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Identification of Chlamydial Iron-Responsive Proteins During Intracellular Growth

A dissertation
presented to
the faculty of the Department of Microbiology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

by
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August 2008

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ABSTRACT

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by

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Chlamydia trachomatis is an obligate intracellular bacterium and the most prevalent cause of bacterial sexually transmitted disease. Genital chlamydial infections, marked by chronic, intense inflammation, can lead to genital tissue scarring and infertility and is a contributing factor to development of pelvic inflammatory disease and ectopic pregnancy. Iron is required as a cofactor for numerous highly conserved pathways, and nearly all studied organisms rely on iron for growth. In response to iron restriction, the chlamydial developmental cycle arrests at the intracellular reticulate body stage, resulting in a phenomenon termed persistence. Persistence likely plays a role in chlamydial pathogenesis through the expression of virulence factors and antigens in addition to sustaining chronic infection; however, little is known concerning how chlamydiae respond to iron limitation at the molecular level, and no systems for iron acquisition have been identified in Chlamydia. This dissertation presents an investigation into the chlamydial response to iron restriction. Chlamydial heat shock protein 60 (cHsp60) has been implicated in development of the more severe disease sequelae and has been found to increase in expression following iron restriction; however, three cHsp60 homologues were identified following the sequencing of the chlamydial genome. Here, iron restriction is shown to increase expression of cHsp60-2 but not the two other homologs, cHsp60-1 or –3. Next, in order to investigate an
alternate model for restricting iron availability to chlamydiae, a cell line with inducible expression of recombinant ferroportin, a eukaryotic iron efflux protein, was examined. Lastly, 10 chlamydial proteins differentially expressed during growth in iron-restricted host cells were identified by proteomic analysis of radiolabeled proteins followed by mass spectrometry analysis; transcripts encoding 5 iron responsive proteins were examined across a timecourse of infection and revealed increased transcript levels at 18 and/or 24 hours post infection. Together, these studies have examined the molecular response of chlamydiae to reduced iron availability and have underlined the importance for pathways involved in protection against oxidative damage and adaptation to stress.
DEDICATION

This dissertation is dedicated to Jane E. Raulston, Ph.D.
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**CHAPTER 1**

**INTRODUCTION**

*Chlamydia trachomatis* is a Gram-negative, obligate intracellular bacterial pathogen that infects the mucosal epithelium. *C. trachomatis* is divided into the trachoma and lymphogranuloma venereum (LGV) biovariants, and the trachoma biovar is further separated into serovariants A-K, based on antiserum specificity to the variable region of the major outer membrane (MOMP). Serovars A-C infect the conjunctiva, causing trachoma, the leading cause of preventable blindness worldwide (Thylefors and others 1995), while trachoma serovars D-K are non-invasive pathogens of the genital tract. The LGV biovar causes an invasive sexually transmitted disease (STD) found primarily in tropical areas of the world.

Chlamydia infection is the number one bacterial STD in the world, with 92 million new infections each year (World Health Organization 2001). Over 1 million new cases of chlamydial infection in the United States were reported to the Centers for Disease Control and Prevention in 2006, triple that of the next most common bacterial STD, *Neisseria gonorrhoeae*; the estimated total number of actual new chlamydial infections in the US is 3 million per year (Centers for Disease Control and Prevention 2007). The chlamydial infection rate among adolescent females (24.1% to 27%) is alarming, especially considering the potential for long-term effects on reproductive health (World Health Organization 2001).
Non-invasive chlamydial genital infections initially cause urethritis or cervicitis; sequelae include prostatitis and epididymitis in men, and salpingitis, endometritis, pelvic inflammatory disease, infertility, and ectopic pregnancy in women (Schacter 1999). The initial infection is often asymptomatic, which allows infections to go undetected and thus untreated, providing further spread. Invasive genital infections caused by LGV progress from infection of the genital mucosa through the basal membrane to infection of macrophages. Next, macrophages carry chlamydiae to the inguinal lymph nodes, where a severe lymphoadenopathy can develop.

Another human chlamydial pathogen of note is *Chlamydophila pneumoniae*, a respiratory pathogen responsible for a “walking pneumonia”. *Chlamydophila pneumoniae* was reclassified from *Chlamydia pneumoniae* following ribosomal RNA phylogenetic analysis. Not identified until the 1980s, *C. pneumoniae* is a ubiquitous pathogen, with a world-wide seroprevalence of 60-70% (Schachter 1999). After culturable *C. pneumonia* was recovered from atherosclerotic plaques, numerous studies have implicated infection with *C. pneumoniae* and the progression of heart disease (Saikku and others 1988; Hoymans and others 2007).

The first line treatment for a *C. trachomatis* genital infection is a single 1 g oral dose of azithromycin or 100 mg of doxycycline twice daily for 7 days (Centers for Disease Control and Prevention 2006). Antibiotic resistance has never been identified in human chlamydial strains; however, a naturally-occurring tetracycline resistant strain of the porcine chlamydial pathogen, *Chlamydia suis*, has been isolated (Lenart and others 2001).
Chlamydiae exhibit a biphasic developmental cycle involving transition between the elementary body (EB) and the reticulate body (RB). The EB (0.3 μm) is the infectious, metabolically inert form responsible for extracellular stability, attachment, and entry (Figure 1.1A). In order to fulfill this role, the outer membrane is stabilized by cross-linking of outer membrane proteins via disulfide bonds, and the chromosome is condensed by chromatin-like proteins (Abdelrahman and Belland 2005). EB enter the host cell by receptor-mediated endocytosis; primary binding may involve heparin sulfate on the host cell surface, followed by a secondary, irreversible binding step. Although secondary attachment involves an unknown chlamydial ligand and eukaryotic receptor, numerous candidates have been proposed; evidence also indicates chlamydiae may use multiple uptake pathways, depending on chlamydial strain and host cell type (Dautry-Varsat and others 2004; Dautry-Varsat and others 2005). Upon attachment, the chlamydial protein Tarp is secreted into the host cell cytosol via the putative type three secretion system, resulting in the recruitment of actin and may play a role in entry or early events following entry (Clifton and others 2004).

After entry, the EB convert within the endosomal vacuole into the metabolically active 1 μm RB form. The chlamydial endosome, which following chlamydial modification is known as the inclusion, is altered in order to change from the endocytic pathway to the exocytic pathway, thus avoiding lysosomal fusion and consequent acidification (Fields and Hackstadt 2002). Inclusions undergo homotypic fusion (in most strains) and traffic to the perinuclear region. After undergoing a 6-14h lag phase, RB replicate by binary fission (Figure 1.1B). In response to an unknown cue, the RB convert asynchronously back to EB, which after host cell lysis or extrusion of the
inclusion (Hybiske and Stephens 2007) allows the spread of infectious progeny to new cells. The duration of the chlamydial developmental cycle differs by species and serovar; that of the *C. trachomatis* trachoma biovar is approximately 60-72 h, the LGV biovar is ~48h, and *C. pneumoniae* is ~96 h.

![Figure 1.1](image)

**Figure 1.1.** Transmission electron microscopy of (A) EB attaching to the epithelial cell surface and entering via nascent endocytotic vesicles, and (B) RB growing inside an inclusion at 36 hpi (photomicrographs courtesy of Jane Raulston).

The biphasic cycle can be interrupted at the RB stage in a phenomenon termed persistence (Figure 1.2). During persistence, the RB cease to divide, become extremely enlarged, and accumulate chromosomes. Infectivity is severely attenuated, because infectious EB are not produced. Great interest in chlamydial persistence has developed due to its potential role in producing chronic infections and pathogenesis, particularly because persistent chlamydiae may be refractory to antibiotic killing, allowing the continuation and spread of infection even after the patient has received treatment, and increased expression of virulence factors by persistent chlamydiae may
contribute to a prolonged inflammatory response (Hogan and others 2004). Pathogenesis in *Chlamydia* is considered to be immune-mediated, although whether this results from an antigen-dependent delayed-type hypersensitivity, autoimmunity, or a chronic, intense inflammatory response to infection is debated (Stephens 2003).

There have been numerous inducers of persistence identified, including interferon-γ exposure, penicillin exposure, amino acid (tryptophan) limitation, viral co-infection, and, most relevant to this dissertation, iron limitation (Raulston 1997; Hogan and others 2004; Deka and others 2006). A study by Raulston (1997) established the induction of chlamydial persistence via iron restriction, using the pharmaceutical agent Desferal, which is used to treat patients with iron overload disorders (Raulston 2006).
The iron-chelating compound Desferal is derived from *Streptomyces pilosus* (Muller and Raymond 1984).

The first paper investigating the effect of iron restriction on chlamydiae examined *Chlamydophila* (formerly *Chlamydia* *psittaci*), a strain that infects avian species, following exposure to Desferal. In *C. psittaci*-infected Desferal-exposed macrophages, no decrease in inclusion number was found (Murray and others 1991). However, when Raulston (1997) examined *Chlamydia trachomatis*-infected HEC-1B cells (a human endometrial epithelial carcinoma cell line) exposed to Desferal, inclusion numbers were not reduced, but inclusion size decreased and alterations in chlamydial (i) morphology, (ii) infectivity, and (iii) protein expression profile were observed.

Chlamydiae exposed to Desferal exhibited an enlarged, aberrant RB morphology typical of that seen in reported persistent chlamydial infections from exposure to interferon-γ or penicillin (Beatty and others 1993). Chlamydial infectivity dropped 8-fold in Desferal-exposed cultures, as determined by the percentage of cells containing inclusions after inoculation using progeny EB at a given dilution (Raulston 1997). Furthermore, supplementing Desferal-exposed, infected cultures with iron-loaded transferrin restored infectivity of progeny EB. To determine if iron restriction altered the chlamydial proteome, chlamydiae were radiolabeled in cultures with or without Desferal, and EB were harvested and Renografin purified. EB proteins were solubilized and separated by two-dimensional polyacrylamide electrophoresis (2D-PAGE), a method that separates proteins based on isoelectric point (pI) and molecular weight, thus producing a two-dimensional array of proteins. In comparing spot intensities between 2D gels from Desferal-exposed and unexposed cultures, 19 protein species were found
to increase in expression; of these proteins, Hsp60 (heat shock protein 60), YtgA (a putative ABC metal transporter), Pgk (phosphoglycerate kinase), and YscN (associated with the type III secretion system apparatus) were identified (Raulston 1997). The discovery that YtgA and Hsp60 are iron responsive was significant, because YtgA is a putative component of an ABC iron transporter (Raulston and others 2007) and Hsp60, a chaperone responsible for folding newly synthesized or denatured proteins into the correct secondary structure, has repeatedly been implicated in a role in the inflammatory damage of chlamydial pathogenesis (LaVerda and others 1999).

The requirement of chlamydiae for iron is not surprising because iron is a cofactor required for a number of ubiquitous cellular metabolic processes in both bacterial and eukaryotic cells, including cytochromes, ribonucleotide reductase, RNA polymerase III, amino acid hydroxylases, superoxide dismutase, and catalase (Wooldridge and Williams 1993; Andrews and others 2003). Iron serves as an electron bank, donating or accepting electrons in order to catalyze oxidation and reduction reactions. All examined organisms require iron except lactobacilli (Archibald 1983; Weinberg 1997) and Borrelia burgdorferi (Posey and Gherardini 2000), which substitute alternative transition metal cofactors cobalt and manganese for iron.

Numerous barriers stand between pathogens and the iron they require from the host. In aqueous, aerobic environments, the primary state of iron is ferric hydroxide, which has a solubility of $1.4 \times 10^{-9}$ M at pH 7 (Schaible and Kaufmann 2004). Additionally, although iron is required for life, free iron can catalyze the formation of damaging reactive oxygen species via the Fenton reaction, which damage lipids, proteins, and nucleic acids:
\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \]

(Wooldridge and Williams 1993; Van Ho and others 2002; Masse and Arquin 2005). In order to minimize the catalysis of reactive oxygen species, mammals produce iron-binding proteins, including transferrin, that chelate iron in the blood; lactoferrin, that binds iron in mucosal secretions; and ferritin, the primary intracellular iron storage protein (Levenson and Tassabehji 2004; Oates and Ahmed 2007).

Control of iron level also plays a role in innate immunity, as iron availability is a primary requirement for infection; general limitation of free iron helps to prevent infection, and increased limitation occurs in response to infection (Kadner 2005; Sritharan and others 2006; Markel and others 2007). For example, macrophages express the protein ferroportin following activation in order to reduce intracellular iron; ferroportin is the only cellular iron efflux protein in mammals and is also expressed in enterocytes and hepatocytes (Ganz 2005).

Because iron availability is a primary limitation to infection, bacteria have evolved numerous virulence factors in response to limited iron availability. Reduced iron availability is known to induce toxin expression by pathogens, including the shiga toxin in *Shigella dysenteriae*, the shiga-like toxin in *E. coli*, exotoxin A in *Pseudomonas aeruginosa*, and diptheria toxin in *Corynebacterium diptheriae* (Litwin and Calderwood 1993; Hantke 2001). Additionally, pathogens possess numerous mechanisms to obtain iron from host stores. Most pathogens express siderophores, compounds that directly chelate iron from host iron-protein complexes with high affinity (\(K_D\) value of \(10^{22}-10^{50}\)). Over 500 different siderophores have been identified and can originate from bacteria, yeast, and fungi (Ratledge and Dover 2000); however, no homologs for enzymes...
responsible for siderophore synthesis have been identified in the *C. trachomatis* genome (Stephens and others 1998).

Another bacterial scheme for obtaining iron is the expression of receptors for host iron-containing compounds, such as human heme, hemoglobin, transferrin, and lactoferrin. Receptors specific for transferrin have been identified in several pathogens, including *Neisseria meningitidis* (Schryvers and Morris 1988), *Pasteurella haemolytica* (Ogunnariwo and Schryvers 1990), *Staphylococcus aureus* (Modun and others 1994), *Moraxella catarrhalis* (Campo and others 2004), *and Borrelia burgdorferi* (Carroll and others 1996); acquisition of iron from transferrin has also been implicated in the iron acquisition strategies of the obligate intracellular pathogens *Coxiella* (Howe and others 2005) and *Ehrlichia* (Barnewall and others 1997). Another iron acquisition strategy by pathogens during intracellular growth involves accessing ferritin or heme, which has been shown to be important for *Neisseria meningitidis* (Larson and others 2004), *Mycobacterium tuberculosis* (Olakanmi and others 2002), and the intracellular fungus *Histoplasma capsulatum* (Hwang and others 2008). Additionally, the intracellular protozoan *Leishmania* appears to rely on the reduction of ferric to ferrous iron followed by internalizing the solubilized iron via a ferrous iron transporter (Huynh and Andrews 2008).

While no gene homologues for proteins involved in conserved iron-uptake mechanisms (i.e. siderophores or receptors for eukaryotic iron-binding proteins such as transferrin) were identified following sequencing of the *C. trachomatis* serovar D genome (Stephens and others 1998), it is likely that chlamydiae actively obtain iron from the host due to the low concentration of soluble iron in the eukaryotic cell and the fact
that the inclusion membrane is known to prohibit diffusion of tracer molecules as small as 520 Da (Heinzen and Hackstadt 1997). Scidmore and Hackstadt showed that incubation of infected cells with $^{55}\text{Fe}$-labeled human transferrin resulted in radiolabeled EB, although at low levels (Scidmore and Hackstadt 1995), and Raulston demonstrated that the addition of human transferrin to infected cells grown under iron-deficient conditions restored the infectivity of EB (Raulston 1997). Interestingly, a recent report has demonstrated that reduced expression of transferrin or Rab11, a protein involved in trafficking of transferrin-positive vesicles, reduces the production of infectious EB (Heuer and others 2006). These observations make it tempting to hypothesize chlamydiae obtain iron from transferrin; however, while transferrin-positive vesicles have been found adjacent to the chlamydial inclusion, researchers have been unable to detect the transferrin-transferrin receptor complex in the inclusion membrane, either by transmission electron microscopy (TEM; Scidmore and others 1996; Taraska and others 1996) or fluorescence microscopy (van Ooij and others 1997). The identification of a chlamydial transferrin receptor would make a promising potential vaccine candidate; numerous studies have investigated the use of the transferrin receptor as a vaccine target for *Neisseria meningitidis* (Banerjee-Bhatnagar and Frasch 1990; Danve and others 1993; Pintor and others 1996; Schryvers and Stojiljkovic 1999).

If iron-containing compounds were trafficked into the inclusion and bound to the chlamydial outer membrane, chlamydiae would still need to transport the iron compounds across both the outer and inner chlamydial membranes. The TonB-ExbB-ExbD system is a conserved complex that is required for transport of iron complexes across the Gram-negative outer membrane into the periplasm, providing the energy
required to move the iron compounds across the outer membrane and into the periplasm, where the iron is extracted and moved into the cytoplasm (Larson and others 2002). Genes encoding accessory proteins involved in the TonB transport system were identified in the chlamydial genome (\textit{tolB}, \textit{tolQ}, \textit{exbD}), but no homologue was found for TonB itself (Stephens and others 1998).

Although the iron acquisition pathway used by chlamydiae is undefined, a homolog to the primary iron regulatory protein in Gram-negative bacteria, the transcriptional repressor Fur, has been identified. Under elevated iron levels, activated Fur binds to specific regions in the promoter region of regulated genes, termed Fur boxes, preventing transcription of that gene; when iron levels are lowered, Fur becomes deactivated and loses its association with DNA, allowing transcription of the regulated gene to resume. The chlamydial Fur homolog, termed DcrA (divalent cation-dependent regulator), has demonstrated the ability to bind \textit{E. coli} Fur boxes (Wyllie and Raulston 2001). Binding of purified \textit{E. coli} Fur protein to a chlamydial library has identified several putative DcrA binding sites in the chlamydial genome (Rau and others 2005).

While genetic manipulation via gene knockout and directed mutation is the standard method of investigating virulence genes, this approach is not currently feasible in \textit{Chlamydia} due to problems inherent in its biphasic, obligate intracellular lifestyle, including difficulties in isolating discrete clonal colonies (Tam and others 1994). The goal of the research detailed in this dissertation has been to further characterize the chlamydial response to iron restriction at the molecular level. While analysis of transcription can provide critical information regarding transcriptional regulation, we chose to pursue the question of how chlamydiae respond to iron limitation through a
primarily proteomic approach because protein expression represents the end-stage of regulation. The first study (Chapter 2) examined expression of the three Hsp60 homologs following an iron restrictive shock to identify which homolog or homologs were iron responsive. In the second study (Chapter 3), an established cell line capable of inducible expression of the iron-exporting protein ferroportin was evaluated for its potential use as a model for chlamydial iron restriction; this was examined via TEM, infectivity of progeny EB, and host cell iron level. In the final study (Chapter 4), chlamydial proteins demonstrated to be iron-responsive during intracellular growth were identified, and the transcript levels encoding a subset of these proteins were measured in the presence or absence of iron restriction over a timecourse of infection.
CHAPTER 2

CHLAMYDIAL HSP60-2 IS IRON RESPONSIVE IN CHLAMYDIA TRACHOMATIS SEROVAR E-INFECTED HUMAN ENDOMETRIAL EPITHELIAL CELLS IN VITRO

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Abstract

Chlamydial heat shock proteins 60kDa (cHsp60s) are known to play a prominent role in the immunopathogenesis of disease. It is also known that several stress-inducing growth conditions, such as heat, iron deprivation, or exposure to interferon-γ, result in the development of persistent chlamydial forms that often exhibit enhanced expression of cHsp60. We have shown previously that the expression of cHsp60 is greatly enhanced in Chlamydia trachomatis serovar E propagated in an iron-deficient medium. The objective of this work was to determine which single cHsp60 or combination of the three cHsp60 homologs encoded by this organism responds to iron limitation. Using monospecific polyclonal peptide antisera that recognize only cHsp60-1, cHsp60-2, or cHsp60-3, we found that expression of cHsp60-2 is responsive to iron deprivation. Overall, our studies suggest that the expression of cHsp60 homologs differs among the mechanisms currently known to induce persistence.

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Introduction

Studies have consistently shown that there is a correlation between the production of chlamydial 60-kDa heat shock protein (cHsp60) antibodies in chlamydiae-infected patients and adverse disease consequences. These observations appear to be universal for *Chlamydia* species and their disease presentations. Early studies of whether cHsp60 plays a role in immunopathogenesis involved analyses of serum antibodies from female patients presenting with *Chlamydia trachomatis*-associated tubal infertility (7, 52); elevated levels of anti-cHsp60 in the sera of these patients were significantly associated with disease. A separate group of investigators described the contribution of cHsp60 in a guinea pig model of trachoma; an intense mononuclear cell inflammatory response was observed after conjunctival inoculation of cHsp60 following resolution of a primary ocular infection with *Chlamydia psittaci* GPIC (37). Patients with coronary artery disease often have serological evidence of previous infection by *Chlamydia pneumoniae* (45), and cHsp60 has been directly identified in human atheromatous tissue (31). Most recently, *C. pneumoniae* and *Chlamydia pecorum* cHsp60s have been implicated in urogenital tract disease in koalas (*Phascolarctos cinereus*), leading to infertility and death; chlamydiae are the most commonly recognized disease agents in the threatened koala population (23, 24).

Studies to determine the role of cHsp60 and immunopathogenesis are still being performed. Recently, workers have examined the initial interactions of cHsp60 with host cells that induce an inflammatory response. For example, cHsp60 interacts with Toll-like receptor 4 that stimulates the proliferation of human vascular smooth muscle cells (47), activates macrophages, and activates endothelial cells (8). The interaction
between cHsp60 and Toll-like receptor 4 also leads to apoptosis in primary human trophoblasts, placental fibroblasts, and a trophoblast cell line by both caspase-dependent and -independent pathways (18). cHsp60 and other microbial ligands can also activate mononuclear cells by binding to CD14, the monocyte receptor for lipopolysacharide (32). Although cHsp60 clearly plays a prominent role in chlamydial pathogenesis, it is not the only molecule involved. The genetically linked cHsp10, encoded by the groES gene upstream of groEL-1, is also associated with disease complications (6, 23, 27, 33). Moreover, several studies have demonstrated that genetic predisposition plays a significant role in chronic chlamydial disease (10, 12, 38). Perhaps most interesting is the fact that cHsp60 has been used in a human trial involving women at high risk for C. trachomatis infection; cHsp60 was used to stimulate the patients’ peripheral blood mononuclear cells to produce gamma interferon (IFN-γ), and the results indicated that a protective response against incidental infection developed (11).

Our laboratory is involved in identifying and analyzing C. trachomatis proteins that respond to iron restriction, as well as the mechanisms involved (42, 43, 55); cHsp60 is one of several proteins whose expression increases significantly during iron limitation in vitro (43). It is known that iron sources and the availability of iron fluctuate in menstruating women due to the cyclic pressures of estrogen and progesterone (1, 29); active or persistent C. trachomatis organisms in the reproductive tract are therefore likely to respond to this dynamic environment using transcriptional, translational, or posttranslational mechanisms to alter the production of specific chlamydial proteins. While we are not involved in direct studies of persistent chlamydiae, which have been
defined as viable but non-cultur able organisms (3), iron deprivation is one of several modes for induction of persistent chlamydiae (22, 39, 43). In women with tubal factor subfertility, cHsp60 is a serological marker for persistence (15) along with chlamydial proteasome/protease-like activity factor (48). However, the result of recent studies with C. psittaci (22) and C. pneumoniae (39) indicate that cHsp60 is not a general marker for persistence.

When the complete sequence of the C. trachomatis serovar D chromosome became available, one of many surprises was that there are three open reading frames (ORF) that code for groEL-related proteins (49). These ORF are positioned in separate regions of the chromosome and designated as follows: CT110 or groEL-1, encoding cHsp60-1; CT604 or groEL-2, encoding cHsp60-2; and CT755 or groEL-3, encoding cHsp60-3. Only groEL-1 is preceded by groES. Matching cHsp60s in different Chlamydia species appears to be conserved in the sequences that are currently available. For example, the predicted level of amino acid sequence identity between cHsp60-1 in C. trachomatis serovar D and cHsp60-1 in C. pneumoniae AR39 is 91%. However, there are considerable differences between cHsp60-1, cHsp60-2, and cHsp60-3 in a given species or serovariant. In C. trachomatis serovar D, the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-2 are 23% and 19%, respectively; the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-3 are 18% and 20%, respectively; and the levels of amino acid identity and similarity between cHsp60-2 and cHsp60-3 are 17% and 15%, respectively (28, 35, 49).

Although the majority of previous studies clearly involved cHsp60-1, as confirmed by sequence analysis, certain studies, including our study (43), generated new questions
concerning the extent to which each cHsp60 responds to a given microenvironment, especially a microenvironment leading to chlamydial persistence. Thus, the purpose of this study was to determine which cHsp60 is iron responsive in C. trachomatis serovar E.

Materials and methods

Bacterial strains, eukaryotic host cells, and growth

Stock inocula of C. trachomatis serovar E/UW-5CX EB were generated in McCoy cell fibroblasts and titrated to determine their infectivity. Polarized human endometrial epithelial cells (HEC-1B) were used as host cells in iron deprivation experiments and were maintained in Eagle’s minimal essential medium containing 2 mM glutamine and 5% (vol/vol) heat-inactivated fetal bovine serum at 37°C. For induction of iron deprivation, chlamydiae-infected cultures were allowed to grow to 36 hrs post-inoculation (hpi) and one-half of the samples were exposed to 500 µM Desferal for 30 min and 1 hr and 2 hrs.

Escherichia coli LMG194 (pBAD/HisA) was used to engineer and overexpress each cHsp60. The recombinants expressing cHsp60-1, cHsp60-2, and cHsp60-3 were designated as E. coli LMG194 (pJER516), E. coli LMG194 (pJER517), and E. coli LMG194 (pJER518), respectively. Each recombinant E. coli was grown in reduced medium (Invitrogen, Carlsbad, CA) containing 0.2% (wt/vol) glucose and 100 µg/ml ampicillin (Sigma Genosys, The Woodlands, TX) to the mid-log phase (A600, 0.4 to 0.6) at 37°C. Cultures were subsequently centrifuged, washed, and resuspended in prewarmed glucose-free medium. Arabinose was then added to each culture for 4 h of
induction. Maximum expression of cHsp60-1 and cHsp60-2 in E. coli LMG194 (pJER516) and E. coli LMG194 (pJER517) required 0.002% (wt/vol) arabinose, whereas maximum expression of cHsp60-3 in E. coli LMG194 (pJER518) required 20% (wt/vol) arabinose.

DNA amplification, cloning, and sequence analysis

The primers used for the PCR amplification of the chlamydial groEL genes were designed using the genome sequence of C. trachomatis serovar D (49). The reactions were carried out with an Expand High Fidelity PCR system kit (Roche, Nutley, NJ) in the presence of 0.5 pmol of forward, 0.5 pmol of reverse primer, and 10-fold (1:10-1:1,000) dilutions of C. trachomatis serovar E DNA template. After 35 cycles of amplification, the PCR products were cleaned-up using a QIAquick PCR purification kit (Qiagen, Germantown, MD); the sizes and concentrations of the purified products were monitored by agarose gel electrophoresis in the presence of ethidium bromide and by determining the optical density, respectively. All PCRs were done in duplicate to reduce introduction of errant nucleotides. The PCR products were then directionally cloned into the pBAD/HisA vector (Invitrogen, Carlsbad, CA) under the control of the araC promoter with a N-terminal six-histidine tag for recombinant protein detection and used to transform E. coli LMG194 by the traditional CaCl₂ method (46). For each groEL gene, the recombinant plasmids from three clones were purified using the Concert nucleic acid purification kit (Invitrogen) and then sequenced to verify in-frame cloning and to determine the complete nucleic acid sequences.
Peptide antibodies

The predictive amino acid sequences of *C. trachomatis* serovar E Hsp60-1, Hsp60-2, and Hsp60-3 were aligned using the EditSeq and MegAlign software from DNAStar, Inc. (Madison, WI). Peptides that were 17 to 21 residues long, detailed by Giles *et al.* (21), were commercially synthesized, the purity was assessed by analytical high-pressure liquid chromatography and mass spectroscopy, and each peptide was subsequently used to immunize two female New Zealand White rabbits (Sigma-Genosys, The Woodlands, TX). The results of enzyme-linked immunosorbent assays were provided by the manufacturer to ensure reactivity.

The majority of antiserum from each bleed was immediately stored at -20°C upon receipt. One milliliter of antiserum from each bleed was kept at 4°C to determine the Western blot reactivities of the crude preimmune and immune sera against total protein from HEC-1B cells and *E. coli* LMG194 as controls and arabinose-induced recombinant *E. coli* LMG194 (pJER516), *E. coli* LMG194 (pJER517), and *E. coli* LMG194 (pJER518). Immune sera exhibiting the most selective reactivity with the intended Hsp60 homolog were then placed on protein A columns (ImmunoPure immobilized protein A, Pierce, Rockford, IL) to purify immunoglobulin G, and Western blotting was performed to determine the degree of monospecificity and the reduction in the cross-reactivity with other *E. coli* proteins. Antiserum against the peptide from cHsp60-3 required a further step of adsorption against whole cells of arabinose-induced *E. coli* LMG194 (pJER516) expressing cHsp60-1. A monoclonal antibody reagent (Sigma-Genosys, The Woodlands, TX) against the polyhistidine tag was also used in this study.
Protein quantitation, sodium dodecyl sulfate-polyacrilamide gel electrophoresis, Western blotting, and chemiluminescence

The total protein concentrations of samples were determined using the Micro BCA Assay (Pierce, Rockford, IL). Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I, and 10 mM CaCl$_2$) and subjected to three freeze-thaw cycles. After the final thaw, samples were centrifuged at 8,000 X $g$ for 10 min, and each supernatant was combined with denaturing sample buffer and heated at 100°C for 5 min. The proteins were resolved in small-format 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes for Western blotting. Preliminary separations were conducted in large-format 12.5% polyacrylamide gels loaded with 1 mg of protein to accommodate multiple blots for screening and titrating antisera.

For Western blotting, membranes were blocked with Blotto-plus (5% [wt/vol] dry non-fat milk in phosphate-buffered saline, 0.1% [vol/vol] Tween 20, and 10% [vol/vol] heat-inactivated fetal bovine serum), and washing was performed with phosphate-buffered saline containing 0.1% (vol/vol) Tween 20. Various dilutions were examined for the polyclonal peptide antisera generated against each of the cHsp60s, and a monoclonal antibody against the poly-His tag (Sigma-Genosys, The Woodlands, TX) was also used as a control. Specific signals were then detected either (i) by a colorimetric assay with an anti-rabbit alkaline phosphatase-conjugated secondary antibody and Western Blue substrate (Promega, Madison, WI), or (ii) by chemiluminescence using an anti-rabbit horseradish peroxidase conjugate, the SuperSignal West (Pierce) solution, and Kodak X-OMAT AR film.
Electron microscopy

Samples of *C. trachomatis*-infected polarized HEC-1B cells at 36 hpi were exposed to 500µM Desferal for 30 min and 1 h and 2 h; mock-exposed samples were used as controls. Each sample was immediately washed, fixed, processed, and embedded in Epon-araldite and Lowicryl (Polysciences, Inc.) for high-contrast electron microscopy and immunoelectron microscopy, respectively, as described by Giles *et al.* (21).

Visualization and image capture were done using a Philips Tecnai 10 transmission electron microscope (FEI Company, Hillsboro, Oregon) operating at 80 kV.

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database under the following accession numbers: AY447001 for *C. trachomatis* serovar E *groEL-1*, AY447002 for *C. trachomatis* serovar E *groEL-2*, and AY447003 for *C. trachomatis* serovar E *groEL-3*.

Results

Amplification and nucleotide sequence analysis of *C. trachomatis* serovar E *groEL* ORF

DNA that was the expected size was amplified with all dilutions of the *C. trachomatis* serovar E *groEL* DNA templates except the *groEL-3*/CT755 template (Fig. 2.1A, lanes 3). Figures 2.1B and 2.1C show the results of a change in primer strategy, and the data revealed that CT755 was present along with the flanking sequences. Further analysis showed that a missing cytosine residue in the initial sequence of the
CT755 in serovar E at position 25 was responsible for the lack of primer hybridization and amplification (Fig. 2.1D). As determined by comparison with the published sequence of CT755 in serovar D, a frameshift placed the ORF back into frame by insertion of a cytosine residue at position 30 in serovar E. The final results were confirmed using a new set of primers (Figure 2.1E). The nucleotide sequences of *C. trachomatis* serovar E *groEL-1, groEL-2,* and *groEL-3* are 99.7%, 98.5%, and 99.2% identical to their counterparts in *C. trachomatis* serovar D, with only 6-, 25-, and 8-bp differences, respectively.
Figure 2.1 PCR amplification of C. trachomatis serovar E groEL. (A) Initial attempt to amplify groEL-1, groEL-2, and groEL-3 (lanes 1, lanes 2, and lanes 3, respectively) from C. trachomatis serovar E DNA template and primers based on the sequence of C. trachomatis serovar D. (B) Strategy used to amplify groEL-3 and flanking sequences. (C) Result of amplification of groEL-3 and flanking sequences. Lanes 1 through 5 contained areas indicated in panel B, and amplification of groEL-1 was used as a control. (D) Difference in the starting sequences of C. trachomatis serovars D and E. (E) Amplification of C. trachomatis serovar E groEL-1, groEL-2, and groEL-3 with redesigned primers for groEL-3.

Specificity of the peptide antisera

Monospecificity was achieved for anti-cHsp60-1 and anti-cHsp60-2 with purification of immunoglobulin G alone (Fig. 2.2A). Antiserum against cHsp60-3 initially exhibited faint cross-reactivity with Hsp60-1 that was removed by cross-adsorption against whole cells of arabinose-induced E. coli LMG194 (pJER516) expressing cHsp60-1. A control using an anti-histidine monoclonal antibody (Sigma-Genosys, The Woodlands, TX) was included.
Iron-responsiveness of cHsp60s

Next, the peptide antibodies were used to examine C. trachomatis-infected cells with or without 500 µM Desferal, which were exposed for 30 min and 1 h and 2 h, beginning at 36 hpi (Fig. 2.2B). Preliminary experiments using the standard 50 µM Desferal for 96 h resulted in induction of a persistence-like state (43); these initial experiments indicated that only cHsp60-2 responds to iron deprivation beginning at 36 hpi (data not shown). Therefore, we changed the strategy in a manner analogous to application of heat, cold, or acid shock in other bacteria. Figure 2.2B confirms that cHsp60-2 is the primary cHsp60 that responds to iron limitation. Notably, cHsp60-2 is a target of proteolysis during cell lysis even in the presence of protease inhibitors (several combinations of inhibitors were tested). The data also showed that cHsp-1 is strongly expressed, but there was little or no difference between the expression in the absence of Desferal and the expression in the presence of Desferal; cHsp60-3 expression was delayed, but again, there was little difference between expression in the absence of Desferal and the expression in the presence of Desferal.
Figure 2.2 Specificity of peptide antisera and response of cHsp60-2 to iron deprivation. (A) Samples used for Western blotting included uninfected HEC-1B cells, *E. coli* LMG194 alone, and arabinose-induced recombinants *E. coli* LMG194 (pJER516), *E. coli* LMG194 (pJER517), and *E. coli* LMG194, representing cHsp60-1, cHsp60-2, and cHsp60-3, respectively. An anti-His tag monoclonal was used as a control (upper left panel). (B) Samples included uninfected HEC-1B cells (control) (lanes C), cells mock exposed for 30 min and 1 h and 2 h, and cells exposed to Desferal for 30 min and 1 h and 2 h. One milligram of protein was loaded onto preparative gels (A), whereas 15 µg was loaded into each lane in panel B. Arrowheads indicate the position of cHsp60. The asterisk indicates the position of a major proteolytic product of cHsp60-2, and the circle indicates the position of a cross-reactive protein in HEC-1B cells.
Immunoelectron microscopy

To confirm that *C. trachomatis* cHsp60-2 is iron responsive, the final experiment involved examination of thin sections by immunoelectron microscopy. Multiple images were captured, saved as TIFF files, and printed; 2 µm square grids were used to enclose 10 to 15 randomly selected squares containing chlamydial reticulate bodies (RB) on prints of each sample, and the gold particles in each box were counted (Fig. 2.3).

Figure 2.3A, C, and E represent chlamydial RB in HEC-1B cells at 36 hpi (mock exposure); Figure 2.3B, D, and F illustrate RB at 36 hpi in cells that were exposed to 500µM Desferal for 1 h. Consistent with the results of the Western blot analyses, cHsp60-1 was strongly expressed, but there was not a significant difference between mock-exposed chlamydiae and chlamydiae exposed to Desferal (Fig. 2.3A and B); the number of particles in Figure 2.3A and B for cHsp60-1 were 19 (+/- 8) and 18(+/- 6), respectively. Likewise, for weakly expressed cHsp60-3 there was no difference between mock exposure and exposure to Desferal; the numbers of gold particles in both Fig. 2.3E and F were 5 (+/- 2). However, there was a significant difference (*P* < 0.001) between expression of cHsp60-2 between mock exposure and exposure to Desferal (Fig. 2.3C and D), as determined by Student’s two-tailed *t* test; the numbers of gold particles in Fig. 2.3C and D were 4 (+/- 2) and 10 (+/- 3), respectively.
Figure 2.3 Immunolabeling transmission electron microscopy showing the response of cHsp60-2 to iron limitation. *Chlamydia*-infected HEC-1B cells at 36 hpi were either not exposed to Desferal (A, C, and E) or exposed to 500 µM Desferal (B, D, and F) for 1 h and labeled using a 1:100 (vol/vol) dilution of anti-cHsp60-1 (A and B), a 1:20 (vol/vol) dilution of anti-cHsp60-2 (C and D), or a 1:40 (vol/vol) dilution of anti-cHsp60-3 (E and F). A 15nm gold-conjugated anti-rabbit serum (Amersham Biosciences) was used at a 1:200 (vol/vol) dilution for visualization. Bars = 0.5 µm. The arrowheads indicate gold particles.
Discussion

In this report we show conclusively that *C. trachomatis* serovar E Hsp60-2 is the primary cHsp60 that exhibits enhanced expression in response to iron restriction. In a larger context, the specificity of cHsp60-2 expression as a result of iron limitation indicates that the mechanisms for development of chlamydial persistence have unique signatures. This is an emerging concept in the study of chlamydial pathogenesis. The expression of cHsp60s, as determined by either protein expression or transcript analysis, has been examined in several models of persistence (2, 4, 17, 19, 22, 39).

Belland and colleagues (4) conducted a comprehensive microarray study of *C. trachomatis* serovar D transcription and compared standard growth and growth of IFN-γ-mediated persistent chlamydiae in HeLa 229 cells. None of the *groEL* transcripts varied significantly for the first 24 h; however, by 48 hpi, transcription of *groEL-1* increased 2.8-fold due to tryptophan depletion by IFN-γ. Tryptophan is an essential amino acid for *C. trachomatis*. A separate group of investigators examined transcription using quantitative real-time PCR for three distinct modes of persistence, exposure to IFN-γ, penicillin G, and iron depletion, in *C. psittaci* growing in HEp-2 cells (22). At 24 hpi, *groEL-1* was upregulated only in the penicillin G model of persistence; IFN-γ persistence actually showed a significant downregulation of *groEL-1*. Downregulation of *groEL-1* was also observed for 48 hpi for *C. psittaci* persistence induced by iron deprivation; *groEL-2* was not examined in this study. Using a different stress environment, Karunakaran and colleagues (28) examined transcription using a heat shock model. HeLa 229 cells were infected with *C. trachomatis* serovar D for 18 h and subsequently subjected to a 10-min heat pulse at 45°C. mRNA was quantified using a microarray
procedure, and the results showed that there was a >5-fold increase in groEL-1 transcripts; the quantities of the groEL-2 and groEL-3 transcripts did not change. In studies of protein expression, expression of C. trachomatis serovar A chSp60-1, as determined using Western blotting and an anti-chSp60-1 monoclonal antibody, was enhanced in an in vitro model of IFN-γ-mediated persistence (2). For C. pneumoniae chSp60-1, there was a twofold increase in expression at 48 hpi with the following three different models of persistence and/or stress: (i) IFN-γ exposure, (ii) iron deprivation, and (iii) heat shock (39).

Our findings are more consistent with results reported by Gerard and colleagues (20). These investigators quantified mRNA for each groEL homolog in C. trachomatis serovar K using real-time reverse transcription-PCR with the following systems: (i) active infection in HEp-2 cells, (ii) persistent infection in human monocytes, and (iii) synovial tissue from patients with Chlamydia-associated arthritis. In active HEp-2 cell infection, all groEL transcripts were present beginning at 8 hpi, and the levels increased throughout chlamydial development; groEL-3 was transcribed at the highest levels. In the monocyte persistence model, the levels of groEL-1 and groEL-3 transcripts were low, whereas the level of the groEL-2 transcripts increased threefold over 3 days as the organisms entered the persistent state. Findings for the synovial tissues also showed that the levels of groEL-2 transcripts were high. Comparisons with this model of C. trachomatis serovar K persistence in monocytes may not be entirely legitimate because our model involves C. trachomatis serovar E, a less invasive organism, in epithelial cells, but the observations are intriguing nonetheless.
From the standpoint of immunopathogenesis, the importance of cHsp60s in disease has been the subject of several excellent reviews (9, 13, 14, 16, 26, 30, 34, 40). Our previous work, performed with the antisera generated in this study, showed that cHsp60-2 and cHsp60-3 but not cHsp60-1 escape from chlamydial inclusions via vesicle eversion, a process that is exacerbated by exposure to azithromycin (21, 44). The vesicles are thought to interact with host cell antigen presentation and to contribute to the inflammatory response. Studies of heat shock proteins, in general, are being performed because heat shock proteins carry antigens and deliver peptides to the major histocompatibility complex, thus priming the adaptive immune response by inducing specific B and T cells in the absence of adjuvants (41). Heat shock proteins also participate in the innate immune response by stimulating the production of chemokines (41). In one study, cHsp60 serum antibodies were shown to serve to be the best predicting factor for tubal factor infertility (51). Mapping of cHsp60-1 peptide epitopes in human sera has been done (50, 54); it may be worthwhile to investigate whether peptides of cHsp60-2 and/or cHsp-3 contribute to the generation of specific immunoglobulins.

GroEL proteins are essential for bacterial growth and ensure that newly synthesized proteins are functional; expression of GroEL proteins increase in response to a variety of stresses, including heat shock and nutrient deprivation (56). Structural studies of cHsp60s have shown that although the primary amino acid sequences of cHsp60s differ from the primary amino acid sequences of other organisms, amino acid residues involved in binding polypeptides are conserved (28). It is also clear that cHsp60-1 is negatively regulated by the interaction of a negative regulator, HcrA, with a CIRCE
element in the operator regions of the groES-groEL-1 and dnaK operons; HrcA does not appear to regulate groEL-2 or groEL-3 (53). Although the results of studies of C. trachomatis serovar K persistence and synovial fluid support the hypothesis that there is regulation of chlamydial groEL-2 at the level of transcription, there appears to be no Fur/DcrA binding site in upstream sequences. There is only a partial Fur box approximately 300 bp downstream in C. trachomatis serovar E groEL-2; determining whether DcrA binds to groEL-2 sequences is part of a separate project in our laboratory. A likely alternative mechanism for enhanced expression of cHsp60-2 may involve small RNAs that regulate genes posttranscriptionally or by stabilization of mRNA; numerous iron-responsive proteins in other bacteria are known to be regulated in this fashion (25). Chlamydiae code for several small RNAs (5). Finally, the increased level of cHsp60-2 might also involve protein stability. For example, when E. coli GroEL is bound to an unfolded substrate in vitro, the complex remains stable at 25°C for more than 2 weeks; at 43°C, the half-life is 1.5 h (36).

Overall, the results of this study and our previous work (21, 43) strongly indicate that cHsp60-2 should be considered a potential mediator of immune-mediated damage, and they mechanistically indicate that not all modes of chlamydial persistence are identical.

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References


CHAPTER 3

EXAMINATION OF AN INDUCIBLE EXPRESSION SYSTEM FOR LIMITING IRON AVAILABILITY DURING CHLAMYDIA TRACHOMATIS INFECTION

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Abstract

The obligate intracellular bacterium *Chlamydia trachomatis* requires iron in order to complete its developmental cycle. Addition of an iron-chelating drug, Desferal (deferoxamine mesylate), to infected cell culture causes *Chlamydia* to enter persistence. Here, we explore the ability of a stably-transfected cell line with inducible over-expression of the eukaryotic iron efflux protein ferroportin to starve *C. trachomatis* serovar E for iron. Ferroportin-induced iron removal is perhaps a more direct method of removing iron from the intracellular compartment versus exposure to an exogenous chemical chelator. Following induction, ferroportin-green fluorescent protein (Fpn-GFP) was detected in the plasma membrane, and cells expressing Fpn-GFP remained viable throughout the timescale required for *Chlamydia* to complete its developmental cycle. Following Fpn-GFP induction in infected cells, chlamydial infectivity remained unchanged, indicating chlamydiae were not in persistence. Ferritin levels indicate only a small decrease in cellular iron following Fpn-GFP expression relative to cultures exposed to Desferal. These data indicate that expression of Fpn-GFP in chlamydiae-infected cells is not capable of reducing iron below the threshold concentration needed to cause chlamydiae to enter persistence.

Introduction

*Chlamydia trachomatis* serovar E, an obligate intracellular bacterium, is the leading cause of bacterial sexually transmitted disease in the United States. *C. trachomatis* causes chronic, often asymptomatic infections, and can lead to more serious sequelae including endometritis and pelvic inflammatory disease.

Members of the genus *Chlamydia* (and the reclassified *Chlamydophila*) exhibit a unique biphasic developmental cycle, switching between the intracellular, replicative reticulate body (RB) and the extracellular, metabolically inert elementary body (EB). The canonical developmental cycle is interrupted when chlamydiae enter persistence. While the formulation of a firm molecular definition is ongoing, chlamydial persistence is characterized by a non-culturable, but viable, state in which RB do not undergo maturation into EB. Cellular division ceases, RB become enlarged, and transcription and protein expression profiles are altered. Several inducers of persistence have been observed, the most studied being tryptophan deprivation via interferon-γ exposure and incubation in the presence of penicillin. Persistence is a phenomenon of medical importance because persistent chlamydiae are refractory to antibiotics and may upregulate destructive virulence factors.

Iron starvation has been observed to induce persistence in chlamydiae. Upon depletion of intracellular iron stores with the iron-chelating drug Desferal (defereroxamine mesylate), chlamydiae demonstrate delayed development, decreased infectivity of progeny EB, and the appearance of enlarged RB, which is reversible upon supplementing the medium with an iron source [1-4]. The existence of an iron-dependent repressor [5,6] and differential transcription and protein expression profiles
The reliance of chlamydiae on iron availability is not surprising, as iron is the most important metal in biological systems and is required by nearly all organisms studied. This ubiquitous reliance of life on iron has evolved because the chemical properties of iron allow it to function as a biocatalyst and electron carrier when incorporated into proteins. While life depends on iron, iron in an aerobic environment poses some difficulties. Ferric iron (the form predominating in an aerobic environment) has a solubility of only \(10^{-18}\) M at pH 7.0, and free iron can generate damaging free radicals via the Fenton reaction [9]. Thus, organisms tightly sequester iron with high-affinity binding proteins. Major iron-binding proteins in mammals include transferrin, lactoferrin, heme, and ferritin.

Because free iron is so scarce in the host, iron availability is a primary limitation to bacterial colonization, and many pathogens possess virulence factors regulated by iron availability, including siderophores, receptors for iron-binding proteins, iron transporters, and exotoxins [10,11].

It is clear that iron availability fluctuates in the endometrium of menstruating women [12-15] and, thus, chlamydiae inhabiting this environment experience a modulation in iron availability. The importance of characterizing iron-restricted chlamydiae lies not only in elucidating mechanisms with which chlamydiae acquire iron from within its intracellular environment but also in identification of iron deprivation-associated virulence factors involved in chlamydial pathogenesis.

Although Desferal creates an iron-restricted environment for chlamydiae, addition of an exogenous chemical may have pleiotropic effects on the host cell. In order to use
a more elegant and direct system for starving chlamydiae for iron, we explored the use of a cell line with inducible iron starvation via over-expression of the eukaryotic iron efflux protein ferroportin. Ferroportin (IREG1, MTP, Slc 11a3) is the major iron exporter of enterocytes, macrophages, and hepatocytes and functions to secrete intracellular iron into the bloodstream [16,17]. With the idea that cells over-expressing ferroportin could be used in chlamydial iron studies, we obtained a stably-transfected cell line with an inducible gene expressing a ferroportin-green fluorescent protein fusion (Fpn-GFP). This cell line was capable of generating a drop in intracellular iron level following induction of Fpn-GFP, measured by a decrease in ferritin, the major intracellular iron storage protein [18].

**Materials and Methods**

**Cell culture**

Human Embryonic Kidney cells (HEK293) and a stably transfected cell line expressing a ferroportin-green fluorescent protein fusion (HEK293-Fpn-GFP) were used to examine the effect of Fpn-GFP expression on chlamydial infectivity. In brief, the HEK293-Fpn-GFP cell line has two introduced elements: a stably expressed ecdysone receptor and an ecdysone inducible promoter upstream of the recombinant ferroportin gene fused with a GFP tag. The ecdysone analog, ponasterone A, is used to induce expression of Fpn-GFP.

HEK293 cells were maintained in Dulbecco’s Modified Essential Medium (D-MEM) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS), 2 mM GlutaMax (Gibco), 1 µg/ml ciprofloxacin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.
Medium for HEK293-Fpn-GFP cells is formulated as above, along with 0.4 mg/ml Zeocin (Invitrogen) and 1 mg/ml G418. Media supplemented with 10 μg/ml ponasterone A (A.G. Scientific, San Diego, CA) was used for induction of Fpn-GFP expression. HEK293, HEK293-Fpn-GFP, and C. trachomatis inoculum were confirmed to be free from Mycoplasma contamination by the VenorGem PCR test (Sigma-Aldrich).

Fluorescence microscopy

HEK293 and HEK293-Fpn-GFP cells were grown to subconfluency on 6-well Costar Transwell filter insert plates (Corning), and medium was replaced with medium plus or minus ponasterone. After 12, 24, or 36 hours (h), filter inserts were washed, cut out, and fixed with fresh 4% (wt/vol) paraformaldehyde for 30 minutes. After washing, filters were mounted on glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL). A FITC band pass filter was used to visualize GFP fluorescence on a Zeiss Axiovert S 100 microscope. Images were captured with a Zeiss AxioCam digital camera using AxioCam software v1.1.6. Identical exposure settings were used for all images (exposure time=1 second, contrast=3, resolution=1280x1024).

Live/dead cell viability assay

Eukaryotic cell death was measured via the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) at 12, 24, and 36 h following addition of ponasterone A. Controls included HEK293 cells without ponasterone. Host cells were grown in duplicate on Costar filters and processed according to the protocol. The previously described fluorescence microscope, camera, and capture software were used with a FITC long
pass filter. The percentage dead cells of the total cells was determined by counting six fields per filter.

**Chlamydial infectivity**

Cultures of HEK293 and HEK293-Fpn-GFP cells were grown in triplicate in 25 cm² tissue culture flasks (Corning). All antibiotics were removed 24 h prior to infection. *C. trachomatis* serovar E/UW-5/CX was used in all infections. The host cells were infected with *C. trachomatis* inoculum titrated to infect 80% of cells and were subsequently exposed to ponasterone at 36 hours post-infection (hpi). Controls consisted of chlamydiae-infected cells incubated in medium without ponasterone A. EB were harvested at 64 hpi, and were diluted 1:10 and used to infect fresh host cells grown on glass coverslips. Six fields per replicate were counted to calculate percent infectivity.

**TEM analysis of chlamydial morphology**

Samples were collected for thin-section transmission electron microscopy (TEM). Host cells were infected as above and immediately exposed to medium with or without ponasterone (HEK293-Fpn-GFP cells) or with or without 50 µM Desferal (HEK293 cells). At 48 hpi, cells were processed and embedded in Epon-Araldite 812 resin (Polysciences, Inc.) as described previously [19]. Gold thin sections were cut on an Ultracut T Microtome (Leica) and examined using a Tecnai 10 (FEI) transmission electron microscope operating at 60-80 kV.
Ferritin assay

The Spectro Ferritin Assay (Ramco Laboratories, Stafford, TX), an enzyme immunoassay procedure, was used to measure ferritin content of samples. HEK293-Fpn-GFP cells were incubated in flasks with medium with or without ponasterone A for 48 hours, and HEK293 cells were incubated in medium with 0, 5, 10, 25, or 50 µM Desferal (Sigma-Aldrich) for 48 hours. Following incubation, cell monolayers were washed three times in Dulbecco’s PBS and collected in lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 1% (vol/vol) Triton X-100, and a general protease inhibitor cocktail [Sigma-Aldrich]). Ten to 20 µg of protein was loaded into each well, in triplicate, in 96-well plates. The protein concentration of samples was determined by BCA assay (Pierce) and was used to standardize ferritin concentrations for variations in protein load. The entire experiment was repeated three times, with at least three replicates per run. A two-tailed Student’s t-test was used to determine significance for a change in ferritin concentration.

Results

Timeframe for Fpn-GFP expression

In order to coordinate Fpn-GFP expression with the chlamydial developmental cycle, it was necessary to establish the timecourse of Fpn-GFP expression in transfected HEK293 cells following addition of the inducer, ponasterone A. Fluorescence microscopy was used to detect the green fluorescence of Fpn-GFP following induction. Fluorescence was clearly visible beginning 12 hours following induction, reached a maximum at 24 hours, and was stable through 36 hours (Fig. 3.1).
Green fluorescence was also visible in the plasma membrane of the transfected cells, the correct final location of this protein. Addition of ponasterone A to the HEK293 cell line showed only background fluorescence similar to uninduced cells.

Figure 3.1  Fpn-GFP expression is visible by green fluorescence at the plasma membrane of induced HEK293-Fpn-GFP cells. HEK293-Fpn-GFP (A, B) and untransfected HEK293 (C, D) cells were incubated in medium containing the inducer ponasterone A for 12, 24, and 36 hours, along with a negative control lacking ponasterone A. Images were taken with fluorescence (A, C) and light (B, D) microscopy. Bar = 50 µm.
Host cell viability

The effect of Fpn-GFP expression on cell viability was unknown, and because healthy host cells are an obvious necessity for chlamydial development, cell viability for the remainder of the chlamydial developmental cycle following induction with ponasterone A needed to be verified. Incubation of either the HEK293 or the HEK293-Fpn-GFP cell lines with ponasterone A through 36 hours had no effect on viability (Fig. 3.2). The HEK293 cell population had an average of 6.5% dead cells (standard deviation [s.d.] 2.9) across all incubation times, while the HEK293-Fpn-GFP had an average of 28.5% dead cells (s.d. 5.4). Because the data showed there is no increase in cell death as a result of Fpn-GFP expression, this rules out the possibility that differences in infectivity after induction could be the result of host cell death.

Figure 3.2  Cell death following addition of ponasterone A as a function of time. HEK293 and HEK293-Fpn-GFP were incubated with 10 μg/ml ponasterone A for 12, 24, or 36 hours or in the absence of inducer. Cell death was calculated as a percentage of dead cells to total cells measured using a live/dead assay.
Infectivity of progeny EB harvested from induced versus uninduced cells

Decreased infectivity is a hallmark of chlamydial iron restriction. In order to evaluate the ability of Fpn-GFP expression to starve chlamydiae for iron, an infectivity assay was conducted to detect a change in infectivity in EB harvested from induced cells versus uninduced cells. Because iron restriction interrupts the normal chlamydial developmental cycle, it was hypothesized that a Fpn-GFP-mediated drop in intracellular iron would significantly decrease infectivity of EB; however, no drop in infectivity was seen between EB harvested from induced and uninduced HEK293-Fpn-GFP cells (Fig. 3.3). The only difference in infectivity was between EB harvested from HEK293-Fpn-GFP and HEK293 cells. Average infectivity of EB harvested from HEK293-Fpn-GFP (with and without addition of ponasterone A) was 25% (s.d. 6) versus 39% (s.d. 7) for HEK293.

Figure 3.3 Expression of Fpn-GFP in induced HEK293-Fpn-GFP infected cells does not reduce infectivity of progeny elementary bodies (EB). EB were harvested from infected host cells grown in the presence or absence of inducer, and infectivity was titrated. Chlamydial infectivity is expressed as a ratio to HEK293 minus inducer.
Analysis of chlamydial morphology

We also examined infected cells at 48 hours post infection (hpi) by thin section transmission electron microscopy. Persistent inclusions are marked by aberrant morphology, including enlarged RB and membranous blebbing [3]. Contrasting with infected HEK293 cells containing normal chlamydial inclusions (Fig. 3.4A), HEK293 cells exposed to Desferal showed the beginnings of this characteristic morphology (Fig. 3.4B,C,D). HEK293-Fpn-GFP cells plus (4E) or minus ponasterone A (4F) induction showed no notable differences in chlamydial morphology, although inclusions consistently appeared less robust compared to those in HEK293 cells, regardless of ponasterone exposure.
Figure 3.4 Transmission Electron Microscopy (TEM) visualization of chlamydial inclusions plus or minus exposure to 50 µM Desferal or with or without Fpn-GFP induction.  

(A) Typical mid-cycle inclusions in HEK293 cells.  
(B,C,D) Desferal-exposed HEK293 cells with inclusions exhibiting aberrant morphology, including enlarged reticulate bodies (RB), smaller inclusions, and membranous blebs. Image D shows a high magnification view of chlamydial envelope ghosts and membranous ghosts. 

Inclusions in HEK293-Fpn-GFP cells uninduced (E) and induced (F) show similar morphology.  
Bars: (A,B,D,E,F) 5 µm; (C) 2 µm.
Quantitation of intracellular ferritin following Desferal exposure or induction of Fpn-GFP

Because progeny EB from induced HEK293-Fpn-GFP cells did not show the anticipated drop in infectivity, we compared the change in ferritin level in cells exposed to Desferal, which is known to result in decreased chlamydial infectivity, to that of cells expressing Fpn-GFP. To assure Desferal does not induce toxicity in HEK293 cells, the Live/dead assay was used to verify there was no increase in cell death following 48 h exposure to 50 µM Desferal versus mock-exposed cells. Expression of ferritin, the primary intracellular iron storage protein, is tightly regulated by iron level, and is, therefore, a useful target for measuring relative iron levels.

HEK293 cells were exposed to various concentrations of Desferal for 48 hours. Ferritin content showed a dose-responsive drop in relation to Desferal concentration, falling from 28.2 ng/ml (in cells not exposed to Desferal) to 3.2 ng/ml (in cells exposed to 50 µM desferal (Fig. 3.5A). Each increase in Desferal concentration led to a statistically significant drop in ferritin level (p< 0.01). Following incubation of HEK293-Fpn-GFP cells for 48 hours, average ferritin levels dropped from 49.3 ng/ml (s.d. 10.4) to 38.2 ng/ml (s.d. 8.3), a change that is not statistically significant (Fig. 3.5B). It is notable that ferritin levels naturally fluctuate, which likely explain the disparity between overall ferritin levels between HEK293 and HEK293-Fpn-GFP cells.
Figure 3.5 Ferritin content of host cells following exposure to Desferal or expression of Fpn-GFP. (A) Ferritin levels of HEK293 cells following 48 h exposure to 0, 5, 10, 25, or 50 µM Desferal. Asterisk indicates a significant change (p < 0.01). (B) Ferritin levels of HEK293-Fpn-GFP cells, incubated plus or minus the inducing agent ponasterone A for 48 h.

Discussion

Because there is no established genetic system in *Chlamydia*, transfected eukaryotic host cells represent one way to use genetic tools with this intracellular bacterium. In many expression systems, tetracycline is used as the inducing agent. While the primary effect in other applications is a decreased risk for bacterial contamination of the culture, tetracycline is lethal for *Chlamydia*. In this study, we found
no decrease in chlamydial infectivity when the inducer ponasterone was incubated with infected HEK293 cells. Thus, ponasterone-inducible systems are a viable option for creating host genetic systems for *Chlamydia*, and this is the first study known to these authors to use a ponasterone-inducible system in *Chlamydia*.

Currently, the addition of the iron-chelating drug Desferal is the sole approach used to study iron starvation in *Chlamydia*, and this method has proved capable of causing chlamydiae to enter a persistent state. While Desferal is apparently effective in starving chlamydiae for iron, it would be advantageous to have an additional method for starving chlamydiae of iron, as the addition of a chemical agent may have pleiotropic effects on cell viability and protein expression. For example, Desferal was found to penetrate and cause direct damage to *Pneumocystis carinii*, independent of an iron effect, and is now being considered as a treatment for patients with *Pneumocystis* infections due to its toxic effect on the pathogen [20].

Over-expression of eukaryotic ferroportin was successfully used to starve a facultative intracellular pathogen of iron in at least one other study. In a transient ferroportin expression system, a drop in available iron was successful in limiting intracellular growth of *Salmonella enterica* [21].

The goal of this project was to evaluate use of the HEK293-Fpn-GFP cell line to create an iron-starved environment following infection with *Chlamydia*. Ultimately, we found that the Fpn-GFP system used in the present study was not capable of reducing iron below the threshold level chlamydiae require for normal development. Infectivity of EB harvested from induced host cells did not demonstrate a drop in infectivity, and, upon comparing ferritin levels in ponasterone-induced HEK293-Fpn-GFP cells with...
HEK293 cells exposed to Desferal, we found that Fpn-GFP induction did not lead to a statistically significant decrease in host cell ferritin.

It is not surprising that Desferal produces a greater drop in ferritin level than Fpn-GFP expression. Desferal is a high affinity intracellular iron chelator that can strip iron from all intracellular iron stores, ferritin being the major source. In iron studies where it is undesirable to cause the chlamydiae to enter persistence through complete iron restriction, the HEK293-Fpn-GFP cell line may be useful for generating a small decrease in intracellular iron.

The data from Chlosta et al. (2006) suggests either *Salmonella* is more sensitive to iron restriction than is *Chlamydia*, or the transient HeLa cell expression system used by Chlosta et al. created a greater drop in intracellular iron than did the HEK293-Fpn-GFP system used in the current study. Although Chlosta et al. detected a decrease in iron availability using a *Salmonella* iron-sensing promoter assay, they did not use a quantitative measure of intracellular iron. This would have allowed comparison of the relative efficacy of iron efflux in their Fpn expression system to that of the HEK293-Fpn-GFP system used in this study.

The HEK293-Fpn-GFP cells used in this study were previously used to elucidate the relationship between ferroportin and the peptide hormone hepcidin [18]. The experimental procedure used by Nemeth et al. (2004) included supplementing the medium with iron prior to Fpn-GFP expression. Expression of Fpn-GFP caused ferritin levels to return to the pre-supplementation level, a much greater decrease than seen in the current study. This may mean that the Fpn-GFP model requires a higher initial concentration of intracellular iron for effective efflux. Because the critical factor for
restricting iron from an intracellular pathogen is iron level rather than the change in iron level, supplementing the iron level prior to Fpn-GFP induction would have been ineffective in this study. Thus, we conclude inducing HEK293-Fpn-GFP is ineffective in producing an iron-restricted environment during C. trachomatis serovar E infection such that the chlamydial developmental cycle is altered.

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References


CHAPTER 4

IDENTIFICATION OF IRON RESPONSIVE PROTEINS EXPRESSED BY *CHLAMYDIA TRACHOMATIS* RETICULATE BODIES DURING INTRACELLULAR GROWTH

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Abstract

The obligate intracellular bacterium *Chlamydia trachomatis* serovar E is the most prevalent cause of bacterial sexually transmitted disease. With an established requirement for iron, the developmental cycle arrests at the intracellular reticulate body stage during iron restriction resulting in a phenomenon termed persistence. Persistence has implications in natural infections for altered expression of virulence factors and antigens in addition to a potential role in producing chronic infection. In this study, chlamydial proteins in iron-restricted infected HEC-1B were radiolabeled during mid-developmental cycle growth, harvested, and separated using two-dimensional polyacrylamide electrophoresis (2D-PAGE). Of ~250 radiolabeled protein species visualized, densitometric analysis revealed 25 proteins that increased in expression under iron restriction compared to iron-sufficient control samples; 10 protein species identified by mass spectrometry are involved in the oxidative damage response (alkyl hydroperoxide reductase, 6-phosphogluconlactonase, and acyl carrier protein synthase), transcription (RNA polymerase subunit alpha and transcription anti-termination factors NusA and NusG), protein modification (peptide deformylase and trigger factor), and virulence (CADD). Transcript-level expression patterns of *ahpC*, *devB*, *cadd*, *fabF*, and *ct538* were measured by quantitative PCR throughout the developmental cycle, and each gene examined demonstrated a significant but small mid-cycle increase in transcript level in iron-restricted cultures compared to iron-replete controls. Taken together, these data suggest that the primary response of chlamydiae to reduced iron availability is to increase expression of proteins involved in protection against oxidative damage via iron-catalyzed generation of reactive oxygen species and
adaptation to stress by increasing expression of transcriptional machinery and other stress responsive proteins.
Introduction

*Chlamydia trachomatis* is an obligate intracellular pathogen of human genital and ocular mucosal epithelia. *C. trachomatis* serovars D-K are the number one cause of bacterial sexually transmitted disease worldwide with 92 million new infections a year (62); in 2006, more than 1 million new cases were reported to the Centers for Disease Control and Prevention in the United States alone (10). While genital infections caused by the non-invasive serovars D-K are commonly asymptomatic and limited to the lower genital tract, an ascending infection marked by inflammation-induced damage can develop and lead to severe sequelae including endometritis and pelvic inflammatory disease.

*Chlamydiae* exhibit a biphasic developmental cycle marked by conversion between the extracellular, infectious form termed the elementary body (EB) and the intracellular, metabolically active reticulate body (RB). The developmental cycle can be interrupted by induction of persistence in which division of the RB ceases and conversion to the EB is prevented. Identified inducers of persistence include interferon-γ or penicillin exposure and nutrient deficiency. Although chromosomal replication continues, cellular division ceases, RB become enlarged, and transcription and protein expression profiles are altered; additionally, persistent chlamydiae are refractory to killing by some antibiotics and expression of antigens continues during the prolonged development cycle (21). Persistence has become an issue of interest in chlamydial pathogenesis due to the potential role in establishing chronic infection, which can result to the development of more severe disease outcomes.
Iron is required as a cofactor in numerous ubiquitous cellular metabolic processes in both bacterial and eukaryotic cells, including cytochromes, ribonucleotide reductase, RNA polymerase III, amino acid hydroxylases, superoxide dismutase, catalase, and peroxidase (4, 61). Free iron concentrations are kept low in biological systems because unbound iron catalyses free oxygen radicals through the Fenton reaction, which results in damage to lipids, proteins, and nucleic acids (32, 54, 61). In mammals, iron-binding proteins, such as transferrin, lactoferrin, and ferritin, chelate free iron, and transport of iron into cells is tightly regulated in response to intracellular iron level (28, 38). Additionally, control of intracellular iron level plays a role in innate immunity through general limitation of iron to prevent infection and increased limitation in response to infection (24, 31, 48). Indeed, all examined microbial organisms have an absolute requirement for iron except lactobacilli (5, 59) and *Borrelia burgdorferi* (40), which use alternative transition metal cofactors including cobalt and manganese.

Further, reduced iron availability is known to induce virulence factors in pathogens, such as the shiga toxin in *Shigella dysenteriae*, the shiga-like toxin in *E. coli*, exotoxin A in *Pseudomonas aeruginosa*, and diptheria toxin in *Corynebacterium diptheriae* (19, 29).

Iron has been shown to be necessary for normal chlamydial development, and iron restriction leads to chlamydial persistence. When intracellular iron is chelated by deferoxamine mesylate (Desferal) in vitro, the development cycle of *C. trachomatis* arrests at the RB stage and cell division ceases; also, enlarged aberrant chlamydial forms are visible by transmission electron microscopy (42). It is clear that iron restriction induces persistence rather than killing the chlamydiae, as these effects on the chlamydiae are reversible upon addition of an iron source. Similar observations in
response to iron restriction have been made in *Chlamydophila pneumoniae* (1, 15) and *Chlamydophila psittaci* (16).

The first investigation to describe the chlamydial requirement for iron identified several iron responsive protein species by separation of radiolabeled protein from purified EB (42). Notably, two proteins demonstrated in that study to be iron responsive have garnered interest due to their immunogenicity in *Chlamydia*-infected patients: cHsp60-2 (26) and YtgA (43). During menstruation, iron levels in endometrial cells fluctuate, and thus bacteria infecting these cells encounter an additional level of iron limitation (2, 3, 11, 25); therefore, the response of chlamydiae to reduced iron availability has dramatic health implications, especially considering the highest incidence of chlamydial infections occurs in females between 15 and 24 years of age (10).

While the chlamydial requirement for iron is well established, the identification of processes and pathways upregulated under iron restriction have been hindered by the lack of a direct system for genetic manipulation in *Chlamydia* and the lack of identifiable homologues to proteins involved in conserved iron responses in other bacteria, such as pathways for siderophore production. The goal of the present work was to identify proteins increasingly expressed in metabolically-active chlamydiae growing under iron restriction, followed by analysis of the corresponding transcripts for a subset of these proteins, in order to better elucidate the chlamydial response to iron restriction.
Materials and Methods

Host cells, chlamydiae, and iron restriction conditions

Human endometrial carcinoma epithelial HEC-1B cells (HTB-113, ATCC) were grown at 37°C with 5% CO₂ in Dulbecco’s Modified Essential Medium (D-MEM, Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (HyClone, ) and 2 mM GlutaMax (Gibco, Logan, UT) in 6-well plates.

For iron restriction, HEC-1B cells were incubated 24 hours prior to infection in medium containing 5% FBS plus or minus 50 µM of the iron chelator Desferal (deferoxamine mesylate, Sigma-Aldrich, St. Louis, MO). Cultures were incubated with a C. trachomatis serovar E inoculum titrated to infect 80% of host cells. After a 1 h adsorption step at 35°C, inoculated HEC-1B cells were returned to medium plus or minus Desferal containing 0.5 µg/ml cycloheximide, conditions that have previously been shown to induce persistence in C. trachomatis (42). In some protein analysis experiments, Percoll-purified EB, prepared using a standard protocol, were used. HEC-1B and C. trachomatis stocks were routinely tested for Mycoplasma contamination by the VenorGem PCR test (Sigma-Aldrich).

Host cell iron level determination via ferritin assay

The Spectro Ferritin enzyme immunoassay (Ramco Laboratories, Stafford, TX) was used to measure ferritin levels in infected HEC-1B protein samples collected at 24 hours post-infection (hpi), according to the manufacturer’s instructions. All samples were assayed in triplicate, and ferritin levels were normalized for variations in protein concentration (BCA assay,
A two-tailed Student’s t-test was used to determine significance for a change in ferritin concentration.

**Protein radiolabeling**

Protein radiolabeling was conducted in *Chlamydia*-infected HEC-1B cells between 22 and 24 hpi. Cultures were incubated in D-MEM lacking methionine and cysteine and supplemented with 5% FBS, 2 mM GlutaMax, 40 μg/ml cycloheximide, plus or minus 50 μM Desferal, and 100 μCi/ml $^{35}$S-labeled methionine and cysteine (Redivue PRO-MIX, GE Healthcare, Buckinghamshire, UK) for 2 h at 35°C. Following labeling, monolayers were washed with PBS and harvested in deionized water containing 1% CHAPS and a general protease inhibitor cocktail (Pierce) and stored at -20°C.

**Sample preparation and protein separation**

Radiolabeled protein samples collected from iron-restricted or normally grown *C. trachomatis*-infected HEC-1B cells were separated via two-dimensional polyacrylamide electrophoresis (2D-PAGE). Briefly, following desalting using Zeba Desalt spin columns (Pierce), samples were assayed for protein concentration by the BCA assay (Pierce) and the degree of radioisotope incorporation for each sample was determined by scintillation of TCA-precipitated protein from each sample. Protein samples were solubilized in Bio-Rad (Redman, WA) rehydration/sample buffer (8M urea, 50 mM DTT, 2% CHAPS, 0.2% Bio-Lyte 3/10 carrier ampholytes, 0.001% Bromophenol Blue). Protein loads for quantitative gels were equalized to one million cpm and 300 μg total
protein; preparative gels for mass spectrometry identification were loaded with 2 mg of total protein from unlabeled Percoll-purified EB and spiked with 250,000 cpm from a labeled protein sample to facilitate matching of EB protein spots with radiolabeled proteins of interest.

First dimensional isoelectric focusing (IEF) was conducted on a PROTEAN IEF cell with 17 cm isolated pH gradient (IPG) strips with a pH range of 4-7 (Bio-Rad). The pH range of 4-7 was selected because this range encompassed the majority of labeled proteins and allowed for better resolution compared to a wider range separation. The focusing conditions were as follows: 50 µA limit per IPG strip; 250 V maximum for 15 min, 10,000 V maximum for 2h, and 10,000 V maximum for 60,000 volt-hours.

Following IEF, strips were equilibrated for 20 min in equilibration buffer 1 (6 M urea, 2% SDS, 375 mM Tris-HCl, 2% DTT, 30% glycerol), followed by 20 min in equilibration buffer 2 (same formulation except 2.5% iodoacetamide substituted for DTT). Separation in the second dimension was done using PROTEAN II 10% Tris-HCl Ready Gels (Bio-Rad). Gels were run at 24 mA per gel for approximately 5 hours.

Gels for quantitative analysis were fixed in 25% methanol and 10% acetic acid, incubated with Amplify fluorographic reagent (GE Healthcare) for 30 minutes, vacuum dried, and exposed to phosphor screen for 14 days. Phosphor screens were scanned with the Bio-Rad Molecular Imager FX using Quantity One software (Bio-Rad). Preparative gels were fixed in 50% methanol and 7% acetic acid, stained with GelCode Blue Coomassie (Pierce), vacuum dried, and exposed to X-ray film for 14 days. Two replicate gels were run from each of four biological replicates.
Protein spot quantitation and identification

Protein spots were matched and analyzed for differential expression using Dymension software (Syngene, Fredrick, MD). Protein spot intensities were normalized to the total valid spot intensity for each gel. Proteins were deemed iron responsive if (i) the average density of the protein spot from iron-restricted samples showed a two-fold or greater increase over control samples and (ii) \( P \) value < 0.05 using Student’s two-way \( t \)-test.

Protein spots demonstrating a significant increase in density between the iron-replete and iron-restricted samples were excised from preparative gels and submitted for identification via liquid chromatography-tandem mass spectrometry (LC-MS/MS). True matches between detected peptides and protein identities were affirmed by a Mowse score indicating a \( P < 0.05 \) and by comparing predicted versus observed pI and molecular weight.

Quantitative PCR analysis

Transcript levels of \( ahpC, \) \( devB, \) \( cadd, \) \( fabF, \) and \( ct538 \) were measured using quantitative reverse transcription PCR (qRT-PCR). HEC-1B cells were grown in 6-well plates and iron-starved 24 hours prior to infection as described above. Following infection, monolayers were collected at 6, 12, 18, 24, 36, and 48 hpi, along with uninfected control samples. After washing with PBS, each monolayer was scraped with a rubber policeman into RLT lysis buffer (Qiagen, Valencia,CA), passed through a 20-gauge needle 10 times, and stored at -80°C. Samples were collected at least in triplicate on separate occasions. Collected samples were split into two aliquots for
separate RNA and DNA purifications. RNA was purified using the RNeasy Mini kit, including an on-column DNase step (Qiagen), and genomic DNA (gDNA) was purified in parallel using the QIAamp Blood DNA Mini kit (Qiagen). RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and RNA and DNA concentrations were determined by spectrophotometer reading at 260 nm. An equal amount of total RNA for each sample was reverse transcribed using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), following the conditions recommended by the manufacturer.

qPCR was conducted on samples in triplicate using the Bio-Rad iCycler. Reaction mixtures contained 1x Platinum Quantitative PCR Supermix-UDG (Invitrogen), 0.8x SYBR green (Cambrex BioScience, Rockland, ME), 5 mM MgCl₂, 300 nM concentrations of each primer, and 1 µl of sample in a total volume of 50 µl. Primer sequences for each gene are given in Table 4.1. The specificity of each primer set was confirmed by sequencing of the amplicons. To correct for loading variations, chlamydial chromosome copy numbers were normalized to total DNA. Assay validation and quantitative analyses were performed using a standard curve method as described previously (18). The amplification efficiencies of all assays ranged from 94% to 102%. Controls included (i) uninfected samples to demonstrate specificity of primers for chlamydial targets and (ii) RT minus samples, in which the reverse transcription was omitted in order to determine chlamydial gDNA contribution to the qPCR signal for corresponding RT plus samples. Chlamydial genome copy number for each experimental sample was determined by qPCR using the ct538 primer set against gDNA. Transcript levels are presented as copy number per chlamydial genome.
Table 4.1  *C. trachomatis*-specific oligonucleotide primers used in qPCR analyses.\(^a\)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Amplicon length</th>
</tr>
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<tbody>
<tr>
<td>ahpC</td>
<td>CCAGTTAGCTGGACAAACCATTCCG</td>
<td>CGTTCCATTGACGAGGAAATTGCGT</td>
<td>88 bp</td>
</tr>
<tr>
<td>fabF</td>
<td>GGAATTAGTGGAGTGCGAACA</td>
<td>GGATCAACTCGTCTAGCTTGT</td>
<td>128 bp</td>
</tr>
<tr>
<td>devB</td>
<td>ACGAAGATGTAAGCTGGAAAGTA</td>
<td>TGCCTATCCGATACGAAGATTG</td>
<td>98 bp</td>
</tr>
<tr>
<td>cadd</td>
<td>GACAACACAAACCTCTG</td>
<td>GTAGAGGCGCATCGAAGA</td>
<td>83 bp</td>
</tr>
<tr>
<td>ct538</td>
<td>GTTTGACAGCTACACCCTCTG</td>
<td>AACGTCTACGAAACCCTCA</td>
<td>91 bp</td>
</tr>
</tbody>
</table>

\(^a\)Primers were designed using the published *C. trachomatis* serovar D sequences.

Results

Host cell ferritin content

In order to demonstrate that intracellular iron levels decreased in response to Desferal exposure and, therefore, chlamydiae were restricted for iron, host cell ferritin levels were measured by a immunoassay. Measurement of ferritin content is a commonly used method to indirectly determine iron levels in eukaryotic cells because ferritin expression is regulated by cellular iron level. Ferritin content was measured in samples collected from mock- and Desferal-exposed HEC-1B cultures infected with *C. trachomatis* serovar E for 24 h (Fig. 4.1). Ferritin levels in Desferal-exposed samples averaged 119 ng/ml compared to 241 ng/ml in mock-exposed samples. This two-fold difference between Desferal-exposed and unexposed samples was statistically significant, thereby validating this model for iron restriction of *Chlamydia*-infected cells. Additionally, exposure of uninfected cells to Desferal as above resulted in a similar drop in ferritin level (data not shown).
Figure 4.1 Effect of Desferal exposure on *Chlamydia trachomatis* serovar E-infected HEC-1B ferritin levels. Ferritin levels were measured by enzyme immunoassay in infected cultures exposed to 50 µM Desferal or mock-exposed. Samples with a significant difference (*P* < 0.05) from mock-exposed host cells are marked by asterisks. The average of 4 independent experiments are presented (+/- SD).

Separation of chlamydial proteins and analysis of differential protein expression in response to iron availability

In order to identify differences in protein expression levels between chlamydiae growing in iron-replete or restricted conditions, *C. trachomatis* serovar E-infected cells, growing in the presence or absence of the iron chelator Desferal, were pulse labeled at 22-24 hpi. This labeling window was chosen because it is during logarithmic growth (21), which coincides with an increased demand for micronutrients such as iron. Host cells were starved for iron 24 h prior to infection to ensure chlamydiae did not obtain iron stores early in infection. At 22 hpi, medium for each sample was switched to the radiolabeling media, the infection was allowed to continue for 2 hours of labeling, and protein samples were collected, processed, and separated via 2D-PAGE. This
methodology for generation of proteomic maps in *C. trachomatis* were adapted from the pioneering chlamydial proteomic studies by Vandahl, Birkelund, and Christiansen (57).

Approximately 250 protein spots were visible via radiography (Fig. 4.2). Using Dymension 2D-PAGE analysis software, 25 proteins were determined to increase in expression during iron-restricted growth relative to iron-sufficient growth, with a threshold of ≥2-fold expression difference and a *P* < 0.05 given by Student's two-way *t*-test (Fig. 4.3).

![Figure 4.2](image)

Figure 4.2  Representative two-dimensional electrophoretic maps of *C. trachomatis* proteins radiolabeled 22-24 hpi under iron-sufficient (A) and iron-restricted (B) growth conditions. Proteins were separated on 17 cm immobilized pH gradients (pI 4-7) in the first dimension and by SDS-PAGE (10%) in the second dimension. Identified iron responsive protein spots are labeled with arrows. Isoelectric point (pI) and molecular weight are indicated.
Identification of iron responsive proteins by mass spectrometry

Protein spots from preparative 2D gels containing purified EB protein were matched to corresponding spots on radiographs, excised, and submitted for identification via LC-MS/MS. Of the 25 spots found to be more highly expressed in iron-restricted cultures, 10 proteins were expressed at a high enough level to allow mass spectrometric identification (Table 4.2; Fig. 3).
### Table 4.2  Identified *C. trachomatis* serovar E iron responsive proteins

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>ORF</th>
<th>Protein name</th>
<th>Description</th>
<th>UniProt accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold increase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pI / MW predicted&lt;sup&gt;c&lt;/sup&gt;</th>
<th>pI / MW observed&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism/</td>
<td>CT603</td>
<td>AhpC</td>
<td>alkyl hydroperoxide reductase (thio-specific antioxidant peroxidase)</td>
<td>O84608</td>
<td>6.4</td>
<td>4.8 / 22 kDa</td>
<td>4.8 / 22 kDa</td>
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<tr>
<td>biosynthesis</td>
<td>CT186</td>
<td>DevB</td>
<td>6-phosphogluconolactonase</td>
<td>O84189</td>
<td>2.1</td>
<td>5.3 / 29 kDa</td>
<td>5.3 / 28 kDa</td>
</tr>
<tr>
<td></td>
<td>CT770</td>
<td>FabF</td>
<td>acyl carrier protein synthase</td>
<td>O84775</td>
<td>2.6</td>
<td>5.4 / 45 kDa</td>
<td>5.6 / 42 kDa</td>
</tr>
<tr>
<td></td>
<td>CT353</td>
<td>Def</td>
<td>peptide deformylase</td>
<td>O84357</td>
<td>3.6</td>
<td>5.7 / 21 kDa</td>
<td>5.8 / 21 kDa</td>
</tr>
<tr>
<td></td>
<td>CT707</td>
<td>RopA</td>
<td>trigger factor (peptidyl prolyl cis-trans isomerase)</td>
<td>O84713</td>
<td>3.7</td>
<td>5.0 / 50 kDa</td>
<td>5.2 / 50 kDa</td>
</tr>
<tr>
<td>Protein</td>
<td>CT507</td>
<td>RpoA</td>
<td>RNA polymerase subunit alpha</td>
<td>Q46449</td>
<td>3.1</td>
<td>5.3 / 42 kDa</td>
<td>5.4 / 40 kDa</td>
</tr>
<tr>
<td>modification</td>
<td>CT097</td>
<td>NusA</td>
<td>transcription anti-termination factor</td>
<td>O84099</td>
<td>2.1</td>
<td>5.2 / 49 kDa</td>
<td>5.5 / 50 kDa</td>
</tr>
<tr>
<td></td>
<td>CT320</td>
<td>NusG</td>
<td>transcription anti-termination factor</td>
<td>O84322</td>
<td>5.9</td>
<td>5.3 / 21 kDa</td>
<td>5.3 / 18 kDa</td>
</tr>
<tr>
<td>Virulence</td>
<td>CT610</td>
<td>CADD</td>
<td><em>Chlamydia</em> protein Associating with Death Domains</td>
<td>O84616</td>
<td>3.4</td>
<td>4.9 / 27 kDa</td>
<td>5.0 / 26 kDa</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>CT538</td>
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<td>hypothetical protein</td>
<td>O84543</td>
<td>2.9</td>
<td>5.2 / 27 kDa</td>
<td>5.3 / 27 kDa</td>
</tr>
</tbody>
</table>

<sup>a</sup>Accession number for *C. trachomatis* serovar D proteins.

<sup>b</sup>Fold increase in spot volume in iron-restricted versus iron-sufficient growth conditions as determined by analysis with Dymension 2D software. Average value from 2 replicate gels from each of 4 independent experiments.

<sup>c</sup>Calculated using ExPASy Compute pI/MW tool.

Interestingly, 9 of these iron responsive proteins have been assigned a putative function by homology to genes known in other bacteria. AhpC (alkyl hydroperoxide reductase subunit C), a protein involved in protecting the cell from oxidative damage, showed a 6.4-fold increase in expression by iron-restricted versus iron-replete chlamydiae, the highest degree of iron responsiveness of the proteins identified in this study. Two proteins involved in biosynthesis pathways were also identified as iron responsive, DevB (6-phosphogluconolactonase) and FabF (3-oxoacyl-[acyl-carrier-protein] synthase), for which a 2.1-fold and a 2.6-fold increase in expression was found, respectively, in response to iron restriction.
Two chlamydial proteins with putative roles related to protein modification, i.e. RopA (trigger factor or peptidyl prolyl cis-trans isomerase) and Def (peptide deformylase), were found to be iron responsive, with 3.7-fold and 3.8-fold increased levels in iron-starved samples compared to control samples. Three proteins involved in transcription were also identified. Indeed, in response to iron restriction, RpoA (RNA polymerase subunit alpha) increased 3.1-fold, while the transcription elongation proteins NusA and NusG increased 2.1-fold and 5.9-fold, respectively. In addition, putative virulence factor CADD (Chlamydia protein associating with death domains) expression was found to increase 3.4-fold. Lastly, the uncharacterized protein CT538 demonstrated a 2.9-fold increase in expression.

Transcriptional analysis of selected genes

qPCR was used to examine transcript levels of a subset of the identified iron-responsive proteins, i.e. *ahpC*, *devB*, *fabF*, *cadd*, and *ct538*. These transcripts were selected due to their potential role in the oxidative response (*ahpC*, *devB*, and *fabF*), the putative role of *cadd* in chlamydial virulence, and the fact that *ct538* is uncharacterized. Transcript levels were measured at 6, 12, 18, 24, 36, and 48 hpi in iron-restricted and in mock-exposed control chlamydiae using qPCR. Analysis of transcript levels normalized to chlamydial chromosome copy number revealed that the transcript levels for all five genes examined peaked at 18 hpi (Fig. 4.4). It is not surprising that the level of these transcripts would be highest mid-cycle, considering the relatively high expression of these proteins during radiolabeling, 22-24 hpi.
Figure 4.4. Chlamydial transcript levels at selected times throughout the developmental cycle from infected cultures grown under normal or reduced iron conditions. Times at which transcripts are significantly increased in iron-restricted (D+) versus iron-sufficient (noD) growth are marked by asterisk.

Mid-cycle levels increased for each transcript in samples from iron-restricted chlamydiae compared to samples from iron-replete chlamydiae at either 18 or 24 hpi ($P < 0.05$; Fig. 4.4). Transcript levels of cadd reached a higher peak level at 18 hpi in
samples from iron-restricted cultures compared to iron-replete cultures. In contrast, *ahpC*, *devB*, *fabF*, and *ct538* transcript levels peaked at similar levels under both growth conditions at 18 hpi; however, in samples from iron-restricted chlamydiae, higher levels of these transcripts were detected at 24 hpi compared to samples from iron-replete chlamydiae. Because protein expression differences between iron-restricted and iron-replete samples were higher than transcript level differences, the duration of heightened transcript level may be more important for regulation of protein expression under iron restriction than changes in maximal transcript levels.

**Discussion**

The goal of this study was to identify chlamydial proteins showing increased expression during intracellular growth in iron-restricted compared to iron-replete host cells. Little is known about chlamydial responses to altered iron availability, aside from the requirement for sufficient iron. In addition to helping to characterize the chlamydial iron stimulon, identifying proteins increasingly expressed during iron restriction also defines modulation of protein expression during chlamydial persistence. In the current study, 10 *C. trachomatis* serovar E iron responsive proteins were identified by 2D-PAGE coupled with mass spectrometry.

The primary iron regulatory protein in Gram-negative bacteria is the transcriptional repressor Fur, which acts via binding, in the presence iron, to specific regions in the promoter region of regulated genes termed Fur boxes. Of the approximately 90 Fur-regulated genes in *Escherichia coli*, 60 of these encode siderophore-related proteins, 13 encode virulence factors and toxins, and 18 encode
proteins involved in iron storage and the oxidative stress response (19). In the present study, *C. trachomatis* iron responsive proteins involved in oxidative stress response and virulence were identified, but none appear to be directly involved in iron acquisition. This may be surprising considering the obvious necessity for upregulating iron acquisition during iron limitation; however, no genes encoding conserved iron acquisition mechanisms, such as siderophores, were identified following the sequencing of the chlamydial genome (50), although a putative component of an ABC metal transporter, YtgA, has been identified in *Chlamydia* (43).

The lack of obvious iron-uptake system homologs in *Chlamydia* suggests that these organisms (i) possess conserved iron acquisition genes with highly divergent sequences or (ii) use alternative or novel iron acquisition systems. The pathways by which intracellular organisms acquire iron are much less defined compared to those used by extracellular bacteria, but it appears to involve alternative systems. For example, iron reductases are believed to play a role in iron acquisition by *Listeria monocytogenes* through the removal and solubilization of iron from iron binding proteins like heme, ferritin, and transferrin (12). Interestingly, a recent study has shown that reduced expression of transferrin or Rab11, a protein involved in trafficking of transferrin-positive vesicles, leads to a decrease in the production of infectious EB (20).

An analog to Fur, termed DcrA, was identified in *C. trachomatis* serovar E, and has shown the ability to bind *E. coli* Fur boxes (63). Putative DcrA binding sites have been identified in the chlamydial genome by binding of purified *E. coli* Fur protein (41). Notably, binding sites for DcrA have been identified upstream of genes encoding two proteins found to be iron responsive in the current study, AhpC and FabF.
Mukhopadhyay et al. (35) conducted a proteomic analysis of *Chlamydothila pneumoniae* that examined protein expression modulation by three inducers of persistence: iron restriction, heat shock, and interferon-γ exposure. Proteins increasingly expressed under iron restriction but not following heat shock or interferon-gamma exposure were involved in cofactor biosynthesis, cellular processes, and translation, including one protein identified as iron responsive in the current study, AhpC. In another study, interferon gamma exposure was shown to increase expression of AhpC (36) and RpoA (34) in *C. pneumoniae* and to upregulate transcription of *ahpC* in *C. trachomatis* (6). Interferon gamma has pleiotropic effects on the host cell including tryptophan limitation, synthesis of nitric oxide species, and iron restriction (23). While these studies may indicate AhpC and RpoA are increasingly expressed as a part of a non-specific stress response, it is possible that expression of these proteins is induced by the iron limitation component of the host cell’s response to interferon-γ.

The close connection between the response to iron availability and oxidative stress in bacteria and eukaryotes is well established. AhpC belongs to a family of antioxidant proteins that are responsible for the reduction of peroxides and is expressed in response to oxidative stress. In *E. coli*, expression of AhpC is controlled by the regulator of oxidative stress OxyR (64). *Bacillus subtilis* and *Campylobacter jejuni* possess a Fur homologue named PerR that regulates genes involved in the oxidative-stress response, including *ahpC* (8, 55). Furthermore, decreased iron availability has been shown to modulate expression of *ahpC* transcription and/or AhpC protein expression in numerous organisms, including *Helicobacter pylori* (33), *Corynebacterium diptheriae* (52), *Campylobacter jejuni* (56), and *Francisella tularensis*.
Two chlamydial proteins involved in biosynthetic pathways, DevB and FabF, were found to be iron responsive in the present study. DevB catalyzes the hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate, the second step of the pentose phosphate pathway (PPP), while FabF catalyzes the chain-elongation step of type II fatty-acid biosynthesis. Like AhpC, the PPP is also involved in the response to oxidative conditions, through the generation of NADPH, which serves as an electron source for reductases and antioxidants. Another PPP enzyme, glucose-6-phosphate dehydrogenase (G6PD), is regulated by the *E. coli* _soxR_ system involved in oxidative defense (53). G6PD mutants of *E. coli* (37), *Salmonella typhimurium* (30), and mouse stem cells (39) have extreme sensitivity to oxidative damage. In addition to a role in oxidative protection, the PPP may be more important for generation of energy under iron restriction because iron-containing species such as cytochromes are required for the TCA cycle.

FabF is also likely iron responsive due to a role in oxidative damage defense because increased expression of other enzymes involved in fatty acid biosynthesis appear to be involved in the iron restriction and oxidative responses in other bacteria. For example, FabH expression is involved in hydrogen peroxide resistance and is co-regulated with siderophore production in *Pseudomonas syringae* (51), while *Francisella tularensis* (27) and *Campylobacter jejuni* (22) increase expression of FabG under iron restriction.

Two proteins responsible for protein modification were found to be iron responsive in this study: RopA and Def. RopA is a chaperone involved in protein folding.
in conjunction with DnaK (14) and appears to have a role during oxidative and other stresses. *Streptococcus mutans* upregulates transcription of *ropA* under low iron (44), and *ropA* mutants are less tolerant to acid or oxidative stress than the wild type strain (60). RopA expression may be required for correct processing of other iron responsive proteins. In *E. coli* lacking RopA and DnaK, numerous proteins were incorrectly folded, including two proteins identified as iron responsive in the current study, FabF and RpoA, as well as 6-phosphogluconate dehydrogenase, an enzyme that functions with DevB in the PPP (58).

Def catalyzes the removal of the formyl group from the N-terminal L-methionine of nascent peptides. Altered expression of Def could serve as a regulatory mechanism for substrate proteins, as formylated proteins are inactive or because it is an iron-requiring enzyme. The transcription of *def* has been shown to increase during iron restriction of *Francisella tularensis* (13). In a *Staphylococcus aureus* study, induction of the TCA cycle resulted in increased secretion of formylated δ-toxin relative to the typical deformylated form (47), which was hypothesized to be a result of the increased consumption of iron by enzymes required for the TCA cycle because Def has a di-iron center that is required for activity. Thus, increased expression of Def by iron-restricted chlamydiae may reflect the production of inactive enzyme, which could activate a feedback loop to increase expression. Additionally, Def activity may play a role in the immunological response to chlamydial infection, as formylated protein has been shown to be a chemoattractant to neutrophils (45).

Three proteins required for transcription were identified. RpoA, as part of RNA polymerase, catalyzes the transcription of DNA into RNA. NusA and NusG are
transcription elongation proteins that bind to RNA polymerase and the transcription terminator Rho in order to regulate transcriptional activity by modulating pausing, arrest, termination, and anti-termination of RNA polymerase (7). Increased expression of these transcription factors may be involved in regulating transcription under iron restrictive or oxidative stress, as numerous transcripts encoding proteins involved in transcription and translation, including RpoA, were upregulated following iron restriction of *Neisseria meningitidis* (17) and *Francisella tularensis* (27).

The finding that CADD is iron responsive is significant because it is involved in induction of host cell death via interaction with the death domains of Fas (49) and possibly enzymatic action as a redox protein (46). CADD is one of several identified factors chlamydiae express that is involved in induction or inhibition of host cell death. Inhibition of apoptosis has been hypothesized to promote chronic infections (i.e. persistence), while activation of cell death is thought to be the chlamydial strategy to prevent a dying host cell from entering necrosis, which is pro-inflammatory (9). In the case of iron restriction, chlamydiae may sense this as a sign of a dying host cell or a cellular response to interferon-γ. Notably, a microarray study showed *cadd* transcript levels of *C. trachomatis* serovar D increased in response to interferon-γ exposure by 2.85-fold (6).

In summary, the analysis of chlamydial protein expression alterations during iron restriction at mid developmental cycle growth shows an increase in expression of proteins involved in protection against oxidative damage and response to stress by increasing expression of transcriptional machinery and other stress responsive proteins. Further, this has been matched with an increased level of transcript encoding five of the
identified proteins during mid-developmental cycle. While the mechanisms used by chlamydiae to obtain iron remain elusive, the concept that chlamydiae have a reliance on iron availability is clearly indicated by the demonstrated metabolic alterations associated with iron restriction.

**Acknowledgements**

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Intracellular growth provides a protected environment for pathogens that have evolved mechanisms to invade the host cell; however, obtaining required nutrients and avoiding cellular defense mechanisms provides challenges to intracellular survival. Intracellular iron levels are carefully controlled in eukaryotic cells due to the reactive nature of free iron, and, furthermore, detected infections activate pathways to further restrict available iron. Chlamydiae require iron in order to complete a productive development cycle because reduced iron availability induces persistence. Extending the knowledge of how Chlamydia respond to iron limitation is critical because of the potential role in pathogenesis as well as to contribute to the understanding of the response of intracellular bacteria to iron restriction.

The focus of this lab has been characterizing the response of C. trachomatis serovar E to iron, including protein expression alterations (Raulston 1997; LaRue and others 2007), transcriptional response and regulation (Wyllie and Raulston 2001; Rau and others 2005), and potential iron acquisition pathways (Raulston and others 2007). In this dissertation, evidence has been presented that (i) chlamydiae increase expression of cHsp60-2 in response to iron restriction, (ii) Desferal-exposure of host cells, but not induction of a recombinant ferroportin protein, resulted in a drop in the intracellular iron level sufficient to induce the altered, chlamydial persistent state, and (iii) 10 chlamydial proteins showing increased expression during iron-restricted growth.
have been identified, and an increased transcript level of a subset of genes encoding these proteins was also observed under iron restriction.

The first study investigated the expression of each of three chlamydial heat shock protein 60 homologs (cHsp60-1, cHsp60-2, and cHsp60-3) during iron restriction of *Chlamydia*-infected, polarized human endometrial epithelial cells using peptide antisera generated to be specific for each (LaRue and others 2007). Chlamydial Hsp60 has a long history of being implicated in chlamydial pathogenesis through the correlation of patients developing more severe disease outcomes with the presence of antisera reactivity against the protein; thus, the finding that cHsp60 exhibited an increase in expression following iron restriction was intriguing (Raulston 1997). The sequencing of the chlamydial genome identified three cHsp60 homologs (Stephens and others 1998), which begged the questions as to whether each homolog had a different function and which homolog or homologs were iron responsive.

Gene fragments specific for each gene were PCR-amplified and cloned into a pBAD vector for expression in *Escherichia coli* (Figure 2.1), and antisera were generated by inoculation of rabbits with these peptides. To generate protein samples for Western blot, chlamydiae were grown in host cells exposed to Desferal or mock-exposed control. For this study, a short exposure to a high concentration of Desferal was used (500 μM, compared to 50 μM, for 30 min to 2 h prior to collecting protein samples), in order to provide an iron restriction “shock” that may be more appropriate for analysis of these heat shock proteins.

Each antiserum was confirmed to be specific for the correct cHsp60 homolog (Figure 2.2A). Western blots labeled with polyclonal monospecific antibodies generated
against each cHsp60 homolog revealed that while cHsp60-1 and Hsp60-3 expression remained constant, a dramatic increase in expression of cHsp60-2 occurred 30 min following Desferal exposure, an effect that diminished at 1 and 2 h following exposure (Figure 2.2B). These results were confirmed by immunoelectron microscopy of infected host cells. Increased second affinity labeling of antibody-conjugated gold particles was observed in samples probed with antiserum against cHsp60-2 exposed to Desferal 1 h compared to the mock-exposed control, while antisera against cHsp60-1 and cHsp60-3 showed no change between Desferal and mock-exposed growth (Figure 2.3). Thus, this paper demonstrates that expression of cHsp60-2 increases under iron-restricted growth.

In the second study, a novel model for studying iron restriction in Chlamydia was investigated, at the suggestion of a colleague, Dr. Jerry Kaplan. While Desferal exposure has been shown to induce iron restriction in chlamydiae (Raulston 1997), exposure of host cells and bacteria to this type of exogenous chemical can result in pleiotropic effects, perhaps undetected, on the model system. In the case of Desferal, it is known to have toxic effects on Pneumocystis carinii independent of an iron effect (Clarkson and others 2001). Thus, a project to develop an additional and complimentary iron restriction model was undertaken.

We obtained from Dr. Kaplan HEK293 cells stably transfected with a ferroportin-green fluorescent protein fusion (Fpn-GFP), which is inducible by addition of the insect hormone analog ponasterone (Nemeth and others 2004). Ferroportin is critical for iron homeostasis in mammals, as it is the only known cellular iron efflux protein (Ganz 2005).
In order to determine the timing of Fpn-GFP expression following induction, a fluorescence time course was conducted. Expression of Fpn-GFP, marked by the appearance of fluorescence, appeared at 12 h after induction, intensified at 24 h, and persisted through 36h -- the last time examined (Figure 3.1). Additionally, cell viability was monitored in order to verify that induction of Fpn-GFP did not increase cell death, which would influence infectivity determination (Figure 3.2). Induction did not increase cell death, although the Fpn-GFP-transfected cell line showed a higher rate of death than the untransfected control cell line.

Iron-restricted chlamydiae enter persistence that prevents maturation of RB into EB. If the expression of Fpn-GFP in infected cells caused chlamydiae to be restricted for iron, there should be a decreased level of infectivity from progeny EB. Unfortunately, induction of Fpn-GFP in infected cells did not reduce the infectivity of progeny EB and, thus, chlamydiae did not enter persistence (Figure 3.3). Additionally, transmission electron microscopy revealed Fpn-GFP induction did not lead to the appearance of aberrant RB following Fpn-GFP expression (Figure 3.4), as did Desferal exposure.

Because expression of the Fpn-GFP was confirmed by fluorescence microscopy, we investigated whether or not iron levels were altered by Fpn-GFP expression by measuring levels of ferritin. Ferritin is the primary eukaryotic intracellular iron storage protein, and its levels are tightly regulated by iron level, making it a convenient and effective target for assaying relative cellular iron levels in eukaryotes. Contrary to the observation of the Kaplan laboratory (Nemeth and others 2004), we saw no statistically significant decrease in iron level following Fpn-GFP induction as measured by ferritin
expression (Figure 3.5B), while, as expected, Desferal-exposure led to a dose-dependent decrease in ferritin levels (Figure 3.5A). While it is possible that the Fpn-GFP-transfected cell line lost the ability to express functional protein during cell passage (while retaining GFP fluorescence), the most likely explanation for our differing results is the use of iron citrate by the Kaplan laboratory to pre-load host cells with iron prior to inducing Fpn-GFP expression. The recombinant Fpn-GFP protein may only function efficiently when intracellular iron levels are high; however, pre-loading host cells with iron is not a biologically relevant model for studying iron restriction of chlamydiae. In a recent publication, the Kaplan laboratory demonstrated a decreased ability of chlamydiae to multiply in cells expressing Fpn-GFP, again following pre-incubation of host cells with ferric citrate (Paradkar and others 2008). While not their intention, Paradkar and others have demonstrated that increased iron availability (via ferric citrate pre-incubation) is beneficial to chlamydial replication compared to basal iron levels (reached through induction of Fpn-GFP).

The third study used proteomic tools to identify chlamydial proteins showing increased expression during intracellular growth in iron-restricted host cells, followed by analysis of transcripts encoding a subset of these proteins. The model for iron restriction of chlamydiae consisted of pre-starving HEC-1B cells with 50 µM Desferal 24 h prior to infection and maintaining this exposure until lysates were collected. Although iron-responsive proteins have been identified in C. trachomatis by a proteomic approach previously, the goals and approach of the current study differs. The primary objective of Raulston (1997) was to establish that chlamydiae require iron, and one way in which this was demonstrated was through identifying that alterations could be found in the
5proteome following iron restriction. EB were Renografin-purified from iron restricted cultures and the end point levels of proteins radiolabeled throughout the developmental cycle were examined. The current study’s approach is more focused on protein expression profile during intracellular growth; RB proteins were radiolabeled over a limited duration and harvested during mid-developmental cycle to preserve expression profiles of proteins expressed specifically during iron restricted growth.

In order to demonstrate Desferal-exposed chlamydiae were indeed starved for iron, the ferritin assay was again used to investigate the iron level of the model system. Analysis revealed an approximately 50% decrease in ferritin level following incubation in Desferal-exposed HEC-1B cells, validating this model for restricted iron to chlamydiae (Figure 4.1).

To generate protein expression profiles of chlamydiae grown in iron-restricted and iron-replete host cells, chlamydial proteins were metabolically radiolabeled from 22 to 24 hpi. The timing of radiolabeling is a critical consideration because only de novo protein synthesis is detected and protein expression profiles should fluctuate throughout the developmental cycle according to the stage-specific needs of chlamydiae. This timeframe was selected because it is during exponential growth of chlamydiae, when the need for iron would be greatest. Furthermore, a shorter labeling period is preferable, because longer incubations tend to allow greater eukaryotic incorporation of radiolabeled amino acids, even in the presence of the eukaryotic protein synthesis inhibitor cycloheximide.

Following collection and processing of protein samples, proteins were separated by 2D-PAGE, gels were dried and exposed to phosphor screens, and the screens were
scanned by phosphorimager. Figure 4.2 shows the protein profiles generated under iron-replete and iron-restricted conditions. Dymension 2D analysis software was used to match protein spots across replicates and to measure spot volumes. Fold-change of iron responsive proteins was determined by analysis of two replicate gels from each of four independent experiments.

In order to obtain protein identities for species showing an increase in expression following iron-restricted growth, spots were excised from preparative gels and submitted for mass spectrometric analysis. Preparative gels consisted of a reduced amount of radiolabeled sample along with a maximal load (2-3 mg of total protein per gel) of unlabeled Percoll-purified EB. Because mid cycle-expressed proteins would be most highly expressed in RB, purified RB would be the most logical starting material, however, the lower purity of Renografin-purified RB limited the amount of chlamydial protein that could be gel separated due to the relatively high abundance of eukaryotic protein. The radiolabeled and purified EB sample was separated by 2D-PAGE, Coomassie stained, dried, and exposed to x-ray film. Coomassie-stained spots corresponding to spots on x-ray film were excised and sent to the Columbia University Protein Core Facility for identification by liquid chromatography-tandem mass spectrometry.

Of the 25 chlamydial proteins shown to increase in expression during iron-restricted growth, 10 proteins were identified by mass spectrometry (Table 4.2). Mowse scores, which indicate the confidence level that peptides matching the identified protein is not random, confirmed the protein identification for each sample, and these results were further checked by matching to predicted isoelectric points and molecular weights
for these proteins. Figure 4.3 shows images of each protein from a representative 2D gel along with the normalized spot volumes as provided by Dymension.

In order to investigate changes in mRNA levels in the chlamydial response to iron restriction, specific qPCR primers were designed for transcripts encoding five of the proteins identified as iron responsive in this study: AhpC, DevB, FabF, CADD, and CT538 (Table 4.1). The genes *ahpC*, *devB*, and *fabF* were selected because of their iron-responsiveness in other bacteria and their involvement with the oxidative stress response. Transcription of *cadd* was analyzed because its putative role in chlamydial virulence through modulation of host cell death, and *ct538* was examined because it is uncharacterized and may hold a significant function under iron restriction.

In addition to regulation at the transcriptional level, such as via Fur, regulation at the post-transcriptional level is a well-defined mechanism used to regulate iron-responsive gene expression in eukaryotes, in which iron regulatory proteins bind to iron responsive elements on untranslated regions of regulated mRNA species; binding at the 5’ tail prevents translation of the transcript, while binding the 3’ tail confers increased stability to the transcript (Levenson and Tassabehji 2004). Evidence for similar mechanisms is emerging in bacteria as well, such as the small RNA RyhB in *E. coli*, which directs the degradation of several iron-requiring proteins during iron restriction (Kadner 2005; Masse and Arguin 2005).

RNA and DNA were separately purified from iron-restricted or iron-replete *Chlamydia*-infected cell lysates collected at 6, 12, 18, 24, 36, and 48 hpi. qPCR was used to measure reverse transcribed cDNA or genomic DNA, and chlamydial transcript levels were normalized to chlamydial chromosome copy number. Chlamydial
chromosomes were also normalized to total DNA content of samples, to account for host cell number variation and DNA isolation efficiency. Transcript levels for all five examined genes peaked at 18 hpi (Fig. 4.4), which corresponds to high expression of these proteins during radiolabeling, conducted at 22-24 hpi.

Mid-cycle transcript levels increased for each transcript in samples from iron-restricted chlamydiae compared to samples from iron-replete chlamydiae at either 18 or 24 hpi (Fig. 4.4). While transcript levels of $ahpC$, $devB$, $fabF$, and $ct538$ peaked at similar levels under both growth conditions at 18 hpi, samples from iron-restricted chlamydiae demonstrated a delayed decrease in transcript level at 24 hpi compared to samples from iron-replete chlamydiae. Transcript levels of $cadd$ were higher at 18 hpi in samples from iron-restricted cultures compared to iron-replete cultures.

Protein expression differences between iron-restricted and iron-replete samples were higher than transcript level differences, suggesting that the duration of heightened transcript level may be more important for regulation of protein expression under iron restriction than changes in maximal transcript levels. Whether this transcript pattern is the result of derepression due to inactivation of DcrA under reduced iron levels or increased transcript stability is unclear and should be explored further.

This dissertation project has begun to address the protein-level response of $C. trachomatis$ to an iron-limited environment. While the pathways used by chlamydiae to obtain iron have not been elucidated, the findings of this project support the concept that chlamydiae require iron and the restriction of iron availability alters their metabolic activity. Because several iron responsive proteins identified in this dissertation possess functions involved in the response to oxidative stress, it is apparent that the chlamydial
responses to iron restriction and oxidative stress are connected. This is not surprising, because the response to iron restriction and oxidative stress are inter-related and even co-regulated in bacteria and eukaryotes (Hantke 2001; Cairo and others 2002; Raulston 2006). The reason for this connection likely lies in that iron uptake is upregulated upon iron restriction, introducing an increase of iron in the labile pool; this necessitates upregulating oxidative damage defense mechanisms in light of the increase of reactive species. Also, co-regulation of these responses could be due to induction of a general stress response, considering the plurality of effects iron starvation would have on cellular pathways of the bacteria. Additionally, the chlamydial response to reduced iron and oxidative damage may be linked as an adaptation to the immune response to infection. Interferon-γ is known to play an important role in the immune response to chlamydial infection, and exposure of host cells to this compound triggers production of nitric oxide and reduction of iron levels. Furthermore, some chlamydial strains, particularly LGV, are capable of infecting macrophages, which actively remove iron from intracellular compartments using the Nramp1 efflux pump (Forbes and Gros 2001), and expose invading bacteria to an oxidative burst; co-regulation of these pathways would be an efficient adaptation. Taken together, these data indicate we are only beginning to understand how chlamydiae have adapted to satisfying the requirement for iron in the intracellular compartment, and more details will be required at the molecular level before we can construct a coherent model of the chlamydial response to iron restriction.
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