Chlamydia trachomatis YtgA is an iron-binding periplasmic protein induced by iron restriction

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Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium that is the causative agent of common sexually transmitted diseases and the leading cause of preventable blindness worldwide. It has been observed that YtgA (CT067) is very immunogenic in patients with chlamydial genital infections. Homology analyses suggested that YtgA is a soluble periplasmic protein and a component of an ATP-binding cassette (ABC) transport system for metals such as iron. Since little is known about iron transport in *C. trachomatis*, biochemical assays were used to determine the potential role of YtgA in iron acquisition. ⁵⁹Fe binding and competition studies revealed that YtgA preferentially binds iron over nickel, zinc or manganese. Western blot and densitometry techniques showed that YtgA concentrations specifically increased 3–5-fold in *C. trachomatis*, when cultured under iron-starvation conditions rather than under general stress conditions, such as exposure to penicillin. Finally, immuno-transmission electron microscopy provided evidence that YtgA is more concentrated in *C. trachomatis* during iron restriction, supporting a possible role for YtgA as a component of an ABC transporter.

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INTRODUCTION

Chlamydia trachomatis is a Gram-negative intracellular pathogen that is the causative agent of the most common sexually transmitted bacterial infections, leading to 4 million diagnosed cases per year in the United States (Adderley-Kelly & Stephens, 2005). It is also the primary cause of preventable blindness in the developing world, responsible for an estimated 5.9 million cases of blindness or severe vision loss (Thylefors *et al.*, 1995; West, 2004). Genital infections can progress to a chronic stage that could result in pelvic inflammatory disease in women, fertility problems, ectopic pregnancy, and possible sterility in a portion of the infected population (Gray-Swain & Peipert, 2006). The insidiousness of these infections is due, in part, to the fact that these obligate intracellular bacteria have evolved a sophisticated biphasic developmental cycle within their eukaryote host cells (Abdelrahman & Belland, 2005). This cycle alternates between metabolically inert, infectious elementary bodies (EB), which can attach to and enter targeted host epithelial

cells (Dautry-Varsat *et al.*, 2005; Hackstadt, 1999), and metabolically active reticulate body forms (RB) (Abdelrahman & Belland, 2005), protected within an inclusion that utilizes host organelles and transport mechanisms to reroute metabolites and macromolecules required for replication to the inclusion (Scidmore, 2006).

During infection, *C. trachomatis* can transition into persistence, defined as a viable but non-cultivable enlarged RB growth stage, resulting in a long-term relationship with the host cell. Researchers have found that *C. trachomatis* can be diverted to enter a state of persistence *in vitro* by the addition of antibiotics or the cytokine interferon γ , or by starving the organisms of essential nutrients, such as amino acids or iron (Beatty *et al.*, 1994; Hogan *et al.*, 2004). When organisms enter the persistent state, the RB do not appear to form complete septa during division; rather they form enlarged, pleomorphic RB that are inhibited in binary fission and differential maturation into EB (Hogan *et al.*, 2004; Raulston, 2006). In the case of iron-restricted persistence, once the iron-limited conditions have been removed, the aberrant organisms recover and return to normal metabolically active RB (Raulston, 1997).

At this time, we have almost no information on how *C. trachomatis* transports or utilizes iron (Raulston, 2006).

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Abbreviations: RB, reticulate bodies; EB, elementary bodies; ABC, ATP-binding cassette; h.p.i., hours post-infection; PAR, 4-2-pyridylazoresorcinol monosodium salt hydrate; Desferal, deferoxamine mesylate; TEM, transmission electron microscopy.

The genome lacks genes homologous with those encoding proteins involved in known siderophore biosynthesis pathways (Stephens *et al.*, 1998). The genome does suggest that *C. trachomatis* has a need for iron, encoding systems such as a complete respiratory chain that requires iron for activity (Raulston, 2006; Stephens *et al.*, 1998). Due to the reaction of RB to iron restriction and the evidence for an iron requirement in its genome, it is presumed that iron is important for the survival and pathogenesis of *C. trachomatis*, as it is for virtually all bacteria (Raulston, 2006).

In this study, we have investigated *C. trachomatis* serovar E YtgA (CT067), a protein with strong homology to periplasmic proteins in ATP-binding cassette (ABC) transport systems. Two different metal-binding techniques identified iron as the preferred metal for YtgA, a finding supported by competitive binding assays. Further, YtgA is increasingly expressed under iron-restricted conditions. Monospecific polyclonal rabbit serum was generated from purified recombinant chlamydial YtgA and used to label *C. trachomatis* YtgA proteins within fixed, infected HEC-1B samples. Transmission electron microscopy (TEM) coupled with immunogold labelling provided evidence that YtgA is more concentrated in the chlamydial RB periplasm during normal growth conditions. Western blotting of samples collected from iron-restricted and penicillin-exposed cultures showed that YtgA is upregulated to a greater extent under iron restriction than under another inducer of persistence. Our results identify YtgA as an iron-binding protein that is influenced specifically by iron availability, and may be part of an ABC transporter system.

METHODS

Bacterial strains and cell lines. Experiments were performed using a human urogenital isolate of *C. trachomatis* serovar E/UW-5/CX, originally obtained from S. P. Wang and C.-C. Kuo (University of Washington, Seattle, WA, USA), that has been maintained for years in our laboratory. *C. trachomatis* EB were harvested, titrated for infectivity, and stored as described previously (Guseva *et al.*, 2007; Tam *et al.*, 1992; Wyrick *et al.*, 1996). The human endometrial epithelial cell line HEC-1B (HTB-113; ATCC) was propagated at 37 °C in a humidified 5% CO₂ atmosphere in Minimal Essential Medium (MEM, Invitrogen) supplemented with 2 mM glutamine (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Hyclone).

Isolation and purification of recombinant YtgA protein. *C. trachomatis* serovar E *ytgA* was cloned into the expression vector pBAD/HisA (Invitrogen) and sequence fidelity verified by DNA sequencing. The resulting protein expression plasmid (pJER009) was grown in *Escherichia coli* TOP10 to an OD₆₀₀ of 0.6, and then induced with 0.002% arabinose to express recombinant YtgA, following the manufacturer's instructions (Invitrogen). After induction, the bacteria were pelleted, washed in warm PBS and resuspended in a small volume of PBS. The bacteria were lysed by French cell press (Thermo Fisher Scientific), and the lysate was cleared of membrane debris by centrifugation at 22 000 g for 30 min. The clarified lysate was treated with increasing concentrations of ammonium sulfate (10, 20, 30, 40, 60 and 80% saturation), with the resulting precipitates

collected and resuspended in PBS. Otherwise, isolation and purification of recombinant polyhistidine-tagged YtgA from induced, pelleted *E. coli* was completed using the B-PER 6xHis Spin Purification kit (Pierce). Each fraction was dialysed overnight in a 3.5K MWCO Slide-A-Lyser cassette (Pierce) at 4 °C in PBS. Equal concentrations of protein (determined by BCA assay; Pierce) were resolved on small format 12% Bistris NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (Whatman). Membranes were probed with mouse anti-polyhistidine monoclonal antibodies and visualized using Western Blue stabilized substrate for alkaline phosphatase (Promega). The YtgA-containing fractions were pooled, resolved on large-format 4–12% SDS-PAGE protein gels, and the gel slice containing YtgA was cut from the gel. The purified recombinant YtgA was electroeluted, concentrated by lyophilization, and resuspended in PBS to a final concentration of 2 mg ml⁻¹.

Generation of primary sera. The purified recombinant YtgA was used by Sigma-Genosys to generate polyclonal antibody sera. Two New Zealand White rabbits were pre-bled and injected on days 0, 14, 28, 42, 56 and 70 with the provided recombinant YtgA. The rabbits were periodically bled by Sigma-Genosys (days 49, 63 and 77), and the sera were tested for reactivity by Western blotting. The serum with the highest affinity and lowest avidity for *C. trachomatis* YtgA was treated with the ImmunoPure IgG kit (Pierce) to remove non-specific antibodies. The resulting monospecific polyclonal anti-YtgA rabbit serum was used in all Western blot and immunogold labelling techniques.

Metal binding assay for YtgA. A high-throughput metalloprotein assay has previously been described by Hogbom *et al.* (2005). Briefly, 20 µl 8 M urea was added to the wells of a non-transparent 96-well plate with optical bottom (NUNC). PBS containing 1 nM, 0.5 nM or 0 nM solutions of ferric chloride (Fe), manganese chloride (Mn), nickel chloride (Ni) or zinc chloride (Zn), were added to control wells. Purified recombinant YtgA (10 µg per well) was added to experimental wells. A Modulus Microplate Luminometer (Turner Biosystems) was used to take an initial background reading of the 96-well plate, with an integration time of 0.5 s. Next, 50 µl luminol solution [11 mM luminol, 500 mM NaCO₃, 230 mM H₂O₂ (Chelex-treated, filtered)] was injected into the appropriate control and experimental wells. Sample luminescence was read at an integration of 0.5 s, at 5 s and 1 min time-points following luminol injection. Then, 25 µl of the metal chelator 4-(2-pyridylazo)resorcinol monosodium salt hydrate (PAR) was injected into the appropriate control and experimental wells, and the luminescence was read at the same integration as described above following PAR injection. After 2 h incubation at room temperature, plates were centrifuged at 3000 g for 2 min, and the absorbance was read at 492 nm using a Spectramax microtitre plate reader (Molecular Devices).

⁵⁹Fe binding and competition assays for recombinant YtgA proteins. The ⁵⁹Fe binding assay was performed as described by Suyama *et al.* (2006). Briefly, 50 µg of either recombinant YtgA, human transferrin (Sigma-Aldrich) or BSA was suspended in 0.5 ml Tris/HCl buffered saline. The proteins were mixed with 6.6 µl ⁵⁹FeCl₃ (92 500 Bq ml⁻¹; Perkin Elmer). After a 10 min incubation at 37 °C, the protein and supernatant fractions were separated on Microcon YM-10 filter devices (Millipore). The radioactivity of the protein and supernatant fractions from each sample was determined on an LS6500 scintillation counter (Beckman). Competition assays were performed by adding 6.6 µl non-radioactive FeCl₃, ZnCl₂, or MnCl₂ (each 25 µmol ml⁻¹) to the reaction mixtures prior to incubation. The results are expressed as the percentage of ⁵⁹Fe in the protein or supernatant fraction (Suyama *et al.*, 2006).

In vitro YtgA protein concentration in normal and iron-limited conditions. HEC-1B cells were grown in 150 cm² tissue culture flasks

(Corning) at 35 °C under a humidified 5% CO₂ atmosphere until the cells reached 90% coverage of the flask surface. Host cells were inoculated with a diluted titre of *C. trachomatis* EB crude stock, determined to yield at least 80% infected cells (80% Ct titre). After 1 h incubation at 35 °C, the cell monolayers were washed with PBS, then top-off medium [MEM supplemented with 10% FBS, 2 mM glutamine, 0.5 µg cycloheximide ml⁻¹ and 25 µg gentamicin ml⁻¹ (Sigma-Aldrich)] was added to each flask (referred to as time 0), and the infected flasks were moved back to 35 °C. At 23 hours post-infection (h.p.i.), flask cultures were either mock-exposed or exposed to 50 µM deferoxamine mesylate (Desferal). At 0, 24, 36, 48, 60 and 72 h.p.i., mock-exposed or Desferal-exposed infected HEC-1B cells were scraped into 2.5 ml PBS. Cell samples were denatured, and equal concentrations of proteins from each sample (determined by BCA assay) were resolved on 12% (v/v) Bistris NuPAGE gel cassettes (Invitrogen). Proteins were transferred to nitrocellulose membranes (Whatman), probed with primary anti-YtgA rabbit serum, and detected with the Super Signal West Pico chemiluminescent kit (Pierce Biotechnology) and Hyclone CL film (Denville Scientific). In addition, β-actin was used as a loading control and probed using monoclonal anti-β-actin antibodies (mAb 1501; Millipore). Densitometry of the YtgA bands was performed using Quantity One v. 4.5 (Bio-Rad). Data were graphed on Graphpad Prism v. 4. (Graphpad Software).

YtgA induction in normal, iron-restricted, and penicillin G-induced persistent *C. trachomatis*. HEC-1B cells were cultured in 150 cm² tissue culture flasks (Corning) at 35 °C in a humidified 5% CO₂ atmosphere, until the cells reached 95% coverage of the flask surface. Host cells were inoculated with 80% Ct titre, and incubated at 35 °C for 1 h. After the incubation period, the cell monolayers were washed with PBS, and then top-off medium (as described above) was added to each flask (time 0). The infected cultures were incubated at 35 °C in 5% CO₂. At 5, 11, 17 and 23 h.p.i., samples were mock-exposed, exposed to 500 µM Desferal, or exposed to 20 U penicillin G ml⁻¹. At 0, 6, 12, 18 and 24 h.p.i., flasks of HEC-1B cells from each experimental condition were scraped into 2.5 ml PBS. Samples were denatured in Laemmli 2 × sample buffer (Roskams & Rodgers, 2002), and total protein concentration from each sample was established using the BCA assay. The proteins were resolved, transferred to nitrocellulose or PVDF, and detected by Western blotting using the Super Signal West Pico chemiluminescent kit and Hyclone CL film. Resulting films were scanned, and YtgA band densitometry was performed using Quantity One v. 4 software. Results (expressed as counts per mm²) were graphed using Graphpad Prism v. 4.

Immunogold labelling of YtgA in *C. trachomatis*. Polarized HEC-1B cells grown on Transwell polycarbonate tissue culture inserts (Costar) were infected with *C. trachomatis* by adsorption for 2 h, and subsequently incubated at 35 °C in Dulbecco's Modified Essential Medium with high glucose (Invitrogen) containing 2 mM glutamine, 5% heat-inactivated FBS, 0.5 µg cycloheximide ml⁻¹, and with or without 50 µM Desferal (Raulston, 1997). At 48 h.p.i., samples were washed, processed, and embedded in Lowicryl (Polysciences) for immunoelectron microscopy, as described previously (Giles *et al.*, 2006; Wyrick *et al.*, 1994). Thin sections were mounted on Formvar-coated gold grids (EM Sciences), and the primary anti-*C. trachomatis* YtgA rabbit serum was diluted 1:10 or 1:50 and visualized with a 1:200 dilution of 15 nm or 5 nm gold-conjugated second-affinity antibodies (Amersham Biosciences). Samples were post-stained using a 4% (w/v) uranyl acetate solution in 50% (v/v) ethanol. Electron micrographs were taken on a Tecnai 10 transmission electron microscope (Philips) with an accelerating voltage of 80 kV. Immunogold-labelled YtgA (both 5 and 15 nm gold particles) were enumerated from random micrographs. Results were graphed using GraphPad Prism v. 4.

Statistics. Unpaired two-tailed *t*-tests were performed in conjunction with *F* tests for all statistical analyses using Graphpad Prism v. 4. A *P*-value less than 0.05 was considered statistically significant. All values are expressed as mean ± SEM.

RESULTS

Sequence analysis of YtgA (CT067)

The confirmed amino acid sequence of YtgA from *C. trachomatis* serovar E, deduced after DNA sequencing (GenBank accession DQ164839; Raulston *et al.*, 2007), was used to examine the predicted secondary structure of the protein using SOSUI (Mitaku group). The SOSUI program predicted that the protein had the hydrophilic motifs of a soluble protein. A 28 aa signal sequence was also identified at the protein's N terminus (underlined sequence in Fig. 1). When the YtgA amino acid sequence was used to perform a conserved domain search (NCBI), our submitted sequence was found to be highly homologous to tyrosine-rich metal-binding motifs of ABC transporter periplasmic proteins (boxed sequences in Fig. 1). These metal-binding motif sequences strongly resembled iron-, zinc- and manganese-binding sites from the ABC transporter periplasmic binding proteins TroA from *Treponema pallidum* (Hardham *et al.*, 1997) and YfuA from *Yersinia pestis* (Gong *et al.*, 2001).

YtgA has iron-binding capabilities

As the sequence of YtgA indicated conserved metal-binding motifs, we sought to determine which metals were candidates for binding. We evaluated the ability of YtgA to bind iron, nickel, manganese and zinc, based on a metalloprotein binding assay described by Hogbom *et al.* (2005). This assay utilized luminescence and absorbance to track the behaviour of certain metal salts in the presence of luminol and the metal chelator PAR. The assay was performed on YtgA, which was compared with the behaviour of individual metal salt controls (Fe, Mn, Ni, Zn), to determine candidates for any metal(s) that may bind to the protein (Table 1). The YtgA luminescence and

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MSFFHTRKYKLILRGLLCLAGCFMLNSCSSSRGNQPADESIYVLSMNRMICDCVS
RITGDRVKNIVLIDGADPHSYEMVKGDGEDRMAMSQILFCNGLGLEHSASLRKHL
EGNPVKVVDLGQRLLNKNCFDLLEEFGFDPHWTDMRVWGA AVKEMAAALIQQ
FPQYEEFQKNADQILSEMEELDRWAARSLSIPEKNRYLVTHNAFSYFTRRYL
SSDAERVSGEWSRCSPEGLAPEAQISIRDIRRVVEYISANDVEVFLEDTLNQDA
LRKIVSCSKSGQKIRLAKSPLYSDNVCDNYFSTFQHNYRTITEELGGTVLE
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Fig. 1. Analysis of the deduced amino acid sequence of YtgA from *C. trachomatis* serovar E. The amino acids corresponding to metal-binding domains are boxed. The 28 aa signal sequence is underlined. Alignments were performed using NCBI Conserved Domain Search.

Table 1. Metal-binding luminescence assay

Metal salts FeCl_3 , MnCl_2 , NiCl_2 and ZnCl_2 were used as controls. Luminescence was measured before the addition of luminol (background), after the addition of luminol, and after the addition of the metal chelator PAR to each well. Results are expressed as the change of arbitrary luminescence units from the previous sample manipulation. Absorbance was measured at 492 nm.

	Background	Change from background after addition of luminol	Change from luminol after addition of PAR	A_{492}
YtgA	1.13×10^5	3.02×10^7	-2.95×10^7	0.313
Iron	2.21×10^4	6.26×10^7	-6.25×10^7	0.350
Manganese	1.83×10^4	2.25×10^7	5.46×10^7	0.993
Nickel	2.69×10^4	1.16×10^8	-1.16×10^8	0.486
Zinc	2.15×10^5	1.71×10^8	-1.71×10^8	0.589

absorbance data most closely resembled the iron control data, indicating that iron may bind to YtgA. The other metals tested exhibited data trends that were too dissimilar to the YtgA experimental data to suggest that they bound to YtgA.

Binding and competition assays of YtgA using ^{59}Fe

To confirm the observations from the metalloprotein assay, an *in vitro* YtgA binding assay, using ^{59}Fe ($^{59}\text{FeCl}_3$) as the substrate, was employed. When incubated with YtgA in a reaction mixture, over 92 % of the ^{59}Fe was detected in the protein fraction of three separate experiments (Fig. 2a). Radiolabelled iron also bound strongly to the positive control, human transferrin, which retained over 92 % of the ^{59}Fe in the assay. The YtgA binding of ^{59}Fe was

significantly greater than the negative control, BSA, which only retained 8 % of the available ^{59}Fe in the protein fraction.

We further analysed the strength of iron binding to YtgA by performing competition assays using non-radiolabelled metals. The recombinant YtgA protein mixtures were pre-treated with solutions of unlabelled FeCl_3 , ZnCl_2 , or MnCl_2 (each $25 \mu\text{mol ml}^{-1}$) for 5 min before the addition of ^{59}Fe (Fig. 2b). When unlabelled iron was present in the reaction mixture, the amount of ^{59}Fe bound to the recombinant YtgA dropped significantly – over 80 % of the ^{59}Fe was in the supernatant fraction. This result was statistically significant ($P < 0.0001$). The fact that the unlabelled iron competed with or blocked the ^{59}Fe from binding the YtgA further supported our hypothesis that YtgA is an iron-binding protein. Unlabelled zinc or manganese had no

Fig. 2. Binding studies of YtgA utilizing radiolabelled ^{59}Fe . The metal-binding preference of recombinant YtgA was examined using (a) ^{59}Fe binding experiments and (b) competitive binding assays. The white bars signify percentage of ^{59}Fe retained by the protein fraction, and the black bars signify the percentage of ^{59}Fe in the supernatant fraction. BSA, bovine serum albumin; YtgA, recombinant YtgA and ^{59}Fe with no competitor; YtgA+(metal), recombinant YtgA and ^{59}Fe mixed with unlabelled iron (Fe), manganese (Mn) or zinc (Zn). Each bar represents the mean $\pm$ SEM of three separate experiments. Graphs were constructed and SEM calculated in Graphpad Prism v. 4.

significant effect on the binding efficiency of radiolabelled iron to YtgA (86 % binding in the presence of Zn; 90 % binding in the presence of Mn; Fig. 2b), despite the higher concentration of the unlabelled metals in the reaction mixture. These data confirmed that YtgA had a significantly higher preference for iron compared to other metals in the same solution.

Specificity of the anti-chlamydial YtgA primary rabbit serum

Protein gels and Western blots were performed to confirm the specificity of the monospecific polyclonal rabbit serum generated commercially against purified *C. trachomatis* serovar E hexahistidine-tagged YtgA. The serum positively identified the recombinant YtgA in arabinose-induced *E. coli* JER009 lysate (Fig. 3a, b, lane 2), purified recombinant YtgA at 37 kDa (Fig. 3a, b, lane 3), and YtgA in a *C. trachomatis* serovar E lysate (Fig. 3a, b, lane 4). Uninfected HEC-1B cells served as both a negative control and a non-specific binding control (Fig. 3a, b, lane 5). There was no visible background binding of the rabbit serum to uninfected HEC-1B protein extract (Fig. 3b, lane 5), confirming the high affinity and very low avidity of the primary rabbit serum to be used for TEM and Western blot techniques.

Increase in YtgA concentration by iron restriction

To examine YtgA concentrations throughout the chlamydial developmental cycle, HEC-1B cells infected with *C. trachomatis* serovar E were either mock-exposed or exposed to the iron chelator Desferal (50 μ M) at 23 h.p.i. Desferal was previously documented to reduce the epithelial cell intracellular ferritin level significantly (Dill *et al.*, 2009). Protein samples from both cell culture growth conditions were removed throughout the chlamydial developmental cycle (0, 24, 36, 48, 60 and 72 h.p.i.), and equal protein concentrations from mock-exposed or Desferal-exposed samples at all time-points were resolved

on protein gels. The YtgA bands were labelled and visualized by Western blot methods. It was noted first that the primary rabbit serum used to label the *C. trachomatis* YtgA did not show non-specific background binding when the negative control was examined (Fig. 4a, HEC lane). *C. trachomatis* organisms exposed to 50 μ M Desferal at 23 h.p.i. displayed an increase in YtgA protein after 1 h (Fig. 4a, 24(+) lane) as compared to mock-exposed organisms (Fig. 4a, 24(-) lane). This result indicates a relatively quick induction of YtgA after the bacteria experience iron restriction. Further, YtgA protein concentrations appeared to be higher at every time in the iron-restricted (+) cultures, suggesting that iron has a direct effect on native YtgA concentration. Densitometry (Fig. 4b) was also employed to measure each YtgA band from each Western blot and compare the relative concentration in each sample. From 36 h.p.i. onwards, *C. trachomatis* grown in iron-limited conditions exhibited at least three- to fivefold higher YtgA protein concentrations than *C. trachomatis* from mock-treated cell cultures. β -Actin, used as a loading control for Western blotting, remained constant throughout 48 h.p.i., but decreased by 72 h.p.i. under both treatment conditions.

YtgA protein expression during the early *C. trachomatis* developmental cycle, under normal, iron-limited and penicillin G cell culture conditions

While YtgA was previously detectable at 24 h.p.i., expression at earlier time-points had not been examined. *C. trachomatis*-infected HEC-1B cells were cultured under normal conditions until an hour before proteins were harvested. At that time (5, 11, 17 and 23 h.p.i.), cultures were either exposed to 500 μ M Desferal, mock-exposed with PBS, or exposed to 20 U penicillin G ml^{-1} (Fig. 5). Under these experimental conditions, YtgA was not detected above background noise until 18 h.p.i. At 18 h.p.i., the YtgA protein concentration in the iron-restricted cultures was increased 2.4-fold, as compared to

Fig. 3. Specificity of the anti-*C. trachomatis* YtgA monospecific polyclonal rabbit serum. Protein samples resolved by PAGE (a) followed by Western blotting (b) were used to examine the specificity of the commercially generated rabbit serum. Lanes: 1, molecular mass protein standards (15 μ g); 2, *E. coli* JER009 lysate containing induced recombinant YtgA (0.5 μ g); 3, purified recombinant YtgA (1 μ g); 4, *C. trachomatis* bacterial lysate (20 μ g); 5, uninfected HEC-1B cell lysate (20 μ g). The 37 kDa molecular mass standard is indicated.

Fig. 4. YtgA concentration differences in *C. trachomatis*-infected HEC-1B cells during normal or iron-restricted growth. Western blot (a) and densitometry (b) methods were used to analyse the concentration of YtgA protein in samples at various times (h.p.i.) in the developmental cycle. Samples were either mock-exposed (–) or exposed to 50 μM Desferal (+). Lanes were loaded with 20 μg protein per well. β -Actin served as a loading control. Densitometry results are from one representative experiment. M, protein standards; Ct, *C. trachomatis* bacterial lysate; HEC, uninfected HEC-1B cell lysate.

mock-exposed or penicillin G-exposed samples. This trend continued at 24 h.p.i., where iron-restricted cultures again displayed greater concentrations of YtgA protein when compared to mock-exposed or penicillin G-exposed cultures. These results imply that YtgA concentration increased specifically in response to iron limitation and not to other stressors, such as penicillin.

Identification of YtgA in normal *C. trachomatis* cell culture by TEM

The anti-chlamydial YtgA rabbit serum was employed as the primary antibody to label native YtgA in *C. trachomatis* serovar E-infected cells by immunoelectron microscopy. All of the TEM photomicrographs displayed (Fig. 6a–g) are

Fig. 5. YtgA concentrations in the early developmental cycle of *C. trachomatis*, cultured under normal, iron-restricted, or penicillin G-exposed conditions. Lanes were loaded with 10 μg protein per well. YtgA protein samples under each growth condition are labelled and shown from the Western blot in the boxes above the appropriate columns. β -Actin loading controls are also shown. Densitometry results of the YtgA bands are from one representative experiment. Black bars represent normal conditions (N); light grey bars represent iron-restricted growth conditions (I); dark grey bars represent penicillin G exposure (P).

Fig. 6. Ultrastructural location of YtgA proteins in normal (a–c) or iron-restricted (d–g) conditions. (a), (b), and (d–f) are labelled with antibody-conjugated 15 nm gold particles; (c) and (g) are labelled using antibody-conjugated 5 nm gold particles. Black arrowheads highlight immunogold particles. White arrowheads highlight the outer membrane (OM), inner membrane (IM) and periplasm (P) in (a). Bars: 100 nm (a, b), 500 nm (c–g). Each micrograph is representative of numerous micrographs examined.

illustrative examples that are representative of results observed from examining nearly 200 micrographs of *C. trachomatis*-infected HEC-1B cells. The YtgA proteins expressed in normal *C. trachomatis* serovar E bacteria in host cell inclusions, visualized by 15 nm gold particles conjugated to the mouse anti-rabbit secondary antibody (Fig. 6a–c), appeared to localize primarily in the periplasm of the RB at 48 h.p.i.; lower concentrations of labelled YtgA were observed in the cytoplasm. The YtgA proteins did not appear to be localized exclusively to either the outer or inner membrane, but were found equally throughout the periplasm. These data support the SOSUI solubility prediction and suggest that YtgA is likely untethered when in the periplasm.

Further labelling experiments were performed utilizing 5 nm gold particle-conjugated secondary antibodies for finer resolution and to differentiate between the inner membrane, periplasm and outer membrane areas of the

bacteria, since 15 nm gold particles were large enough to occasionally obscure the protein location. Total 5 nm gold particles were enumerated and categorized based upon their location within the cell (Fig. 7a). The gold particles were further sorted by chlamydial developmental form. To expedite gold particle counting, an RB was defined as any chlamydial body larger than 500 nm in diameter, and an EB was considered to be any chlamydial body smaller than 500 nm in diameter. In reality, intermediate bodies (IB) undergoing development may be included in either category based solely upon their size. Under normal conditions, the mean percentages of total gold particles visualized in the four designated RB locations were: 38 % cytoplasm, 32 % inner membrane, 12 % periplasm and 16 % outer membrane (Fig. 7a). By comparison, the distribution of gold particles localized to EB under these conditions was: 30 % cytoplasm, 31 % inner membrane, 3 % periplasm and 36 % outer membrane (Fig. 7a).

Fig. 7. Localization of YtgA in EB and RB during normal or iron-restricted growth conditions. Each bar represents the mean number of gold particle-bound YtgA per morphological area of *C. trachomatis* serovar E grown in (a) normal conditions ($n=4$) or (b) iron-restricted conditions ($n=3$). Graphs were constructed in Graphpad Prism v. 4. Error is expressed as SEM. IM, inner membrane; OM, outer membrane.

Increase of YtgA protein in iron-restricted persistent RB

Immunogold-labelled samples of *C. trachomatis*-infected host HEC-1B cells at 48 h.p.i., grown under iron-restricted conditions (50 μ M Desferal), were examined by TEM to determine if iron restriction affects where YtgA localizes in enlarged polymorphic RB. During the initial stages of iron restriction, the RB were observed to be in the process of septum formation (Fig. 6d), but could not complete division. YtgA could be found in the periplasm in these 'pre-aberrant RB', but was also found in the cytoplasm with greater regularity. Once *C. trachomatis* reached the typically enlarged, pleomorphic RB stage of persistence (Fig. 6e–g), most of the YtgA remained in the cytoplasm and the concentration in the periplasm dropped significantly. Enlarged pleomorphic RB also appeared to have a greater concentration of total YtgA proteins (5.14 ± 2.14 particles per organism) than RB grown under normal conditions (1.69 ± 0.84 particles per organism), when the 15 nm gold particles were tallied. These results further confirm that YtgA is upregulated under iron-limited conditions.

Again, using 5 nm gold particles to determine more accurately where YtgA localizes in iron-restricted conditions (Fig. 6g), the mean percentages of gold particles enumerated in RB were: 59% cytoplasm, 22% inner membrane, 10% periplasm and 15% outer membrane (Fig. 7b). The cytoplasmic gold particle count in pleomorphic RB represented an increase of YtgA within the cytoplasm of affected bacteria when compared to bacteria cultured under normal growth conditions (Fig. 6a, b). Under iron-restricted conditions, the mean percentages of gold particles labelling EB were: 14% cytoplasm, 30% inner membrane, 28% periplasm and 26% outer mem-

brane (Fig. 7b). There was not an increase in cytoplasmic YtgA in the iron-restricted EB compared to iron-restricted pleomorphic RB, as seen under normal growth conditions (Fig. 7a). Furthermore, there was an increase of YtgA in the periplasm of iron-restricted EB versus the periplasm of EB under normal conditions.

DISCUSSION

Iron is an essential cofactor of many enzymes due to its redox potential (Visca *et al.*, 2002), and is required by bacteria for a functional respiratory chain, tricarboxylic acid cycle and oxidative defence systems. In eukaryotic cells, iron in the cytosol is targeted to: (i) proteins and enzymes for immediate use, (ii) specific RNA structures for iron-regulation, and (iii) storage in the protein shell of ferritin. Outside of the cell, iron is tightly sequestered in lactoferrin, transferrin and haemoglobin (Raulston, 2006). It is noteworthy that mammalian hosts do not have sufficient free iron to support bacterial growth (Hanks *et al.*, 2005; Shouldice *et al.*, 2005). If left unbound, iron can catalyse the production of toxic oxygen and hydroxyl radicals, leading to damage of lipids, proteins and nucleic acids (Andrews *et al.*, 2003; Braun & Braun, 2002; Clarke *et al.*, 2001). Bacteria avoid the detrimental effects of iron by limiting its availability, restricting uptake, and sensing adequate iron levels (Clarke *et al.*, 2001). In addition, new findings report that *C. trachomatis* responds to limited iron availability by increasing expression of proteins involved in protection against oxidative damage (Dill *et al.*, 2009). *C. pneumoniae* protein levels under iron-limited conditions have also been examined, showing that iron stress induced observable changes in expression in at least six proteins (Wehrl *et al.*, 2004). Bacterial strategies to obtain iron from

the environment during iron restriction include reduction of ferric to ferrous ions, direct acquisition of iron from iron-binding proteins, and the uptake of iron from ferric siderophores (Braun & Braun, 2002; Visca *et al.*, 2002).

C. trachomatis has been shown to transport substrates into its cytoplasm or move substrates from the host cell into the inclusion to supply its nutritional needs, including purine nucleotides, uracil, sphingomyelin and glycoproteins (Hackstadt *et al.*, 1995; Hatch *et al.*, 1982; Scidmore *et al.*, 1996; Stephens *et al.*, 1998; Tipples & McClarty, 1993; Tjaden *et al.*, 1999). Like most pathogenic organisms, *C. trachomatis* also appears to be dependent upon the host cell for iron, due to a need implied by its genome, and by the persistent-like state that the RB enter in iron-restricted conditions (Raulston, 1997). *C. trachomatis* may obtain its required iron from ferritin, the large macromolecular protein complexes used by eukaryotic cells to store iron and protect themselves from rampant production of oxygen radicals (Raulston, 2006). However, multiple elegant experiments by Scidmore *et al.* (1996) have clearly demonstrated that labelled transferrin–transferrin receptor-containing early endosomes are juxtaposed to but not fused with EB-containing endosomes; furthermore, there was no evidence that transferrin was released into the lumen of the developing chlamydial inclusion.

Other bacteria use compounds called siderophores to remove iron from iron-storing macroproteins; no homologues to siderophore biosynthesis pathway genes have been identified in the *C. trachomatis* genome (Raulston, 2006; Stephens *et al.*, 1998), nor was there evidence of YtgA secretion in this study (unpublished data). *C. trachomatis* also appears to be missing a TonB analogue (Raulston, 2006), which would span the periplasm and is crucial in energy transfer to substrate-specific outer-membrane transporters used to bring iron–siderophore complexes into the periplasm of Gram-negative bacteria (Postle & Kadner, 2003). Considering these apparent gaps in the genome, one could speculate that *C. trachomatis* would need a reductase on the inclusion membrane to transport ferrous iron from the eukaryotic cytosol into the inclusion.

ATP-binding cassette (ABC) transport systems play a role in the acquisition of iron and iron-complexes, amino acids, sugars, peptides and other essential nutrients (Clarke *et al.*, 2001). They consist of a soluble periplasmic protein, a transmembrane protein permease composed of one or more gene products, and an ATP-binding lipoprotein (Clarke *et al.*, 2001; Hanks *et al.*, 2005). The soluble periplasmic protein binds the targeted molecule and changes conformation to close around the substrate (Clarke *et al.*, 2001). The periplasmic binding protein then moves to and binds the transmembrane protein permease in a receptor–ligand mechanism (Clarke *et al.*, 2001). ATP binds to the ATP-binding lipoprotein, creating a conformational change in the permease complex that transports the substrate into the cytoplasm (Higgins & Linton, 2004; Jones & George, 2002). In the case of iron, molecules

are bound to and coordinated in the periplasmic binding protein by a combination of water, phosphate and a group of amino acids including histidine, tyrosine, and glutamic acid (Shouldice *et al.*, 2005). In other pathogenic bacteria, ABC transport systems that transport iron, zinc and manganese into the cytoplasm include Tro from *Treponema pallidum* (Hardham *et al.*, 1997), Yfe from *Yersinia pestis* (Bearden & Perry, 1999), Fbp from *Neisseria meningitidis* (Chen *et al.*, 1993), Psa from *Streptococcus pneumoniae* (Novak *et al.*, 1998) and Yfu from *Y. pestis* (Gong *et al.*, 2001).

The YtgA protein does have high homology to periplasmic binding proteins of ABC transport systems. *ytgA*, a gene of 978 bp, resides in an operon with *ytgB* (CT068), *ytgC* (CT069) and *ytgD* (CT070). YtgB and YtgC have predicted membrane-spanning domains, and probably form the pore of the ABC transporter; however, it is not known how many proteins of each form the pore complex. It is also not clear how many YtgD proteins are associated with each YtgB/YtgC pore. When compared to other ABC transport periplasmic binding proteins, YtgA contains similar metal-binding motifs (histidine, tyrosine) to other metal-binding periplasmic proteins, suggesting a role for YtgA as an iron-binding protein for a metal transporter. Our studies have established that YtgA primarily binds iron, as supported both by the radiolabelled iron-binding studies and by the luminescence/absorbance experiments detailed in this report. Competition assays using high concentrations of non-radiolabelled metals to compete against radiolabelled metals for YtgA binding further corroborate iron as the preferred metal for YtgA. While it is possible that YtgA could bind other metals *in vivo*, our methods *in vitro* identified only iron as the protein's targeted metal.

The Belland group (Belland *et al.*, 2003) has identified mRNA transcripts for YtgA as early as 8 h during normal chlamydial growth. Under similar growth conditions, YtgA was not readily detected in this study until 18 h.p.i., which is probably due to a difference in the sensitivity of the techniques employed. However, if a high concentration of Desferal (500 µM versus 50 µM) was added to normally growing chlamydiae in HEC-1B cells at 17 h.p.i., within 1 h the amount of YtgA detected by Western blot was essentially doubled, confirming that YtgA is responsive to iron availability. In contrast, there was only a minimal increase in YtgA expression following 1 h of penicillin exposure.

Past work by the Rockey group (Bannantine & Rockey, 1999) had localized YtgA to the chlamydial membrane through immunofluorescence microscopy. These findings were confirmed by our experiments using TEM with immunogold (5 and 15 nm) labelling techniques. Further, by exploiting higher resolutions available from electron microscopy compared to confocal microscopy, we identified soluble YtgA protein unbound to either the inner or outer membranes. It is worth noting that once the chlamydial RB were exposed to 50 µM Desferal to induce

iron restriction, forcing the change to large aberrant pleomorphic bodies, YtgA proteins were observed with higher frequency in the cytoplasm compared to normal RB. Further, periplasmic-labelled YtgA dropped considerably in the large pleomorphic bodies, as if the proteins could no longer be reliably routed to the periplasm. These observations were supported by the tally of 5 nm gold particles in both RB and EB.

In summary, the work presented in this study identifies YtgA as an iron-binding protein that is influenced specifically by iron levels. Secondary structure analysis and the *C. trachomatis* genome strongly suggest that YtgA is part of an ABC transporter system, due to its location within an ABC transporter operon (Stephens *et al.*, 1998). The work presented in this study provides further evidence that this is likely the case. We have determined that YtgA preferentially binds iron over other metals, consistent with the specificity of metal-transporting ABC systems. Using immuno-TEM, we have shown that YtgA localizes to the periplasm and membrane region of *C. trachomatis*, the regions of interest for metal acquisition and ABC transporter function. Finally, we have demonstrated that the YtgA protein concentration increases specifically in response to the stress of iron restriction, consistent with the activity of ABC transporter function under critical substrate-limiting conditions. Taking these results together, we propose that YtgA is an iron-binding protein involved in an ABC transporter system in *C. trachomatis*.

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