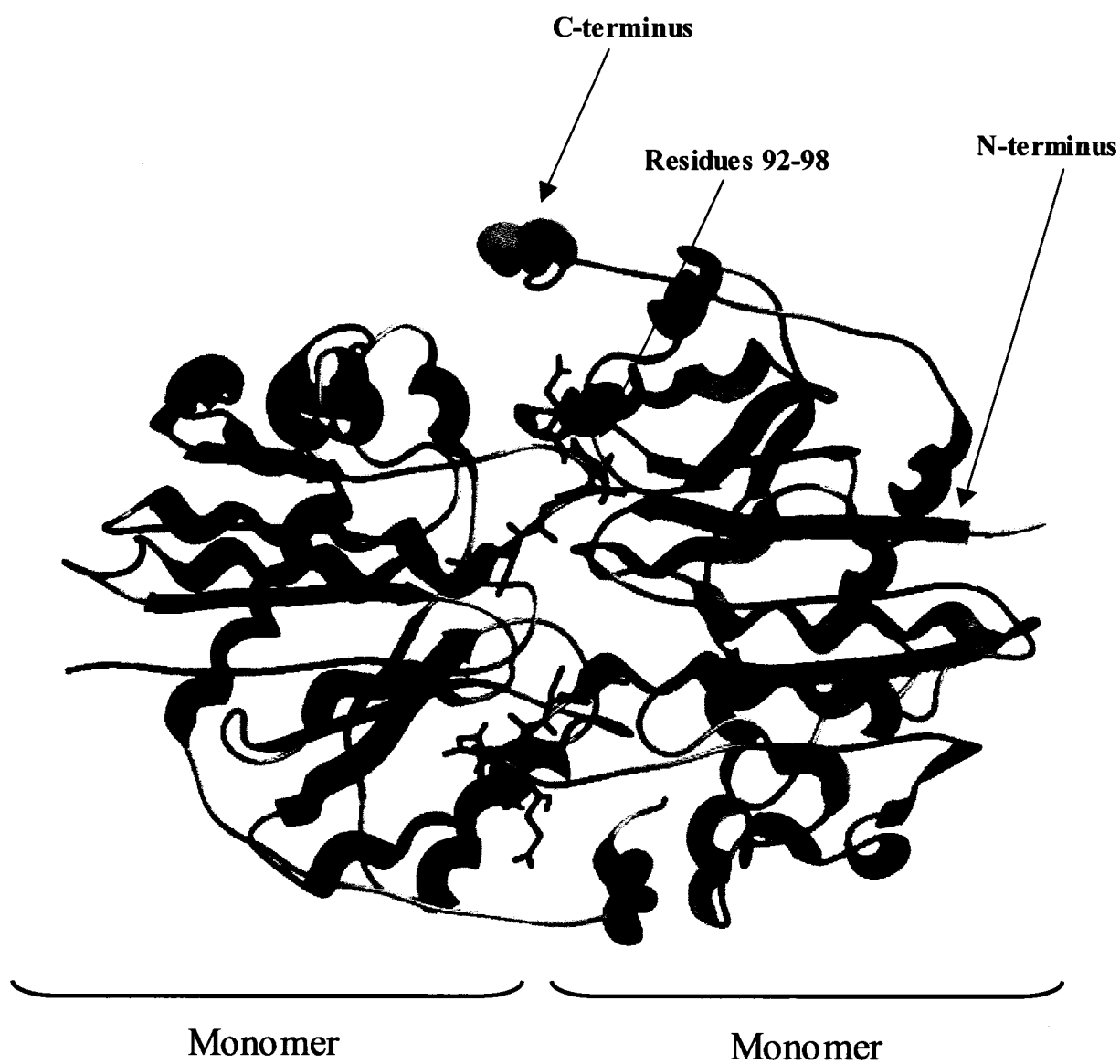


Figure 17. Ribbon diagram representation of a NifH dimer from the nitrogenase complex of *Azotobacter vinelandii* (PDB# 1N2C) (Schindelin *et al.*, 1997). The residues displayed in blue style (amino acids 92-98) represent a loop region involved in mediating subunit contact through polar interactions in each NifH monomer. Ribbon diagrams generated using MOLMOL (version 2K.2).



3.1.2 Phenotype of *MinD_{Ng-3aaNT}* and *MinD_{Ng-loop}* mutant protein expression in *E. coli*

PB103

In order to assess the functionality of the two mutant proteins, *MinD_{Ng-3aaNT}* and *MinD_{Ng-loop}*, I examined their effects on the morphology of wild type *E. coli* PB103 (Table 1). Previous work from our laboratory demonstrated that gonococcal MinD is active in *E. coli* and can therefore be used as an indicator of *MinD_{Ng}* functionality (Szeto *et al.*, 2001; Ramirez-Arcos *et al.*, 2002). As shown in Figure 18B, overexpression of wild type *MinD_{Ng}* encoded on pSR3 (*MinD_{Ng}*) (Table 1) resulted in filamentous *E. coli* cells, indicative of cell division inhibition and functional *MinD_{Ng}*, while bacteria transformed with the negative control plasmid, pUC18, exhibited normal *E. coli* short rod morphology, as previously described (Figure 18A) (Szeto *et al.*, 2001). Similarly, overexpression of the mutant *MinD_{Ng-loop}* from pJS7 (Table 1) resulted in cells with a filamentous morphology (Figure 18C), characteristic of cell division inhibition at all potential division sites arising from functional MinD. In contrast, overexpression of the mutant *MinD_{Ng-3aaNT}* from pSIA11 (Table 1) resulted in cells with a minicell morphology (Figure 18D), indicative of cell division at cell poles and disruption of the ability of the protein to totally inhibit cell division. The absence of *MinD_{Ng-3aaNT}* function was not due to low protein expression levels since expression of wild type and truncated proteins was found to be similar as determined by Western blotting (Figure 18E and F). The faint band in lane 1 of Figure 18E, and lane 2 of Figure 18F represents background levels of resident *E. coli* MinD, which was detected by anti-*MinD_{Ng}* antisera. Remaining lanes show overexpression of MinD and its derivatives in *E. coli* PB103 (Table 1). Overall, these studies suggest that the loop mutant was still able to function in *E. coli* while the N-terminal truncation mutant had lost function.

Figure 18. Expression of wild type, MinD_{Ng-loop} and MinD_{Ng-3aaNT} in *E. coli* PB103. (A) Cells transformed with pUC18 control show a typical short rod morphology. (B) pSR3 (*minD_{Ng}*) transformants display a filamentous phenotype. (C) pJS7 (*minD_{Ng-loop}*) transformants display filamentous phenotype. As indicated by the black arrow, (D) pSIA11 (*minD_{Ng-3aa}*) transformants display minicell morphologies. Scale bar in (A) represents 5 μ m and all figures are at the same magnification. (E) Western blotting using anti-MinD_{Ng} antisera confirms gonococcal protein overexpression in cells transformed with pSR3 (*minD_{Ng}*) (lane 2), and pSIA11 (*minD_{Ng-3aa}*) (lane 3) relative to background expression of native *E. coli* MinD in strain PB103 (lane 1). (F) Western blotting using anti-MinD_{Ng} antisera confirms gonococcal protein overexpression in cells transformed with pJS7 (*minD_{Ng-loop}*) (lane 1), relative to background expression of native *E. coli* MinD in strain PB103 (lane 2).

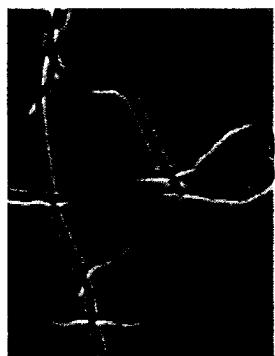
A



B



C



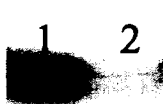
D



E



F



3.1.3 Yeast Two-Hybrid Analysis of *MinD_{Ng-3aaNT}* and *MinD_{Ng-loop}*

In *Saccharomyces cerevisiae*, the transcriptional activator GAL4 is composed of two subunits, the activation domain (AD) and DNA binding domain (BD). When these units are brought in close proximity, transcription of *lacZ* encoding β -galactosidase occurs (Brown, 2002). By exploiting this, I utilized the yeast two-hybrid system to determine whether *MinD_{Ng-3aaNT}* and *MinD_{Ng-loop}* were able to self interact and/or interact with the other Min proteins. It is important to note that this technique is prone to limitations. False negatives can arise if the hybrid proteins are not expressed stably in yeast, if the hybrid proteins impede the natural folding patterns of the proteins, or simply obscure their interaction sites. In contrast, if a protein contains DNA binding activities false positive results can arise from autoactivation of reporter transcription (Brown, 1999). To circumvent this, each yeast vector was first transformed singly into the yeast reporter strain to ensure that their encoded fusion proteins were unable to activate the β -galactosidase reporter gene by themselves.

The gene encoding each mutant was cloned into yeast two-hybrid vectors pGAD424 (yeast GAL4 AD) and pGBT9 (yeast GAL4 BD). The presence of β -galactosidase activity demonstrated that wild type *MinD_{Ng}* displayed self-interaction, and interacted with *MinE_{Ng}*, as previously reported (Szeto *et al.*, 2001; Ramirez-Arcos *et al.*, 2002) (Table 3). Although yeast two-hybrid assays have shown that MinC and MinD from *E. coli* and *B. subtilis* can interact with each other (Huang *et al.*, 1996; Marston and Errington, 1999), our laboratory has not been able to detect an interaction between gonococcal MinC (*MinC_{Ng}*) and *MinD_{Ng}*, possibly due to *MinC_{Ng}* instability in the yeast. Interestingly, our laboratory has designed a chimera containing amino acids 1-99 of *E. coli* MinC and amino acids 103-237 of *N. gonorrhoeae* MinC that interacts with *MinD_{Ng}* (Ramirez-Arcos *et al.*, 2004). Despite the

above shortcomings, I have detected an interaction between MinD_{Ng} and *E. coli* MinC (MinC_{Ec}) using the yeast two-hybrid assay (Table 3), which was subsequently used to investigate whether my mutations in MinD_{Ng} would affect interaction with MinC.

Similar to wild type MinD_{Ng}, yeast colony lift and liquid β -galactosidase assays demonstrated that MinD_{Ng-loop} had retained interactions with itself, MinE_{Ng}, and MinC_{Ec}. In contrast to wild type protein, MinD_{Ng-3aaNT} had lost interaction with itself, wild type MinD_{Ng}, and MinE_{Ng} while retaining interaction with MinC_{Ec} (Table 3). Thus, yeast two-hybrid analysis of MinD_{Ng-3aaNT} suggests that even though this mutant could not form dimers it was still capable of interacting with MinC.

3.1.4 Oscillation and localization studies of GFP-MinD_{Ng-3aaNT} and GFP-MinD_{Ng-loop}

Previous studies have demonstrated the ability of GFP-fusions to wild type MinD_{Ng} to oscillate within their bacterial hosts in a MinE-dependent manner (Ramirez-Arcos *et al.*, 2002). To determine whether MinD_{Ng-3aaNT} and MinD_{Ng-loop} retain intracellular mobility, I constructed plasmids pSIA17 and pJS9 (Table 1) encoding GFP-MinD_{Ng-3aaNT} and GFP-MinD_{Ng-loop} fusion proteins, respectively, along with MinE_{Ng} provided *in cis* to stimulate MinD_{Ng} oscillation. In collaboration with Szeto (former PhD student; Dillon Laboratory), localization studies were carried out in *E. coli* PB114 ($\Delta minCDE$) (Table 1) to eliminate any interference from endogenous *E. coli* Min proteins. As previously established, *E. coli* cells transformed with the plasmid pSR15 (Table 1), which contains wild type GFP-MinD_{Ng} *in cis* with MinE_{Ng}, displayed oscillation of the GFP-MinD_{Ng} fusion protein. In rods approximately 2.0–2.5 μ m in length the pole-to-pole-to-pole movement of GFP-MinD_{Ng} took ~30 seconds (Figures 19A and B). As expected, in the absence of MinE_{Ng}, GFP-MinD_{Ng} localized along the periphery of the cell without any evidence of oscillatory movement (Figure 19C) (Szeto

Table 3. Yeast two-hybrid analysis of the interactions of MinD_{Ng} and its N-terminal truncation derivatives with the Min system.

Fusion to GAL4-BD ^a	Fusion to GAL4-AD ^b	Intensity of colony ^c	B-galactosidase activity ^d
MinD _{Ng}	MinD _{Ng}	+++	8.8 ± 0.3 ^f
MinD _{Ng-loop} ^e	MinD _{Ng-loop}	+++	21.8 ± 0.6 (Szeto <i>et al.</i> , 2004)
MinD _{Ng-3aaNT}	MinD _{Ng}	-	0.03 ± 0.01
MinD _{Ng-3aaNT}	MinD _{Ng-3aaNT}	-	0.03 ± 0.06
MinE _{Ng}	MinD _{Ng}	+++	8.3 ± 0.6
MinE _{Ng}	MinD _{Ng-loop}	+++	31.2 ± 0.2 (Szeto <i>et al.</i> , 2004)
MinE _{Ng}	MinD _{Ng-3aaNT}	+/-	0.16 ± 0.02
MinC _{Ec}	MinD _{Ng}	+++	411.7 ± 0.8
MinC _{Ec}	MinD _{Ng-loop}	+++	76 ± 1 (Szeto <i>et al.</i> , 2004)
MinC _{Ec}	MinD _{Ng-3aaNT}	+++	57 ± 10

^aGAL4-DNA binding domain

^bGAL4-activation domain

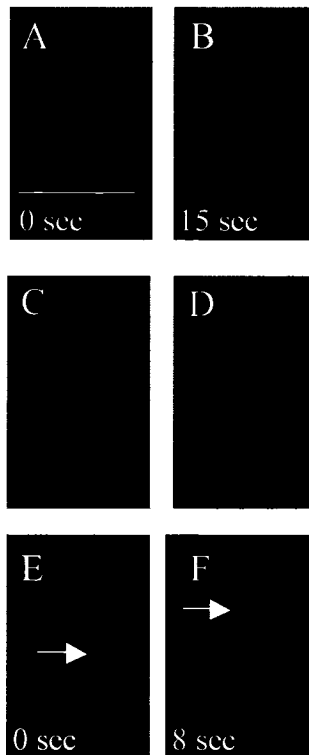
^cPlus signs indicate the intensity of color relative to positive control interactions

^dActivity is measured in Miller units

^eMinD_{Ng-loop} contains R92L, D93L, and K94I substitutions

^fDeviation in β-galactosidase activity was calculated from at least two separate transformations for each interaction tested

Figure 19. Localization of wild type, MinD_{Ng-loop}, and MinD_{Ng-3aaNT} in the presence of MinE_{Ng} in *E. coli* PB114. The average oscillation period (from one pole to the other and back) of wild type GFP-MinD_{Ng} (A, B) was approximately 30 seconds. In the absence of MinE_{Ng} (C), GFP-MinD_{Ng} localizes as a smooth, unbroken fluorescent signal along the inner cell periphery without evidence of oscillatory movement. In contrast to wild type, raw image of GFP-MinD_{Ng-loop} (D) shows the fusion protein distributed along the entire inner periphery of the cell and lacking any obvious pole to pole oscillation (Szeto *et al.*, 2005). Even though much of GFP-MinD_{Ng-3aaNT} (E, F) is distributed to the cytosol it still exhibited pole-to-pole oscillation with one period requiring only 15 seconds. Scale bar in (A) represents 5 μ m and all figures are at the same magnification (Szeto *et al.*, 2004).



et al., 2004). In contrast to wild type GFP-MinD_{Ng}, cells expressing GFP-MinD_{Ng-loop} did not display any fusion protein movement, and the fluorescent signal appeared to be distributed along the entire inner periphery of the cell (Figure 19D) (Szeto *et al.*, 2005). Initially, GFP-MinD_{Ng-3aaNT} did not appear to oscillate in *E. coli* PB114 cells since much of the fluorescent signal was distributed throughout the cytoplasm. However, closer inspection of the images revealed faint oscillatory signals shifting from pole to pole with an average period of ~20 seconds (Figures 19E and F) (Szeto *et al.*, 2004); significantly less than that observed for GFP-MinD_{Ng}. Recently, Shih *et al.*, (2003) reported that the three Min proteins are organized into membrane-associated helical cytoskeletal substructures, and the observed pole-to-pole oscillation of MinC/MinD/MinE appears to occur by the dynamic redistribution of these proteins within this helical framework. Indeed, raw fluorescent images of wild type GFP-MinD_{Ng} revealed fusion protein localized as helical structures extending along the longitudinal axis of the cells (Figure 20). Moreover, image enhancement showed dynamic bands composed of accumulated GFP-MinD_{Ng} signals and weaker bands in areas of lower fusion protein accumulation (Figure 20B) (Szeto *et al.*, 2005). Image enhancement of GFP-MinD_{Ng-loop} revealed mutant protein localized as coiled structures along the cell length (Figure 20D) (Szeto *et al.*, 2005). However, in contrast to wild type, there was no evidence of oscillation since a uniform fluorescent signal was distributed throughout the entire helical substructure which extended along the entire length of the cell (Figure 20C) (Szeto *et al.*, 2005). Thus, unlike GFP-MinD_{Ng}, which accumulated at specific regions of the helical substructure, GFP-MinD_{Ng-loop} had lost its topological specificity since its localization did not have a preference for a specific region along the helical array.

Figure 20. Localization of GFP-MinD_{Ng} variants in the presence of MinE_{Ng} in *E. coli* PB114. (A) Raw image of GFP-MinD_{Ng} bands typically seen in longer *E. coli* PB114 transformants (Szeto *et al.*, 2005). (B) Enhanced image of (A), showing GFP-MinD_{Ng} localizing as coils, with distinct accumulation in specific areas. In contrast to wild type, raw image of GFP-MinD_{Ng-loop} (C) shows the fusion protein distributed along the entire inner periphery of the cell and lacking any obvious pole to pole oscillation. Moreover, image enhancement (D) reveals that even though the protein can still assemble into higher ordered structures, MinD_{Ng-loop} lacks topological specificity since the protein is recruited along the entire length of the scaffold, including the midcell location (Szeto *et al.*, 2005). Raw image of GFP-MinD_{Ng-3aaNT} (E) shows a fusion protein distributed uniformly in the cytosol. (F) Enhanced image of (E) revealed that a small proportion GFP-MinD_{Ng-3aaNT} localized as polymeric structures (Szeto *et al.*, 2004). Scale bar in (A) represents 5 μ m and all figures are at the same magnification.

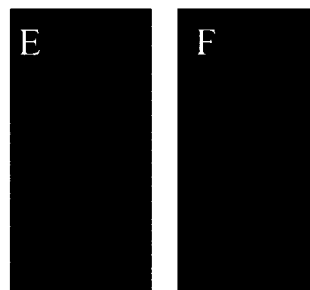
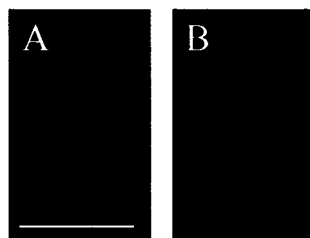


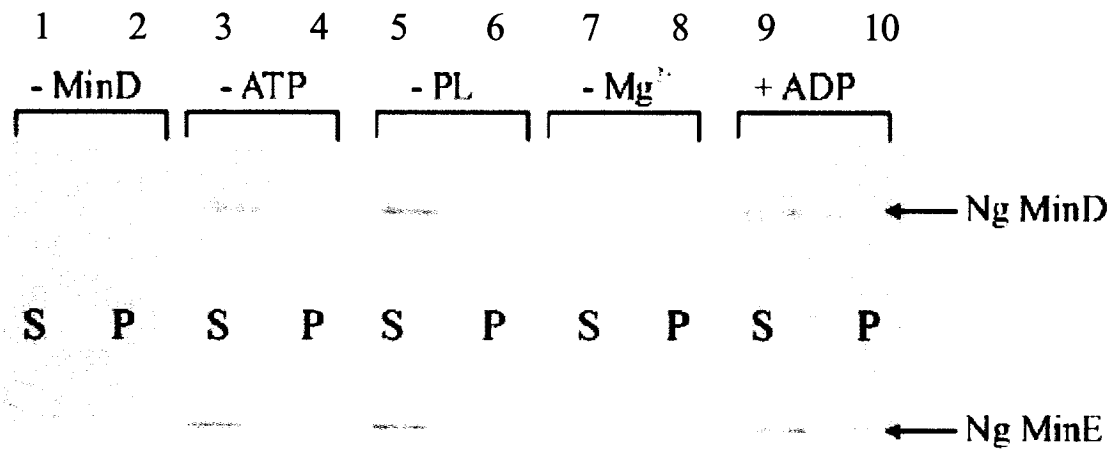
Image contrast enhancement of GFP-MinD_{Ng-3aaNT} revealed a small proportion of fusion protein localized as polymeric structures (Figure 20F) (Szeto *et al.*, 2004). Hence, while the majority of the protein is cytosolic (Figure 20E) (Szeto *et al.*, 2004), MinD_{Ng-3aaNT} retained its ability to localize to the helical substructure and in contrast to MinD_{Ng-loop} displayed faint oscillatory signals.

3.1.5 *MinD_{Ng} associates with phospholipids in the presence of ATP and Mg²⁺*

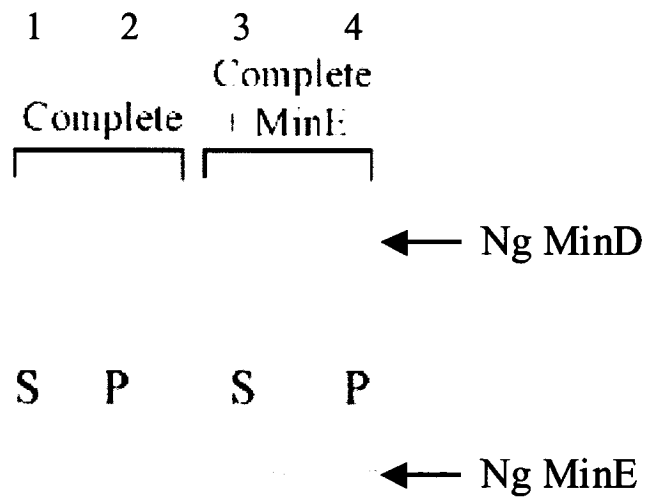
In order to assess the ability of purified wild type and mutant MinD_{Ng} proteins to bind phospholipid membranes *in vitro*, I developed lipid vesicle sedimentation assays. In these assays purified MinD_{Ng} was incubated with or without phospholipid vesicles in the presence of ATP and MgCl₂. After centrifugation the supernatant and pellet fractions were analyzed by SDS-PAGE. As shown in Figure 21 vesicle bound protein would localize to the pellet fraction, whereas protein that does not bind to the lipid vesicle would appear in the supernatant. As expected, MinD_{Ng} binding to the phospholipid vesicles required both ATP and Mg²⁺ (Figure 21B). In contrast, ADP was not able to promote membrane binding even in the presence of Mg²⁺ (Figure 21A; lane 10). MinE_{Ng} was also found to localize to the pellet fraction, but only in the presence of membrane-bound MinD_{Ng}, supporting the model that MinE can only localize to the membrane via interactions with MinD. I then extended the analysis by comparing the association of MinD_{Ng} with the membrane in the presence or absence of MinE_{Ng}. In the presence of MinE_{Ng}, greater amounts of MinD_{Ng} was recovered in the supernatant relative to the pellet. In contrast, in the absence of MinE_{Ng}, approximately equal amounts of MinD_{Ng} were recovered in both the pellet and the supernatant fractions (Figure 21B; lanes 1-4).

Figure 21. Lipid vesicle sedimentation assay results for wild type MinD_{Ng}. Reaction mixtures were prepared as described in the Materials and Methods. (A) Sedimentation of MinD_{Ng} (3 μ M) and MinE_{Ng} (3 μ M) was examined either in the absence of MinD_{Ng}, ATP (1 mM), total *E. coli* phospholipids (PL) (300 μ g/ml), or MgCl₂ (2 mM), or in the presence of ADP (1 mM). After a 30 minute incubation, 20 μ l aliquots of the supernatant (S) and pellet (P) fractions were electrophoresed on a 12% SDS-PAGE gel. In all reactions protein localized exclusively to the soluble fraction, indicating no interaction between MinD and the lipid vesicles. (B) Sedimentation of MinD_{Ng} with 'complete' reaction mixtures (ATP, MgCl₂, and phospholipids) examined in the presence or absence of MinE_{Ng}. The presence of MinD in the pellet fraction in both cases indicates an interaction with the lipid membrane. In the presence of MinE_{Ng}, a slight reduction in the amount of MinD_{Ng} localized to the pellet is shown.

A



B



In addition, the localization properties of MinD_{Ng} bearing a (K16Q) mutation in its Walker A ATP binding motif MinD_{Ng-K16Q}, was examined since our laboratory has previously shown that this mutation diminishes MinD dimerization, as assessed by yeast two hybrid assays (Ramirez-Arcos *et al.*, 2002). In contrast to wild type MinD, regardless of the presence or absence of MinE_{Ng}, this mutant protein was recovered entirely in the supernatant fraction (Figure 22A; lanes 1-4).

To elucidate the *in vitro* effect of mutations within residues 92-94 of MinD_{Ng}, I purified MinD_{Ng-loop} for vesicle sedimentation assays. In contrast to the K16Q mutation, MinD_{Ng-loop} retained its ability to localize to the membrane (Figure 22B; lanes 1-2) possibly by maintaining its ability to self associate despite the simultaneous substitution of the conserved polar residues with hydrophobic residues. Interestingly, although MinE_{Ng} and MinD_{Ng-loop} could still interact with each other (Figure 22B; lane 4), the addition of MinE_{Ng} did not decrease the fraction of membrane bound MinD_{Ng-loop}.

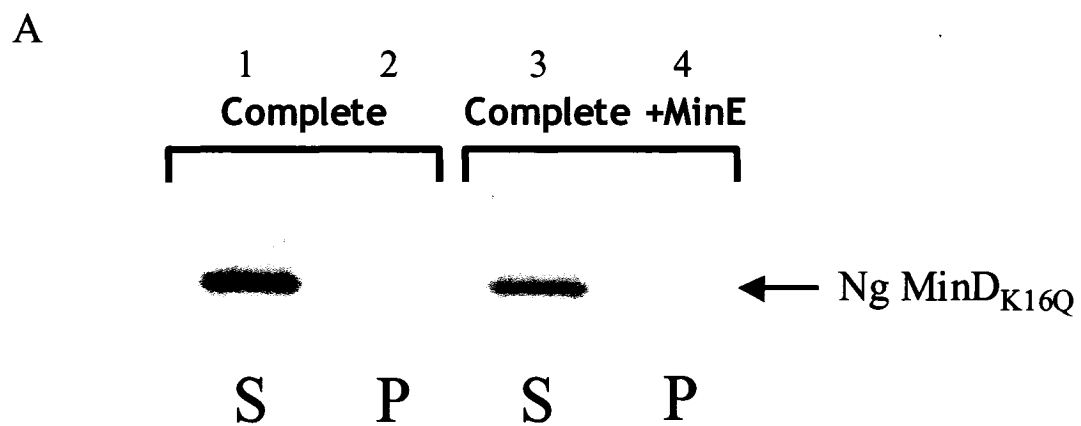
3.1.6 ATPase activity of purified wild type and mutant MinD proteins

To explore the role of the ATPase activity in the rapid oscillation of MinD_{Ng-3aaNT} and the lack of topological specificity of MinD_{Ng-loop}, I measured the release of inorganic phosphate from MinD_{Ng} proteins in the presence of phospholipids and ATP. Since C-terminal His-tagged MinD_{Ng} was still able to inhibit cell division in *E. coli* C41 (DE3) (Szeto *et al.*, 2004) it was not necessary to remove the tag for my studies. Similarly, His-tagged MinE_{Ng} was also active since it caused obvious minicell phenotype in *E. coli* (Szeto *et al.*, 2004) and hence it was used in my assays without removal of the His-tag.

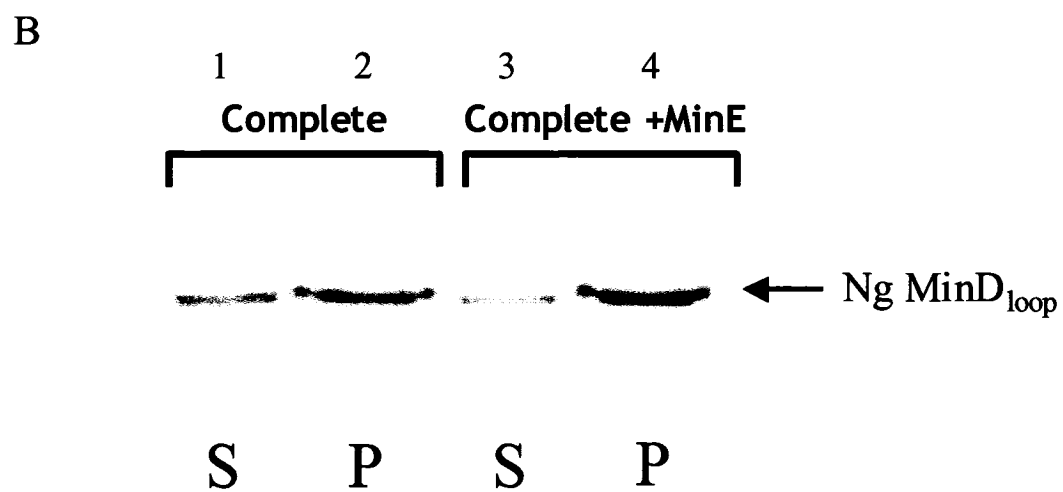
In the absence of MinE_{Ng}, wild type MinD_{Ng} displayed a basal level of ATPase activity as shown by the fact that over a 90 minute period the rate of ATP hydrolysis was

Figure 22. Lipid vesicle sedimentation assay results for MinD_{Ng-K16Q}, and MinD_{Ng-loop}.

(A) Sedimentation of MinD_{Ng-K16Q} in the presence of ATP, phospholipids and MgCl₂ reveals the inability of this protein to localize to the membrane regardless of the presence/absence of MinE_{Ng} since it localizes entirely to the supernatant fraction. In contrast, sedimentation of MinD_{Ng-loop} (B) reveals the ability of this protein to localize to the membrane.



← Ng MinE

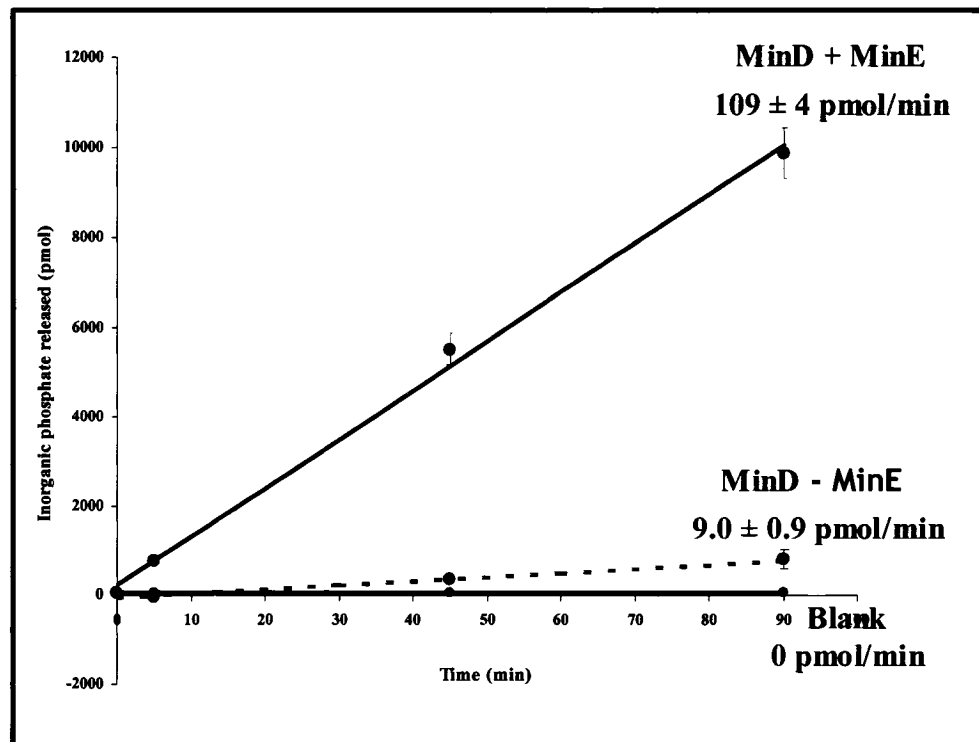


← Ng MinE

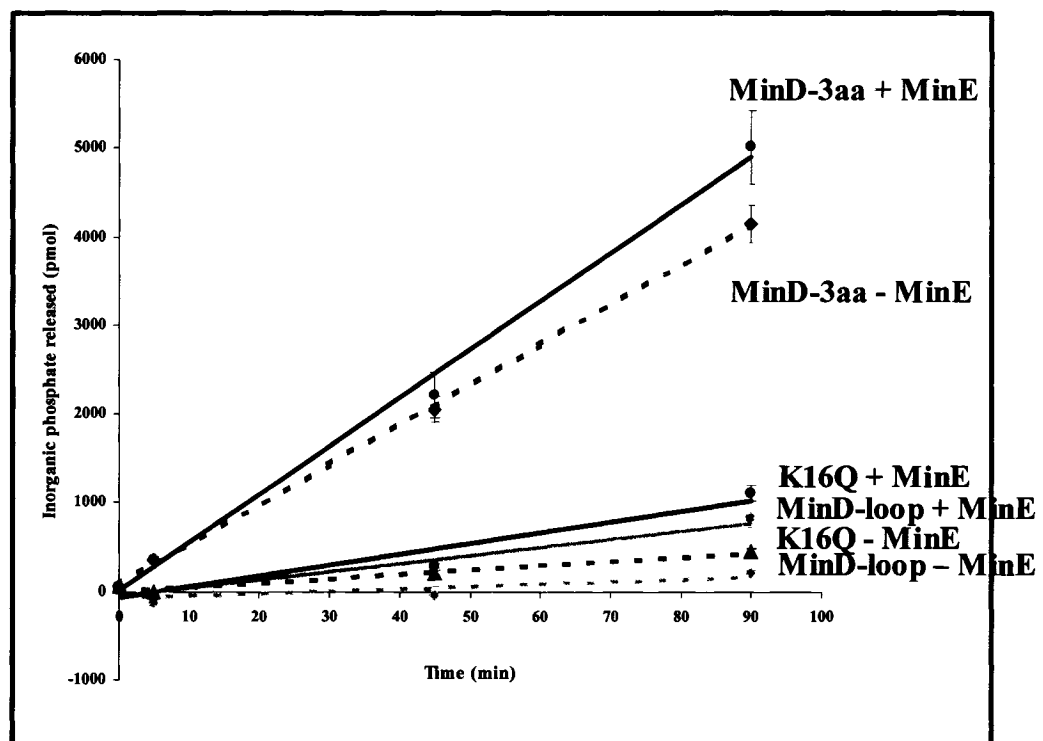
only $\sim 9.0 \pm 0.9$ pmol/minute (Figure 23A), comparable to the negative control MinD_{Ng-K16Q} (Figure 23B). The initial rate of ATP hydrolysis determined by the slope of the best fit line revealed that the addition of MinE_{Ng} stimulated the ATPase activity of MinD_{Ng} almost 12-fold in the presence of phospholipid vesicles since the rate of ATP hydrolysis (slope) in the presence of MinE was $\sim 109 \pm 4$ pmol/minute (Figure 23A). Despite the addition of MinE_{Ng}, the ATPase activities of MinD_{Ng-K16Q} ($\sim 13 \pm 2$ pmol/minute) – the negative control – and MinD_{Ng-loop} ($\sim 8 \pm 1$ pmol/minute) were comparable to basal wild type MinD ATPase activity of $\sim 9.0 \pm 0.9$ pmol/minute (Figure 23B). Interestingly, similar to MinD_{Ng-K16Q}, the addition of MinE_{Ng} did not increase the level of inorganic phosphate released from MinD_{Ng-loop} (Figure 23B). However, unlike MinD_{Ng-K16Q}, the inability of MinE_{Ng} to stimulate MinD_{Ng-loop} ATPase activity cannot be attributed to a deficiency in membrane binding since a significant fraction of MinD_{Ng-loop} was recovered in the pellet (Figure 22B; lane 4). After 90 minutes the blank reaction, absence of both MinD and MinE, did not release any measurable amount of Pi such that the rate of ATP hydrolysis was ~ 0 pmol/minute. I also investigated the ATPase activity of the MinD_{Ng-3aaNT} mutant. In striking contrast with ATPase results on all other MinD mutants, I found that in the absence of MinE_{Ng}, MinD_{Ng-3aaNT} displayed greater ATPase activity than wild type MinD_{Ng} (Figure 23B). In fact, after 90 minutes, MinD_{Ng-3aaNT} in the absence of MinE_{Ng}, had released up to 20 times more inorganic phosphate than wild type MinD_{Ng}. This is astonishing since MinD_{Ng-3aaNT}, which did not appear to dimerize or interact with MinE according to yeast two-hybrid results (Table 3) is nonetheless capable of hydrolyzing ATP and oscillating approximately two times faster than wild type MinD_{Ng}. Even more surprising was the fact that the addition of MinE_{Ng} did not cause a significant increase in the ATPase activity of MinD_{Ng-3aaNT} (Figure 23B).

Figure 23. Plot of the amount of inorganic phosphate released over time by (A) wild type and (B) mutant MinD proteins. In a typical reaction mixture, the following components were added sequentially: 22 μ l reaction buffer, MinD_{Ng} protein (3.0 μ M), ATP (1 mM), total *E. coli* phospholipids (0.5 mg/ml) and MgCl₂ (2 mM). The reaction mixtures were incubated at room temperature (23°C) for 5 minutes after which depending on the reaction MinE_{Ng} (3 μ M) or buffer was added. Over a 90 minute period, the amount of inorganic phosphate released due to ATP hydrolysis was monitored using a malachite green based method. The initial rate of ATP hydrolysis was determined by linear regression utilizing the method of least squares. Reactions were performed at least twice for each time point tested and the standard deviation in the initial rate of ATP hydrolysis calculated.

A



B

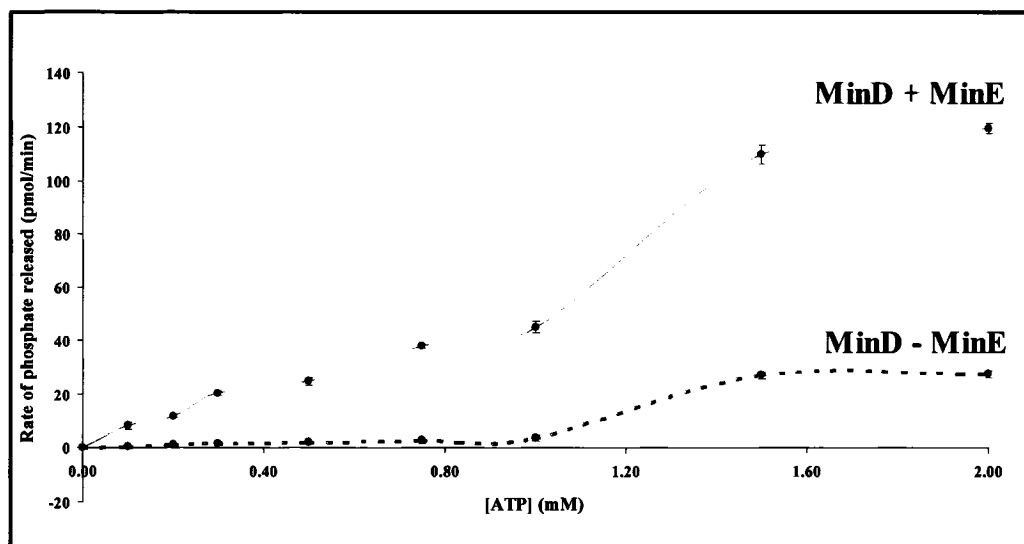


3.1.7 *MinD_{Ng} ATPase activity displays cooperative behavior*

MinD_{Ng} is an allosteric enzyme since it binds ATP and its ATPase activity is significantly enhanced by interaction with MinE_{Ng}. In order to understand the mechanism of MinE stimulation of ATPase activity, I examined MinD_{Ng} ATP hydrolysis rate as a function of ATP concentration in the presence and absence of MinE. As described above, I monitored the amount of inorganic phosphate released at a range of ATP concentrations (from 0.25 mM to 3.0 mM) over time while keeping the concentration of all other required components (i.e. lipids, MgCl₂, MinE) constant. A plot of the phosphate released versus time yielded linear relationships; the slope of this line corresponds to the initial reaction velocity for MinD_{Ng} under the given conditions. For instance, the rate of ATP hydrolysis for wild type MinD_{Ng} at 0.5 mM and 1.0 mM substrate (ATP) concentrations was determined to be $\sim 25 \pm 3$ and $\sim 45 \pm 7$ pmol/minute respectively, showing that MinD ATPase activity increases as the ATP concentration increases, as expected (Appendix; Figure 1A). This analysis was also performed in the absence of MinE_{Ng} yielding much lower rates than obtained in the presence of MinE_{Ng}. For example, ATP hydrolysis rates for wild type MinD_{Ng} at ATP concentrations of 0.2 mM and 0.3 mM were $\sim 1.18 \pm 0.03$ and $\sim 1.77 \pm 0.01$ pmol/minute respectively (Appendix; Figure 1B). The findings from these results suggest that in the presence of MinE_{Ng} and phospholipid vesicles, MinD_{Ng} ATPase activity is significantly enhanced.

As shown in Figure 24, a plot of the initial reaction rate versus substrate concentration produces a sigmoidal, or S-shaped curve both in the presence and absence of MinE_{Ng}. The sigmoidal shape of these curves reflects the fact that MinD_{Ng} is an allosteric enzyme with positive substrate cooperativity and therefore does not follow simple Henri-Michaelis-Menten behavior that would produce the more familiar hyperbolic curve

Figure 24. Plot of the rate of ATP hydrolysis at varying ATP concentrations by wild type MinD_{Ng} in the presence and absence of MinE_{Ng}. Reaction rates were measured as shown in Figure 23 either in the presence (red) or absence (blue) of MinE_{Ng} (3 μ M). The sigmoidal, or S-shaped curves suggest that MinD_{Ng} is an allosteric enzyme consistent with the finding that it displays positive substrate cooperativity. Lines connecting the points are drawn in for the purpose of visualization. Each reaction was performed at least twice and the standard deviation in the initial rate of ATP hydrolysis at each substrate concentration calculated.



characteristic of single-site enzymes. Considering that homology modeling suggests that two molar equivalents of ATP will be bound by and hydrolyzed by each MinD dimer, ATP binding at one site must change the activity and/or affinity of the second binding site for ATP.

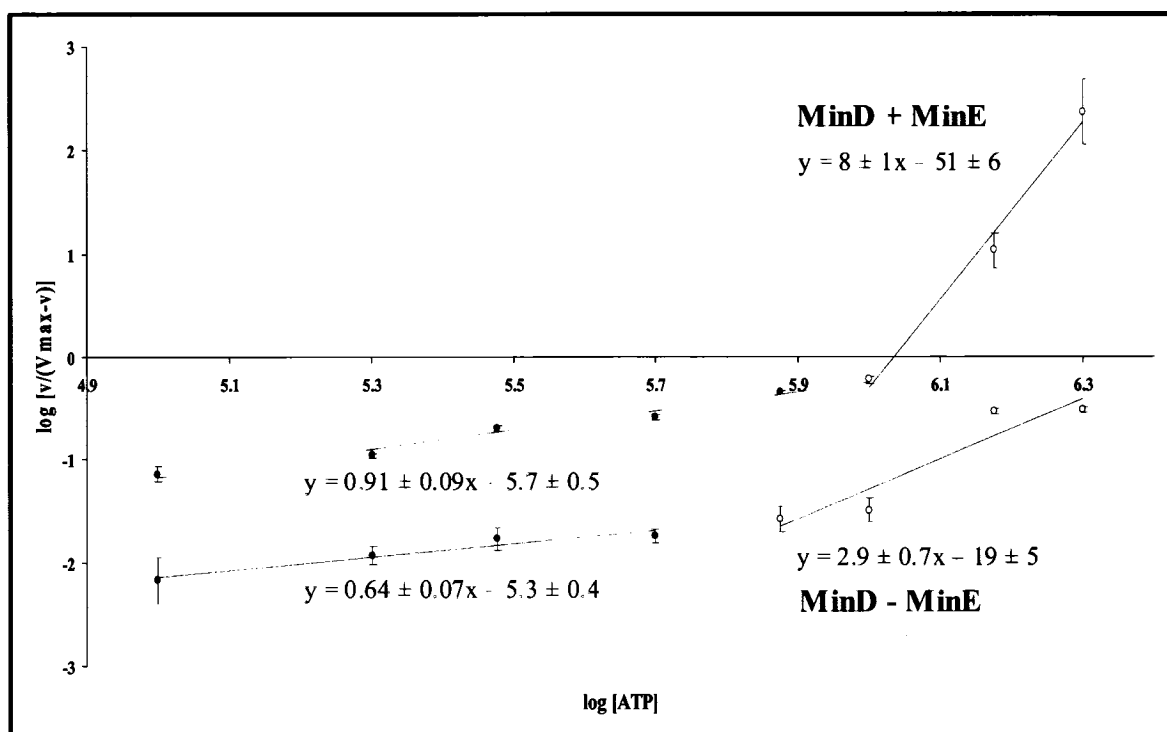
In order to investigate the mechanism of MinE_{Ng} stimulation of MinD_{Ng} ATPase activity, I have also determined the ATP dependence of the initial rate in the absence of MinE_{Ng}. The maximal rate of MinD activity in the absence of MinE is approximately 5-fold lower than that in the presence of MinE_{Ng} (Figure 24). The sigmoidal shape of the curve is preserved suggesting that positive substrate cooperativity is not dependent on MinE_{Ng}, but is an intrinsic property of MinD ATPase activity. In addition, the substrate concentration at which half-maximal activity is obtained ([ATP]_{0.5}) is approximately the same in the absence and presence of MinE at ~1.4 mM.

Since the different sigmoidal curves obtained in the presence and absence of MinE_{Ng} may also be due to a difference in the degree of cooperativity, I analyzed the rate data in terms of a Hill plot (Figure 25). An equation originally derived by Hill for a binding reaction with positive cooperativity:

$$v = V_{\max} [S]^h / K' + [S]^h$$

where v is the initial reaction rate, $V_{\max}$ is maximal rate, S is substrate, h is Hill coefficient which provides a measure of cooperativity and K' is the substrate concentration at half the maximal rate (Cornish-Bowden, 2004). The velocity equation can be converted to a useful linear form by plotting $\log (v/V_{\max} - v))$ as a function of $\log [S]$. The slope of this line

Figure 25. Hill plot for the data from the rate of ATP hydrolysis at varying ATP concentrations by wild type MinD_{Ng} in the presence and absence of MinE_{Ng}. The maximal activity determined from Figure 24 and the rate of ATP hydrolysis at the specific ATP concentration was utilized to calculate $\log [v/(V_{\max} - v)]$ and plotted as a function of $\log [ATP]$. The slope of the best fit lines determined by linear regression utilizing the method of least squares provides an estimate of the lower limit for the number of ligand-binding sites on each molecule of MinD_{Ng}, in the presence (red) and absence (blue) of MinE_{Ng}. Each reaction was performed twice and the log of the standard deviation in $v/(V_{\max}-v)$ calculated.



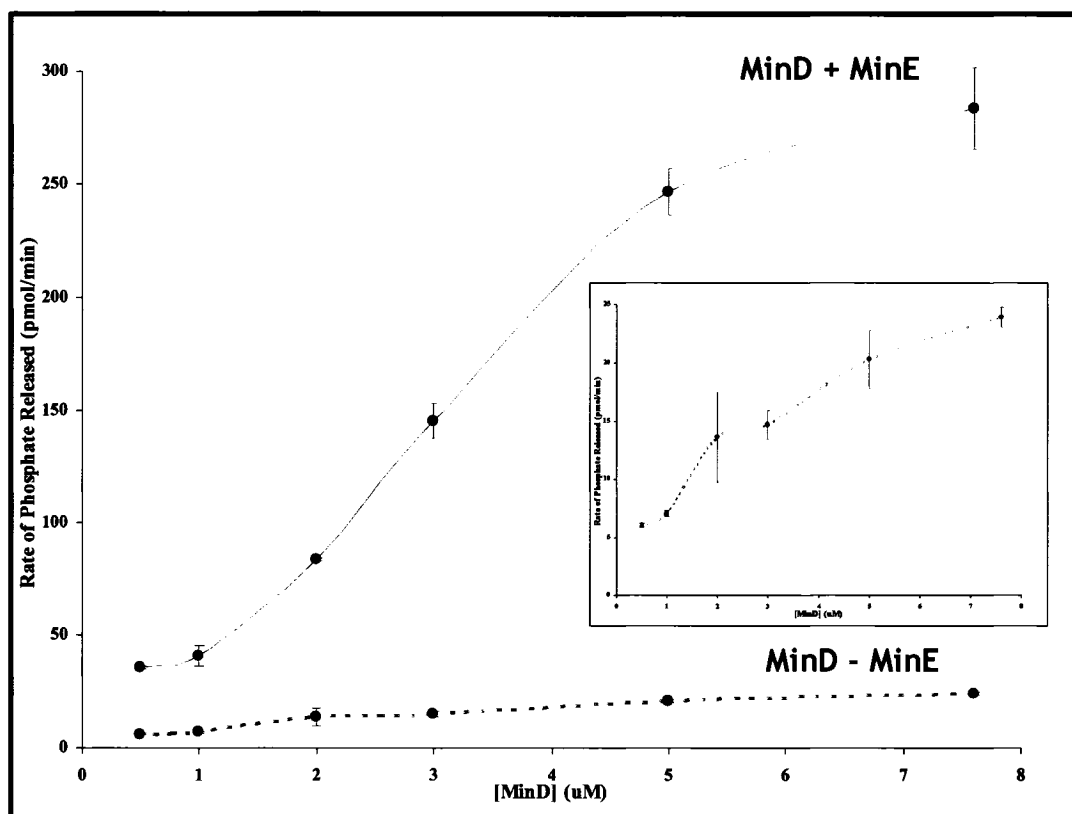
commonly known as the Hill coefficient (h) or an index of cooperativity provides a lower limit for the number of ligand-binding sites in each enzyme complex (Cornish-Bowden, 2004). It appears that at low substrate concentrations MinD behaves in a typical Henri-Michaelis-Menten fashion, while at higher concentrations there is evidence for cooperativity (Figure 25). Our results suggest that in the absence of MinE there are two independent binding sites, while in the presence of MinE there is a minimum of eight binding sites. In the presence of MinE there are more than two communicating active sites thereby providing strong evidence that MinD polymerization has occurred. Formation of higher-order cooperatively active oligomers of MinD suggests that MinD activity should be highly dependent on concentration.

3.1.8 *Specific activity of MinD_{Ng}*

To investigate the concentration dependence of MinD activity in the presence and absence of MinE, I measured the amount of Pi released at different MinD concentrations while keeping the concentration of all other required components (i.e. lipids, MgCl₂, ATP, and MinE) constant. As observed in the ATP concentration dependence study, linear correlations were observed between inorganic phosphate release and time, allowing the calculation of initial rate constants from the slope of these lines. For instance, the rates of ATP hydrolysis for wild type MinD_{Ng} at 0.5 μ M and 2.0 μ M protein concentrations were $\sim 36 \pm 6$ and $\sim 84 \pm 5$ pmol/minute respectively (Appendix; Figure 2A). Plots of the initial reaction rate as a function of protein concentration produced sigmoidal curves characteristic of positive cooperativity both in the presence and absence of MinE_{Ng} (Figure 26).

At the highest MinD_{Ng} concentration measured, the ATPase activity was found to be approximately 12-fold higher in the presence of MinE which is larger than the increase

Figure 26. Plot of the rate of ATP hydrolysis as a function of varying wild type MinD_{Ng} concentration in the presence and absence of MinE_{Ng}. All reactions were performed in the presence of total *E. coli* phospholipids (500 $\mu\text{g/ml}$), 10 mM MgCl_2 , and 1mM ATP in the presence and absence of MinE_{Ng} (10 μM). In the presence of MinE_{Ng} (red), half-maximal activation occurred at approximately 3.5 μM MinD_{Ng} while in the absence of MinE_{Ng} (blue) it occurred approximately at 2.5 μM MinD_{Ng}, as depicted in the inset. Lines connecting the points were drawn in for the purpose of visualization. All reactions were performed twice and the standard deviation in the initial rate of ATP hydrolysis at each MinD_{Ng} concentration calculated.



observed in the ATP concentration dependence studies. This may be due to the higher concentration of MinE that was used in this series of measurements, since at 10 μ M there was always a greater than 1:1 molar ratio of MinE:MinD present. In the ATP concentration series there was 3 μ M MinE present to give a molar ratio of 1:1. The larger degree of MinE-activation observed when MinE was present at higher molar ratios suggests that MinD stimulation will also be dependent on MinE concentration, a possibility to be investigated in the future.

3.1.9 Specific activity of *MinD_{Ng}* mutants

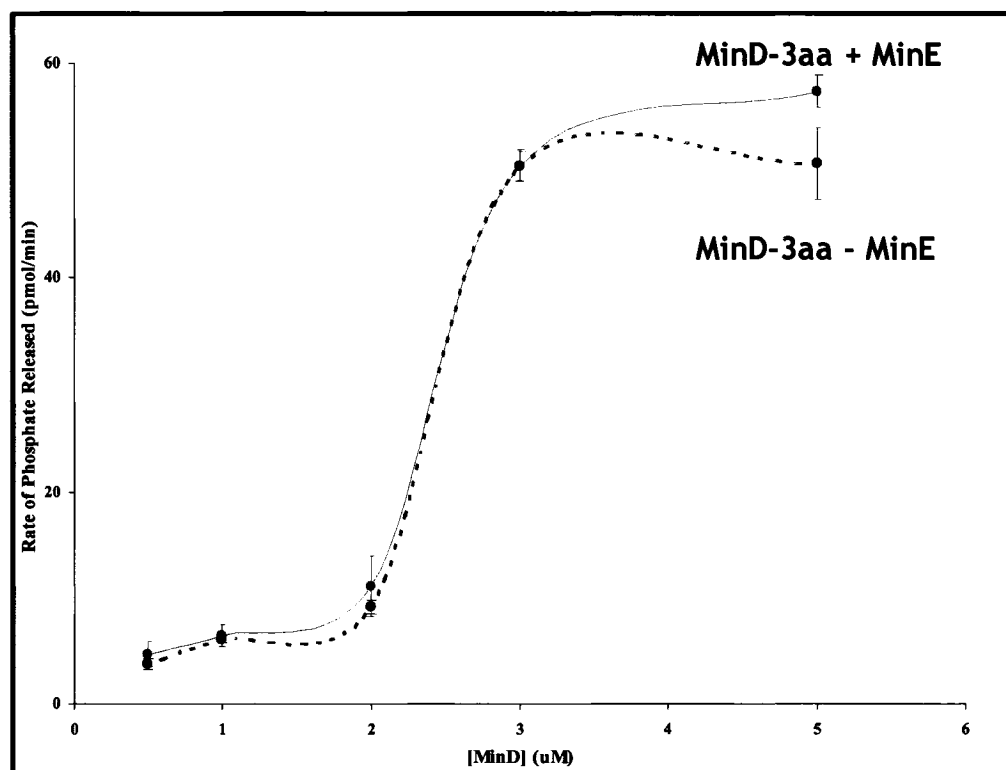
To investigate the effect of the MinD mutants on ATPase activity ATP hydrolysis rates were measured for MinD_{Ng-K16Q}, MinD_{Ng-loop}, and MinD_{Ng-3aaNT}. From these studies, I found that MinD_{Ng-K16Q} and MinD_{Ng-loop} had extremely low basal ATPase activity that was not significantly stimulated by MinE_{Ng} (Appendix; Figure 3). This was not surprising since MinD_{Ng-K16Q} vesicle sedimentation assays (Figure 22A) show that this mutant cannot bind phospholipid vesicles even in the presence of ATP. Since MinD ATPase activity requires membrane localization (Hu *et al.*, 2002; Lackner *et al.*, 2003), the lipid binding deficiency would be expected to prevent activation of MinD_{Ng}. In contrast, the ATPase activity of MinD_{Ng-loop}, could not be stimulated even though this mutant protein bound ATP as well as phospholipid vesicles (Figure 22B) suggesting a more direct role for the loop residues in ATP hydrolysis.

Similar to the results obtained with the two MinD mutants, the ATPase activity of MinD_{Ng-3aaNT} was not stimulated by MinE_{Ng}. However, unlike the other two mutant proteins, but similar to wild type, activity was found to be highly dependent on MinD concentration, with a sigmoidal rate dependence on MinD concentration. Half-maximal occurred at

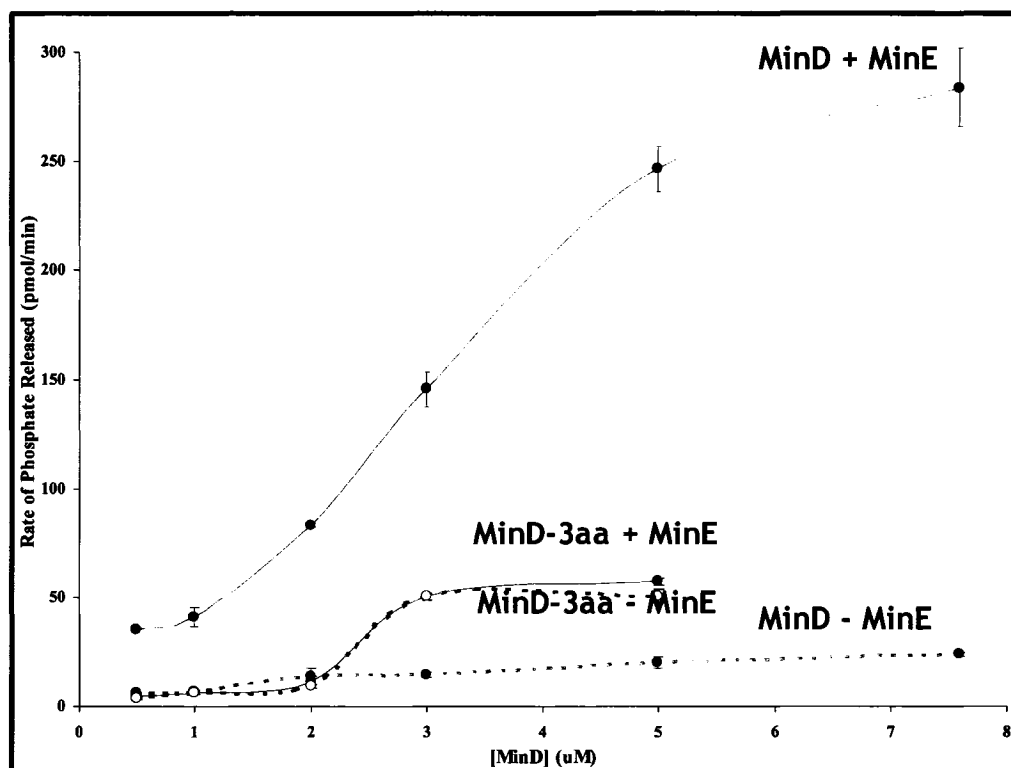
approximately 2.5 μ M MinD_{Ng-3aaNT} (Figure 27A) similar to that observed for wild type MinD in the absence of MinE. Surprisingly, the maximal activity obtained by this mutant in the absence of MinE was 2-3 times larger than the wild type activity (Figure 27B). However, in the presence of MinE_{Ng} the maximal rate and half-maximal activation for MinD_{Ng-3aaNT} did not change. This suggests that MinE is not binding to this mutant, which is consistent with the yeast two-hybrid data showing reduced MinE binding activity by this mutant. Thus, these results from the *in vitro* ATPase assay show an increased MinE independent ATPase activity for MinD_{Ng-3aaNT}.

Figure 27. Plot of the rate of ATP hydrolysis as a function of varying MinD_{Ng-3aaNT} concentration in the presence and absence of MinE_{Ng}. All reactions were performed as described for wild type. In the presence of MinE_{Ng} (red), half-maximal activation occurred at approximately 2.5 μ M MinD_{Ng} and in the absence of MinE_{Ng} (blue), half-maximal activation occurred at approximately 2.5 μ M MinD_{Ng-3aaNT}. (B) Comparison of MinD concentration dependence of ATP hydrolysis rates between wild type and MinD_{Ng-3aaNT} as a function of protein concentration shows a difference in both maximal and half maximal activity as well as basal ATPase activity. Curves are the same as those shown in Figure 26 and Figure 27A. Lines connecting the points were drawn in for the purpose of visualization. Reactions were performed at least twice and the standard deviation in the initial rate of ATP hydrolysis at each enzyme concentration calculated.

A



B



3.2 *Sample development for future NMR studies of MinD*

To obtain a high quality structure via NMR, sample concentrations of about 0.5 mM are typically required, with a sample volume of 0.4 to 0.6 ml. Since several different NMR spectra must be recorded over a three to six week period, each concentrated sample must be stable for weeks at room temperature with respect to aggregation, precipitation, and degradation (Clore and Gronenborn, 1998). Published results from structural proteomics consortia identify poor protein solubility as one of the main bottlenecks for structural studies (Yee *et al.*, 2002). In fact, estimates show that 33 to 50% of non-membrane proteins over-expressed in *E. coli* are not soluble, and approximately 25 to 57% of the remaining soluble proteins aggregate or precipitate during concentration (Christendat *et al.*, 2000; Yee *et al.*, 2002; Yee *et al.*, 2003). Thus, for structure determination ideal conditions for expression, purification, and structural analysis must be empirically determined to produce a concentrated and stable protein sample.

3.2.1 *Design of His-tagged constructs*

In order to produce a high-level protein expression system for MinD_{Ng} that could be easily isolated from native *E. coli* proteins, I initially designed plasmids encoding wild type MinD_{Ng} with either a C-terminal (pSC9) or N-terminal (pSA1) hexahistidine tag (Table 1; Figure 14A). Purification of these wild type constructs revealed that the C-terminal His-tag led to MinD_{Ng} samples of superior purity and yields compared to those of N-terminus His-tagged MinD. For this reason all future MinD expression constructs were made with C-terminal His-tags. Specifically, a His-tagged 15-amino acid C-terminal truncation mutant (pSA2) and a His-tagged full-length K16Q mutant (pSC10) were also made (Figure 14B). Since GFP studies as well as vesicle sedimentation assays (Figure 22A) had shown these

mutants to be deficient in membrane localization I wanted to test whether higher protein solubility could be achieved for structural study of these mutants. In fact, based on parallel large-scale protein purifications, the protein yield from MinD_{Ng-15aaCT-6XHis} (pSA2) was approximately 25% higher than that from MinD_{Ng-6XHis} (pSC9) (Figure 28) (Table 1).

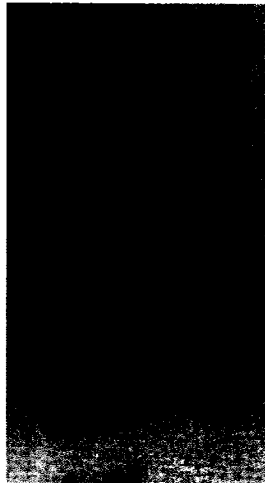
3.2.2 *Optimize expression levels of recombinant MinD and develop large-scale purification protocol*

Growth and induction conditions can have a significant impact on the expression levels of recombinant proteins in *E. coli*. Generally, non-disulfide containing recombinant globular proteins can be straightforwardly expressed in *E. coli* in a soluble form; however, at high expression levels these proteins can also aggregate and form insoluble inclusion bodies. Thus, to maximize the amount of expressed protein that localizes to the soluble fraction, the effects of overexpressing MinD in *E. coli* C41 were monitored by microscopy. Cells subject to increasing induction times with 2.0 mM IPTG display a gradual increase in filamentation, indicative of cell division inhibition due to increased overexpression of MinD_{Ng-6XHis} (Figure 29). Furthermore, soluble fractions monitored by SDS-PAGE revealed that varying IPTG concentrations did not have an obvious effect on MinD_{Ng-6XHis} expression levels (data not shown). Optimization of the purification procedure was also done to choose the most appropriate purification reagents and methods to maximize the yield and purity of MinD that could be obtained from a single chromatography step. Ultimately a purification protocol was developed such that a single round of nickel affinity using Ni-IDA resin could generate greater than 90% pure His-tagged MinD (Figure 30A; lane 8). Also, comparison of purification products from wild type and mutant MinD constructs showed that the C-terminal six-His tag could be generated with increased purity and yields compared to those N-terminal six-His tagged proteins (Figure 30B).

Figure 28. Comparative nickel affinity chromatography purification of MinD_{Ng-6XHis}, versus MinD_{Ng15aaCT-6XHis}. (A) MinD_{Ng-6XHis}: lane 1, 60 mM imidazole wash; lane 2, MinD_{Ng-6XHis}. (B) MinD_{Ng15aaCT-6XHis}: lane 1, 45 mM imidazole wash; lane 2, MinD_{Ng15aaCT-6XHis}. Based on parallel large-scale protein purifications performed for these two constructs, the protein yield from MinD_{Ng-15aaCT-6XHis} (~8 mg) was approximately 25% higher than that from MinD_{Ng-6XHis} (~6 mg).

A

1 2



B

1 2

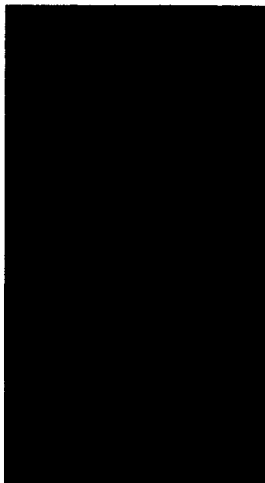


Figure 29. Effects of overexpressing MinD_{Ng-6XHis} in *E. coli* C41 (DE3). (A) Cells were transformed with MinD_{Ng-6XHis} and induced with 2.0 mM IPTG. Induced cells were fixed and visualized by phase contrast microscopy. (i) Uninduced cells show normal short rod morphology. Cells subjected to increasing incubation times after addition of IPTG (ii) 1 hour, (iii) 2 hours, (iv) 4 hours, and (v) 6 hours display a gradual increase in filamentation, indicative of cell division inhibition due to increased overexpression of MinD_{Ng-6XHis}. Scale bar in (i) represents 5 μ m and all figures are at the same magnification.

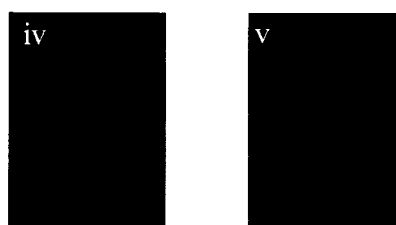
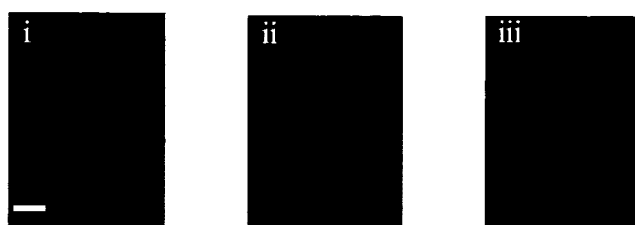
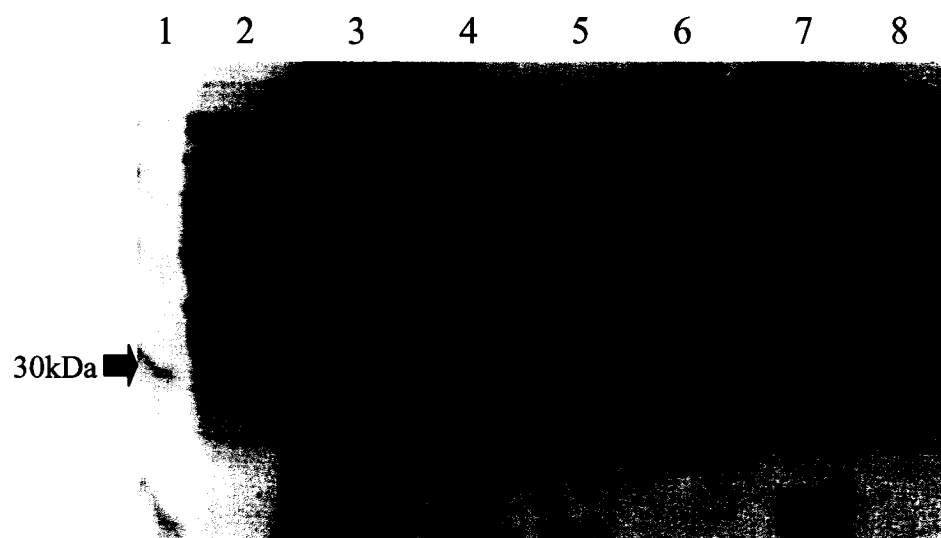
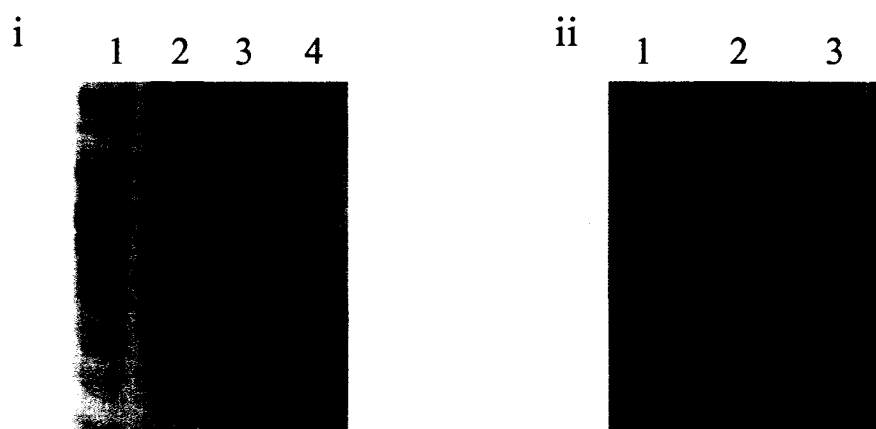


Figure 30. Wild type MinD_{Ng} purification. (A) Nickel affinity chromatography purification of the C-terminally His-tagged MinD_{Ng}-6XHis: lane 1, protein ladder; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, flow through; lane 5, 5 mM imidazole wash; lane 6, 30 mM imidazole wash; lane 7, 60 mM imidazole wash; lane 8, MinD_{Ng}-6XHis. (B) Comparative nickel affinity chromatography purification of 6XHis-MinD_{Ng} versus MinD_{Ng}-6XHis. (i) 6XHis-MinD_{Ng}: lane 1, 5 mM imidazole wash; lane 2, 30 mM imidazole wash; lane 3, 60 mM imidazole wash; lane 4, 6XHis-MinD_{Ng}. (ii) MinD_{Ng}-6XHis: lane 1, 5 mM imidazole wash; lane 2, 60 mM imidazole wash; lane 3, MinD_{Ng}-6XHis. In both cases the mobility of the major product of purification corresponds to a 30 kDa species as expected for these MinD proteins. Note that locating the hexahistidine tag at the C-terminus of MinD (MinD_{Ng}-6XHis) resulted in increased purity and higher protein yields than with a N-terminus tag (6XHis-MinD_{Ng}).

A



B



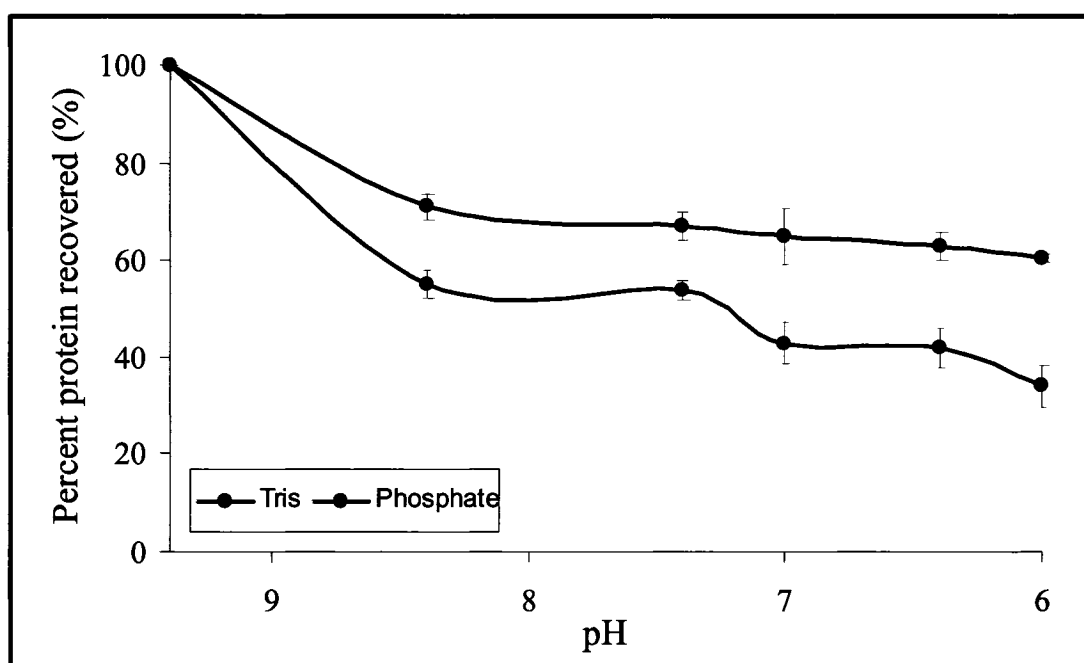
3.2.3 Investigation of the effect of sample conditions on MinD aggregation

Initially, the purified proteins were subjected to a single step dialysis in Tris-Cl and phosphate buffers at varying salt concentrations and pH. In order to assess the solubilization and stabilization capabilities of these buffers after dialysis, the concentration of the remaining soluble protein was measured and compared to the concentration prior to dialysis. The studies described hereafter were performed on MinD_{Ng-15aaCT-6XHis} (pSA2) since the protein yield from MinD_{Ng-15aaCT-6XHis} was approximately 25% higher than that from MinD_{Ng-6XHis} (pSC9) (Figure 28).

After overnight dialysis in 50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, and 10% glycerol (pH 7.4), 31% of MinD_{Ng-15aaCT-6XHis} was lost to aggregation. Altering the pH from a range of 9.4 to 6.0 had negligible effect on increasing protein solubility (Figure 31). In fact, for the analyzed pH range our data suggests that a decrease in pH leads to a decrease in MinD_{Ng-15aaCT-6XHis} solubility. Moreover, our results suggest that MinD is slightly more stable in Tris buffer as opposed to phosphate buffer since after overnight dialysis in 0.1 mM phosphate buffer (pH 7.4) approximately 46% of the protein was lost to precipitation (Figure 31). Ultimately, these studies suggest that MinD proteins have very low initial solubility in both Tris and phosphate based buffers.

Given that an overriding goal of these studies is the production of a MinD sample suitable for solution NMR studies, I was interested in maximizing the solubility in lower pH solutions. Under pH conditions of 7 – 5 exchange of backbone amide protons with solvent protons is reduced, allowing the observation of these structurally important protons in NMR spectra. Although our initial protein solubility studies suggested that acidic pH conditions promoted MinD aggregation, the hypothetical isoelectric point of MinD_{Ng} is ~5.9. Therefore, the

Figure 31. MinD_{Ng-15aaCT-6XHis} proteins have very low initial solubility in both Tris and phosphate buffers. The amount of MinD_{Ng-15aaCT-6XHis} recovered in the soluble fraction after overnight dialysis of a 0.1 mM solution of MinD_{Ng-15aaCT-6XHis} in Tris (blue) [50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, and 10% glycerol] and 0.1 mM phosphate (red) buffers at varying pH are assessed. Percent protein recovered refers to the proportion of MinD remaining in solution after dialysis as determined by the Bradford method of protein concentration determination. In both cases, altering the pH from a range of 9.4 to 6.0 had small effect on protein solubility with a decrease in pH decreasing MinD_{Ng-15aaCT-6XHis} solubility. Lines connecting the points were drawn in for the purpose of visualization. The standard deviation in percent protein recovered was calculated from two separate dialysis experiments.



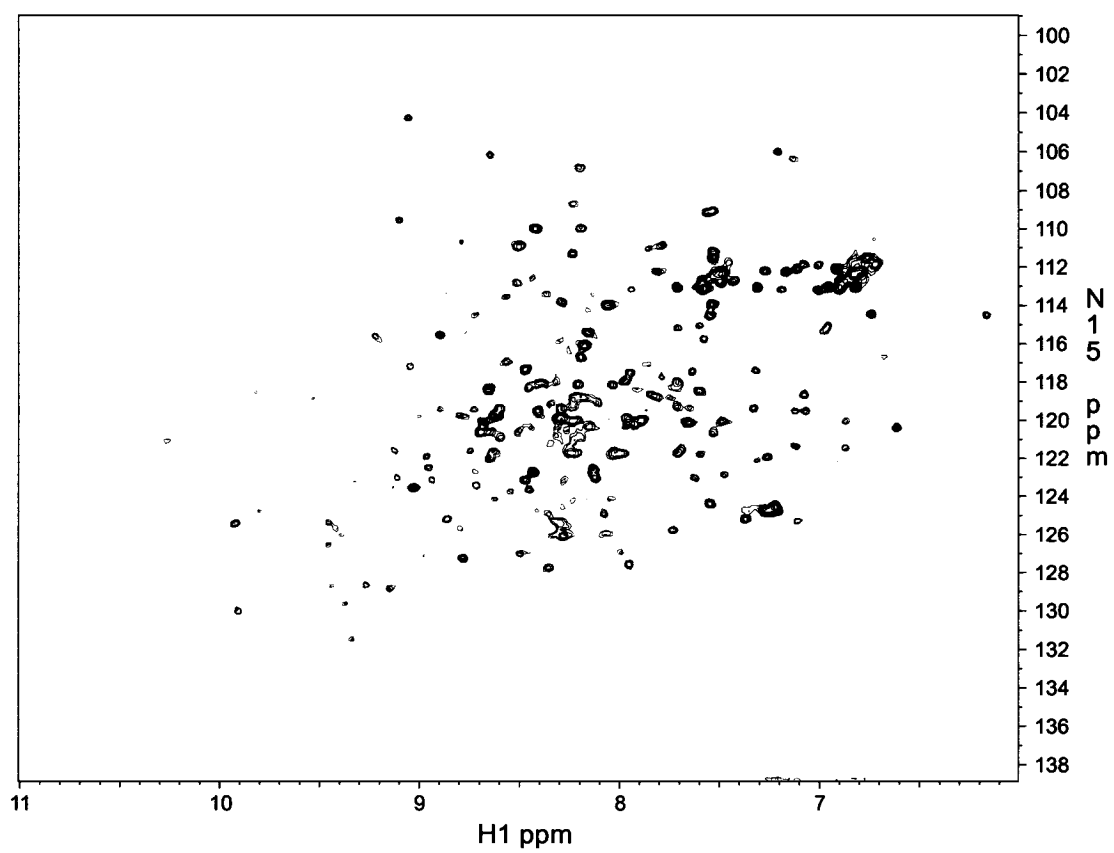
previous dialysis experiments may have exposed the sample to pH conditions equal to the isoelectric point for prolonged time periods. Protein solubility is usually lowest at the isoelectric point due to cancellation of positive and negative charges, creating a neutral species more prone to self-association. Thus, to reduce the tendency for aggregation and precipitation, solubility was investigated using the rapid pH drop method. In this approach the samples were first diluted 2X with ddH₂O to reduce the possibility of aggregation. This was then followed by a rapid introduction of 200 mM acetate pH 5.0 with 200 mM (NH₄)₂SO₄. At this stage, based on qualitative analysis, there was no visible precipitation. Since NMR also benefits from the use of solutions with lower salt concentrations, the sample was also subjected to a second night of overnight dialysis against 20 mM acetate pH 5.0, 50 mM (NH₄)₂SO₄, 1 mM EDTA, 1% glycerol, and 10 mM Arg and then concentrated to the desired volume. Based on this alternative method, I was able to obtain a ~3.0 mg/ml, ¹⁵N labeled MinD_{Ng-15aaCT-6XHis} protein sample. This is a significant improvement from the previously attainable ~0.3 mg/ml ¹⁵N labeled MinD_{Ng-15aaCT-6XHis} sample, considering I am now approaching concentrations that allow me to examine the protein by solution NMR.

3.2.4 Heteronuclear Single Quantum Correlation (1H-15N HSQC) spectra of

MinD_{Ng-15aaCT-6XHis}

Based on the above rapid pH drop protocol, I was able to obtain a 0.1 mM concentration which should be observable by solution NMR. For this purpose a ¹⁵N labeled MinD_{Ng-15aaCT-6XHis} was prepared and a 1H-15N HSQC spectrum was recorded at 500 MHz at 25°C. As shown in Figure 32 signal to noise was extremely low, as expected from the low protein concentration used. However, only 80% of the expected 255 peaks could be observed, suggesting that a larger number of peaks should be observable once higher MinD protein

Figure 32. 1H – 15N HSQC of MinD_{Ng15aaCT-6XHis}. A ¹⁵N labeled 0.1 mM MinD_{Ng-15aaCT-6XHis} protein sample dialysed against 20 mM acetate pH 5.0, 50 mM (NH₄)₂SO₄, 1 mM EDTA, 1% glycerol, and 10 mM was prepared, D₂O added to 10% and a 1H-15N HSQC spectrum was recorded at 500 MHz at 25°C. The chemical shift dispersion suggests that the protein is folded, however, the signal to noise is extremely low, as expected from the low protein concentration used. Approximately 80% of the expected 255 peaks are present, suggesting that a larger number of peaks should be observable once higher MinD protein concentrations are attained.



concentrations are attained. In addition, the range of proton chemical shifts is relatively large suggesting that MinD is folded under these conditions. Overall these preliminary investigations into MinD solubility and NMR feasibility suggest that further optimization of sample conditions should produce NMR spectra of quality sufficient for high resolution structure determination.

4. DISCUSSION

MinD plays a role in determining midcell localization and division patterns in Gram-negative bacteria of varying morphologies, including the coccus *Neisseria gonorrhoeae* which divides in alternating perpendicular planes and the bacillus *Escherichia coli* that divides in parallel planes (Margolin, 2001; Ramirez-Arcos *et al.*, 2001; Szeto *et al.*, 2001). Previously, our laboratory reported that gonococcal MinD could complement an *E. coli minD* mutant and induce cell division arrest in wild type *E. coli* (Szeto *et al.*, 2001). Thus, our laboratory was able to develop a heterologous model for studying gonococcal Min proteins. Furthermore the ability of gonococcal MinD to influence cell division in gonococci, as well as in *E. coli*, is dependent upon its interactions with the other two Min proteins (Szeto *et al.*, 2001; Ramirez-Arcos *et al.*, 2002), MinC and MinD.

Several studies from our laboratory have identified the functional importance of different regions of MinD (Ramirez-Arcos *et al.*, 2002; Szeto *et al.*, 2002; Szeto *et al.*, 2004; Szeto *et al.*, 2005). I and others in the laboratory determined that the conserved N-terminus is involved in dynamic localization and enzymatic activity (Szeto *et al.*, 2004). In addition, we have identified a polar putative loop region of MinD implicated in its proper topological specificity (Szeto *et al.*, 2005). Other reports have highlighted the importance of the conserved C-terminus of MinD in membrane targeting (Szeto *et al.*, 2002; Hu *et al.*, 2003). However, the molecular basis for the altered phenotypes that result from MinD mutations made in these regions are not yet known. For example, MinD mutants that show altered dynamic localization could be deficient in interactions with ATP, the membrane, MinE and/or MinD itself. Therefore additional studies that could help to distinguish between these

possibilities are required to gain insight into the functional role of these regions in MinD oscillation.

An additional facet of MinD activity that remains to be investigated is the possibility that conformational changes in MinD structure might regulate Min protein function (Lutkenhaus, 2001; Szeto *et al.*, 2005; Zhou *et al.*, 2005). However, the nature and the effect of these hypothesized conformational changes on neighboring domains and on ATP hydrolysis have yet to be deciphered. One method that can be used to produce insight into conformational changes in MinD at the atomic level is solution NMR spectroscopy. Therefore, in this investigation, I developed high-level expression systems and purification protocols for NMR sample production. Generation of samples suitable for NMR analyses have the potential to answer questions regarding the conformational changes involved in MinD activation and would also allow the identification of the dimerization interface of bacterial MinD. To complement the proposed structural studies, I have also characterized the interaction between MinD, phospholipids, ATP, and MinE through kinetic studies and vesicle sedimentation assays.

4.1 Loop residues required for ATPase activity and hence protein oscillation

Based on homology modeling and protein sequence alignments, I and others in the laboratory identified a highly conserved polar region of MinD_{Ng} (R92, D93, and K94) that we predicted to lie in a flexible solvent-exposed loop in the structure. A simultaneous substitution of these polar residues (R92L, D93L, and K94I) revealed the importance of this region in both oscillation and topological specificity (Szeto *et al.*, 2005). Similar to wild type MinD, overexpression of MinD_{Ng-loop} was able to induce cell filamentation indicating that the protein retained functionality as a cell division inhibitor upon interaction with MinC; as predicted by

its phenotype the protein retained its ability to self-associate, and to interact with MinC. Surprisingly, despite also showing a significant interaction with MinE, which dissociates MinC from the MinCD complex, the mutant protein no longer oscillated from pole to pole. In fact, fluorescence microscopy of GFP-tagged MinD_{Ng-loop} showed that this mutant localized to the cell periphery in a spiral pattern suggesting assembly along the entire internal membrane bound scaffold, including the midcell location. Thus, in contrast to the behavior of wild type MinD, the mutant protein failed to localize to the cell poles in an oscillatory fashion.

Numerous studies suggest that MinE catalyses the dissociation of MinD from the membrane by stimulating its ATPase activity (Hu and Lutkenhaus, 2001; Lackner *et al.*, 2003; Shih *et al.*, 2003). However, recent work from our laboratory suggests that for this to be possible, the N-terminus of MinE must bind MinD thereby facilitating the recruitment of the latter protein to the coiled array (Eng *et al.*, 2005; in press). This study also provided evidence that once MinD is associated with the coiled array, basal ATPase activity may be sufficient for oscillation. Specifically, it was found that MinE_{Ng-E67L} was able to bind MinD_{Ng}, but very weakly, such that MinD ATPase activity was not stimulated under conditions sufficient for stimulation by wild type MinE_{Ng}. Although results from this *in vitro* study would suggest that MinE_{Ng} does not stimulate MinD_{Ng} ATPase activity *in vivo*, oscillation of MinD_{Ng} was nonetheless still maintained (Eng *et al.*, 2005; in press). However, the rate of oscillation was significantly reduced relative to that of wild type, suggesting that the strength of the interaction between MinD and MinE may be correlated to the extent of ATPase stimulation, and hence MinD oscillation. Based on this data we have proposed that

the period of oscillation is dependent on the binding strength between MinD and MinE (Eng *et al.*, 2005; in press).

Given that the subcellular coiled arrays observed *in vivo* require both MinD and MinE to form, and that GFP-MinD_{Ng-loop} retained its ability to localize to the coil-like structures, it is unlikely that mutations within this selected region affected the interaction between MinD and the N-terminus of MinE. Moreover, the lack of oscillation is probably not due to weaker binding interactions with the mutant MinD protein since the yeast two-hybrid data actually detected a stronger self-association of MinD_{Ng-loop} compared to wild type MinD_{Ng}. Interactions between MinD and MinE were also substantiated in our lipid vesicle binding studies that show membrane localization of MinE in the presence of MinD. Hence, my data suggests that the ability of MinD to bind MinE and localize to a membrane-associated helical scaffold is not sufficient for Min protein dynamism. Rather other MinD functionalities involving the loop region are also required for oscillation.

In our homology model of dimeric MinD, loop residues 92-94 correspond to α -4 (helix 4) in each archaeal MinD monomer. Interestingly, the switch I and II sites commonly present in most nucleotide binding proteins and implicated in protein conformational changes during nucleotide hydrolysis (Vale, 1996) are in close proximity to our designated 'loop' region in the model. In fact, in the *Pyrococcus* crystal structure, the switch I region (residues 40-46) interacts with α -4. Since our drastic mutation replaces a charged group of amino acids with a cluster of hydrophobic ones, it may be that the local environment around switch I was altered in such a manner that a conformational response to MinE binding was not possible. Alternatively, it is possible that residues in the 'loop' region may influence mobility and/or

function of one or both of the switch regions, since it is not uncommon for regions external to switch motifs in P-type ATPases to contribute to activity (Kull and Endow, 2002).

4.2 *MinD N-terminus is required for protein function*

Previously our laboratory showed that a 4 amino acid (MinD_{Ng-4aaNT}) deletion from the N-terminus of MinD is sufficient to abrogate MinD function (Szeto *et al.*, 2001) while a 2 amino acid (MinD_{Ng-2aaNT}) truncation could still oscillate in an *E. coli* background, was able to induce cell filamentation, and retained its ability to dimerize and interact with wild type MinD_{Ng}, MinE_{Ng}, or MinC_{Ng} (Szeto *et al.*, 2004). This led us to propose that the conserved N-terminus region of MinD_{Ng}, although far from the active site, may be involved in regulating MinD ATPase activity, and hence overall MinD function. To test this idea, I constructed a 3-amino acid (MinD_{Ng-3aaNT}) truncation and investigated its functional properties.

In contrast to wild type, a truncation of three amino acids at the N-terminus rendered MinD_{Ng} unable to induce cell filamentation in *E. coli*; hence, the activity of the protein was abrogated. Despite maintaining some interaction with MinC_{Ec}, (10% of wild type signal in β -galactosidase assay) the truncated protein's interactions with itself, wild type MinD_{Ng}, and MinE_{Ng} were significantly compromised (0.3 – 1.9 % of wild type signal) according to yeast two-hybrid studies. While it initially appeared that GFP-MinD_{Ng-3aaNT} localization was almost entirely cytoplasmic, enhancement of raw images revealed that the fusion protein retained some membrane affinity. Interestingly, its oscillation cycles were significantly faster (~30% reduction in average oscillation time) relative to wild type MinD_{Ng} (Szeto *et al.*, 2004). This data may also explain the minicell phenotype observed when this mutant was overexpressed in *E. coli*. One possibility is that the mutant MinD is binding the native MinC, but has a

reduced tendency to bind the membrane due to its reduced ability to dimerize. This would not only lead to accumulation of GFP-MinD_{Ng-3aaNT} in the cytoplasm, but would also sequester MinC away from the native MinD that is also being expressed.

Studies have shown that the extreme C-terminal region of *E. coli* MinD, which contains a highly conserved 8- to 12- residue sequence motif (shared with MinD_{Ng} and other homologues), is essential for membrane localization of the protein (Szeto *et al.*, 2002; Hu *et al.*, 2003); however, our mutant MinD_{Ng-3aaNT} protein has an intact C-terminus and yet, its ability to localize to the membrane *in vivo* was decreased. The “Dimer Trigger Model” of Hu *et al.*, (2003) proposes that, upon binding ATP, MinD dimerizes and undergoes some conformational change that allows specific C-terminal residues to interact with membrane layers. If MinD cannot dimerize then it may have a reduced ability to undergo the conformational change required to bind the membrane. The K16Q Walker A motif mutation of MinD (Hu *et al.*, 2002; Ramirez-Arcos *et al.*, 2002) is another example of a mutation found outside of the C-terminus that results in a loss of membrane affinity. In the case of K16Q, it is known that ATP binding is compromised, preventing dimerization and hence, membrane binding. Similar mutants have also been identified in *B. subtilis* MinD, where mutations to putative ATP-binding residues and residues involved in nucleotide hydrolysis render the protein incapable of localization to the inner membrane (Karoui and Errington, 2001). It is possible that, similar to the proposal for a K16Q mutation (Hu *et al.*, 2003), MinD_{Ng-3aaNT} may be insensitive to ATP-induced conformation changes that are a prerequisite for membrane affinity. In addition, the significant decrease in self-interaction of MinD_{Ng-3aaNT} could be a consequence of reduced ATP-binding, which would undoubtedly result in a corresponding decrease in membrane affinity. Ultimately, these biological studies

suggest that the first three amino acids of MinD may be essential to obtain proper protein configuration that allows MinD protein-protein and/or MinD-phospholipid interactions that are necessary for proper localization.

4.3 *Assembly of MinD on phospholipid vesicles promoted by ATP and Mg^{+2}*

Previous studies have shown that MinD_{Ec} assembles into short, paired filaments in the presence of ATP, and the addition of phospholipid vesicles stimulates self-assembly into longer and thicker bundles at the membrane (Suefuji *et al.*, 2002). The present results are consistent with a similar behavior for gonococcal MinD as I demonstrate that MinD_{Ng}-ATP binding is coupled to membrane binding (Figure 21). Since MinD_{Ng} could only bind phospholipids membranes in the presence of ATP, it is possible that the monomeric form prevents MTS interactions with the membrane through intramolecular interactions between MinD and its own MTS as suggested by the 'dimer trigger' model (Lutkenhaus and Sundaramoorthy, 2003; Zhou and Lutkenhaus, 2003). Further support for this theory is based on the observation that in the presence of ADP, the association of MinD with phospholipids was not observed (Figure 21A). This suggests that the inability of ADP to promote membrane binding may be due to a lack of dimerization, which is prerequisite for the conformational changes that ultimately expose or activate the MinD MTS such that the amphiphatic helix is available for interaction with the lipid bilayer.

In our vesicle sedimentation studies, I obtained *in vitro* evidence suggesting that in the presence of ATP, MinE is recruited to MinD decorated vesicles. In addition, this is the first study to show that in the absence of MinD_{Ng}, MinE_{Ng} does not co-sediment with phospholipid vesicles (Figure 21). Although it has yet to be conclusively established, this data would be consistent with the model where MinE associates with MinD only after MinD

binds the lipid membrane. Since similar experimental conditions have given rise to well-ordered helical polymers in MinD_{Ec} as observed by electron microscopy (Hu *et al.*, 2002), it is possible that similar structures are also being formed in our assay. In addition, I observed that MinE decreases the fraction of membrane-bound MinD, suggesting that MinE_{Ng} catalyses the dissociation of MinD_{Ng} from the membrane, by stimulation of MinD ATPase activity.

4.4 *MinD K16Q is deficient in binding to phospholipid vesicles*

The importance of MinD dimerization for membrane affinity was reiterated with MinD_{Ng-K16Q}. A K16Q mutation in the Walker A motif alters the ATP binding pocket of MinD and inactivates protein function. While an *E. coli* MinD_{K16Q} mutant binds ATP as strongly as wild type protein, it is speculated that this mutant protein is unable to adopt the conformational changes in response to ATP binding (Hu *et al.*, 2002). Since our MinD_{Ng-K16Q} was unable to bind to the membrane, it is possible that MinD_{K16Q} mutants are deficient in conformational changes that allows for proper dimerization, which is a prerequisite for membrane binding and stimulation by MinE. Size exclusion chromatography on purified MinD_{Ng-K16Q} and yeast two-hybrid studies from our laboratory confirmed that this mutant has a reduced ability to self-associate (Szeto *et al.*, 2005). Thus, this mutant has abrogated activity due to an inability to dimerize and subsequently bind the lipid membrane.

In contrast to MinD_{Ng-K16Q}, the MinD_{Ng-loop} protein underwent an ATP-dependent assembly on phospholipid vesicles; however, MinE could not reduce the amount of MinD_{Ng-loop} associated with phospholipid vesicles. It is not likely that the stronger self-association of MinD_{Ng-loop} relative to wild type as assessed by the yeast two hybrid system could have inhibited the disassembly of MinD_{Ng-loop} subunits, since Szeto *et al.*, (2005) characterized

other MinD mutants, such as MinD_{Ng-E103L}, that exhibited higher dimerization affinity, but were still capable of being stimulated by MinE. Therefore the inability of the MinD_{Ng-loop} mutant to oscillate in *E. coli* was not due to a deficiency in ATP-dependent membrane association, but may instead result from the inability of MinE to stimulate its release.

4.5 *MinE stimulates MinD ATPase activity in the presence of phospholipid vesicles*

In vitro studies suggest that the MinE-induced ATPase activity of MinD is the rate-limiting step for Min protein oscillation (Hu and Lutkenhaus, 2002; Suefuji *et al.*, 2002). Moreover, MinD oscillation and ATPase activity are intimately linked since a decrease in ATPase stimulation to 50 – 75% of wild type levels results in a 2-fold increase in the period of oscillation (Hu and Lutkenhaus, 2001). Changes in the intracellular MinD/MinE ratio and mutations within MinE have been shown to affect the MinD oscillation frequency (Raskin and de Boer, 1999A). I observed that at physiological concentrations of wild type MinD_{Ng}, in the presence of MinE_{Ng}, ATPase activity is stimulated by more than a factor of ten relative to that in the absence of MinE_{Ng}, similar to observations made with MinD_{Ec}. Also, *in vivo* studies showed that in the absence of MinE_{Ng}, GFP-MinD_{Ng} localizes along the entire inner cell periphery, and there is no evidence of oscillation or organization into the helical scaffold (Szeto *et al.*, 2004). However, in the presence of MinE_{Ng}, the membrane localization of MinD_{Ng} is dynamic as it promotes localization to the subcellular coiled array and oscillates between the two cell halves (Szeto *et al.*, 2004). Therefore, interactions between MinD and MinE are not only critical for the recruitment of MinD_{Ng} to the coiled array but also in stimulating the subsequent period of oscillation.

These initial kinetic observations suggested that activity of MinD is regulated by MinE but did not provide any insight into how this was occurring. For example, does MinE

binding lead to an increase in the affinity of the MinD active site for the substrate ATP? If this were the case, then it is possible that at higher concentrations of ATP, MinD activity in the presence and absence of MinE would be the same. Alternatively, MinE binding may improve the catalytic ability of MinD, which would lead to significant activation by MinE at any ATP concentration. Hence, in order to resolve this issue and gain additional insight into the mechanism by which MinE stimulates MinD ATPase activity, I examined the rate of MinD activity as a function of substrate concentration.

4.6 *MinD_{Ng} displays positive cooperativity with respect to ATP*

As shown in Figure 24, the ATPase activity of MinD displayed positive cooperative behavior, consistent with the finding that it dimerizes (Szeto *et al.*, 2001) and forms higher-order complexes (Hu and Lutkenhaus, 2001; Lackner *et al.*, 2003) in the presence of ATP. Our results suggest that, with increasing substrate concentration, the turnover rate of ATP by MinD increases both in the presence and absence of MinE. The sigmoidal shape with respect to ATP indicates that MinD does not follow typical Henri-Michaelis-Menten kinetics characteristic of single-site enzymes. Instead our results indicate that the binding of ATP at one site of a MinD complex increases the affinity of one or more additional binding sites in the same complex. Since it is known that ATP binding promotes MinD dimerization, it is likely that MinD-ATP dimer forms cooperatively which manifests as cooperativity in ATP hydrolysis. In addition, the association of MinD with membrane may involve multiple types of oligomerization states (i.e., dimer and various polymer), which would lead to additional contributions to the observed cooperative behavior of MinD. Interestingly, the sigmoidal shape of the curve was preserved even in the absence of MinE_{Ng}. This implies that positive cooperativity is not dependent on MinE, but is rather an inherent property of MinD ATPase

activity. Also, the finding that half-maximal activity occurs at approximately 1.5 mM both in the presence and absence of MinE suggests that MinE_{Ng} binding does not affect MinD affinity for ATP. In other words, the significant increase in activation observed in the presence of MinE is not due to an increased affinity of ATP but rather an increase in the rate of ATP hydrolysis.

Results from our *in vitro* ATPase assays demonstrated that the maximal rate of MinD activity in the presence of MinE is approximately 6-fold higher than in the absence of MinE. Since the half-maximal ATP concentration was not affected it appears that MinE binding appears to increase the catalytic activity but not the substrate affinity. In other words, MinE seems to change the properties of MinD such that it can hydrolyze ATP more efficiently. Although it is not yet possible to determine the exact mechanisms by which MinE achieves this, it is possible that MinE binding induces conformational changes in the active site environment which could alter the chemical properties of the active site residues.

The activation of MinD ATPase activity by MinE cannot be understood unless we first understand the mechanism of ATP hydrolysis by MinD. In the MinD-AMPPCP crystal structure, Hayashi *et al.*, (2001) observed a water molecule, which is aligned for an in-line nucleophilic attack on the γ -phosphate. Interestingly, the water molecule occupies the same position as the γ -phosphate in the MinD-ADP complex and is hydrogen bonded to the carboxyl group of Asp40 and the main chain amide proton of Ala121, residues that fall within the switch I and switch II regions, respectively. In the well-characterized NIP structure, Asp from the other subunit within the dimeric protein functions as a general base for ATP hydrolysis by activating the attacking water molecule while Lys from the Walker A motif stabilizes the leaving group (Schindelin *et al.*, 1997). Thus for ATP hydrolysis it

appears that one subunit from the dimeric protein interacts with the nucleotide while the partner subunit stabilizes the nucleotide intermediate (Schindelin *et al.*, 1997). An identical role for these residues in MinD ATP hydrolysis is likely considering the high conservation of Walker motifs in MinD homologues as well as other P loop proteins (Leipe *et al.*, 2002).

In MinD, the Walker A motif that is responsible for direct interactions with phosphate groups of bound ATP contains a lysine residue that forms critical salt bridge and hydrogen bonding interactions with residues in the well-conserved helix 7 (Hayashi *et al.*, 2001). Also, this α -helix was identified as important for MinE interactions (Hayashi *et al.*, 2001; Ma *et al.*, 2004; Zhou *et al.*, 2005). More specifically, D152 in the *E. coli* MinD was shown to be required for interactions with a 31-residue peptide from the MinE N-terminus. Disruption of these interactions by mutagenesis of helix 7 residues facing the Walker A motif in *E. coli* MinD eliminates MinE stimulation of ATPase activity (Zhou *et al.*, 2005), suggesting that this helix either forms part of the MinE interaction surface and/or couples MinE binding to enhanced ATP hydrolysis. Hence, it is possible that the effects of MinE binding are transmitted from helix 7 to the Walker A motif residues which, together with the other conserved loops known as the switch I and switch II, form a cavity in which the substrate ATP cozily sits (Sakai *et al.*, 2001). Changes in the α 7-Walker A interaction could disrupt interactions between the Walker A motif and the two conserved switch loops leading to changes in the charge and/or shape of the active site that could affect the reactivity. For example, it may be that conformational changes induced by the binding of MinE to helix 7 could stabilize the hydrogen bonding interactions between the water molecule and the carboxyl group of Asp40 in switch I and main chain imino group of Ala121 in switch II to facilitate the ionization of bound water into a nucleophilic hydroxyl ion.

Another factor that needs to be considered for cooperative systems is that differences in the sigmoidal curves may also reflect changes in the degree of cooperativity. Since the minimum number of potential substrate binding sites on the enzyme complex and the degree of cooperativity among them can be quantified by h , the Hill coefficient (Copeland, 2000), I analyzed the rate data in terms of the Hill plot (Figure 25). Interestingly, our results show that at low substrate concentrations there is no evidence of cooperativity, while at higher concentrations cooperativity exists. One could argue that the non-cooperative behavior ($h \sim 1$) observed at low substrate concentrations is simply due to the fact that the Hill equation is at best an approximation and regardless of the enzyme and its substrate one should expect the observations to tend towards a slope of one at the extremes (Cornish-Bowden, 1995). Having said that, it is possible that the absence of cooperativity at low substrate concentrations can be attributed to the low concentration of the MinD-ATP complex which could be too low for MinD polymer formation. In support of this, Hu *et al.*, (2002) showed that the ability of MinD to form packed helical arrays on the surface of phospholipid vesicles is dependent on MinD concentration and displays a critical concentration around 3 μM , which is similar to the estimated *in vivo* concentration (de Boer *et al.*, 1991).

According to the Hill analyses, in the absence of MinE it would appear that there are at least two non-equivalent active sites in the MinD complex, suggesting that, in the absence of MinE, the minimal functional MinD oligomer is dimeric. In support of this, Mileykovskaya *et al.*, (2003) obtained Hill coefficients of about 2 for MinD binding to liposomes in the presence of ATP, suggesting that MinD-ATP oligomerizes at least to the level of dimers on the liposome surface. In contrast, our data suggests that in the presence of MinE, the oligomeric enzyme has a minimum of eight binding sites ($h \sim 8$). This increase in

cooperativity upon MinE stimulation suggests that the nature of interactions between MinD is changed by the presence of MinE, potentially indicating an increase in the polymerization state. However, it cannot be assumed that the Hill coefficient is a straightforward indicator of the number of actual binding sites in a complex, since it is an index of cooperativity, with the degree of cooperativity being considered to increase as h increases (Cornish-Bowden, 1995). Since it is common for oligomers to have two or more additional binding sites with weaker inter-site cooperativity, it would be incorrect to state that the oligomeric enzyme has exactly eight substrate-binding sites.

In aggregate, our results provide new insight into the activation of MinD ATPase activity by MinE. Specifically, I have obtained evidence that MinE promotes the formation of MinD polymers with an extensive network of catalytic sites that positively regulate each other. In the absence of MinE, cooperativity is greatly reduced, with the dimer being the potential basic unit of MinD catalysis. These results are consistent with *in vivo* fluorescence imaging demonstrating that functional MinE is required for formation of MinD coiled arrays (Shih *et al.*, 2003, Eng *et al.*, 2005; in press). *In vitro* studies have revealed that MinE_{Ec} promotes the bundling of MinD_{Ec} filaments under certain conditions, and it was recently suggested that MinE might act as a cross-linker for polymerized MinD (Suefuji *et al.*, 2002). Since MinD filaments can form *in vitro* in the absence of MinE, it is possible that the increase in cooperativity may be due to functional coupling between MinD fibers in the bundled polymer. The concept of protein bundling being critical for function is not unique. For instance, Koppelman *et al.*, (2004) characterized a FtsZ mutant whose altered bundling *in vivo* abrogated the protein's ability to initiate cell division.

4.7 *Oligomeric state of MinD has higher activity*

The sigmoidal curves observed with respect to saturating concentration of ATP support the fact that MinD forms dimers and higher ordered structures. Since oligomerization is favored by higher concentrations of MinD, I investigated whether the oligomeric state of MinD has higher activity. Consistent with the ATP concentration dependence studies, increasing protein concentration – both in the presence and absence of MinE_{Ng} – produced sigmoidal curves characteristic of positive cooperativity, indicating self-interaction of MinD. Moreover, this cooperative behavior indicates that two or more active sites are functionally linked in a way that is favored by increasing MinD concentrations. Given that MinD is known to form a dimer in the presence of ATP (Hu *et al.*, 2003; Szeto *et al.*, 2005), and that higher order structures may also be formed *in vitro* (Hu *et al.*, 2002; Suefuji *et al.*, 2002; Shih *et al.*, 2003), the observed cooperative behavior of the MinD ATPase indicates that MinD oligomerization strongly favors ATPase activity. This concentration dependence of catalytic activity in cases of saturating amounts of ATP and lipids suggests that self-association may further activate the enzyme. These results correlate closely with a study on the *E. coli* MinD enzyme activity by Hu and Lutkenhaus (2001). Additional support for our argument stems from the general belief that MinD is active as a dimer, but not as a monomer (Szeto *et al.*, 2002; Hu *et al.*, 2003). While I acknowledge the dimer is more active, I am not convinced that any of these studies demonstrate that the monomer is strictly inactive. In support of this, in the present study, as well as in Szeto *et al.*, (2004), I showed that the monomeric mutant (MinD_{Ng-K16Q}) has greatly reduced activity, however the reduction may be due to the inability to bind its substrate. Having said this, it is tempting to argue that the extent of filamentation is proportional with the tendency to hydrolyze ATP and be released from the membrane;

thus, providing a potential mechanism by which the length of cooperatively acting MinD filaments can be regulated by MinE. According to this hypothesis, longer filaments promote greater ATPase activity, leading to an increased tendency for MinD to be released from the lipid membrane and hence the MinD polymer.

In the presence of MinE half-maximal activation was found to occur at approximately 3.5 μM MinD_{Ng}, while in the absence of MinE_{Ng} it occurred approximately at 2.5 μM MinD_{Ng}. This small difference in half-maximal MinD concentrations potentially reflects a difference in the nature of the MinD oligomer that might be forming in the presence and absence of MinE. For instance, the affinities could be reflecting two different types of equilibrium systems i.e. the equilibrium is monomer-dimer in the absence of MinE and monomer-polymer in the presence of MinE.

It is interesting to note that at the highest MinD_{Ng} concentration assayed, the rate of ATPase activity was approximately 15-fold higher than that in the absence of MinE_{Ng} which is considerably larger than the extent of activation observed in our ATP dependence concentration studies. Comparison of the ATP- and MinD-dependent rate profiles provides evidence that the MinD:MinE ratio also modulates activity. Specifically the activity of 3 μM MinD with 1 mM ATP with 3 μM MinE (Figure 24) was significantly lower than that for the equivalent reaction run with 10 μM MinE (Figure 26). This difference in activation can be attributed to differences in the MinE:MinD molar ratio. In fact, it has been shown for MinE_{Ec} that MinD ATPase activity increases cooperatively with increasing MinE concentration (Suefuji *et al.*, 2002). This indicates that the rate profiles will also depend on the molar ratio of MinD and MinE, providing another mechanism by which MinD activity, filament growth and MinD oscillation can be modulated. In fact, an increased MinE:MinD ratio has been

shown to decrease the period of oscillation, while a decreased ratio has been shown to extend the oscillatory cycle (Raskin and de Boer, 1999B). Since there is evidence that the period of oscillation is linked to the rate of ATP hydrolysis by MinD (Raskin and de Boer, 1999B), a decrease in the MinE:MinD molar ratio should decrease ATPase activity leading to a slower oscillatory cycle. The corollary to this is that an increased MinE:MinD molar ratio would increase the ATPase activity thereby speeding oscillation. Studies on our 'loop' mutant substantiate this idea since the inability of MinE_{Ng} to effectively stimulate the *in vitro* ATPase activity of MinD_{Ng-loop} accounts for the absence of clearly distinguishable oscillations of GFP-MinD_{Ng} in spite of the fact that MinE is binding membrane-bound MinD (Szeto *et al.*, 2005).

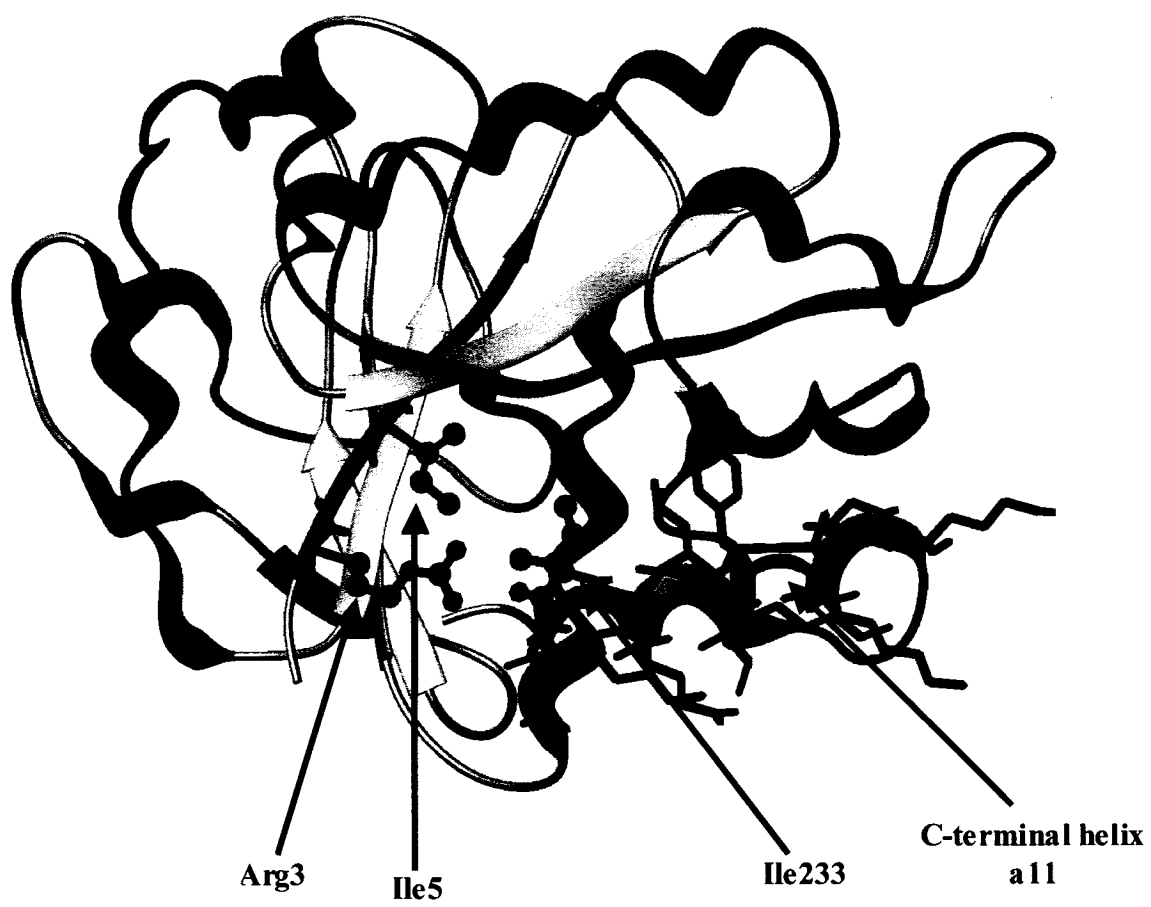
4.8 Regions of MinD important for MinE stimulation of ATPase activity

Results with our MinD_{Ng-loop} mutant (R92L/D93L/K94I), showed that although dimerization, MinE and MinC binding and membrane localization were all retained, this mutant had decreased basal ATPase activity that could not be stimulated by MinE binding (Szeto *et al.*, 2004). While this provides insight into the failure of this mutant to oscillate *in vivo*, a new question is raised by these results: Given that the loop is not predicted to be part of the enzyme active site, why can't MinD_{Ng-loop} efficiently catalyze ATP hydrolysis, and, why does MinE binding fail to stimulate its activity? As aforementioned it is possible that the drastic mutation could have altered the local environment around switch I such that a response to MinE binding was not possible. This is not difficult to fathom considering that mutating amino acid residues 92-94 significantly reduced affinity for MinC raising the possibility that this region is proximal to switch I. Structural studies of this mutant should help to elucidate what role this part of the protein may play in MinE stimulation of ATP hydrolysis.

Another region of MinD that is important for MinE stimulation of ATPase activity is the N-terminus, since I found that the 3-aa N-terminal truncation mutant possesses basal ATPase activity that is more than 2-fold greater than that of the wild type basal activity that was not stimulated by MinE binding. Moreover, I found that MinD_{Ng-3aaNT} had reduced interactions with MinE_{Ng} as determined by yeast two-hybrid studies, but still exhibited faster oscillation than wild type MinD_{Ng} (Szeto *et al.*, 2004).

It would be interesting to determine how a mutation made to a region of MinD that is not part of the catalytic or MinE-binding site could exhibit a higher basal rate of ATPase activity. However, examination of x-ray structures of MinD homologues provides some insight into the potential affect of a 3 amino acid truncation at the N-terminus. Specifically, interactions between the N-terminal residues and the C-terminal helix ($\alpha 11$) appear to be occurring that should normally stabilize the protein fold. Although in all X-ray crystal structures the $\alpha 11$ C-terminus appears to be relatively mobile, all C-terminal structures contain a conserved hydrophobic residue (I232 in Af, I233 in Pf/Ph) that is within van der Waals contact with the highly conserved amino terminal residue I5. Acidic amino acids flanking this residue are also oriented towards the N-terminus, potentially providing charge complementarity to the conserved N-terminal R3 residue. This complimentary pattern of hydrophobic and charged residues at N- and C- termini is conserved in bacterial sequences suggesting that this type of interaction also occurs in *E. coli* and *N. gonorrhoeae* MinD proteins. Deletion of the first 3 residues would be predicted to remove this interaction and destabilize the protein (Figure 33). In fact, I do observe lower solubility for this mutant during purification relative to wild type MinD possibly due to the destabilizing phenomenon. Since I would expect non-local effects to arise from this destabilization, it is possible that the

Figure 33. Ribbon diagram of archaeal MinD_{Ph} (PDB# 1ION) (Sakai *et al.*, 2001) highlighting N-terminal residues that may interact with the C-terminal helix (α 11). All C-terminal Archeal structures contain a conserved hydrophobic residue (I232 in Af, I233 in Pf/Ph) that is within van der Waals contact with the highly conserved amino terminal residue I5. In addition, we propose that acidic amino acids flanking I233 provide charge complementarity to the conserved N-terminal R3 residue since they are oriented towards the N-terminus. This suggests that interactions between the two termini could be critical in stabilizing the protein fold.



increased basal activity results from the disruption of an inhibitory interaction in the MinD active site, that may normally be disrupted by MinE binding. This proposal is in accordance with previous studies on MinD_{Ec} that suggest an inhibitory interaction between the MinD helix 7 and the P-loop that is alleviated by MinE binding. Nevertheless it is not yet possible to conclusively identify the mechanism of MinE activation of MinD activity with the current available data. However, it should be possible to test this hypothesis by generation of destabilizing mutations made to residues at the end of the C-terminal helix which should have a similar phenotype.

In order to establish whether the effect of a 3-aa truncation is to influence cooperativity and/or maximal activity of MinD, I measured its activity over a range of MinD concentrations, as I had done for wild type MinD_{Ng}. The activity was highly dependent on MinD concentration, with a sigmoidal rate dependence on MinD concentration suggesting that cooperativity is maintained. I propose that the N-terminus of MinD is involved in regulating the enzymatic activity of this protein since the maximal activity of MinD_{Ng-3aaNT} is approximately 5-fold lower. In support of this there are several examples of ATPases in which the N-terminus is involved in regulating enzymatic activity (Fernandez *et al.*, 1995). For instance, sequential deletion of as few as 3 amino acid residues from the epsilon subunit of the chloroplast ATP synthase resulted in significant increases in the ATPase activity of the holoenzyme (Shi *et al.*, 2001).

Although MinD_{Ng3aa-NT} exhibited higher basal activity, I observed that MinE_{Ng} did not further stimulate its ATPase activity. The most straightforward and simple explanation for this is the disrupted or reduced interaction with MinE, consistent with our yeast two-hybrid data (Szeto *et al.*, 2004). In fact, we recently observed that the extent of MinD_{Ng} ATPase

stimulation depends on the binding strength between MinD_{Ng} and the C-terminus of MinE_{Ng}. Since binding affinities are proportional to relative populations of bound and free states of a protein, an increase in MinE binding affinity means that the population of MinE-bound MinD will increase and therefore the relative amount of MinD in the MinE-stimulated state will increase. More specifically, our laboratory has showed that MinE_{Ng} does not stimulate MinD_{Ng} ATPase activity unless the interaction between the two exceeds a certain binding threshold (Eng *et al.*, 2005; in press). If the lack of stimulation is due to reduced MinE binding, then further work including ATPase assays with increasing MinE concentrations could help to determine whether MinE can stimulate MinD ATPase activity at higher concentrations. In addition, it would be interesting to perform ATP dependent studies on this mutant as it could identify a difference in the degree of cooperativity in the presence and absence of MinE_{Ng}.

4.9 Preparation for NMR studies of MinD

Our laboratory is interested in elucidating the conformational changes that MinD undergoes during the various stages of its functional cycle. Given the unique nature of this protein and its importance for normal cell viability our long-term goal is to design compounds that can inhibit its action. However, since no structural information on the MinD dimer is available, it is not yet known what residues are involved in forming the binding interface. Thus, for the rational development of molecules that can interfere with normal cell division in pathogenic bacteria a molecular description of interactions involving Min proteins and the structural basis underlying their function is required.

To produce a high-level protein expression system for MinD_{Ng}, I designed plasmids encoding wild type MinD with either an N-terminal or C-terminal hexahistidine tag. I

observed that a hexahistidine tag fused to the C- terminus of MinD_{Ng} results in increased purity and protein yields. It is possible that the high solubility of the poly-His tag could counter balance the tendency of the hydrophobic residues to aggregate. In terms of increased purity the archaeal MinD monomer crystal structures provide a plausible explanation. Based on the position of the amino terminus, it is possible that the hexahistidine tag might be too close to in sequence to folded, buried N-terminus of MinD, thus, reducing the efficiency and strength of coordination between the histidine residues and the immobilized Ni⁺² ions. In contrast, the C-terminal region is thought to be structurally disordered since it was not resolved in the archael X-ray crystal structure (Hayashi *et al.*, 2001), which would undoubtedly make the hexahistidine tag more readily accessible. Placement of the His-tag onto a disordered C-terminus should improve its accessibility, enabling a stronger interaction with the nickel resin relative to the N-terminal hexahistidine tag.

Based on parallel large-scale purifications, I note that the protein yield from our C-terminal truncation (MinD_{Ng15aaCT-6XHis}) construct was approximately 25% higher relative to wild type. The fact that MinD gains membrane affinity through its activated C-terminal membrane targeting sequence (Szeto *et al.*, 2002; Hu and Lutkenhaus, 2003), offers a possible explanation. Our 15 amino acid C-terminal truncation mutant is unable to bind to the membrane (Szeto *et al.*, 2002), thereby allowing us to achieve a higher amount in the soluble fraction upon lysis.

I have been able to heterologously express MinD_{Ng} with a C-terminal His tag in minimal media at high yield in *E. coli*. A single round of nickel affinity chromatography is sufficient to generate His-tagged MinD that is > 90% pure. From one litre of minimal media, I obtained ~ 40 mg of His-tagged MinD. In the absence of nucleotide, these samples can be

concentrated to ~0.15 mM at neutral pH. Based on the aforementioned rapid pH drop method, I have increased sample concentrations approximately 10-fold. This is a significant accomplishment considering I am now approaching concentrations that allow us to examine the protein by solution NMR. At this point, on a ^1H - ^{15}N HSQC spectra, I am detecting approximately 80% of the expected 255 peaks.

Due to the molecular mass of MinD_{Ng} (~29.6 kDa), after optimization of sample conditions, it will be necessary to prepare ^2H , ^{15}N , and ^{13}C labeled samples to reduce the number of available spin relaxation pathways that degrade NMR signal sensitivity and resolution in slowly tumbling proteins. Using a cryoprobe-equipped spectrometer, transverse-relaxation optimized backbone assignment experiments can be performed. Once assigned, secondary backbone chemical shifts can be calculated to determine the secondary structure and compared with the known X-ray structures. Ultimately this will pave the pathway for elucidating the structure of the proposed dimerization interface and the complex conformational changes that regulate Min protein function.

5. CONCLUSIONS

5.1 *Min protein oscillation*

The dynamic oscillation of MinD involves a membrane association-dissociation cycle that is dependent on MinD ATPase and MinE. I show that in the presence of ATP and Mg^{+2} , MinD associates with phospholipid vesicles and forms the horseshoe like structure in one half of the cell. The association of MinD with the membrane appears to be cooperative and is dependent on MinD concentration. The present results confirm this suggestion since I show that (i) the binding of ATP at one site of MinD complex increases the affinity of one or more additional binding sites in the same complex; and (ii) cytoplasmic MinD has two binding sites while the membrane bound oligomeric MinD appears to have a minimum of eight binding sides.

The shortening of the horseshoe like structure and its eventual reappearance in the other half of the cell is initiated by MinE stimulation of MinD ATPase. It appears that MinD-ATP dimers that form cooperatively manifest as cooperativity in ATP hydrolysis. While MinE is not involved in the observed cooperative behaviour it appears to increase the catalytic activity of MinD ATPase; thereby, regulating and promoting the release of membrane bound MinD and its subsequent oscillation and reassembly at the opposite cell pole.

5.2 *Contributions of this research*

Based on a combination of qualitative and quantitative *in vitro* assays, my research has provided insight into the mechanism by which nucleotide binding and hydrolysis control the localization of MinD, and how MinE can promote higher reaction rates in MinD. A novel finding, my results suggest that $MinD_{Ng}$ undergoes a conformational change upon $MinE_{Ng}$ binding that increases the catalytic activity but not the substrate concentration at half maximal activity. Results from lipid binding and functional assays revealed that the

recruitment of MinD_{Ng} to the membrane requires Mg²⁺ and ATP. This is the first study to show that in the absence of MinD_{Ng}, MinE_{Ng} does not co-sediment with phospholipid vesicles. In addition, concurrent research with mutant MinD_{Ng} proteins established that dimerization is critical for membrane localization, and interactions between MinD and MinE alone are not sufficient for MinE_{Ng} to effectively stimulate the *in vitro* ATPase activity of MinD_{Ng}. As well, these biochemical studies highlight the importance of the N-terminus of MinD_{Ng} in regulating MinD protein-protein and/or MinD-phospholipid interactions that are necessary for proper localization.

My research into the mechanism of MinE_{Ng} stimulation of MinD_{Ng} ATPase activity is the first study to show that MinD displays positive substrate cooperativity with respect to ATP. Moreover, my data is the first to suggest that MinE may be an allosteric regulator of MinD, and acts to increase the degree of cooperativity in MinD activity. Since MinD forms polymeric filaments that change in morphology in the presence of MinE and ATP, my results suggest that the change in filament structure could promote greater functional cooperativity in MinD. One possibility is that longer filaments have a higher tendency to hydrolyze ATP and be released from the membrane, providing an additional mechanism by which the length of cooperatively acting MinD filaments can be regulated by MinE.

An additional objective of this thesis was to develop a system for the production of samples suitable for analysis by solution NMR such that structural studies on MinD could be initiated. After optimization of purification and sample conditions a 10-fold improvement in the maximum concentration of MinD that can be maintained in solution was obtained. I was the first to prepare a ¹⁵N labeled MinD_{Ng-15aaCT-6XHis} sample, yielding a NMR spectrum where 80% of the expected 255 peaks could be observed.

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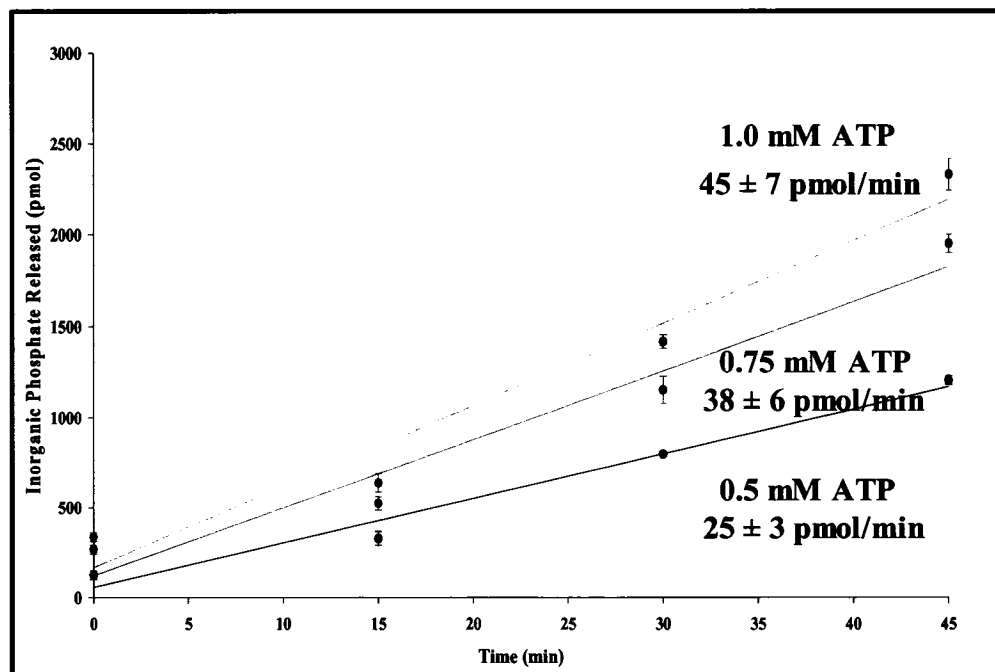
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APPENDIX

Figure 1. Plot of the amount of inorganic phosphate released by wild type MinD_{Ng} at varying ATP concentrations in the presence (A) and in the absence (B) of 3 μ M MinE_{Ng}.

The slope of inorganic phosphate released as a function of time at varying ATP concentrations gives a measure of the initial reaction rate of ATP hydrolysis of wild type MinD. MinD_{Ng} (3 μ M) was incubated with total *E. coli* phospholipids (500 μ g/ml), 10 mM MgCl₂, and varying ATP concentrations. To avoid clustering, graphs are drawn in for 3 representative substrate concentrations. The initial rate of ATP hydrolysis was determined by linear regression utilizing the method of least squares. All reactions were performed at least twice for each time point tested and the standard deviation in the amount of inorganic phosphate released at each time point calculated.

A



B

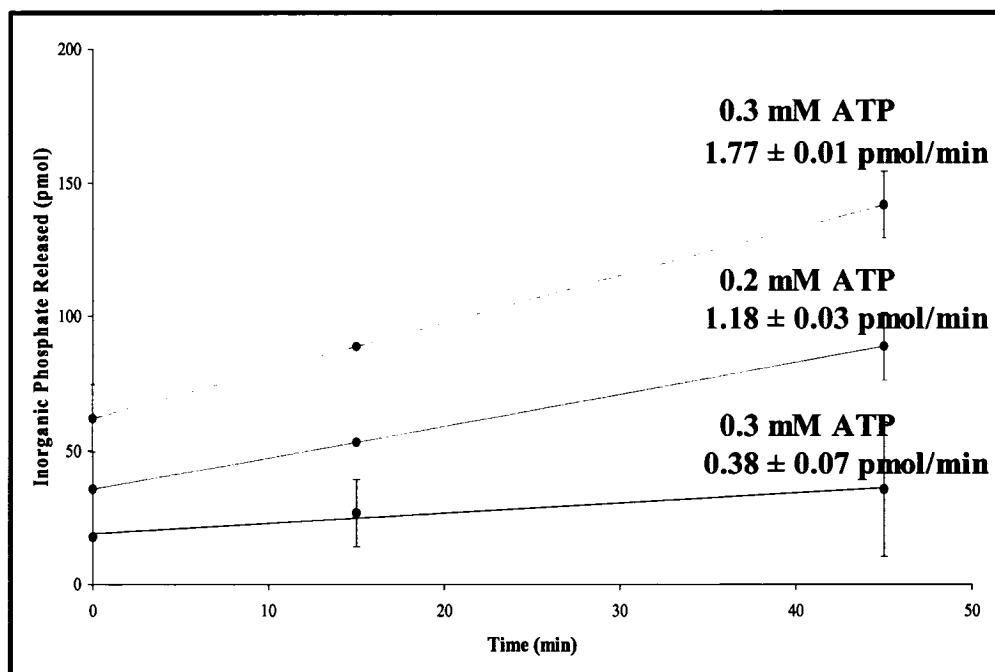
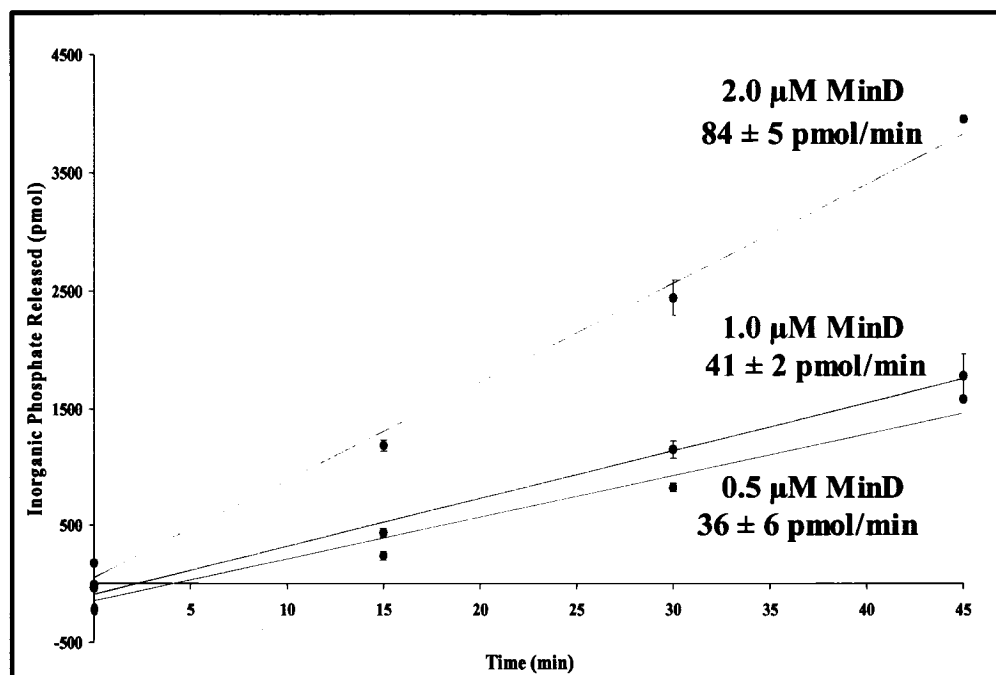


Figure 2. Plot of the amount of inorganic phosphate released as a function of varying wild type MinD_{Ng} concentration in the presence (A) and in the absence (B) of 10 μ M MinE_{Ng}. To avoid clustering, graphs are drawn in for 3 representative substrate concentrations. The initial rate of ATP hydrolysis was determined by linear regression utilizing the method of least squares. All reactions were performed at least twice for each time point tested and the standard deviation in the amount of inorganic phosphate released at each time point calculated.

A



B

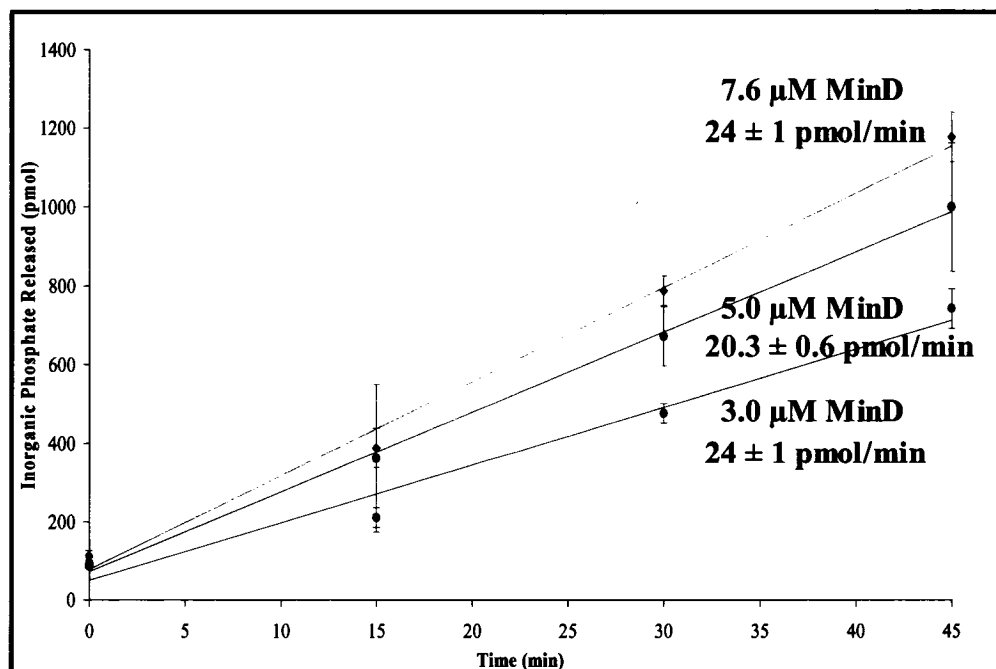
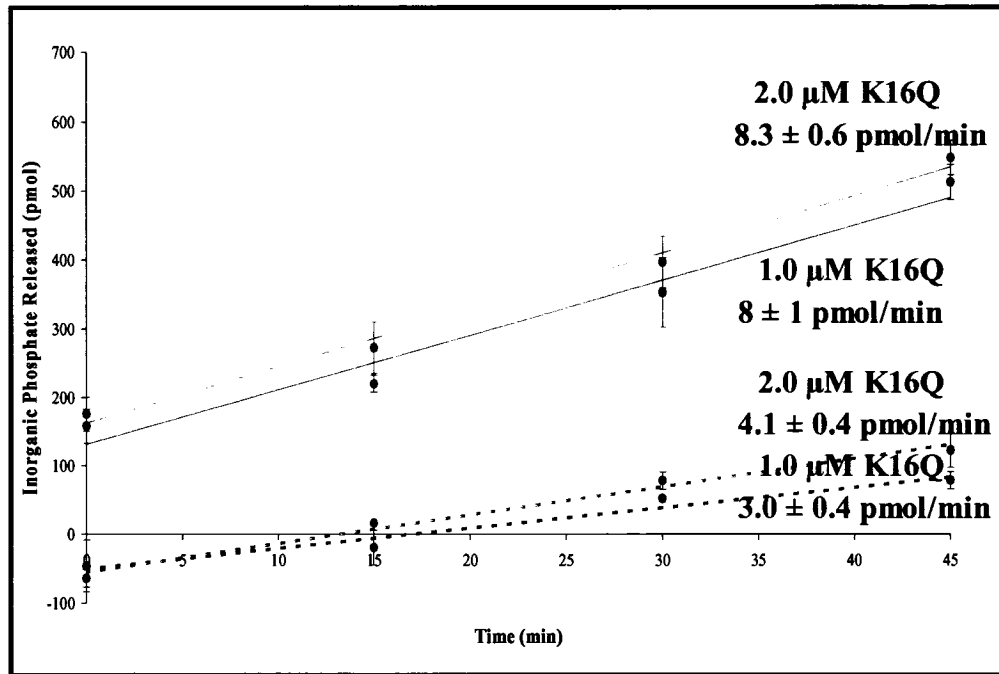
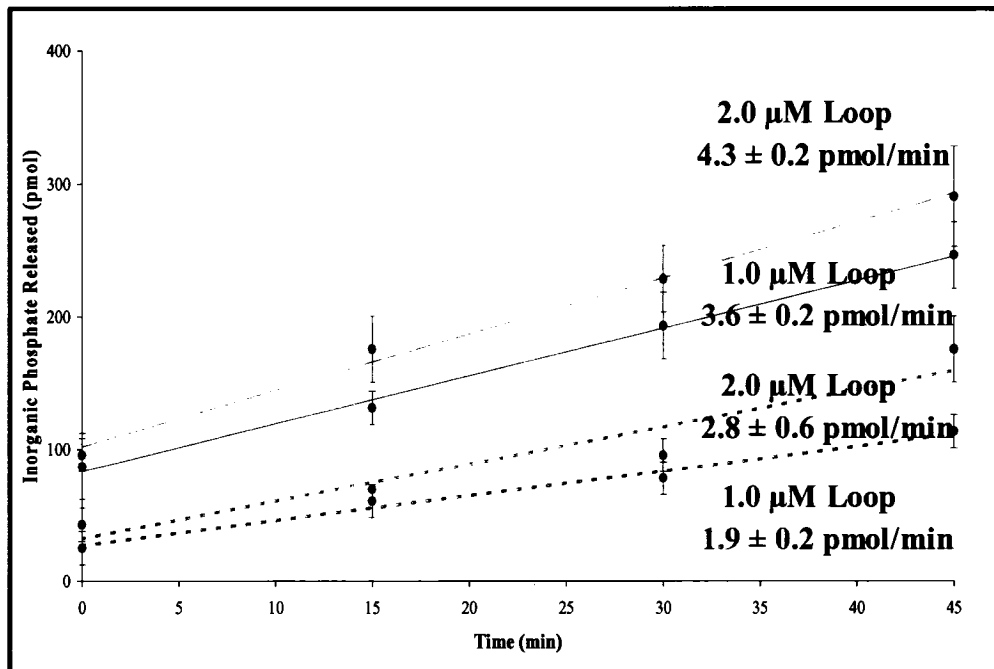


Figure 3. Plot of the amount of inorganic phosphate released as a function of MinD_{Ng-K16Q} and MinD_{Ng-loop} in the presence and absence of MinE_{Ng}. (A) ATPase activity of MinD_{Ng-K16Q} in the presence (solid lines) and absence (broken lines) of MinE_{Ng}. (B) ATPase activity of MinD_{Ng-loop} in the presence (solid line) and absence (broken line) of MinE_{Ng}. Note that in contrast to wild type MinD_{Ng}, the addition of MinE_{Ng} did not cause a significant increase in ATP hydrolysis. To avoid clustering, graphs are drawn in for 2 representative substrate concentrations. The initial rate of ATP hydrolysis was determined by linear regression utilizing the method of least squares. All reactions were performed at least twice for each time point tested and the standard deviation in the amount of inorganic phosphate released at each time point calculated.

A



B



Sudeep Acharya
36 Rodeo Drive
Nepean, Ontario
K2J 4Z5
Phone: (W) 613-562-5800 Ext. 6560
(H) 613-823-8468
Email: sacha017@uottawa.ca

EDUCATION

- 2003-Present* M.Sc. Biochemistry
Department of Biochemistry, Microbiology and Immunology
University of Ottawa, Ottawa, Ontario
Thesis title: Structural and functional analysis of the cell division site determinant MinD_{Ng}.
- 1999-2003* B.Sc.Honours in Biopharmaceuticals
Department of Chemistry
University of Ottawa, Ottawa, Ontario
Thesis Title: Analysis of the N-terminal domain of the *Neisseria goorrhoeae* cell division site determinant MinD: To divide or not to divide.

GRADUATE COURSES

- BCH 8101 Physical and Chemical Methods in Biochemistry (Completed A+)
BCH 8102 Biochemistry of Lipids and Membranes (Completed A+)
BCH 8212 Seminar (Completed)
BCH 8213 Seminar (progress)
BCH 7999 THESIS RESEARCH (PROGRESS)

HONOURS AND DISTINCTION

- University of Ottawa Summer Research Award (2003)
University of Ottawa Merit Scholarship (2002-2003)

University of Ottawa Biopharmaceutical Director's Scholarship (2001-2002)

University of Ottawa Entrance Scholarship (1999)

Governor General's Bronze Medal recipient (1999)

UNIVERSITY ACTIVITIES

05/2003 – 08/2003 Research Associate for Dr. Jo-Anne R. Dillon
Department of Biochemistry, Microbiology and Immunology
University of Ottawa

09/2002 – 05/2003 Honours student for Dr. Jo-Anne R. Dillon
Department of Biochemistry, Microbiology and Immunology
University of Ottawa

PUBLICATIONS

Eng, N.F., Szeto, J., **Acharya, S.**, Tessier, D., and Dillon, J.R. (2005). Characterization of gonococcal MinE shows two domains critical in bacterial cytokinesis. In press. Res Microbiol. April 2005.

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