Identification of iron-responsive proteins expressed by *Chlamydia trachomatis* reticulate bodies during intracellular growth

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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 cells were radiolabelled during mid-developmental cycle growth, harvested, and separated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Of ~250 radiolabelled protein species visualized, densitometric analysis revealed 25 proteins that increased in expression under iron restriction compared to iron-sufficient control samples; ten protein species identified by mass spectrometry are involved in the oxidative damage response (alkyl hydroperoxide reductase, 6-phosphogluconolactonase 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 (*Chlamydia* protein associating with death domains, CADD). Transcript-level expression patterns of *ahpC*, *devB*, *cadd*, *fabF* and *ct538* were measured by quantitative RT-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-catalysed generation of reactive oxygen species and adaptation to stress by increasing expression of transcriptional machinery and other stress-responsive proteins.

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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 (WHO, 2001); in 2006, more than one million new cases were reported to the Centers for Disease Control and Prevention in the USA alone (CDC, 2007). 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 , penicillin exposure and nutrient deficiency. Although chromosomal replication continues, cellular division ceases, RBs become enlarged, and transcription and protein expression profiles are altered; in addition, persistent chlamydiae are refractory to killing by some antibiotics and expression of antigens continues during the prolonged development cycle (Hogan *et al.*, 2004). Persistence has become an issue of interest in chlamydial pathogenesis due to the potential role in

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Abbreviations: EB, elementary body; gDNA, genomic DNA; MOMP, major outer-membrane protein; p.i., post-infection; RB, reticulate body.

establishing chronic infection, which can result in 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 (Andrews, 2000; Wooldridge & Williams, 1993). 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 (Masse & Arguin, 2005; Van Ho *et al.*, 2002; Wooldridge & Williams, 1993). 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 (Levenson & Tassabehji, 2004; Oates & Ahmed, 2007). In addition, 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 (Kadner, 2005; Markel *et al.*, 2007; Sritharan *et al.*, 2006). Indeed, all examined microbial organisms have an absolute requirement for iron, except lactobacilli (Archibald, 1983; Weinberg, 1997) and *Borrelia burgdorferi* (Posey & Gherardini, 2000), which use alternative transition metal cofactors, including cobalt and manganese. Furthermore, reduced iron availability is known to induce virulence factors in pathogens, such as the Shiga toxin in *Shigella dysenteriae*, the Shiga-like toxin in *Escherichia coli*, exotoxin A in *Pseudomonas aeruginosa*, and diphtheria toxin in *Corynebacterium diphtheriae* (Hantke, 2001; Litwin & Calderwood, 1993).

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 developmental cycle of *C. trachomatis* arrests at the RB stage and cell division ceases; also, enlarged aberrant chlamydial forms are visible by transmission electron microscopy (Raulston, 1997). 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* (Al-Younes *et al.*, 2001; Freidank *et al.*, 2001) and *Chlamydophila psittaci* (Goellner *et al.*, 2006).

The first investigation to describe the chlamydial requirement for iron identified several iron-responsive protein species by separation of radiolabelled protein from purified EB (Raulston, 1997). Notably, two proteins demonstrated in that study to be iron responsive have attracted interest due to their immunogenicity in *Chlamydia*-infected patients: cHsp60-2 (LaRue *et al.*, 2007) and YtgA (Raulston *et al.*, 2007). During menstruation, iron levels in endometrial cells fluctuate, and thus bacteria infecting these cells encounter an additional level of iron limitation

(Anderson *et al.*, 2001; Andrews, 2000; Cohen *et al.*, 1987; Kelter *et al.*, 1996); therefore, the response of chlamydiae to reduced iron availability has dramatic health implications, especially considering that the highest incidence of chlamydial infections occurs in females between 15 and 24 years of age (CDC, 2007).

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 begin 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.

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) supplemented with 10% (v/v) fetal bovine serum (HyClone) and 2 mM GlutaMax (Gibco) in six-well plates.

For iron restriction, HEC-1B cells were incubated 24 h prior to infection in medium containing 5% FBS plus or minus 50 µM of the iron chelator Desferal (deferrioxamine mesylate, Sigma-Aldrich). 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 cycloheximide ml⁻¹, conditions that have previously been shown to induce persistence in *C. trachomatis* (Raulston, 1997). In some protein analysis experiments, Percoll-purified EBs, 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) was used to measure ferritin levels in infected HEC-1B protein samples collected at 24 h post-infection (p.i.), according to the manufacturer's instructions. All samples were assayed in triplicate, and ferritin levels were normalized for variations in protein concentration (BCA assay, Pierce). A two-tailed Student's *t*-test was used to determine significance of a change in ferritin concentration.

Protein radiolabelling. Protein radiolabelling was conducted in *Chlamydia*-infected HEC-1B cells between 22 and 24 h p.i. Cultures were incubated in D-MEM lacking methionine and cysteine and supplemented with 5% FBS, 2 mM GlutaMax, 40 µg cycloheximide ml⁻¹, plus or minus 50 µM Desferal, and 100 µCi ml⁻¹ (3.7 MBq ml⁻¹) ³⁵S-labelled methionine and cysteine (Redivue PRO-MIX, GE Healthcare) for 2 h at 35 °C. Following labelling, monolayers were washed with PBS and harvested in deionized water containing 1% CHAPS and a protease inhibitor cocktail (Pierce) and stored at -20 °C.

Sample preparation and protein separation. Radiolabelled protein samples collected from iron-restricted or normally grown *C. trachomatis*-infected HEC-1B cells were separated via 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 counting of TCA-precipitated protein from each sample. Protein samples were solubilized in Bio-Rad rehydration/sample buffer (8 M 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 10^6 c.p.m. and 300 µg total protein; preparative gels for mass spectrometry identification were loaded with 2 mg total protein from unlabelled Percoll-purified EB and spiked with 250 000 c.p.m. from a labelled protein sample to facilitate matching of EB protein spots with radiolabelled 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 labelled 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 2 h, and 10 000 V maximum for 60 000 V h.

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 h.

Gels for quantitative analysis were fixed in 25 % methanol and 10 % acetic acid, incubated with Amplify fluorographic reagent (GE Healthcare) for 30 min, 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 quantification and identification. Protein spots were matched and analysed for differential expression using Dymension software (Syngene). Protein spot intensities were normalized to the total valid spot intensity for each gel. Proteins were deemed iron responsive if (i) the mean density of the protein spot from iron-restricted samples showed a twofold or greater increase over control samples and (ii) the *P* value was <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 $P < 0.05$ and by comparing predicted versus observed pI and molecular mass.

Quantitative PCR analysis. Transcript levels of *ahpC*, *devB*, *cadd*, *fabF*, *ct538*, *ompA*, *euo* and *omcB* were measured using quantitative reverse transcription PCR (qRT-PCR). HEC-1B cells were grown in six-well plates and iron-starved 24 h prior to infection as described above. Following infection, monolayers were collected at 6, 12, 18, 24, 36 and 48 h p.i., along with uninfected control samples. After washing with PBS, each monolayer was scraped with a rubber policeman into RLT lysis buffer (Qiagen), 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 an Agilent 2100 Bioanalyser (Agilent Technologies), 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), following the conditions recommended by the manufacturer.

qRT-PCR was conducted on samples in triplicate using the Bio-Rad iCycler. Reaction mixtures contained $1 \times$ Platinum Quantitative PCR Supermix-UDG (Invitrogen), $0.8 \times$ SYBR green (Cambrex BioScience), 5 mM MgCl_2 , 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 1, except for *euo* and *omcB* (Belland *et al.*, 2003a) and *ompA* (Gomes *et al.*, 2005). 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 (Guseva *et al.*, 2007). 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 qRT-PCR signal for corresponding RT plus samples. Chlamydial genome copy number for each experimental sample was determined by qRT-PCR using the *ct538* primer set against gDNA. Transcript levels are presented as copy number per chlamydial genome.

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 an immunoassay. Measurement of ferritin content is a commonly used method to indirectly determine iron levels in eukaryotic cells since 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. 1). Ferritin levels in Desferal-exposed samples averaged 119 ng ml^{-1} compared to 241 ng ml^{-1} in mock-exposed samples. This twofold difference between Desferal-exposed and unexposed samples was statistically significant, thereby validating this model for iron restriction of *Chlamydia*-infected cells. In addition, exposure of uninfected cells to Desferal as above resulted in a similar drop in ferritin level (data not shown).

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 labelled at 22–24 h p.i. This labelling window

Table 1. *C. trachomatis*-specific oligonucleotide primers used in qRT-PCR analyses

Primers were designed using the published *C. trachomatis* serovar D sequences.

Gene	Forward primer (5–3')	Reverse primer (5–3')	Amplicon length (bp)
<i>ahpC</i>	CCAGTTAGCTGGACAAACCATTCG	CGTTCCATTGACGAGGAATTGCGT	88
<i>fabF</i>	GGAATTAGTGGAGTGCGAACA	GGATCAACTCGTCTAGCTTGT	128
<i>devB</i>	ACGAAGATGTAGAAGCTGGAAGTA	TGCGGTATCCATACGAAAGATTG	98
<i>cadd</i>	GACAACTACAACAAGTTCCTG	GTTAGAGGCATCGCAAGA	83
<i>ct538</i>	GTTTGACAGCTACCACTCTG	AACGTCTACGCAAACCTCA	91

was chosen since it is during exponential growth (Hogan *et al.*, 2004), which coincides with an increased demand for micronutrients such as iron. Host cells were starved for iron 24 h prior to infection to ensure that chlamydiae did not obtain iron stores early in infection. At 22 h p.i., medium for each sample was switched to the radiolabelling medium, the infection was allowed to continue for 2 h of labelling, and protein samples were collected, processed, and separated via 2D-PAGE. This methodology for generation of proteomic maps in *C. trachomatis* was adapted from the pioneering chlamydial proteomic studies by Vandahl *et al.* (2004).

Approximately 250 protein spots were visible via radiography (Fig. 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. 3).

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 2; Fig. 3).

Interestingly, nine 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. The uncharacterized protein CT538 demonstrated a 2.9-fold increase in expression. Of note, no significant difference in spot intensity of the major outer-membrane protein (MOMP) was found between iron-restrictive and iron-replete growth.

Transcriptional analysis of selected genes

qRT-PCR 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 of *ompA*, *euo* and *omcB* were used as controls for

Fig. 1. Decrease in intracellular host iron levels following Desferal exposure versus mock exposure as measured by ferritin concentration. Ferritin levels of *C. trachomatis* serovar E-infected HEC-1B cells were measured by enzyme immunoassay following exposure to 50 μ M Desferal or mock-exposed. Means $\pm$ SD of four independent experiments; $P < 0.05$.

Fig. 2. Representative two-dimensional electrophoretic maps of *C. trachomatis* proteins radiolabelled 22–24 h p.i. 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 labelled with arrows, along with MOMP for reference. Isoelectric point (pI) and molecular mass are indicated.

Fig. 3. Representative 2D-PAGE of iron-responsive proteins and non-responsive MOMP with relative expression levels. Raw images of spots from samples collected from iron-restricted (Des+) or iron-sufficient (Des-) *Chlamydia*-infected HEC-1B cells were combined for each protein spot prior to adjusting contrast in Adobe Photoshop in order to preserve the relative spot intensities. Graphs indicate normalized volume of protein spots.

Table 2. Identified *C. trachomatis* serovar E iron-responsive proteins

Functional group	ORF	Protein name	Description	UniProt accession*	Fold increase†	pI/mol. mass (kDa)	
						Predicted‡	Observed
Metabolism/biosynthesis	CT603	AhpC (TSA)	Alkyl hydroperoxide reductase (thio-specific antioxidant peroxidase)	O84608	6.4	4.8/22	4.6/22
	CT186	DevB (6PGL)	6-Phosphogluconolactonase	O84189	2.1	5.3/29	5.3/28
	CT770	FabF	Acyl carrier protein synthase	O84775	2.6	5.4/45	5.6/42
Protein modification	CT353	Def	Peptide deformylase	O84357	3.8	5.7/21	5.8/21
	CT707	RopA	Trigger factor (peptidyl prolyl <i>cis-trans</i> isomerase)	O84713	3.7	5.0/50	5.2/50
Transcription	CT507	RpoA	RNA polymerase subunit alpha	Q46449	3.1	5.3/42	5.4/40
	CT097	NusA	Transcription anti-termination factor	O84099	2.1	5.2/49	5.5/50
	CT320	NusG	Transcription anti-termination factor	O84322	5.9	5.3/21	5.3/18
Virulence	CT610	CADD	<i>Chlamydia</i> protein associating with death domains	O84616	3.4	4.9/27	5.0/26
Hypothetical	CT538	–	Hypothetical protein	O84543	2.9	5.2/27	5.3/27

*Accession number for *C. trachomatis* serovar D proteins.

†Fold increase in spot volume in iron-restricted versus iron-sufficient growth conditions as determined by analysis with Dymension 2D software. Mean value from two replicate gels from each of four independent experiments.

‡Calculated using ExPASy Compute pI/Mw tool.

comparison, since these should not be regulated by iron availability. It is noteworthy that equivalent levels of gDNA were detected in Desferal-exposed versus mock-exposed cultures. Transcript levels were measured at 6, 12, 18, 24, 36 and 48 h p.i. in iron-restricted and in mock-exposed control chlamydiae using qRT-PCR. Analysis of transcript levels normalized to chlamydial chromosome copy number revealed that the transcript levels for all five iron-responsive genes examined peaked at 18 h p.i. (Fig. 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 radiolabelling, 22–24 h p.i. The transcript level of *ompA* was highest at 24 h p.i., *euo* at 12–18 h p.i. and *omcB* at 36 h p.i., reflecting the timing of peak transcript level previously described for these transcripts, mid-, early- and late-developmental cycle, respectively (Belland *et al.*, 2003a).

Mid-cycle levels increased for each iron-responsive transcript in samples from iron-restricted chlamydiae compared to samples from iron-replete chlamydiae at either 18 or 24 h p.i. ($P < 0.05$; Fig. 4). Transcript levels of *cadd* reached a higher peak level at 18 h p.i. 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 h p.i.; however, in samples from iron-restricted chlamydiae, higher levels of these transcripts were detected at 24 h p.i. compared to samples from iron-replete

chlamydiae. Since 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. No significant differences in transcript levels for *ompA*, *euo* or *omcB* between cultures grown in the presence or absence of Desferal were found at any time, validating the differences measured for the iron-responsive transcripts.

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, ten *C. trachomatis* serovar E iron-responsive proteins were identified by 2D-PAGE coupled with mass spectrometry. While iron-responsive proteins involved in oxidative stress response and virulence were identified, none appear to be directly involved in iron acquisition. This may be surprising considering the obvious necessity

Fig. 4. Chlamydial transcript levels at selected times throughout the developmental cycle from infected cultures grown under normal (noD, ■) or low-iron (D+, ◇) conditions. hpi, hours post-infection. The y-axes indicate mRNA copies per chlamydial genome (normalized by amount of gDNA). Times at which transcripts are significantly increased in iron-restricted versus iron-sufficient growth are marked by an asterisk. Transcripts of *ompA*, *euo* and *omcB*, which should not be regulated in response to iron availability, are shown for reference.

for upregulating iron acquisition during iron limitation; however, no genes encoding conserved iron-acquisition mechanisms, such as siderophores, were identified follow-

ing the sequencing of the chlamydial genome (Stephens *et al.*, 1998), although a putative component of an ABC metal transporter, YtgA, has been identified in *C.*

trachomatis (Raulston *et al.*, 2007). YtgA has previously been shown to increase in expression during iron restriction; however, in ongoing studies on expression of YtgA, differential expression does not appear to occur until 36 h p.i. (unpublished data).

The lack of obvious iron-uptake system homologues 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 utilized by extracellular bacteria, but the process 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 such as haem, ferritin and transferrin (Deneer *et al.*, 1995). 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 EBs (Heuer *et al.*, 2006).

An analogue to Fur, the primary iron regulatory protein in Gram-negative bacteria, termed DcrA, was identified in *C. trachomatis* serovar E, and has shown the ability to bind *E. coli* Fur boxes (Wyllie & Raulston, 2001). Putative DcrA-binding sites have been identified in the chlamydial genome by binding of purified *E. coli* Fur protein (Rau *et al.*, 2005). 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.* (2006) conducted a proteomic analysis of *Chlamydophila pneumoniae* that examined modulation of protein expression by three inducers of persistence: iron restriction, heat shock and exposure to interferon- γ . Proteins increasingly expressed under iron restriction but not following heat shock or interferon- γ exposure were involved in cofactor biosynthesis, cellular processes and translation, including one protein identified as iron responsive in the current study, AhpC. 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. Decreased iron availability has been shown to modulate expression of *ahpC* transcription and/or AhpC protein expression in numerous organisms, including *Helicobacter pylori* (Merrell *et al.*, 2003), *Corynebacterium diphtheriae* (Tai & Zhu, 1995), *Campylobacter jejuni* (van Vliet *et al.*, 1998) and *Francisella tularensis* (Lenco *et al.*, 2007).

Two chlamydial proteins involved in biosynthetic pathways, DevB and FabF, were found to be iron responsive in the present study. DevB catalyses the hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate, the second step of the pentose phosphate pathway (PPP), while FabF catalyses 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 defence (Tsaneva & Weiss, 1990). G6PD mutants of *E. coli* (Nunoshiba *et al.*, 1995), *Salmonella typhimurium* (Lundberg *et al.*, 1999) and mouse stem cells (Pandolfi *et al.*, 1995) have extreme sensitivity to oxidative damage. FabF is also likely iron responsive due to a role in oxidative damage defence, since 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* (Taguchi *et al.*, 2006), while *F. tularensis* (Lenco *et al.*, 2007) and *Campylobacter jejuni* (Holmes *et al.*, 2005) increase expression of FabG under iron restriction.

The finding that CADD is iron responsive is significant since it has been implicated in induction of host-cell death via interaction with the death domains of Fas (Stenner-Liewen *et al.*, 2002). 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 (Byrne & Ojcius, 2004). 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 (Belland *et al.*, 2003b).

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. Furthermore, this has been matched with an increased level of transcript encoding five of the identified proteins during mid-developmental cycle. While the mechanisms utilized 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.

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