

ABSTRACT

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COVER SHEET

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Identification of Two Binding Partners for the Bacterial Actin Homolog MreB

Paul Buske, CALS Senior Honors Thesis

Abstract:

A distinguishing characteristic of prokaryotes was long thought to be the lack of a cytoskeleton similar to eukaryotes consisting of actin filaments, microtubules, and intermediate filaments. However, recent work has shown the existence of bacterial proteins homologous to the eukaryotic cytoskeleton. One such protein, MreB (murein cluster e), encircles the bacterial cell and exhibits structural homology to actin. Because of the homology between MreB and actin and the many interacting proteins with which actin has in its various cellular functions, we hypothesized MreB also interacts with many proteins in the bacterial cell. Preliminary interaction tests using light scattering fluorometry and co-sedimentation assays show interactions between purified *E. coli* MreB and the His-tagged proteins NusG and EF-Tu, with work still to be done on identification of other interacting partners and the biochemical nature of these interactions.

Introduction:

Understanding the mechanisms and different molecules involved in cell division, motility, and structure holds key information in the study of life. Essential to the organization and structure of eukaryotic cells, the cytoskeleton consists of a dynamic network of actin filaments, microtubules (polymers of tubulin), and intermediate filaments. These proteins are vital regulators and essential coordinators of many eukaryotic cellular processes, including cell shape determination (morphogenesis), division, segregation, polarity, phagocytosis, movement, and macromolecular trafficking. Biochemical characterization of the polymerization of actin has led to the discovery of the forces behind cell motility (Loisel et al., 1999). Research done on the proteins responsible for cell division has led to great advances in fighting cancer and other diseases that involve uncontrollable cell division. Knowledge about the proteins involved in cell growth and division in bacterial cells can also lead to new insight and development of antibiotics to combat disease-causing bacteria, such as those responsible for cholera, meningitis and tuberculosis.

Viewed by many as only belonging to eukaryotic cells, cytoskeletal proteins were extensively studied for decades. Prokaryotes like the bacterium *E. coli*, it was believed, only

needed cell walls made of peptidoglycan to maintain their shape (Doolittle and York, 2002). For decades, microscopic and biochemical studies failed to detect cytoskeletal elements in bacteria (Carballido-Lopez, 2006). However, it has now been shown that structural and functional homologues of tubulin (FtsZ) and actin (MreB) exist in many eubacteria and some archaea (van den Ent et al., 2001). A homologue for the intermediate filaments was also identified as crescentin in *Caulobacter crescentus* (Ausmees et al., 2003). Amino acid sequence similarity between these proteins and their eukaryotic counterparts is extremely low (e.g. only ~15% between MreB and actin), explaining their recent discovery (Bork et al., 1992). However, nearly all of the homology between MreB and actin lays in nearly identical ATP-hydrolyzing domains, an indication that they are evolutionary related (Doolittle and York, 2002). Despite low sequence identity, the solved crystal structure of MreB from *Thermotoga maritima* shows it nearly superimposable with actin (van den Ent et al., 2001).

Actin is one of the most abundant proteins in eukaryotic cells, present in its 43 kDa monomeric form (G-actin). Upon addition of salt and ATP, actin monomers polymerize into linear filaments (F-actin) *in vitro* (Korn et al., 1987). Spontaneous assembly of actin monomers into filaments is unfavorable due to instability in actin dimers and trimers, but once small oligomers of actin form, filaments grow rapidly (Pollard and Borisy, 2003). The filaments polymerize noncovalently from both ends, with each end having different affinities for monomers. The resulting filaments develop an intrinsic polarity in the form of a slow-growing end (pointed end) and a fast-growing end (barbed end) (Carballido-Lopez, 2006). Actin microfilaments are 3 to 6 nm in diameter and flexible and *in vivo* they rarely occur in isolation but, rather in cross-linked aggregates and bundles (Carballido-Lopez, 2006). In the cell, actin polymerization is an extremely regulated process controlled both by nucleotide

binding and hydrolysis and by the action of a number of actin binding proteins that can cross-link, nucleate, cleave, bundle, stabilize, or destabilize the filaments (Pollard and Borisy, 2003).

Because of the homology between actin and MreB, recent work has been aimed at determining their similarities to better understand the evolutionary steps that led from the cytoskeleton in the last common ancestor to MreB in prokaryotes and to actin in eukaryotes. MreB has been shown to spontaneously polymerize in the presence of ATP, similar to actin (van den Ent et al., 2001). Our lab has worked to further extend the understanding of MreB polymerization using purified MreB from *Thermotoga maritima*. Electron microscopy has demonstrated the ability of MreB to polymerize into long, linear filaments (van den Ent et al., 2001). It undergoes reversible polymerization like that of actin, and polymerization conditions have been determined to be 20mM KCl, 1mM MgCl₂, 1mM EGTA, and 10mM imidazole at pH 7.0 and 20°C in the presence of ATP (Bean and Amann, unpublished). Polymerization is temperature dependent, with the rate and extent of polymerization increasing as temperature increases. A divalent cation (either Mg²⁺ or Ca²⁺) is not required for polymerization, but reactions with divalent cation polymerize much better. Divalent cation concentrations over 5mM are inhibitory to polymerization. The presence of salt also affects polymerization, as increasing concentrations of KCl decrease the rate and extent of MreB polymerization. Polymerization rate and extent also decreases at pHs above 7.0, while conditions with a pH below 6.0 are inhibitory to polymerization. Polymerization can also be inhibited by the novel compound S-benzylisothiourea, also known as A22 (Iwai et al., 2002).

The role of MreB inside actual cells has also been defined. Specifically, work on MreB has shown that it was responsible for the rod shape in *E. coli* (Doi et al., 1988), and a

mutation in the MreB gene resulted in spherical cells in colonies of *E. coli* cells (Lee et al., 2003). Immunofluorescence microscopy experiments show that MreB acts as the protein responsible for the shape of *Bacillus subtilis*, encircling the entire cytoplasm in a spiral shape and assembling into helical filaments which lay just beneath the cell surface, suggesting the formation of a genuine actin-like cytoskeleton (Jones et al., 2001). MreB may also coordinate the movement of PBPs during the making of the cell wall (Figge et al., 2004), and it also is essential for the segregation of the bacterial chromosome during cell division (Gitai et al., 2005).

Because of the functional similarities between actin and MreB and the many different proteins actin interacts with inside the eukaryotic cell (Pollard and Borisy, 2003), it is thought that MreB may also have interacting partners within the bacterial cell. Possible partners include MreC and MreD. Related to MreB, the MreC and MreD genes exist on the same operon along with MreB and two other genes (Burger et al., 2000). The roles of the MreC and MreD proteins have not yet been determined, but the analysis of the amino acid sequence in MreC suggests that it is membrane associated along with MreD (Lee et al., 2003). Using prior research on MreB and laboratory observations on the location of MreC in the cell membrane, a model proposed by Kruse, Bork-Jensen, and Gerdes suggests that MreC and MreD form a complex in the membrane that interacts with the MreB filaments, using the MreB as a locator for proper positioning (2005) (Fig. 1).

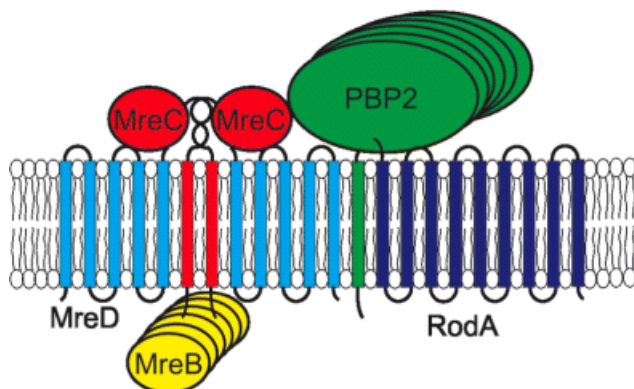


Fig. 1 - Proposed bacterial transmembrane model, reproduced from Kruse, Bork-Jensen, Gerdes (2005).

The cell division proteins (FtsA, FtsI, FtsK, FtsW, and FtsZ) are also strong candidates as possible interacting partners due to the role of MreB in cell division and chromosome segregation. Many of the Fts proteins have been shown to interact with each other using a bacterial two-hybrid system (Karimova et al., 2005), but further protein interactions have not been studied. Furthermore, recent work using a genetic screening of the *E. coli* genome has shown 18 other possible interacting partners with MreB (Butland *et al.*, 2005).

Because of the shared ATP-hydrolyzing domain and structural similarity between MreB and actin, we have hypothesized some of these 25 proteins exhibit genuine, specific interactions with MreB much like actin demonstrates with its binding partners in eukaryotic cells. Specifically, proteins from the genes *malT*, *nusG*, *gatY*, *gyrA*, and *tufB* chosen from the Butland study were tested for their ability to interact with MreB. We demonstrate here that preliminary *in vitro* tests show interactions between MreB from *E. coli* and the proteins NusG and EF-Tu (*tufB*).

Methods:

Cloning and Purification of Target Proteins

The genes *malT*, *nusG*, *gatY*, *gyrA*, and *tufB* were amplified from *E. coli* genomic DNA and cloned into the IPTG-inducible pET-23a vector (Novagen[®]) at the NdeI (5') and XhoI (3') sites. The vector includes a region encoding a 6-His residue C-terminal fusion to allow for purification by Ni²⁺ affinity chromatography. Plasmids were then transformed into *E. coli* C43(DE3) cells and plated on agar medium containing ampicillin to select for cells that took up the plasmid. Plates were incubated at 37°C for 12–24 hours. From the original colonies grown, *E. coli* cultures were grown at 37°C in 125mL LB broth with ampicillin and

induced with 0.375mM IPTG at an $OD_{600} = 0.7$, which indicated the amount of bacteria present in the culture.

The cells were then harvested by centrifugation after 3 hours of growth with IPTG and frozen at -20°C . Cell pellets were then homogenized in 1XNiG8 buffer (1XNiG8= 5mM Tris-Cl pH 8.0, 0.02% sodium azide, 0.2mM ATP, 300mM NaCl, 10mM imidazole pH 8.0, 0.1mM CaCl_2). Homogenized cells were then sonicated to lyse the cells and break up protein aggregates. The lysate was then cleared by ultracentrifugation at 125,000g at 4°C for 30 minutes. The resulting supernatant was then run through a Ni^{2+} affinity chromatography column. The column was then washed first with 1XNiG8 and a second wash of 1XNiG8 + 25mM imidazole. The His-tagged proteins were then eluted from the column with 10 x 5mL aliquots of 1XNiG8 + 200mM imidazole, pH 8.0. Each 5mL elution was collected separately, and samples analyzed by SDS-PAGE to determine the elutions containing the greatest concentration of purified protein. The proteins were then placed in dialysis tubing and immersed in 1000mL 1XCaG8 buffer (1XCaG8 = 5mM Tris-Cl pH 8.0, 0.02% sodium azide, 0.2mM ATP, 300mM NaCl, 10mM imidazole pH 8.0, 0.1mM CaCl_2 , 0.5mM dithiothreitol) to remove excess NaCl and imidazole from solution.

Expression and Purification of *E. coli* MreB

The gene for native *E. coli* MreB was amplified from genomic *E. coli* DNA by PCR. Same procedures from previous section were used here; however, the His-tag from the vector was not included. From the native gene, a form of MreB expressing a N-terminal six histidine tag was also cloned and amplified by PCR (MreB + N-His) and cloned into the IPTG-inducible pET-46 vector (Novagen[®]). Plasmids were then transformed into *E. coli* C43(DE3) cells. As before, cells were then plated on agar medium containing ampicillin and

incubated at 37°C for 12–24 hours. From the original colonies grown, *E. coli* cultures were grown at 37°C in 125mL LB broth with ampicillin and induced with 0.375mM IPTG at an $OD_{600} = 0.7$, which indicated the amount of bacteria present in the culture.

The cells were then harvested by centrifugation and frozen at -20°C. Cells expressing MreB + N-His were then homogenized and sonicated in 1XNiG8 buffer, and the cell extract cleared by centrifugation at 125,000g at 4°C for 30 min. The resulting supernatant containing soluble MreB + N-His was purified using Ni^{2+} affinity chromatography exactly as described above for other His-tagged proteins. Purified protein elutions were then dialyzed in 1XCaG8 buffer to remove excess salt.

E. coli MreB native protein was purified to its useable form as follows: Cell pellets expressing *E. coli* MreB native were homogenized and sonicated in 1XCaG8 buffer + 5mM DTT. The resulting extract was then cleared by ultracentrifugation at 100,000g at 4°C for 30 minutes. The resulting supernatant was poured off and saved. The pellet was re-suspended in 1XCaG8 buffer + 5mM DTT and consequently homogenized and sonicated. The re-suspended pellet was then centrifuged under the same conditions as before. The resulting supernatant from this second spin was then poured off and combined with the saved supernatant from the first spin. The combined sups were then used to make a polymerization reaction with 15mM MES pH 6.5 and 4mM $CaCl_2$. The polymerization reaction was left on ice at 4°C for 16-24 hours overnight.

The ensuing polymerization reaction was then centrifuged at 100,000g at 20°C for 30 minutes. The pellet contained polymerized MreB native was then re-suspended in 1XCaG8 buffer + 5mM DTT. To depolymerize the protein, the re-suspended pellet was dialyzed in 1XCaG8 buffer + 5mM DTT + 5mM Tris HCL for 72 hours with fresh buffer exchanged

every 24 hours. The dialyzed and depolymerize protein was then centrifuged again at 100,000g at 4°C for 30 minutes. The resulting supernatant was then saved and used in a second polymerization reaction with 15mM MES pH 6.5 and 4mM CaCl₂ and allowed to react on ice at 4°C for 16-24 hours.

The second polymerization reaction was then centrifuged as the first one and the depolymerization process repeated exactly as above. The resulting depolmyerized MreB native proteins was again spun at 100,000g at 4°C for 30 minutes, and the resulting supernatant used as the finalized purified form of *E. coli* MreB native.

Light Scattering Interaction Tests

MreB polymerization was monitored over time by use of light scattering fluorometry under varying conditions (pH, KCl, MgCl₂, CaCl₂) to determine those best for polymerization. Light intensity was measured at an angle of 90° from the axis of incident light at 400nm. Purified *E. coli* MreB + N-His was used for all tests due to better readability in the fluorometer. All reactions were performed at 20°C unless otherwise noted and allowed to react for 20 minutes. To test for protein interactions with MreB, the same concentrations (5µM) of individual ligand proteins and MreB + N-His were mixed under MreB polymerization conditions (0mM KCl, 1mM MgCl₂, 10mM MES, pH 6.5) and light scattering observed over time. As a control, light scattering was also observed under MreB polymerization conditions for ligands alone. Light scattering data was collected using a fluorometer from ISS and VINCI 1.4.5 software and analyzed using Microsoft Excel and Kaleidagraph.

Co-sedimentation Assays

Purified *E. coli* MreB native was mixed at 5 μ M with various concentrations of (0-15 μ M) ligand protein under MreB polymerization conditions. Reactions were incubated at room temperature for 30 minutes and then subjected to centrifugation at 100,000g for 10 minutes. Supernatants were carefully decanted and pellets re-suspended in ddH₂O in a volume equal to the reaction volume (150 μ l). Supernatants and re-suspended pellets were then analyzed by SDS-PAGE to determine binding between MreB and any ligands. As controls, reactions containing ligand alone and MreB alone under MreB polymerization conditions were performed to ensure any pelleting of ligands were due to an interaction with MreB.

Results:

Expression and Purification of MreB and Other Proteins

Overexpression of *E. coli* MreB native and a form of *E. coli* MreB with an enterokinase cleavable N-terminal His-tag ((H)₆-D-D-D-D-K) in the IPTG-inducible pET-46 vector was successful (Fig. 2A). The N-terminal His-tagged MreB was originally intended to be adhered to Ni²⁺ affinity chromatography beads while commercially available enterokinase enzyme cleaved off the extra His-tag, leaving MreB in its native form. However, the enterokinase did not cut at the proper cleavage site resulting in many small proteolytic fragments of the *E. coli* MreB + N-His (data not shown).

Consequent purification of *E. coli* MreB native through the multiple polymerization-depolymerization cycles, described in the Methods, yielded a mostly pure form of the protein at 36 kDa in the final depolymerization spin supernatant (Fig. 2B). This ability of the protein to polymerize upon centrifugation and consequently depolymerize via dialysis has been

demonstrated in eukaryotic actin. However, a few contamination bands were still present, most noticeable at 45 kDa. This was the final MreB native form used in all consequent experiments. Purification of *E. coli* MreB + N-His by Ni²⁺ affinity chromatography produced high concentrations of pure protein with no other contamination present (Fig. 2C).

High expression levels were also observed in cells overexpressing the His-tagged proteins from the *E. coli* genes *malT*, *nusG*, *gatY*, *gyrA*, and *tufB*. Subsequent purification via Ni²⁺ affinity chromatography then yielded purified forms of the proteins. The only noticeable contamination band was observed to be present with *E. coli* EF-Tu (*tufB*) at 21 kDa (data not shown).

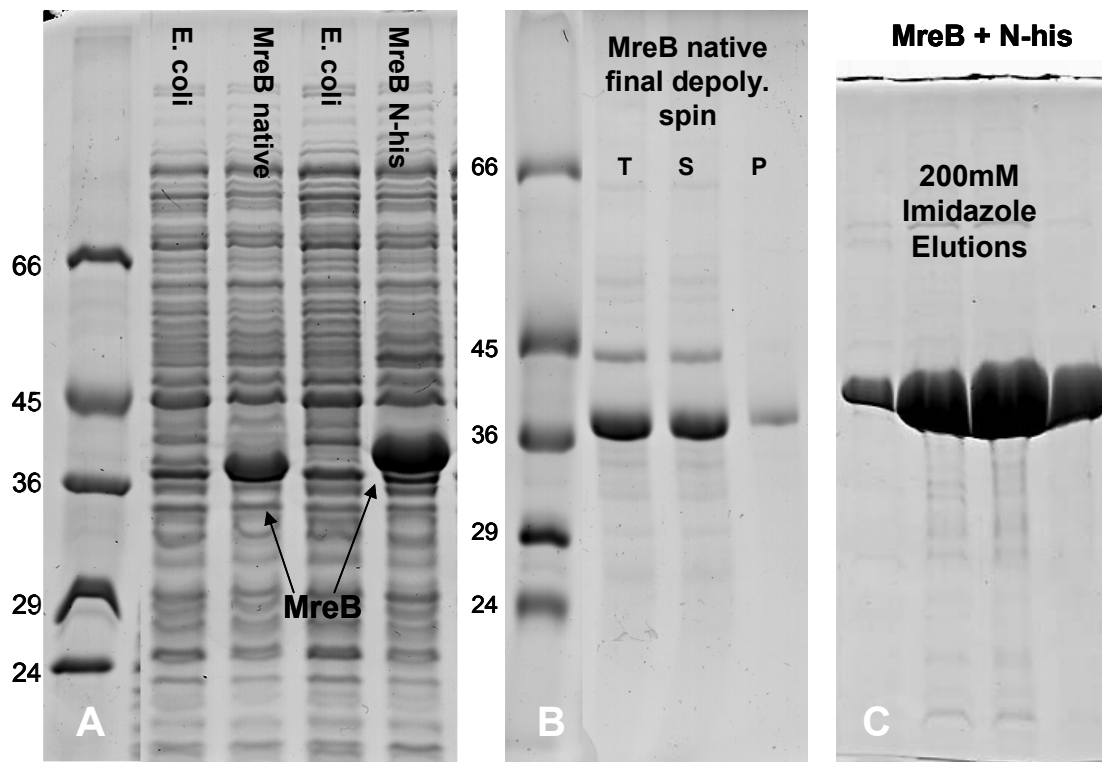


Figure 2. Expression and Purification of *E. coli* MreB A) Overexpression of MreB native and N-His forms in *E. coli* cells pre- and post-induction. B) Purification of MreB native. Final purified form is in supernatant, labeled “S”. C) Purification of MreB + N-His by Ni²⁺ affinity chromatography.

Polymerization of *E. coli* MreB

Conditions for the polymerization for *E. coli* MreB were determined by use of light scattering fluorometry. An increase in light scattering over time indicates polymerization. Monomers or small oligomers of MreB will not scatter much light from the light source as it passes through the solution and reaction cuvette undetected. Once monomers start to polymerize and form filaments, the light from the source will refract off of the filaments and scatter through the cuvette where it is detected by the machine. Based on our previous studies of *T. maritima* MreB, we tested conditions with varying pH, KCl concentration, and divalent cation content (MgCl₂ or CaCl₂) and concentration. Purified *E. coli* MreB + N-His was used in most fluorometry tests because it was in a highly purified form, although similar behavior was observed in the native form as well (data not shown). Tests were conducted by taking purified *E. coli* MreB + N-His stored in 1XCaG8 buffer and adding the necessary components to the protein in a reaction vessel and then transferring the contents to the cuvette in which the light scattering is measured. Multiple tests were then repeated, changing one variable while holding the others constant.

The first variable tested was the pH dependence of polymerization. KCl and MgCl₂ concentration were kept at 40mM and 1mM, respectively, based on polymerization conditions for *T. maritima* MreB. MreB concentration was kept at 5μM and the pH was varied from 6.0 – 7.0 and light scattering measured. The plotted data showed no increase in polymerization over time at pH 7.0. The light scattering counts stayed constant at approximately 1×10^4 (Fig. 3). Light scattering at pH 6.0 started at just under 1×10^6 light scattering counts and increased only slightly (Fig. 3). The high starting value at this pH is not ideal, as a solution of monomeric MreB should show little light scattering. This likely

indicated a pH-induced change in protein secondary and tertiary structure causing the protein to aggregate immediately instead of showing true filament formation and polymerization.

The pH 6.5 conditions showed a much improved polymerization curve (Fig. 3). Counts started low at about 2×10^4 and rapidly increased to just over 2×10^5 counts by 600 seconds of reaction time. Equilibrium was then reached, with counts holding steady the remaining length of the reaction showing more like a typical *T. maritima* MreB or actin polymerization curve.

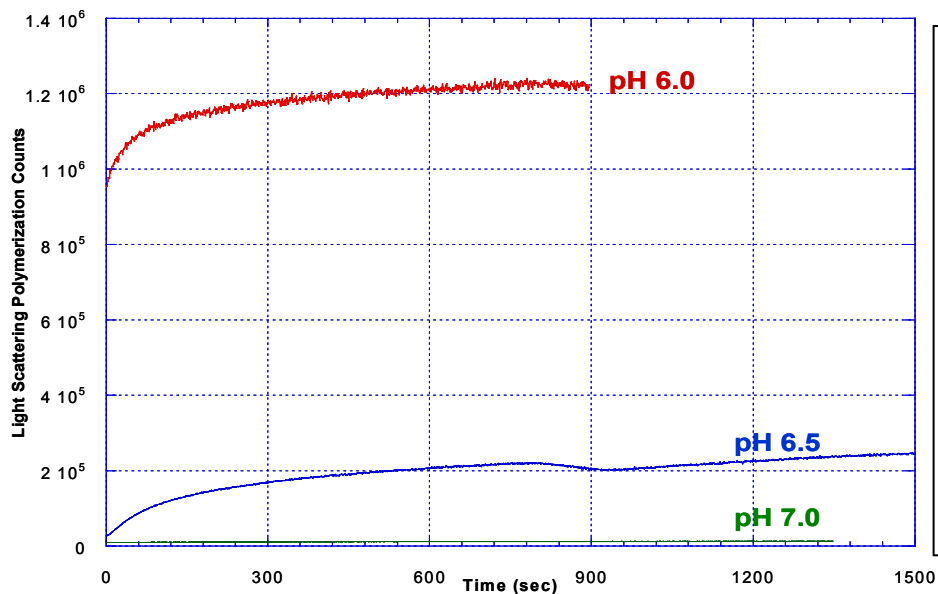


Figure 3. pH dependence of MreB polymerization. Light scattering of $5 \mu\text{M}$ *E. coli* MreB + N-His at 40mM KCl and 1mM MgCl_2 was measured at varying pHs. Resulting plots of light scattering versus time were compiled on the same graph. pH 6.5 was determined to be the best condition for polymerization.

Once the proper pH conditions were determined, the dependence of KCl concentration on polymerization was determined. Again, light scattering fluorometry was performed on $5 \mu\text{M}$ *E. coli* MreB + N-His at pH 6.5 and 1mM CaCl_2 while KCl concentration was varied from 0 - 300mM . The resulting plots show the greatest increase in light scattering over time to occur at 0mM KCl and then a steady decrease in light scattering over time as the KCl concentration increases (Fig. 4). KCl is inhibitory to the polymerization of MreB.

Because the difference between 0mM and 20mM KCl at the highest light scattering value

was so great (4×10^5 counts), 0mM KCl was determined to be the best salt condition for MreB polymerization.

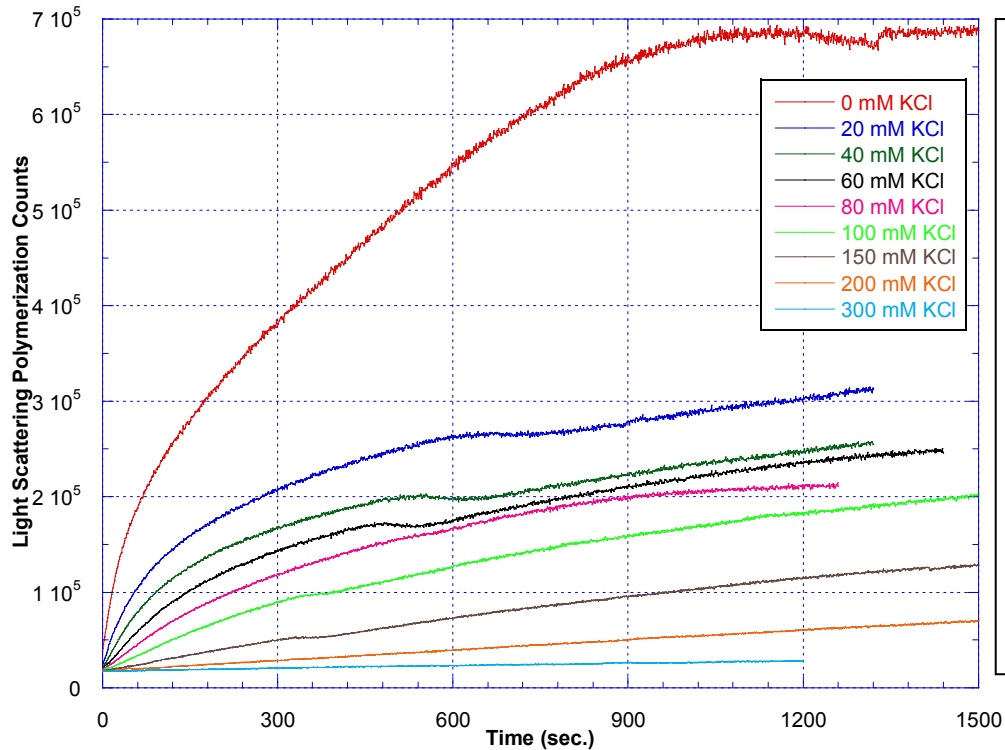


Figure 4. KCl Effects on MreB Polymerization. Light scattering of $5 \mu\text{M}$ *E. coli* MreB + N-His at pH 6.5 and 1mM CaCl_2 was measured at varying KCl concentrations. Resulting plots of light scattering versus time were compiled on the same graph. Salt has an inhibitory effect on MreB polymerization. 0mM KCl was determined to be the best condition for polymerization, showing the greatest amount of light scattering over time.

Because Mg^{2+} and Ca^{2+} were both used as divalent cations in different polymerization experiments, we decided to test whether there were any noticeable differences in MreB polymerization caused by the use of one cation over the other. Light scattering fluorometry was performed on $5 \mu\text{M}$ *E. coli* MreB + N-His at pH 6.5 and 0mM KCl at varying concentrations of both MgCl_2 and CaCl_2 from 0-2000 μM . Because the 1xCaG8 buffer used to store *E. coli* MreB + N-His already contains Ca^{2+} ions, EGTA is added to all MgCl_2 experiments to a concentration of 1mM to chelate all Ca^{2+} ions and ensure the only divalent cations available to the MreB are Mg^{2+} .

For both MgCl_2 and CaCl_2 , light scattering increases over time as the concentration of divalent cation increases (Fig. 5). Only a slight difference was observed in total light

scattering between MgCl_2 and CaCl_2 (8×10^5 versus 1×10^6 counts, respectively, for $2000 \mu\text{M}$). The largest difference was seen in the rate at which equilibrium was reached. It only took approximately 60 seconds to reach 1×10^6 counts for $2000 \mu\text{M}$ CaCl_2 , while it took nearly 900 seconds to reach the maximum counts for $2000 \mu\text{M}$ MgCl_2 . However, the overall pattern of MreB polymerization observed for both MgCl_2 and CaCl_2 was very similar and because both MgCl_2 and CaCl_2 promoted polymerization, both were used interchangeably as the necessary cation for MreB polymerization. 1mM divalent cation concentrations were determined to be best for polymerization to be consistent with experiments conducted on *T. maritima* MreB.

From the experiments testing polymerization of *E. coli* MreB + N-His under various conditions, we determined polymerization conditions to be 0mM KCl, 1mM divalent cation (Mg^{2+} or Ca^{2+}) and pH 6.5. These were the conditions used for the interaction tests with MreB and the target His-tagged *E. coli* proteins.

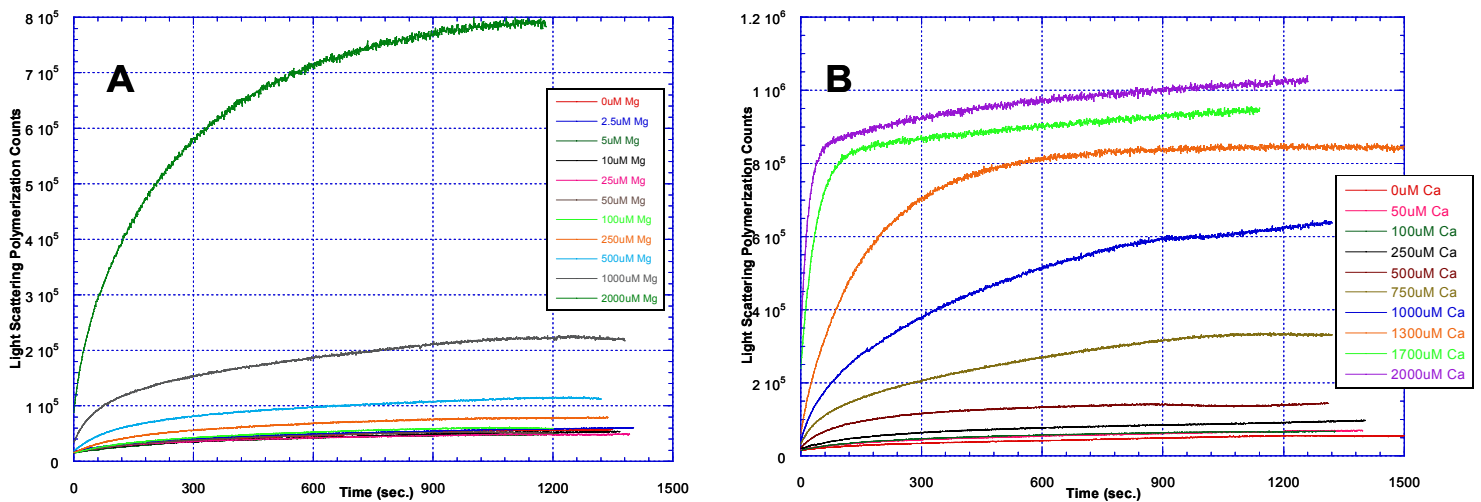


Figure 5. Divalent Cation Effects on MreB Polymerization. Light scattering of $5 \mu\text{M}$ *E. coli* MreB + N-His at 0mM KCl and pH 6.5 with variable concentrations of MgCl_2 or CaCl_2 . A). MgCl_2 at various concentrations. Polymerization increases in order as concentration does. B). CaCl_2 at various concentrations. Polymerization shows same pattern as that with MgCl_2 , except total light scattering counts are increased and rate at which equilibrium is reached is also greater. Patterns for both cations are similar, and either can be used in polymerization experiments.

MreB Interacts with His-tagged NusG and EF-Tu

Once polymerization conditions for *E. coli* MreB were determined, high force co-sedimentation assays between MreB and the target His-tagged proteins from the genes *malt*, *nusG*, *gatY*, *gyrA*, and *tufB* were performed to test for putative interactions. All of the His-tagged proteins stayed soluble in the supernatant under MreB polymerization conditions except GatY, which came down completely in the pellet (Fig. 6A). Upon addition of *E. coli* MreB native, only NusG and EF-Tu were observed to interact with MreB (Fig. 6B). GatY also was present in the pellet with MreB, but because it pelleted alone, any co-sedimentation with MreB was determined to be due to the properties of GatY and not any true interaction with MreB.

As preliminary tests showed *E. coli* NusG + His and EF-Tu + His to be the only target proteins showing a possible interaction with *E. coli* MreB native, more experiments were focused on confirming these interactions. Another co-sedimentation assay was performed with EF-Tu + His held at 5 μ M mixed with varying concentrations of *E. coli* MreB native under MreB polymerization conditions. This experiment was intended to better determine the molar ratio of each when binding occurs, but instead yielded a surprising result. A noticeable contaminant protein was present with EF-Tu + His at approximately 21 kDa, and as the concentration of MreB in the reaction increase, the amount of this contaminant protein in the pellet with MreB appeared to increase (Fig. 7).

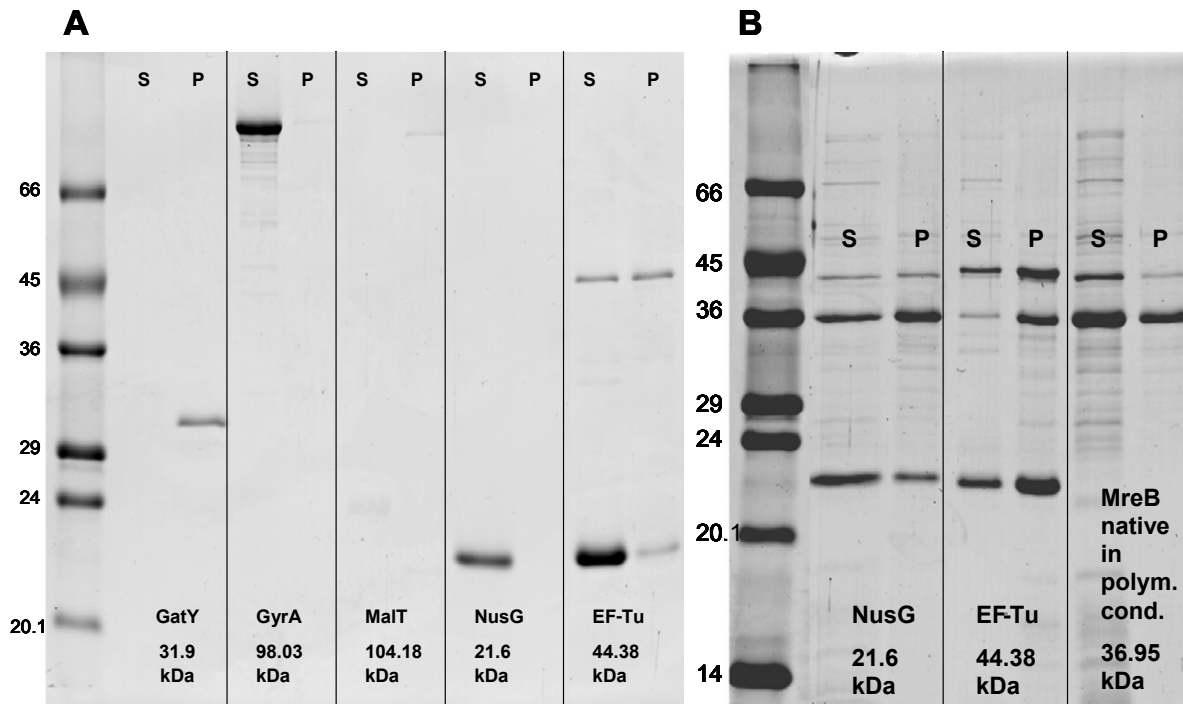


Figure 6. Co-sedimentation of *E. coli* GatY + His, GyrA + His, MalT + His, GatY + His, NusG + His, EF-Tu + His with *E. coli* MreB native. A) Coomassie Blue stained SDS-PAGE gels of supernatants (S) and pellets (P) following a 30 minute incubation and 100,000 x g centrifugation of 5 μ M target proteins in MreB polymerization conditions (pH 6.5, 0mM KCl, 1mM MgCl₂) in absence of MreB. B) Coomassie Blue stained SDS-PAGE gels of supernatants (S) and pellets (P) following a 30 minute incubation and 100,000 x g centrifugation of 5 μ M target proteins in MreB polymerization conditions (pH 6.5, 0mM KCl, 1mM MgCl₂) in presence of 5 μ M MreB. Only NusG + His and EF-Tu + His pellet with MreB. MreB also pellets in absence of any ligand (far right).

The rise in the amount of contaminant present with MreB in the pellet as MreB concentration increased was confirmed by densitometric analysis of Coomassie Blue-stained gels of the supernatant and pellet fractions shown in Figure 6 (Fig. 8). Upon analysis by mass spectroscopy, this contaminant band was determined to be *E. coli* NusG + His (data not shown), providing more evidence of an interaction between *E. coli* NusG + His and *E. coli* MreB native.

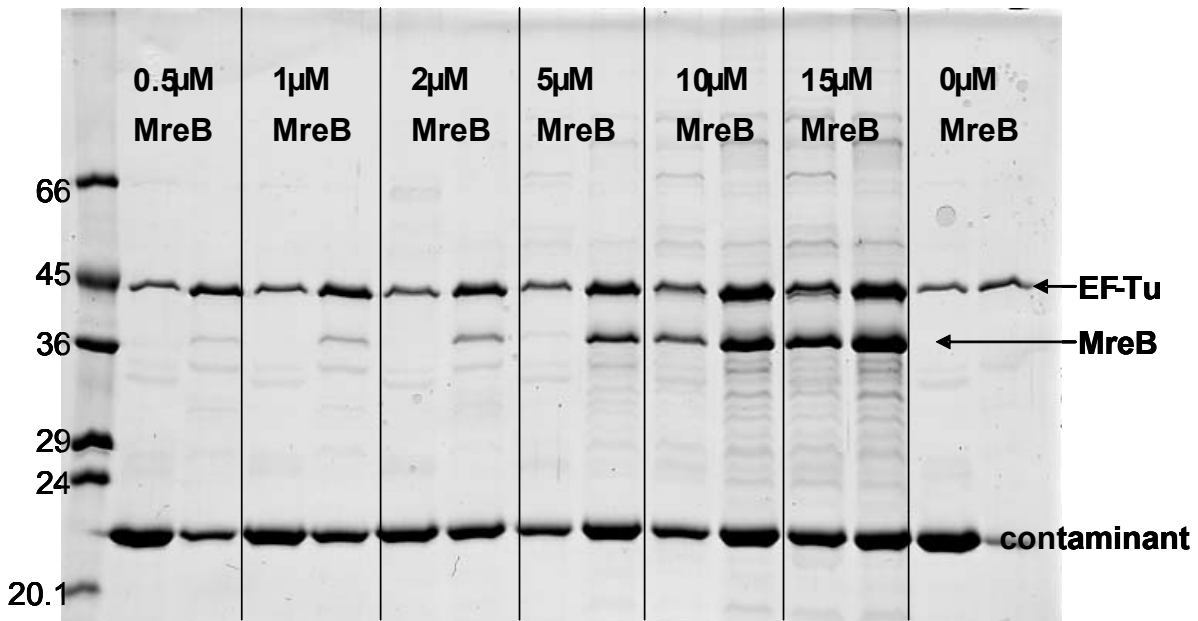


Figure 7. *E. coli* EF-Tu + His binding the MreB native. Coomassie Blue stained SDS-PAGE gels of supernatants (S) and pellets (P) following a 30 minute incubation and 100,000 x g centrifugation of 5 μM EF-Tu + His in MreB polymerization conditions (pH 6.5, 0mM KCl, 1mM MgCl₂) in presence of increasing concentrations of MreB native. Contaminant protein at ~21 kDa is present with EF-Tu + His and shows increasing pelleting as the concentration of MreB native increases. Contaminant was later determined to be *E. coli* NusG + His.

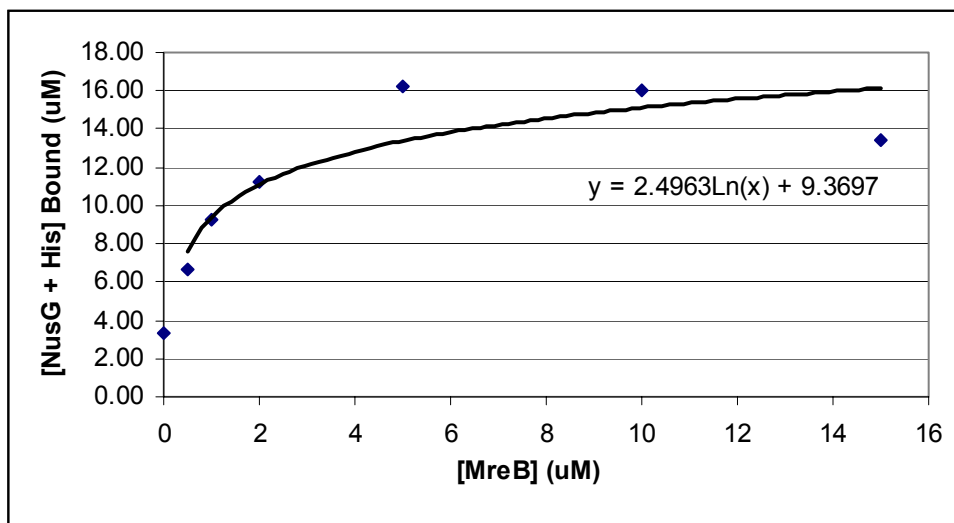


Figure 8. *E. coli* NusG + His binding to MreB native. Increasing concentrations of MreB native were incubated with 5 μM *E. coli* NusG + His and centrifuged at 100,000 x g. The amount of NusG + His bound was determined densitometrically from Coomassie Blue-stained gels and plotted relative to the concentration of MreB present in the reaction. A general pattern of increased binding of NusG + His per concentration of MreB is observed.

Various concentrations (0- 5 μ M) of *E. coli* MreB + N-His were mixed with 5 μ M *E. coli* NusG + His under MreB polymerization conditions and light scattering observed over time. As controls, light scattering of NusG + His under MreB polymerization conditions in the absence of MreB and *E. coli* MreB + N-His in the absence ligand was measured. As the concentration of MreB increased, light scattering (and therefore MreB polymerization) also increased (Fig. 9). These results are similar to the co-sedimentation assays that showed increased interaction between NusG + His and MreB as the concentration of MreB increased. The light scattering tests confirmed the results of the co-sedimentation assays, and both experiments provide preliminary evidence of an interaction between *E. coli* MreB and *E. coli* NusG + His.

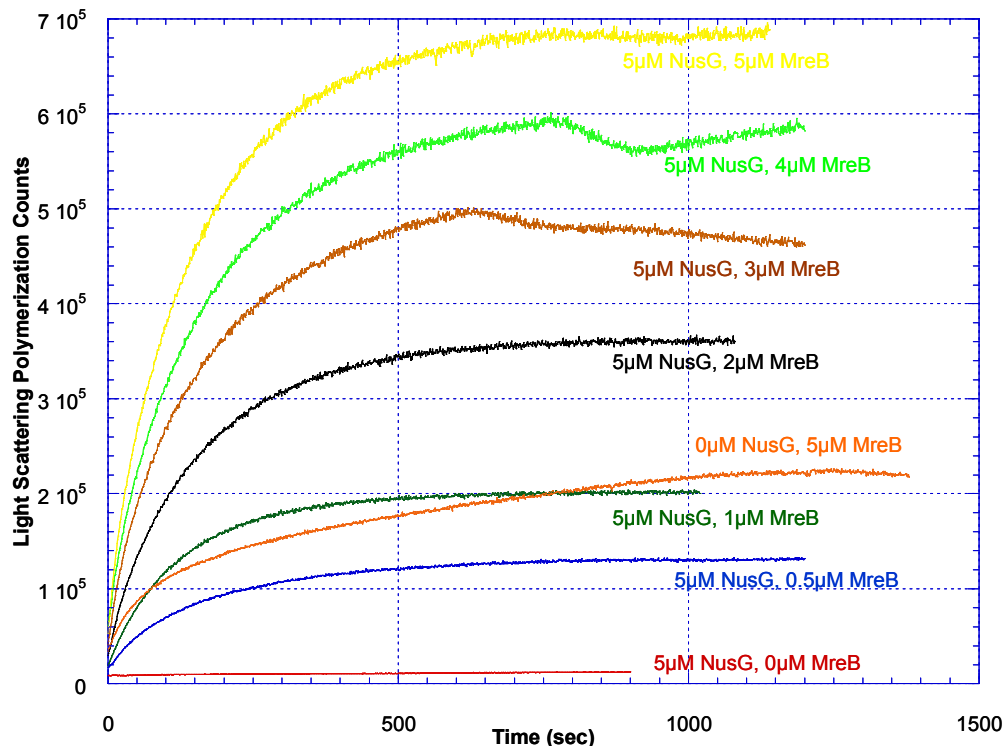


Figure 9. Light scattering of 5 μ M *E. coli* NusG + His and variable concentrations *E. coli* MreB + N-His under MreB polymerization conditions (pH 6.5, 0mM KCl, 1mM MgCl₂). Nearly three-fold increase in light scattering observed at 5 μ M NusG and MreB reaction versus 5 μ M MreB alone shows possible interaction.

Discussion:

The proteins with which the actin homolog MreB interacts with *in vivo* in *E. coli* have yet to be identified. The proteins from the genes *malT*, *nusG*, *gatY*, *gyrA*, and *tufB* were hypothesized to interact with MreB based on a list from a preliminary genetic screen of the protein interactions within the *E. coli* genome (Butland *et al.*, 2005). Here we showed through preliminary interaction tests that MreB interacts with the His-tagged forms of NusG and EF-Tu (*tufB*) *in vitro*. We were also able to determine the polymerization conditions for *E. coli* MreB (pH 6.5, 0mM KCl, and 1mM MgCl₂) and demonstrate the ability of the protein to undergo reversible polymerization, much like eukaryotic actin.

Purification of the native form of *E. coli* MreB was needed before any interaction tests and proved to be very difficult. Though the protein was soluble and able to undergo polymerization and depolymerization cycles, contaminant bands visible by SDS-PAGE were always present in each of the purification steps. The form of *E. coli* MreB native used in all experiments contained a noticeable 45kDa contaminant (Fig. 1B) but was the most highly purified form we were able to attain. Later analysis by mass spectroscopy showed this contaminant to be EF-Tu. As MreB and EF-Tu co-sediment together in the polymerization purification, we have more evidence of possible interaction between MreB and EF-Tu.

Because of the EF-Tu contaminant, we developed a form of MreB with an enterokinase-cleavable N-terminal His tag. We originally intended to purify the N-His form using Ni²⁺ affinity chromatography and then remove the tag using enterokinase to yield native protein; however, the enterokinase proved to be unspecific in its proteolytic activity and produced an unusable form of MreB. The N-His form of MreB was then used because of its high purity.

Light scattering fluorometry was used to establish conditions needed for MreB polymerization. Because of its high purity, *E. coli* MreB + N-His was used in the experiments. As our lab has done to determine the properties of *T. maritima* MreB polymerization, we tested various pHs, KCl concentrations, and divalent cation contents and concentrations to determine the environment best for *E. coli* MreB polymerization. Being close to physiological conditions, pH 6.5 was preferred. KCl proved to be inhibitory to polymerization, most likely interfering with specific interactions between side chains at the interface of binding monomers. Increasing divalent cation concentrations promoted polymerization, similar to that observed in actin and *T. maritima* MreB. Binding of Ca^{2+} or Mg^{2+} to MreB monomers might induce conformational changes in the monomers necessary for polymerization, a property observed in actin (Frieden, 1983). The different polymerization rates observed between reactions using Ca^{2+} or Mg^{2+} could be explained by different conformational changes induced by the two cations. However, since no crystal structure exists of *E. coli* MreB, it is difficult to accurately conclude how cation binding affects monomer structure and ultimately filament formation during polymerization.

Interaction tests done under MreB polymerization conditions showed that of the five proteins examined, His-tagged GatY, GyrA, and MalT did not exhibit any binding to MreB. Co-sedimentation assays showed binding of MreB to *E. coli* NusG + His and *E. coli* EF-Tu + His. Light scattering fluorometry confirmed the binding assays, showing increased light scattering and MreB polymerization in the presence of NusG + His. An increase in MreB polymerization in the presence of a ligand indicates the ligand is binding to MreB and promoting the addition of MreB monomers to existing filaments. Light scattering tests were

not performed to test the interaction between EF-Tu + His and MreB because of the presence of a contaminant protein that was determined to be *E. coli* NusG + His.

The exact nature of MreB binding to *E. coli* NusG + His and *E. coli* EF-Tu + His has yet to be determined. These tests have only shown that an interaction is most likely occurring, but more experiments are needed to determine the stoichiometry of binding and the type of binding (whether NusG or EF-Tu promote monomer nucleation, bundle filaments, or are structurally supported by MreB). It is also difficult to draw conclusions about how possible interactions translate to an *in vivo* function. NusG is necessary for Rho-dependent transcription termination, and EF-Tu is a transcriptional elongation factor. MreB is known to interact with RNA polymerase (RNAP) and possibly serve as a scaffold for RNAP to facilitate chromosome segregation (Kruse et al., 2006). It is also possible that as MreB assembles into helices that span the cell just under the cytosolic membrane, it also serves as support for NusG and EF-Tu as they carry out their cellular functions. Still, many more experiments *in vitro* and *in vivo* are needed to develop a better understanding of how MreB functions in the cell.

Many of these experiments were done using His-tagged proteins because of the quick and easy purification procedure. Ideally, all experiments will be done on the native forms of proteins because it is possible that the His-tags on His-tagged proteins are part of an interaction that would not normally occur in native proteins. Also, a purification procedure that yields completely purified *E. coli* MreB native still must be determined. The experiments performed with the slightly contaminated *E. coli* MreB gave a very preliminary indication into how *E. coli* MreB polymerizes and interacts with other proteins; however, the contaminant protein could still affect interactions and polymerization that we cannot directly

detect. Much work is still to be done to determine the dynamics of MreB in *E. coli*, but the field is still very young and promises to yield exciting results in the future.

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