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Publication Date
2012

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Modes of Nutrient Transport Across the
Chlamydia trachomatis Inclusion

by

Sara Irene Ruiz

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Richard S. Stephens, Chair
Professor Suzanne M.J. Fleiszig
Professor Patricia C. Zambryski

Spring 2012

ABSTRACT

Modes of Nutrient Transport Across the
Chlamydia trachomatis Inclusion

by

Sara Irene Ruiz

Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Richard S. Stephens, Chair

Chlamydia trachomatis is a gram-negative obligate intracellular bacterium that has a significant public health toll. During infection, the bacteria alternate between a metabolically inert, infectious form (elementary body, EB), and a metabolically active, non-infectious form (reticulate body, RB). The process through which the bacteria alternate between the two forms, called the developmental cycle, occurs within the confines of a parasitophorous vacuole termed the inclusion. The inclusion represents an important aspect of chlamydial pathogenesis as it is the mediator between the host cell and the bacteria.

In order to establish and maintain infection, all pathogens must be able to acquire nutrients from the host. Vacuolar intracellular pathogens have developed three ways in which to acquire nutrients: (1) intercept vacuoles from the endosomal/lysosomal pathway or through the autophagosomal pathway, (2) have open channels that allow for free exchange between the host cytoplasm, and (3) possess a transport system. The inclusion of *C. trachomatis* does not acquire nutrients via fusion of host vesicles within the endosomal/lysosomal or autophagosomal pathways. It also does not possess an open channel as studies have shown the inclusion to exclude molecules larger than 520Da. These studies demonstrate that the two previously mentioned methods of nutrient acquisition are not utilized by *C. trachomatis* and suggest there exists a transport system within the chlamydial inclusion. No transport system within a bacterial vacuole has been reported, although it has been documented in parasitic vacuoles. It is intriguing to note that RBs align themselves along the inner inclusion membrane. Specifically there is a unique structural morphology indicative of a large macromolecular complex located at the junction between RB and integral to the inclusion membrane. This leads to the hypothesis that the chlamydial inclusion possesses a nutrient transport complex that links the RBs to the inclusion that is essential for nutrient acquisition.

Methionine and glucose analog localization was monitored in *C. trachomatis* infected HeLa cells. It was found that each analog exhibited a unique localization pattern that was dependent upon host and bacterial factors. Methionine was sequestered in the bacteria, while glucose was found in the lumen of the inclusion. This work was carried

out in conjunction with the development of a novel protocol to isolate inclusions from the host cell. This would provide a means for direct manipulation of the inclusion, allowing for more detailed analysis of nutrient acquisition.

Dedicated to my parents

Charles and Susan Ruiz

for giving me life and purpose

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ACKNOWLEDGEMENTS

Graduate school has definitely been an adventure full of twists and turns. I truly am grateful for the experiences I've had, both good and bad. They have shaped me into the person and scientist I am today. Of course, this is no singular feat, and numerous people were instrumental to my experience at Berkeley and scientific development.

First and foremost, I am eternally grateful to my research mentor, Dr. Richard Stephens. You allowed me the independence to grow within your lab, and pushed me to do better than I thought possible. I will carry the multitude of lessons throughout my career and know that any success I encounter first started with your teachings. Thank you to the Stephens Lab, both past and present members. Nathan Sepulveda and Meghan Zuck, it has been wonderful to have people in the lab once again! I need to also give special recognition to Dr. Kevin Hybiske. You trained me during my rotation in the Stephens Lab and helped me more times than I can count as my dissertation work progressed.

I am thankful that I chose to come to the Infectious Disease program. The emotional support all of the students have provided at one point or another has been wonderful. There is, of course, that special group of peers, whom I have had the privilege of calling my friends through these many years. Isa Arias, thank you for your support and encouragement, and especially for making me pancakes the morning of my qual! Danielle Augustin, you were always there to be my scientific sounding board and more importantly, my friend whom could make me laugh regardless of the situation. Alex Chang, I can't even list all of the ways you have helped me, but know that I am so grateful for our lab breaks that kept me sane even when we were working 12-hour days. You went above and beyond whenever I asked for help, and were able to sense when I truly needed the help of a scientist or a friend. Anna Groat, you encouraged and challenged me to overcome any obstacle I encountered in grad school. Thank you for being my friend both inside and outside the lab.

Although the support network I have had through this program has been wonderful, it is nothing compared to my home support. I never would have gotten to this stage in my life without my amazing family; my mother Susan Ruiz, father Charles Ruiz, and brother Nicholas Ruiz. Nicholas you showed me through example that if you truly love something you throw all your effort into that, and no matter how frustrating it may get, it will pay off at some point. To my dear parents, there are truly no words I can say. Everything I am and will be is because of the wonderful foundation you gave me. All those sacrifices you made so that I could have the best education possible did not go unappreciated. I remember when you bought me my first microscope, which led me to this wonderful path. Thank you.

Finally, I need to thank my amazing partner, Nick Vaughan. You were my rock at home. You made sure that I did not let the frustration and stress overshadow the progress. It was through your daily encouragement that I believed in myself enough to finish what I started. You always reminded me to enjoy the journey I am on because it will pay off. Thank you for your amazing patience, love, and laughter through it all.

CHAPTER 1
THE CHLAMYDIAL INCLUSION AND ITS ROLE IN NUTRIENT ACQUISITION

Chlamydia trachomatis
Chlamydia is defined as gram-negative obligate intracellular bacterium. There are numerous species of *Chlamydia* that can infect humans, including *C. pneumoniae* and *C. psittaci*. However, the species that has the greatest public health impact is *C. trachomatis* which is further divided into 3 biovars.

Trachoma is the world's leading cause of preventable blindness and is typically caused by serovars A, B, Ba, and C. The sexually transmitted disease of *Chlamydia* is the most commonly reportable disease in the United States which is predominately associated with serovars D-K. Women infected with *Chlamydia* tend to present as asymptomatic and become infertile due to lack of proper treatment. Lastly, lymphogranuloma venereum (LGV) is caused by serovars L1-L3 and also presents as a STD, but develops into an overall systemic infection that involves inflammation of the inguinal lymph nodes. Regardless of the biovar, all serious sequelae of chlamydial disease are caused by an immunopathological response to chronic or repeated infection. The hallmark of the bacterium *Chlamydia* is a bi-phasic developmental cycle that takes place within a parasitophorous vacuole termed the inclusion (Schacter, 1999).

Developmental cycle
There are two forms of the bacteria: an infectious, yet non-metabolically active form known as the elementary body (EB), and a non-infectious, metabolically active form called the reticulate body (RB). The cycle is initiated when an EB attaches to the host cell via an unknown host receptor. The bacteria are then internalized into a nascent phagosome. Within two hours of uptake, the EBs begin to convert to RBs due to an unknown signal and extensively modify the phagosome to create an inclusion that is completely independent of the host endosomal and lysosomal pathway (Heinzen *et al.*, 1996). The RBs multiply via binary fission and align themselves along the inner leaflet of the inclusion membrane until approximately 18 hours post-infection (hpi) when they revert back to EBs. This process continues until approximately 72hpi when the bacteria are released via lysis of the host cell or extrusion, allowing for the bacteria to disseminate (Hybiske and Stephens, 2007) (Figure 1.1). Although the signals for initiation of the different stages of the developmental cycle have yet to be determined, there are 3 distinct temporal gene clusters. The early genes are associated with macromolecular synthesis and inclusion formation. The mid-genes begin at 6-12hpi and are thought to be involved with growth and multiplication of RBs, along with metabolism. Finally, the late genes are required for reversion of the RB back to the EB and exit (Shaw *et al.*, 2000). Although the entire developmental cycle occurs within the inclusion, there are many key questions yet to be answered concerning this vacuole including how necessary host nutrients are able to traverse the membrane (Moulder, 1991).
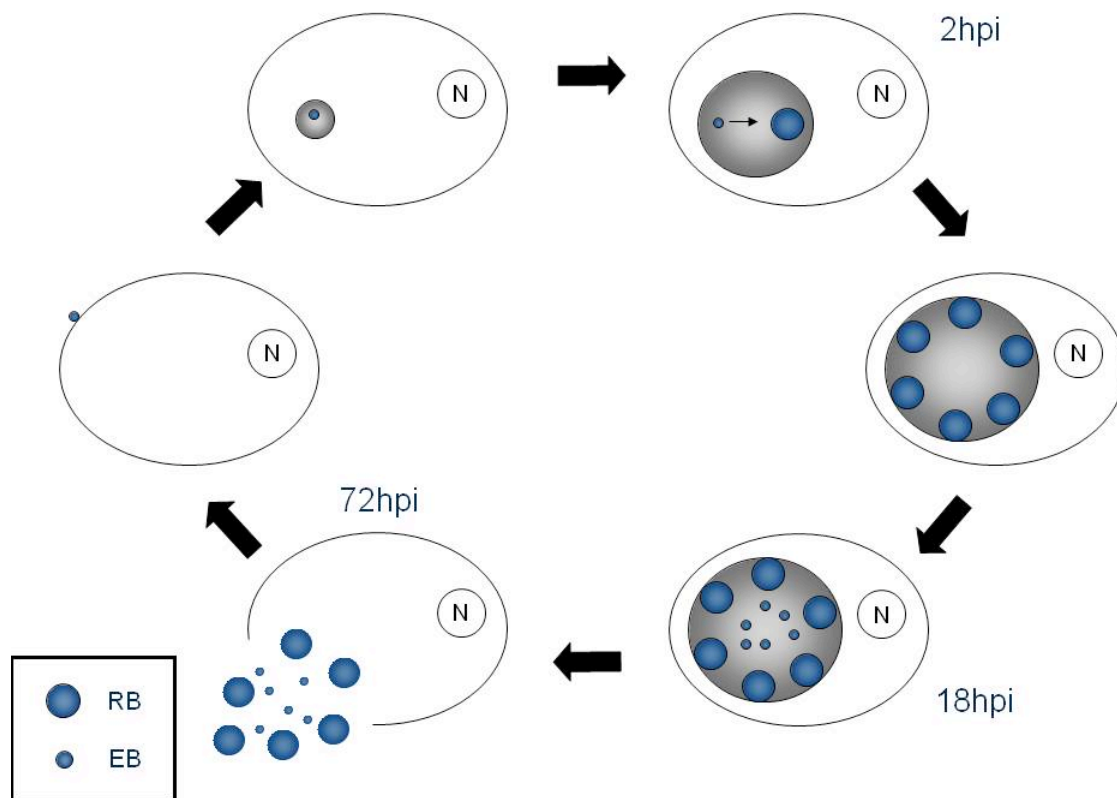
Figure 1.1. The chlamydial developmental cycle.

The cycle is initiated by attachment of the EB to the host cell followed by internalization into a nascent phagosome. The EB converts to the RB, and undergoes binary fission until 18hpi whereupon RBs revert to EBs. This continues until 72hpi at which point the host cell either lyses or extrudes the bacteria allowing for dissemination.

Inclusion

The inclusion is a distinct niche within the host cell that *C. trachomatis* establishes early during infection. Many bacteria will enter a singular host cell resulting in numerous nascent phagosomes, but within 2hpi the phagosomes have fused forming one inclusion (Ridderhof and Barnes, 1989). Although fusogenic with other inclusions, it separates itself early from the host endosomal and lysosomal pathway (Heinzen *et al.*, 1996). There is also no accumulation of endoplasmic reticulum or Golgi markers (Taraska *et al.*, 1996). The inclusion does not fuse with autophagosomes, although MAP-LC3 and calreticulin, which are markers for autophagosomes, are reported to localize to the inclusion membrane (Al-younes *et al.*, 2004). The ability of the inclusion to avoid fusion with the endosomal and lysosomal pathway is dependent on bacterial protein synthesis and is only localized to the inclusion (Scidmore *et al.*, 1996; Eissenberg and Wyrick, 1981). The inclusion is not freely permeable and prevents anything larger than 520Da to traverse the membrane (Heinzen and Hackstadt, 1997; Kleba and Stephens, 2008). This results in the inclusion allowing only cytoplasmic ions to freely enter the interior creating an environment that is similar to the host cytoplasm. The turgid structure of the inclusion that is observed is thought to result from osmotic pressure (Greishaber *et al.*, 2002).

The phospholipid composition of the inclusion membrane mimics that of the eukaryotic plasma membrane (Hatch and McClarty, 1998). The inclusion is thought to be able to grow and acquire phospholipids via accumulation of sphingomyelin from anterograde vesicular trafficking (Hackstadt *et al.*, 1995). The overall growth and replication of *Chlamydia* is dependent on the host's ability to synthesize sphingolipids (van Ooij *et al.*, 2000). The process of accumulation is also dependent on bacterial protein synthesis, energy, and temperature (Scidmore *et al.*, 1996; Hackstadt *et al.*, 1996). Sphingomyelin is acquired specifically from the GBF1 and CERT host pathways. CERT is recruited to the inclusion by a chlamydial specific protein localized to the membrane known as IncD (Elwell *et al.*, 2011; Derré *et al.*, 2011).

There are numerous *Chlamydia* proteins on the inclusion membrane, which are termed Incs. They share a common bi-lobed hydrophobicity domain that consists of approximately 50 amino acids. Upon a comprehensive scan of the *C. trachomatis* genome, 50 potential Incs were identified (Bannantine *et al.*, 2000). To date, 22 of the predicted Incs are found localized to the inclusion membrane, 7 are in the lumen, and 21 are not identified (Li *et al.*, 2008). The majority of the Incs are not assigned a function or binding partner, except for IncA and IncG.

IncA was first identified in *C. psittaci* and is found on the cytoplasmic face of the inclusion allowing it to be phosphorylated by an unknown host protein (Rockey *et al.*, 1995; Rockey *et al.*, 1997). It is observed that fibers extending from the inclusion membrane into the host cytosol express IncA (Brown *et al.*, 2002). It is thought that IncA plays a role in the homotypic fusogenic properties of the inclusion. Evidence for this role is demonstrated by natural variants of *C. trachomatis* that were no longer fusogenic, and lacked only IncA (Rockey *et al.*, 2002; Suchland *et al.*, 2000). Further analysis shows that microinjection of IncA antibody recapitulates the non-fusogenic

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phenotype (Hackstadt *et al.*, 1999). It is noted that IncA possesses a SNARE like motif which has the potential to recruit host SNAREs that are essential for vesicular trafficking (Delevoye *et al.*, 2008).

IncG binds to the host protein 14-3-3β; however, a function for this interaction is not yet determined (Scidmore and Hackstadt, 2001). A few other Incs have been analyzed based upon their expression pattern. It is shown that all Incs except for IncA are transcribed within 2hpi. IncA, IncE, and IncG have an even distribution pattern within the inclusion membrane by 18hpi, while IncD and IncF display a punctate staining pattern. Intriguingly, IncD, IncE, IncF, and IncG are enriched in sites where the RBs align themselves along the inner inclusion membrane leaflet (Scidmore-Carlson *et al.*, 1999). This differential staining pattern leads to speculation that Incs may be involved with chlamydial nutrient acquisition given that they are present only around the metabolically active form of the bacteria.

Nutrient requirements

Chlamydia is intricately tied to the host cell for obtaining all essential nutrients. It is shown that the bacterium possesses an incomplete set of genes for amino acids biosynthesis, and thus has no *de novo* production capabilities. Therefore, it is solely dependent on the host for acquisition of amino acids (Stephens *et al.*, 1998). An overall reduction of the amino acid pools in an infected host cell leads to an aberrant chlamydial developmental cycle (Coles *et al.*, 1993; Harper *et al.*, 2000). There are varying requirement for individual amino acids. Valine, leucine, phenylalanine, glutamine, histidine, and methionine are all essential for proper progression through the developmental cycle, while aspartic acid, glycine, glutamic acid, and proline are non-essential (Allan and Pearce, 1983; Karayiannis and Hobson, 1981).

In addition, *Chlamydia* requires a carbon source from the host cell utilizing both glutamate and glucose. The genome encodes transporters for both of these carbon sources, although the location of these transporters is not known (Stephens *et al.*, 1998). There is an increased overall glucose consumption noted in infected host cells, along with an increase in the expression of the glucose transporter GLUT1 (Ojcius *et al.*, 1998). Decreasing the glucose level within an infected host cell leads to an aberrant developmental cycle that produces *Chlamydia* with reduced infectivity (Harper *et al.*, 2000).

Furthermore, *Chlamydia* was originally thought to obtain all of its required adenosine triphosphate (ATP) from the host cell. However, it is shown that the bacteria are able to synthesize ATP on a reduced level and thus are not solely dependent on mitochondrial ATP. Although the majority of the ATP consumed by *Chlamydia* is from the host, it does not deplete enough from the host pools to change the overall energy charge (Tipples and McClarty, 1993). There is a noted increase in ATP production by the host corresponding to when the bacteria are mid-way through the developmental cycle (Ojcius *et al.*, 1998). Although there is a greater understanding of the nutrients that *Chlamydia* obtains from the cell, there are still questions regarding the route of transport into the bacteria housed within the inclusion.

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Nutrient acquisition
Vacuolar pathogens, although protected from the potential harsh environment of the host cytoplasm, have a multitude of challenges to overcome, including how to access necessary nutrients from the host cytoplasm given that metabolites are not in a form that easily traverses membranes (McClarty, 1994). Other vacuolar pathogens utilize a continuous open channel, intercept host vesicular trafficking, and/or possess specific transporters within the parasitophorous membrane in order to surmount the membrane barrier (Fields and Hackstadt, 2002).

The chlamydial inclusion does not have an open channel since nothing larger than 520Da is able to traverse the membrane (Heinzen and Hackstadt, 1997). It is also known that the inclusion does not intercept the host endosomal, lysosomal, or autophagosomal pathways (Heinzen *et al.*, 1996; Taraska *et al.*, 1996; Al-younes *et al.*, 2004). There is new evidence that lysosomes are crucial for nutrient uptake by creating more free amino acids in the host cytosol during the course of infection; however, it is not a direct interaction between the inclusion and lysosomes (Ouellette *et al.*, 2011). It is suggested that multivesicular bodies (MVB) play a role in nutrient acquisition given that they are essential for sphingolipid and cholesterol trafficking. MVB markers co-localize to the inclusion membrane, but a direct interaction is not observed (Beatty, 2006).

Hypothesis
The metabolically active form of *Chlamydia* (RB) is found to encode for numerous transporters, permeases, and translocases (Saka *et al.*, 2011). There are also 13 predicted ABC transporters that are presumed to primarily be involved with amino acid and oligopeptide transport within the bacteria. No transport orthologs are found to localize to the inclusion membrane (Stephens *et al.*, 1998).

There is a growing body of literature to suggest that the RBs are directly linked to the host cytosol. Electron microscopy images show surface projections emanating from the RB through the inclusion membrane and into the host cytosol (Matsumoto, 1982). Upon closer inspection, the RBs in contact with the inclusion membrane have a unique morphology that appears more rigid in structure. Within the periplasmic space between the inner and outer leaflet of the inclusion membrane, there is electron dense structures that are only found where the RBs have an intimate association with the inclusion membrane that project into the host cytosol (Peterson and de la Maza, 1988). Recent live microscopy captures RBs that wiggle and detach, thus prompting conversion to EBs (Wilson *et al.*, 2009). It is also intriguing that IncD, IncE, IncF, and IncG are enriched only in the sites where the RBs meet the inclusion membrane (Scidmore-Carlson *et al.*, 1999). Given that there is not a demonstrable open channel on the inclusion membrane nor interaction with host pathways that would yield nutrients, the hypothesis that there is a specific transporter located at the inclusion membrane was further analyzed.

Scope

The success of a pathogen to propagate and subsequently cause infection is inextricably linked to its ability to acquire nutrients. Previous work demonstrates that the chlamydial inclusion is unable to obtain nutrients by either intersecting host pathways or via open channels, thus suggesting a novel transport mechanism (Heinzen *et al.*, 1996; Taraska *et al.*, 1996; Al-younes *et al.*, 2004; Heinzen and Hackstadt, 1997). The work presented herein examines the transport of amino acids, particularly methionine, glucose, and ATP to the inclusion. Chapter 2 examines the localization patterns of these required nutrients. Chapter 3 provides a more in-depth analysis of specifically methionine and glucose transport with the usage of a variety of inhibitors and kinetic studies. Given that the inclusion is so intricately tied to the host cell, Chapter 4 discusses the development of a novel protocol to isolate inclusions in order to provide a means for direct manipulation. The final chapter provides a discussion of the presented work and what it adds to the chlamydial field.

CHAPTER 2
NUTRIENT UPTAKE LOCALIZATION WITHIN THE CHLAMYDIAL INCLUSION

ABSTRACT

The gram-negative, obligate intracellular bacterium *Chlamydia trachomatis* acquires all of its essential nutrients from the host cell, although the mechanism through which these nutrients access the bacteria within the inclusion has yet to be elucidated. The inclusion of *C. trachomatis* does not acquire nutrients via fusion of host vesicles within the endosomal/lysosomal or autophagosomal pathways, nor does it possess an open channel within the inclusion membrane. Utilizing fluorescent analogs of required nutrients allowed for tracking from the host cytoplasm into the inclusion via microscopy. A methionine analog localized exclusively to the individual bacteria in a temperature dependent manner. Conversely, a functional glucose analog was found to evenly distribute throughout the inclusion lumen as well as the bacteria. These studies suggest that there is a dual nutrient transport system located within the inclusion membrane.

INTRODUCTION

Chlamydia trachomatis is intricately linked to the host cell due to its obligate intracellular nature and reduced genome size. While it is encased in a parasitophorous vacuole, termed the inclusion, during the entire course of its developmental cycle, it needs to access the host cytosol for nutrients. This creates a unique challenge given that the nutrients must pass the inclusion membrane and bacterial membranes, yet metabolites within the host are not in a form that can readily traverse membrane (McClarty, 1994).

Changes in the overall concentration of amino acids and glucose in an infected cell leads to an aberrant developmental cycle and results in bacteria with reduced infectivity (Coles *et al.*, 1993; Harper *et al.*, 2000). The primary carbon source utilized by the bacteria is glutamate and glucose. This accounts for the bacteria encoding a complete glycogen synthesis and degradation system. However, the bacteria are severely limited in their ability to synthesize amino acids or their precursors (Stephens *et al.*, 1998). The pervading thought within the field was that *Chlamydia* was a true ATP parasite. However, it is shown that the bacteria are not solely dependent on mitochondrial ATP, and appear to be able to undergo minimal ATP generation. While the bacteria steal ATP from host pools, it is not enough to change the overall energy charge of the infected cell (Tipples and McClarty, 1993). The genome of *C. trachomatis* is found to encode proteins that mimic ADP/ATP translocases within *Rickettsia* and mammalian systems (Stephens *et al.*, 1998). Also, the bacteria possess an amino acid transporter, BrnQ, that has a demonstrable affinity for methionine (Braun, 2008). To date, no transporters are observed to localize to the inclusion membrane.

Cellular nutrient transport is governed by two groups of transporter superfamilies: solute carriers (SLC) and ATP binding cassettes (ABC) transporters. The SLC group is predominately utilized for amino acids and nucleotides transport (Naknishi and Tamai, 2011). SLC amino acid transporters are typically coupled with Na^+, K^+, or H^+ to allow for nutrient transport, while ABC transporters are coupled with the hydrolysis of ATP to allow for the movement of substrates against a concentration gradient (Hundal and Taylor, 2009). The transporter family GLUT facilitates glucose transport. These transporters are energy independent and allow for bi-directional flow of glucose (Thorens and Mueckler, 2010). ATP is generated within the mitochondria and is able to enter organelles via an anitporter that uses AMP and ADP (Hirschberg *et al.*, 1998). To date, no amino acid or ATP transport mechanism is found in proximity to the inclusion membrane. The only glucose host complex associated with the inclusion membrane is CERT, and it is observed to transfer sphingomyelin to the inclusion allowing for continual growth of the membrane (Elwell *et al.*, 2011; Derré *et al.*, 2011).

Vacuolar pathogens have a unique challenge of accessing necessary nutrients from the host cytosol. While some pathogens escape their phagosome such as the bacterium *Listeria*, others remain within the confines of a vacuole throughout the course of infection. Such pathogens have developed three modes of nutrient transport between the host and parasitophorous vacuole: 1) continuous open channel, 2) interaction with the endosomal/lysosomal pathway, and 3) specific transporter on the membrane (Fields

and Hackstadt, 2002). It is known that *C. trachomatis* does not have an open pore given that nothing larger than 520Da is able to traverse the inclusion membrane (Heinzen and Hackstadt, 1997). Also, the inclusion separates itself early from the endosomal/lysosomal pathway (Heinzen *et al.*, 1996; Taraska *et al.*, 1996). This leads to the last option of a specific nutrient transporters located at the inclusion membrane. Upon observation of the inclusion membrane utilizing electron microscopy, projections emanating from the metabolically active form of the bacteria, reticulate bodies (RBs) can be observed at the juncture site where they align themselves along the inner leaflet of the inclusion membrane projecting though the membrane and into the host cytosol. There is also a defined morphological change at this site denoted as a rigid membrane structure (Matsumoto, 1982; Peterson and de la Maza, 1988).

Taken together, this study hypothesized that there is a macromolecular complex linking the RBs to the host cytosol directly. This will be examined via microscopically tracking amino acids, glucose, and ATP analogs in infected HeLa cells. It is expected that the localization patterns of these required nutrients will begin to address if there is a direct link from the RB to the host cytosol. If there is a direct linkage, the nutrients would be expected to localize exclusively to the individual bacteria rather than the lumen of the inclusion (Figure 2.1).
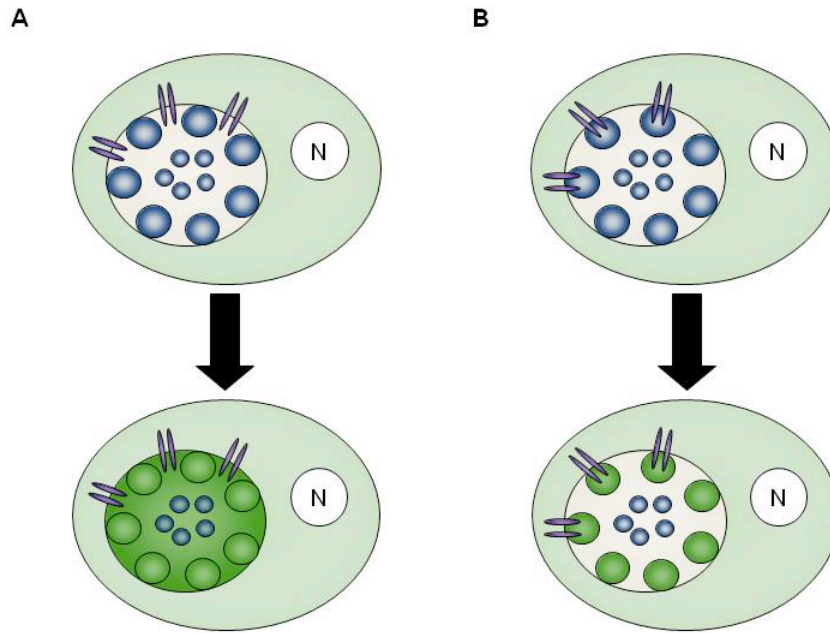
Figure 2.1. Schematic of potential nutrient localization.
This study reviews the hypothesis that there is a direct transport machinery from the host cytosol to the RBs that bypasses the lumen of the inclusion. This will be analyzed by tracking nutrient analogs via fluorescent microscopy. Accumulation of the nutrient in the lumen of the inclusion would mean that the acquisition is via a route unlinked to from the RBs (A). However, if the macromolecular complex is present and responsible for transport, the analog would preferentially localize to the bacteria housed within the inclusion (B).

MATERIALS AND METHODS

Bacterial cultures and tissue culture conditions
HeLa cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Hyclone, Logan, UT). The cells were incubated at 37°C with 5% CO_2. Cells were routinely screened for *Mycoplasma* via PCR. *C. trachomatis* serovar L2/434/Bu was cultured in L929 cells as previously described (Koehler *et al.*, 1992).

C. trachomatis infections
Infections with the *C. trachomatis* serovar L2/434/Bu were carried out as previously described (Koheler *et al.*, 1992). In brief, HeLa cells were plated at a density of 10^5 cells/mL the previous night. Cells were then washed twice with Hank's buffered saline solution (HBSS; Hyclone, Logan, UT) and then incubated with the *C. trachomatis* seed preparation in RPMI 1640 with 10% FBS for 2 hours at 24°C. Following the infection, the cells were washed twice with HBSS and fresh culture media was added. The infected cells were incubated either 24 or 48 hours post-infection (hpi) at 37°C with 5% CO_2.

Amino acid and ATP uptake
C. trachomatis infected HeLa cells were plated on 12mm coverslips in 24 well plates and the infection was allowed to progress to either 24 or 48hpi. At that time, cells were washed twice with HBSS and left in the HBSS for 30 minutes incubating at 37°C. The analogs were then added to their respective wells in HBSS. Click-it AHA (Life Technologies, Grand Island, NY) was utilized as the methionine analog at 50μM. 3-azido-L-Lysine (Jena Bioscience, Germany), 4-azido-L-Phenylalanine (Jena Bioscience, Germany), and 3-azido-L-Alanine (Jena Bioscience, Germany) were added at a final concentration of 5mM. 8-azido-ATP (Jena Bioscience, Germany) was utilized as a final concentration of 50μM.

Upon addition of the appropriate amino acid or ATP analog, the cells were placed at 37°C, 25°C, or 4°C depending on their experimental condition and allowed to incubate for 2 hours. Following this incubation, the cells were washed twice with HBSS and fixed with methanol for 15 minutes at room temperature. The samples were then blocked with 5% Bovine Serum Albumin (BSA; Sigma, St. Louis, MO) in HBSS. The coverslips were then exposed to Click-it cell reaction buffer (Life Technologies, Grand Island, NY) which allowed for a secondary (Alexa Fluor 488 Alkyne) to bind to the azido group on each of the analogs. This reaction took place at room temperature for 30 minutes.

Following the Click-it reaction, the samples were again blocked with 5% BSA in HBSS and had *C. trachomatis* IncG Rabbit polyclonal antibody (Antibodies Incorporated, Davis, CA) added for one hour at room temperature. The coverslips were then washed in HBSS and exposed to Alexa Fluor 546 anti-Rabbit (Life technologies, Grand Island, NY) with DAPI (2.5μg/mL) for an hour at room temperature. The coverslips were then mounted to glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL).

Microscopy was carried out on a Zeiss epifluorescent inverted microscope (Zeiss, Germany) with a 40X air objective. A Hamamatsu Digital camera c10600 Orca-R^2 (Hamamatsu Photonics, Japan) was utilized to capture the image. Volocity 6.0 (Perkin Elmer, Walkan, MA) allowed for optimization of the captured image.

Glucose uptake

HeLa cells were infected with *C. trachomatis* on 35mm glass bottom culture dishes (MatTek Corporation, Ashland, MA). The infection was allowed to progress to 24 or 48hpi at 37°C with 5% CO_2. The cells were then washed twice with HBSS and left in the solution for 30 minutes at 37°C. At that point, 10μM of the glucose analog, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; Life Technologies, Grand Island, NY) was added for two hours and incubated at 37°C, 25°C, or 4°C. The cells were then washed once with HBSS and all solution removed. The cells were live imaged utilizing a Zeiss epifluorescent inverted microscope with a 40X air objective. A Hamamatsu Digital camera c10600 Orca-R^2 captured the image and Volocity 6.0 allowed for optimization of the image.

RESULTS

Methionine uptake was localized to the bacteria and was dependent on temperature.

In order to examine the existence of a complex linking the RBs to the host cytosol a methionine analog was introduced into infected HeLa cells at 24 and 48hpi. An overall reduction of amino acid levels resulted in the formation of aberrant bacteria forms (Coles *et al.*, 1993). Methionine depletion was determined to be sufficient to cause an abnormal developmental cycle and thus was chosen for this study (Allan and Pearce, 1983).

At 37°C, it was observed that the methionine analog was localized solely to the bacteria housed within the inclusion (Figure 2.2). The analog was tracked every 15 minutes after addition to the infected monolayer up to one hour and for every hour up to four hours, and it was found to never accumulate within the lumen of the inclusion (data not shown). The analog localized to the bacteria sequestered in the inclusion within 15 minutes. This uptake process was temperature sensitive. When the monolayers were incubated at 25°C or 4°C, the phenotype was lost, and there was no accumulation of the analog. This was not due to an overall inability of cellular uptake by the analog given that the cell retained the same fluorescent intensity during these temperature conditions. These observations held true for 48hpi *C. trachomatis* infected HeLa cells. Given the size of the fluorescent bacteria, it was presumed that the stained bacteria are the metabolically active form, reticulate bodies (RBs).

A pore localized to the inclusion membrane would be expected to be temperature independent, as it allows for bi-directional passage based upon a concentration gradient. This is exemplified by the *T. gondii* pore at the parasitophorous vacuole which was found to be independent of temperature (Schwab *et al*., 1994). Given that methionine transport to the chlamydial inclusion demonstrated temperature sensitivity, this ruled out the possibility of a pore or other freely traversed conduit was responsible for methionine acquisition during the developmental cycle.
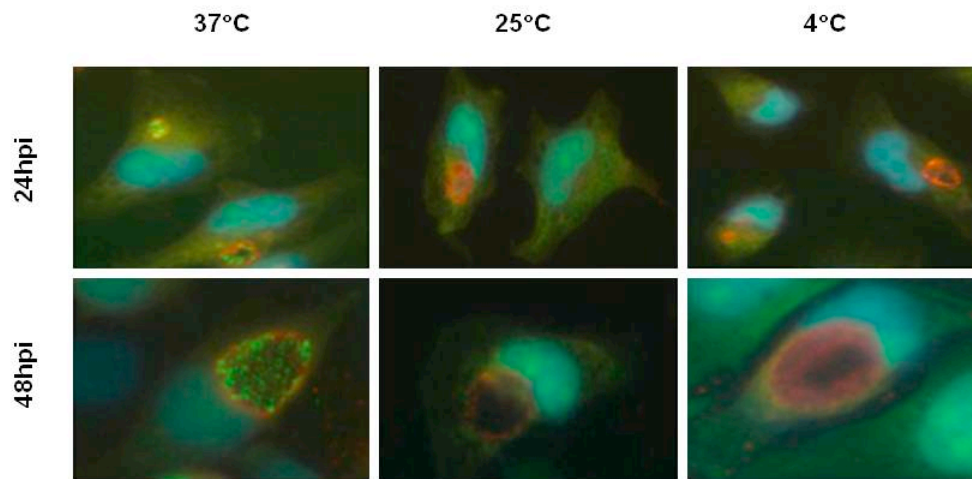
Figure 2.2. Methionine analog was localized to the bacteria and not the lumen of the inclusion.
L2 infected HeLa cells were loaded with a methionine analog (green) at 24 or 48hpi. The cells were then fixed with methanol and stained for a chlamydial inclusion marker, IncG (red), and the nuclear stain, DAPI (blue). It was observed that regardless of the timepoint, the analog preferentially localized to the bacteria and not the lumen of the inclusion. This localization pattern was dependent on temperature.

Other amino acid analogs were not transported to either the lumen or bacteria within the inclusion.

To further examine amino acid transport to the inclusion, analogs of leucine, alanine and phenylalanine were added to infected HeLa cell monolayers (Figure 2.3). Each of these amino acids has been shown to be required in order to undergo a successful developmental cycle (Allan and Pearce, 1983; Karayiannis and Hobson, 1981). Although uptake was observed within the host cell, there was no demonstrable transport to the inclusion. This was true regardless of the temperature (37°C, 25°C, and 4°C) examined, although the host cell was able to acquire all amino acid analogs independent of temperature (data not shown). Lack of transport did not definitively prove absence of uptake during the normal course of the developmental cycle. It was unkown if amino acids are acquired by *Chlamydia* as polypeptides. This could have explained the lack of transport to the inclusion since the analogs were free amino acids. It was also not known if the tag potentially interferes with normal trafficking. Given the confounding data, and the lack of a way to test or overcome these potential obstacles, further analysis of these analogs was not undertaken.
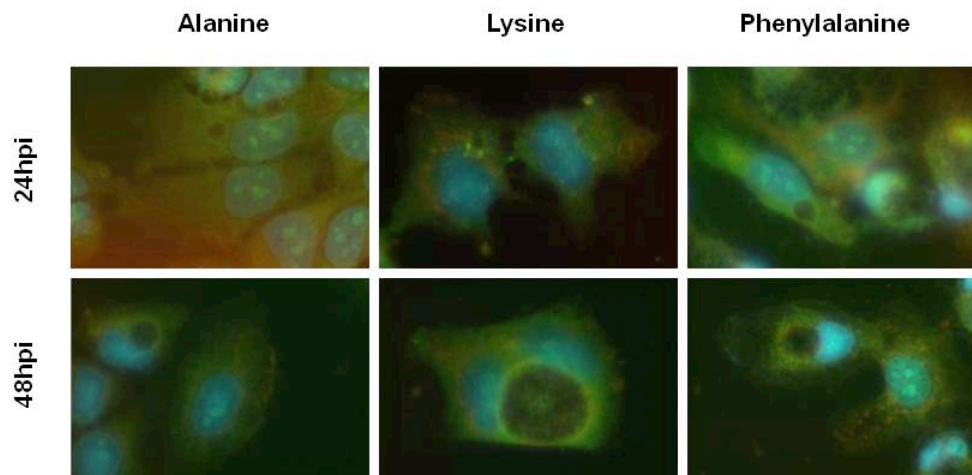
Figure 2.3. Other amino acids did not yield a definitive trafficking pattern.
The remaining amino acids (green) were pulsed into the chlamydial infected HeLa cells at 24 and 48hpi. The cells were subsequently stained with IncG (red) and DAPI (blue).

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The ATP analog did not localize to the chlamydial inclusion.
It is demonstrated that *C. trachomatis* obtains ATP from the host cell. However, to what extent is not determined. Although there is not an overall depletion of the energy charge of an infected cell, there is a noted increase in ATP production that occurs mid-developmental cycle (Tipples and McClarty, 1993; Ojcius, 1998). An analog of ATP was introduced to L2 infected HeLa cells at 24 and 48hpi at 37°C. There was no visible localization to the inclusion although present within the host cell (Figure 2.4). This could have been due to an inherent property within the analog. Further analysis was not warranted given the lack of a usable ATP system.
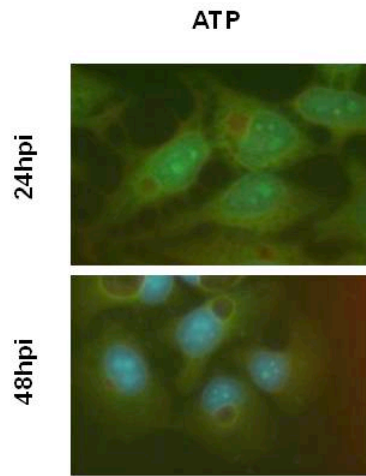
Figure 2.4. The ATP analog did not traffic to the inclusion.
An analog of ATP (green) was added to an infected monolayer of cells at 24 and 48hpi. The monolayer was then fixed with methanol and subsequently stained with IncG (red) and DAPI (blue). The inclusion remained void of the analog at both timepoints.

Glucose transported to the lumen of the inclusion.
Glucose is an important nutrient that *Chlamydia* acquire from the host cell in a yet to be determined route. The glucose analog 2-NBDG is utilized to study glucose transport in mammalian, yeast, and bacterial systems. It actively competes with D-glucose, but not L-glucose and can be utilized by the cell in place of endeogenous glucose (Blodgett *et al.*, 2011; Yoshioka *et al.*, 1996; Yoshioka *et al.*, 1996b). The analog was added to infected HeLa cells at 24 and 48hpi at varying temperatures and visualized via fluorescent microscopy (Figure 2.5). 2-NBDG could not be fixed, and thus required live imaging. Due to this, there was no chlamydial marker. It was found that at 37°C, the analog was present in the cell and trafficked to the lumen of the inclusion. The analog was visualized every 15 minutes up to an hour and every hour for 4 hours. The analog was never sequestered in the bacteria exclusively (data not shown), and was evident in the lumen of the inclusion within an hour. 2-NBDG uptake was completely abrogated in host cells at 25°C and 4°C. Therefore further examination on temperature dependence for uptake within the inclusion exclusively was not performed (data not shown).
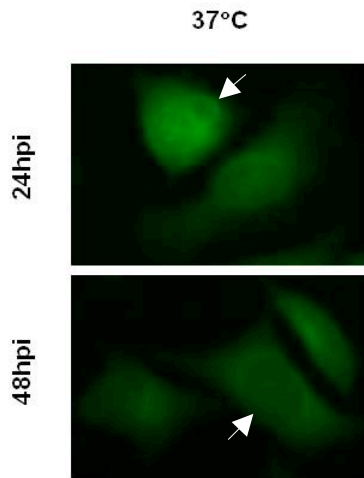
Figure 2.5. The glucose analog 2-NBDG trafficked to the lumen of the inclusion.
2-NBDG was added to infected HeLa cells and visualized with live fluorescent
microscopy. Examination of the localization revealed that the analog appeared in the
lumen of the inclusion. Arrows indicate the location of the inclusion in the infected cell.

DISCUSSION

Chlamydia garners its nutrients by usurping the host pools in an unknown route. Although it is shown that RBs contain numerous transporters, including ABC type oligopeptide transporters and an amino acid transporter that has a high affinity for methionine, no transporter is observed to localize to the inclusion membrane (Saka *et al.*, 2011; Braun *et al*., 2008). Vacuolar pathogens have devised multiple means in order to access host nutrient either by escaping the vacuole, interacting with the endosomal or lysosomal pathways, possessing a pore in the parasitophorous vacuole, or having an embedded nutrient transporter (Fields and Hackstadt, 2002). Previous studies of *Chlamydia* rule out interception of host vesicles and the usage of a pore (Heinzen *et al.*, 1996; Taraska *et al.*, 1996; Heinzen and Hackstadt, 1997). The hypothesis that there is a specific nutrient transport located within the inclusion membrane is further supported by electron microscopy images that show the RBs aligning themselves along the inner leaflet of the inclusion membrane and electron dense projections emanating from the bacteria into the host cytosol (Matsumoto, 1982; Peterson and de la Maza, 1988). In conjunction with this, it is intriguing that many of the Inc proteins display a punctate staining pattern, and specifically IncD, IncE, IncF, and IncG are enriched at the RB juncture site (Scidmore-Carlson, *et al.*, 1999).

Amino acid, glucose, and ATP analogs that could be tracked via fluorescence microscopy were utilized to determine the existence of a complex that bypasses the inclusion lumen for nutrient acquisition purposes. The amino acids methionine, alanine, leucine, and phenylalanine were chosen since it was previously shown that individually depleting each led to an abnormal developmental cycle (Allan and Pearce, 1983). Glucose is known to be one of two carbon sources utilized by the bacteria (Stephens *et al.*, 1998). There is also a marked increase in glucose consumption and expression of the glucose transporter GLUT1 upon infection (Ojcius *et al.*, 1998). ATP is not solely obtained from the host cell, but overall ATP production does increase during the course of the developmental cycle (Tipples and McClarty, 1993; Ojcius *et al.*, 1998).

The methionine analog was observed to localize preferentially to the bacteria within the inclusion in a temperature dependent manner; however, glucose was found to be retained within the lumen of the inclusion. Temperature dependency for glucose could not be assessed given that the cell was unable to uptake the glucose at temperatures other than 37°C. The remaining amino acids and ATP did not yield interpretable results. This could be due to a number of factors in regards to the construction of the analog. It was unknown if the tag added to the molecule interferes with its normal processing within the cell or if it is cleaved. Given these possibilities, further analyses of the amino acids (phenylalanine, leucine, and alanine) and ATP were not pursued.

The temperature dependency observed with methionine transport suggested that this process was neither due to simple diffusion nor utilization of a pore. When fluorescent probes are introduced into *Toxoplasma gondii* infected cells, the parasitophorous vacuole fluoresces regardless of temperature due to the existence of an open channel at the membrane (Schwab *et al.*, 1994). Therefore, the data presented here reinforced

that there is not a pore or channel within the inclusion membrane. It was surprising to find that glucose and methionine each exhibited a different localization pattern. Methionine uptake supported the hypothesis that there was transport machinery linking the host cytosol directly to the RBs that bypassed the lumen of the inclusion. The analog was never found within the lumen of the inclusion regardless of the time observed. This was in stark contrast to glucose, which was visualized throughout the lumen of the inclusion. Taken together, this data suggested there were at least two modes of uptake and transporters that allowed nutrients to enter the inclusion. This has yet to be demonstrated in a bacterial system. However, *Plasmodium falciparum* has multiple transport systems within its parasitophorous vacuole (Baumeister *et al.*, 2006; Levitan, 1994; Lev *et al.*, 1995). It would be intriguing to further explore the dependency of bacterial and host proteins on transport. Given that the Type Three Secretion machinery encoded by *Chlamydia* is ancient in comparison to what is typically found in other bacteria, it is postulated that the needle allows for bi-directional secretion and uptake between the bacteria and the host cell (Hueck, 1998). The following chapter will address these lingering questions and better define the transport of methionine and glucose to the inclusion.

CHAPTER 3
DEFINING THE ROLE OF BACTERIA AND HOST IN METHIONINE AND GLUCOSE UPTAKE

ABSTRACT

The ability of a pathogen to produce or steal nutrients from the host cell is paramount to a successful infection. The gram-negative obligate intracellular bacterium *Chlamydia trachomatis* is known to acquire nutrients from host pools through an unknown mechanism. It is observed that a methionine analog is able to enter the bacteria within the chlamydial inclusion, bypassing the lumen. The glucose analog 2-NBDG was found throughout the lumen of the inclusion. This differential localization pattern denotes at least two distinct modes of uptake employed by *Chlamydia*. In order to better define these separate acquisition pathways, inhibitors to general host and bacterial protein synthesis, Type Three Secretion System (TTSS), and mitochondrial ATP generation were added to chlamydial infected HeLa cells. Methionine transport was found to require bacterial protein synthesis and a functional TTSS. This process was discovered to be host ATP dependent. The transport system was further analyzed and found to be uni-directional. Unlabelled methionine was able to compete with the methionine analog, thus showing that the route of uptake with the analog mirrored that of endogenous methionine. Other amino acids (lysine, alanine, and glycine) did not compete with the methionine analog, denoting specificity within the transport machinery. The transport of glucose to the lumen of the inclusion was discovered to be dependent on bacterial protein synthesis, including the Type Three Secretion apparatus, and host protein synthesis. Accumulation of the host glucose transporter GLUT1 was discovered around the inclusion. This provided further evidence that glucose and methionine are acquired via two distinct pathways.

INTRODUCTION

Pathogens that do not escape their parasitophorous vacuole face a unique challenge. The bacterium or parasite is within a nutrient poor environment and in order to access the host pools, two membrane barriers must be crossed. *Coxiella, Legionella,* and *Mycoplasma* possess a system in which they intersect either the endosomal, lysosomal, or autophagic pathways in order to obtain necessary nutrients (Sinai and Joiner, 1997). *Toxoplasma gondii* and *Plasmodium falciparum* both possess an open pore, allowing for free traversal between the parasitophorous vacuole lumen and the host cytosol (Schwab *et al.*, 1994; Desai *et al.*, 1993). *P. falciparum* is unique in that it also has a nutrient transport system localized to its vacuole membrane (Woodrow *et al.*, 2000).

It is documented that *T. gondii* requires glucose, amino acids, and nucleotides from the host cell (Fox *et al.*, 2004; Pfefferkorn *et al.*, 1986; Krug *et al.*, 1989; Schwartzman *et al.*, 1982). It is able to access these necessary nutrients via a pore embedded within the membrane of its vacuole that allows anything smaller than 1900Da to traverse. This enables free exchange of the needed nutrients between the host cell and the lumen of the vacuole, while waste products can easily exit. The pore is not temperature nor energy dependent (Schwab *et al.*, 1994). It is also observed that there are tethers that attach the vacuole to the mitochondria, which is speculated to provide another means by which to access nutrients (Coppens *et al.*, 2006). It is intriguing that a V-type H^+ pump was found to localize to the parasitophorous vacuole membrane. The pump is shown to generate an internal electrochemical gradient that is hypothesized to be used for nutrient uptake (Moreno *et al.*, 1998). A similar pump is also found in the *P. falciparum* membrane (Saliba and Kirk, 2001).

The vacuole of *P. falciparum* shares many of the same properties as described for *T. gondii*. It requires glucose, purines, and amino acids from the host cell during the course of infection (Diveo *et al.*, 1985; Frances *et al.*, 1994). There is also a channel that allows for free exchange between the host cytosol and the lumen of the vacuole (Desai *et al.*, 1993). However, a nutrient transport system is also defined within the membrane of the vacuole. There is a glucose transporter, PfHT1, that shares 29.8% amino acid homology with the mammalian GLUT1 (Woodrow *et al.*, 2000). Overall, it is known that nutrient transport through the membrane of a parasitophorous vacuole is dependent on both pathogen and host proteins (Baumeister *et al.*, 2006; Levitan, 1994; Lev *et al.*, 1995).

The bacterium *C. trachomatis* resides within a unique parasitophorous vacuole termed the inclusion. It is known that the bacteria are heavily dependent upon host nutrients in order to progress successfully through its developmental cycle (Coles *et al.*, 1993; Harper *et al.*, 2000; Ojcius *et al.*, 1999; Tipples and McClarty, 1993). Unlike the other bacteria that reside within a vacuole, it does not intercept host pathways for nutrient access (Heinzen *et al.*, 1996; Taraska *et al.*, 1996). It also does not possess an open pore, as nothing larger than 520Da can bypass the inclusion membrane (Heinzen and Hackstadt, 1997; Kleba and Stephens, 2008). It is shown that tracking methionine and glucose in infected cells yielded two distinct localization patterns. Methionine

preferentially traffics to the bacteria, while glucose is localized to the lumen of the inclusion. This suggests a novel transport system within the inclusion membrane. Inhibitor studies and competition studies were performed in order to better define the requirements for methionine and glucose transport.

MATERIALS AND METHODS

Bacterial cultures and tissue culture conditions

HeLa cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Hyclone, Logan, UT). The cells were incubated at 37°C with 5% CO_2. Cells were routinely screened for *Mycoplasma* via PCR. *C. trachomatis* serovar L2/434/Bu was cultured in L929 cells as previously described (Koehler *et al.*, 1992).

C. trachomatis infections

Infections with the *C. trachomatis* serovar L2/434/Bu were carried out as previously described (Koheler *et al.*, 1992). In brief, HeLa cells were plated at a density of 10^5 cells/mL the previous night. Cells were then washed twice with Hank's buffered saline solution (HBSS; Hyclone, Logan, UT) and then incubated with the *C. trachomatis* seed preparation in RPMI 1640 with 10% FBS for 2 hours at 24°C. Following the infection, the cells were washed twice with HBSS and fresh culture media was added. The infected cells were incubated either 24 or 48 hours post-infection (hpi) at 37°C with 5% CO_2.

Methionine uptake with inhibitors

C. trachomatis infected HeLa cells were plated on 12mm coverslips in 24 well plates and the infection was allowed to progress to either 24 or 48hpi. At the appropriate timepoint, the cells were washed twice with HBSS. Chloramphenicol (Sigma, St. Louis, MO) was added to a final concentration of 100μg/mL in RPMI 1640 for 30 minutes at 37°C and then in HBSS for 30 minutes incubating at 37°C. The same was done for an hour treatment of cycloheximide (Sigma, St. Louis, MO) with a final concentration of 100μg/mL. The Type Three Secretion System (TTSS) inhibitor C1 (gifted by Dr. Kevin Hybiske, UC Berkeley, CA) was added to the cells at 50μM in RPMI 1640 for 2.5 hours at 37°C and then in HBSS for 30 minutes. The mitochondrial ATP inhibitor carbonyl cyanide 4-(trifluoromethoxy) phenylhdrazone (FCCP, Sigma, St. Louis, MO) was added for 7.5 hours at 10μM in RPMI 1640. The cells then had the same concentration of FCCP added to HBSS for 30 minutes while incubating at 37°C. Click-it AHA (Life Technologies, Grand Island, NY) was introduced at 50μM in HBSS with the appropriate inhibitor. The coverslips were incubated for 2 hours at 37°C.

Following this incubation, the cells were washed twice with HBSS and fixed with methanol for 15 minutes at room temperature. The samples were then blocked with 5% Bovine Serum Albumin (BSA; Sigma, St. Louis, MO) in HBSS. The coverslips were exposed to Click-it cell reaction buffer (Life Technologies, Grand Island, NY) which allowed for a secondary (Alexa Fluor 488 Alkyne) to bind to the azido group on each of the analogs. This reaction took place at room temperature for 30 minutes.

The samples were again blocked with 5% BSA in HBSS and incubated with *C. trachomatis* IncG Rabbit polyclonal antibody (Antibodies Incorporated, Davis, CA) for one hour at room temperature. The cell monolayers were then washed in HBSS and

exposed to Alexa Fluor 546 anti-Rabbit (Life technologies, Grand Island, NY) with DAPI (2.5μg/mL) for an hour at room temperature. The coverslips were then mounted to glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL).

Microscopy was carried out on a Zeiss epifluorescent inverted microscope (Zeiss, Germany) with a 40X air objective. A Hamamatsu Digital camera c10600 Orca-R^2 (Hamamatsu Photonics, Japan) was utilized to capture the image. Volocity 6.0 (Perkin Elmer, Walkan, MA) allowed for optimization of the captured image.

Recovery from chloramphenicol treatment

HeLa cells infected with *C. trachomatis* serovar L2 for 24 hpi were washed twice with HBSS and treated with 100μg/mL chloramphenicol in RPMI 1640 for one hour at 37°C as outlined previously. The cells were then washed twice with HBSS and Click-it AHA was added as previously described 0 minutes, 15, and 30 minutes after removal of chloramphenicol. The cells were incubated with Click-it AHA for 15 minutes, instead of the previous 2 hours and subsequently fixed, stained, and imaged as previously described. Volocity 6.0 software was utilized to measure the mean fluorescence within a field. A two-tailed Student's t-test was performed.

Decay of the methionine analog

Click-it AHA was pulsed into *C. trachomatis* 24hpi HeLa cells as previously described. After the 2-hour incubation, 100μg/mL chloramphenicol was added for one hour, 3 hours, and 5 hours after the Click-it AHA labeling. The cells were subsequently fixed, stained, and imaged as previously described.

Isolation of Perfringolysin O from *E. coli*

All chemicals were purchased for Sigma (St. Louis, MO) unless otherwise noted.

An *E. coli* strain producing 6x His-tag Perfringolysin O (PFO) was provided by Dr. Dan Portnoy (UC Berkeley, CA). The *E. coli* was grown overnight in 25mL LB and 30μg/mL kanamycin with shaking at 37°C. The inoculate was split into 500mL LB with 30μg/mL kanamycin and incubated with shaking for 90 minutes at 37°C. The bacterial cultures were then treated with 1mM IPTG and grown an additional six hours at 30°C with shaking. The cultures were centrifuged for 20 minutes at 5,000rpm. The supernatants were drained and the pellets stored overnight at -80°C.

The bacterial pellets were thawed and resuspended in Sonication Buffer (0.02% sodium azide, 50mM phosphate acetate buffer pH 8.0, 1M sodium chloride, 10mM β-mercaptoethanol, and 1mM PMSF) and sonicated on ice. The lysate was centrifuged for 20 minutes at 12,000rpm. The cleared lysate with then mixed with equilibrated Ni-NTA resin (Qiagen, Valencia, CA) and an additional 1mM PMSF. The slurry was placed at 4°C for an hour on a stir plate. The resin was then allowed to settle in a column and 300mL Sonication Buffer passed through. The column was then washed with 100mL Wash Buffer (50mM phosphate acetate buffer pH 6.0, 1M sodium chloride, 0.1% Tween-20, 10% glycerol, 20mM imidazole, and 0.02% sodium azide) and subsequently 20mL of Wash Buffer without Tween-20 or glycerol. The protein was eluted with 6mL

Elution Buffer (50mM phosphate acetate buffer pH 6.0, 1M sodium chloride, 800mM imidazole, 10mM β-mercaptoethanol, and 0.02% sodium azide). The eluate was placed in a 3-15mL dialysis cassette, MWCO 10K (Pierce, Rockford, IL) for two overnight incubations at 4°C in Storage Buffer (50mM phosphate acetate buffer pH 6.0, 1M NaCl, 1mM EDTA, 5mM DTT, and 0.02% sodium azide). The recovered dialyzed eluate was filter sterilized at 0.45μm (Nalgene, Rochester, NY) and the absorbance read at 280nm to calculate concentration. The protein was then stored at 4°C.

Selective permeabilization with PFO
The procedure was carried out as outlined in Kleba and Stephens, 2008. In brief, infected HeLa cell monolayers were incubated at 4°C for 15 minutes. The cells were then washed twice with cold HBSS and a cold 1% PFO with HBSS solution applied. This sat at 4°C for 15 minutes and was then washed twice with cold HBSS to remove any excess PFO monomers. The monolayers were subsequently incubated at 37°C for one hour.

Competition study
C. trachomatis infected HeLa cells were plated on 12mm glass coverslips in 24 well plates and the infection was allowed to progress to 24hpi. In order to assess the inclusion solely and not the host cell, selective permeabilization with PFO was performed as described earlier. During the final incubation at 37°C, Click-it AHA was added at 50μM along with either 50μM of cold or room temperature DL-methionine (Sigma, St. Louis, MO). Glutamine, alanine, and lysine were all purchased from Sigma (St. Louis, MO). They were added invidually at 50μM with 50μM Click-it AHA and incubated at 37C for 2 hours. The cells were then processed as described previously.

Glucose localization with inhibitors
HeLa cells were infected with *C. trachomatis* on 35mm glass bottom culture dishes (MatTek Corporation, Ashland, MA). The infection was allowed to progress to 24 or 48hpi at 37°C with 5% CO_2. Chloramphenicol was added to a final concentration of 100μg/mL in RPMI 1640 for 30 minutes at 37°C and then in HBSS for 30 minutes at 37°C. The same was done for an hour treatment of cycloheximide with a final concentration of 100μg/mL. The TTSS inhibitor C1 was added to the cells at 50μM in RPMI 1640 for 2.5 hours at 37°C and then in HBSS for 30 minutes. The cells were then washed twice with HBSS and left in the solution for 30 minutes at 37°C with the appropriate inhibitor. At that point, 10μM of the glucose analog, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; Life Technologies, Grand Island, NY), was added for two hours and incubated at 37°C in the presence of the inhibitor. The cells were then washed once with HBSS and all solution removed. The cells were live imaged utilizing a Zeiss epifluorescent inverted microscope with a 40X air objective. A Hamamatsu Digital camera c10600 Orca-R[2] captured the image and Volocity 6.0 allowed for optimization of the image.

GLUT1 and 2 staining

Infected HeLa cells were treated with chloramphenicol, cycloheximide, and C1 at 24hpi or 48hpi as previously described. The monolayers were then fixed with methanol for 15 minutes at room temperature and blocked with 5% BSA in HBSS. Mouse monoclonal GLUT1 (Abcam, Cambridge, MA) and mouse monoclonal GLUT2 (Abcam, Cambridge, MA) antibodies were added at 0.02mg/mL and 0.05mg/mL respectively along with Rabbit polyclonal IncG for one hour at room temperature. The cells were then washed and blocked again with 5% BSA in HBSS. Anti-rabbit Alexa Fluor 546 and anti-mouse Alexa Fluor 488 (Life Technologies, Grand Island, NY) along with DAPI were then incubated for an hour at room temperature. Coverslips were then mounted and visualized as described in previous sections.

RESULTS

Methionine uptake was dependent on both host and chlamydial factors.
It is shown that methionine preferentially localizes to the bacteria within the chlamydial inclusion. Previous data demonstrated that the mechanism was not a pore due to the temperature dependency of methionine transport to the bacteria. The roles host and chlamydial proteins had with the transport of methionine was examined in order to better define the transport machinery. This would provide insight into the origin of the machinery and also if it is a channel (ATP independent) or a pump (ATP dependent).

This study found that the host protein synthesis inhibitor cycloheximide showed no effect on the uptake pattern (Figure 3.1). The methionine analog was still able to traffic preferentially to the individual bacteria within the inclusion. The pattern remained the same even when cycloheximide was added for up to eight hours (data not shown). However, upon addition of chloramphenicol, an inhibitor of bacterial protein synthesis, the methionine was not able to enter either the bacteria or lumen of the inclusion. The cell was unaffected by the treatment, as it still was able to acquire the methionine analog. This halt in transport to the bacteria was observed within 30 minutes of chloramphenicol treatment, and it was never found in the lumen of the inclusion (data not shown). Methionine transport was also abrogated upon inhibition of the chlamydial TTSS and host mitochondrial ATP generation. None of these inhibitors affected the ability of the host cell to uptake the methionine analog. The effects of these inhibitors were observed regardless of the stage in the developmental cycle. To ensure that the uptake of methionine was completely halted and not simply delayed, the analog was tracked up to 8 hours with no change observed (data not shown).

This data suggested that the transport machinery is bacterial in origin given that shut down of chlamydial protein synthesis halted transport. The chlamydial TTSS system was also involved with transport. It is interesting to note that IncA, IncB, and IncC are shown to be secreted by TTSS (Subtil *et al.*, 2001). Taken together, the TTSS system may have been involved by secreting the necessary chlamydial protein to the inclusion membrane to allow for methionine transport. The dependency on host ATP for successful transport to *Chlamydia* implies that acquisition was governed by a pump at the inclusion membrane.
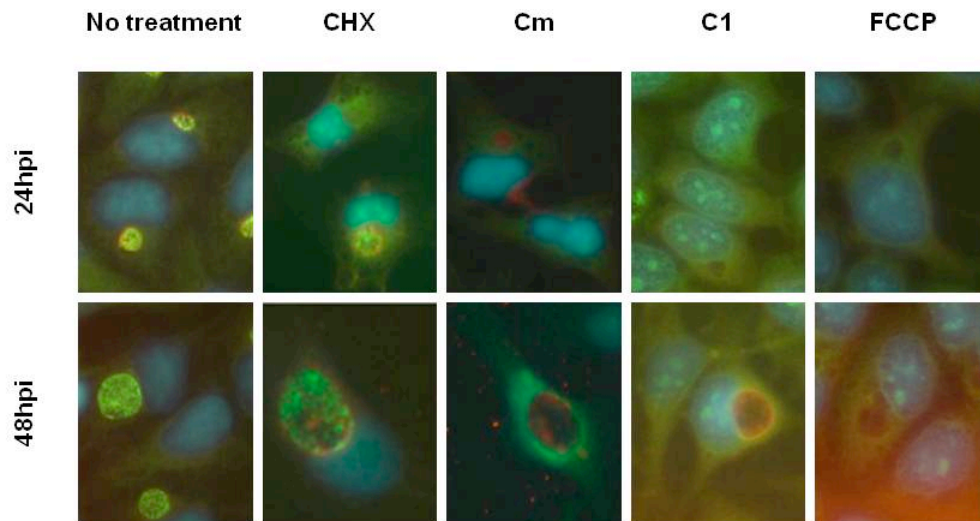
Figure 3.1. Methionine transport was regulated by both host and bacterial factors.
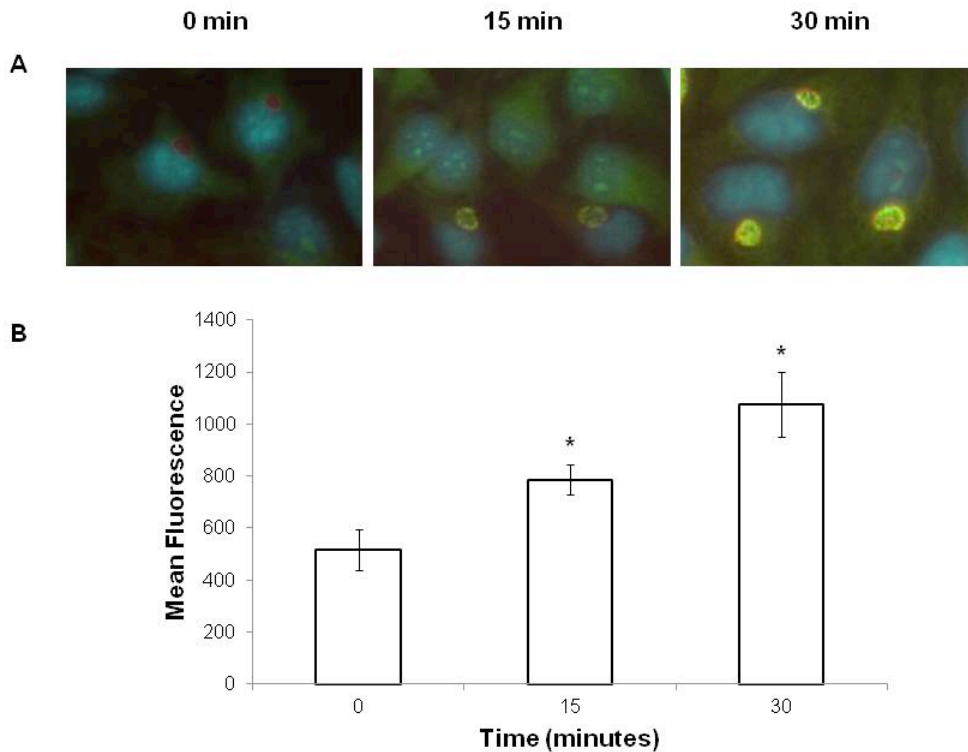*C. trachomatis* infected HeLa cells were exposed to cycloheximide (CHX), chloramphenicol (Cm), a Type Three Secretion System inhibitor (C1), and mitochondrial ATP inhibitor (FCCP) at either 24hpi or 48hpi. The methionine analog was then added. The cells were subsequently fixed and stained for methionine (green), the inclusion membrane marker IncG (red), and the nuclear stain DAPI (blue).

The chlamydial protein(s) contributing to methionine transport were rapidly generated and had a short half-life.

The chlamydial inclusion is known to undergo extensive modification that is dependent upon bacterial protein synthesis shortly after entry into a host cell (Scidmore *et al.*, 1996). The half-life of chlamydial proteins has yet to be directly determined but anecdotal evidence implies a rapid turnover rate. Chloramphenicol treatment was able to completely halt methionine transport after a 30 minute treatment (data not shown). The recovery rate for chloramphenicol was determined in order to ensure that the observed absence of methionine accumulation was not due to detachment of the RBs from the inclusion membrane. Given that methionine localizes to the bacteria, it is likely that the transport machinery links the RBs and the host cytosol. If the RBs become detached from the inclusion membrane, they are hypothesized to begin reversion to EBs (Wilson *et al.*, 2009). Detachment of the RBs would thus negate any conclusions to be drawn about bacterial protein synthesis dependency on the system.

C. trachomatis 24hpi HeLa cells were treated with chloramphenicol for an hour. The drug was then removed and the methionine analog was added. Transport was allowed to progress and subsequently fixed and stained at the appropriate timepoint. It was observed that within 15 minutes after removal of chloramphenicol, methionine transport was resumed (Figure 3.2 A). For a more refined analysis, the accumulation of methionine was quantified by measuring the fluorescence intensity within the inclusions. The methionine approximately doubled in intensity between each timepoint (Figure 3.2 B). Fluorescence intensity observed at 0 minutes was due to background from the host cell. At each timepoint thereafter, the fluorescence doubled in a stastically significant manner.

The halt in methionine transport was a reversible process, and thus not due to the RBs detaching from the inclusion membrane or any other gross abnormality in the developmental cycle brought about by the treatment. This also demonstrated that the bacterial proteins responsible for transport are rapidly generated, given that the process was resumed within 15 minutes of the drug removal. Taken together, this data provided further evidence that the chlamydial inclusion is constantly undergoing extensive modification directed by the bacteria (Scidmore *et al.*, 1996; Carabeo *et al.*, 2003).

Figure 3.2. Methionine transport resumed within 15 minutes of chloramphenicol removal.

C. trachomatis serovar L2 infected HeLa cells were treated with chloramphenicol. The cell monolayer was then washed and the methionine analog (green) added and counterstained with IncG (red) and DAPI (blue). Fluorescence was seen accumulating within 15 minutes of removal, and remained consistent after 30 minutes (A). The fluorescence intensity of 3 random fields for all inclusions within each timepoint was calculated (B). Each timepoint had a statistically significant increase in fluorescence according to a two-tail Student's t-test ($p < 0.05$).

The transport of methionine was not bi-directional.
It has been hypothesized that the TTSS in *C. trachomatis* could act as a bi-directional straw, allowing for chlamydial effectors to access the host cytosol while necessary host molecules can enter the inclusion (Hueck, 1998). Given previous data that methionine transport was dependent on a viable TTSS (Figure 3.1), methionine was tracked for its ability to exit the bacteria.

Chlamydia infected HeLa cells were pulsed with the methionine analog, and subsequently treated with chloramphenicol. A loss in fluorescence intensity over time would indicate a mechanism in which the analog could be transported to and from the bacteria within the inclusion. However, it was observed that fluorescence did not diminish over 5 hours in a statistically significant manner, indicating that transport is not bi-directional (Figure 3.3 B). The analog remained sequestered in the bacteria throughout all timepoints observed (Figure 3.3 A).

This demonstrates that the transport mechanism of methionine is not a pore or straw-like conduit, such as been hypothesized with the TTSS (Hueck, 1998). This also provides further proof for the notion that the transport machinery is a pump given the prior evidence of ATP dependency, and now the uni-directiotional capability of methionine transport.
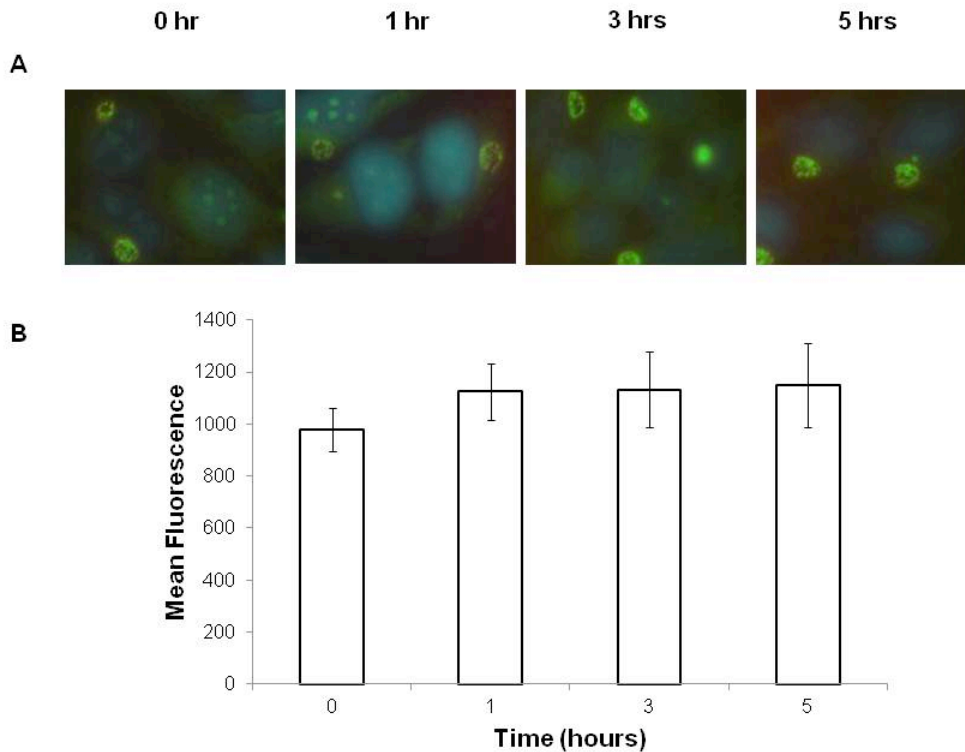
Figure 3.3. Preloaded methionine did not exit from the bacteria.
Methionine analog was introduced to *Chlamydia* infected HeLa cells and subsequently
treated with chloramphenicol for 1, 3, and 5 hours. Localization of methionine analog
(green) within the inclusion (red) was tracked. DAPI marked the nucleus of the cell
(blue). Visually, the analog did not exit the bacteria during the treatment (A). This was
further analyzed via quantification of mean fluorescence intensity within the inclusions of
3 random fields for each timepoint (B). None of the timepoints were statistically different
according to a two-tailed Student's t-test ($p > 0.05$).

Methionine analog was transported via the same pathway as unlabelled methionine.

The host cell is the first barrier for the methionine analog to traverse. If the analog is unable to first enter the host cell, trafficking to the inclusion cannot be monitored. In order to ensure that the methionine analog was trafficked in a similar manner to endogenous methionine, competition studies were performed utilizing unlabelled methionine. The same concentration of unlabelled methionine and the analog were added onto infected HeLa cells. No fluorescence was detected in the cell (data not shown). The unlabelled methionine and analog competed at the host plasma membrane; however, this did not ensure that the same would hold true at the inclusion membrane.

In order to circumvent this issue, selective permeabilization was utilized. It had previously been shown that *Chlamydia* infected cells exposed to the pore forming toxin Perfringolysin O (PFO) allows for delivery to the host cytosol without affecting the chlamydial inclusion (Kleba and Stephens, 2008). PFO can be temperature controlled thus allowing for selective permeabilization of the host cell plasma membrane, while the inclusion remains intact. Therefore, PFO was added to an infected monolayer of HeLa cells in the presence of both unlabelled methionine and the analog to directly test the specificity of the transport machinery at the inclusion membrane. PFO treatment had no affect on methionine transport. The analog was visualized in the individual bacteria, and not the lumen of the inclusion.

It was previously shown that methionine transport was temperature dependent. Therefore, cold methionine would be expected to be transported slower than pre-warmed methionine. If the methionine analog was utilizing the same transport pathway as unlabelled methionine, there would only be a decrease in fluorescence upon addition with pre-warmed methionine. As can be observed in Figure 3.4, cold methionine did not compete with the methionine analog. The methione analog was still visualized within the bacteria. However, upon addition of pre-warmed methionine the analog no longer localized to the bacteria or lumen of the inclusion. This demonstrates that the methionine analog is transported in the same manner as unlabelled methionine.
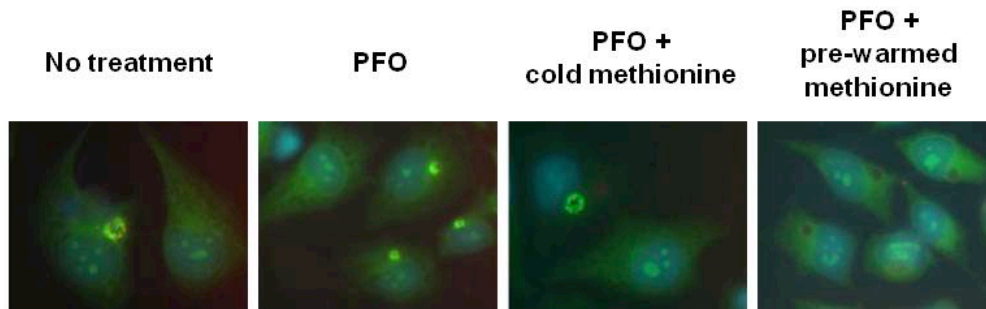
Figure 3.4. Unlabelled methionine competed with methionine analog.
To bypass the influence of the host cell, selective permeabilization via Perfringolysin O (PFO) was performed on infected HeLa cells. Cells were exposed to both the methionine analog and cold or pre-warmed unlabelled methionine and subsequently visualized with the methionine analog (green), IncG (red), and DAPI (blue). It was observed that only pre-warmed methionine had an effect on the methionine analog uptake.

Nutrient transport machinery was specific to methionine.
It is unknown if the transporter for methionine is for all amino acids, a subset of amino acids, or specifically methionine. Given that the machinery is not a pore, it is likely that there is a specificity at some level as seen with *P. falciparum* nutrient complex for glucose transport (Woodrow *et al.*, 1999). Unlabelled lysine (basic side chain), alanine (nonpolar side chain), and glutamine (uncharged side chain) were all added to *Chlamydia* infected HeLa cells individually in the presence of the methionine analog. The cells were treated with PFO in order to subvert the influence of the plasma membrane. Selective permeabilization allowed for the analogs to be delivered directly to the host cytoplasm, which allowed for direct testing at the inclusion membrane interface.

Unlabelled methionine was found to compete with the analog denoted by the lack of analog found in the inclusion. None of the other amino acids, regardless of their side chain group, competed with the methionine analog (Figure 3.5). This resulted in trafficking of the analog to the bacteria sequestered within the inclusion. This demonstrates a high level of specificity within the transporter since no other amino acid was able to compete with the methionine analog.

It was surprising that alanine and methionine would not utilize the same pathway given that they both are grouped as nonpolar side chain amino acids. It is interesting to note that RBs possess a transporter that has a high affinity for methionine, BrnQ (Braun *et al.*, 2008). Although this transporter has never been reported to localize to the inclusion membrane, it is noteworthy that it only is expressed by RBs and is of bacterial origin. This would coincide with the methionine data of a transporter with a specificity for methionine that is bacterial in origin linking the RBs to the host cytosol.
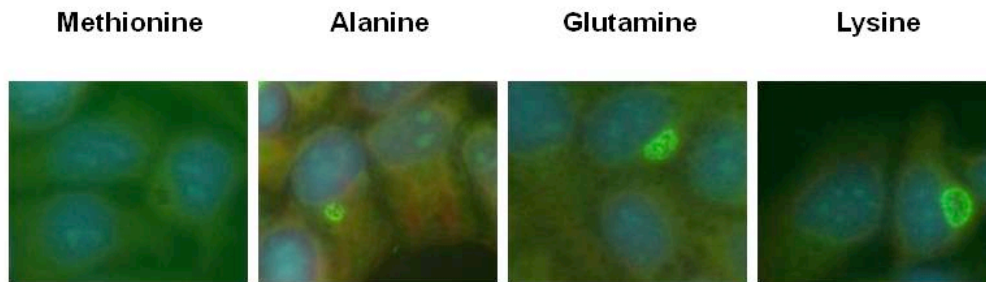
Figure 3.5. Other amino acids did not compete with the methionine analog.
Unlabelled methionine, alanine, glutamine, and lysine were added with the methionine
analog to infected HeLa cells. The analog (green) was only found in the inclusion (red)
with amino acids other than methionine. Nuceli were stained with DAPI (blue).

Glucose transport was dependent on both host and bacterial protein synthesis.
It was previously shown that glucose trafficked to the lumen of the inclusion (Figure 2.5). The parameters of glucose transport were further explored in relation to the contribution by host and chlamydial proteins. Given that glucose displayed a localization pattern that differed from methoinine (inclusion lumen versus individual bacteria), it is expected that the requirements for transport potentially will differ.

Cycloheximide treatment resulted in an inhibition of glucose localization; however, the analog was still observed within the lumen of the inclusion (Figure 3.5). Chloramphenicol treatment appeared to completely abrogate the accumulation of the analog within the inclusion. This is in stark contrast to methionine uptake, which was found to be completely independent of host protein synthesis. The TTSS inhibitor did not fully halt the accumulation of the glucose analog. In order to ensure that the inhibitor phenotypes were not due to a delay of uptake rather than a complete halt, analog localization was tracked for 8 hours with no change in the phenotype (data not shown).

Cycloheximide and chloramphenicol treatments were added one hour prior to addition of the glucose analog. Shutdown of bacterial protein synthesis resulted in no accumulation of glucose transport, implying that the responsible proteins are short-lived and rapidly turn over. The cycloheximide data was confounded as less uptake of the analog was observed in the host cell during treatment. Therefore, it is unknown if the reduced transport to the lumen of the inclusion was due to solely the affects of the drug on *Chlamydia* or because of the affects on the host cell. However, reduction, and not a complete halt in the ability of *Chlamydia* to acquire glucose from the host cell during cycloheximide treatment would account for the ability of the bacteria to flourish in cells treated with cycloheximide in the media during the entire course of infection (Ripa and Mardh, 1977). This shows the bacteria are still able to scavenge glucose while the host cell is at a reduced functional compacity.
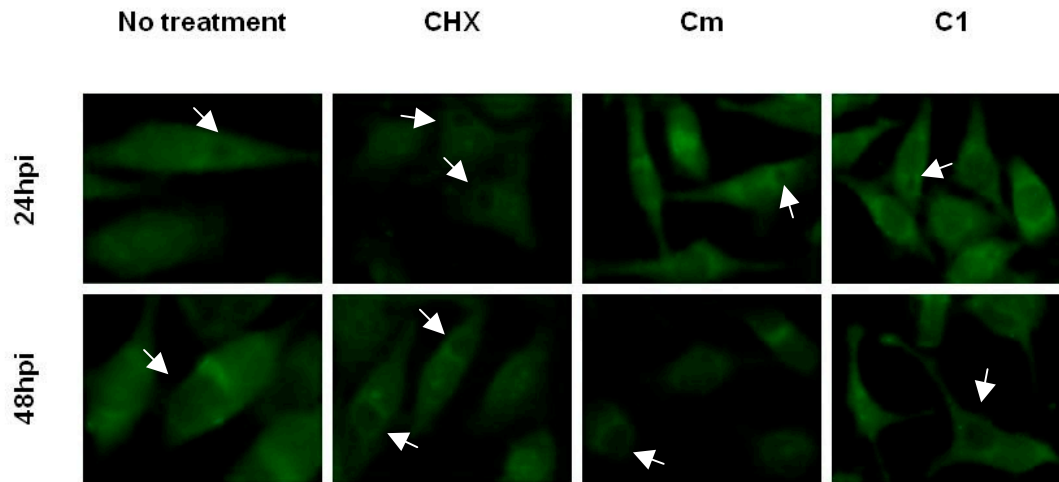
**Figure 3.6. Glucose localization was dependent on host and chlamydial proteins.** Glucose analog (green) was added to 24hpi and 48hpi HeLa cells. The monolayers were also exposed to cycloheximide (CHX), chloramphenicol (Cm), and Type Three Secretion System inhibitor (C1) to explore the dependency on overall bacterial and host protein synthesis, and specifically *Chlamydia* TTSS. The inclusions are denoted by arrows.

GLUT1 localized to the chlamydial inclusion membrane.
The glucose analog utilized during these studies is known to be transported via GLUT1 and GLUT2 (Millon *et al.*, 2011; Yamada *et al.*, 2000). The *Chlamydia* genome does not encode any homologs to either of these transporters. Given that 2-NBDG is able to enter the lumen of the inclusion in a process that is dependent to some degree on host protein(s) (Figure 3.6), the localization of GLUT1 and GLUT2 in proximity to the chlamydial inclusion was explored.

GLUT1 was discovered to localize at the inclusion membrane at both 24 and 48hpi, with the staining pattern became more defined at 48hpi (Figure 3.7). Infected HeLa cells were exposed to the host protein synthesis inhibitor cycloheximide, bacterial protein synthesis inhibitor chloramphenicol, and an inhibitor of chlamydia/ TTSS. The inhibitors reduced the amount of GLUT1 around the inclusion, but did not completely halt its recruitment. GLUT2 was also tested and visualized for its localization pattern. However, there was no accumulation around the inclusion at any point (data not shown).

It is expected that GLUT1 is derived from the host and recruited to the inclusion via bacterial factors. This would account for the inhibition of GLUT1 recruitment and glucose accumulation when cells are exposed to cycloheximide and chloramphenicol. The lack of a complete halt to trafficking could be due to the proteins involved or the robust interaction between the host and bacterial proteins. This would emulate the recruitment of host CERT for sphingomyelin to the inclusion membrane by the chlamydial protein IncD (Derré, 2011).
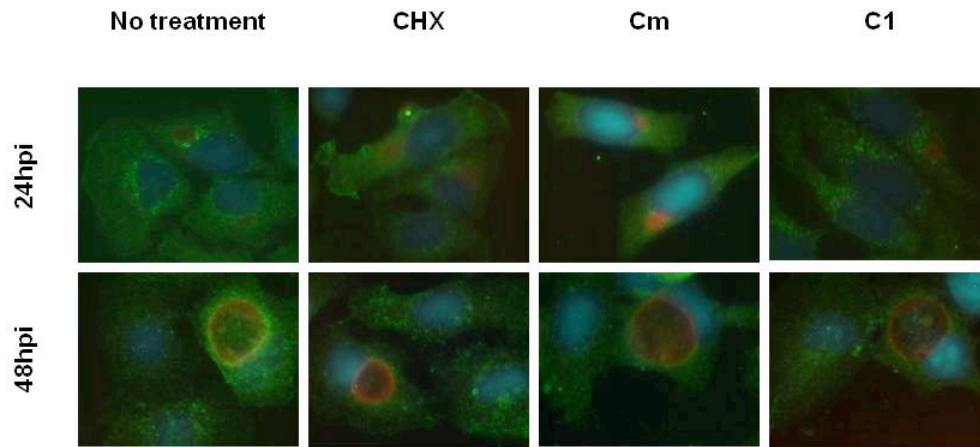
Figure 3.7. The glucose transporter GLUT1 accumulated around the chlamydial inclusion.
C. trachomatis infected HeLa cells were treated with cycloheximide (CHX), chloramphenicol (Cm), and TTSS inhibitor (C1) and subsequently stained with GLUT1 (green), IncG (red), and DAPI (blue). No treatment showed an accumulation of the transporter around the inclusion, while the accumulation was reduced upon inhibitor treatment.

DISCUSSION

Parasitophorous vacuoles cut off the pathogen from the host cell concomitantly hiding it from detection, but also sequestering it from necessary molecules. Those vacuoles formed by bacteria have been documented to actively intercept host pathways to acquire nutrients; however, the chlamydial inclusion does not employ this tactic (Sinai and Joiner, 1997; Heinzen *et al.*, 1996; Taraska *et al.*, 1996; Al-Younes *et al.*, 2004). Another common method of nutrient acquisition is to have a pore (Schwab *et al.*, 1994; Desai *et al.*, 1993). Although *Chlamydia* does not have a pore in its inclusion, this study suggests that it has at least two nutrient transport systems located within its inclusion membrane (Heinzen and Hackstadt, 1997).

Methionine transport from the host cell to the inclusion is via a direct pathway to the metabolically active form of the bacteria or RBs. This process was found to be dependent upon chlamydial protein synthesis, specifically the TTSS. It is postulated that the machinery of the TTSS within *Chlamydia* is ancient in nature, and thus may act as a straw-like conduit, allowing for bi-directional transport (Hueck, 1998). Methionine transport was found to not be bi-directional, and hence, a novel transport system could be responsible for the uptake pattern.

There are two groups of transport superfamilies that facilitate amino acid transport within mammalian cells: the solute carriers (SLC) and the ATP-binding cassette (ABC) transporters (Nakanishi and Tamai, 2011). SLCs move amino acids by coupling with ionic gradients while ABC transports need ATP (Jones *et al.*, 2009). The transport mechanism responsible for methionine uptake was discovered to be dependent upon ATP, supporting the notion that the chlamydial inclusion has an ABC transporter. Although no ABC transporter has been found to localize to the inclusion membrane, the genome encodes for 13 ABC transporters (Stephens *et al.*, 1998). Upon further analysis, these transporters are located exclusively to the RBs (Saka *et al.*, 2011). This provides further credence to the original hypothesis, that there is a macromolecular conduit linking the RBs, which converge at the inner leaflet of the inclusion membrane, and the host cytosol that bypasses the lumen of the inclusion.

The transport machinery is specific for methionine; no other amino acid was able to compete with the methionine analog. Although this level of specificity has yet to be documented for amino acid transport in parasitophrous vacuoles, *P. falciparum* has a glucose transporter (Woodrow *et al.*, 1999). *Chlamydia* RBs have a transporter with a high affinity for methionine, BrnQ (Braun *et al.*, 2008). Given that the methionine transporter is dependent on bacterial protein synthesis, it could be speculated that BrnQ could be part of the machinery linking the RBs to the host cytosol.

Glucose was found to localize to the lumen of the inclusion in a process regulated by both host and bacterial protein synthesis. This is in stark contrast to the localization of methionine. These localization patterns and the observed differential requirements between methionine and glucose belie a dual mode of transport. *P. falciparum* nutrient transport most closely mimics that of glucose since it is also dependent on host and

pathogen proteins (Baumeister *et al.*, 2006; Levitan, 1994; Lev *et al.*, 1995). In conjunction, there is an identified glucose transporter within the parasitophorous vacuole of *P. falciparum* that shares close homology with mammalian GLUT1 (Woodrow *et al.*, 1999).

The GLUT family transports glucose within cells in an energy independent and bi-directional manner (Thorens and Mueckler, 2010). The chlamydial genome does not contain any homologs of GLUT, but upon upon infection, HeLa cells increase their expression of GLUT1 (Ojcius *et al.*, 1998). It was found that GLUT1 localized to the inclusion membrane in a process dependent upon bacterial and host protein synthesis and the TTSS. Although the bacterial proteins responsible for this localization are unknown, it is intriguing that the inclusion membrane proteins, Incs, have been implicated in recruitment of host factors to the inclusion membrane. Recently, it was shown that IncD recruits host CERT to the inclusion in order to usurp sphingomyelin (Derré *et al.*, 2011). It has also been demonstrated that the each Inc has a unique expression and staining pattern (Scidmore-Carlson *et al.*, 1999). The role of Incs, if any, on glucose transport would be an interesting follow up to these experiments. Although a successful system for transformation of *Chlamydia* has been published, it has yet to be seen if knock-out studies can be performed (Wang *et al.*, 2011).

Both transport systems involved bacterial proteins that are rapidly turned over. *Chlamydia* has been known to extensively modify the nascent phagosome in order to establish the inclusion separated from the lysosomal pathway (Scidmore *et al.*, 1996). This appears to be an ongoing phenomenon that allows *Chlamydia* to direct its host for its own gain. These studies are hampered because of how intricately tied the inclusion is to the host cell. The following chapter will introduce a novel protocol to isolate inclusions in order to allow for direct manipulation without the effects of the host cell.

CHAPTER 4
A NOVEL PROTOCOL TO ISOLATE CHLAMYDIAL INCLUSIONS

ABSTRACT

Studies pertaining to the chlamydial inclusion have been limited in the questions that can be addressed due to the currently available tools in the field. Historically, inclusions were perceived to be inherently fragile, and thus impossible to remove from the host cell. Previous attempts to isolate inclusions involved homogenization of infected cells combined with sucrose gradients. These resulting inclusions were found to have numerous fractures throughout the membranes. A novel protocol was developed to isolate chlamydial inclusions from host cells utilizing a pore forming toxin whose activity can be temperature controlled. The released inclusions were found to retain their overall morphology after treatment and remained stable outside the host cell. The bacteria within the inclusion were found to be infectious up to 12 hours post-release, and retained the same level of housekeeping gene expression. This new technique bypasses many issues that have hampered inclusion studies thus far because it enables direct manipulation and characterization of the isolated chlamydial inclusions.

INTRODUCTION

The *Chlamydia* inclusion establishes itself early as a distinct niche within the host cell. It interacts extensively with the host cell during the course of its developmental cycle, perhaps best illustrated by its accumulation of sphingomyelin. It intercepts the exocytic pathway to acquire sphingomyelin in order to allow the inclusion membrane to expand (Hackstadt *et al.*, 1995). This process is dependent on bacterial protein synthesis, energy, and temperature (Scidmore *et al.*, 1996; Hackstadt *et al.*, 1996). It was recently demonstrated that this accumulation is due to the host pathways GBF1 and CERT (Elwell *et al.*, 2011). The chlamydial inclusion membrane protein IncD is responsible for recruitment of the CERT pathway (Derré *et al.*, 2011). Due to the intricate relationship between the host and *Chlamydia*, the inclusion has yet to be fully studied as an entity without host contribution.

C. psittaci inclusions were isolated via homogenization and differential centrifugation. The resulting inclusions were severely fractured and unstable. It was found that sucrose gradient, a common method used for vacuole isolation, promoted lysis of the inclusion, while Bovine Serum Albumin (BSA) was protective (Matsumoto, 1981). As a result of this study, the field deemed the inclusion as inherently fragile. It conjunction, it was observed that micro-injection of an infected cell led to a collapsed inclusion (Heinzen and Hackstadt, 1997). No published attempts to isolate inclusions have been released since Matsumoto in 1981.

In 2008, Kleba and Stephens reported utilizing selective permeabilization to access potential chlamydial effector proteins in the host cytosol. During this study, it was shown that upon treatment with the pore forming toxin Perfringolysin O (PFO), the inclusion remained impervious to fluorescent dyes, denoting that it had not been affected by the treatment. A related pore forming toxin, Streptolysin O (SLO) has been applied to cells infected with *T. gondii* and the parasitophorous vacuole was able to be isolated (Nyalwidhe and Lingelbach, 2006). It had also been shown that SLO treatment does not interfere with *T. gondii* protein synthesis (Baumeister *et al.*, 2001).

PFO and SLO are both members of the cholesterol dependent cytolysins. These toxins form β-barrels and are secreted by gram-positive bacteria. PFO is secreted by the bacterium *Clostridium perfringes*. In order to create a pore, PFO binds cholesterol via its C-terminus. This binding signals structural changes through all domains resulting in the pre-pore complex. This initiates the insertion of two amphipathic β–hairpins into the membrane. These hairpins span the membrane and create a large transmembrane β–barrel (Heuck *et al.*, 2010). This pore is composed of 35-50 monomers that results in 100-200 β–barrels. This toxin in particular was attractive for usage in selective permeabilization because it is temperature sensitive; at 4°C, monomers are only able to bind, thus allowing for excess to be washed away, before increasing the temperature to 37°C and initiating formation of the pore (Tweten, 2005).

This study developed a novel protocol to isolate chlamydial inclusion from the host cell. PFO was preferred to SLO due to it being able to function over a wider range of pHs

and is able to perform in the presence of oxygen without any genetic modifications (Bhakdi *et al.*, 1994). The protocol consisted of incubating chlamydial infected HeLa cells at 4°C, which allowed for the PFO monomers to bind. The excess monomers were then washed away, and the cells place at 37°C. If enough PFO was added, the plasma membrane falls away, releasing the inclusion (Figure 4.1). It is hoped that this protocol will become another tool for the field to utilize to directly study the inclusion.
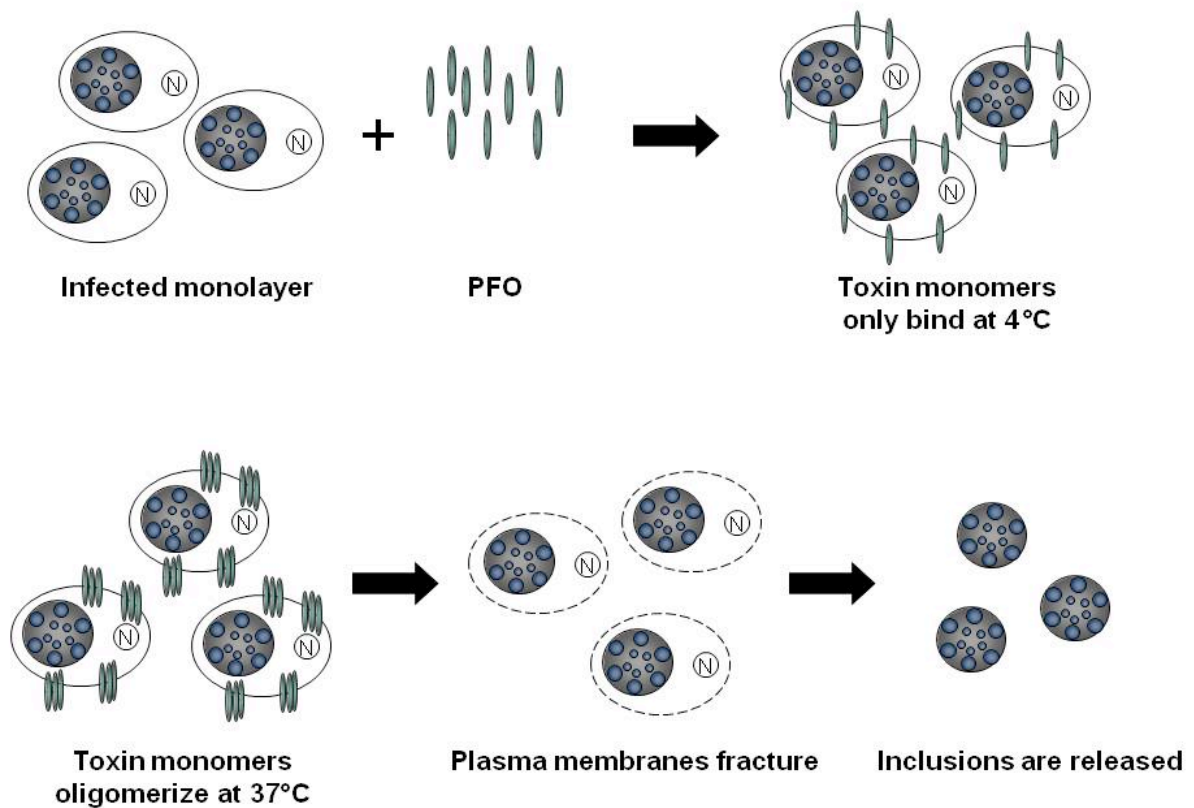
Figure 4.1. Schematic of Perfringolysin O (PFO) activity.
PFO binds the cholesterol rich domains of the plasma membrane. When monomers are added at 4°C, they are only able to bind. The excess monomers are subsequently washed away, and the cell monolayer is placed at 37°C. This allows for the bound monomers to oligomerize, thus creating the pore. If a high enough concentration of PFO is added, the plasma membranes fracture and release the inclusions.

MATERIALS AND METHODS

Bacterial cultures and tissue culture conditions

HeLa cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Hyclone, Logan, UT). The cells were incubated at 37°C with 5% CO_2. Cells were routinely screened for *Mycoplasma* via PCR. *C. trachomatis* serovar L2/434/Bu was cultured in L929 cells as previously described (Koehler *et al.*, 1992).

C. trachomatis infections

Infections with the *C. trachomatis* serovar L2/434/Bu were carried out as previously described (Koheler *et al.*, 1992). In brief, HeLa cells were plated at a density of 10^5 cells/mL the previous night. Cells were then washed twice with Hank's buffered saline solution (HBSS; Hyclone, Logan, UT) and then incubated with the *C. trachomatis* seed preparation in RPMI 1640 with 10% FBS for 2 hours at 24°C. Following the infection, the cells were washed twice with HBSS and fresh culture media was added. The infected cells were incubated either 24 or 48 hours post-infection (hpi) at 37°C with 5% CO_2.

Isolation of Perfringolysin O from *E. coli*

All chemicals were purchased for Sigma (St. Louis, MO) unless otherwise noted.

An *E. coli* strain producing 6x His-tag Perfringolysin O (PFO) was provided by Dan Portnoy (UC Berkeley, CA). The *E. coli* was grown overnight in 25mL LB and 30μg/mL kanamycin shaking at 37°C. The inoculate was split into 500mL LB with 30μg/mL kanamycin and incubated shaking for 90 minutes at 37°C. The bacterial cultures were then treated with 1mM IPTG and grown an additional six hours at 30°C shaking. The cultures were centrifuged for 20 minutes at 5,000rpm. The supernatants were drained and the pellets stored overnight at -80°C.

The bacterial pellets were thawed and resuspended in Sonication Buffer (0.02% sodium azide, 50mM phosphate acetate buffer pH 8.0, 1M sodium chloride, 10mM β-mercaptoethanol, and 1mM PMSF) and sonicated on ice. The lysate was centrifuged for 20 minutes at 12,000rpm. The cleared lysate with then mixed with equilibrated Ni-NTA resin (Qiagen, Valencia, CA) and an additional 1mM PMSF. The slurry was placed at 4°C for an hour on a stir plate. The resin was then allowed to settle in a column and 300mL Sonication Buffer passed through. The column was then washed with 100mL Wash Buffer (50mM phosphate acetate buffer pH 6.0, 1M sodium chloride, 0.1% Tween-20, 10% glycerol, 20mM imidazole, and 0.02% sodium azide and subsequently 20mL of Wash Buffer without Tween-20 or glycerol. The protein was eluted with 6mL Elution Buffer (50mM phosphate acetate buffer pH 6.0, 1M sodium chloride, 800mM imidazole, 10mM β-mercaptoethanol, and 0.02% sodium azide). The eluate was placed in a 3-15mL dialysis cassette, MWCO 10K (Pierce, Rockford, IL) for two overnight incubations at 4°C in Storage Buffer (50mM phosphate acetate buffer pH 6.0, 1M NaCl, 1mM EDTA, 5mM DTT, and 0.02% sodium azide). The recovered dialyzed eluate was

filter sterilized at 0.45μm (Nalgene, Rochester, NY) and the absorbance read at 280nm to calculate concentration. The protein was then stored at 4°C.

Selective permeabilization with PFO
The procedure was carried out as outlined in Kleba and Stephens, 2008. In brief, infected HeLa cell monolayers were incubated at 4°C for 15 minutes. The cells were then washed twice with cold HBSS and a cold 50% PFO with HBSS solution applied. This sat at 4°C for 15 minutes and then washed twice with cold HBSS to remove any excess PFO monomers. The monolayers were subsequently incubated at 37°C for one hour in either intracellular buffer or cytosolic extract with DNase (Promega, Madison, WI), RNase (Life Technologies, Grand Island, NY), and 5% BSA.

Intracellular buffer contained 110mM K-gluconate, 20mM potassium chloride, 0.1mM calcium chloride, 2.7mM potassium phosphate, 1mM magnesium sulfate, 10mM HEPES, 10mM MES, 10mM glucose, 2mM magnesium acetate, 2mM GTP, 5mM ATP, and 1mM glutathione. All chemicals were purchased from Sigma (St. Louis, MO). Cytosolic extract was prepared by taking a monolayer of uninfected HeLa cells and scraping until dislodged. The cells were then sonicated on ice and centrifuged to remove cellular debris.

Texas Red Dextran integrity assessment
Coverslips with 24hpi *C. trachomatis* HeLa cells underwent selective permeabilization as stated previously. During the one hour incubation at 37°C, Texas Red Dextran 3,000MW (Life Technologies, Grand Island, NY) was added to the solution at a concentration of 50μM in HBSS. After the one hour incubation, the monolayers were methanol fixed for 15 minutes at room temperature. The samples were then blocked with 5% Bovine Serum Albumin (BSA; Sigma, St. Louis, MO) in HBSS and subsequently incubated with *C. trachomatis* IncG Rabbit polyclonal antibody (Antibodies Incorporated, Davis, CA) for one hour at room temperature. The cell monolayers were then washed in HBSS and exposed to Alexa Fluor 488 anti-Rabbit (Life technologies, Grand Island, NY) with DAPI (2.5μg/mL) for an hour at room temperature. The coverslips were then mounted to glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL).

Microscopy was carried out on a Zeiss epifluorescent inverted microscope (Zeiss, Germany) with a 40X air objective. A Hamamatsu Digital camera c10600 Orca-R^2 (Hamamatsu Photonics, Japan) was utilized to capture the image. Volocity 6.0 (Perkin Elmer, Walkan, MA) allowed for optimization of the captured image.

Release of cytosolic GFP
HeLa cells expressing cytosolic GFP were provided by Dr. Kevin Hybiske (UC Berkeley). A dilution curve of PFO was added to a cell monolayer. PFO permeabilization was performed as described previously with the one hour incubation being carried out in HBSS. The sample was then centrifuged for 10 minutes at 1000rpm. The supernatant was examined via a fluorometer (Turner Biosystems, Madison, WI). Each sample was performed in triplicate.

PFO titration on chlamydial inclusions
C. trachomatis infected HeLa cells underwent a dilution curve of PFO selective permeabilization as previous described. The solution used during the one hour incubation was intracellular buffer with 5% BSA. The resulting preparation was then fixed in 4% formaldehyde for 30 minutes at room temperature. The solution was centrifuged at 300g for 10 minutes at 4°C. The pellet was resuspended in 5% BSA in HBSS for 15 minutes at room temperature. Rabbit polyclonal IncG (Antibodies Incorporated, Davis, CA) and the plasma membrane marker Syndecan 4 (Abcam, Cambridge, MA) were incubated with the sample for one hour, room temperature. The cells were washed with 5% BSA in HBSS and stained with anti-Rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 546. The sample was then run on Cytopeia FLUX Sorter (Franklin Lane, NJ).

Immunofluorescence, confocal, and transmission electron microscopy
Infected HeLa cells were exposed to 0.12mg/mL PFO as described previously. For immunofluorescence staining and confocal the inclusions were released into intracellular buffer with 5% BSA, while the electron microscopy samples were in cytosolic extract with 5% BSA. The resulting cells were fixed in 4% formaldehyde and processed as outlined in "PFO titration on chlamydial inclusions." For immunofluorescence, HeLa cells transformed with a PALM-YFP construct were used. After PFO release, the sample was stained with IncG and visualized on a Zeiss epifluorescent inverted microscope (Zeiss, Germany) with a 20X air objective. A Hamamatsu Digital camera c10600 Orca-R^2 (Hamamatsu Photonics, Japan) was utilized to capture the image. Volocity 6.0 (Perkin Elmer, Walkan, MA) allowed for optimization of the captured image. Samples for confocal were prepared with wild-type HeLa cells and stained with IncG. For electron microscopy, inclusions were fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.1M sodium cacodylate buffer pH 7.4, post fixed in 2% osmium tetroxide in the same buffer, and block stained in 2% aqueous uranyl acetate, dehydrated in acetone, infiltrated, and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT). Samples were ultrathin sectioned on a Reichert Ultracut S ultramicrotome and counter stained with 0.8% lead citrate. Grids were examined on a JEOL JEM-1230 transmission electron microscope (JEOL USA, Inc., Peabody, MA) and photographed with the Gatan Ultrascan 1000 digital camera (Gatan Inc., Warrendale, PA).

Inclusion stability assay
All chemicals are from Sigma (St. Louis, MO) unless otherwise noted.

C. trachomatis infected HeLa cells went through PFO treatment at 24hpi. The inclusions were released into intracellular buffer with either 0% BSA, 0.5% BSA, 1% BSA, 5% BSA or a protease inhibitor cocktail. The cocktail consisted of 10mg/mL each of antipain, leupeptin, pepstatin A, and chymostatin along with 1mM E64. The suspension was collected and centrifuged at 300g for 10 min. The pellet was resuspended in the appropriate condition and incubated at 37°C until the designated timepoint.

The samples were then centrifuged and both the pellet and supernatant treated with 2X SDS sample buffer (4X Tris-Cl/SDS pH 6.8, 20% glycerol, 0.2M DTT, and 0.001% bromophenol blue). The protein samples were boiled for 5 minutes and loaded onto a 15% SDS-PAGE gel. The gel was then transferred onto nitrocellulose (Hercules, CA). The membrane was blocked with 5% milk in PBS tween for one hour at room temperature. The chlamydial antibody MOMP (Santa Cruz Antibody, Santa Cruz, CA) was incubated overnight at 4°C. The membrane then underwent another block with 5% BSA in PBS tween for an hour. The membrane was developed on Kodak film (Rochester, NY) with GE Healthcare developer.

Infectivity of released inclusions
Inclusions were isolated from 24hpi HeLa cells as previously described. The inclusions were released into either intracellular buffer or cytosolic extract, both with 5% BSA. The inclusions were collected via centrifugation after the initial one hour incubation and placed in the designated solution. The inclusions were incubated at 37°C until the designated time, at which point they were sonicated on ice and added directly to a fresh HeLa cell monolayer. The newly infected cells were incubated at 37°C until 24hpi. The cells were then stained with the chlamydial stain merifluor (Meridian Bioscience, Cincinnati, OH) and imaged on a Zeiss epifluorescent inverted microscope. Three random fields were counted within one monolayer. The experiment was done in triplicate.

RESULTS

Perfringolysin O (PFO) selective permeabilization released chlamydial inclusions.
It was previously demonstrated that selective permeabilization with the cholesterol dependent cytolysin, PFO, could be utilized to access the host cytosol without affecting the chlamydial inclusion (Kleba and Stephens 2008). PFO was added to GFP expressing HeLa cells, and was found to have a titratable activity (Figure 4.2 A). The amount of fluorescence in the supernatant remained the same after 0.027mg/mL PFO, indicating that this was a sufficient concentration to release GFP from all cells.

GFP is a much smaller molecular than a chlamydial inclusion, thus a dilution curve of PFO was applied to 24hpi HeLa cells (Figure 4.2 B). The resulting sample was then stained for an inclusion marker and plasma membrane marker and analyzed via FACS. It was observed that a higher concentration of PFO was needed for greater release of inclusions. PFO concentrations higher than 0.135mg/mL resulted in loss of all populations within the sample. This is more than likely due to an inability to wash away excess monomers at those high concentrations. Infected monolayers treated with the higher concentration of PFO were stained as described in Kleba and Stephens (2008). The inclusions remained impervious to a small dextran as previously published (data not shown).
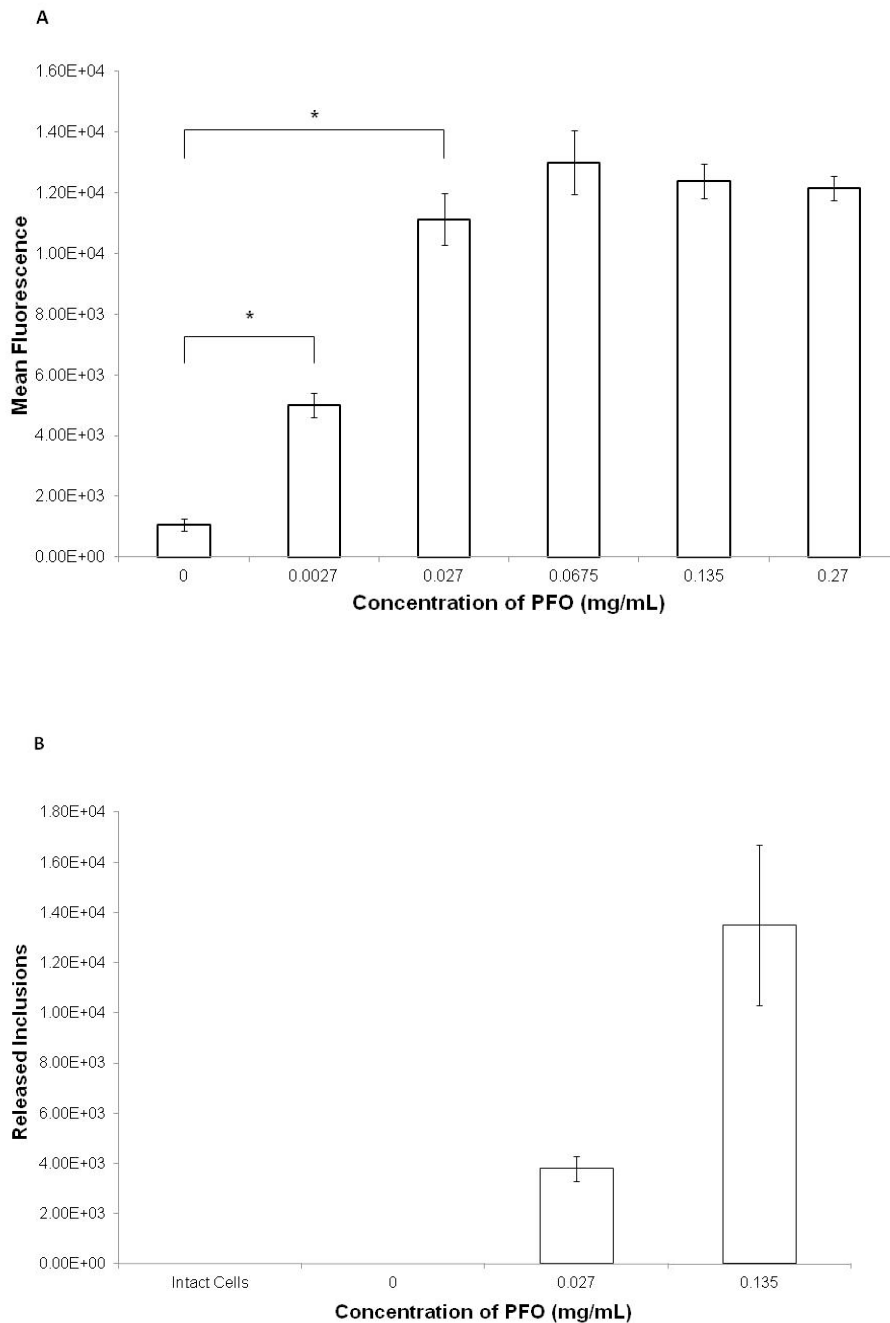
Figure 4.2. Selective permeabilization with PFO was able to release inclusions.
PFO was applied to a monolayer of HeLa cells expressing cytosolic GFP (A). The cells
were centrifuged and the supernatant measured for fluorescence. The optimal
concentration of PFO to release inclusions was explored by FACS (B). *Chlamydia*
infected HeLa cells were exposed to PFO and subsequently stained with a plasma
membrane marker and chlamydial inclusion marker. The sample underwent FACS
analysis. Statistical significance was calculated by a Student's t-test (p<0.05).

Released chlamydial inclusions retained their inherent morphology.
Previous work to isolate inclusions resulted in numerous membrane fractures and an overall abnormal structure of the bacteria. The RBs appeared detached from the inner leaflet of the inclusion membrane and were shurken in comparison to their normal structure (Matsumoto, 1981). The PFO method exerts less force on the chlamydial inclusions than physiological methods of vacuolar release that were employed previously.

Released inclusions were visualized via immunofluorescence staining for an overall assessment of the PFO treatment (Figure 4.3 A). It was observed that although inclusions were released, denoted by staining with chlamydial marker IncG and without plasma membrane staining, there were very few inclusions within a given field. Confocal microscopy was used to begin to ascertain the morphology of the inclusion membrane after release. IncG staining remained punctate and the overall shape of the inclusion was retained (Figure 4.3 B and C). Transmission electron microscopy gave a refined and detailed overview of the released inclusions (Figure 4.3 D-F). The RBs and EBs retained within the inclusions appeared unchanged upon treatment. There was a mixed population of inclusions that had intact membrane (Figure 4.3 D), and ones that had loss their integrity (Figure 4.3 E and F). It was also noted that as demonstrated in Figure 4.3 A, there were not many inclusions within a field. It was also interesting that the inclusions were not turgid in nature, as is typically observed within an infected cell.
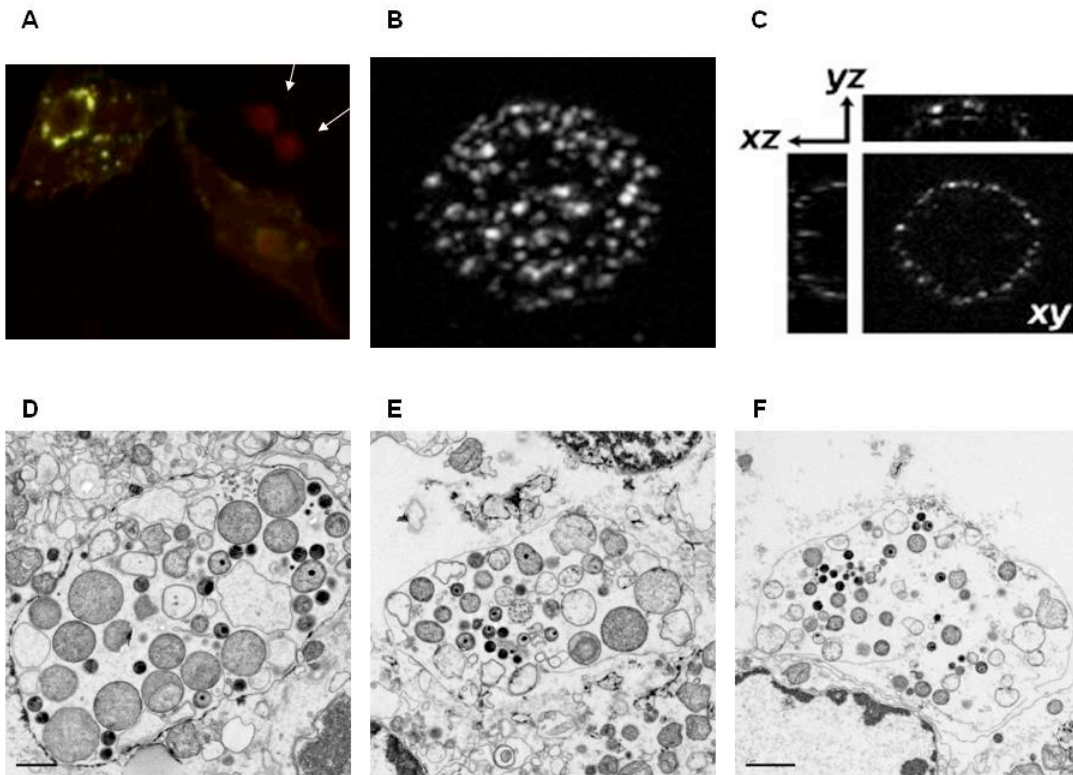
Figure 4.3. Released chlamydial inclusions.
HeLa cells transformed with PALM-YFP were treated with PFO (A). The plasma membrane (green) can be observed to be absent from two inclusions (red), denoted by arrows within a field. PFO treated sample stained with IncG and viewed with confocal microscopy, showed the distinct punctate staining pattern of IncG (B, C). Transmission electron microscopy on infected cells treated with PFO show an overall normal appearance (D-F).

Inclusions stability can be controlled upon release.
The stability of released inclusions needed to be defined to ascertain the usefulness of the PFO protocol. Previous studies used turbidity as a marker for inclusion lysis (Matsumoto, 1981). Immunoblotting was chosen over this method since it allowed for direct testing of inclusion stability and is more sensitive. Lysed inclusions would release bacteria into the supernatant, while intact inclusions allowed for the bacteria to remain in the pellet fraction.

Immunoblots were probed with an antibody specific to the bacteria (MOMP) was used to detect bacterial release from the inclusions (Figure 4.4). Differing BSA concentrations were utilized based upon the previous published work showing it was protective (Matsumoto, 1981). With no BSA, detectable signal was in the supernatant, denoting lysis of the inclusions. However, as BSA is added into the system, signal is retained in the pellet. It was postulated that a byproduct of the PFO protocol is release of proteases from the host cell. This may account for the BSA protective phenomenon, and thus a protease inhibitor cocktail was also tested for inclusion stability. It provided the same amount of protection as the higher concentrations of BSA.
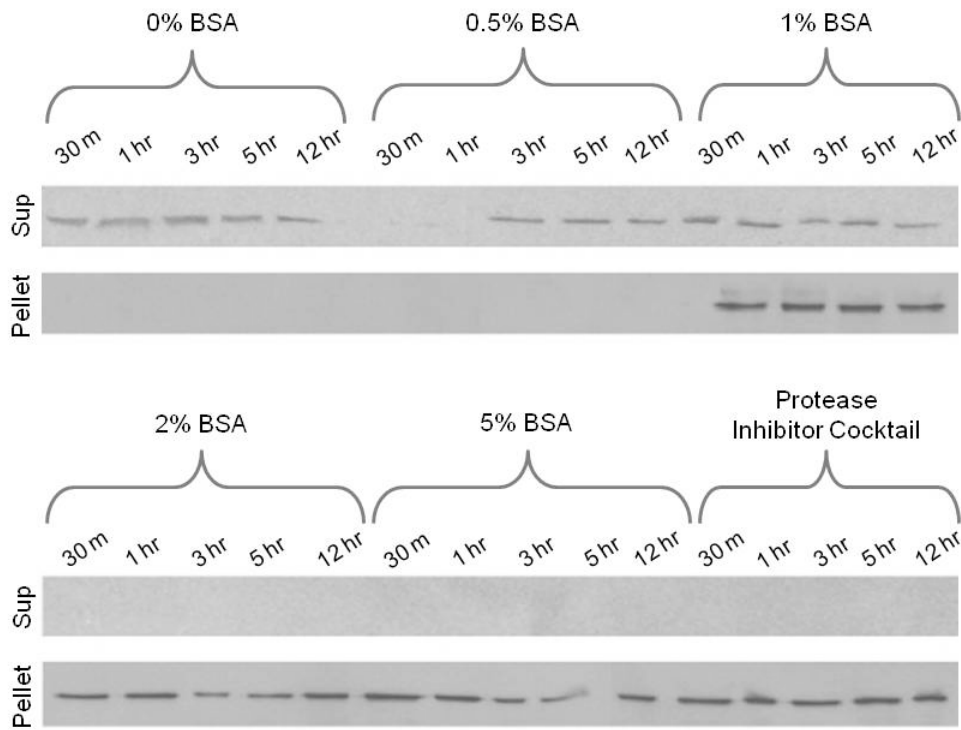
Figure 4.4. Cell free inclusions can be stabilized up to 12 hours.
Differing concentration of BSA or a protease inhibitor cocktail were added to intracellular buffer during PFO treatment. The samples were then incubated at 37°C until the designated time, at which intact inclusions were pelleted. The supernatant containing any bacteria from lysed inclusions and the pellet were run through a 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed for MOMP, a protein found exclusively on the bacteria.

Cell free inclusions yielded infectious bacteria up to 12 hours post-release.
Although the released inclusions remained stable and had a general unremarkable morphology after PFO treatment, the viability of the bacteria housed within was unknown. Infectivity was chosen as a means to assess the general viability of the bacteria housed within the released inclusions.

The inclusions had been kept in intracellular buffer, a solution that mimicked the host cytosol, for previous experiments. However, given the ever expanding understanding of the interactions between the host cell and the inclusion, it was thought that a cytosolic extract would provide a more natural and robust environment for the inclusions. Infected HeLa cells were treated with PFO and the inclusions released into either intracellular buffer or cytosolic extract, both containing 5% BSA. The inclusions were then lysed via sonication and added to a fresh HeLa monolayer at either 0, 12, 24 hours post-release. In intracellular buffer there was very little to no detectable infectivity of the bacteria across all timepoints (Figure 4.5). Those inclusions released into the cytosolic extract yielded a higher infectious rate at 0 hour post-release, and even saw an increase between 0 and 12 hours post-release. This was lost past the 12 hour mark.

This demonstrated that although the bacteria are initially viable upon release and for up to 12 hours, this system is not conducive for the developmental cycle to continue. The need for the inclusions to be released into cytosolic extract to retain any infectivity demonstrates the underlining complex interplay between host and bacteria. This assay only measures the effect on EBs since they are the infectious form. However, given that there is no infectivity beyond 12 hours post-release, the RBs must not be replicating, and the initial increase in infectivity is due to greater conversion to the EB form.
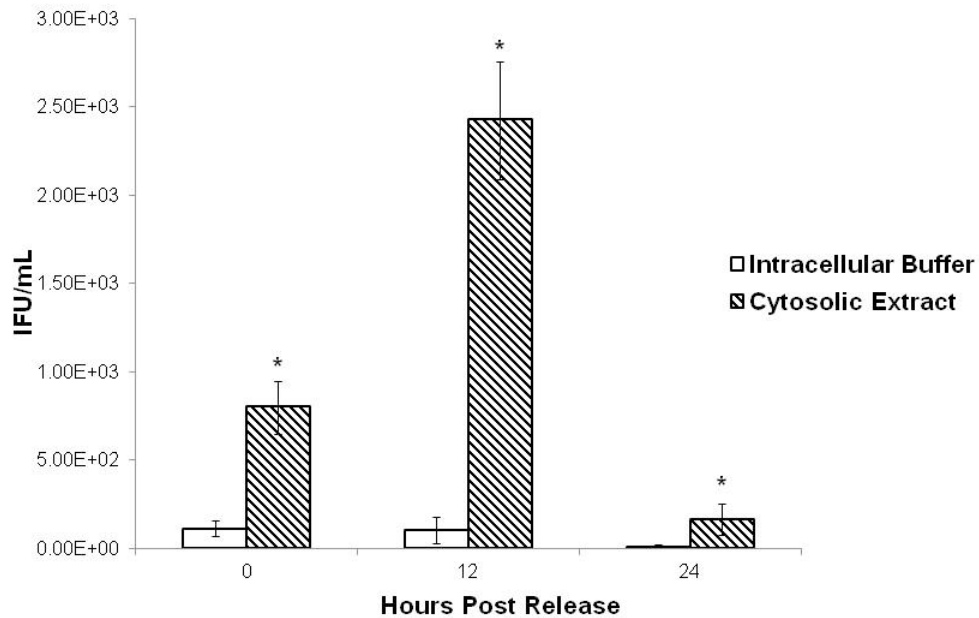
Figure 4.5 Bacteria from isolated inclusions were viable when incubated in cytosolic extract.
Chlamydiae harvested from released inclusions were placed on a HeLa monolayer. Infectivity was assessed at 24hpi by counting the number of inclusions within 3 random fields. Inclusions placed in cytosolic extract resulted in more infectious units than intracellular buffer. Statistical significance was calculated using a Student's t-test ($p<0.05$)

DISCUSSION

The chlamydial inclusion is an important mediator between host-cell interactions given that the bacteria reside within its confines during the entire developmental cycle. However, many questions remain that will require direct manipulations of the inclusion. The pervading notion in the field is that this is an impossible feat given the inherent fragile nature of the inclusion. Previous isolation attempts utilizing homogenization and differential centrifugation led to not only fractured inclusion membranes, but aberrant bacterial forms (Matsumoto, 1981). This was only re-enforced with the observation that micro-injection of an infected cell leads to a collapsed inclusion (Heinzen and Hackstadt, 1997). The novel approach of utilizing selective permeabilization to isolate host cytosol was intriguing due to its ability to not permeabilize the inclusion (Kleba and Stephens, 2008). Although a successful transformation system for *Chlamydia* is reported, a method to directly manipulate the inclusion is still needed (Wang *et al.*, 2011).

Unlike previous attempts to isolate inclusions, the overall structure of the bacteria was retained. However, there was still an issue with fracturing the inclusion membrane. It is also unknown why the inclusions are not turgid in nature as is reported when they are housed within a host cell. One potential issue if this protocol were to be utilized for proteomics, is the quantity of inclusions released. To address this concern, the protocol could be modified to use more starting material, or PFO binding could be optimized. It has been observed that PFO does not bind all cells at the same frequency. In order to circumvent this, cholesterol could be added to the plasma membrane prior to PFO treatment.

Despite the drawbacks to this protocol, it is important to stress that the released inclusions are stable for a period of time. Both BSA and a protease inhibitor cocktail can stabilize the inclusions, emphasizing that it is not the inclusions that are inherently fragile, but rather disrupting the host cell leads to release of proteases. This could account for the majority of lysis. It was necessary to address the functionality of the isolated inclusions. The EBs retained their infectious properties up to 12 hours post-release. The significant increase noted could be due to either more conversion from the RBs to the EB form, or RBs actively replication thus producing more EBs.

It would be of extreme importance to the field to create a cell free growth system. Development of a protocol to isolate numerous inclusions that are stable and metabolically active is a necessary first step. It is hoped that optimization of this protocol to increase the number of inclusions released, and also a suitable growth medium would yield such a system.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

SUMMARY

In order for the developmental cycle to progress, *Chlamydia* must obtain metabolic precursors such as amino acids, glycolytic and tricarboxylic substrates, and nucleotides from the host (Gresihaber *et al.*, 2002; Hatch, 1975; Hatch, 1975b; Kalman *et al.*, 1999; McClarty, 1994; Tipples and McClarty, 1993). Upon examination of the chlamydial genome, many peptide transporters were encoded, but no evidence has been found that they localize to the inclusion membrane (Stephens *et al.*, 1998). It is significant to note that the RBs align themselves along the inner inclusion membrane and at this junction there is a delineated double membrane that is different from the usual morphology of the RB cell envelope (Peterson and de la Maza, 1988).

Intracellular pathogens that reside within vacuoles have been found to acquire nutrients via three distinct modes. One is to intersect a host pathway such as the endosomal/lysosomal or autophagic pathway. This is the method employed by *Coxiella burnetii* which fuses with vacuoles from the endosomal/lysosomal pathway (Heinzen *et al.*, 1996). Another method to obtain nutrients is for the vacuole to have open channels allowing for free exchange with the host cytosol. *Toxoplasma gondii* obtains nutrients in this manner (Schwab *et al.*, 1994). The last system vacuolar pathogens potentially acquire nutrients through the vacuole would be via a transport mechanism. No active transport system has been reported in bacterial vacuoles although it has been documented in vacuoles that house parasites (Heinzen and Hackstadt, 1997; Saliba and Kirk, 2001).

The work presented has shown that the chlamydial inclusion exhibits two modes of transport for methionine and glucose. Both were dependent on bacterial protein synthesis, including Type Three Secretion System, but only glucose required host protein synthesis. The methionine transport appeared to be uni-directional and dependent on mitochondrial ATP. This leads to the hypothesis that methionine transport could be governed by an ABC transporter that the RBs possess. Glucose transport is thought to involve host GLUT1 due to its recruitment to the inclusion membrane. GLUT1 co-localization with the inclusion membrane was also dependent on bacterial and host protein synthesis.

Previous attempts to isolate chlamydial inclusions utilized homogenization and sucrose gradients resulting in fractured inclusions (Matsumoto, 1981). It was found that utilizing the pore forming toxin Perfringolysin O (PFO) was able to permeabilize the host cell, while leaving the inclusion intact (Kleba and Stephens, 2008). *T. gondii* parasitophorous vacuoles have been isolated utilizing a pore-forming toxin, Streptolysin O (SLO). In this study it was found that SLO formed pores that would cause the cell membrane to fall away, leaving the vacuole intact (Nyalwidhe and Linelbach, 2006). This approach was utilized to isolate chlamydial inclusions with the modification that SLO was replaced with PFO. It was shown utilizing a variety of microscopy techniques that PFO was able to release inclusions that retained their intrinsic characteristics.

However, some inclusion membranes were fractured during the process and the overall number of inclusions released was minimal. The resulting isolated inclusions were stable for up to 12 hours post-release upon the addition of BSA or a protease inhibitor cocktail. This insinuates that the inclusions are not inherently fragile, but rather were being degraded by host proteases. The EBs remained infectious upon release, but it soon diminished, while the RBs did not replicate. This is the first step in developing a viable protocol to isolate inclusions.

FUTURE WORK

Although advances were made to better define the nutrient transport mechanism *Chlamydia* utilizes, this is just cursory to what has yet to be examined. First and foremost, more trackable nutrient analogs need to be tested including amino acids and nucleotides, in order for any conclusions to be drawn about overall nutrient acquisition. Unfortunately at this time, there are few such compounds commercially available. However, the advent of Click-it technology implies that an ever increasing catalog of compounds could be available shortly. The role of host GLUT1 should be further explored. This could be accomplished by a transient knock out system for mammalian GLUT1 and monitoring of inclusion formation and the developmental cycle. The dependency of mitochondrial ATP on glucose transport should also be addressed. The proteome of the inclusion membrane needs to be analyzed in order to ascertain any host or bacterial protein in origin that could be responsible for nutrient acquisition. Unfortunately, experimentation is severely limited until a technique to isolate stable and metabolically active inclusions is developed.

Although the PFO method is the first step in this process, much work is still needed. The efficiency of this technique needs to be greatly increased. This could be done by increasing the binding of PFO, and making it more uniform throughout a cell monolayer. A specialty media that would allow for the inclusions to remain stable and metabolically active needs to be developed. This would be an extremely difficult endeavor given the complexity associated between the inclusion and host; however, with the first report of cell free growth of the obligate intracellular bacterium *Coxiella burnetii*, it is not a totally impossible feat (Omsland *et al.*, 2009).

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