Functional analysis of actin-like protein MreB and alternative sigma factor RpoN in chlamydial developmental cycle

By

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Date approved: 04/22/13

Abstract

Chlamydia trachomatis is an obligate intracellular pathogen of immense public health impact and is responsible for diverse disease states leading to blindness, sterility, atherosclerosis etc. in humans. Its unique biphasic developmental cycle is essentially linked to its pathogenesis. MreB, an actin-like protein, is known to be present in most rod-shaped organisms as a part of the cell elongation machinery. The role of MreB in *Chlamydia* is unknown, even though this gene is conserved. Recent studies have attributed diverse roles to MreB in different organisms. Our study focusses on the possible role of MreB in *Chlamydia trachomatis* using different approaches and techniques.

To understand the functional role of MreB, we sought two strategies, *in vitro* and *in vivo* analysis. Purified recombinant chlamydial MreB was subjected to a polymerization reactions using ATP and Mg^{2+} ions in the presence and absence of a known inhibitor of MreB polymerization i.e. A22 and measured using laser light scattering. We observed that MreB scatters light even in the absence of ATP almost at a similar rate as in its presence. The presence of A22 increased the rate of light scattering as opposed to inhibiting it. When the MreB sequence from *Chlamydia trachomatis* was compared to that of other organisms, some amino acid substitutions in the conserved regions of the nucleotide binding pocket which also coincides with the A22 binding sites were evident in chlamydial MreB which could contribute to this inexplicable phenomenon. *Chlamydial* A22 mutant had a mutation outside the nucleotide binding pocket, confirming the possibility of other targets of A22 in *Chlamydia.* Quantitative RT-PCR at different time points through the developmental cycle of *Chlamydia trachomatis* revealed that the expression of MreB increases at later time points proposing its role in RB to EB conversion. Confocal images from immunofluorescence assay showed that MreB appeared as distinct puncta

concentrated at the center of the inclusion which increased in number with time. Summarizing these observations, it could be said that MreB is involved in the RB to EB conversion in *Chlamydia.*

Chlamydia is known to code for an alternative sigma factor, RpoN, but the role is unknown. We performed *in vitro* studies to elucidate the role of sigma 54 (RpoN) in *Chlamydia trachomatis L2/434/Bu.* Using EMSA it was observed that in the presence of RNA core polymerase, chlamydial sigma 54 could bind to the predicted RpoN promoters. The quantitative RT-PCR analysis showed a pattern which confirms its role in transcription of the predicted targets *CT652.1* and *CT683.* Elucidating its role *in vivo* using modified shuttle vector is the next step to study its role in the chlamydial developmental cycle.

This work is dedicated to my family for their

unconditional love and support

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I would like to thank my parents Vidya and Ashwin, as well as my grandparents for their confidence in me. They receive my deepest gratitude and love for their dedication and the many years of support, that provided the foundation for my education. I would also like to thank Jay for his love and encouragement. He has been my biggest strength and my best critic. Lastly, I want to thank my sister, Ruiya, who has been my teacher, my inspiration and my closest friend. She'll always be loved and missed.

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I. Chlamydial introduction

A. Chlamydial history and public health:

In 1907, the causative agent for trachoma, an eye infection leading to preventable blindness, was discovered and incorrectly termed as '*Chlamydozoa'* which in Greek meant mantle or cloak. Neither considered protozoa nor bacteria these were later in the 1964 designated as bacteria (1). Now known as *Chlamydia trachomatis,* it is the causative agent of trachoma in more than 84 million people across the world and over 90 million new cases of sexually transmitted disease each year (2). *Chlamydia trachomatis* belongs to the order *Chlamydiales* and has been characterized as an obligate intracellular pathogen of eukaryotic cells with a very unique developmental cycle (3). These are gram-negative spherical shaped eubacteria that consist of an inner and an outer membrane (4)

Among the *Chlamydiales, Chlamydia psittaci*, *Chlamydia pneumonia* and *Chlamydia trachomatis* are the three main species of importance to public health (5). *C. psittaci* is a zoonotic pathogen that causes psittacosis, a flu-like illness often accompanied by pneumonia in birds, by infecting the respiratory tract and can spread to humans (6). *C. pneumoniae* infects the upper respiratory tract in humans resulting in a host immune response which causes pneumonia and bronchitis (7, 8) and is at times associated with coronary heart disease and atherosclerosis (9).

C. trachomatis also causes ocular infections which may lead to blindness. The ocular infections are caused by serovars A, B, Ba, and C. The scarring of the cornea and infected conjunctiva is the main cause of blindness in 1-3 million people in endemic areas (10). Trachoma in areas of hyperendemicity is treated with single dose of azithromycin to reduce the overall chlamydial in community and prevent risk of re-infection.

C. trachomatis also causes sexually transmitted infections in women affecting infertility worldwide. Serovars D through K cause the genital infections and the LGV (lymphogranuloma) strain (L1-L3) are characterized by genital ulcerations. *C. trachomatis* infects the cervix in women causing infection which is asymptomatic in nature and is also a cause of pelvic inflammatory disease (PID), ectopic pregnancy and sterility in women (11). About $1.5 billion is spent annually for detection, treatment and prevention of C. trachomatis infections in the U.S. (12)

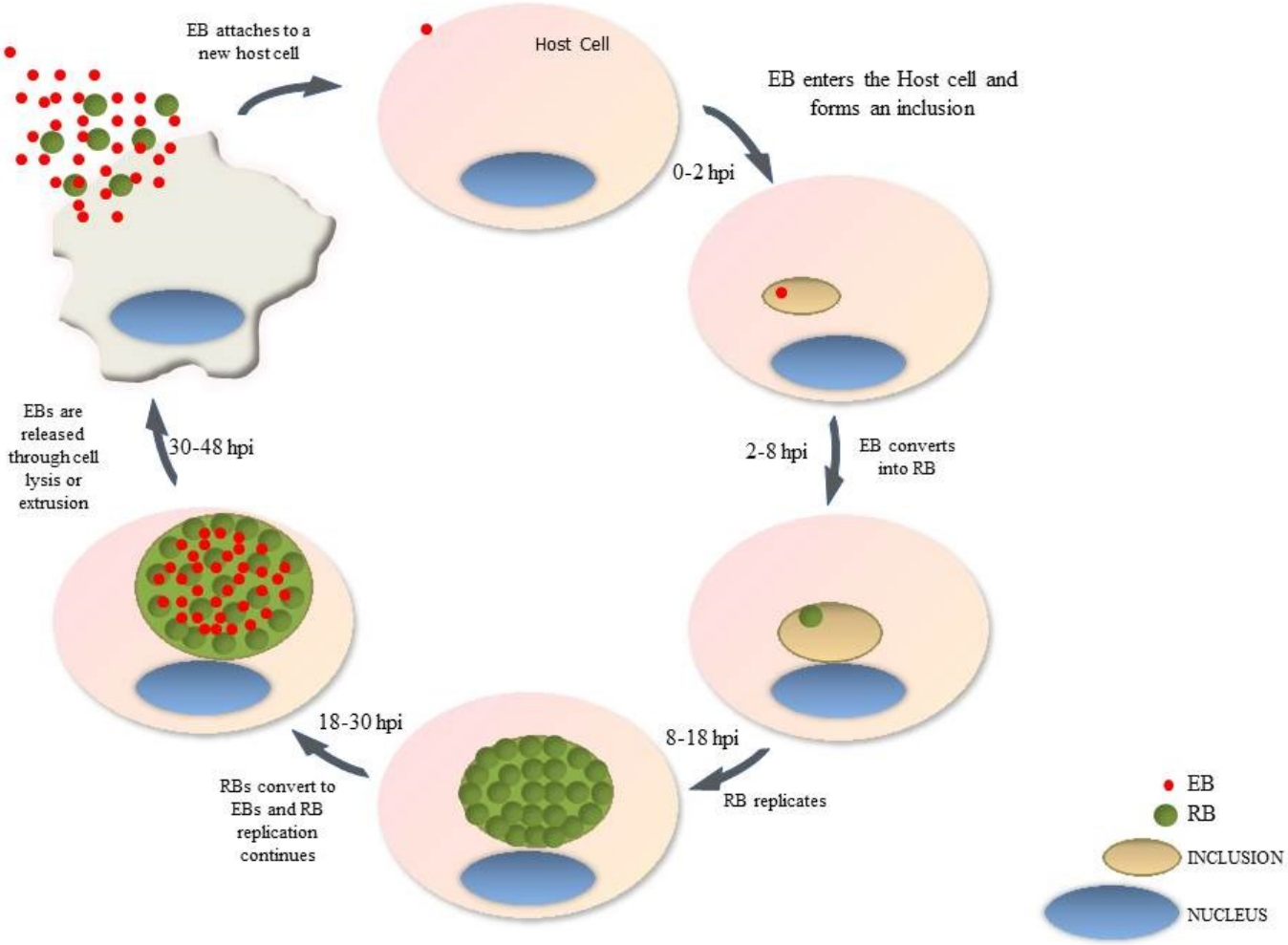
B. Chlamydial developmental cycle:

Chlamydia has a unique developmental cycle, which sets it apart from other bacteria. The developmental cycle involves a reticulate body (RB) and an elementary body (EB), which are phenotypically distinct (13). The metabolically inactive elementary body (EB) is responsible for propagation of infection to new host cells. EBs are spherical bodies which survive in extracellular environments and are known to be spore-like bodies in *Chlamydia* (14). They have a highly cross-linked surface which is rich in disulphide bonds and a very condensed DNA.

The elementary body attaches to a host cell and is internalized through a membrane bound vacuole (15) (Fig 1). This parasitophorous vacuole, termed a chlamydial inclusion, prevents fusing with lysosomes, facilitating survival inside the host cell. Upon entry, the EBs which are 0.2-0.3 μm in size are then converted into RBs which are larger (0.5-1.6 μm) in size. This process is completed within 2-8 hours post infection (hpi) and these metabolically active

Figure 1: Developmental cycle of *Chlamydia trachomatis*

Infection of a host cell with *C. trachomatis* initiates with an EB entering a host cell to form a parasitophorous vacuole termed an inclusion. The EB is then converted into a non-infectious RB which is metabolically active. RB starts replicating within the inclusion by binary fission. RBs asynchronously start converting back to EBs which are the infectious, metabolically inactive form. EBs are released by host cell lysis or extrusion which infect the neighboring host cells.


EB attaches to a new host cell
Host Cell
EB enters the Host cell and forms an inclusion
0-2 hpi
2-8 hpi
EB converts into RB
8-18 hpi
RB replicates
18-30 hpi
RBs convert to EBs and RB replication continues
30-48 hpi
EBs are released through cell lysis or extrusion
EB
RB
INCLUSION
NUCLEUS

forms, i.e. RBs, start replicating within the inclusion by binary fission. RBs are the non-infectious form and are fragile. Between 18-24 hpi these RBs start asynchronously converting back into EBs (13). Exact mechanisms/signals behind this process are yet to be defined. The infectious forms (EBs) then burst out of the host cell by the process of extrusion or host cell lysis (16), completing one life cycle of a chlamydial EB.

II. Chlamydial actin-like protein MreB

a. Bacterial cell wall and the role of MreB

Bacterial cytoskeletal proteins are known to have diverse sequences and functions through different species which include scaffolding peptidoglycan synthesis, cell shape, cell polarity and chromosome segregation. (17). Bacteria contain homologs for eukaryotic actin, tubulin and intermediate filaments such as MreB, FtsZ and Crescentin, respectively (18). The presence of MreB in different species of bacteria and the similarity of their sequence to eukaryotic actin, suggest that the MreB-like proteins have an actin-like role in bacterial cell morphogenesis (19).

MreB orthologues are found in most rod-shaped, helical and spiral-shaped bacteria though there are a few exceptions and are generally absent in spherical shaped bacteria (20) (18). MreB null mutants grow as enlarged spherical cells in organisms like *E. coli* (21), *B. subtilis* (19) and *Caulobacter crescentus* (22).

Actin contains a nucleotide binding pocket at the central core that interacts with ATP/GTP and hydrolyses it for controlling self-polymerization (23) forming filamentous structures *in vitro* (24). Initial reports suggested that MreB polymers assembled as continuous helical structures around the axis of many rod shaped bacteria like *B. subtilis* (19) and *E. coli*. But recent report suggests that it's found in patches around the cytoplasmic membrane (25). MreB polymers form bundles which were believed to determine the cell shape but these polymers are highly dynamic structures and possibly not stable enough to support a sturdy cell shape (24) Although most recent studies indicate that MreB is found in patches which scaffolds the peptidoglycan elongation machinery and could also be involved in cytokinesis (26) (27) (28).

b. MreB and Peptidoglycan in *Chlamydia*:

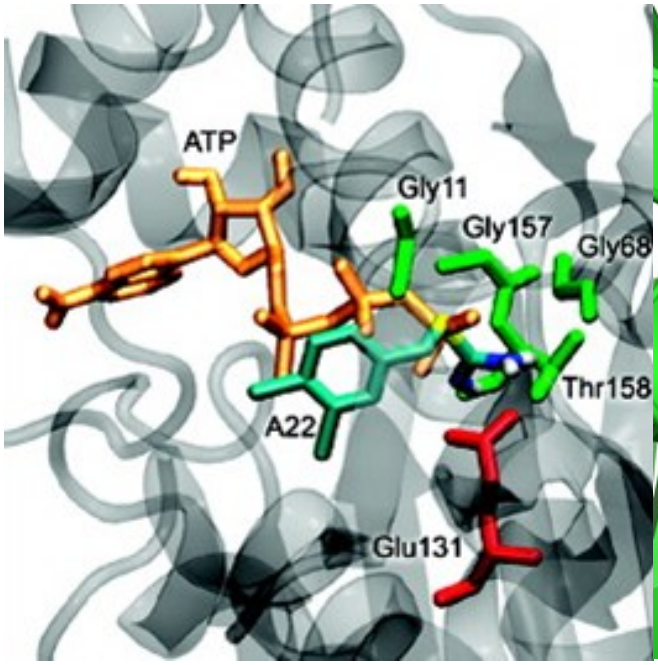
Due to evolutionary adaptation, obligate intracellular bacteria have a smaller genome as compared to other free living organisms and only code for essential genes necessary for parasitism (29). Similar feature has been observed in *Chlamydia* which has a reduced genome size of 1.1 Mb as compared to free living *E. coli* with ~4.6 Mb genome size (30). Although *Chlamydia* encodes genes for the cell wall precursors, efforts to prove the presence of peptidoglycan have failed so far (31). Surprisingly, *Chlamydia* are susceptible to antibiotics against cell wall synthesis like penicillin (32). On treatment with these antibiotics, the developmental cycle is arrested with enlarged RB forms which continue to remain in this non-infectious form until the antibiotic is withdrawn. This has been termed as the chlamydial anomaly (33).

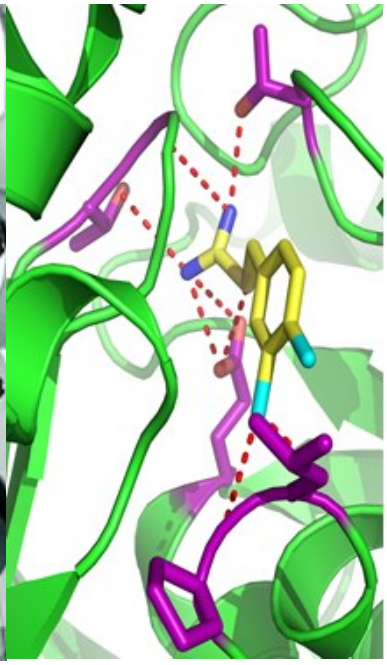
MreB has been known to scaffold peptidoglycan synthesis in many non-spherical organisms and is integral in maintaining their shape (26). *Chlamydia* encodes a few rod-shape determining proteins like PBP2, RodA and MreB (30). Given the spherical shape, it is unusual that MreB is present in *Chlamydia.* FtsZ, an essential cell division protein in most bacteria, is found to be absent in *Chlamydia* (34) (30). A definitive role of MreB has not yet been determined although an intriguing hypothesis of its involvement in cell division has been proposed stating that MreB may substitute for FtsZ as the central co-ordinator of the cell division machinery (35). Chlamydial MreB shares a 54% sequence identity to the *T. maritima* MreB and has conserved the nucleotide-binding pocket required for polymerization.

Figure 2: Predicted A22 binding site in MreB

2A shows predicted A22 binding sites in the nucleotide binding pocket of MreB from *T. maritima*. ATP is shown in its crystal position, demonstrating the competition between the binding sites of its β- and γ-phosphates and A22. 2B shows predicted interactions of A22 with similar residues in the nucleotide binding pocket of chlamydial MreB.

A





B

A small molecule, A22 (S-3,4-dichlorobenzyl isothiourea), has been shown to inhibit MreB polymerization in many non-spherical organisms (36) (37). It not only causes loss of the helical MreB structures but also causes cell shape alterations (37). This small compound has been a very valuable chemical tool to better understand the role of MreB in bacteria. A22 is said to competitively bind to the nucleotide binding pocket of MreB, inhibiting it polymerization. When *Chlamydia trachomatis is* treated with A22, it results in aberrant RBs and hence MreB is assumed to be involved in the process of cell division in *Chlamydia* (35). Also, when *Chlamydia* is treated with this compound, the EB formation is affected and no progeny are produced (observations courtesy Lindsay Sammons). The exact mechanism of how A22 affects chlamydial MreB is yet to be determined and so is the role of MreB. Computational predictions have shown residues that might be involved in A22 binding in MreB from *T. maritima* (38) (Fig. 2A) and similar predicted sites were seen in chlamydial MreB (Fig. 2B -Courtesy: Dr. Michael Barta). Recently it has been observed that A22 might have other cellular targets in bacteria (39). Thus, it is possible that A22 has a different cellular target in *Chlamydia* other than MreB. Here we conduct *in vitro* studies to elucidate the possibility of A22 affecting MreB polymerization in *Chlamydia trachomatis.* We also used a genetics approach using chemical mutagenesis to isolate A22-resistant mutants to check if chlamydial MreB was a direct target of A22. *In vivo* localization and quantitative RT PCR studies were performed to study the possible role of MreB in *Chlamydia trachomatis.*

Materials and methods:

Cloning of pET21b/ pT7HMT:

The plasmid pT7HMT [variant of the commercially available expression vector pET28 (Novagen)] (40) with Kanamycin resistance, was obtained from Dr. Michael Barta. The vector pT7HMT (5.4 Kb) was digested with BamHI restriction enzyme, and full length MreB sequence was ligated into the vector using the infusion kit (Clontech,USA) and transformed into α-Select Gold Efficiency cells (Bioline,USA). Colony was selected and sequenced to confirm insert using MreB specific primers [Forward primer 5'-GGGGTCGACAGGATCCATGACCCCA-3' and reverse GCGGGCTAGCGGATCCTCATACTAAA-3']. Selected colony was then transformed into the expression cells, BL21(DE3) (Agilent Technologies). Plasmid purification was done using the Miniprep Kit (Qiagen,Valencia Ca) and a clone containing full length MreB was verified for protein expression by western blot. The C-terminal His-tag (MreB full length) and the N-terminal His-tag (MreB full length) constructs were made by Shauna Moore and Lindsay Sammons, respectively.

Expression and protein purification by denaturation-renaturation protocol:

Both the C-terminal His-tag and N-terminal His-tag constructs (pET21b vector) were expressed in Acella™ Chemically Competent Cells (Edge BioSystems). Cultures grown at 37° C in Luria Broth with 100 μg/mL ampicillin were induced with IPTG of final concentration of 1 mM at OD_{600} of 0.6 and grown overnight at 15° C. The clone, MreB-pT7HMT, was expressed in presence of kanamycin (50 μg/mL). Overexpressed recombinant protein was purified under denaturing conditions, as it was found in the insoluble fraction. Pellets of induced bacterial cells

were obtained by centrifugation at 8,000 x g for 10 minutes. The pellet was resuspended in 50 mL wash buffer (10mM HEPES, pH 7.2, 5mM EDTA and 0.1% Triton X-100) and sonicated on ice for 2 minutes and centrifuged at 15,000 x g for 30 minutes. The pellet was then washed 3 times with wash buffer and resuspended in 5mL phosphate buffer (pH 6.8) with 6 M guanidine hydrochloride for 1 hour on ice. The supernatant containing the desired recombinant protein was obtained after a final centrifugation at 39,000 x g for 20 minutes. The protein was purified from this supernatant using a cobalt resin column and washed and eluted according to the manufacturer's instructions (TALON His-Tag Purification Resin, Clontech). The denatured protein was refolded by step wise dialysis in refolding buffer (30 mM Tris-HCl pH 7.6, 200 mM KCl, 1 mM EDTA, 5 mM DTT, 10% [v/v] glycerol) with decreasing concentrations of urea (6, 3, 2, 0.5, 0, 0 M) for 4 hours each at 4° C. Refolded protein was divided into 100 µl aliquots and stored at -20° C. Alternatively the rapid-refolding was used where denatured protein was diluted tenfold, rapidly, by injecting it into a beaker with constantly stirring refolding buffer (20 mM Tris-HCl pH 7.6, 500 mM NaCl) and then loaded on the Ni-NTA resin to purify the recombinant protein using manufacturer's instructions (GE healthcare).

SDS-PAGE analysis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini-PROTEAN Tetra Cell System (Bio-Rad Laboratories, Hercules, CA) using a 12 % separating gel and 5 % stacking gel.

Analytical size exclusion chromatography:

About 0.1 mg/mL of refolded MreB was applied to a Sephacryl S200 BIORAD equilibrated with 20 mM Tris-HCl (pH 8) and 500 mM NaCl. Previously, to generate a standard

curve, a protein standard containing Thyroglobin (670 kDa), IgG (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), B12 (13.5 kDa) (BIO-RAD, Hercules, CA) were used.

Transmission electron microscopy sample preparation and imaging:

3 µM concentration of refolded MreB was used for TEM imaging. 10µl of protein sample was laid on to a Carbon coated copper grid and was set aside for 5 minutes at room temperature. Excess sample was drained and the sample was stained with 2% UA (20 µls) for 20 mins. Sample was imaged using FEI Tecnai F20 XT Field Emission Transmission Electron Microscope.

In-vitro polymerization assay:

MreB polymerization assay was performed as previously described by Bean and Aman (41) with specific modifications. The reaction, in a final volume of 800 µl, contained 5 µM MreB, 200 µM ATP, 5 mM $MgCl_2$, 1 mM EGTA, and 10 mM imidazole buffer, pH 7. The non-protein components were first mixed. Separately, MreB was mixed with $1/9^{th}$ volume of 10× cation exchange buffer (1 mM $MgCl_2$, 10 mM EGTA) on ice and incubated for 1 min. By mixing these two solutions, polymerization reaction was initiated at room temperature and monitored for 20 min. Polymerization was observed using Laser light scattering at 532 nm wavelength. All the experiments were performed in triplicate. To study the effect of A22 on polymerization, A22 (dissolved in DMSO) was added to a final concentration of 10, 50, 100 and 300 µM, to the non-protein half of the polymerization reaction. DMSO without A22 was used as a control to show that DMSO didn't affect the polymerization reaction.

In-vivo localization:

Anti-MreB Antibody purification:

a. Using affinity purification on nitrocellulose membrane:

15-20µg of protein (MreB) was separated on a SDS-PAGE gel. The protein was electrophoretically transferred to a nitrocellulose membrane. Ponceau staining of the membrane was performed to identify protein bands. Vertical strips of the membrane containing the protein (approximately 1 cm in width) were cut. In a 50 mL conical tube, the membrane was blocked with PBS/Tween. Anti-MreB antiserum (from rabbit) in PBS/Tween was added and incubated overnight at RT while rocking. The membrane was washed 3X with PBS/Tween (10 minutes each) and 2X with PBS (10 minutes each). The strips were then cut into small squares and placed into a new 50 mL conical. 3 mLs of 100 mM Glycine (pH 2.5) was added and rocked at RT for 15 minutes. The reaction was neutralized with 300 µls of 1M Tris (pH 8.0) and dialyzed overnight in cold PBS. The purified antibodies were then assayed for reactivity.

b. Using coupling resin:

Anti-MreB antibodies were also purified using AminoLink Plus Coupling Resin (Thermo Scientific) according to the manufacturer's instructions. Purified Antibodies were then assayed for reactivity.

Chlamydial infection and EB seed Prep:

In a spinner flask, one liter RPMI (Mediatech Inc, Manassas, VA) supplemented with 10 µg/mL gentamycin and 5 % fetal bovine serum (FBS) was used to grow L929 mouse fibroblasts. Cells were grown to 8 x10^5 cells/mL. At a multiplicity of infection (MOI) of ~1, cells were infected with *C.trachomatis* L2/434/Bu and transferred into 250 mL sterile centrifuge tubes at 48

hpi. Cells were centrifuged at 1380 x g for 10 minutes at 15^0C, the supernatant was removed and pellet was then washed with 200 mL Hank's Balanced Salt Solution (Life technologies). Cells were resuspended in 50 mL HBSS and 40 mL sterile Oak Ridge tubes. The cells were sonicated on setting 6 (Sonic Dismembrator 100), 6 times for 15 seconds each on ice. After centrifugation at 150 x g for 10 minutes at 15^0C, the supernatant was transferred to 40 mL Oak Ridge tubes. This supernatant was again centrifuged at 28,000 x g for 30 minutes at 4^0C. The pellet was resuspended in 2 mL SPG buffer and sonicated two times for 3 seconds each on setting 4 (Sonic Dismembrator 100) on ice. Aliquots of the chlamydial seed were stored at -80 ^{0}C.

Anti-MreB immunoblot:

Immunoblot was performed by separating MreB on a SDS-PAGE gel followed by electrophoretic transfer to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk solution in PBS/Tween at RT for 1 hour. 1:50 dilution of affinity purified primary anti-MreB antibody (from rabbit) was used for primary staining by rocking at RT for 1 hour followed by 3 washes of 10 minutes each at RT using PBS/Tween. Secondary antibody (diluted 1:5000) was used for secondary staining by rocking at RT for 1 hour. The membrane was imaged using Odyssey Infrared imager (LI-COR Biosciences).

MreB localization in *Chlamydia trachomatis* using immunofluorescence assay:

In an µ-Slide eight-well plate (IBIDI, Munich Germany), L929 mouse fibroblast cells were plated at the concentration of 1.2 x 10^5 cells/mL in RPMI (Mediatech, Inc.) containing 10 µg/mL Gentamycin and 5 % FBS. ~90% of the plated host cells were infected *C. trachomatis L2/434/Bu* and fixed using methanol at 18 hpi, 30 hpi and 48 hpi. Fixed samples were stained

using specific primary and secondary antibodies for visualization. Fixed cells were incubated with purified primary rabbit anti-MreB antibodies and diluted to 1:10 in PBS and mouse anti-MOMP antibodies (diluted to 1:500 in PBS) for 1 hour at RT. Cells were washed 3 times for 10 minutes each with PBS and then incubated with Alexa fluor 568-labeled (Life Technologies) goat anti-rabbit secondary antibody (diluted 1:1000), and Alexa fluor 488-labeled (LifeTtechnologies) anti-mouse secondary antibody (diluted 1:1000) for one hour at RT in dark. Cells were then washed 3 times with PBS for 10 minutes each to remove excess secondary antibodies. Cells were then stained with DAPI (diluted 1:1000) using Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories) for 10 minutes at RT. Samples were visualized using Olympus/3I Spinning Disk Confocal microscope (MAI facility, KU).

Expression profiling:

RT-PCR (Quantitative gene expression):

Total RNA was isolated from *C. trachomatis L2/434/Bu*-infected L929 cells every 6 hpi through 36 hpi using TRIzol® Reagent (Life Technologies, Grand Island, NY). Isolated RNA was treated with DNase using the TURBO DNA-free kit (Life Technologies) according to the manufacturer's protocol. Concentration of RNA samples were determined by spectrophotometry. For each RNA sample, cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instruction. Primer pairs for each gene target (Table 1) were designed and verified for efficiency using serially diluted cDNA as a template for PCR. Resulting C_T values were used to generate standard curves using the StepOnePlus software (Applied Biosystems, Life Technologies) and confirmed that primer efficiency was suitable for relative quantification using the comparative

Table 1: Oligonucleotide sequences used in real time RT-PCR amplification.

Gene	Forward Primer sequence	Reverse Primer sequence
CT009 (rodZ)	5'-AAGCAGGACATCTCGGGAAA-3'	5'-TCAGCAATCTATCCCCATCCA-3'
CT046 (hctB)	5'-AGTAGCTTCAACAAAAAAATCTTCC-3'	5'-CCAACTGTGAGCTGTACGAGAAC-3'
CT190 (gyrB)	5'-ATCACGGTCATCTTCAAAAACAATC-3'	5'-CGACTTTACAGGTGCTTGGTCC-3'
CT510 (secY)	5'-GGACGAATGACACGGCTTTTTAC-3'	5'-TACCCAAGGCACTCCAAACAGC-3'
CT709 (mreB)	5'-TTCCGGGAATGTTGGTATCG-3'	5'-GCCACAACGGAAGGTTCACT-3'
CT726 (rodA)	5'-CCCTTCCAACCATCACCAAA-3'	5'-TCGCCAGATTTCCATCCTTG-3'
CT075 (dnaN)	5'-CTTTTCCCCTGTGATCTCCA-3'	5'-GAAAAGGGCCACTTGTTTGA-3'
CT545 (dnaE)	5'-CGATCACGGGAATTTGTTTGGC-3'	5'-GTTGGCAACTCGGCTTTTTC-3'
CT507 (rpoA)	5'-ACCAAAAGCAACTCGCAACAT-3'	5'-CTTTTGAAGCGGTCAATCCTG-3'

C_T method ($2^{-\Delta\Delta C_T}$) (42). Applied Biosystem's StepOnePlus Real-time PCR system was used for relative quantification of target genes. To verify that RNA samples were free of contaminating

DNA, real-time PCR was performed using respective RT-negative reactions for each RNA sample. Relative quantification was performed for each gene target and for each time point using the comparative C_T method provided by the StepOnePlus (Applied Biosystems). Reactions were set up in duplicate with each mixture containing 500 nmol of each primer and Fast SYBR Green Master Mix (Applied Biosystems). Endogenous control, *secY* (CT510) (43), was used as the internal sample normalizing control for all target genes and to reveal patterns of expression throughout the developmental cycle, relative quantification values were calibrated against the 6-hpi time point for each gene target.

Forward genetics:

EMS mutagenesis:

L929 mouse fibroblast cells were plated at a concentration of 4.9 X 10^6 cells/mL in a T75 flask with 10 mL RPMI (Mediatech Inc, Manassas, VA) supplemented with 10 µg/mL gentamycin and 5 % FBS. Cells were infected with *C. trachomatis L2* and at 19 hpi were treated with EMS (diluted in PBS) for an hour at RT. The concentrations of EMS used were 4 mg/mL, 12 mg/mL, 18 mg/mL and 20 mg/mL. After washing with PBS 3 times, 10 mL of RPMI (Mediatech Inc) containing 10 µg/mL gentamycin and 5 % FBS was added and incubated at 37^0C to recover for 48 hours.

RESULTS:

1. Purification of recombinant chlamydial MreB:

To study the potential polymerization properties of the actin-like chlamydial protein MreB, recombinant MreB was expressed in *E. coli* and purified using Ni-NTA resin. Native purification of MreB was not possible as most of the overexpressed protein remained in the insoluble fraction. Also obtaining nucleotide free MreB was necessary for studying its polymerization properties. Using the denaturation-renaturation protocol, nucleotide free protein could be purified.

In the presence of 6 M Guanidine hydrochloride, MreB was obtained in the denatured form and potentially refolded by subsequent removal of the denaturing agent by dialysis. Distinct band of the recombinant MreB was observed by Coomassie blue staining of the SDS-PAGE gel (Fig 3A and 3B). Expected band was observed around 45,000 kDa. Fig 3A shows the MreB expressed using the pT7HMT construct while Fig 3b shows recombinant C-terminal His-tagged MreB and N-terminal His-tagged MreB expressed from the vector pET21b. Upon refolding, MreB was found to pellet when subjected to high speed centrifugation suggesting that MreB might be forming polymers/aggregates on refolding even in the absence of any nucleotide. The purified protein samples were centrifuged and the supernatant potentially containing monomeric MreB was used for further polymerization assays.

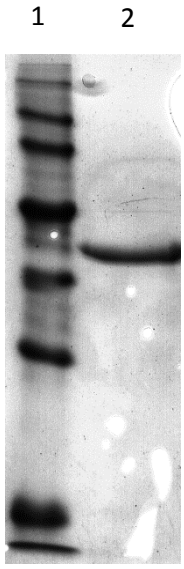
2. MreB forms oligomers *in-vitro*:

Prokaryotic MreB has been shown to form protofilaments *in-vitro* using electron microscopy in *T. maritima* (44). The only crystal structure of MreB available is from *T. maritima.* Monomeric MreB in *T. maritima* is known to form long rigid polymers *in-vitro* which are a ~3.3

Figure 3: Protein Purification of recombinant MreB.

Recombinant MreB purifies as a monomer but can form polymers easily. A) MreB purified from pT7HMT vector. Lane 1 shows protein marker while Lane 2 shows purified recombinant MreB B) MreB purified from pET21B. Lane 1 shows the protein marker, Lanes 2-4 show different aliquots of C-terminal His-tagged MreB, and Lanes 5-7 show different aliquots of N-terminal His-tagged MreB.

A



B

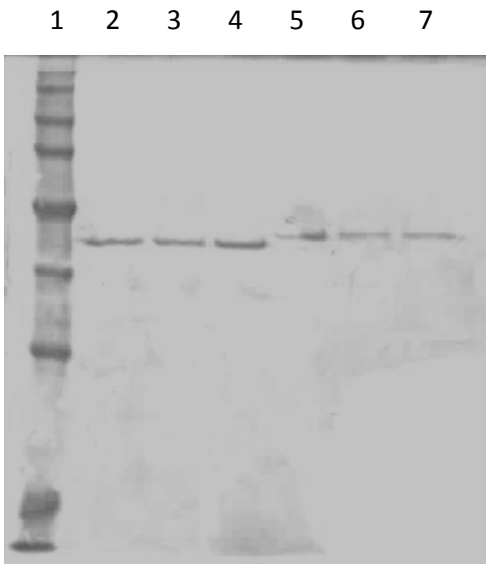
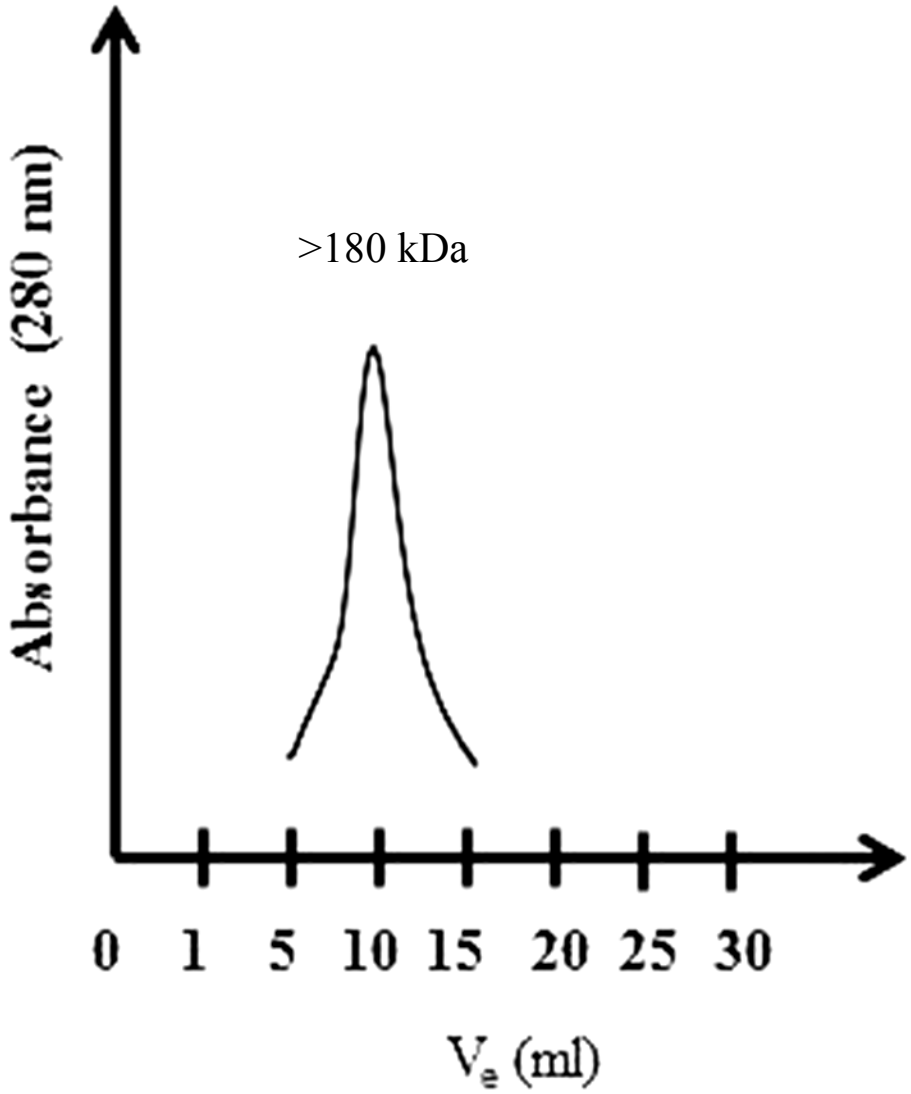


Figure 4: Recombinant MreB purifies as an oligomer.

To determine the *in vitro* oligomeric state of the purified recombinant MreB (before centrifugation), analytical size exclusion chromatography was used. The protein was eluted at a high molecular size that was much larger than the monomeric size of the protein, i.e. 45 kDa.


Absorbance (280 nm)
>180 kDa
0
1
5
10
15
20
25
30
Ve (ml)

μm in length and these protofilaments are also known to form sheets through lateral contact in organisms like (44).When purified recombinant full length MreB (before centrifugation) was subjected to size exclusion chromatography, the protein was observed to form oligomer of a much larger molecular weight (Fig 4). Monomeric full length MreB has a molecular weight of ~ 40 kDa and the His-tag with the linker was ~5 kDa. These oligomers were observed to be more than 180 kDa which would contain about 4-5 monomeric MreB.

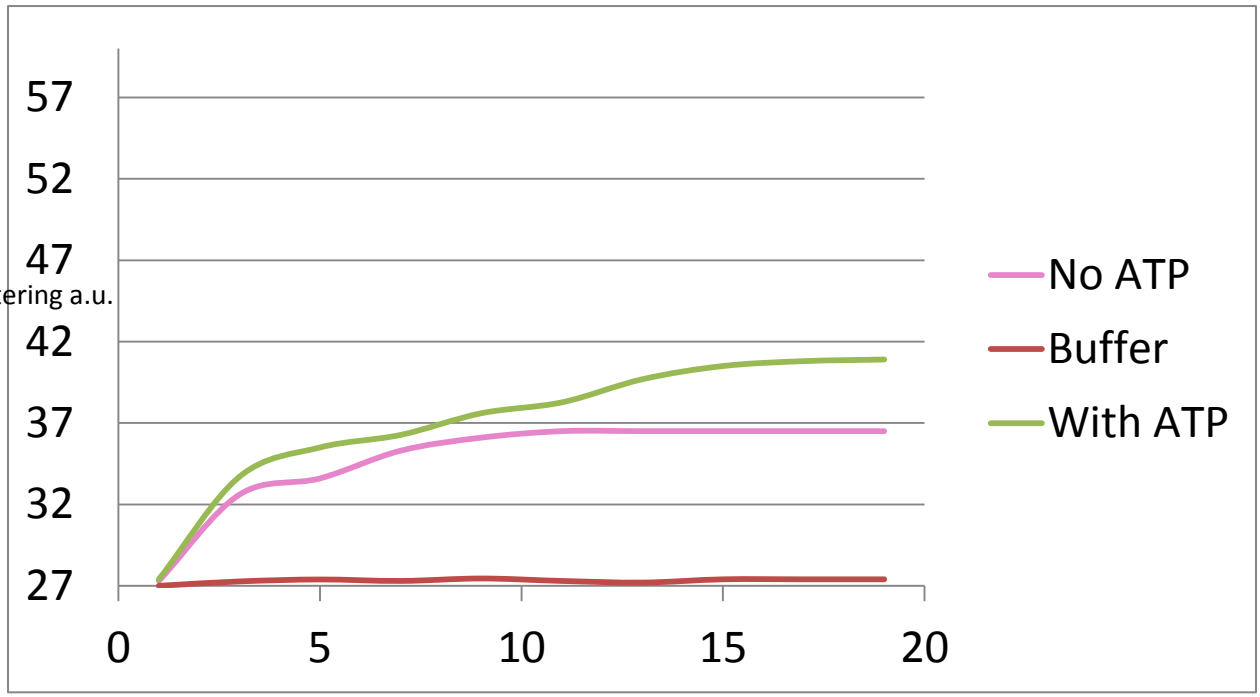
3. MreB shows time dependent increase in light scattering:

Recently, *in-vitro* polymerization studies with MreB from *Chlamydia pneumoniae* showed that chlamydial MreB has the ability to polymerize in solution (45). MreB has also been shown to form filamentous polymers *in vitro* from other organisms as well.

To investigate the role of MreB in *Chlamydia trachomatis,* standard polymerization assay with slight modifications were used to study its polymerization properties. Polymerization was performed at pH 7.4 using standard conditions at room temperature, and polymerization was observed using laser light scattering (B&W TEK INC.) The wavelength of laser was 535nm. Laser was controlled using the Flex v2.10 software, and the images were captured using Measurements and automation explorer (version 3.1). Light scattering was quantified every two minutes using Adobe Photoshop and graph was plotted with time on X-axis and light scattering units (a.u.) on the Y-axis. Buffer without MreB was the negative control for light scattering. An increase in the light scattering units was observed when MreB was added to this polymerization reaction with ATP (Fig 5). Increase in light scattering was also observed without adding ATP. Such a property of MreB to polymerize *in vitro* in the absence of a nucleotide has been previously reported in other organisms including *Chlamydia pneumoniae* (45).

Figure 5: MreB polymerizes in-vitro

Laser light scattering assays were performed at 532 nm using 5 μM chlamydial MreB *in vitro.* Light scattering was measured in arbitrary units (a.u.)



Laser light scattering a.u.

Time in minutes

4. MreB polymerization is not inhibited by S-(3,4-dichlorobenzyl) isothiourea (A22):

A22 is a compound which converts rod-shaped *E. coli* cells into spherical shaped cells (46) It also has been shown to inhibit the polymerization of MreB in *T. maritima,* which is ATP dependent *in vitro* (38). Recently it was found that A22 couldn't inhibit the polymerization of MreB of *C. pneumonia in vitro* (45).

We studied the effect of different concentrations of A22 on MreB polymerization *in vitro* from *C. trachomatis* using laser light scattering. As seen in *C. pneumonia* previously (45), MreB polymerization wasn't impaired in *C. trachomatis* by A22 *in vitro* (Fig 6). On the contrary, it was observed that the rate of polymerization increased as the concentration of A22 increased. To test whether the presence of A22 is the cause of light scattering, an A22 control (polymerization buffer with 300 μM A22 and no protein) was used. It was observed that A22 doesn't increase light scattering with time. As a positive control, polymerization of MreB was observed in the presence of ATP and in the absence of A22.

5. TEM imaging reveals MreB polymerizes in-vitro:

Refolded MreB was observed to form oligomers *in vitro* using size exclusion chromatography and it has been difficult to differentiate aggregates from polymers. To confirm the state of oligomers, transmission electron microscopy was used to determine the ultrastructure of MreB *in-vitro*.

TEM processing and imaging were used to prepare samples and test MreB polymers versus aggregates *in vitro*. MreB has been seen to form aggregates as well as filaments ranging between 40-50 nm in length and about 10 nm in width in *Caulobacter crescentus (47)*. MreB polymers observed in *T.maritima* were about 3.3 μm in length.

Figure 6: A22 doesn't inhibit MreB polymerization *in vitro*

Influence of increasing concentrations of A22 was observed on MreB polymerization *in vitro.*

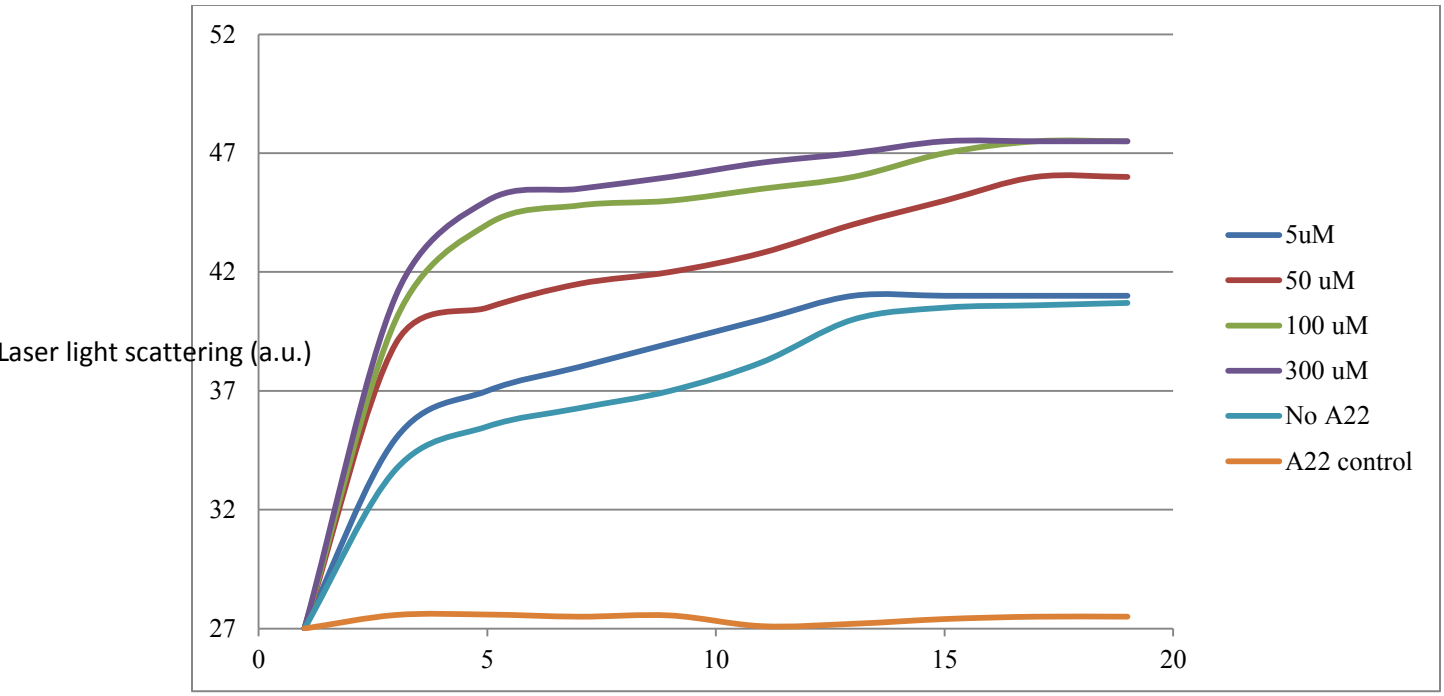
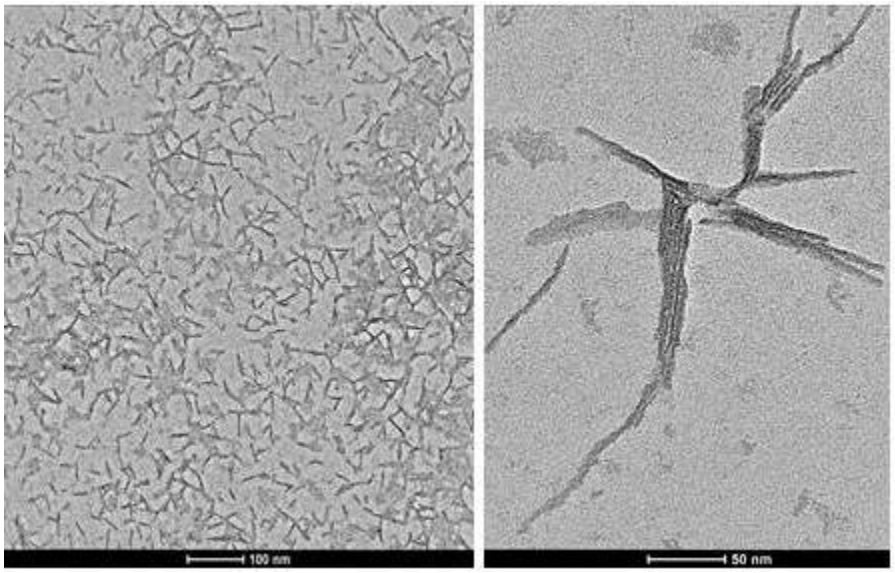

52
47
42
37
32
27
0
5
10
15
20
Laser light scattering (a.u.)
Time in minutes
5uM
50 uM
100 uM
300 uM
No A22
A22 control

Figure 7: TEM images reveal MreB polymers

Electron microscopy of refolded MreB on carbon-coated copper grids was performed. A) At the concentration of 3 μM of refolded MreB, filaments were observed ranging from 40-80 nm in length, at low magnification. The protein sample contained 500 mM NaCl and no ATP. B) At higher magnitude the filaments were seen to form a number of bundled protofilaments.

A



B

Aggregate like structures were at times observed on the EM grids. But one of the samples at the concentration of 3μM revealed rod-like polymeric structures which were 40-80 nm in length and ~8 nm in width (Fig 7). The buffer contained no ATP and 500 mM NaCl at pH 7.4 and the sample was prepared at RT using aforementioned TEM sampling protocol. These observed polymers were shorter than those observed in *T. maritima.* MreB polymers also exhibited branched structures and protofilaments in lateral conformation forming sheet-like structures. These polymers were only observed once although multiple attempts with the same conditions and the protein concentration were used.

Previous MreB polymerization studies done in *Chlamydia pneumoniae* were performed using light scattering experiments. Techniques like transmission electron microscope haven't been used to verify if these light scattering studies were due to polymerization or aggregation. Here our aim was to verify these polymerization properties of MreB not just using laser light scattering but also using TEM to visualize these polymers/aggregates. Though the initial attempt to visualize such polymers was successful, we were not successful in observing any polymers using TEM in any of the consecutive samples. Another possibility for the lack of visualization of polymers by TEM could also be optimization of the technique of attachment of the protein to the grid, uranyl acetate staining, or TEM imaging.

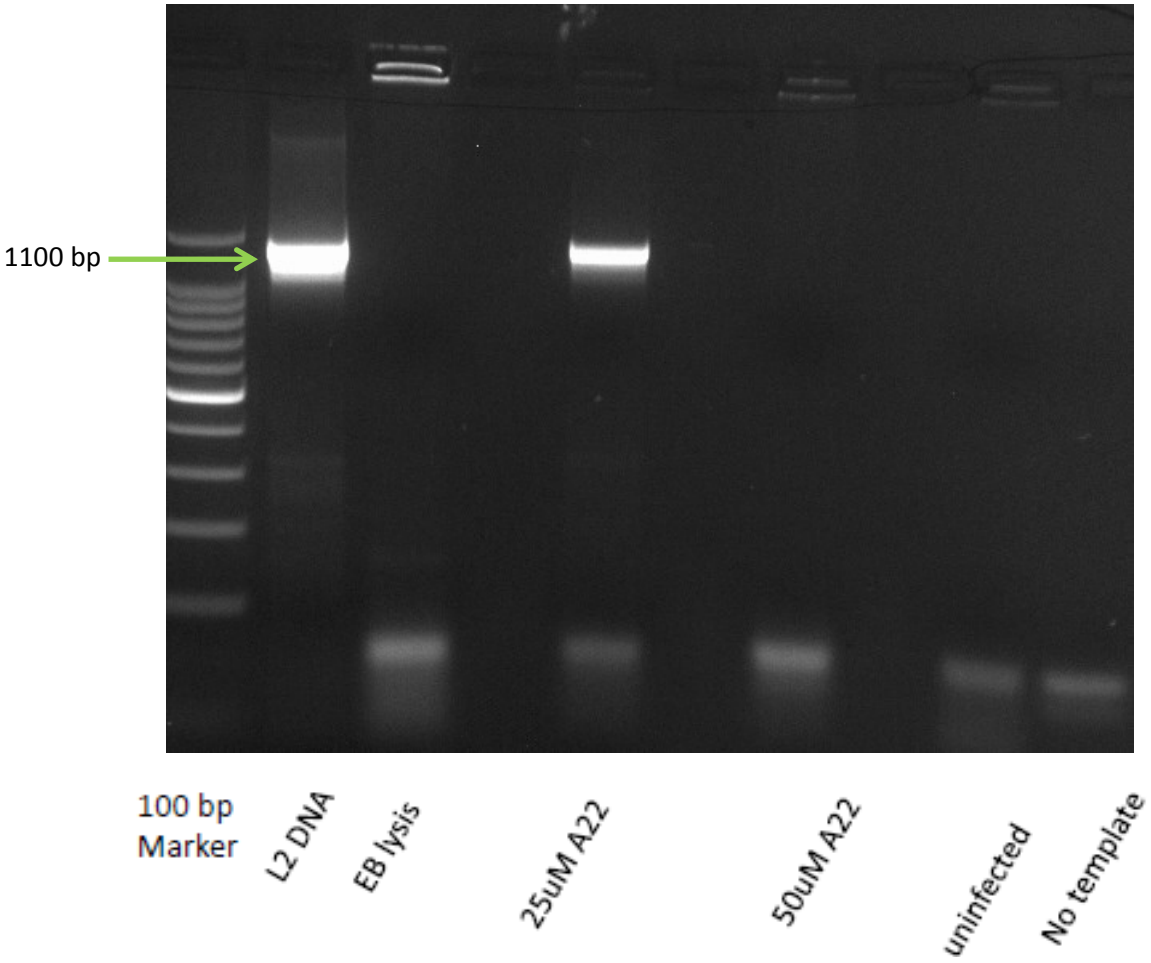
6. Chemical mutagenesis yields isolates with lower sensitivity to A22:

Alternatively, a genetic approach was used to isolate MreB mutants using chemical mutagenesis. Chemical mutagenesis using EMS (ethyl methyl sulfonate) has been previously used to induce random mutations in *Chlamydia* (48). *Chlamydia trachomatis* was treated with EMS between 18-19 hpi, when RBs start replicating. After

undergoing 72 hours of recovery and replication, selective pressure was used to isolate A22 resistant organisms in the presence of the drug. Two different concentrations of A22 at which inhibition of progeny formation was observed were used. It was observed that at higher concentration of A22 (50 µM) no chlamydial inclusions were observed. At 25 µM A22, a few chlamydial inclusions were observed, which were subjected to multiple passaging in presence of A22. Using glass beads, cell lysis was performed and these isolates were purified and stored at -80^{0}C. These isolates were then used to detect any alterations in the *mreB* sequence. *mreB* specific primers were used to amplify this gene and was detected on a 1.5 % agarose gel (Fig 8). The amplified gene was sequenced. When the sequence of the mutant and the wild type were compared, a single amino acid substitution was found (E273K). This mutant doesn't propagate in the presence of higher amount of A22 (50 µM). It is possible that these organisms growing and propagating in the presence of 25 µM A22 were not resistant but tolerant to the drug.

Figure 8: PCR amplification of A22 tolerant *Chlamydia trachomatis*

Lysates from EMS treated A22 tolerant infections were used to amplify the *mreB* gene and checked on a 1.5% agarose gel. The 1100 bp fragment was observed in the L2 cDNA (positive control) and the 25 μM lysate. The amplified fragment was further sequenced for mutations.


1100 bp
100 bp Marker
L2 DNA
EB lysis
25uM A22
50uM A22
uninfected
No template

This mutation was found to be in helix 8 (H8) of MreB. This helix is known to be associated with lateral contact between MreB protofilaments. The resistance/tolerance to A22 could also be attributed to a target other than MreB, and genome-wide sequencing of these mutants would give a better idea of the A22 targets in *Chlamydia trachomatis.*

7. MreB detected by immunoblotting

Immunoblotting (western blotting) is an assay used to detect the presence of specific proteins using antibodies (49). To check the specificity of the purified anti-MreB antibodies, a western blot analysis was performed with *E. coli* lysates of recombinant chlamydial MreB and native MreB in EB lysate. These rabbit anti-MreB antibodies were purified from the anti-MreB antiserum using affinity purification and coupling resin protocols as mentioned in materials and methods. Antibodies purified using both protocols showed similar reactivity.

A distinct MreB band was observed in the EB lysate at a molecular weight of 40 kDa (Fig 9. A bright band was detected with the recombinant MreB protein overexpressed in *E. coli* cells. Different dilutions of the purified antibodies were used, and 1:50 (Anti-MreB antibodies: PBS) was found to be the appropriate dilution. This dilution was further used for immunofluorescence assay.

8. MreB appear as distinct puncta

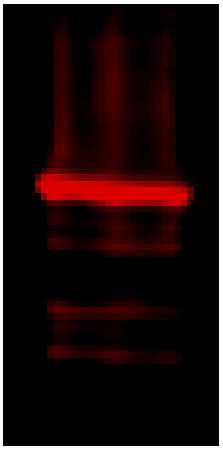
Immunofluorescence assay has been used to detect proteins in cell culture using antibodies specific to protein of interest (50). There are limitations to visualize localization of proteins in *Chlamydia* using immunofluorescence given the size. Nevertheless localization of the protein of interest within an inclusion is possible. Also, using commercially available antibodies against known outer membrane proteins in *Chlamydia,* protein of interest could be

Figure 9: Western blot with Anti-MreB antibodies show specificity to recombinant as well as native MreB

Anti-MreB antibodies were purified and used to study specificity to recombinant and native MreB. Lane 1 shows cell lysate from *E. coli* cells over-expressing recombinant chlamydial MreB. Lane 2 shows native MreB from EB lysate which was detected by the purified antibodies.

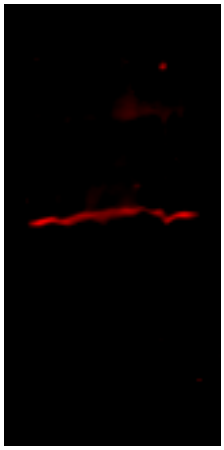
Anti-MreB Immunoblot

Recombinant MreB



E. coli over-expressing chlamydial MreB

Native MreB



EB lysate

studied for co-localization analysis.

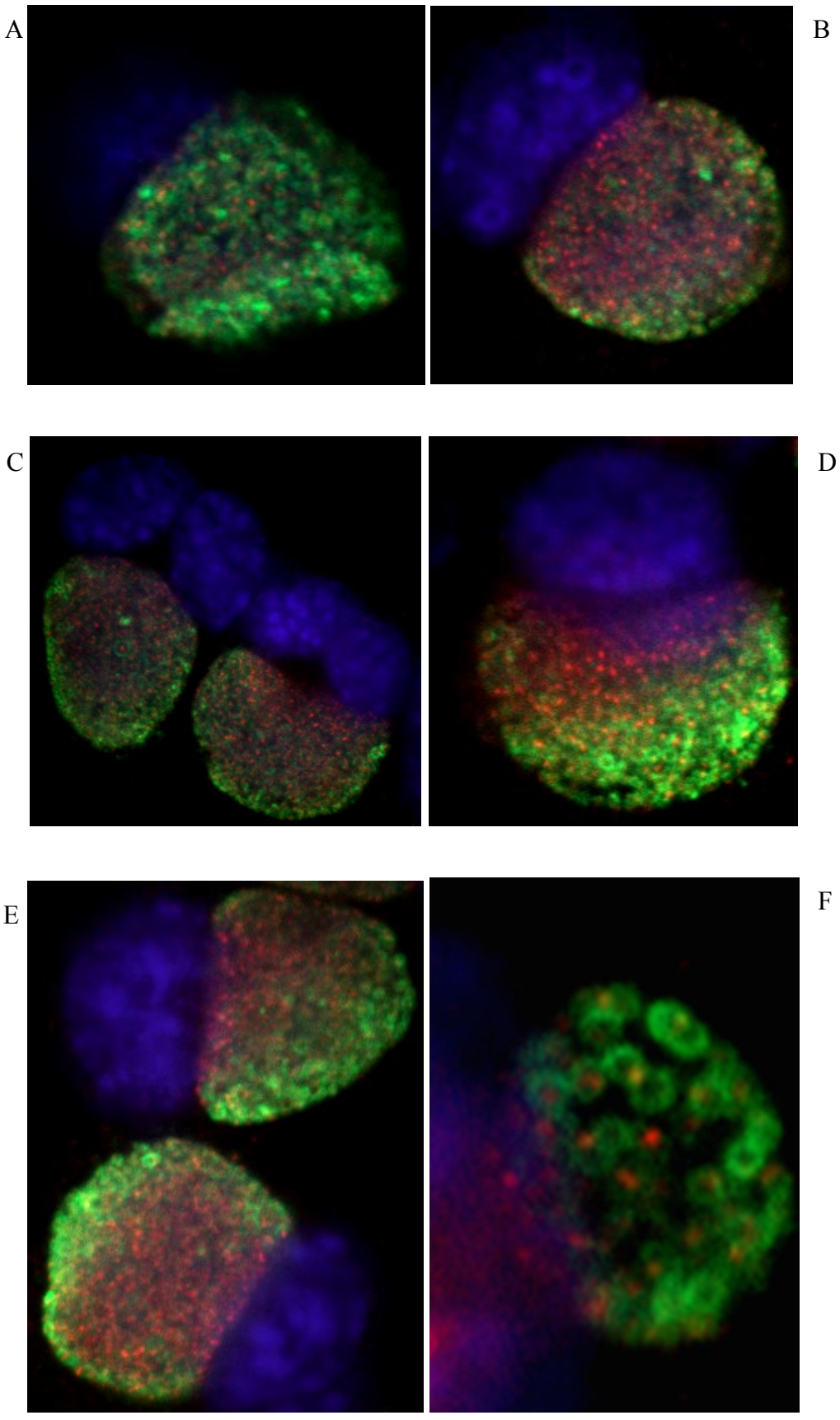
Using IFA and confocal imaging at 100X magnification, it was observed that chlamydial MOMP (major outer membrane protein) and MreB don't co-localize (Fig 10). This shows that MreB might be localizing to an area other that the outer membrane. Samples fixed at a later time point in infection (30-40 hpi) showed MreB to be more concentrated in the center of the inclusion as opposed to MOMP which was brighter at the periphery. MreB appeared as puncta while MOMP appeared as distinct spherical entities (Fig 10 A-E). Also inclusions at an early time point (18 hpi) showed MOMP stained RBs but relatively lesser MreB staining (Fig 10 F). Hence this complies with the observation that MreB levels increase at a later time point in the chlamydial developmental cycle.

9. Relative quantitation expression pattern of MreB

The role of MreB in most other bacteria appears to be associated with peptidoglycan synthesis during elongation of rod shaped bacteria (26). This is typically in concert with RodZ and other peptidoglycan machinery. Bacterial two-hybrid analysis supports chlamydial RodZ interacting specifically with MreB (observations by Kyle Kemege). To elucidate the possible role of MreB in *Chlamydia*, the expression pattern of *mreB* was studied using quantitative RT-PCR.

Figure 10: Immunofluorescence assay to detect MreB

IFA performed at 100X magnification with purified anti-MreB antibodies and anti-MOMP show no co-localization. MreB (red) appear as puncta while MOMP (green) appears to be spherical shaped structures. Also MreB was observed to be concentrated mostly in the center of the inclusion (A-E). IFA at an earlier time point showed smaller inclusion with more MOMP staining than MreB (F).

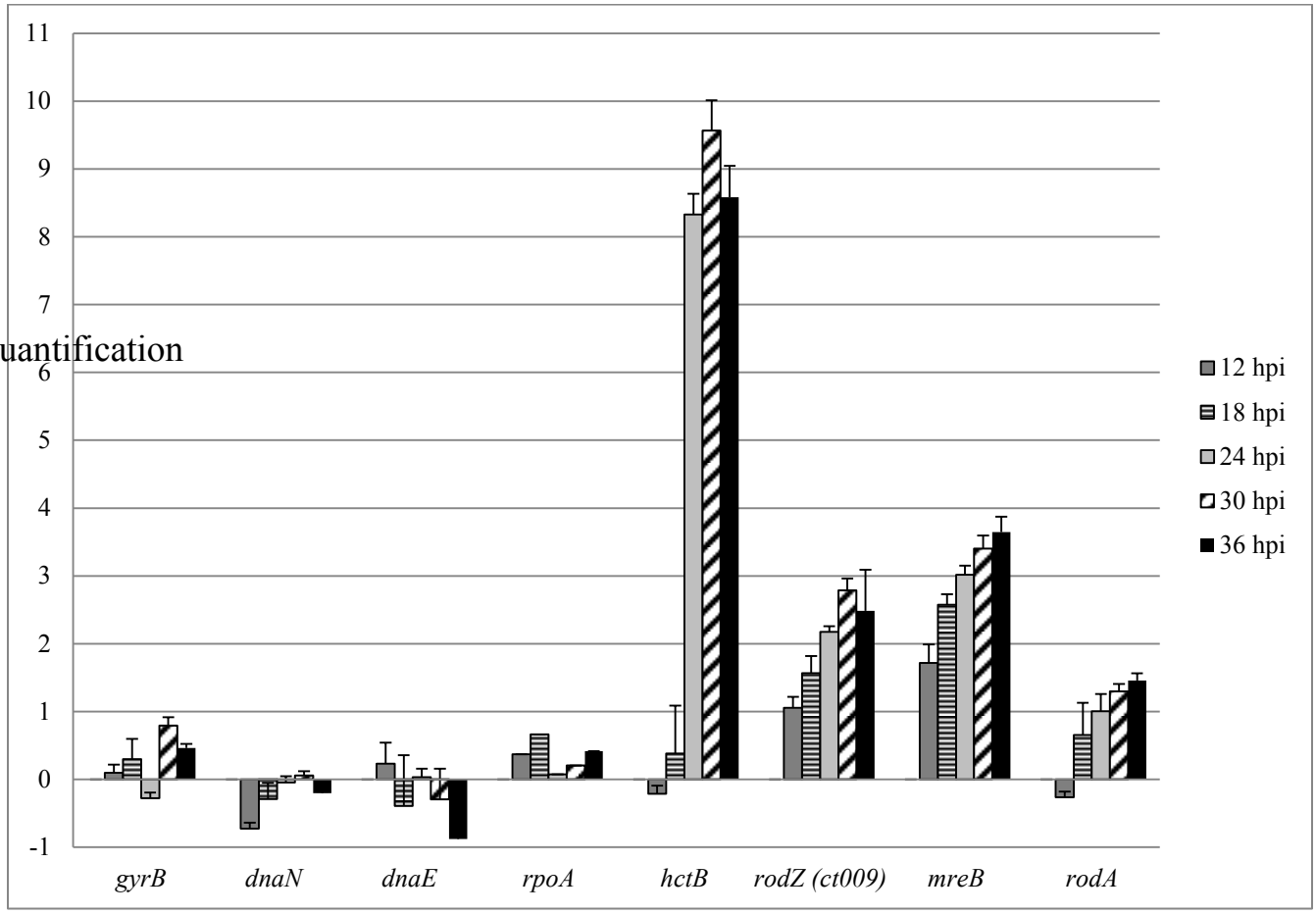

A
B
C
D
E
F

Gene expression analysis was performed on *Chlamydia*-infected L929 cells at 6-hour increments for 36 hpi to study the transcription patterns of MreB and associated throughout the chlamydial developmental cycle. A 4-fold increase in *mreB* transcription was revealed between 12 to 24 hpi. Similarly, *CT009 (rodZ)* was expressed at 12 hpi and upregulated 4-fold by 24 hpi. Expression levels remained relatively constant during the late stage of development (24-36 hpi) for both these genes. *rodA,* which is a cell membrane protein in other bacteria, is upregulated by 2-fold between 6-18 hpi and stays constant throughout the rest of the developmental cycle. *hctB,* which is expressed late in the developmental cycle and has been known to be involved in the RB to EB conversion process, was observed to be expressed at high levels at 24 hpi and remains fairly constant through 36 hpi, as expected. Controls for constitutive gene expression patterns of genes associated with transcription (*rpoA)* and genes associated with DNA replication (*gyrB, dnaN and dnaE)* were used (Fig 11).

These qRT-PCR values are normalized to the expression levels of *secY* which is an inner cell membrane protein and is known to be constitutively expressed throughout the chlamydial developmental cycle. Hence these qRT-PCR values are not indicative of quantity of expression of individual gene transcripts but pattern of expression of each gene transcript relative to its expression at 6 hpi and to the expression at s*ecY* transcript levels. It was observed that the expression pattern of *mreB* was upregulated at later time points indicating a possible role in BR to EB conversion.

Fig 11: Quantitative gene expression analysis

Total RNA was isolated at 6, 12, 18, 24, 30, and 36 hours post-infection from L929 cells infected with *Chlamydia trachomatis L2*. Complimentary DNA was synthesized and used as a template for relative gene expression quantification of *CT009 (rodZ), mreB,* and *rodA*. Transcript quantity was transformed to a $\log_2$ ratio. The constitutive control *secY* (CT510) was used to normalize expression. The constitutively expressed gene, *gyrB* (CT190) and the *hctB* (CT046) (RB-to-EB conversion associated control) were included. Expression patterns of *dnaN*, *dnaE* (associated with DNA replication) and *rpoA* (associated with transcription) were used as constitutive controls for different cellular functions. 6-hpi time point for each gene was used to calibrate expression ratios. Error bars represent the standard deviation of duplicate samples.



2^{-} Relative Quantification

Target

Discussion:

The aim of this project was to study the role of MreB in *Chlamydia trachomatis L2/434/Bu.* MreB is known to form polymers or aggregates *in vitro*. During purification of recombinant MreB, it was observed that the overexpressed protein stayed in the pellet and wasn't soluble. Hence denatured form of recombinant MreB had to be purified followed by refolding (Fig 3). MreB contains a nucleotide binding pocket, and there is a possibility that recombinant MreB purified under native conditions could already be in the nucleotide bound conformation. Thus denatured purification ensured that the purified MreB was not in its nucleotide bound state.

MreB polymers are known to scatter light and polymerization was studied using laser light scattering technique. MreB was observed to be temperature sensitive and as the temperature increased from 4^0C to room temperature, MreB started forming visible precipitates. To confirm that these precipitates were polymers and not aggregates, TEM was used to visualize these structures (Fig 5). Polymers were observed to be ~8 nm in width and 40-80 nm in length. Previously similar sized structures were seen with *Caulobacter* MreB (47) but much larger polymers were observed with *T. maritima* MreB. Even at 4^0C, MreB tends to form an oligomer observed by analytical size exclusion chromatography. Standard polymerization reaction conditions were used in the polymerization experiments and time dependent polymerization was observed in presence of ATP (200 μM). Surprisingly polymerization was also observed in absence of ATP but at a slightly lower rate.

Even though more than 50% identity is seen between MreB sequences of *Chlamydia trachomatis, Bacillus subtilis* and *Thermogota maritima,* they show different dependence on nucleotides for polymerization and also their susceptibility to A22 which is known to be an

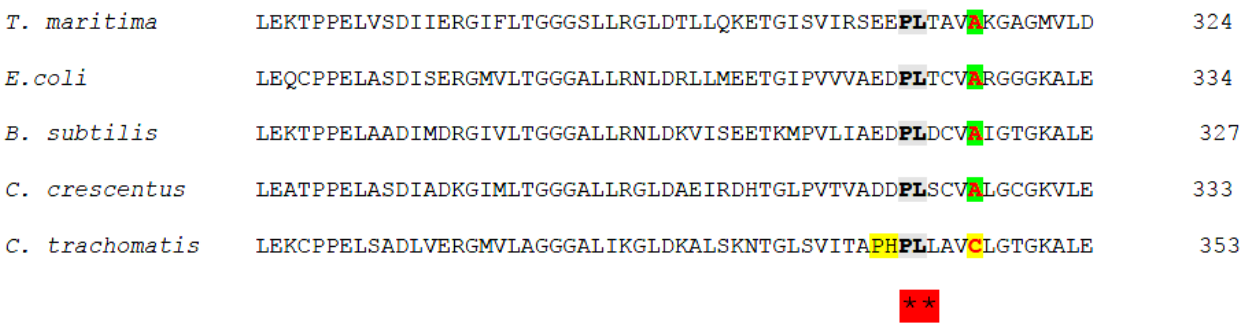
inhibitor of MreB polymerization. Though MreB from *Thermogota maritima* has been seen to polymerize in the presence of ATP (41), MreB from *Caulobacter crescentus* (47), *Bacillus subtilis* (51) and *Chlamydia pneumoniae* (45) was seen to polymerize independent of ATP *in vitro*. Studies in *Chlamydia pneumoniae* (45) also suggest that MreB polymerization changes with cation ($MgCl_2$) concentration. This is indicative of the possibility that stability of MreB must be dependent on cellular conditions like pH, cation availability and interactions with other proteins involved in similar processes as MreB. Actin bound to Mg-ATP under normal physiological conditions is known to have a stable conformation and the equilibrium shifts from a monomer to polymers when exposed to internal/external stimuli and cellular events.

Studies in *Caulobacter crescentus* also show that energy input or ATP hydrolysis is not required for MreB's contribution to cell wall synthesis or its assembly into clusters on the membrane(52). MreB belongs to the superfamily of Hsp70 and one of the features of this superfamily is that the proteins have retained the nucleotide binding pocket but for functions other than polymerization. One of the properties of this family of proteins is that ATP binding and hydrolysis triggers conformational changes that mediate the interaction with other proteins involved in the same function (53). It is possible that MreB uses ATP not to polymerize but to interact with other proteins.

A22 is known to competitively bind to the nucleotide binding pocket of MreB, thus inhibiting its polymerization in *T. maritima in vitro* (38). Susceptibility of A22 to MreB changes for different organisms. *E. coli* lose their rod shape and become coccid on treatment with A22, which also results in slow growth of the organism (46). MreB from *B. subtilis* shows susceptibility to A22 only at a very high concentration (51) while *Caulobacter crescentus* doesn't show any inhibition by A22 (47). Polymerization of chlamydial MreB too doesn't seem

to be affected by the presence of A22 but appears to promote it with an increasing concentration of A22 (Fig 7) even though we're not entirely ruling out the possibility that these might be MreB aggregates and not structured polymer. It has been observed that A22 could bind to MreB in a non-specific manner, inducing aggregation *in vitro* (38). If chlamydia MreB is not a target for A22, it is possible that A22 binds non-specifically, which would result in increased light scattering. MreB is known to interact with other proteins and hence these *in vitro* studies might not be an accurate representation of its functions *in vivo*.

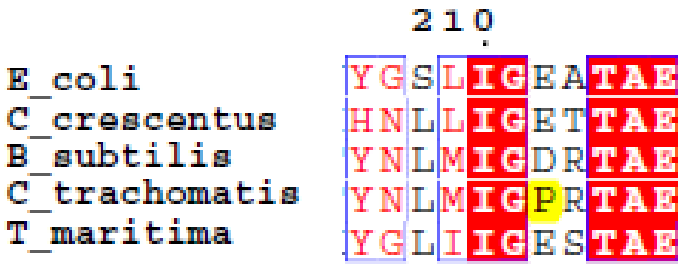
When sequences of MreB were compared from different organisms, a few amino acid substitutions were seen in *Chlamydia trachomatis* as compared to the conserved sequences in other organisms. The D/E at positions 346 and 347 in *Chlamydia trachomatis* is replaced by a Proline (P) and a Histidine (H) respectively. These amino acid substitutions are in the beta sheet structure of the protein right next to the nucleotide binding site which is also one of the A22 binding sites. Proline is known to induce kinks or bulges in a protein structure and it is possible that this substitution changes the nucleotide binding pocket interactions, affecting nucleotide binding or A22 binding.



Oxidative stress is known to disrupt actin structure in eukaryotes by modifying the sulfhydryl group of the cysteine residue (54). The alanine residue is substituted by a cysteine at

position 353 in *Chlamydia trachomatis*. This residue might be responsible for disulphide linkages which change the structure or the interaction of MreB with the nucleotides or with A22.

It was observed in *Caulobacter crescentus* that one of the A22 mutants had an amino acid substitution E213G (E212 in *E. coli*) (52) and this mutations is in the nucleotide binding pocket in the *T. maritima* MreB crystal structure. This amino acid present on the subdomain II is known to form a salt bridge with K49 on subdomain I on the opposite side of the nucleotide binding cleft in *T. maritima*.



These *in vitro* studies gave some interesting results though the role of MreB in *Chlamydia trachomatis* could still not be elucidated. Looking at alternate functions of MreB in other organisms like involvement in spore formation, it could be hypothesized that MreB in *Chlamydia trachomatis* could also play a role in formation of the spore-like EB. Quantitative RT-PCR is a technique to study gene expression patterns and has been applied to study expression patterns in *Chlamydia.* The developmental cycle of *Chlamydia trachomatis* has been divided into three temporal classes of gene expression i.e. i) early genes (6-12 hpi), ii) mid-cycle genes (12-24 hpi) and iii) late genes (post 24 hpi) (55) (56). The early genes are mostly involved in the EB to RB conversion, few genes in the mid-cycle associated with the RB growth and cell division and many of the late genes in converting the RBs back to EBs.

To hypothesize the possible function of *mreB* in *Chlamydia,* expression patterns of this gene transcript was compared to that of other genes involved in different cellular functions like cell division, transcription, DNA replication and RB to EB conversion (Fig 11). *hctB* transcript levels are upregulated at 24 hpi and are exclusively known to be involved in RB to EB conversion. This represents the time-point where morphological changes occur from RBs to EBs. Strikingly, *CT009* and *mreB* are upregulated by 4-fold between 12-30 hpi. Whereas other gene transcripts like *gyrB,* which is a part of the replication machinery; *dnaN,* which is a part of the DNA polymerase III and *dnaE*, a catalytic α subunit of DNA polymerase III acting as a DNA helicase, are constitutively expressed throughout the developmental cycle. *rpoA* encodes the α-subunit of RNA polymerase involved in transcription and is expressed constitutively as well. Cellular processes such as DNA replication, transcription, translation and protein processing are active early in the infectious process and are expressed throughout the life cycle in *Chlamydia* (57). Hence the levels of these proteins involved in these processes show constitutive expression when compared to the endogenous control gene, *secY*. These data supports the possibility that *mreB* might be a part of cellular processes involved in the conversion of RBs to EBs.

A recent report suggests a lateral transfer of the peptidoglycan synthesis related genes between C*hlamydiae* and *Streptomyces* (58). *Streptomyces coelicolor* use MreB to generate infectious particles, i.e. spores (59). In *Streptomyces,* MreB and RodZ are known to interact and are a part of the cell morphogenic complex involved in spore cell wall formation. Studying the pattern of relative gene expression of *mreB* and *CT009 (rodZ)* in *Chlamydia,* it is possible that these protein a part of the cell envelope and are involved in EB formation. MreB and RodZ are known to be a present in rod shaped organisms and some chlamydial relatives like the *Rhabdochlamydia* spp. produce rod-shaped EBs (1). Hence it is possible that these proteins are

conserved and assigned a different function like the process of EB formation, even though EBs of *Chlamydia trachomatis* are not rod-shaped. *Vibrio parahaemolyticus* remodels MreB by breaking the filaments into fragments to form spherical stationary phase cellular structures under stressful conditions (60).

Even though peptidoglycan has not been detected in *Chlamydia,* the presence of some proteins involved in the peptidoglycan machinery is intriguing. MreB in some organisms is known to scaffold the peptidoglycan machinery during cell wall formation and it is possible that MreB in chlamydia might be a part of the peptidoglycan synthesis machinery facilitating the conversion from RB to EB. RodA is known to be a cell integral membrane protein in many rod-shaped bacteria, which is also involved in cell division, but its role in *Chlamydia* is unclear. *rodA* transcripts were observed to be expressed at 18 hpi and to remain constant through 36 hpi. These comply with the expected observation of cell division proteins to be constitutively expressed in this analysis. Thus comparing the relative gene expression pattern of *mreB* to other genes involved in different cellular functions suggests these possible multiple roles in the chlamydial life cycle.

Compiling the data, we hypothesize would be that *Chlamydia* could have an altered MreB structure which forms short polymers in the absence of ATP. These short polymers could be responsible for EB formation. This phenomenon could be a possible mechanism used by *Chlamydia* to form the infectious particles (EBs). As observed, MreB could form polymers even in the absence of ATP and hence might be an adaptation to form short MreB polymers in EBs during an energy deficient environment. We observed that A22 enhances the polymerization/aggregation of chlamydial MreB *in vitro,* which might be a representation of formation of long MreB polymers or aggregates *in vivo.* These might not desirable for EB

formation hence stalling the developmental cycle at the aberrant RB stage and inhibiting the production of any progeny (EBs). The other hypothesis could be that ATP hydrolysis alters the conformation of MreB and hence its interaction with other proteins involved in the process of RB to EB conversion. These possible roles of MreB in *Chlamydia* can be examined by further investigations.

Alternative sigma factor RpoN:

Introduction:

1. Bacterial sigma factors:

Bacterial transcription is mediated by DNA-dependent RNA polymerase comprising of a core enzyme with 2 α and 2 β subunits along with a sigma factor, which facilitates specific promoter recognition. While σ^{70} (the major sigma factor) is linked to recognition of promoters of majority of the genes during exponential growth of most of the organisms (61), alternative sigma factors often regulate transcription of certain genes influenced by environmental conditions (62). σ^{54}, an alternative sigma factor, is known to be associated with processes like nitrogen regulation, formate and acetoacetate catabolism, amino acid transport, etc.(63). In many bacterial pathogens it plays an important role in virulence and pathogenesis (64). It has also being assigned to various other functions like electron transport, chemotaxis, heat shock and flagellation (65).

σ^{54} is known to have a unique mechanism of regulation (66), where the σ^{54}-holoenzyme occupies a promoter and is transcriptionally inactive and needs an activator to initiate transcription (67). In some organisms, σ^{54} binds to its promoters even in the absence of RNA polymerase core enzyme (68). Activators of σ^{54} belong to the NtrC subfamily of phosphorylated response regulators (69). In the two-component system of response regulators, NtrB autophosphorylates in response to environmental limitations and transfers this phosphate group to NtrC, which activates the NtrC oligomer and binds to an enhancer region upstream of the σ^{54} promoter site (70) (71). The physical bending of DNA enables NtrC to interact with the σ^{54}-holoenzyme (72), and the activated NtrC catalyzes ATP hydrolysis to form an open complex

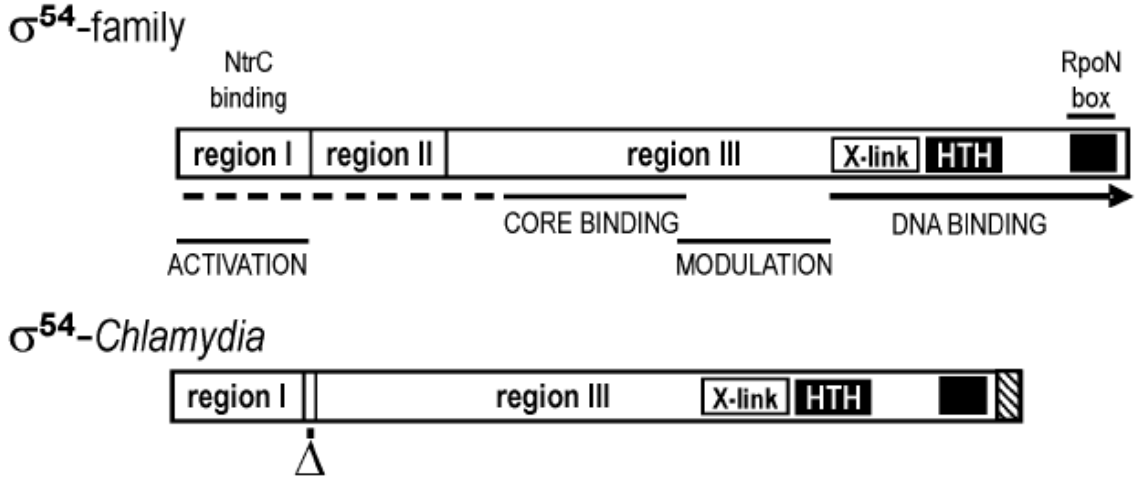
which initiates transcription (73). σ^{54} promoters have a consensus binding sequence across microbial species (65) i.e. TGGCAC-N_5-TTGCA located -24 to -12 upstream of the transcriptional start site of the genes transcribed by σ^{54} (74).

2. Chlamydial sigma factors:

Based on homology to other organisms, σ^{54} in *Chlamydia* is known to have three conserved regions (75). Chlamydial σ^{54} has conserved sequences except for certain deletions in region II (Fig. 12). *Klebsiella pneumoniae* lost its ability to bind DNA in absence of RNA polymerase core enzyme when this region was partially deleted (76). Thus this deletion in region II could be contributed to the loss of DNA binding ability of *C. trachomatis* in absence of RNA polymerase core enzyme.

Figure 12: Chlamydial σ^{54}

Chlamydial σ^{54} showing conserved sequences except for certain deletions in region II. [Adapted from Wan, C (2004) Bioinformatic Identification and Functional Characterization of sigma 54 Promoters in *Chlamydia trachomatis*]


σ54-family
NtrC binding
RpoN box
region I
region II
region III
X-link
HTH
CORE BINDING
DNA BINDING
ACTIVATION
MODULATION
σ54-Chlamydia
region I
region III
X-link
HTH
Δ

Three major classes of transcription factors are detected in *Chlamydia* i) RpoD or Sigma 66 (homolog of sigma 70), ii) RpsD or Sigma 28 and iii) RpoN or sigma 54. In *Chlamydia trachomatis L2,* CT267 which is a transcriptional factor, has been characterized to be homologous to the integration host factor in other bacteria (77) and through genome sequencing we now know that *Chlamydia* retains most of the components required in assembly of polymerase and transcription. The genome sequence of *C. trachomatis* revealed σ^{54} as a part of the RNA polymerase machinery along with the activators of σ54, CtcB-CtcC (two-component system homologous to NtrB/NtrC in other bacteria) (30). CtcB exhibited autophosphorylation and this phosphate is transferred to CtcC, which shows pathway conservation (78).

Though the role of σ^{54} is unknown in chlamydial species, promoters of two gene targets i.e. *CT652.1 and CT683* have been identified using computational prediction. Both are designated to code for hypothetical proteins and hence studying the function of σ^{54} in chlamydial gene regulation remains to be determined.

Recently a shuttle vector based on a chlamydial plasmid was developed, which contained the GFP (green fluorescent protein) as a positive control for expression (79). Recent work in our laboratory has been focused on developing genetic and molecular tools for precise control of gene expression in *Chlamydia*. Using these tools widespread functional and biological studies could be performed in *Chlamydia* and allow for a better understanding of its developmental cycle

Thus, the aim of this project was to carry out *in vitro* studies confirming the binding of σ^{54} holoenzyme to its predicted promoters and *in vivo* analysis by designing and isolating transformants expressing a dominant negative CtcC and studying the change in the pattern of different genes associated with σ^{54}.

MATERIALS AND METHODS

***In vitro* analysis**:

Construction of expression vectors pET21b:

To overexpress the recombinant chlamydial RpoN (σ^{54}), pET21b was digested with BamHI and HindIII restriction enzymes and then ligated with full length σ^{54} sequence. (This work was done by Yamini Mutreja). The plasmid was then transformed into Acella™ Chemically Competent Cells (Edge Biosystems) using the infusion kit (Clontech, USA). Plasmid was isolated using the Miniprep Kit (Qiagen,Valencia Ca) and sequenced. Isogenic clone was determined and used for overexpression and purification of recombinant RpoN.

Expression and protein purification by denaturation-renaturation protocol:

Cultures grown at 37^0C in Luria Broth with 100 μg/mL ampicillin were induced with IPTG of final concentration of 1 mM at OD_{600} of 0.6 and grown overnight at 15° C. Overexpressed recombinant protein was purified under denaturing conditions as it was found in the insoluble fraction. Pellets of induced bacterial cells were obtained by centrifugation at 8000 x g for 10 minutes. The resuspended pellet in 50 mL wash buffer (10mM HEPES, pH 7.2, 5mM EDTA and 0.1% Triton X-100) was sonicated on ice for 2 minutes and centrifuged at 15,000 x g for 30 minutes. The pellet was then washed 3 times with wash buffer and resuspended in 5 mL phosphate buffer (pH 6.8) with 6 M guanidine hydrochloride for 1 hour on ice. The supernatant containing the desired recombinant protein was obtained after a final centrifugation at 39,000 x g for 20 minutes. The protein was purified from this supernatant using a cobalt resin column, washed and eluted according to the manufacturer's instructions (TALON His-Tag Purification Resin, Clontech). The denatured protein was refolded by step wise dialysis in refolding buffer (30mM Tris-HCl pH 7.6, 200 mM KCl, 1mM EDTA, 5 mM DTT, 10% [v/v] glycerol) with

decreasing concentrations of urea (6, 3, 2, 0.5, 0, 0 M) for 4 hours each at 4° C. Refolded protein was divided into 100 µl aliquots and stored at -20° C. Alternatively, the rapid-refolding was used where denatured protein was diluted ten times, rapidly, by injecting it into a beaker with constantly stirring refolding buffer (20 mM Tris-HCl pH 8, 500 mM NaCl) and then loaded on the Ni-NTA resin to purify the recombinant protein using manufacturer's instructions (GE healthcare).

SDS PAGE analysis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini-PROTEAN Tetra Cell System (Bio-Rad Laboratories, Hercules, CA) using a 12% separating gel and 5% stacking gel.

Analytical size exclusion chromatography:

About 0.1 mg/mL of refolded RpoN was applied to a Sephacryl S200 BIORAD equilibrated with 20 mM Tris-HCl (pH 8) and 500 mM NaCl. Previously, to generate a standard curve, a protein standard containing thyroglobin (670 kDa), IgG (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and B12 (13.5 kDa) (BIO-RAD, Hercules, CA) were used.

Circular Dichroism analysis:

Recombinant RpoN was dialyzed into the CD buffer (30mM Tris pH 7.6, 500mM NaF, 1mM EDTA and 5mM DTT). The protein was used at a concentration of 0.3 mg/mL. Circular dichroism spectra was acquired using JASCO J-815 Spectropolarimeter. Wavelength scans were collected at a scan rate of 50 nm/min between the ranges of 190-260 nm. JASCO secondary structure estimation software was used to calculate secondary structures.

Electrophoretic Mobility Shift Assay:

To detect DNA binding ability of RpoN in the presence of core polymerase, electrophoretic mobility shift assay was performed. Promoter sequences of *CT683* (Forward Primer 5'IRD800/TCG TGA TTG GCA TGG TTT TTG CTC CTG TAA AAG GTA AGG TAT, Reverse primer ATA CCT TAC CTT TTA CAG GAG CAA AAA CCA TGC CAA TCA CGA) and *CT652*.1 (Forward Primer 5'IRD800/GTT ATT AAT TAG AGC TGG CAC ACT TTT TGC TCC TAG TAA AGA TGA, Reverse primer TCA TCT TTA CTA GGA GCA AAA AGT GTG CCA GCT CTA ATT AAT AAC) were labeled with IR800 and annealed to obtain double-stranded oligonucleotides. The DNA binding reaction was performed with 10 ng of DNA in 40 mM Tris-HCl (pH 8.0) 0.1 mM EDTA, 100 mMNaCl, 250 mM KCl, 1 mM DTT and 10% glycerol and incubated at 37^0C for 5 minutes. 10 μM of purified recombinant σ^{54} and 2 μM of *E. coli* core RNA polymerase **(**Epicentre Biotechnologies, Chicago) was added to this reaction and further incubated for 10 minutes at 37^0C. The reaction was analyzed on a 6% TBE gel (INVITROGEN) in 0.5X TBE buffer at 150 V. The DNA was visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Expression profiling:

RT-PCR (Quantitative gene expression):

Total RNA was isolated from *C. trachomatis*-infected L929 cells every 6 hpi through 36 hpi using TRIzol® Reagent (Life Technologies, Grand Island, NY). Isolated RNA was treated with DNase using the TURBO DNA-free kit (Life Technologies) according to the manufacturer's protocol. Concentration of RNA samples were assessed by spectrophotometry. For each RNA sample, cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instruction. Primer pairs for

Table 2: Oligonucleotide sequences used in real time RT-PCR amplification.

Gene	Forward Primer sequence	Reverse Primer sequence
CT046 (*hct*B)	5'-AGTAGCTTCAACAAAAAAATCTTCC-3'	5'-CCAACTGTGAGCTGTACGAGAAC-3'
CT190 (*gyr*B)	5'-ATCACGGTCATCTTCAAAAACAATC-3'	5'-CGACTTTACAGGTGCTTGGTCC-3'
CT510 (*sec*Y)	5'-GGACGAATGACACGGCTTTTTAC-3'	5'-TACCCAAGGCACTCCAAACAGC-3'
CT609 (*rpo*N)	5'-TAGGCAACCTCTCCCCAGAA-3'	5'-GGAAAAGGTGTTCGGAGACG -3'
CT652.1	5'-GCAGTCGGAAAGAGCGTACA-3'	5'-GCTATCGCGGCTATCGTC-3'
CT683	5'GGAAGAGGCGGAAAAGCA-3'	3'-AGCCCAGCTTCCGAGTCTAA-3'

each gene target were designed (Table 2) and were verified for efficiency using serially diluted cDNA as a template for PCR. Resulting C_T values were used to generate standard curves using the StepOnePlus software and confirmed that primer efficiency was suitable for relative quantification using the comparative C_T method ($2^{-\Delta\Delta C_T}$) (42). Applied Biosystem's StepOnePlus Real-time PCR system was used for relative quantification of target genes. To verify that RNA samples were free of contaminating DNA, real-time PCR was performed for the housekeeping gene, *secY* (CT510), using respective RT-negative reactions for all samples. Relative quantification was performed for each gene target and for each time point using the comparative C_T method provided by the StepOnePlus software. Reactions were set up in duplicate with each mixture containing 500 nmol of each primer and Fast SYBR Green Master Mix (Applied Biosystems). Endogenous control, *gyrB* (CT190), was used to normalize ratios for all the target genes and relative quantification values were calibrated against the 6-hpi time point for each gene target.

In vivo functional analysis:

Cloning of CtcC wild type in pTL2E:

Primers were designed named 315 [5'-CTT TAA GAA GGA GAT ACC GGT ATG TCG ATA GAA CAC ATT CTT ATT ATT G-3'] and 316 [5'-TCA CTT CAC AGG TCA ACC GGC CGT TAT AAG AGA GCG AGC ATA GAA GG-3']. Using the Finzyme PCR program [98° C (15 seconds); 60° C (30 seconds); 72° C (1 minute)] and Phusion High Fidelity PCR reagents (New England BioLabs), CtcC WT gene (CT468) from *Chlamydia trachomatis L2/434/Bu* was amplified. The size of the PCR product was confirmed using gel electrophoresis and PCR product was purified using the PCR purification kit (QIAquick, QIAGEN). The plasmid pTL2E

(designed by Dr. Jason Wickstrum) was cut using the restriction enzyme EagI. The CtcC WT gene was ligated into the vector pTL2E using the IN-FUSION PCR cloning kit (Clontech) and transformed into alpha-select gold efficiency competent cells (Bioline). Positive clones were identified using PCR with primers 100 (NNNNNNGGGCCCTTTAGAGCTTGACGGGGAAAGCCGG) and 126 (GAGTTATTTTACCACTCCCT) (from Dr. Jason Wickstrum) to identify clones with CtcC WT gene inserts. Plasmids were isolated using the miniprep kit (QIAquick, QIAGEN) and sequenced. Colony #6 was chosen for further experiments and glycerol stocks were prepared.

Site directed mutagenesis of CtcC gene:

Primers were designed for site-directed mutagenesis of CtcC WT gene. The sequence of the primers were CtcC (CT468) mutagenesis Forward Primer 5'CTT TCG ATC TGA TTA TTT CCG CTA TGA ATA TGC CTG ATG GTT C-3'and CtcC (CT468) mutagenesis Reverse Primer 5'- GAA CCA TCA GGC ATA TTC ATA GCG GAA ATA ATC AGA TCG AAA G-3'. PCR was performed using the QuickChange II XL site-directed mutagenesis kit (Stratagene, Cedar Creek) by following the manufacturer's instructions. The aspartic acid (D) residue 54 of CtcC in the wild-type gene was replaced with an alanine (A). The CtcC WT gene cloned into pTL2E was used as the template for mutagenesis. These plasmids were transformed into XL10-Gold ultracompetent cells (Agilent technologies) and mutation was confirmed by sequencing. Mutant #3 was selected for further experiments and glycerol stocks were made.

Transformation of CtcC WT and CtcC D54A mutant in *Chlamydia trachomatis L2/434/Bu:*

Transformation reaction was set up using *C. trachomatis L2* seed. 3 µg of plasmid prep in sterile water was mixed with 50 µLs of 2X $CaCl_2$ (20mM Tris pH7.4, 100mM $CaCl_2$) buffer and final

volume was made up to 100 µLs using sterile water. This reaction was incubated at RT for 30 mins. Host cells were resuspended in 1X $CaCl_2$ (10mM Tris pH7.4, 50mM $CaCl_2$) buffer at a final concentration of 2 X 10^7 cells/mL. 100 µLs of these cells were added into the reaction mixture and incubated at RT for 20 minutes. This transformation reaction was added to a 6-well plate (100 µLs per well) along with 2 mLs of RPMI (with 5% FBS and 10 µg/mL gentamycin). The reaction was incubated at 37^0C, 5% CO_2 for 40-48 hours. The cells were further scraped using 1X SPG buffer and lysed by vortexing with glass beads for 45 seconds. The lysate was centrifuged at 1380 X g 5 minutes, and the supernatant was used to infect the next round of host cells and incubated for 2 hours at RT. After aspirating the inoculant, RPMI (with 5%FBS and 10 µg/mL gentamycin, 1 µg/mL Ampicillin and 1 µg/mL cyclohexamide) was added, and the reaction was incubated at 37^0C, 5% CO_2 for 30-40 hpi. The protocol was repeated for 5 more passages to further select for transformants.

Results:

1. Expression and Purification of recombinant RpoN using denaturation-renaturation protocol

To characterize the function of RpoN in *Chlamydia,* recombinant RpoN was expressed in *E. coli* and purified using Ni-NTA resin. As the recombinant RpoN remained in the insoluble fraction, denaturation-renaturation protocol was used.

In the presence of 6 M Guanidine hydrochloride, RpoN was obtained in the denatured form and then refolded by gradual removal of guanidine hydrochloride by dialysis. Commassie staining of SDS-PAGE was used to visualize the purified RpoN (Fig. 13) and a 50 kDa band was observed. Refolded protein maintained solubility and was further purified using FPLC and assessed for secondary structure using CD spectrometry.

2. RpoN purifies as a monomer

RpoN is a protein with an estimated mass of 48,000 Da and is expected to be present as a monomer. Purified refolded recombinant RpoN was subjected to size exclusion chromatography. A peak was obtained corresponding to 50 kDa, confirming the expected size of the purified protein (Fig. 14). Protein samples from corresponding wells were collected and used for functional assays.

3. CD spectroscopy confirms proper refolding of recombinant RpoN

Recombinant RpoN was purified using the denaturation-renaturation protocol, and it was important to determine its secondary structure to ensure proper refolding of the protein. The CD spectrometry showed that recombinant RpoN purified from *Chlamydia*

Figure 13: Protein Purification of recombinant RpoN.

Lane 1 shows the broad range protein molecular marker (Promega). SDS-PAGE analysis of refolded RpoN stained using Commassie blue shows a 50 kDa band in Lane 2.

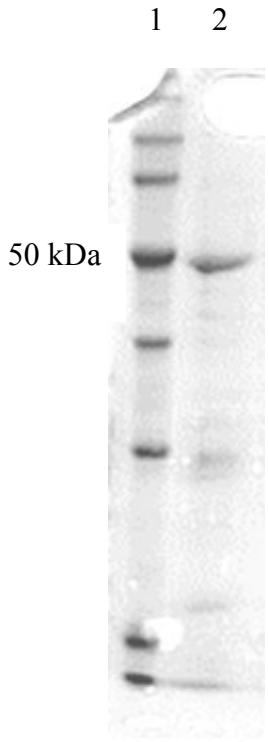

1
2
50 kDa

Figure 14: RpoN purifies as a monomer *in vitro*

Analytical size exclusion chromatography was used to further purify recombinant RpoN based on the molecular weight. The protein was eluted at a molecular size of ~50 kDa, which is its expected molecular weight.

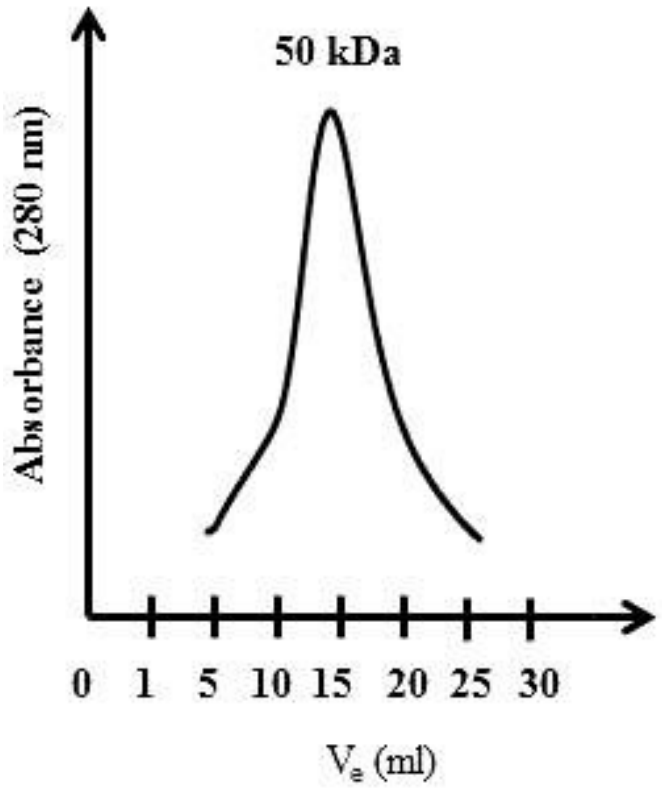
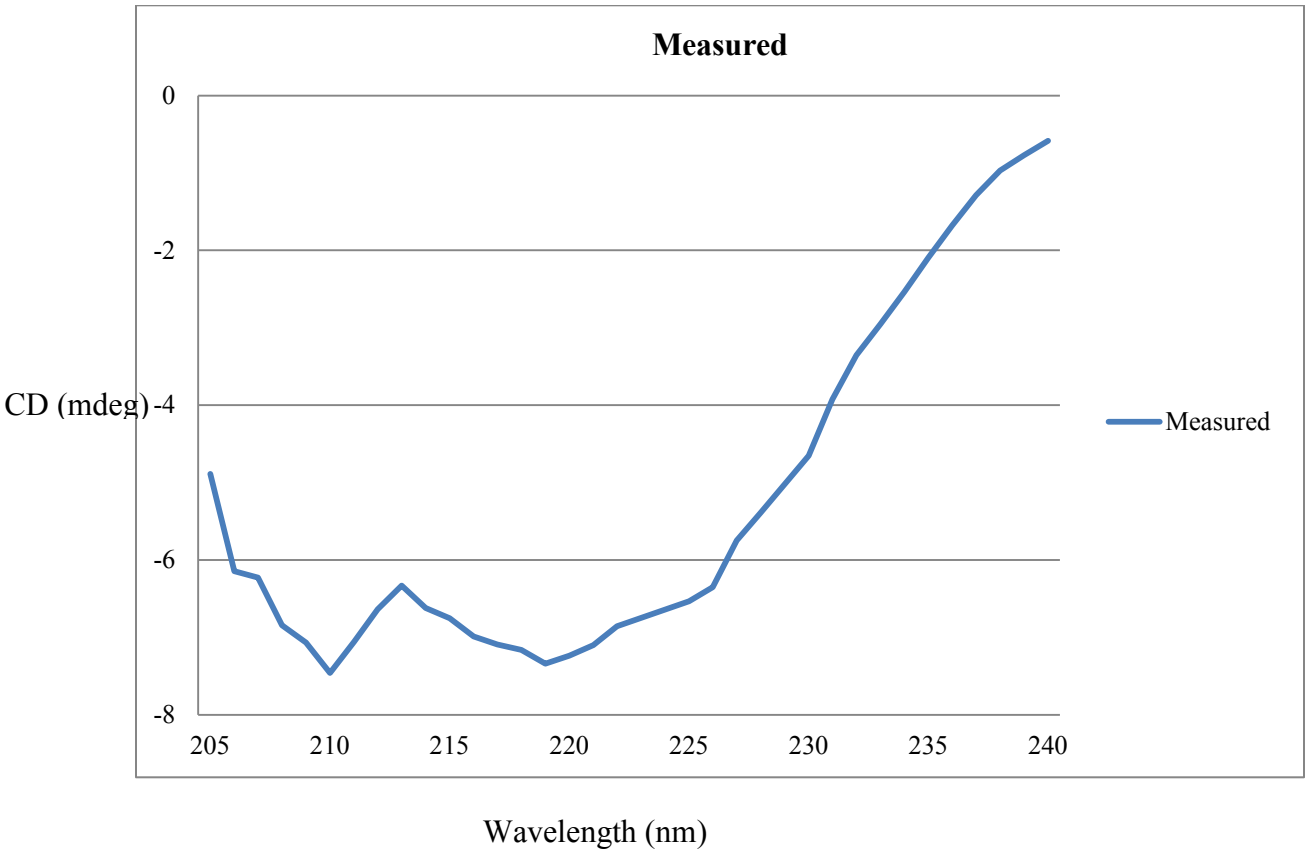

50 kDa
Absorbance (280 nm)
0 1 5 10 15 20 25 30
Ve (ml)

Figure 15: CD spectroscopy reveals secondary structure of chlamydial RpoN

Circular dichroism spectra of purified refolded recombinant RpoN was used to predict the secondary structure. Proportions were calculated based on the CD profile obtained using the JASCO secondary structure estimation software.


Measured
0
-2
-4
-6
-8
CD (mdeg)
205
210
215
220
225
230
235
240
Wavelength (nm)
Measured

trachomatis L2 had 32.8% α-helix, 20.3% β-sheets, 15.3% turns and 31.5% random coils (Fig 15). The alternative sigma factor (RpoN) from *K. pneumoniae* was considered as a reference for comparison of the secondary structure (80). The secondary structure of RpoN from *K. pneumonia* estimates about 35% of α-helices and β-sheets with 30% of β-turns and random coils. The purified chlamydial RpoN showed significant structural similarity and it could be said that the refolding was successful with stable protein available for functional studies.

4. Predicted promoters of chlamydial σ^{54} show a shift with sigma 54-holoenzyme

The function of σ^{54} in *Chlamydia spp.* is yet unknown. The promoter region of the two hypothetical proteins CT652.1 and CT683, located 100 bps upstream of the TSS, contained the σ^{54} consensus binding sequences (81). To test if these promoters bind to the recombinant σ^{54}-holoenzyme from *Chlamydia trachomatis,* EMSA was performed in presence of *E. coli* RNA core polymerase (Epicentre Biotechnologies**,** Madison, WI). Chlamydial σ^{54} did not result in mobility shift of *CT652.1 or CT683,* whereas σ^{54}-holoenzyme produced shifts for both promoters (Fig 16). Hence these results suggest that chlamydial sigma 54 is capable of binding to these promoters only in the presence of RNA core polymerase. An oligomeric sequence with no RNA core polymerase binding sequence was used as a negative control. No shift was observed when σ^{54}-holoenzyme was added to the negative control.

5. Quantitative RT-PCR:

Previously microarray analysis was performed to study the global effects of different genes in *Chlamydia* (55). These studies give us a good idea of the relative expression of many genes and hence their pattern of expression in the chlamydial developmental cycle can be determined.

Figure 16: RpoN binds to DNA in presence of RNA core polymerase

EMSA to test chlamydial RpoN binding to promoter sequences of *CT652.1* and *CT683.* A) Lane 1 shows promoter sequence of *CT652.1*, Lane 2 shows core polymerase bound to the promoter, Lane 3 shows promoter with sigma 54 and Lane 4 shows promoter bound to σ^{54}-holoenzyme. B) Lane 1 shows promoter sequence of *CT683*, Lane 2 shows core polymerase bound to the promoter, Lane 3 shows promoter with σ^{54} and Lane 4 shows promoter bound to σ^{54}-holoenzyme. c) Negative control showing no shift with RNA core polymerase, σ^{54} or with σ^{54}-holoenzyme. Random oligomeric sequence with no core binding region was used as a control.

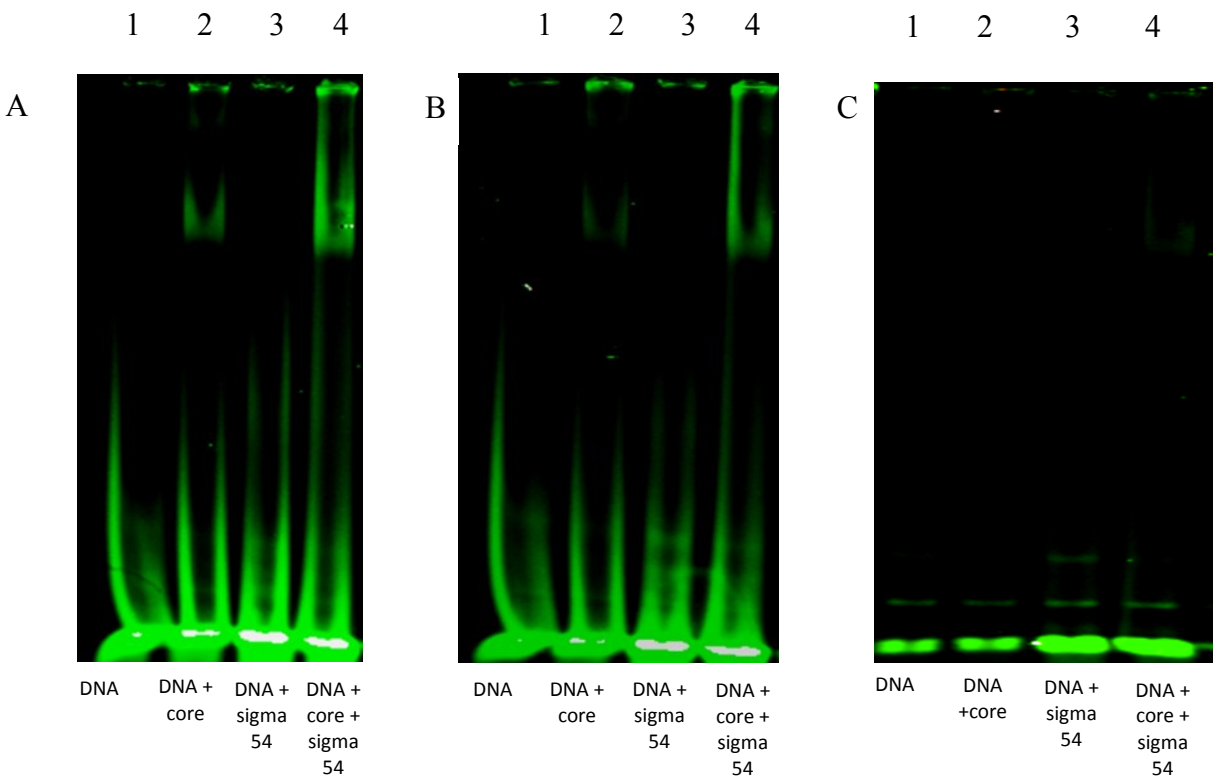
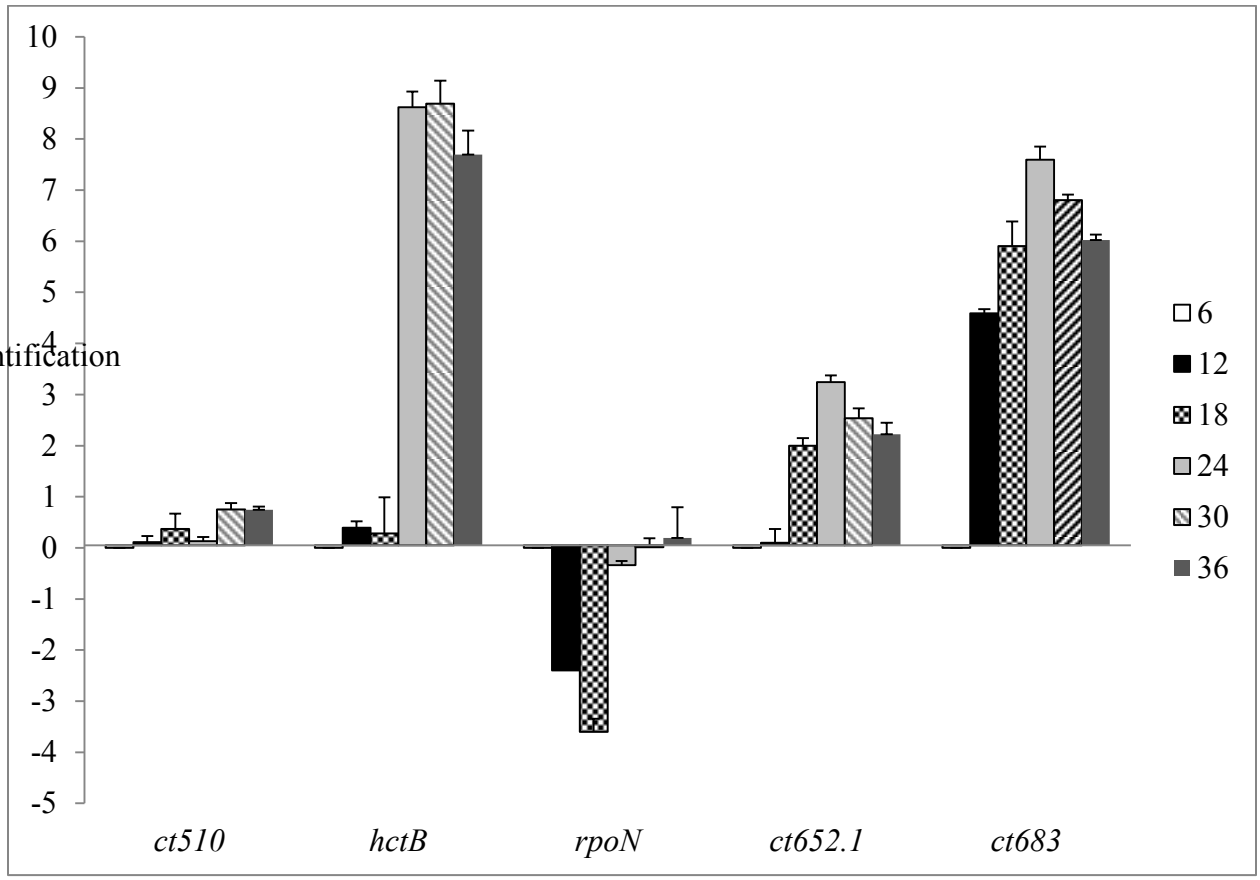

1 2 3 4
A
DNA
DNA + core
DNA + sigma 54
DNA + core + sigma 54
1 2 3 4
B
DNA
DNA + core
DNA + sigma 54
DNA + core + sigma 54
1 2 3 4
C
DNA
DNA +core
DNA + sigma 54
DNA + core + sigma 54

Figure 17: Quantitative gene expression analysis

Total RNA was isolated at 6, 12, 18, 24, 30 and 36 hours post-infection from L929 cells infected with *Chlamydia trachomatis L2* using TRIzol® Reagent. Complimentary DNA was synthesized and used as a template for relative gene expression quantification. Transcript quantity was transformed to a $\log_2$ ratio. The endogenous control *gyrB* (CT190) was used to normalize expression. The constitutively expressed gene *secY* (CT510), and *hctB* (CT046) (RB-to-EB conversion associated control) were included. 6-hpi time point for each gene was used to calibrate the ratio of expression. Error bars represent the standard deviation of duplicate samples.



Relative Quantification

Target

Relative gene expression levels were studied of *rpoN,* and its predicted targets *CT652.1* and *CT683* (Fig 17). Data revealed *rpoN* to be expressed constitutively with the exception of 12-24 hpi, where the expression seemed to be repressed. *CT652.1* contains a predicted σ^{54} promoter and shows elevated gene transcript levels (6 fold) at 24 hpi and remains fairly constant through 36 hpi. The other predicted σ^{54} promoter, *CT683*, is upregulated 8-folds at 24 hpi. *CT683* is known to code for a tetratricopeptide-motif-containing protein.

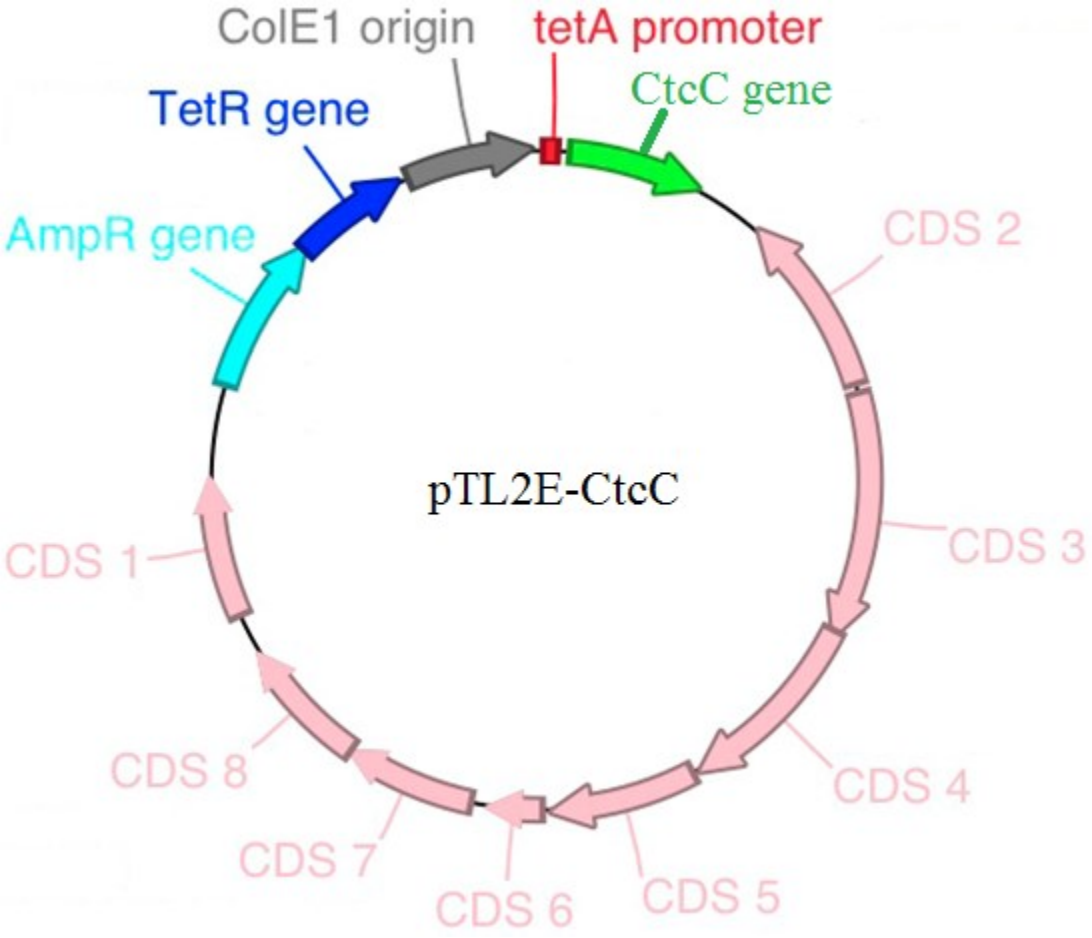
6. CtcC WT and D54A mutant shuttle vectors:

CtcB-CtcC are known to be encoded by the *C. trachomatis* genome and are hypothesized to be a part of the two-component response regulators responsible for σ^{54} activation. *In vitro* studies have revealed that CtcB has the ability to autophosphorylate and transfer this phosphate group to CtcC which then activates the transcription of genes regulated by σ^{54}. CtcC in *C. trachomatis* lacks the DNA binding domain. It has been shown that replacing the Aspartic acid (D) residue 54 in the predicted phosphorylation site of CtcC in the wild-type gene with an Alanine (A), failed to transfer this phosphate group from CtcB to CtcC (78).

Thus the aim of this study was to induce similar mutation in the CtcC WT gene and by incorporating this into the shuttle vector, study its effects *in vivo.* The CtcC WT gene from *C. trachomatis L2* was integrated into the modified shuttle vector designed by Dr. Jason Wickstrum (Fig. 18). This shuttle vector with the CtcC WT gene was then used to obtain the CtcC D54A mutant using the site-directed mutagenesis kit as mentioned earlier. The shuttle vector contains a gene for ampicillin resistance as a selection marker for transformation. It is expected that in the presence of anhydrous tetracycline the CtcC gene would be transcribed.

Figure 18: Genetic manipulation of CtcC in *Chlamydia trachomatis*

CtcC wild-type gene was ligated into pTL2E with an EagI restriction site. The vector contains an Ampicillin resistance gene, which is the marker for selection of transformants. In the presence of anhydrous tetracycline, the CtcC gene is transcribed and expressed. Point mutation in the CtcC WT gene was obtained by using the site-directed mutagenesis kit.


ColE1 origin
tetA promoter
CtcC gene
TetR gene
AmpR gene
CDS 2
pTL2E-CtcC
CDS 3
CDS 1
CDS 8
CDS 4
CDS 7
CDS 6
CDS 5

Hence the pTL2E-CtcC wild type and pTL2E-CtcC D54A mutant were obtained in *E. coli* (α-select cells). Attempts at transforming *Chlamydia* with these vectors haven't been successful but further attempts with minor changes would be made in the future. pBR325::L2 with constitutive GFP expression was used as a positive control for the transformation protocol/conditions. GFP positive *Chlamydia* transformed with this vector could be obtained but no transformants could be seen for both CtcC WT and CtcC mutants under the same conditions.

Discussion:

Previously, the major sigma factor σ^{66} was characterized (82) and recent sequencing of the *Chlamydia trachomatis* genome revealed two alternative sigma factors: σ^{54} and σ^{28} (30). σ^{28} has been linked to the RB to EB conversion as it has been demonstrated to transcribe two middle to late stage genes in *Chlamydia* like *hctB* (83). In contrast to the identified σ^{28} gene targets, little is known about the σ^{54} gene targets. The only literature available is based on prediction of promoters upstream of two genes i.e. *CT652.1* and *CT683* (81).

Cloning of RpoN from *Chlamydia trachomatis L2/434/Bu* was carried out to express the recombinant protein and then purify it for further functional assays. As the purified protein was found to be in the insoluble fraction, denaturation procedure was adopted to purify the protein (Fig 13). Hence CD spectroscopy was used to ensure proper refolding of the protein. The CD spectroscopy analysis revealed 32.8% α-helix, 20.3% β-sheets, 15.3% turns and 31.5% random coils (Fig. 15), which was a good indication of the secondary structure of the refolded RpoN. CD analysis of *K. pneumoniae* revealed similar percentages of predicted secondary structure (80), hence reassuring the refolding of the purified RpoN (sigma 54). Size exclusion chromatography revealed that the purified RpoN was present in a monomeric form at the size of 50 kDa (Fig 14)

and helped to determine whether the purified recombinant protein was monomeric, which was further used in functional studies.

EMSA is a traditionally used technique to study protein-DNA binding. Studying *in vitro* transcription assays with proteins like σ^{54} (RpoN), which need a two-component system of activators for transcription, failed to work. Thus, EMSA is a good alternative to study molecular interactions of proteins with DNA. As stated earlier, the region II in *Chlamydia* is missing, which is also observed in other organisms like *Aquifex aeolicus, Rhodobacter capsulatus* (84, 85). Although these organisms lack the region II and are less efficient in the promoter DNA melting, they have been shown to be functionally active (86).

The EMSA with promoters of two genes, *CT652.1* and *CT683,* showed no shift when sigma 54 was added but sigma 54 was able to only recognize and bind to these promoters in the presence of *E. coli* RNA polymerase core enzyme (Fig. 16). *CT652.1* codes for a protein which is conserved through *Chlamydia spp.* but has unknown function. *CT683* is also conserved through *Chlamydia spp.* and has been classified as O-linked N-acetylglucosamine transferase and a type III secretion chaperon (30) and contains 3 tetratricopeptide domains. Tetracopeptide repeat domains are known to be involved in protein-protein interactions associated with protein transport.

Relative gene expression levels were compared between r*poN* and its predicted targets *CT652.1* and *CT683*. Data showed *rpoN* to be almost constitutively expressed except for 12-18 hpi, where it seems to be repressed. CT652.1 contains a predicted sigma 54 promoter and shows elevated gene transcript levels (6 fold) at 24 hpi and remains almost constant through 36 hpi. The other predicted σ^{54} target, CT683, is upregulated 6-fold between 12-24 hpi. CT683 codes for a

TPR-motif-containing protein. Proteins with TPR domains have been shown to have diverse cellular functions and many have been demonstrated to play a role in cell cycle control, signal transduction, protein transport, chaperone function, and transcription (87). Proteins with TPR domains have also associated with type three secretion systems (TTSS). σ^{54} is also known to be associated with bacterial exterior and virulence in other organisms and hence might be associated with any of these functions in *Chlamydia* (88). The pattern of expression of CT652.1 and CT683 seem to be quite similar confirming its regulation by a common sigma factor.

In vitro studies are insufficient to assign any function to RpoN because the predicted gene targets code for hypothetical proteins. Thus with recent advancements in manipulating chlamydial genes has made it possible to construct shuttle vectors and induce targeted gene mutations in *Chlamydia* (79). The modified shuttle vector (pTL2E) with CtcC wild type and the D54A mutant, if expressed in chlamydial cells, would be expected to affect the normal functioning of RpoN, therefore affecting its transcriptional targets. This vector obtained from *E. coli* cells are transformed into *Chlamydia* and is under a transcriptional regulation of anhydrous tetracycline. Controlled overexpression of the wild type CtcC and the dominant negative mutant of CtcC (D54A) would assist in studying the possible function of CtcC and thus RpoN. Using the inducible expression of the gene of interest in the presence of anhydrous tetracycline, dominant mutant CtcC could be expressed at different time points in the chlamydial developmental cycle. Changes in the phenotype or in the developmental cycle would thus suggest a role of RpoN in *Chlamydia*. Though several attempts to acquire these transformants haven't been successful yet, some modifications maybe required. Also a possible reason could be that the modified shuttle vector at times shows leaky expression of the targeted protein even in the absence of anhydrous tetracycline and this minute level of the protein could be toxic for the cell. Another reason could

be the difference in the methylation pattern on the shuttle vector because it is produced in *E. coli* and then transformed into chlamydial cells. Thus these methods need to be refined to better understand the role of RpoN in *Chlamydia.*

References:

1. Horn M (2008) Chlamydiae as symbionts in eukaryotes. *Annual review of microbiology* 62:113-131.
2. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, & Piot P (1998) Global prevalence and incidence estimates of selected curable STDs. *Sexually transmitted infections* 74 Suppl 1:S12-16.
3. Stephenson J, Hopwood J, Babiker A, Copas A, & Vickers M (2003) Recent pilot studies of chlamydia screening. *Sexually transmitted infections* 79(4):352.
4. McCoy AJ & Maurelli AT (2006) Building the invisible wall: updating the chlamydial peptidoglycan anomaly. *Trends in microbiology* 14(2):70-77.
5. Belland R, Ojcius DM, & Byrne GI (2004) Chlamydia. *Nature reviews. Microbiology* 2(7):530-531.
6. Schachter J (1990) Chlamydial infections. *The Western journal of medicine* 153(5):523-534.
7. Blasi F, Centanni S, & Allegra L (2004) Chlamydia pneumoniae: crossing the barriers? *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 23(4):499-500.
8. Grayston JT (2000) Background and current knowledge of Chlamydia pneumoniae and atherosclerosis. *The Journal of infectious diseases* 181 Suppl 3:S402-410.
9. Belland RJ, Ouellette SP, Gieffers J, & Byrne GI (2004) Chlamydia pneumoniae and atherosclerosis. *Cellular microbiology* 6(2):117-127.
10. Resnikoff S*, et al.* (2004) Global data on visual impairment in the year 2002. *Bulletin of the World Health Organization* 82(11):844-851.
11. Gerbase AC, Rowley JT, & Mertens TE (1998) Global epidemiology of sexually transmitted diseases. *Lancet* 351 Suppl 3:2-4.
12. Gaydos CA*, et al.* (2010) Performance of the Abbott RealTime CT/NG for detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *Journal of clinical microbiology* 48(9):3236-3243.
13. Abdelrahman YM & Belland RJ (2005) The chlamydial developmental cycle. *FEMS microbiology reviews* 29(5):949-959.
14. Matsumoto A (1973) Fine structures of cell envelopes of Chlamydia organisms as revealed by freeze-etching and negative staining techniques. *Journal of bacteriology* 116(3):1355-1363.
15. Hybiske K & Stephens RS (2007) Mechanisms of Chlamydia trachomatis entry into nonphagocytic cells. *Infection and immunity* 75(8):3925-3934.
16. Hybiske K & Stephens RS (2007) Mechanisms of host cell exit by the intracellular bacterium Chlamydia. *Proceedings of the National Academy of Sciences of the United States of America* 104(27):11430-11435.
17. Cabeen MT & Jacobs-Wagner C (2010) The bacterial cytoskeleton. *Annual review of genetics* 44:365-392.

18. Vats P, Yu J, & Rothfield L (2009) The dynamic nature of the bacterial cytoskeleton. *Cellular and molecular life sciences : CMLS* 66(20):3353-3362.
19. Jones LJ, Carballido-Lopez R, & Errington J (2001) Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell* 104(6):913-922.
20. Carballido-Lopez R (2006) The bacterial actin-like cytoskeleton. *Microbiology and molecular biology reviews : MMBR* 70(4):888-909.
21. Kruse T, Bork-Jensen J, & Gerdes K (2005) The morphogenetic MreBCD proteins of Escherichia coli form an essential membrane-bound complex. *Molecular microbiology* 55(1):78-89.
22. Figge RM, Divakaruni AV, & Gober JW (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus. *Molecular microbiology* 51(5):1321-1332.
23. Pollard TD (1990) Actin. *Current opinion in cell biology* 2(1):33-40.
24. Shih YL & Rothfield L (2006) The bacterial cytoskeleton. *Microbiology and molecular biology reviews : MMBR* 70(3):729-754.
25. White CL, Kitich A, & Gober JW (2010) Positioning cell wall synthetic complexes by the bacterial morphogenetic proteins MreB and MreD. *Molecular microbiology* 76(3):616-633.
26. Jermy A (2011) Bacterial physiology: MreB takes a back seat. *Nature reviews. Microbiology* 9(8):560-561.
27. Wang S, Arellano-Santoyo H, Combs PA, & Shaevitz JW (2010) Actin-like cytoskeleton filaments contribute to cell mechanics in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 107(20):9182-9185.
28. Mattei PJ, Neves D, & Dessen A (2010) Bridging cell wall biosynthesis and bacterial morphogenesis. *Current opinion in structural biology* 20(6):749-755.
29. Moran NA (2002) Microbial minimalism: genome reduction in bacterial pathogens. *Cell* 108(5):583-586.
30. Stephens RS*, et al.* (1998) Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. *Science* 282(5389):754-759.
31. Hatch TP (1996) Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? *Journal of bacteriology* 178(1):1-5.
32. Barbour AG, Amano K, Hackstadt T, Perry L, & Caldwell HD (1982) Chlamydia trachomatis has penicillin-binding proteins but not detectable muramic acid. *Journal of bacteriology* 151(1):420-428.
33. Chopra I, Storey C, Falla TJ, & Pearce JH (1998) Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited. *Microbiology* 144 (Pt 10):2673-2678.
34. Kalman S*, et al.* (1999) Comparative genomes of Chlamydia pneumoniae and C. trachomatis. *Nature genetics* 21(4):385-389.
35. Ouellette SP, Karimova G, Subtil A, & Ladant D (2012) Chlamydia co-opts the rod shape-determining proteins MreB and Pbp2 for cell division. *Molecular microbiology* 85(1):164-178.
36. Iwai N, Fujii T, Nagura H, Wachi M, & Kitazume T (2007) Structure-activity relationship study of the bacterial actin-like protein MreB inhibitors: effects of substitution of benzyl group in S-benzylisothiourea. *Bioscience, biotechnology, and biochemistry* 71(1):246-248.
37. Iwai N*, et al.* (2004) Structure-activity relationship of S-benzylisothiourea derivatives to induce spherical cells in Escherichia coli. *Bioscience, biotechnology, and biochemistry* 68(11):2265-2269.
38. Bean GJ*, et al.* (2009) A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB. *Biochemistry* 48(22):4852-4857.
39. Wu X, Iwai N, & Chen WL (2011) Effects of S-(3,4-dichlorobenzyl) isothiourea on different cellular events in the cyanobacterium Anabaena sp. strain PCC 7120. *Research in microbiology* 162(4):375-381.

40. Geisbrecht BV, Bouyain S, & Pop M (2006) An optimized system for expression and purification of secreted bacterial proteins. *Protein expression and purification* 46(1):23-32.
41. Bean GJ & Amann KJ (2008) Polymerization properties of the Thermotoga maritima actin MreB: roles of temperature, nucleotides, and ions. *Biochemistry* 47(2):826-835.
42. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.
43. Hefty PS & Stephens RS (2007) Chlamydial type III secretion system is encoded on ten operons preceded by sigma 70-like promoter elements. *Journal of bacteriology* 189(1):198-206.
44. van den Ent F, Amos LA, & Lowe J (2001) Prokaryotic origin of the actin cytoskeleton. *Nature* 413(6851):39-44.
45. Gaballah A, Kloeckner A, Otten C, Sahl HG, & Henrichfreise B (2011) Functional analysis of the cytoskeleton protein MreB from Chlamydophila pneumoniae. *PloS one* 6(10):e25129.
46. Iwai N, Nagai K, & Wachi M (2002) Novel S-benzylisothiourea compound that induces spherical cells in Escherichia coli probably by acting on a rod-shape-determining protein(s) other than penicillin-binding protein 2. *Bioscience, biotechnology, and biochemistry* 66(12):2658-2662.
47. Dye NA*, et al.* (2010) The assembly and interactions of MreB in the maintenance of cell shape in Caulobacter crescentus. p 1 online resource.
48. Nguyen BD & Valdivia RH (2012) Virulence determinants in the obligate intracellular pathogen Chlamydia trachomatis revealed by forward genetic approaches. *Proceedings of the National Academy of Sciences of the United States of America* 109(4):1263-1268.
49. Gallagher S & Chakavarti D (2008) Immunoblot analysis. *Journal of visualized experiments : JoVE* (16).
50. Bayer PM, Fabian B, & Hubl W (2001) Immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA) in autoimmune disease diagnostics--technique, benefits, limitations and applications. *Scandinavian journal of clinical and laboratory investigation. Supplementum* 235:68-76.
51. Mayer JA & Amann KJ (2009) Assembly properties of the Bacillus subtilis actin, MreB. *Cell motility and the cytoskeleton* 66(2):109-118.
52. Dye NA, Pincus Z, Fisher IC, Shapiro L, & Theriot JA (2011) Mutations in the nucleotide binding pocket of MreB can alter cell curvature and polar morphology in Caulobacter. *Molecular microbiology* 81(2):368-394.
53. Liberek K, Skowyra D, Zylicz M, Johnson C, & Georgopoulos C (1991) The Escherichia coli DnaK chaperone, the 70-kDa heat shock protein eukaryotic equivalent, changes conformation upon ATP hydrolysis, thus triggering its dissociation from a bound target protein. *The Journal of biological chemistry* 266(22):14491-14496.
54. Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, & Colombo R (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free radical biology & medicine* 31(12):1624-1632.
55. Nicholson TL, Olinger L, Chong K, Schoolnik G, & Stephens RS (2003) Global stage-specific gene regulation during the developmental cycle of Chlamydia trachomatis. *Journal of bacteriology* 185(10):3179-3189.
56. Belland RJ*, et al.* (2003) Genomic transcriptional profiling of the developmental cycle of Chlamydia trachomatis. *Proceedings of the National Academy of Sciences of the United States of America* 100(14):8478-8483.
57. Shaw EI*, et al.* (2000) Three temporal classes of gene expression during the Chlamydia trachomatis developmental cycle. *Molecular microbiology* 37(4):913-925.

58. Griffiths E & Gupta RS (2002) Protein signatures distinctive of chlamydial species: horizontal transfers of cell wall biosynthesis genes glmU from archaea to chlamydiae and murA between chlamydiae and Streptomyces. *Microbiology* 148(Pt 8):2541-2549.
59. Mazza P*, et al.* (2006) MreB of Streptomyces coelicolor is not essential for vegetative growth but is required for the integrity of aerial hyphae and spores. *Molecular microbiology* 60(4):838-852.
60. Chiu SW, Chen SY, & Wong HC (2008) Localization and expression of MreB in Vibrio parahaemolyticus under different stresses. *Applied and environmental microbiology* 74(22):7016-7022.
61. Lonetto M, Gribskov M, & Gross CA (1992) The sigma 70 family: sequence conservation and evolutionary relationships. *Journal of bacteriology* 174(12):3843-3849.
62. Hengge-Aronis R (2000) A role for the sigma S subunit of RNA polymerase in the regulation of bacterial virulence. *Advances in experimental medicine and biology* 485:85-93.
63. Reitzer L & Schneider BL (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in Escherichia coli. *Microbiology and molecular biology reviews : MMBR* 65(3):422-444, table of contents.
64. Studholme DJ & Buck M (2000) The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS microbiology letters* 186(1):1-9.
65. Merrick MJ (1993) In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N). *Molecular Microbiology* 10(5):903-909.
66. Wang L & Gralla JD (1998) Multiple in vivo roles for the -12-region elements of sigma 54 promoters. *Journal of bacteriology* 180(21):5626-5631.
67. Morett E & Segovia L (1993) The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *Journal of bacteriology* 175(19):6067-6074.
68. Buck M & Cannon W (1992) Specific binding of the transcription factor sigma-54 to promoter DNA. *Nature* 358(6385):422-424.
69. Shingler V (1996) Signal sensing by sigma 54-dependent regulators: derepression as a control mechanism. *Molecular microbiology* 19(3):409-416.
70. Porter SC, North AK, Wedel AB, & Kustu S (1993) Oligomerization of NTRC at the glnA enhancer is required for transcriptional activation. *Genes & development* 7(11):2258-2273.
71. Wyman C, Rombel I, North AK, Bustamante C, & Kustu S (1997) Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science* 275(5306):1658-1661.
72. Su W, Porter S, Kustu S, & Echols H (1990) DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. *Proceedings of the National Academy of Sciences of the United States of America* 87(14):5504-5508.
73. Popham DL, Szeto D, Keener J, & Kustu S (1989) Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* 243(4891):629-635.
74. Barrios H, Valderrama B, & Morett E (1999) Compilation and analysis of sigma(54)-dependent promoter sequences. *Nucleic acids research* 27(22):4305-4313.
75. Merrick M, Gibbins J, & Toukdarian A (1987) The nucleotide sequence of the sigma factor gene ntrA (rpoN) of Azotobacter vinelandii: analysis of conserved sequences in NtrA proteins. *Molecular & general genetics : MGG* 210(2):323-330.
76. Southern E & Merrick M (2000) The role of region II in the RNA polymerase sigma factor sigma(N) (sigma(54)). *Nucleic acids research* 28(13):2563-2570.
77. Zhong J, Douglas AL, & Hatch TP (2001) Characterization of integration host factor (IHF) binding upstream of the cysteine-rich protein operon (omcAB) promoter of Chlamydia trachomatis LGV serovar L2. *Molecular microbiology* 41(2):451-462.

78. Koo IC & Stephens RS (2003) A developmentally regulated two-component signal transduction system in Chlamydia. *The Journal of biological chemistry* 278(19):17314-17319.
79. Wang Y*, et al.* (2011) Development of a transformation system for Chlamydia trachomatis: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS pathogens* 7(9):e1002258.
80. Missailidis S, Cannon WV, Drake A, Wang XY, & Buck M (1997) Analysis of the architecture of the transcription factor sigma N (sigma 54) and its domains by circular dichroism. *Molecular microbiology* 24(3):653-664.
81. Mathews SA & Timms P (2000) Identification and mapping of sigma-54 promoters in Chlamydia trachomatis. *Journal of bacteriology* 182(21):6239-6242.
82. Koehler JE, Burgess RR, Thompson NE, & Stephens RS (1990) Chlamydia trachomatis RNA polymerase major sigma subunit. Sequence and structural comparison of conserved and unique regions with Escherichia coli sigma 70 and Bacillus subtilis sigma 43. *The Journal of biological chemistry* 265(22):13206-13214.
83. Yu HH & Tan M (2003) Sigma28 RNA polymerase regulates hctB, a late developmental gene in Chlamydia. *Molecular microbiology* 50(2):577-584.
84. Cannon W*, et al.* (1996) Purification and activities of the Rhodobacter capsulatus RpoN (sigma N) protein. *Molecular microbiology* 21(2):233-245.
85. Studholme DJ, Wigneshwereraraj SR, Gallegos MT, & Buck M (2000) Functionality of purified sigma(N) (sigma(54)) and a NifA-like protein from the hyperthermophile Aquifex aeolicus. *Journal of bacteriology* 182(6):1616-1623.
86. Buck M, Gallegos MT, Studholme DJ, Guo Y, & Gralla JD (2000) The bacterial enhancer-dependent sigma(54) (sigma(N)) transcription factor. *Journal of bacteriology* 182(15):4129-4136.
87. Blatch GL & Lassle M (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *BioEssays : news and reviews in molecular, cellular and developmental biology* 21(11):932-939.
88. Francke C*, et al.* (2011) Comparative analyses imply that the enigmatic Sigma factor 54 is a central controller of the bacterial exterior. *BMC genomics* 12:385.