

**THE OPTIMIZATION OF TRANSFORMATION
EFFICIENCY IN BACTERIUM *CHLAMYDIA MURIDARUM***

BY

IKRAM HAFIZ

**A THESIS SUBMITTED TO
THE SCHOOL OF GRADUATE STUDIES
RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE (M.S)
GRADUATE PROGRAM IN
PHYSIOLOGY AND INTEGRATIVE BIOLOGY
WRITTEN UNDER THE DIRECTION OF
DR. HUIZHOU FAN
AND APPROVED BY**

NEW BRUNSWICK, NEW JERSEY

JANUARY 2021

ABSTRACT OF THE THESIS

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**Thesis Director:
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Chlamydia trachomatis is an obligate intracellular microbe that is responsible for trachoma and chlamydia in much of the developing world, but is difficult to manipulate due to its biphasic developmental cycle. The current protocol for transforming *Chlamydia trachomatis* is a very time consuming process, yielding limited numbers of successful transformants only after multiple passages of infection which can take up to 48 hours per passage, while also requiring a highly time consuming purification process for elementary bodies. *Chlamydia trachomatis*' genetically conserved and faster growing cousin, *Chlamydia muridarum* (MoPn) was used as a model organism to test the influence of elementary body (EB) purity and concentration of plasmid on transformation efficiency. Ultimately, the experimentation was inconclusive; higher amounts of plasmid in the initial transformation did not yield a higher number of transformants nor yield them in earlier generations when compared to the control groups. Furthermore, the purity of infectious elementary bodies (EBs) were not essential for transformational competence; both ultra-purified EBs that were isolated via a density gradient and partially purified *Chlamydia muridarum*

displayed successful transformants. Further experimentation would be needed with higher sample sizes to ensure statistical significance, and trials on *Chlamydia trachomatis* itself would have to be performed to ensure consistency across both species. Finally, trimethoprim, banzal-N-acylhydrazones (BAH), and other antibiotics should continue to be screened as alternative selective agents due to their differences in mechanisms of action.

ACKNOWLEDGEMENTS

I would like to extend my utmost thanks to Dr. Huizhou Fan as both program director and member of my graduation committee. He has been extremely kind, patient, and understanding despite my rather tenuous masters track. At a time when I was overcome by problems that I later found to have been out of my control, it was Dr. Fan who stepped up as a gentle mentor to guide me through all of my woes. His constant stern yet friendly attitude has served to help refine my abilities as a researcher and focus upon what matters most instead of secondary distractions. Dr. Fan is a paradigm of what every research mentor should strive to become, and it is only through his kindness that I was able to persist through my health problems and pursue my masters despite all of the road blocks. I am truly grateful for having had the opportunity to learn from him as a mentor.

It is also difficult to express how much I appreciate my fellow lab members, Dr. Wurihan Wurihan, Dr. Huirung Zhang, Mr. Malhar Desai, Alec Webber, and Mr. Sanjeevan Vellapan, for taking the time to walk me through my experiments in a hands on fashion while still maintaining their own tenuous work. Every member of the lab was always extremely kind and patient with me despite any sort of headaches I had caused. My time spent with them, however brief, is something that I will continue to cherish as a period of support when I was not feeling my best. From Dr. Fan to everyone else, the environment had an atmosphere that felt like a small family; I can only hope to end up in such a welcoming group of coworkers and friends in the future.

I would also like to apologize to my friends and family from Maryland. The whole masters

process for me has taken a lot longer than it realistically should have, but none of them wavered in their support. I spent many nights contemplating my future and suitability for continuing the program and while I never actively mentioned my doubts to my family, they were kind enough to let me live at home and develop new skills while I regained enough confidence to return back to work. While I was out of state, I missed all too many family gatherings and familiar faces in a way that I realize I took for granted.

Furthermore, to the members of my thesis defense committee: Dr. Joseph Fondell and Dr. Loren Runnels, I appreciate them taking the time out of their busy schedule to read over my presentation and teach me in my graduate courses over the years. I would like to thank Dr. Runnels in particular, as he was kind enough to take me in for a lab rotation on short notice, and allowed me to use his centrifuge when the one in the Fan lab was broken.

Finally, I would like to extend my appreciation for my primary care provider, Dr. Nan Wang. Dr. Wang listened to my problems and took the time to explore every realm of possibility which could be causing my symptoms. Despite the typical demographics, Dr. Wang recommended me to a sleep specialist and I was finally diagnosed with sleep apnea. Dr. Wang represents the type of physician I hope to one day emulate that understands the individual nature of medicine where statistics do not define the individual and makes every effort to take care of their patients.

My masters has been a long and arduous journey, and my only wish is to have been able to work at 100% from the beginning. With the past now behind me, the most I can do is strive forward to make the most of the future thanks to everyone's continued help and support.

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INTRODUCTION

The bacterium *Chlamydia trachomatis* is an obligate intracellular gram-negative pathogen responsible for its namesake infections: chlamydia, the most widely represented sexually transmitted disease (STD) in the United States [1], and trachoma; the leading infectious cause of blindness in the world with up to 2.8 million cases globally as of 2016 [2] [26]. As an STD, the World Health Organization (WHO) estimates that chlamydia affects about 4.2% of all women aged 15-49 years, and about 2.7% of all men aged 15-49 years [3]. Furthermore, the Centers for Disease Control and Prevention (CDC) reports a total prevalence of chlamydia of 1.7% in the U.S population aged from 14-39 years [4] and 4.7% of sexually active women aged from 14-24 years [5]. Moreover, it is estimated that about only 10% of men and 5-30% of women with confirmed chlamydia infections develop symptoms [6], resulting in lower treatment rate and a potentially much higher infection rate than currently reported. In the case of trachoma, infections commonly occur in 44 countries in Africa, Asia, Central and South America, Australia, and the Middle East. In some areas it is endemic with up to 60-90% of children being affected, and up to 61% of global cases centralized in the sub-Saharan region [7]. Trachoma is spread through direct and indirect contact with an affected patient's eye or nose discharge, and as a result is hyperendemic in areas of poor sanitation [7]. As a pathogen for the globally present chlamydia and trachoma infections, *C. trachomatis* represents a preventable threat that is important to both understand and mitigate for many years in the future.

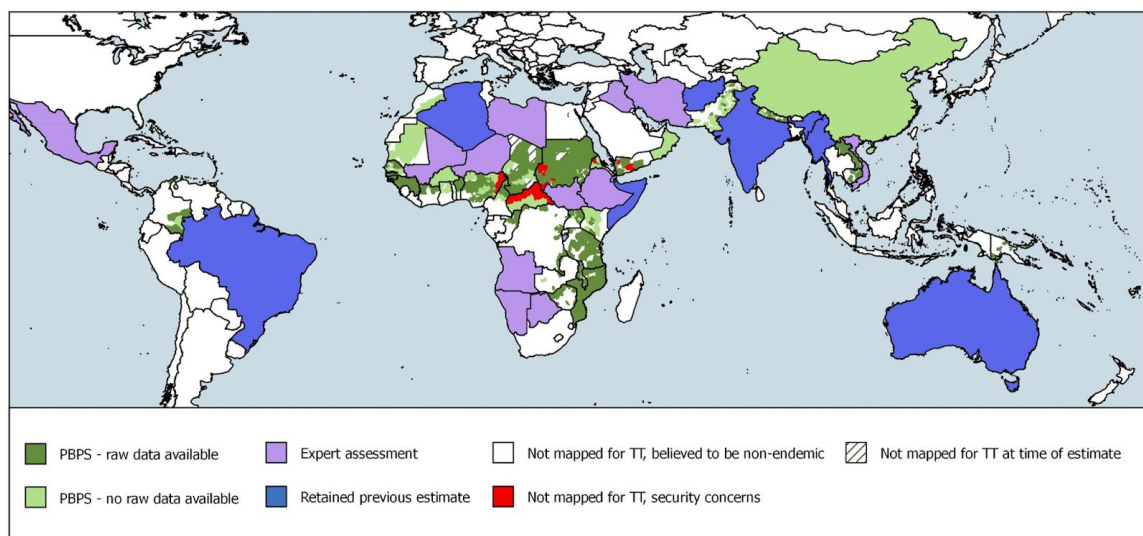


Figure 1. A Global Distribution of Trachoma in 2016 [29]. Note the clustering in areas of high population density as well as much of the developing world.

Due to its asymptomatic nature, *C. trachomatis* is commonly left untreated and can progress into several, more serious complications in both trachoma and chlamydia. In trachoma, the earlier stages of progression have no visible signs of infection [9] and are treated relatively easily with azithromycin and tetracycline eye drops [8]. However, continual infections combined with the resultant inflammation gradually causes an accumulation of scarring among the inside of the eyelid called trachomatous conjunctival scarring [8], requiring at least surgery [8]. As the scarring accumulates, the eyelids turn inwards so that the lashes scratch the eyeball, reaching the trachomatous trichiasis grade of infection and more extensive surgery is required [8]. Finally, the eyeball can become so heavily scratched to where the cornea becomes opaque, and thus the patient becomes irreversibly blind [8].

In *C. trachomatis*' other infection, chlamydia, there is a very similar case where early treatment through broad spectrum antibiotics will cure the disease, but progression results in much more severe symptoms that are irreversible [10]. In women, bacteria initially affect the cervix, and might cause the onset of cervicitis (bleeding, discharge), which may then spread onto the urethra and cause urethritis (dysuria, painful urination) [1]. Further progression of chlamydia to the fallopian tubes and uterus can result in pelvic inflammatory disease (PID) and eventually

infertility or fatal ectopic pregnancy, where the embryo attaches outside of the uterus and is killed off [1]. In males, there are similar symptoms of urethritis, discharge and epididymitis, but a different group of *C. trachomatis* serovars, L1-3, have been found to be much more invasive than the other sexually transmitted groups (D-K) and are associated with the development of proctitis and eventually lymphogranuloma venereum (LGV), especially among men who have sex with men [11]. Patients that have an untreated chlamydia infection may also be more susceptible to acquiring or spreading HIV, the precursor to AIDs [1].

Of particular note, the genome of *C. trachomatis* is highly conserved among all of the different serovars, with ocular and genital tissue tropism determined by small changes in the genome [29]. In particular, the sexually transmitted variants retain synthesis of tryptophan synthase that ocular serovars do not [29]. Tryptophan synthase allows *C. trachomatis* to produce nutrients in a persistent and potentially chronic state while in the presence of interferon- γ , immune cells which kill by depleting tryptophan in the local environment [48]. However, the most important factor involved in the classification of serovars is the genetic variation of the major outer membrane protein (MOMP), a product of gene *ompA*. MOMP are porin and structural proteins that compose up to 60% of the total membrane dry-weight and contain four highly variable surface-exposed domains that are major determinants of immune response and also contribute to adherence to different host surfaces [49].

The Serovars of *Chlamydia trachomatis*

| Serovar | Method of Transmission | Infection | Symptoms |
|----------|---|---|---|
| A-C | Contact to eyes by fomites, Hands, flies | Conjunctivitis | Scarring, Blindness (Trachoma) |
| D-K | Sexual | Urethritis, cervicitis, Endometritis, Discharge Proctitis | Ectopic Pregnancy, Miscarriage, Premature birth, Pelvic Inflammatory Disease (PID), Infertility, Cancer |
| L1,L2,L3 | Sexual | Genital Ulcer, Lymphadenopathy In addition to previous | Lymphogranuloma venereum (LGV) |

Table 1. A Classification of *Chlamydia trachomatis* Based on Tissue Tropism

Considering the role of *C. trachomatis* in the development of the global epidemics: LGV, chlamydia, and trachoma through its various serovars, the need to further increase our understanding of the bacteria and develop new techniques of treatment is becoming increasingly important. The current protocol involves the usage of broad spectrum antibiotics that are also toxic to members of the gut and vaginal microbiome; which have recently become understood to influence many aspects of wellbeing from something as particular as women's health [12], to obesity [13], and even cancer [14]. As research continues to explore the vast systemic effects of the human microbiota, it is imperative that we examine *C. trachomatis* physiology and develop specialized medicine that would avoid the need for harsh unfocused bactericidal agents that could potentially harm the patient.

In order to develop more specialized medicine against *Chlamydia trachomatis*, it is important to understand the unique biphasic developmental cycle that remains consistent through all different forms of infection. The defining characteristic of all *Chlamydia* is their two alternating cellular forms, the infectious yet reproductively dormant elementary body (EB), and the actively replicating reticulate body (RB), both which serve important purposes as they progress through development [15]. The lab's serovar of interest, *C. trachomatis* L2, enters the cell as an EB through clathrin-mediated endocytosis; more specifically through the actions of fibroblast growth factor 2 (FGF2) acting as a bridging molecule to facilitate interactions between EBs and the FGF receptor on the cellular membrane causing endocytosis (steps 1-2 on Figure 2) [16]. About 2 hpi (hours post inoculation) EBs are trafficked to the microtubule organization center via dynein motors and begin the process of forming an inclusion in the perinuclear area (step 2-3).

Subsequently, the EBs begin to convert into RBs until ~6-8 hpi when RB replication begins (step 4). The RBs replicate for ~8 bacterial chromosomal divisions and then re-differentiate back into EBs starting at around 24 hpi, fully completing by ~40–70 hpi (step 5). The gene expression in the period of conversion from an EB-dominating state to RB-dominating state has been of particular interest in microarray studies and have found that some genes only show expression in either particular stage [31-32]. Because of the extensive need for transcriptional level control in the expression of temporally active genes, continued studies have

focused on the interactions of the various subunits comprising the RNA polymerase: two α subunits, a β subunit and a β' subunit [32]. However, the most important findings have been surrounding the regulation and purpose of the three sigma factors encoded by *C. trachomatis* (σ^{28} , σ^{54} , and σ^{66}) which all play a factor in EB - RB interconversion [33-35]. While the targets of σ^{54} have not been well characterized [35], it has been found that a chlamydia specific transcription factor, GrgA (General Regulator of Genes A), regulates both σ^{66} , involved in the expression of the majority of chlamydia genes throughout development [37], and σ^{28} , necessary for several genes late in the developmental cycle [36] (along with σ^{54}) [38]. Furthermore, GrgA itself has been characterized as a promising target for the development of future pharmaceuticals due to its specific nature as well as low possibility of spontaneous resistance to benzal-N-acylhydrazones (BAH) [39], which have been proven to have antichlamydial effects [41].

Once the EBs fully saturate the vacuole (step 6), they can exit the cell and begin affecting neighboring cells through two possible mechanisms; lysis of the cellular host with a series of calcium and protease dependent pathways that result in the breakdown of the plasma and inclusions respectively (step 7), or a packaged release mechanism called extrusion (not shown) [28]. Extrusion is a slow process by which the inclusion pinches, protrudes out of the cell within a cell membrane compartment and detaches by hijacking host proteins that are involved in cytokinesis, leaving behind a residual inclusion that can continue to differentiate. Through inhibition of several different types of cellular factors in *Chlamydia*-infected cells, it was found that actin polymerization, Wiskott-Aldrich syndrome protein, myosin II and Rho GTPase were necessary for extrusion to occur [28]. The whole developmental cycle of *C. trachomatis* takes on average 2-3 days, but there is a degree of variability due to the asynchronous nature of RB maturation. It is due to this distinct biphasic, obligate-intracellular developmental mechanism that the progression of in-vitro methodologies for genetic manipulation has remained elusive until recently, where new techniques have already become an important cornerstone for our current knowledge of the molecular biology controlling *C. trachomatis*.

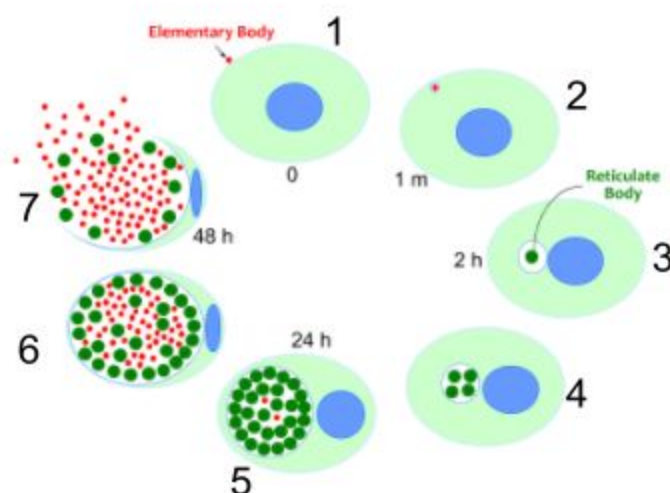


Figure 2. The Development Cycle of *C. trachomatis*
 Image courtesy of Dr. Huizhou Fan

Historically, genetic modification has been performed by manipulating the bacterial process of transformation, wherein an organism modifies its genome through the endocytosis and incorporation of exogenous DNA from its natural environment. In 1928, transformation in bacteria was first demonstrated in the classic work of Frederick Griffith where he demonstrated a “transforming principle” where nonvirulent *Streptococcus pneumoniae* strains could become activated when exposed to heat-killed strains [17]. Progression in new transformative techniques remained stagnant until the 1970s and 1980s when the modern protocols of CaCl_2 exposure and electroporation were established as ways of conferring transformational competency to *Escherichia coli* [18]. Following CaCl_2 (or any divalent cation) exposure, bonds form with the positively charged Ca^{2+} , the negatively charged phosphate backbone, and lipopolysaccharides on the surface of the cell membrane. Afterwards, the cell is heat shocked at $\sim 42^\circ\text{C}$ to depolarize the cell membrane and make the cytosol less negative, allowing the negatively charged LPS and DNA complex to pass through the membrane and into the cytosol. In the alternative technique, electroporation, the cell is shocked to compromise the cell membrane, which allows DNA to pass freely into the cytosol to dramatically increase chances of a successful transformation. Cells are transformed concurrently with a gene of interest, a reporter gene such as GFP or luciferase, as well as a gene that confers penicillin or some form of drug resistance to select for successful transformants and kill off the rest. From a medical perspective, the development of modern

transformation principles have revolutionized the mass production of insulin. Thanks to transformation, *E. coli* cells have been modified to produce human insulin as opposed to previous iterations that were directly taken from cattle and pig, which have been known to cause adverse reactions in patient populations [19]. Adoption of modern transformation principles yielded the first biosynthetic human insulin “Humulin,” and its predecessors which let an estimated 150-200 million people across the world live with insulin therapy in the growing epidemic of diabetes and obesity [20].

Despite the different techniques that have successfully transformed *E. coli*, *C. trachomatis*’ unique physiology as an obligate intracellular organism demonstrates the need for a novel approach. As noted earlier, *C. trachomatis* only actively replicates as a reticulate body (RB) within an isolated host cell vacuole known as an inclusion, making the delivery and uptake of foreign DNA through transformation a difficult task that must be performed early on to maximize the spread of the respective gene. An early attempt at genetic recombination in 1994 involved the attachment of chimeric plasmid pPBW100, constructed from a combination of *C. trachomatis* and *E. coli*, to a chlamydia promoter p7248::cat (chloramphenicol acetyltransferase) cassette and introduced into *C. trachomatis* through electroporation [21]. The experiment resulted in “transient” expression of the plasmid that only rarely persisted after four passages, as determined by in situ and Southern hybridization analyses [21]. Further experimentation with homologous recombination introduced by electroporation was conducted in 2009 on *Chlamydia psittaci*, a *Chlamydia* species associated with avian origin, where a single rRNA operon targeted with a synthetic 16S rRNA allele successfully conferred resistance to kasugamycin and spectinomycin. Despite the successful transformation, the genetic modification was highly inefficient as homologous recombination and transformation yielded only a few successful recombinants who, in turn, displayed changes limited to the 16S rRNA. Ultimately, the early attempts at transformation served more as a proof of concept for the development of *Chlamydia* transformation technique rather than as something with more practical benefits [22].

The first major breakthrough came in 2011 when Yibing Wang from the Clarke Lab successfully developed the foundation for modern *C. trachomatis* transformational protocol by

incorporating shuttle vector pBR325::L2 through CaCl_2 treatment instead of the previously attempted electroporation [23]. For the initial experiment, the Clarke lab introduced both β -lactamase (*bla*) and chloramphenicol acetyltransferase (*cat*) genes that grant resistance to their respective drug (penicillin and chloramphenicol) through direct metabolism. As far as the selective agent was concerned, penicillin was chosen more as a result of eliminating tetracycline and chloramphenicol as possible alternatives. Tetracycline is prescribed as the treatment of choice for *Chlamydia trachomatis* infections, and thus the development of a resistant species would be unfavorable. Despite having resistance from chloramphenicol thanks to the aforementioned (*cat*) gene, it was theorized that mitochondrial stress would negatively affect growth and proliferation and result in less than optimal gene integration. However, this theory was unfounded as the Fan Lab demonstrated in 2013 that the minimal concentration for inhibition of inclusion formation is 0.05 $\mu\text{g/ml}$, much lower than the toxic concentration of 10 $\mu\text{g/ml}$ [30]. Further experimentation with chloramphenicol demonstrated it as an effective selective agent in *C. trachomatis* serovars for which β -lactamase carrying vectors are not permitted as well as an effective secondary selective agent [30].

Historically, progression in the field of *C. trachomatis* research has been limited due to the conservative nature of the studies, and has only recently exploded with a multitude of activity thanks to the establishment of a modern transformational protocol with the Clarke Lab in 2011, allowing researchers to perform various genetic experiments to coincide with the sequencing of *Chlamydia trachomatis*' genome [23]. In particular, genetic sequencing found a number of genes (28%) that code for hypothetical proteins, which require an extensive amount of analysis through either a forward or reverse genetic approach [46]. As a form of reverse genetics, transformation represents an ideal tool for the identification of functions of individual genes. A particular gene in question can be manipulated by a localized knockout, knockin, or silencing mutation introduced by transformation, which in turn will result in a distinct phenotype that can later be analyzed. In contrast, forward genetics represents the classical Mendellian approach where the starting point is a phenotype resulting from nonspecific mutation and the gene(s) responsible are unknown. An organism has so many different genes that to isolate the function of a single gene is extremely

time intensive, especially when the mutant phenotype is not obviously distinguishable. Forward genetics serves better as a way to identify how whole organisms function rather than to identify the function of a single unknown gene of interest. Transformation has become almost synonymous with modern genetic research, and is being used in many different organisms ranging from plants, to *Saccharomyces cerevisiae* to *E. coli* ; it comes at no surprise that following the seminal discovery by Wang et al. in 2011 that further research opportunities exploded to make use of this new technology. Later advances such as Wickstrum et al's inducible gene expression system in 2013 [40] and Mueller et al's fluorescence-reported allelic exchange mutagenesis (FRAEM) in 2016 [47] use transformation as a strong foundation to employ their own methodologies and further accelerate our knowledge of *Chlamydia* function.

To further expand on the experiments performed by Wang et al., the initial transformation protocol was derived from a standard developed for *E. coli* and utilized bacteria treated with ice cold solutions of CaCl_2 followed by heat shock. Subsequent testing found that heat shock did not provide any additional benefits and incubation could be performed at room temperature by incubating purified EBs, plasmid conferring the genes of choice, and competent cells in CaCl_2 . After 3 rounds of selection, the infected cells were exposed to penicillin and examined for growth. Wild-type penicillin-inhibited *C. trachomatis* were killed with no chances for any growth. In contrast, transformants were able to display similar growth kinetics (albeit a slightly extended lag phase) and inclusion morphology as the wild-type *C. trachomatis* in a control environment and also persisted for continuous 8 passages (the end of the experiment), showing permanence of the integrated gene. The pBR325::L2 transformants were then purified and analyzed using a Southern blot which confirmed that no changes aside from DNA incorporation had occurred. As a proof of concept, the RBs isolated from transformed *C. trachomatis* L2/434/Bu and wild type *C. trachomatis* L2/434/Bu were assayed for β -lactamase activity by measuring the hydrolysis of Nitrocefin, a β -lactamase substrate, and found results consistent with transformed *E. coli* controls; a rise in absorbance at 486 nm as time elapsed in the transformants with nothing on the wild-type.

As confirmation that the transformation and drug resistance was not due to some unknown factor found in pBR325:L2, a final set of experiments was conducted with a newly created plasmid that encoded redshifted-GFP in addition to the chloramphenicol acetyltransferase and β -lactamase of the original, and dubbed pGFP::SW2. Ultimately, the experiments showed that their newly found transformational protocol was reproducible and effective, as this final group of transformants demonstrated penicillin-resistance and green fluorescent inclusions 24 hpi after the initial passage.

It was the focus of my work to further examine and optimize the factors contributing to the transformation efficiency of the currently established protocol in *Chlamydia* as it is a very time, money, and laboriously intensive process, both in the transformation itself as well as in the preparatory purification of EBs. For my research, I turned to a closely related cousin to *Chlamydia trachomatis* that had already been successfully transformed by the Zhong lab in 2014: *Chlamydia muridarum* (MoPn), a strain that is very similar but with minor practical differences. MoPn is a species originally known for causing pneumonia when nasally inoculated in members of the family *Muridae* [25], and has been found to mimic the signs of *Chlamydia trachomatis* infections in humans [45]. The developmental cycle of *Chlamydia muridarum* progresses at a faster rate than *Chlamydia trachomatis*' various serovars, releasing inclusion forming units at 24 hpi [43], instead of 30-48 hpi in the genital strains and 48-68 hpi for the ocular strains [44]. With such a rapid rate of growth, passing the respective transformants can progress into subsequent generations with more regularity than similar experiments conducted with *Chlamydia trachomatis*. Furthermore, *Chlamydia muridarum* only naturally infects members of the family *Muridae* (most notably mice and hamsters) and not humans as *Chlamydia trachomatis* does. Because of the difference in the respective hosts, there are no longer any ethical dilemmas in examining tetracycline or any other possible pharmaceuticals as an alternative selective agent. Ultimately, MoPn serves as an ideal model organism to further examine the factors contributing to transformation efficiency in Chlamydiae and seek out ways to optimize it with a myriad of different factors such as alternative selective pressures, levels of plasmid DNA or even changing the purity of the elementary bodies used in the transformation procedure.

RESULTS

As mentioned before, my first experiment was a reevaluation of the previous experiment conducted by the Zhong lab in 2014, where the plasmid pGFP::CM was successfully transformed in *Chlamydia muridarum* and conferred resistance to chloramphenicol and ampicillin [42]. In my experiment, only one colony successfully transformed after five passages of penicillin G supplementation. The lone transformant displayed penicillin-resistant *Chlamydia muridarum* inclusions that readily fluoresced green under microscopy. A further passage displayed no phenotypic change with the colony, and continued to fluoresce in microscopy and grow despite exposure to bacteriostatic conditions, indicating a permanence of the GFP and β -lactamase genes respectively.

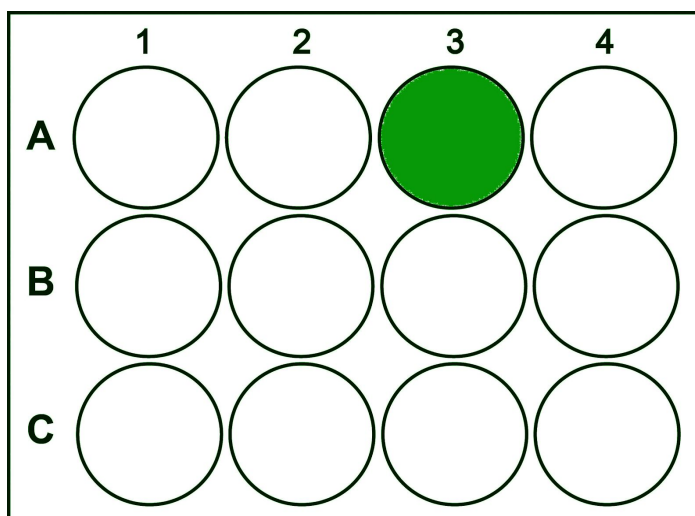


Figure 3. The Growth of *Chlamydia muridarum* Transformed With Plasmid pGFP::CM and Selected With Penicillin G (5th passage)

Image represents a 12-well plate with a confluent monolayer of L929 cells. White indicates no *Chlamydia muridarum* present, Green indicates successful transformant. Wells represent independent lines of cultures passaged and selected with penicillin.

The Effects of Plasmid Concentration on Transformation Efficiency

Following the success of the preliminary test, we examined the effects of increasing the concentration of plasmid in the incubation portion of the initial step of transformation of the procedure, surmising that there might be a less than optimal amount of plasmid exposure. Ultimately, there was no notable difference between the two concentrations of 5 μ g and 15 μ g of

plasmid; fluorescent inclusions emerged at the same generation (passage 4, left side of Figure 4) and remained consistent for the fifth and final passage (right side of Figure 4). The control (5 μ g) had one less transformant than the 15 μ g, but more experimentation is required to determine the statistical significance in the difference between the two samples.

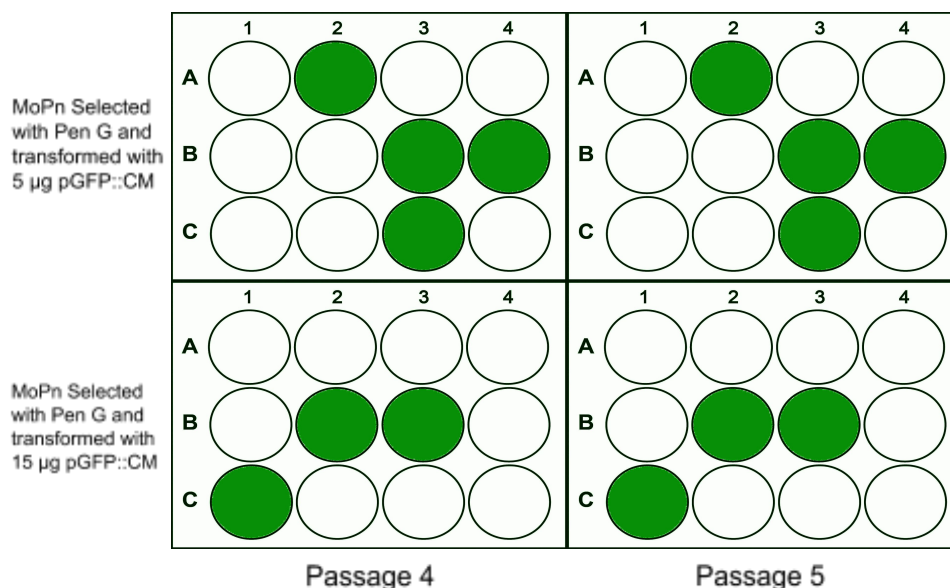


Figure 4. The Effects of Plasmid pGFP::CM Concentration on *Chlamydia Muridarum* Growth When Selected With Penicillin G

Image represents a 12-well plate with a confluent monolayer of L929 cells. White indicates no *Chlamydia muridarum* present, Green indicates successful transformant. Wells represent independent lines of cultures passaged and selected with penicillin.

The Effects of Purification Status of Elementary Bodies on Transformation Efficiency

To continue our exploration of the factors contributing to transformation efficiency, we examined the effects of purification status on EBs to see if purity was a necessity for the emergence and an optimal number of successful transformants. We set the control as the samples following the protocol of purification via MD-76 gradients in the Fields paper, and designated them “highly purified.” In contrast, the experimental sample was merely SPG washed and designated “partially purified.” Initial testing suggested that highly purified elementary bodies were necessary for transformation to occur at all, with none appearing even after four passages in the crude sample at all (top of figure 5). A second trial was conducted and had the exact opposite results, both the highly purified and partially purified samples found similar numbers of

successful transformants with no differences in physiology nor time of emergence, as both began fluorescing at the third passage (bottom of figure 5).

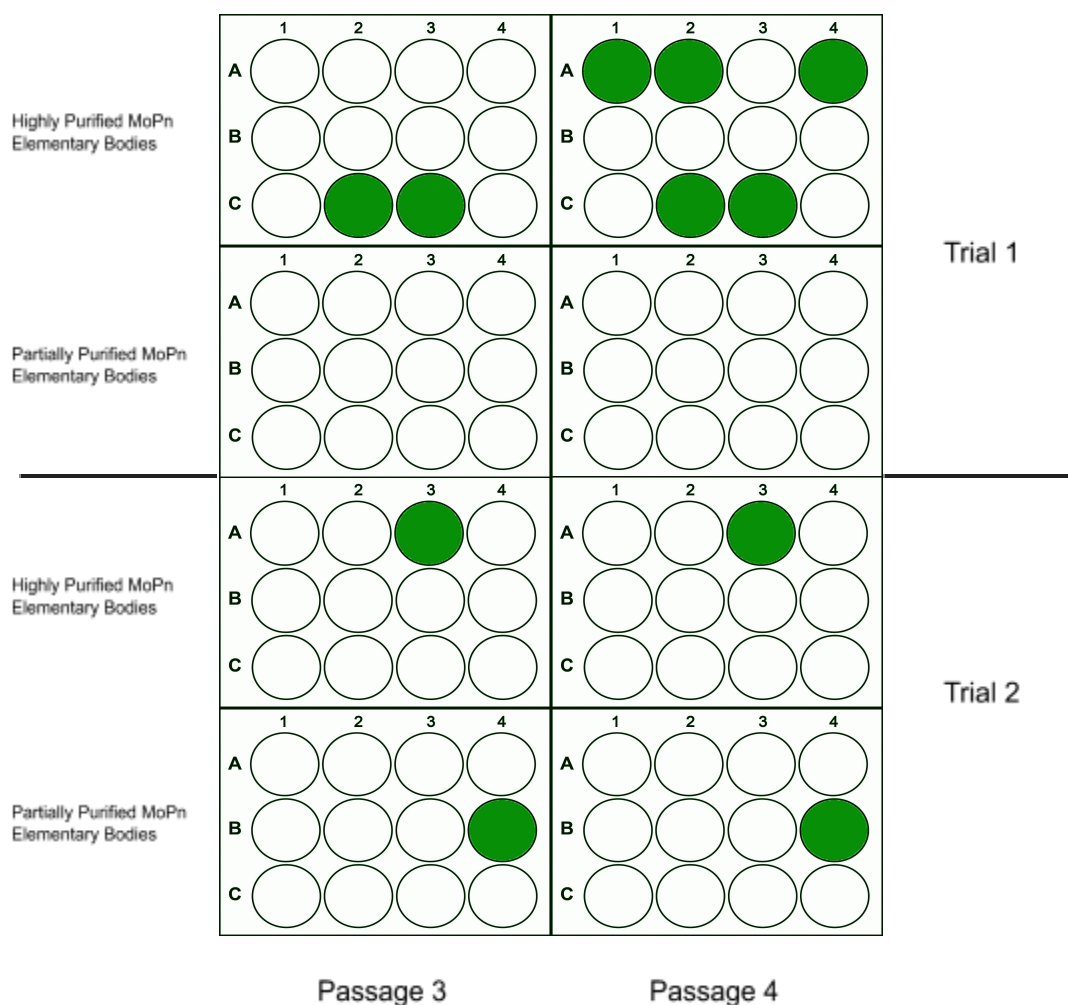


Figure 5. The Effects of Elementary Body Purity on *Chlamydia Muridarum* Growth When Selected With Penicillin G

Image represents a 12-well plate with a confluent monolayer of L929 cells. White indicates no *Chlamydia muridarum* present, Green indicates successful transformant. Wells represent independent lines of cultures passaged and selected with penicillin.

DISCUSSION AND CONCLUSION

Despite the limited amount of trials completed for the experiments, there are still some findings which may prove valuable for further research. With respect to the initial experiment varying the concentration of plasmid from 5 µg to 15 µg the similarity in results suggested that

there is some degree of saturation limit for the exogenous DNA to be incorporated to the MoPn DNA replication cycle as increasing the plasmid amount by 200% only increased successful transformants by 33% (Figure 4). In order to further explore this phenomenon, a more variable amount of DNA exposure should be presented at 1, 3, 5, 10 or 15 μg of pGFP::CM; with 1 and 15 μg representing the extreme values to determine when some sort of asymptotic value for saturation starts making an appearance.

The other factor examined in my experiments was the significance of purity in the infective EB used in the initial incubation procedure, distinguishing between purification status with a sample collected by a separation of elementary bodies (EBs) from reticulate bodies (RBs) using a density gradient, and sample that utilized a crude SPG wash purification with no separation of *C. muridarum* forms. At first appearance, the data suggests that the ultra-high speed purification has a much higher transformation efficiency than the partially purified sample and that ultra purification is necessary to successfully transform colonies at all (top of figure 5). However, the results from the second trial, on the contrary, suggested that both of the populations had similar transformation efficiency and purity is not truly necessary as there were successful transformants (bottom of figure 5). Given the low sample size in my experiments, it is difficult to make any sort of meaningful comparisons between the various populations because all of the data could have been due to random chance. With more trials and data, statistical tests can be performed to determine if the different populations were significantly different from each other and if any such trends would be considered statistically significant with a p-value < 0.05 . Furthermore, especially with regard to the first experiment concerning plasmid concentration, a linear regression would be performed to determine a line of best fit to determine the degree to which plasmid concentration can influence transformation efficiency.

Future researchers can expand upon my initial research with penicillin G and examine the effectiveness of alternative selective agents to *Chlamydia*. I was in the process of conducting titrations with chloramphenicol and trimethoprim to evaluate their effectiveness as selective agents in *Chlamydia trachomatis* with the plasmid pBR325::SW2, however future researchers can just as well evaluate the effectiveness of benzal-N-acylhydrazones (BAH) and or other antibiotics

with different mechanisms of action. While we have already identified several markers for *Chlamydia* transformation such as chloramphenicol and penicillin G, secondary markers to build upon an already transformed sample and further increase selective agency, is becoming more and more important especially with the looming threat of growing numbers antibiotic resistance day by day. Ultimately, additional experimentation would also need to be conducted upon *Chlamydia trachomatis*. Overall, all of my work was conducted upon a model organism instead of the organism itself and while *Chlamydia muridarum* is a good representation of an infection in humans, there is an importance to demonstrate a consistency in effect across both species.

Chlamydia trachomatis remains one of the more ubiquitous infectious agents, it affects 44 different countries and reaches near endemic proportions in areas of sub-Saharan Africa making a thorough understanding of its underlying mechanisms evermore urgent. However, *Chlamydia trachomatis*' unique biphasic developmental cycle makes direct genetic manipulation through traditional techniques very difficult, demonstrating the need for reverse genetic techniques such as transformation, that are hampered by poor efficiency in established protocol. My research involving *Chlamydia trachomatis*', genetically preserved cousin *Chlamydia muridarum*, represents a preliminary exploration of the factors contributing to the transformation of the species. With time, a more optimized protocol can be built off the foundations laid here with an end result of a higher transformation efficiency in *Chlamydia trachomatis* and ultimately a deeper level of understanding of the mysteries that surround this enigmatic bacterium.

MATERIALS AND METHODS

Reagents:

Phosphate Buffered Saline (PBS), Hanks Balanced Salt Solution (HBSS), Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/L) and 110 mg sodium pyruvate, Trypsin (10x stock), cycloheximide, ampicillin, penicillin G, CaCl₂ and gentamicin were purchased from Sigma Aldrich. The pGFP::CM shuttle plasmid was kindly provided by Dr. Guangming Zhong (University of Texas Health Science Center San Antonio Texas). All cell culture related materials such as T150 flasks, 6 / 12 well / 20 ml plates, and

serological pipettes were purchased from Greiner Bio-One. The mini, midi and maxi prep-kits were purchased from Thermo Fisher. The DH5-alpha E. coli, *Chlamydia muridarum* (MoPn), and mouse fibroblast L929 cells were purchased from ATCC.

Cell Culture and Growth Parameters

Mouse L929 cells were maintained in DMEM with high glucose (4.5 g/L), 110 mg sodium pyruvate with 10% FBS and kept in T150 Falcon flasks after being supplemented with 1 µl of cycloheximide and 1 µl/1 ml penicillin. Except for in experiments, culture flasks were placed in a 37°C humidified 5% CO₂ incubator and filled no more than half with loose caps to allow for gas exchange between the interior and exterior of the flasks.

Amplification of Plasmid

10 µl of Plasmid pGFP::CM was added to <1 µl of competent DH5-α cells and iced for 20 minutes. The cells were then heat-shocked in a 42°C water bath for 30 seconds and then iced. 300 µl of LB was then added to each tube and left in a 37°C water bath for 30 minutes. The mixture was then plated onto ampicillin plates, spread evenly using small glass beads and finally left overnight in a large 37°C incubator overnight. A resistant colony was then touched with a p10 pipette tip and then placed in 2 ml of LB for 8 hours. The stock was expanded to 1 L LB of solution, supplemented with ampicillin at a ratio of 1 µl/1 ml medium and finally harvested when confluent as deemed by the spectrophotometer. Finally, the plasmid was isolated using a maxiprep kit at 4°C and the insert confirmed for accuracy using gel electrophoresis.

Isolation of Elementary Bodies

Cell monolayers of 10 flasks at about 85-95% confluence were incubated for 28 hpi (hours post infection) with *Chlamydia muridarum* at time of harvesting. Cells were washed with PBS, and scraped off using a CellLifter. The cells were then suspended with 2 ml SPG and collected by low speed centrifugation at 1200 rpm for 10 mins. The supernatant was then aspirated and the pellet resuspended in 5 ml SPG. The suspensions were then sonicated in small 50 ml glass tubes at

40% intensity. Finally, the disrupted cell suspension was centrifuged again at 1500 rpm for 10 minutes at 4°C degrees to collect excess cellular debris. The rest of the isolation was performed utilizing ultra-high speed centrifugation of MD-76 gradients [27].

***Chlamydia muridarum* Transformation Protocol**

C. muridarum was transformed in a manner similar to Dr. Fields [24], but with slight modifications. 2.6×10^6 EBs and 5 µg of plasmid DNA were mixed with 50 µl CaCl_2 buffer (50 mM CaCl_2) and incubated at room temperature for 30 minutes. Each mixture was suspended in 15 ml of Hanks Balanced Salt Solution (HBSS) and applied to a 12 well plate with a 3.8 cm² monolayer of confluent L929 cells per individual well and infected by centrifugation at 2300 rpm for 1 hour at room temperature herein referred to 0 hpi at termination. Afterwards, the HBSS was aspirated from the wells and replaced with Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/L), 110 mg sodium pyruvate, 10% Fetal Bovine Serum (FBS) and incubated at 37°C with 5% CO₂ without antibiotics. After 7 hpi, the medium was aspirated and medium containing 0.2 µg/ml penicillin G (Pen G) and 1.0 µg/ml cycloheximide was replaced. Infected L929 Cells were harvested approximately 26 hpi with a CellLifter and sonicated in small 50 ml glass tubes at 40% intensity. The resultant fresh EBs were used to infect new 12-well plates with a confluent monolayer of L929 by an hour of centrifugation at 2300 rpm. The process was repeated until successful transformants were identified by fluorescent microscopy. Transformed *C. muridarum* were scraped with a p200 tip, resuspended in SPG, and stored at -80°C for future use.

Transformation protocol (Plasmid concentration variation)

The process was same as above, however two 12 well plates with a 3.8 cm² monolayer of confluent L929 cells per individual well were performed at the same time, with each separate plate having either 5µg or 15µg of pGFP::CM used in the initial incubation period with 50µl of CaCl_2 buffer.

Transformation protocol (Elementary Body purification variation)

The process is the same as above, however each separate 12 well plate utilize either an ultra-purified sample of Elementary Bodies (EBs) or partially SPG purified EBs that did not undergo separation by MD-76 gradients in the initial incubation with 5µg pGFP::CM in CaCl₂. The two samples were obtained from the same initial stock of EBs and controlled for infectivity.

BIBLIOGRAPHY

1. *Detailed STD Facts - Chlamydia*. (2018). *Cdc.gov*.
<https://www.cdc.gov/std/chlamydia/stdfact-chlamydia-detailed.htm>
2. *Hygiene-related Diseases | Hygiene-related Diseases | Hygiene | Healthy Water | CDC*. (2018). *Cdc.gov*. <https://www.cdc.gov/healthywater/hygiene/disease/trachoma.html>
3. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLoS One*. 2015 Dec 8;10(12):e0143304. doi: 10.1371/journal.pone.0143304. PMID: 26646541; PMCID: PMC4672879.
4. *Chlamydia - 2016 STD Surveillance Report*. (2018). *Cdc.gov*.
<https://www.cdc.gov/std/stats16/chlamydia.htm>
5. *Prevalence of Chlamydia trachomatis Genital Infection Among Persons Aged 14–39 Years — United States, 2007–2012*. (2018). *Cdc.gov*.
https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6338a3.htm?s_cid=mm6338a3_w
6. Korenromp EL, Sudaryo MK, de Vlas SJ, Gray RH, Sewankambo NK, Serwadda D, Wawer MJ, Habbema JD. What proportion of episodes of gonorrhoea and chlamydia becomes symptomatic? *Int J STD AIDS*. 2002 Feb;13(2):91-101. doi: 10.1258/0956462021924712. PMID: 11839163.
7. *Trachoma*. (2018). *World Health Organization*.
<http://www.who.int/en/news-room/fact-sheets/detail/trachoma>
8. *Trachoma - Diagnosis and treatment - Mayo Clinic*. (2018). *Mayoclinic.org*.
<https://www.mayoclinic.org/diseases-conditions/trachoma/diagnosis-treatment/drc-20378509>
9. *What is trachoma*. (2018). *World Health Organization*.
<http://www.who.int/trachoma/disease/en/>
10. *CDC – Chlamydia Treatment*. (2010). *Cdc.gov*.
<https://www.cdc.gov/std/chlamydia/treatment.htm>
11. Abdelsamed H, Peters J, Byrne GI. Genetic variation in *Chlamydia trachomatis* and their hosts: impact on disease severity and tissue tropism. *Future Microbiol*. 2013 Sep;8(9):1129-1146. doi: 10.2217/fmb.13.80. PMID: 24020741; PMCID: PMC4009991.
12. Martin DH. The microbiota of the vagina and its influence on women's health and disease. *Am J Med Sci*. 2012 Jan;343(1):2-9. doi: 10.1097/MAJ.0b013e31823ea228. PMID: 22143133; PMCID: PMC3248621.
13. Zacarias MF, Collado MC, Gómez-Gallego C, Flinck H, Aittoniemi J, Isolauri E, Salminen S. Pregestational overweight and obesity are associated with differences in gut microbiota composition and systemic inflammation in the third trimester. *PLoS One*. 2018 Jul 13;13(7):e0200305. doi: 10.1371/journal.pone.0200305. PMID: 30005082; PMCID: PMC6044541.
14. Muls A, Andreyev J, Lalondrelle S, Taylor A, Norton C, Hart A. Systematic Review: The Impact of Cancer Treatment on the Gut and Vaginal Microbiome in Women With a Gynecological Malignancy. *Int J Gynecol Cancer*. 2017 Sep;27(7):1550-1559. doi: 10.1097/IGC.0000000000000999. PMID: 28590950; PMCID: PMC5571893.
15. Stephens AJ, Aubuchon M, Schust DJ. Antichlamydial antibodies, human fertility, and pregnancy wastage. *Infect Dis Obstet Gynecol*. 2011;2011:525182. doi: 10.1155/2011/525182. Epub 2011 Sep 22. PMID: 21949601; PMCID: PMC3178110.
16. Hybiske K, Stephens RS. Mechanisms of *Chlamydia trachomatis* entry into nonphagocytic cells. *Infect Immun*. 2007 Aug;75(8):3925-34. doi: 10.1128/IAI.00106-07. Epub 2007 May 14. PMID: 17502389; PMCID: PMC1952008.
17. Griffith F. The significance of pneumococcal types. *J Hyg (Lond)*. 1966 Jun;64(2):129-i4. doi: 10.1017/s0022172400040420. PMID: 20475871; PMCID: PMC2134723.

18. Asif A, Mohsin H, Tanvir R, Rehman Y. Revisiting the Mechanisms Involved in Calcium Chloride Induced Bacterial Transformation. *Front Microbiol.* 2017 Nov 7;8:2169. doi: 10.3389/fmicb.2017.02169. PMID: 29163447; PMCID: PMC5681917.
19. Quianzon CC, Cheikh I. History of insulin. *J Community Hosp Intern Med Perspect.* 2012 Jul 16;2(2). doi: 10.3402/jchimp.v2i2.18701. PMID: 23882369; PMCID: PMC3714061.
20. Garg SK, Rewers AH, Akturk HK. Ever-Increasing Insulin-Requiring Patients Globally. *Diabetes Technol Ther.* 2018 Jun;20(S2):S21-S24. doi: 10.1089/dia.2018.0101. Epub 2018 Jun 6. PMID: 29873518.
21. Tam JE, Davis CH, Wyrick PB. Expression of recombinant DNA introduced into *Chlamydia trachomatis* by electroporation. *Can J Microbiol.* 1994 Jul;40(7):583-91. doi: 10.1139/m94-093. PMID: 8076253.
22. Binet R, Maurelli AT. Transformation and isolation of allelic exchange mutants of *Chlamydia psittaci* using recombinant DNA introduced by electroporation. *Proc Natl Acad Sci U S A.* 2009 Jan 6;106(1):292-7. doi: 10.1073/pnas.0806768106. Epub 2008 Dec 22. PMID: 19104068; PMCID: PMC2629194.
23. Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, Clarke IN. Development of a transformation system for *Chlamydia trachomatis*: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog.* 2011 Sep;7(9):e1002258. doi: 10.1371/journal.ppat.1002258. Epub 2011 Sep 22. PMID: 21966270; PMCID: PMC3178582.
24. Mueller KE, Fields KA. Application of β -lactamase reporter fusions as an indicator of effector protein secretion during infections with the obligate intracellular pathogen *Chlamydia trachomatis*. *PLoS One.* 2015 Aug 10;10(8):e0135295. doi: 10.1371/journal.pone.0135295. PMID: 26258949; PMCID: PMC4530969.
25. Fan H, Zhong G. Chapter 81 - *Chlamydia trachomatis*. In: Tang Y-W, Sussman M, Liu D, Poxton I, Schwartzman J, editors. *Molecular Medical Microbiology* (Second Edition). Boston: Academic Press; 2015. p. 1449-69.
26. Flueckiger RM, Courtright P, Abdala M, Abdou A, Abdulnafa Z, Al-Khatib TK, Amer K, Amiel ON, Awoussi S, Bakhtiari A, Batcho W, Bella AL, Bennawi KH, Brooker SJ, Chu BK, Dejene M, Dezoumbe D, Elshafie BE, Elvis AA, Fabrice DN, Omar FJ, François M, François D, Garap J, Gichangi M, Goepogui A, Hammou J, Kadri B, Kabona G, Kabore M, Kalua K, Kamugisha M, Kebede B, Keita K, Khan AA, Kiflu G, Yibi M, Mackline G, Macleod C, Manangazira P, Masika MP, Massangaie M, Mduluza T, Meno N, Midzi N, Minnih AO, Mishra S, Mpyet C, Muraguri N, Mwingira U, Nassirou B, Ndjemba J, Nieba C, Ngondi J, Olobio N, Pavluck A, Phiri I, Pullan R, Qureshi B, Sarr B, Seiha D, Chávez GMS, Sharma S, Sisaleumsak S, Southisombath K, Stevens G, Woldendrias AT, Traoré L, Turyaguma P, Willis R, Yaya G, Yeo S, Zambroni F, Zhao J, Solomon AW. The global burden of trichiasis in 2016. *PLoS Negl Trop Dis.* 2019 Nov 25;13(11):e0007835. doi: 10.1371/journal.pntd.0007835. PMID: 31765415; PMCID: PMC6901231.
27. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect Immun.* 1981 Mar;31(3):1161-76. doi: 10.1128/IAI.31.3.1161-1176.1981. PMID: 7228399; PMCID: PMC351439.
28. Hybiske K, Stephens RS. Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc Natl Acad Sci U S A.* 2007 Jul 3;104(27):11430-5. doi: 10.1073/pnas.0703218104. Epub 2007 Jun 25. PMID: 17592133; PMCID: PMC2040915.
29. Carlson JH, Porcella SF, McClarty G, Caldwell HD. Comparative genomic analysis of *Chlamydia trachomatis* oculotropic and genitotropic strains. *Infect Immun.* 2005 Oct;73(10):6407-18. doi: 10.1128/IAI.73.10.6407-6418.2005. PMID: 16177312; PMCID: PMC1230933.
30. Xu S, Battaglia L, Bao X, Fan H. Chloramphenicol acetyltransferase as a selection marker for chlamydial transformation. *BMC Res Notes.* 2013 Sep 23;6:377. doi: 10.1186/1756-0500-6-377. PMID: 24060200; PMCID: PMC3849861.
31. Nicholson TL, Olinger L, Chong K, Schoolnik G, Stephens RS. Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. *J Bacteriol.*

- 2003 May;185(10):3179-89. doi: 10.1128/jb.185.10.3179-3189.2003. PMID: 12730178; PMCID: PMC154084.
32. Belland RJ, Zhong G, Crane DD, Hogan D, Sturdevant D, Sharma J, Beatty WL, Caldwell HD. Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A*. 2003 Jul 8;100(14):8478-83. doi: 10.1073/pnas.1331135100. Epub 2003 Jun 18. PMID: 12815105; PMCID: PMC166254.
 33. Douglas AL, Hatch TP. Expression of the transcripts of the sigma factors and putative sigma factor regulators of *Chlamydia trachomatis* L2. *Gene*. 2000 Apr 18;247(1-2):209-14. doi: 10.1016/s0378-1119(00)00094-9. PMID: 10773461.
 34. Hua Z, Rao X, Feng X, Luo X, Liang Y, Shen L. Mutagenesis of region 4 of sigma 28 from *Chlamydia trachomatis* defines determinants for protein-protein and protein-DNA interactions. *J Bacteriol*. 2009 Jan;191(2):651-60. doi: 10.1128/JB.01083-08. Epub 2008 Oct 31. PMID: 18978051; PMCID: PMC2620819.
 35. Mathews SA, Timms P. Identification and mapping of sigma-54 promoters in *Chlamydia trachomatis*. *J Bacteriol*. 2000 Nov;182(21):6239-42. doi: 10.1128/jb.182.21.6239-6242.2000. PMID: 11029448; PMCID: PMC94762.
 36. Soules KR, LaBrie SD, May BH, Hefty PS. Sigma 54-Regulated Transcription Is Associated with Membrane Reorganization and Type III Secretion Effectors during Conversion to Infectious Forms of *Chlamydia trachomatis*. *mBio*. 2020 Sep 8;11(5):e01725-20. doi: 10.1128/mBio.01725-20. PMID: 32900805; PMCID: PMC7482065.
 37. Bao X, Nickels BE, Fan H. *Chlamydia trachomatis* protein GrgA activates transcription by contacting the nonconserved region of $\sigma 66$. *Proc Natl Acad Sci U S A*. 2012 Oct 16;109(42):16870-5. doi: 10.1073/pnas.1207300109. Epub 2012 Oct 1. PMID: 23027952; PMCID: PMC3479454.
 38. Desai M, Wurihan W, Di R, Fondell JD, Nickels BE, Bao X, Fan H. Role for GrgA in Regulation of $\sigma 28$ -Dependent Transcription in the Obligate Intracellular Bacterial Pathogen *Chlamydia trachomatis*. *J Bacteriol*. 2018 Sep 24;200(20):e00298-18. doi: 10.1128/JB.00298-18. PMID: 30061357; PMCID: PMC6153665.
 39. Zhang H, Vellappan S, Tang MM, Bao X, Fan H. GrgA as a potential target of selective antichlamydials. *PLoS One*. 2019 Mar 1;14(3):e0212874. doi: 10.1371/journal.pone.0212874. PMID: 30822328; PMCID: PMC6396966.
 40. Wickstrum J, Sammons LR, Restivo KN, Hefty PS. Conditional gene expression in *Chlamydia trachomatis* using the tet system. *PLoS One*. 2013 Oct 7;8(10):e76743. doi: 10.1371/journal.pone.0076743. PMID: 24116144; PMCID: PMC3792055.
 41. Bao X, Gylfe A, Sturdevant GL, Gong Z, Xu S, Caldwell HD, Elofsson M, Fan H. Benzylidene acylhydrazides inhibit chlamydial growth in a type III secretion- and iron chelation-independent manner. *J Bacteriol*. 2014 Aug 15;196(16):2989-3001. doi: 10.1128/JB.01677-14. Epub 2014 Jun 9. PMID: 24914180; PMCID: PMC4135636.
 42. Liu Y, Chen C, Gong S, Hou S, Qi M, Liu Q, Baseman J, Zhong G. Transformation of *Chlamydia muridarum* reveals a role for Pgp5 in suppression of plasmid-dependent gene expression. *J Bacteriol*. 2014 Mar;196(5):989-98. doi: 10.1128/JB.01161-13. Epub 2013 Dec 20. PMID: 24363344; PMCID: PMC3957687.
 43. Lyons JM, Ito JI Jr, Peña AS, Morré SA. Differences in growth characteristics and elementary body associated cytotoxicity between *Chlamydia trachomatis* oculogenital serovars D and H and *Chlamydia muridarum*. *J Clin Pathol*. 2005 Apr;58(4):397-401. doi: 10.1136/jcp.2004.021543. PMID: 15790704; PMCID: PMC1770636.
 44. Miyairi I, Mahdi OS, Ouellette SP, Belland RJ, Byrne GI. Different growth rates of *Chlamydia trachomatis* biovars reflect pathotype. *J Infect Dis*. 2006 Aug 1;194(3):350-7. doi: 10.1086/505432. Epub 2006 Jun 22. PMID: 16826483.
 45. De Clercq E, Kalmar I, Vanrompay D. Animal models for studying female genital tract infection with *Chlamydia trachomatis*. *Infect Immun*. 2013 Sep;81(9):3060-7. doi: 10.1128/IAI.00357-13. Epub 2013 Jul 8. PMID: 23836817; PMCID: PMC3754237.
 46. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, Koonin EV, Davis RW. Genome sequence of an obligate

- intracellular pathogen of humans: *Chlamydia trachomatis*. *Science*. 1998 Oct 23;282(5389):754-9. doi: 10.1126/science.282.5389.754. PMID: 9784136.
47. Mueller KE, Wolf K, Fields KA. Gene Deletion by Fluorescence-Reported Allelic Exchange Mutagenesis in *Chlamydia trachomatis*. *mBio*. 2016 Jan 19;7(1):e01817-15. doi: 10.1128/mBio.01817-15. PMID: 26787828; PMCID: PMC4725004.
 48. Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT, Timms P. Chlamydial persistence: beyond the biphasic paradigm. *Infect Immun*. 2004 Apr;72(4):1843-55. doi: 10.1128/iai.72.4.1843-1855.2004. PMID: 15039303; PMCID: PMC375192
 49. Nunes A, Nogueira PJ, Borrego MJ, Gomes JP. Adaptive evolution of the *Chlamydia trachomatis* dominant antigen reveals distinct evolutionary scenarios for B- and T-cell epitopes: worldwide survey. *PLoS One*. 2010 Oct 5;5(10):e13171. doi: 10.1371/journal.pone.0013171. PMID: 20957150; PMCID: PMC2950151.