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Response of *Chlamydia trachomatis* Serovar E to Iron Restriction In Vitro and Evidence for Iron-Regulated Chlamydial Proteins

JANE E. RAULSTON*

Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7290

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Iron is a well-established mediator of virulence in several bacterial pathogens, yet little is known about the role of iron in infectious disease processes caused by obligate intracellular bacterial pathogens. In this study, the effect of iron limitation was examined for the sexually transmitted infectious agent *Chlamydia trachomatis* in an in vitro model of human genital infection using the intracellular iron-chelating reagent deferoxamine mesylate (Desferal). Iron restriction caused a significant reduction in infectivity of *C. trachomatis* elementary bodies (EB) harvested from Desferal-exposed polarized epithelial cells when compared to that of EB harvested from iron-sufficient control cell cultures. Replacement of the Desferal exposure medium with medium containing iron-saturated transferrin restored chlamydial infectivity, whereas replacement with growth medium alone had no effect. The following three prominent morphological features were observed by electron microscopic examination of chlamydia-infected cells exposed to Desferal: (i) inclusions containing chlamydiae greatly delayed in maturation, (ii) substantial blebbing within chlamydial inclusions, and (iii) electron-dense material surrounding inclusions. Protein analyses of highly purified EB by two-dimensional polyacrylamide gel electrophoresis revealed that there were at least 19 candidate iron-repressible proteins in *C. trachomatis* and at least one protein which was iron inducible. One putative iron-repressible protein was confirmed by Western blot (immunoblot) analysis to be the chlamydial heat shock protein 60 (hsp60). The enhanced production of this antigen by chlamydiae as a result of iron limitation is of particular importance since there is a well-documented association between chlamydial hsp60 and destructive immunopathological sequelae in infected patients.

Iron, the most abundant metal in biological systems, is intimately involved in numerous cellular metabolic processes. Since this element is essential for almost all living organisms examined to date, the oxidative properties of free iron necessitate that intracellular concentrations be tightly regulated and complexed with high-affinity iron-binding proteins. Otherwise, iron would interact with reduced forms of oxygen, resulting in free radicals known to cause lipid peroxidation, protein modification, and nucleic acid damage (1, 4, 18, 27).

The competition for iron by bacterial pathogens is a subject of intense examination, and numerous studies have documented key roles for this element in pathogenesis (for selected reviews, see references 9, 15, 21, 30, 40, 49, and 52). Besides the well-recognized example of the relationship between iron and virulence in regulation of toxin production by *Corynebacterium diphtheriae* (49), studies on gram-negative bacterial envelope components involved in iron acquisition suggest that iron-binding proteins may serve a role in host specificity and even in colonization of mucosal sites within a given host. For example, the pathogenic *Neisseria* species and *Haemophilus influenzae*, but not their commensal counterparts, utilize iron from human transferrin (15, 33). Conversely, *Helicobacter pylori* is unable to sequester iron from human transferrin but may capitalize on the supply of iron released from lactoferrin in the acidic compartment of the upper gastrointestinal tract for survival and growth (25). The importance of iron to mucosal pathogens has been heightened by two recent findings. First, specific antibodies against a transferrin-binding protein in the outer membrane of *Neisseria meningitidis* elicits protective immunity in a mouse model of infection (2, 16, 21). Second, a transferrin receptor-deficient mutant strain of *Neisseria gonorrhoeae* was not able to initiate urethritis in a human volunteer challenge study (14).

Chlamydia trachomatis is a major mucosal pathogen. Serovariants (or serovars) A to C infect the conjunctiva to cause trachoma, the leading cause of preventable blindness in the world. Serovars D to K infect the genital mucosae and are the leading cause of bacterially acquired sexually transmitted infections (12). The role of iron in the pathogenesis of chlamydiae at mucosal surfaces has not been examined. Also notably absent in the literature is the role, if any, that iron serves in the disease processes caused by obligate intracellular bacteria such as the chlamydiae. Host cell vesicles containing facultative intracellular bacteria, such as *Salmonella typhimurium* (42) and *Legionella pneumophila* (24), are sufficiently low in iron content as to enhance the production of iron-regulated virulence factors in these bacteria. There is also evidence that iron influences killing or survival of *Legionella pneumophila* and *Listeria monocytogenes* in human monocytes and macrophages, respectively (3, 11). However, *C. trachomatis* classically resides in mucosal epithelial cells. Little is understood about how chlamydiae interact with host cells to obtain nutrients and metabolites, few chlamydial components have been associated with virulence properties, and virtually nothing is known about the mechanisms responsible for host and cellular tropism.

Deciphering iron sequestration by the chlamydiae may be complicated by the unusual growth of these bacteria in epithelial cells in which the chlamydiae exhibit several morphologic and metabolic transitions. The unifying characteristic of the chlamydiae is their developmental cycle, which alternates in a biphasic asynchronous manner between infectious extracellular forms, termed elementary bodies (EB), and noninfectious intracellular forms, termed reticulate bodies (RB). The smaller

* Mailing address: Department of Microbiology and Immunology, University of North Carolina School of Medicine, Campus Box 7290, 804 Mary Ellen Jones Building, Chapel Hill, NC 27599-7290. Phone: (919) 966-5041. Fax: (919) 962-8103. E-mail: raulston@med.unc.edu.

EB form is metabolically inert and appears to have a singular mission in the attachment to and entry into a susceptible mucosal epithelial cell. Once EB are internalized by a receptor-mediated endocytic route in polarized host epithelial cells, certain immediate events take place coincident with EB transformation to RB. The chlamydia-containing vesicles appear to undergo rapid modifications, which include the incorporation of Golgi-derived lipids from the host exocytic pathway (22). Although there is a slight initial decrease in vacuolar pH, vesicles containing viable chlamydiae quickly neutralize their immediate environment and are not subject to acidification via the lysosomal pathway (19, 46). For *C. trachomatis*, individual vesicles fuse and RB begin to divide by binary fission. The growth of a single membrane-bound microcolony, termed an inclusion, can be observed by light microscopy at 18 to 24 h postinfection (hpi). Recent studies indicate that the inclusion membrane is a highly selective barrier which does not allow passive permeation of small molecules (23). Chlamydial RB eventually condense back to infectious EB, which are released after 72 to 96 h to infect new host cells. Intermediate chlamydial forms are observed during transitional phases between EB and RB and during condensation of RB to EB.

Receptor-mediated internalization of iron bound to transferrin is the most well understood route for iron acquisition in eukaryotic cells, although alternative pathways have been identified and are under investigation (1, 18, 41). In a preliminary study, Scidmore and Hackstadt (47) showed that the chlamydiae do obtain iron from transferrin; when ^{55}Fe-loaded transferrin was used as an endosomal marker in chlamydia-infected cells, the EB progeny isolated after one cycle of development were radiolabeled. The utilization of iron by chlamydiae is most likely accomplished by metabolically active RB; yet it is interesting that several investigators (48, 50, 51) have observed that transferrin-containing endosomes are positioned immediately adjacent to developing chlamydial inclusions but are not obviously fused to inclusions. These observations would generally suggest that complex transport mechanisms may be involved to transfer iron across (i) the endosomal membrane, (ii) the inclusion membrane, and (iii) the chlamydial envelope. There is evidence of a functional membrane transport system for the transfer of iron from endosomes to the eukaryotic cell interior (1, 39, 41).

The purpose of this study was to examine whether genital *C. trachomatis* serovar E is affected by iron limitation and to begin to identify specific chlamydial proteins that may be regulated by iron. Since *C. trachomatis* serovars D to K are strictly human pathogens, most of our understanding of chlamydial biology is derived from in vitro cell culture studies. In this report, polarized human endometrial epithelial cells were used as a model for chlamydial infection, and an iron-chelating reagent, Desferal (deferoxamine mesylate: CIBA Pharmaceutical Co., Summit, N.J.), was used to reduce iron availability to the chlamydiae. This compound is well documented to specifically chelate labile pools of intracellular iron in eukaryotic cells, including genital epithelial cells (29, 44). Although in vitro studies are more simplistic, given the absence of an intact immune response or hormonal regulation, the availability and specific sources of iron may very well influence chlamydial mucosal infection in vivo, as is the case with *N. gonorrhoeae*. In addition, it is known that chlamydial attachment and infectivity are substantially increased in estrogen-dominant versus progesterone-dominant human endometrial gland epithelial cells (31). Immunohistological studies on human endometrial tissues obtained from both proliferative and secretory phases reveal a role for estradiol in the regulation of lactoferrin expression (26). There is also evidence from an animal model study that the amount of uterine progesterone is correlated with expression levels of the intracellular iron storage protein, ferritin (58). Taken together, these observations support an investigation into the consequences of the withholding of iron on chlamydial pathogenesis.

MATERIALS AND METHODS

Bacteria and eukaryotic host cells. *C. trachomatis* serovar E/UW-5/CX was provided by C. C. Kuo and S. P. Wang, University of Washington, Seattle. Host cell cultures were routinely determined to be free of *Mycoplasma* contamination by staining with the Hoechst reagent. McCoy cells were used for titrations of chlamydial infectivity by inclusion formation. Chlamydiae in inclusions were stained with either Giemsa stain or with a pool of fluorescein-conjugated monoclonal antibodies against the *C. trachomatis* major outer membrane protein (MOMP; Syva, Palo Alto, Calif.).

Polarized human endometrial epithelial cells were used as the model host cell system for all iron deprivation experiments (56). The human endometrial carcinoma cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.) was grown on collagen-coated (i) Cytodex 3 microcarrier beads in suspension for purification of EB on a large scale (45, 57) and (ii) Transwell polycarbonate membranes (Costar, Cambridge, Mass.) for experiments involving multiple samples (55, 56). A few modifications were necessary for adaptation of the HEC-1B cells to the recently developed microcarrier culture method. Unlike the faster-growing McCoy cells, which reach confluency within 3 to 4 days, HEC-1B epithelial cells require 10 to 14 days of growth on microcarrier beads before infection with *C. trachomatis*. Prior to purification of EB, infected HEC-1B cells require two sequential 15- to 20-min exposures to collagenase and dispase to remove the firmly anchored cells from the beads. Finally, the number of beads was kept low, at 5×10^4 per 125-ml culture, to reduce mechanical shearing of polarized epithelial cells from bead surfaces. Otherwise, the procedure was conducted as outlined previously (45, 57).

Viability experiments. The intracellular iron-chelating reagent Desferal was used to generate an iron-restricted environment for propagation of *C. trachomatis* serovar E. The effect of Desferal, at concentrations of 25, 50, and 100 μM, on EB particle yield and subsequent infectivity was first determined with polarized HEC-1B cells on microcarrier beads. This experiment was conducted on two separate occasions. Cells were infected by adsorption of an EB inoculum for 2 h followed by incubation at 35°C in Eagle's minimal essential medium (MEM) containing 2 mM glutamine, 5% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, Utah), and 0.5 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml, and with or without Desferal. The HEC-1B cell line was not adaptable to growth in serum-free medium. EB were purified with discontinuous Renografin gradients, and particle counts were obtained with a spectrophotometric standard curve derived from particle counting at the level of electron microscopy (54). The infectivity of purified EB was determined by diluting preparations to a multiplicity of 50 EB particles per host McCoy cell and staining for inclusions with Giemsa stain at 48 hpi.

In a subsequent experiment, holotransferrin (hTf; >99% iron saturated and endotoxin-free; Sigma) was added to chlamydia-infected cultures to reverse the effect of Desferal. This experiment was conducted on two separate occasions, and each parameter was assayed in triplicate. Uninfected HEC-1B cells were exposed to 100 μM Desferal or 6 mg of hTf per ml for 72 h and assayed for viability with the LIVE/DEAD Viability/Cytotoxicity Kit of Molecular Probes, Inc. (Eugene, Oreg.). Polarized HEC-1B cells on Transwell polycarbonate tissue culture inserts were infected with *C. trachomatis* by adsorption for 2 h and subsequently incubated at 35°C in Dulbecco's modified essential medium with a high glucose level (DMEM-H; Gibco BRL, Grand Island, N.Y.) containing 2 mM glutamine, 5% FBS, and 0.5 μg of cycloheximide per ml, and with or without 50 μM Desferal. At 24 or 48 hpi, the Desferal exposure medium in certain samples was replaced with fresh DMEM-H without cycloheximide and with or without 6 mg of hTf per ml. Samples were subpassaged at 72 or 96 hpi by (i) removing infected host cells with a rubber policeman, (ii) washing filters with buffer (2SPG; 0.2 M sucrose, 0.02 M phosphate buffer, 5 mM glutamine [pH 7.2]), (iii) centrifuging each sample with respective washes for 30 min at 15,000 × *g* and 4°C, (iv) resuspending each pellet in 200 μl of 2SPG, (v) dispersing each sample by sonication and mixing on a Vortex mixer, and (vi) diluting each sample 50-fold in 2SPG prior to inoculation of McCoy cell monolayers for infectivity assessments. A two-tailed Student's *t* test was used to determine significance at a confidence level of 99% ($P < 0.01$).

Transmission electron microscopy. *C. trachomatis* serovar E-infected and -uninfected polarized HEC-1B cells, on polycarbonate membranes, were examined over (i) a range of Desferal concentrations and (ii) a 72-h time course of infection. Duplicate samples were washed in phosphate-buffered saline (pH 7.2) and fixed in 2% glutaraldehyde–0.5% paraformaldehyde for processing and embedding in Epon-Araldite resin as described in detail by Wyrick et al. (55). Ultrathin sections were stained with uranyl acetate and lead citrate for high-contrast morphology and examined with a Zeiss EM900 transmission electron microscope operated at 60 kV.

Protein and electrophoretic analyses. For metabolic radiolabeling of chlamydiae, polarized HEC-1B cells on microcarrier beads were infected by adsorption

for 2 h and subsequently incubated in Select-Amine MEM (Gibco BRL). Amino acid supplements were complete (≥100 μM) with the exception of L-methionine and L-cysteine, which were present at 10 μM. A final concentration of 10 μCi of [^{35}S]methionine-cysteine (Easytag express protein labeling mix; NEN DuPont Co., Wilmington, Del.) was added to chlamydia-infected cell cultures after the 2-h adsorption period. The distribution of radiolabel in the mixture was routinely 73% L-[^{35}S]methionine–22% L-[^{35}S]cysteine, and Select-Amine MEM was also supplemented with 5% FBS, 0.5 μg of cycloheximide per ml, and 0 or 50 μM Desferal. Mock-infected HEC-1B cells were treated in an identical fashion by exposure to chlamydial dilution buffer (2SPG) for 2 h prior to the addition of medium containing [^{35}S]methionine-cysteine.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to examine total protein profiles of Renografin-purified *C. trachomatis* serovar E EB on four separate occasions; two to three gel profiles of each sample were examined per experiment. EB were suspended in a mixture containing 9 M urea, 4% (vol/vol) Nonidet P-40, 2% (vol/vol) β-mercaptoethanol, and a 2% (vol/vol) mixture of ampholytes (Biorad Bio-Lyte and Pharmacia Pharmalyte; pH 3 to 10) for 2 h at 25°C prior to isoelectric focusing as described previously (43). Tube gels were then loaded with 8×10^7 EB particles, which is approximately 15 (±2) μg (mean ± standard deviation) of total EB protein (bicinchoninic acid microassay; Pierce, Rockford, Ill.). Concentrations of 15 μg of total cellular protein from mock-infected HEC-1B cells were also loaded onto tube gels. After separation of proteins in the first dimension, tube gels were equilibrated for 30 min in a mixture of 0.125 M Tris-HCl (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, 0.01% (wt/vol) bromophenol blue, and 0.8% (vol/vol) β-mercaptoethanol prior to separation in the second dimension on 12.5% polyacrylamide slab gels (28). Low-molecular-weight markers (Bio-Rad) were used for determination of protein mass in kilodaltons; purified trypsin inhibitor (pI 4.6), β-lactoglobulin (pI 5.1), and carbonic anhydrase (pI 5.9) were used as isoelectric markers (Sigma). The pH gradient was also assessed as described previously (43).

Two-dimensional gels were dried onto Whatman paper, covered in Mylar film, and exposed to Phosphor screens (Eastman Kodak, Rochester, N.Y.) for 48 h. Phosphorimaging and densitometric analyses of resolved proteins were accomplished with a Storm 860 phosphorimaging system and ImageQuaNT software from Molecular Dynamics (Sunnyvale, Calif.). In some experiments, EB proteins were examined by silver staining by the procedure outlined by Wray et al. (53).

Western blot analysis. EB proteins resolved by 2D-PAGE were transferred overnight to nitrocellulose for Western blot (immunoblot) analysis. Following a 1-h blocking period in PBS-Tween 20 plus 2.5% gelatin and three washes in PBS-Tween 20, the blots were incubated for 2 h with a mixture of the following primary antibodies: (i) a mouse monoclonal antibody generated against chlamydial hsp60, kindly provided by Richard Morrison (University of Alabama, Birmingham), and (ii) a rabbit-monospecific polyclonal antiserum generated against gel-purified chlamydial hsp70 protein (43). Antibodies were used at a 1:1,000 dilution in PBS-Tween 20. After washing, blots were incubated for 2 h with a mixture of goat anti-mouse and goat anti-rabbit alkaline phosphatase-conjugated secondary antisera; each detection antibody was diluted 1:2,000. Antigenic reactivity was subsequently visualized following reaction with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium.

RESULTS

Effect of iron limitation on infectivity and morphology of *C. trachomatis* serovar E. Polarized human endometrial cells (HEC-1B) were used throughout these studies as target host cells for human genital chlamydial infection, and Desferal was used to generate the iron-restrictive environment. Some investigators report that Desferal, in concentrations greater than 35 μM, is toxic to human monocytes (11), whereas others report that a very high concentration of Desferal, 2.5 mM, has no adverse effect on human macrophages (38). In this study, a 72-h exposure to 100 μM Desferal did not compromise HEC-1B cell viability (data not shown). It is known that eukaryotic cells respond to iron restriction with an immediate increase in the expression of transferrin receptors at the cell surface to enhance iron sequestration from the external growth medium (1, 18). Therefore, as an additional measure to ensure iron restriction, a 0.5-μg/ml concentration of cycloheximide, which has no inhibitory activity to chlamydiae (45), was included in these studies to inhibit host cell protein synthesis and reduce the rate of iron uptake from the growth medium.

In the first set of experiments, the effect of increasing concentrations of Desferal on EB particle yield and infectivity was examined. There were no significant differences in the absolute number of developing inclusions between normal and Desferal-exposed chlamydia-infected cultures (data not shown) ($P < 0.01$). However, inclusions developing in the presence of Desferal appeared smaller by light microscopy. *C. trachomatis* serovar E EB from iron-sufficient and Desferal-exposed cultures were highly purified by Renografin gradient centrifugation at 62 and 70 hpi. After particle counts were obtained, purified EB were diluted to a multiplicity of infection of 50:1 to determine the resultant infectivity in each preparation. The results presented in Table 1 document that the number of EB particles from iron-restricted, infected epithelial cell cultures was less than the number of EB particles purified from iron-sufficient, control cell cultures. Most importantly, the subsequent infectivity of EB following subpassage onto fresh cell monolayers was considerably reduced in a dose-dependent manner as the concentration of Desferal was increased. An examination of the EB preparations by fluorescence microscopy, using monoclonal antibodies generated against the MOMP, confirmed that although more particles were obtained from cultures exposed to 100 μM Desferal than from those exposed to 25 μM desferal, the EB were notably irregular in shape (data not shown).

TABLE 1. Infectivity and total number of EB particles of *C. trachomatis* serovar E harvested from HEC-1B cell cultures exposed to Desferal[a]

Expt no.	Time of harvest (hpi)[b]	Final concn of Desferal (μM)	Total no. of EB particles[c]	% Infectivity on subpassage[d]
1	62	0 (control)	1.8×10^9	56
		25	8.3×10^7	24
		50	3.4×10^8	12
		100	2.0×10^8	7
2	70	0 (control)	1.4×10^9	40
		25	4.2×10^7	21
		50	2.2×10^7	19
		100	3.6×10^8	13

[a] Approximately 5×10^7 polarized HEC-1B cells on porous Cytodex 3 beads were infected by adsorption with an inoculum of *C. trachomatis* serovar E which would result in 50% infected cells. Infected cultures were incubated at 35°C in Eagle's MEM with or without Desferal.

[b] EB were purified by Renografin density gradient centrifugation.

[c] Total EB particles were determined spectrophotometrically by extrapolation from a standard curve of particle counts obtained by electron microscopy.

[d] EB infectivity was determined by the number of inclusions formed per 100 McCoy cells by use of a multiplicity of infection of 50 EB particles per host cell. Values reflect the average inclusion count from 10 microscopic grid fields at ×40 magnification.

To more closely examine the morphological features of chlamydiae propagated in the presence of Desferal, studies were extended to the level of electron microscopy (Fig. 1). Uninfected HEC-1B cells exposed to 100 μM Desferal for 72 h showed that ultrastructural features were well maintained (compare Fig. 1A and B). Chlamydia-infected polarized HEC-1B cells, in the presence or absence of Desferal, were examined at 24, 48, and 72 hpi. A normal 72-h inclusion of *C. trachomatis* serovar E, containing condensed EB and a few RB, is illustrated in Fig. 1C. In the Desferal-exposed cultures, three very prominent morphological features were observed. After a 72-h incubation, chlamydial inclusions contained either (i) an abnormal quantity of membrane blebs, presumably chlamydial in origin (Fig. 1D, enlargement in inset), or (ii) inclusions containing predominantly RB (Fig. 1E). The latter observation reflects a considerable delay in maturation for 72 hpi. In addition, the outer membranes surrounding individual RB in Desferal-exposed cultures were dense and wavy relative to those surrounding normal RB (compare insets in Fig. 1C and

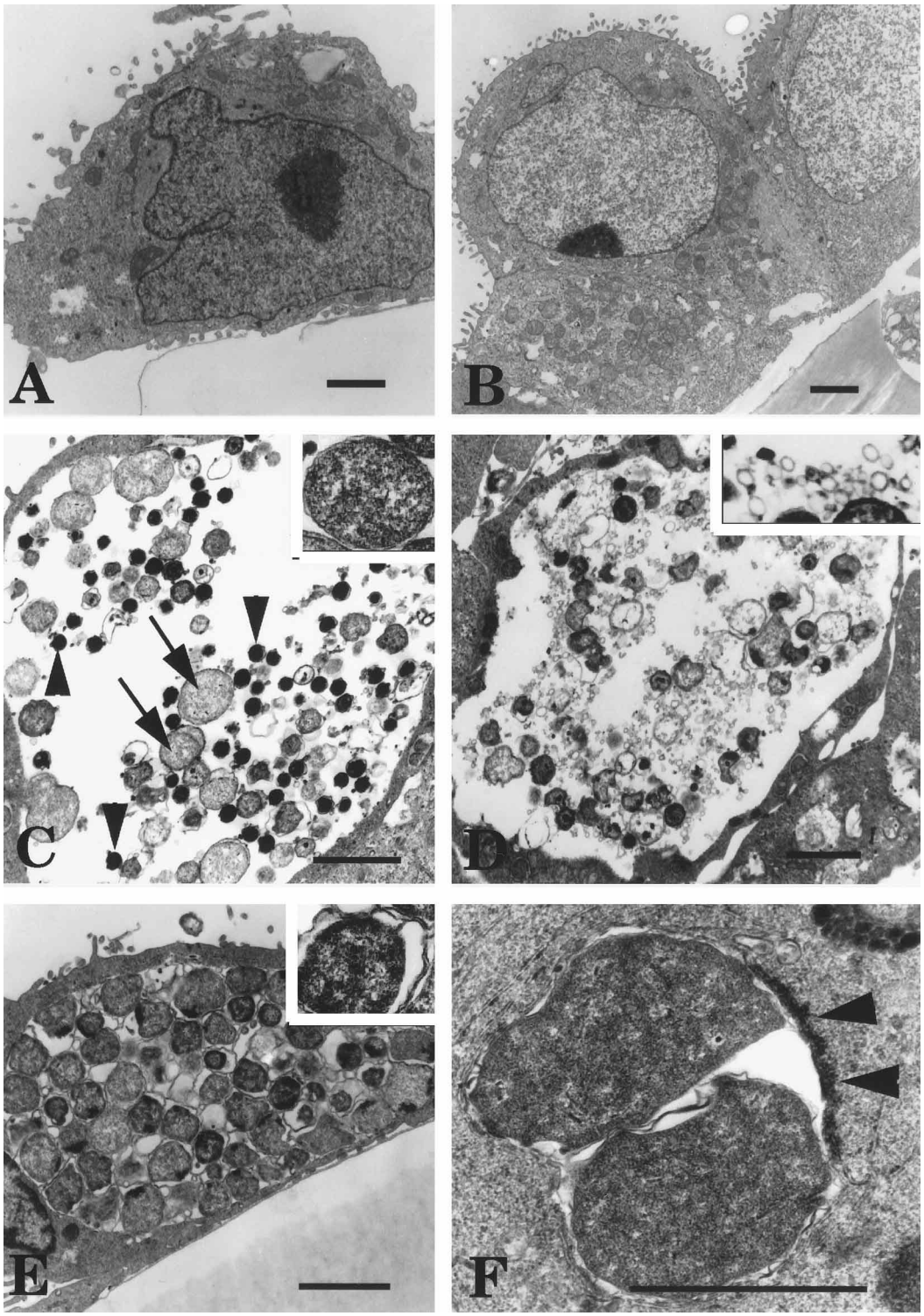

FIG. 1. Ultrastructural analysis of *C. trachomatis* serovar E in polarized HEC-1B cells exposed to the intracellular iron-chelating reagent Desferal. Uninfected (A and B) and *C. trachomatis*-infected (C to F) polarized endometrial epithelial cells were fixed, processed, and embedded for high-contrast transmission electron microscopic examination of ultrathin sections. (A to C) Controls included uninfected HEC-1B cells (A), uninfected HEC-1B cells exposed to 100 μM Desferal for 72 h (B), and *C. trachomatis*-infected cells at 72 hpi (C). In panel C, the typical chlamydial inclusion contains condensed EB (arrowheads) and larger RB (arrows); the inset is an enlargement of a normal RB to illustrate standard envelope ultrastructure. (D and E) *C. trachomatis* inclusions after 72 h of exposure to 100 μM Desferal contained numerous intrainclusion blebs (D; enlargement shown in inset) or predominantly RB (E). RB growing in the presence of Desferal exhibited loose and wavy outer membranes (compare inset in panel E to inset in panel C). (F) Electron-dense material surrounding a small inclusion after 24 h of exposure to 100 μM Desferal is shown (arrowheads). Bars, 2.0 μm.

E), indicating a potential increase in synthesis of the chlamydial envelope in response to Desferal exposure. The third ultrastructural feature was the presence of dense material at the periphery of the inclusion membrane (Fig. 1F); this material was observed surrounding inclusions as early as 24 hpi.

Although the adverse viability and morphological observations provided evidence that the chlamydiae may be affected by iron availability, the question still remained as to whether these bacteria responded specifically to iron limitation versus an indirect metabolic consequence of a compromised host cell environment. To confirm that the observed effects resulted from iron deprivation, experiments were conducted to restore chlamydial viability by replacing the culture medium containing 50 μM Desferal with medium containing 6 mg of iron-saturated hTf per ml, since transferrin is at least one source of iron for the chlamydiae (47). A 6-mg/ml concentration of hTf exceeds the iron-binding capacity of 50 μM Desferal by threefold on an equimolar basis. Importantly, toxicity was not observed in HEC-1B cells exposed to 6 mg of hTf-containing medium per ml for 72 h (data not shown). The medium was replaced to remove cycloheximide so that host cell synthesis, and therefore iron acquisition and utilization, were resumed.

Several intervals of time for the addition of hTf, as well as for subpassage of chlamydiae, were included in this experiment since (i) normal chlamydial development and the appearance of metabolically active RB occur in an asynchronous manner and (ii) electron microscopic examination showed that Desferal delayed chlamydial maturation. To simplify the presentation of these data, a schematic of the experimental design is shown in Fig. 2A. The results of the experiment, shown in Fig. 2B, confirmed that exposure of *C. trachomatis*-infected, polarized HEC-1B cells to 50 μM Desferal for 72 h caused a significant reduction in infectivity (Fig. 2B, compare samples 1 and 5; $P < 0.01$). Most importantly, the addition of hTf at 24 hpi led to a significant recovery of chlamydial infectivity at 72 hpi (Fig. 2B, compare samples 5 and 7), which was more dramatic at 96 hpi (compare samples 9 and 11; $P < 0.01$). There was no significant recovery of infectivity by replacement of the growth medium alone (Fig. 2B, compare samples 5 and 6 and samples 9 and 10; $P < 0.01$). Under normal growth conditions, *C. trachomatis* serovar E inclusions at 48 hpi contain a mixture of EB, RB, and intermediate forms; presumably, only the metabolically active RB would respond to the increased availability of iron. If hTf was added to infected cell cultures at 48 hpi, at least 48 h of further incubation was required for improved recovery of infectious EB (Fig. 2B, compare samples 8 and 12). The removal of cycloheximide also restored competition between chlamydiae and the host cell for metabolites (Fig. 2B, compare samples 1 and 2). Overall, these data suggest that the detrimental effects of Desferal on chlamydial viability and infectivity are due primarily to the withholding of iron from chlamydiae via the eukaryotic host cell.

Two-dimensional analyses of *C. trachomatis* serovar E EB proteins following iron limitation. Since it is well documented that iron restriction leads to the enhanced production of specific proteins, many of which are virulence factors in other bacteria, total protein profiles of highly purified EB from iron-sufficient and iron-limited polarized HEC-1B cells were analyzed by 2D-PAGE. All samples were adjusted by dilution to an equivalent particle-per-particle basis prior to resolution by 2D-PAGE. Since Desferal exposure appeared to alter the ultrastructural features of the chlamydial envelope, one might expect a higher quantity of outer membrane proteins, in particular, in EB purified from iron-limited than from iron-sufficient host cells by this method of comparison.

Representative 2D-PAGE phosphorimages of *C. trachomatis* ^{35}S-labeled EB proteins are presented in Fig. 3 A and B, and enlargements of high-molecular-weight proteins are provided in Fig. 4A and B. Total protein in EB purified from HEC-1B cells under normal growth conditions is shown in Fig. 3A and 4A, and EB protein from cells exposed to 50 μM Desferal is shown in Fig. 3B and 4B. For comparison, total cellular protein from mock-infected polarized HEC-1B cells, in the absence (Fig. 3C) or presence (Fig. 3D) of Desferal, was examined. The total mock-infected HEC-1B cell population was solubilized for 2D-PAGE since there was no detectable host cell material at the Renografin gradient interface where chlamydiae migrate during centrifugation and purification. Although a small degree of labeling was observed for mock-infected HEC-1B cells, even in the presence of 0.5 μg of cycloheximide per ml, there were no exact protein matches between the mock-infected host cells (Fig. 3C and D) and the purified *C. trachomatis* ^{35}S-labeled EB (Fig. 3A and B).

Densitometric values obtained by phosphorimaging confirmed that there was an average 1.5-fold increase in the quantity of the MOMP (40 kDa) in EB from Desferal-exposed cultures compared with that in EB from iron-sufficient cultures

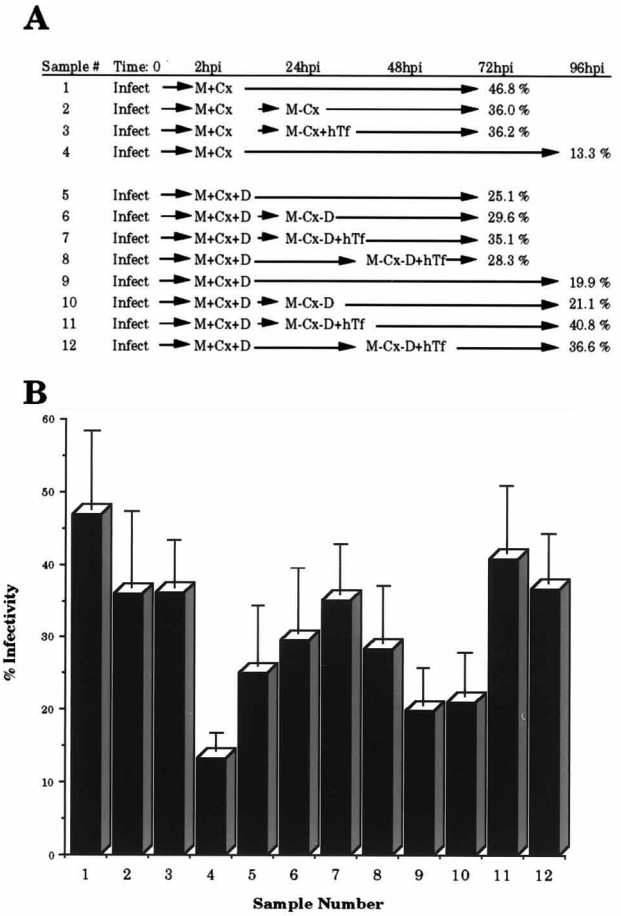

FIG. 2. Recovery of *C. trachomatis* serovar E infectivity, after Desferal exposure of infected polarized HEC-1B cells, by replacement of the culture medium with medium containing iron-saturated transferrin. (A) Schematic of the experimental design. M, DMEM-H (see Materials and Methods); Cx, 0.5 μg of cycloheximide per ml; hTf, 6 mg of hTf per ml; D, 50 μM Desferal. Percentages represent the average number of inclusions per 100 McCoy cells following direct passage of a 1:50 dilution of harvested chlamydiae (i.e., percent infectivity). (B) Presentation of the data in a bar graph format. The error bars represent 1 standard deviation of the data from each sample.

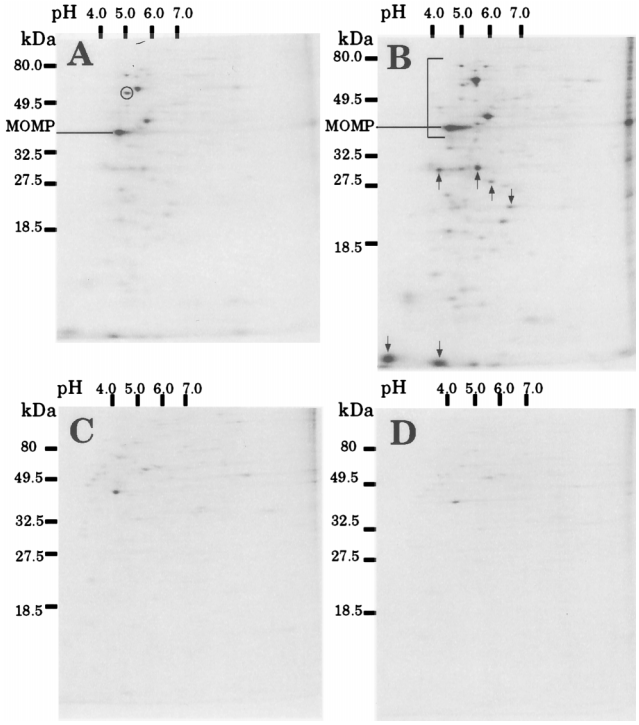

FIG. 3. Detection of putative iron-repressible proteins in purified *C. trachomatis* serovar E metabolically ^{35}S-radiolabeled EB by phosphorimage analysis of 2D-PAGE profiles. Purified EB were obtained from chlamydia-infected polarized HEC-1B cell cultures under normal growth conditions (A) and in the presence of 50 μM Desferal (B). Controls included mock-infected polarized HEC-1B cells unexposed (C) or exposed (D) to 50 μM Desferal for 72 h. Densitometric values of total EB proteins in panel B were compared with proteins in panel A; at least 15 proteins (6 designated by arrows and 9 contained within the bracketed area) were detected in EB from iron-restricted cultures (B) but were either absent or quantitatively threefold less in normal EB (A). One iron-inducible protein is illustrated in panel A (circle).

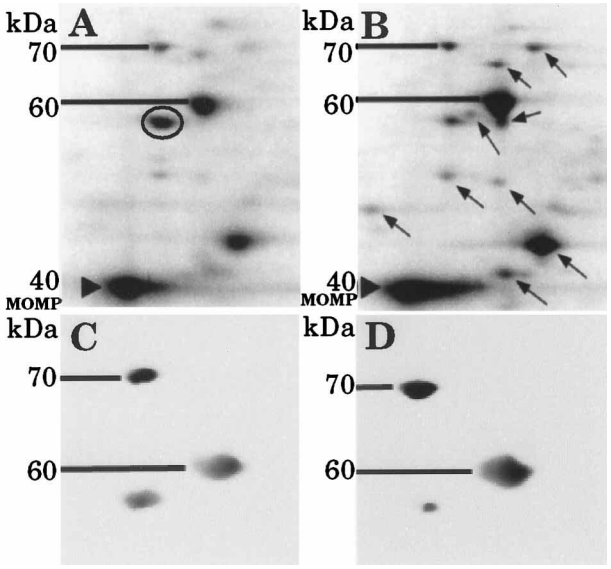

FIG. 4. High-molecular-weight putative iron-repressible proteins and confirmation of one such protein as the chlamydial heat shock protein 60 (hsp60) by Western blot analysis. Proteins were resolved by 2D-PAGE from purified *C. trachomatis* serovar E EB grown in polarized HEC-1B cells under normal conditions (A and C) or in the presence of 50 μM Desferal (B and D). (A and B) Enlarged profiles of ^{35}S-radiolabeled EB proteins detected by phosphorimaging. Iron-repressible proteins are indicated by arrowheads in panel B, and the iron-inducible protein is circled in panel A. (C and D) Western blot reactivity of the *C. trachomatis* hsp60 and hsp70 proteins following transfer of 2D-PAGE proteins to nitrocellulose, incubation with a mouse monoclonal antibody to hsp60 and a rabbit monospecific polyclonal antibody to hsp70, and detection by alkaline phosphatase-conjugated secondary antibodies.

(Fig. 4B and A, respectively). It is not yet known whether the MOMP is regulated by iron at the level of transcription, but for further protein comparisons, the quantity of MOMP was used as an internal control. The candidate iron-repressible chlamydial protein species highlighted in this study (Fig. 3B and 4B) were either (i) present in EB purified from Desferal-exposed cell cultures and absent or undetectable in EB purified from host cells under normal growth conditions or (ii) quantitatively threefold greater in EB from iron-restricted cultures than in EB from iron-sufficient cultures. By these criteria, there were at least 19 chlamydial proteins that exhibited enhanced expression due to Desferal-mediated iron limitation. Three proteins were detectable only by silver staining (data not shown). A summary of size and charge characteristics of the 19 putative iron-repressible chlamydial proteins is provided in Table 2. Interestingly, there was at least one chlamydial protein that was induced under iron-sufficient growth conditions (Fig. 3A; enlarged in Fig. 4A).

The 60-kDa protein. One chlamydial protein that showed a considerable increase in expression as the result of iron limitation was 60 kDa in mass with an acidic isoelectric point (pI) of 5.4 (Fig. 4B). These properties are characteristic of the chlamydial heat shock protein (hsp60) or GroEL (5, 8). Proteins resolved by 2D-PAGE were transferred to nitrocellulose for Western blot analysis to confirm the identity of this protein species. Blots were probed simultaneously with a monoclonal antibody directed against chlamydial hsp60 and monospecific antiserum directed against chlamydial hsp70, as a control, for proper orientation of the blots. The result, illustrated in Fig. 4C and D, confirmed that the 60-kDa protein was indeed the chlamydial hsp60 homolog. Although there was a slight increase in production of the hsp70 protein following iron limitation, the quantitative difference did not meet the criteria outlined above, and, thus, hsp70 was not highlighted as an iron-regulated candidate.

DISCUSSION

The data presented in this study (i) demonstrate that *C. trachomatis* serovar E development and infectivity are adversely affected by iron limitation and (ii) provide evidence for the existence of chlamydial proteins regulated by iron availability. It is known that iron chelation by Desferal in eukaryotic cells is exclusively intracellular and does not remove iron from serum-containing growth medium (29, 41), which was important in this study since HEC-1B cells were not adaptable to growth in serum-free medium. There is some evidence that Desferal may actually cause extrusion of iron from cells into the growth medium (20). One concern regarding the use of Desferal was a documented interference with ribonucleotide reductase activity (13). Iron is part of the active center of this enzyme which reduces ribonucleotide diphosphates to deoxyribonucleotide diphosphates. However, the chlamydiae do not transport deoxyribonucleotides from host cells; instead, these organisms are thought to have their own ribonucleotide reductase (32). Although it is still possible that Desferal compromises chlamydial development by alternative activities on iron-requiring host cell enzymes, the significant recovery of chlamydial infectivity by providing transferrin to the system

TABLE 2. Characteristics of candidate iron-repressible proteins in purified *C. trachomatis* serovar E EB[a]

Molecular mass (kDa)	pI
73[b]	5.2
69	5.8
66	5.4
60	5.4
59[c]	5.1
58[c]	5.5
52	4.9
51	5.5
48[b,c]	6.8
47[c]	4.1
44	5.9
42[c]	5.5
37[b,c]	>9.0
30	5.5
30	4.1
28	6.0
23	6.8
<15	<3.0
<15	4.2

[a] Proteins which were quantitatively threefold greater in EB purified from iron-restricted polarized HEC-1B cell cultures versus EB purified from normal iron-sufficient polarized HEC-1B cell cultures, as determined by densitometric values following phosphorimage analyses, are characterized.

[b] Protein detected by silver staining only.

[c] Protein absent or undetectable in EB harvested from iron-sufficient host cell cultures.

argues that the effects observed are due primarily to reducing iron availability to the chlamydiae.

There is only one additional study on the effect of the withholding of iron on chlamydial development. Murray et al. (37) used Desferal to restrict iron availability to *Chlamydia psittaci* growing in gamma interferon-activated macrophages. By staining and counting inclusions, these authors reported no difference in the numbers of inclusions that developed within iron-restricted and control macrophages. In the present study, there was also no difference in the absolute numbers of *C. trachomatis* inclusions that formed within iron-restricted and iron-sufficient epithelial cells. However, microscopic examination clearly illustrated that *C. trachomatis* inclusion development was impaired in the presence of Desferal. The observation that a higher number of EB particles was obtained from cultures exposed to 100 μM Desferal than from cultures exposed to 25 μM Desferal might suggest that if iron is severely limited, chlamydial development is prematurely terminated, leading to incomplete maturation or condensation of RB to normal infectious EB, whereas under less-extreme conditions of iron restriction, chlamydial development is merely delayed. The most crucial finding was that chlamydial infectivity was reduced as the removal of iron, by increasing the concentration of Desferal, was increased. Taken together, these observations provide a hypothesis that the spread of chlamydial infection in genital epithelial cells may be reduced under conditions of iron limitation. Although this study employed the use of a chemical chelator, evidence from other studies (26, 58) indicates that estradiol and progesterone modulate the sources of, and availability of, iron in endometrial tissue in vivo, respectively.

A decrease in chlamydial infectivity due to iron restriction might initially be viewed as favorable. However, the enhanced production of chlamydial hsp60 due to the withholding of iron raises considerable concern. This antigen provokes an inflammatory response in an animal model of ocular infection (35, 36), and patient antibody production to chlamydial hsp60 is associated with destructive sequelae such as pelvic inflammatory disease, infertility, and ectopic pregnancy (reviewed in reference 10). There is evidence for the existence of abnormally large, persistent chlamydial forms which may contribute to immune damage by providing a steady dose of chlamydial antigen (7). In an in vitro model of *C. trachomatis* persistence mediated by gamma interferon, expression of the hsp60 antigen remains steady while synthesis of other major antigens declines (6). Although unusual chlamydial inclusion development was observed in Desferal-exposed cells in this study, the underlying mechanism appears to differ from that of gamma interferon since (i) the morphology of RB under conditions of iron limitation did not resemble the persistent forms induced by gamma interferon and (ii) expression of the MOMP is increased. Regardless of the mechanism, increased synthesis of chlamydial hsp60 as a result of iron restriction may contribute to the destructive immune-mediated pathology of chlamydial disease.

In other bacteria, iron-repressible proteins are controlled at the level of transcription by a repressor similar to *Escherichia coli* Fur (ferric uptake regulator) protein. In the presence of sufficient iron, Fur protein multimers bind to the promoter region of iron-regulated genes with ferrous iron as a cofactor (30), and a 19-bp DNA Fur-binding consensus sequence has been established (17). For comparison, the upstream sequence regions of available chlamydial GroES/GroEL (hsp60) genes were examined for Fur-binding consensus sequences. The most convincing matches were observed in *C. psittaci* GPIC (35). Although such evidence is indirect, these sequences indicate that, like other bacteria, the chlamydiae may have transcriptional regulatory mechanisms for the expression of specific proteins under iron limitation. Studies to identify a chlamydial iron repressor are presently under way.

At least 19 putative iron-repressible proteins were observed for *C. trachomatis* serovar E by exposure of infected epithelial cells to Desferal. It is possible that additional iron-repressible proteins remain undetected. Alternative iron-chelating compounds have not yet been examined; agents such as α,α′-dipyridyl or phenanthroline, which are lipophilic and preferentially chelate iron from membrane-bound components, may reveal additional iron-repressible chlamydial proteins. Of the chlamydial protein species highlighted in this study, several merit further examination. For example, one protein is 37 kDa in size and has an unusually basic pI; the periplasmic ferric-binding protein (Fbp) of *N. gonorrhoeae* is 37 kDa in size and has a pI of greater than 9.35 (34). In addition, the transferrin- and lactoferrin-binding proteins in other bacteria are consistently 65 to 85 kDa or 100 kDa in size (21), and there are at least three chlamydial proteins with masses of 65 to 85 kDa that exhibit enhanced expression due to iron limitation. This study supports the finding of Scidmore and Hackstadt (47) that transferrin is at least one major source of iron for the chlamydiae. Whether transferrin is an exclusive source of iron or alternative complexes such as lactoferrin or heme provide iron to the chlamydiae is not yet known. Overall, the present study provides a foundation and the feasibility to further pursue specific components subject to iron regulation to better our understanding of the cellular microbiology of chlamydial infection.

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