A unique \textit{Coxiella burnetii} lipoprotein involved in metal binding (LimB)

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\textit{Coxiella burnetii} is the bacterial agent of Q fever in humans. Here, we describe a unique, \textless~7.2 kDa, surface-exposed lipoprotein involved in metal binding which we have termed LimB. LimB was initially identified as a potential metal-binding protein on far-Western (FW) blots containing whole-cell lysate proteins when probed with nickel-coated horseradish peroxidase (Ni-HRP) and developed with a chemiluminescent HRP substrate. The corresponding identity of LimB as CBU1224a was established by matrix-assisted laser desorption ionization-tandem time-of-flight mass spectrometry. BLAST analyses with CBU1224a showed no significant similarity to sequences outside strains of \textit{C. burnetii}. Additional \textit{in silico} analyses revealed a putative 20 residue signal sequence with the carboxyl end demarcated by a potential lipobox (LSGC) whose Cys residue is predicted to serve as the N-terminal, lipidated Cys of mature LimB. The second residue of mature LimB is predicted to be Ala, an uncharged envelope localization residue. These features suggest that CBU1224a is synthesized as a prolipoprotein which is subsequently lipidated, secreted and anchored in the outer membrane. Mature LimB is predicted to contain 45 aa, of which there are 10 His and 5 Cys; both amino acids are frequently involved in binding transition metal cations. Recombinant LimB (rLimB) was generated and its Ni-HRP-binding activity demonstrated on FW blots. Ni-HRP binding by rLimB was inhibited by \textgreater 95\% on FW blots done in the presence of EDTA, imidazole, Ni$^{2+}$ or Zn$^{2+}$, and roughly halved in the presence of Co$^{2+}$ or Fe$^{3+}$. The \textit{limB} gene was maximally expressed at 3–7 days post-infection in \textit{Coxiella}-infected Vero cells, coinciding with exponential phase growth. Two isoforms of LimB were detected on FW and Western blots, including a smaller (\textless~7.2 kDa) species that was the predominant form in small cell variants and a larger isoform (\textless~8.7 kDa) in large cell variants. LimB is Sarkosyl-insoluble, like many omps. The predicted surface location of LimB was verified by immunoelectron and immunofluorescence microscopy using anti-rLimB antibodies. Overall, the results suggest that LimB is a unique \textit{Coxiella} lipoprotein that serves as a surface receptor for divalent metal cations and may play a role in acquiring at least one of these metals during intracellular growth.

\textbf{INTRODUCTION}

\textit{Coxiella burnetii} is a gammaproteobacterium and the agent of Q fever in humans. \textit{C. burnetii} is one of the most infectious pathogens known, with an ID$_{50}$ of 1–10 bacteria in the guinea pig model (Moos & Hackstadt, 1987). Human infections with \textit{C. burnetii} are generally zoonoses acquired by inhalation of contaminated aerosols. Q fever typically presents as an acute, self-limiting flu-like illness accompanied by pneumonia or hepatitis. In roughly 1\% of cases, a severe chronic infection can occur, in which endocarditis is the predominant manifestation (Maurin & Raoult, 1999). These attributes and its past use as a biological weapon component (Regis, 1999) were grounds for classifying \textit{Coxiella} as an ‘HHS Select Agent’.

In nature, \textit{C. burnetii} is an obligate intracellular pathogen and undergoes a developmental cycle that exhibits two distinct cell morphotypes. The infectious small cell variant (SCV) has been described as ‘spore-like’, and it is extremely resistant to environmental stressors, including UV light and desiccation. Shortly after entry into a eukaryotic phagolysosome-like compartment called a parasitophorous vacuole (pH \textless 4.5), the SCV transforms into a ‘vegetative’ morphotype, termed a large cell variant (LCV) (McCaul & Williams, 1981). After 5–6 days of intracellular replication, the LCV transforms back to a SCV that is released from the host cell to complete the developmental cycle (Coleman...
et al., 2004; McCaul & Williams, 1981). In addition to distinct developmental forms, two separate phase variants of the bacterium have also been described. First, wild-type *C. burnetii* is typically 'phase I', in reference to serological reactivity against its 'smooth', long-chain LPS, and must be manipulated in a bio-safety level (BSL)-3 facility. Second, repeated passage in tissue culture or embryonated hens' eggs results in the spontaneous generation of attenuated 'phase II' (PII) organisms that synthesize a 'rough' LPS that can be caused by a chromosomal deletion (Hoover et al., 2002; Vodkin & Williams, 1986), and may be handled in a BSL-2 laboratory. Finally, a number of *C. burnetii* strains [such as ‘Nine Mile’ (NM)] have been isolated from different parts of the world and have been grouped according to their association with acute or chronic disease manifestations of Q fever (Beare et al., 2009; Seshadri & Samuel, 2005).

Despite several fascinating attributes, little is known regarding *Coxiella's* virulence determinants. In fact, LPS is the only bona fide virulence factor described to date (Moos & Hackstadt, 1987), and it has been shown to function as an immune-evasion shield (Shannon et al., 2005). One potential set of virulence factors that has received relatively little attention is the pathogen's consider-able assemblage of lipoproteins. Lipoproteins have long been recognized as virulence determinants in a variety of bacterial pathogens, and a number of them have been developed into vaccine immunogens (Fortney et al., 2005; McCaul & Williams, 1981), and may be handled in a BSL-3 facility. Second, SCVs were prepared and purified from 28-day-old cultures as described previously (Raghavan et al., 2008). SCVs were used to inoculate Vero cell monolayers to produce synchronous infections (Cockrell et al., 2008). Whole-cell lysates of *Coxiella* were obtained by using a freeze-thaw method (Samoïlis et al., 2007).

**METHODS**

**Bacterial strains, cell lines and growth conditions.** NM PII C. *burnetii* (strain RSA 439; clone 4) was propagated in African green monkey kidney (Vero) epithelial cells (CCL-81; American Type Culture Collection), as described previously (Raghavan et al., 2008). SCVs were prepared and purified from 28-day-old cultures as described previously (Raghavan et al., 2008). SCVs were used to inoculate Vero cell monolayers to produce synchronous infections (Coleman et al., 2004). Mixed-cell populations of LCVs and SCVs were harvested from synchronous cultures as for SCVs, but at 10 days post-infection. LCVs were prepared from 72 h synchronous cultures using a digitonin-based protocol (Cockrell et al., 2008). Whole-cell lysates of *Coxiella* were obtained by using a freeze-thaw method (Samoïlis et al., 2007).

**SDS-PAGE and far-Western (FW) blots.** Protein concentrations were determined by using a BCA protein assay kit (Thermo Scientific). Bacterial suspensions were solubilized with Laemmli sample buffer (Laemmli, 1970). Samples were typically boiled for 10 min and centrifuged (1 min, 10 000 g) to remove insoluble material; the resulting supernatant was separated on an SDS-PAGE gel (Laemmli, 1970). Protein bands were visualized by staining gels with Coomassie brilliant blue [CBB; 0.1 % (w/v) in 50 % methanol, 7 % (v/v) acetic acid].

FW blots were prepared using whole-cell lysate proteins (20 μg) from SCVs, LCVs or mixed-cell populations, separated by SDS-PAGE, then transferred to supported nitrocellulose [45 μm pore; GE Osmonics (Towbin et al., 1979)]. Resulting blots were probed with nickel-coated horseradish peroxidase (Ni-HRP; Pierce) and developed with ECL reagents (SuperSignal West Pico; Pierce) according to the manufacturer's instructions. Blots were developed for 5 min in chemiluminescent horseradish peroxidase (HRP) substrate (SuperSignal West Pico; Pierce) according to the manufacturer's instructions. Blots were visualized using an LAS-3000 digital imaging system (FUJIFILM).

Inhibition studies were done using recombinant LimB (rLimB) and FW blots were prepared as described above, except the probing solution contained a potential competitive inhibitor of binding, including EDTA (100 mM) or imidazole (500 mM). In some cases, HRP was used in place of Ni-HRP, at the same final concentration (1 μg ml⁻¹), to demonstrate that LimB binding activity was dependent on the nickel moiety of Ni-HRP. Finally, to qualitatively analyse the relative affinity of LimB for various metal ions, modified FW blots were performed based on a previously published protocol (Zhao & Waite, 2006). First, rLimB (5 μg protein) was separated by SDS-PAGE, transferred to nitrocellulose, blocked and washed as above. Second, blots were incubated (1 h; 25 °C) in TBST containing chloride salts of individual metal ions (FeCl₃·6H₂O, CoCl₂·6H₂O, NiCl₂·6H₂O or ZnCl₂) at a final concentration of 10 mM. Excess metal was subsequently removed by washing three times for 5 min with TBST containing 0.1 mM of the individual metal ion. Third, resulting FW blots were probed and washed as above except solutions contained 0.1 mM of the particular metal ion. Finally, blots were developed and visualized as above.

**Mass spectrometry (MS).** A *C. burnetii* whole-cell lysate (20 μg protein) was separated by using SDS-PAGE [10–15 % acrylamide (w/v)] and stained with CBB, as above. Bands corresponding to Ni-HRP-binding activity on FW blots were excised from gels and subjected to trypsin digestion and MALDI-TOF peptide mass fingerprinting and MALDI-tandem TOF (MALDI-TOF/TOF) peptide sequencing. All MS work and database searches (NRDB1 database using Mascot version 2.2.03 software) were performed by Alphalyse.

**Nucleic acid isolation and rLimB production.** Infected monolayers were harvested at specific time points (0, 3, 5, 6, 7 and 8 days) by replacing culture medium with 2.5 ml TRI reagent (Ambion), briefly incubating (5 min, 25 °C) and scraping. Harvested monolayers were stored (−80 °C) for subsequent nucleic acid isolation. RNA and DNA were isolated from each harvested monolayer sample essentially as

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967
described previously (Raghavan et al., 2008). RNA was isolated from this extract by the addition of 300 µl 100 % ethanol and use of a RiboPure kit (Ambion) according to the manufacturer’s instructions, and then stored at −20 °C. DNA and RNA were removed from these samples by DNase treatment (TURBO DNase kit; Ambion) and an RNA clean-up step (RNeasy kit; Qiagen). Purified RNA was quantified spectrophotometrically (260 nm), and 1 µg RNA from each sample was converted to cDNA with an iScript cDNA kit (Bio-Rad), as instructed. Finally, cDNA was used to analyse limB transcript profiles by quantitative real-time PCR (qRT-PCR).

DNA was isolated using TRI Reagent DNA/protein isolation protocol (Ambion). DNA-containing supernatant was transferred to another microcentrifuge tube and 40 µl HEPES (0.1 M) was added to adjust the pH to 8.2. Isolated DNA was cleaned using a QIAquick PCR purification kit (Qiagen) as instructed and stored at −20 °C until needed.

A portion of Coxiella limB encoding the predicted, mature LimB (i.e. Cys21 to His93) was cloned into pMAL-c5X (NEB) to create pJB202, a plasmid that encodes a LimB/maltose-binding protein (MBP) fusion. Briefly, primers (LimBFpJB202, a plasmid that encodes a LimB/maltose-binding protein microcentrifuge tube and 40 µl HEPES (0.1 M) was added to adjust the pH to 8.2. Isolated DNA was cleaned using a QIAquick PCR purification kit (Qiagen) as instructed and stored at −20 °C until needed.

qRT-PCR and quantitative PCR (qPCR). Primers were designed using Beacon Designer Software (Premier Biosoft). Primers were synthesized (Integrated DNA Technologies) and used to quantify limB mRNA over the course of a synchronous infection by normalizing to the number of C. burnetii genomes, as described previously (Coleman et al., 2004; Raghavan et al., 2008),. The number of genomes in each purified DNA sample at each time point (0, 3, 5, 6, 7 and 8 days) was determined in triplicate qPCR reactions (each triplicate experiment was performed twice, thus resulting in six independent determinations). This was accomplished using an iCycler single-colour real-time PCR detection system (Bio-Rad), SYBR Green supermix (Bio-Rad) and a dotA (CBU_1648) primer set (DOTA-F, GATAGCCTGGAGCCGTGAC; DOTA-R, TTCCTTGGCATGCGATT) by normalization to a standard curve of 10-fold serially diluted (1×10^−5) C. burnetii genomes. Second, cDNA from each time point was used to determine the starting quantity (SQ) mean, a value calculated by iCycler software (MyIQ, v1.0, Bio-Rad) by normalizing limB transcript numbers with a limB primer set (LIMB-F, GGT CCTGT TTGATAAACATG; LIMB-R, GGTGTTAGTTCAGTT GTGATT) to a standard curve generated by dotA qPCR of serially diluted C. burnetii genomes. Finally, the number of limB mRNAs per genome equals the SQ mean divided by the number of genomes × 1000.

Antibodies, Western blots, immunofluorescence and immuno-electron microscopy. Anti-LimB and anti-rCom1 polyclonal antibodies were generated in female New Zealand white rabbits, as described previously (Parrow et al., 2009), using 100 µg purified rLimB or rCom1 (Hicks et al., 2010) for both primary and booster injections. Rabbit anti-MBP polyclonal antibodies were purchased (NEB) and used as a negative control for the LimB fusion protein partner. Mouse monoclonal antibody against Coxiiella elongation factor-Ts (EF-Ts) was used as a negative control for intracellular proteins that may have inadvertently leaked from the bacterium during handling and preparation for microscopy (a generous gift from Robert Heinzen).

Immunoblots were done by transferring un-fixed SDS-PAGE gels to support nitrocellulose and probing overnight with anti-rLimB antibodies (1:5000 dilution) and peroxidase-conjugated goat anti-rabbit antibodies (Sigma), as described previously (Parrow et al., 2009), except ECL reagents were used during development according to the manufacturer’s instructions (Pierce), and signals were visualized with an LAS-3000 digital imaging system (Fujifilm).

Immunofluorescence microscopy to identify surface-exposed proteins was done by the general methods described by Ge & Rikihisa (2007). Bacteria were cultured and harvested from Vero cells at 5 days post-infection, as above, pelleted and washed in PBS containing 0.25 M sucrose (PBSS; pH 7.4). This, and all subsequent steps were done at 25 °C. Bacteria were fixed for 45 min in 2 % (w/v) paraformaldehyde. After quenching in PBS containing 0.1 M glycine and subsequently washing in PBS, bacteria were incubated for 1 h with anti-LimB, anti-Com1, anti-EF-Ts, anti-MBP or pre-immune serum (PIS) at a 1:100 dilution in 0.2 % (w/v) gelatin in PBS (PG buffer). After washing with PG buffer, bacteria were labelled for 1 h with Alexa Fluor 488 goat anti-rabbit or mouse (for anti-EF-Ts) immunoglobulin G (Invitrogen) at a dilution of 1:100 in PG buffer. Bacteria were washed in PG buffer, suspended in PBS and imaged with a BX51 fluorescence microscope and digital acquisition software (DP2-BSW) (Olympus).

To conduct immuno-electron microscopy, bacteria were cultured and harvested at 5 days post-infection as described above, and diluted in PBSS to a concentration of ~1×10^6 genomes ml⁻¹. The suspension was combined with an equal volume (100 µl) of antiserum or PIS [diluted 1:100 in PBS-B (PBS containing 0.01 mg BSA ml⁻¹)] and incubated for 1 h at 37 °C. Cells were then centrifuged (10 min at 10,000 g), the pellet was washed with 300 µl PBSS, and the pellet was suspended in 200 µl PBS-B. An aliquot (100 µl) of the cell suspensions was combined with an equal volume of protein A–gold conjugate [Electron Microscopy Sciences (EMS)] pre-diluted 1:20 in PBS-B. Following a brief incubation (50 min, 37 °C), cells were centrifuged and washed as above, and finally suspended in 30 µl PBS. Half the volume of these suspensions was placed on Formvar-coated TEM grids (EMS; 400 mesh) and incubated for 10 min at 23 °C for adherence. Excess suspension was wicked from the grid surface by using blotting paper, replaced with 15 µl uranyl acetate [2 % (w/v) in H2O, J. T. Baker, Phillipsburg, NJ], and incubated for 3 min at 23 °C. Excess protein A–gold was washed from grids by successive immersions in three beakers containing 2 l dH2O. Finally, grids were air-dried and analysed by using a Hitachi 7100 transmission electron microscope.

Sarkosyl fractionation. Omps of C. burnetii were enriched by Sarkosyl-fractionation of the bacterium. A whole-cell lysate from a mixed-cell population of Coxiella was prepared and the insoluble cell-wall fraction was pelletted by centrifugation. Briefly, the pellet was suspended in 300 µl PBS (pH 7.4), to which 38 ml 0.1 M sodium bicarbonate (pH 11) was added, then incubated on ice for 1 h (with rocking). The mixture was centrifuged (141 000 g for 80 min, 4 °C) and the resulting pellet was extracted with 4 ml sodium N-lauroylsarcosine (Sarkosyl; Sigma-Aldrich) solution (2 %; w/v). The extraction was done on ice for 30 min (with rocking). The Sarkosyl-
insoluble (omp-rich) fraction was isolated by centrifuging the mixture (138 000 g for 1 h, 4 °C). The Sarkosyl-soluble fraction (supernatant) was precipitated in 4 vols acetone for 1 h at −20 °C. The mixture was centrifuged (3000 g for 30 min at 4 °C) to obtain a pellet. Both fractions were analysed by SDS-PAGE and blotting.

**Sequencing, in silico analyses and statistics.** Sequence data were obtained with an automated DNA sequencer (ABI3130x1) and a BigDye terminator cycle sequencing ready reaction kit (ABI). GenBank searches were done by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). In silico tools available at the database of bacterial lipoproteins (DOLOP; http://www.mrc-lmb.cam.ac.uk/genomes/dolop/; Babu et al., 2006) were used to determine if a predicted protein sequence contained a prolipoprotein signal peptide. MacVector genetic software (Version 11.1.1) was used for protein sequence analysis as well as sequence manipulations. Comparative densitometry of FW blot signals was done using ImageJ 1.42q software (http://rsweb.nih.gov/ij/). At least three independent determinations were used to calculate means and standard deviations for all numerical data. Statistical and graphical analyses were done using InStat3 (GraphPad) and/or Excel (Microsoft) software. Statistical significance was determined using Student’s t test, where a P-value of <0.05 was considered significant.

**RESULTS**

**FW blots reveal a ~7.2 kDa component of Coxiella that binds Ni-HRP**

A prominent, chemiluminescent band of ~7.2 kDa was discovered on Ni-HRP-probed FW blots containing whole-cell lysate proteins from a mixed-cell population (10-day-old culture) of *C. burnetii* LCVs and SCVs (Fig. 1a and b). We were curious to determine whether the signal was a result of Ni-HRP binding to a *C. burnetii* constituent or whether it was due to an endogenous peroxidase that was directly acting on the chemiluminescent HRP substrate. To address this question, we performed FW blots as above but without Ni-HRP. The resulting FW blots showed that even with prolonged exposure (5 min) a chemiluminescent band was not detectable Fig. 1(b) (−Ni-HRP), suggesting that the FW blot signal was due to Ni-HRP binding to a *C. burnetii* component, and not due to an endogenous peroxidase. To ensure that Ni-HRP was not binding a contaminating host-cell constituent, Vero cells were cultivated and harvested for Ni-HRP FW blot analyses (Fig. 1c). The resulting FW blots, prepared as above, showed no detectable chemiluminescent signal in Vero host cell lysate proteins (Fig. 1d). Finally, FW blots of *C. burnetii* were prepared and probed with HRP; however, a chemiluminescent signal was not produced in the absence of Ni (data not shown). From these results, we hypothesized that the Ni-HRP was binding to a *C. burnetii* component, that binding was dependent on the nickel moiety of the probe, and that a small, His-rich protein might be responsible.

**MS identifies the Ni-HRP binding protein as CBU1224a**

To identify the *C. burnetii* protein responsible for Ni-HRP binding, MALDI-TOF peptide mass fingerprinting and MALDI-TOF/TOF peptide sequencing were employed; these identified two major peaks in the chromatogram that matched two of the tryptic peptide fragments calculated for GI-153209557, a *C. burnetii*‘putative lipoprotein’. MS sequencing results represented 56 % coverage of the predicted mature, 45 aa lipoprotein. These data allowed us to presumptively identify the Ni-HRP-binding ligand as the protein encoded by the CBU1224a locus of *C. burnetii* (strain RSA 493).

**In silico analysis of CBU1224a reveals a potential lipoprotein signal sequence, a ‘lipobox’ and a His-rich carboxyl-terminus**

Several in silico analyses were performed on CBU1224a and its encoded protein. BLAST was used to search the GenBank...
database for similar sequences. Published genomic sequences of six *C. burnetii* strains (RSA 493, RSA 331, Q177, Q154, Q212 and S1108-111) (Beare et al., 2009) (Seshadri & Samuel, 2005; Seshadri et al., 2003) possess ORFs that are 100 % identical to CBU1224a. In each strain, the 198 bp ORF is flanked by genes encoding haloalkane dehalogenase (111 bp upstream) and an RmuC family protein (105 bp downstream). RmuC (rearrangement mutator) family proteins are hypothesized to play a role in DNA structure/recombination (Slupska et al., 2000) and haloalkane dehalogenase (EC 3.8.1.5) is an enzyme involved in degradation of halocarbons. Based on considerable intergenic spacing, neither of the flanking genes appears to be transcriptionally linked to CBU1224a. Finally, BLAST searches of the non-redundant GenBank database using DNA, as well as the predicted 65 aa residue prolipoprotein and 45 aa mature lipoprotein sequences, did not yield significant similarities outside *C. burnetii*.

We also used in silico tools available on the DOLOP website (Babu et al., 2006) to investigate the putative lipoprotein encoded by CBU1224a. These analyses revealed a potential ‘lipobox’ and characteristic N, H and C prolipoprotein signal peptide regions, supporting the ‘lipoprotein’ annotation for the locus. Specifically, the characteristics of the lipobox are: (i) it consists of 4 aa with the consensus signal peptide region, supporting the ‘lipoprotein’ designation residue (ELR) (circled) immediately following the N-terminal Cys suggests that mature LimB is translocated to the outer membrane, as reported for Gram-negative bacteria. Mature LimB contains ten His (red) and four Cys (orange) residues that may be involved in metal binding.

**Fig. 2.** Predicted features of the CBU1224a-encoded lipoprotein involved in metal binding (LimB). In silico analyses reveal a predicted signal peptide (lower case) containing a potential lipobox (LSGC; blue box), a consensus peptide cleavage site (red line), and an N-terminal Cys (green) in mature LimB (upper case) that is predicted to be covalently modified with diacylglycerol (DAG) and a fatty acid (FA), as observed in other Gram-negative bacteria. The uncharged Ala envelope localization residue (ELR) (circled) immediately following the N-terminal Cys suggests that mature LimB is translocated to the outer membrane, as reported for Gram-negative bacteria. Mature LimB contains ten His (red) and four Cys (orange) residues that may be involved in metal binding.

The high-affinity binding of Ni-HRP observed in FW blots (Fig. 1) is probably explained by the high percentage of His residues located in the C-terminal portion of CBU1224a (Fig. 2). Specifically, the predicted, mature 45 aa lipoprotein contains ten His (22 % of the total amino acids), nine of which are located in the last 20 aa of the protein. Four Cys residues are also found in this region, an observation that is noteworthy for two reasons: (i) Cys residues could be partially responsible for Ni-HRP binding as both His and Cys are known to coordinate binding of transition metals, and (ii) intra- and/or inter-disulfide bonds could contribute to structure or multimerization of the protein, respectively.

**Recombinant protein encoded by CBU1224a binds Ni-HRP**

To unequivocally demonstrate that Ni-HRP binding on FW blots was due to the protein encoded by CBU1224a, a plasmid (pJB202) was constructed to produce a translational fusion with MBP. FW blots were prepared to compare Ni-HRP binding characteristics of *E. coli* BL21 (pJB202) versus *E. coli* BL21 (pMAL-c5X; empty cloning vector). Results clearly show that the recombinant protein binds Ni-HRP (Fig. 3, lanes 4 and 5), like the native protein (Fig. 3, lane 1). In addition, recombinant MBP (rLimB’s fusion partner) does not bind the probe (Fig. 3, lanes 2 and 3). Based upon these results and our in silico analyses, we designated CBU1224a as a lipoprotein involved in metal-binding (LimB).

**rLimB binds divalent cations, which involves His residues**

To further characterize Ni-HRP binding, rLimB was employed in FW blots done in the presence of inhibitors
or divalent metal cations. First, when the metal chelator EDTA was added along with the Ni-HRP probe, a chemiluminescent signal could not be detected, in contrast with untreated controls done in parallel (Fig. 4, EDTA vs none). These results suggest that the Ni moiety of Ni-HRP mediates binding to LimB. Second, when the His analogue imidazole is added along with Ni-HRP, the luminescent signal was reduced by >95% compared with untreated controls (Fig. 4, IMID vs none). These data suggest that LimB His residues are involved in Ni-HRP binding. However, since the signal was not completely inhibited, other residues, such as Cys, may also be involved. Third, we wanted to determine whether LimB was capable of binding other metals. We hypothesized that metal ions could fill LimB binding residues, and inhibit or ‘block’ the binding of Ni-HRP on FW blots. The relative competition capacity of free metal ions could therefore be analysed by FW blots, where a reduction in chemiluminescent signal is directly proportional to the affinity of that particular metal to LimB. To this end, immediately following transfer of proteins to nitrocellulose, we blocked FW blots by incubation in TBST containing chloride salts of four metal ions. FW blots blocked with nickel showed ~95% reduction in their Ni-HRP chemiluminescent signal relative to untreated controls (Fig. 4, Ni2+), further supporting previous results showing that the nickel moiety of Ni-HRP mediates binding to LimB. Interestingly, when blots were blocked with zinc (Fig. 4, Zn2+) the chemiluminescent signal was no longer detectable relative to untreated controls. These data suggest that rLimB binds Ni2+ and Zn2+ with high affinity. In contrast, when FW blots were blocked with cobalt (Co2+) or iron (Fe3+), the chemiluminescent signal was roughly half of untreated controls, suggesting that these metals bind LimB with relatively lower affinity than Ni2+ or Zn2+. Taken as a whole, the results demonstrate that LimB binds a variety of divalent metal cations with a higher affinity for Ni2+ and Zn2+ relative to Co2+ and Fe3+.

### 2-Mercaptoethanol (2-ME) treatment implicates the Cys residues of LimB in metal binding

We were curious whether the Cys residues of LimB also play a role in Ni-HRP binding. As mentioned above, four Cys residues are located in the C-terminal portion of LimB and might bind nickel and/or confer inter- or intra-LimB disulfide bonds. To address these possibilities, cell lysate proteins of *C. burnetii* were prepared with and without 2-ME, and FW blots were done as described above. Resulting blots showed a marked reduction in the chemiluminescent signal of LimB when samples were prepared without 2-ME compared with those with 2-ME (data not shown). Increased binding of Ni-HRP in the presence of 2-ME may be due to increased exposure of His and/or increased availability of thiol ligands as a result of disulfide reduction. Multiple LimB bands that would indicate disulfide-mediated inter-LimBimerization were never observed; however, it is still possible that multimers occur, especially considering the low Ni-HRP binding efficiency observed in the absence of 2-ME.

### limB expression is maximal during exponential phase

To determine whether expression of *limB* changes during the developmental cycle of *C. burnetii*, synchronous infections were initiated and the transcript quantity was analysed over an 8 day time-course using qRT-PCR. Results showed that *limB* mRNA was significantly higher (P<0.05) at 3–7 days post-infection, relative to the initial day (day 0). The *limB* transcripts were highest at 5 days post-infection (~48-fold increase over the day 0 value). A corresponding analysis of *Coxiella* genomes in these samples shows that *limB* expression is highest during exponential phase growth of the bacterium (Fig. 5). Although transcription of *limB* is maximal during exponential phase growth (Fig. 5), the LimB protein can be detected in both LCVs and SCVs (Fig. 6). Interestingly, FW blots and immunoblots prepared from 12.5% acrylamide gels revealed two distinct molecular mass forms of native LimB, with a larger (~8.7 kDa) LimB species dominant in LCVs and an ~7.2 kDa isoform of LimB in SCVs (28 days post-infection) (Fig. 6). Not surprisingly, mixed-cell populations of the bacterium possess both LimB isoforms (Fig. 6a and b, lane 1). Although the two LimB isoforms can be distinguished as a doublet or smear on blots prepared from gradient gels (e.g.
(a) Fig. 1b), resolution of the bands was much clearer when blots were prepared from 12.5% acrylamide gels (Figs 6 and 7). The two LimB isoforms may reflect post-translational modification of LimB, and perhaps cleavage of LimB’s signal sequence and/or lipidation during the protein’s maturation.

**LimB is a surface-exposed omp**

The second residue of mature LimB is predicted to be Ala (Fig. 2). Previous work has shown that the second residue of mature lipoproteins plays an important role in coordinating transport of the protein to its final destination in the cell, thus it is referred to as an envelope localization residue (ELR) (Yamaguchi et al., 1988). Since lipoproteins with an uncharged ELR are translocated to the outer membrane of Gram-negative bacteria, we hypothesized that LimB was surface-exposed in *C. burnetii*. To address the hypothesis, we used three different methods. First, we prepared a Sarkosyl-insoluble fraction of *Coxiella*, a method commonly used to purify omps of Gram-negative bacteria, previously employed for this purpose with *C. burnetii* (Zhang et al., 2005). Analysis of Sarkosyl fractions of a mixed-cell population of *Coxiella* by FW blots and immunoblotting

![Fig. 4. Ni-HRP binding by rLimB is inhibited by EDTA, imidazole and divalent metal cations. (a) FW blots were done in parallel and prepared from the same SDS-PAGE gel (12.5% acrylamide; w/v) using whole-cell lysates of *C. burnetii* (mixed-cell population; 20 μg protein per lane). Blots were incubated in the presence of EDTA, imidazole (IMID) or divalent metal cations, as described in Methods. The resulting FW blots demonstrate a marked reduction in rLimB binding of Ni-HRP in the presence of Co²⁺ or Fe³⁺ (<50% of control), as shown by the reduced chemiluminescent signal (arrow). Binding signal was not detectable on blots incubated with EDTA or Zn²⁺ and was only slightly detectable (<5% of control) if imidazole (IMID) or Ni²⁺ were present. (b) Densitometric analysis of the blots in (a), shown as a percentage of the signal density of the untreated control (None). The assay is representative of two independent sets of experiments.](image4)

![Fig. 5. limB expression is highest during exponential phase growth of *C. burnetii* in synchronous infections of cultured Vero cells. An analysis of *limB* transcript quantities (histogram) relative to the number of genomes (● and line) was determined by qRT-PCR and qPCR, respectively, over the course of an 8 day synchronous infection of Vero cells, as described in Methods. Values represent the mean ± SD from six independent determinations. Asterisks denote a statistically significant increase in *limB* mRNA relative to 0 days (*P*<0.05).](image5)
with anti-rLimB antibodies showed that: (i) both molecular mass forms of LimB were present in the insoluble fraction but absent in the Sarkosyl-soluble fraction, (ii) Ni-HRP was bound by both LimB isoforms and (iii) both protein isoforms were recognized by anti-LimB antibodies (Fig. 7). These data suggest that LimB is an omp.

Second, we employed immunoelectron microscopy with anti-rLimB or control antibodies and live bacteria. Resulting electron micrographs showed uniform protein A–gold binding on the surface of Coxiella treated with either anti-LimB or anti-Com1 (positive control) antibodies (Fig. 8a). In contrast, treatment with anti-MBP antibodies (a control for the fusion partner of the rLimB immunogen), anti-EF-Ts (a control for leaked intracellular proteins) or rabbit PIS from the same animal used to generate anti-rLimB antibodies, showed minor and inconsistent protein A–gold binding to the bacteria (Fig. 8a).

Finally, we employed these antibodies and PIS in immunofluorescence microscopy with Coxiella cells that had been fixed with paraformaldehyde. This procedure was previously employed to detect surface-exposed proteins of Ehrlichia and Anaplasma species (Ge & Rikihisa, 2007). Results of these experiments correlated with the immunoelectron microscopy data, and showed prominent immunofluorescence when Coxiella was treated with anti-rLimB or anti-Com1 antibodies, and minor, sporadic immunofluorescence when negative-control antibodies or PIS were used (Fig. 8b). Taken together, the Sarkosyl insolubility and detection of LimB on the surface of live cells by immunoelectron microscopy and on paraformaldehyde-fixed cells by immunofluorescence microscopy strongly suggest that LimB is a surface-exposed omp in Coxiella.

**DISCUSSION**

In this study, we present data on the discovery and partial characterization of Coxiella LimB, a surface-exposed omp that binds divalent metal cations, including nickel and zinc with high affinity, and cobalt and iron with relatively lower affinity. Although assigning specific roles to LimB requires additional investigation regarding the protein’s structure and ligand-binding characteristics, the results of this study allow us to formulate reasonable hypotheses. First, maximal expression of the limB gene occurs during exponential phase growth, suggesting that LimB and its ligand(s) may be important for replication. Second, LimB’s surface location suggests that its metal ligand is obtained extracellularly. Third, because the replication of Coxiella in nature is restricted to an acidified, lysosome-like
compartment of the host cell, termed a parasitophorous vacuole (PV) (Voth & Heinzen, 2007), it is logical to assume that the ligand(s) is present in the PV niche. Fourth, it is plausible that LimB activity in the PV generates an active competition with the host cell, especially if the ligand is limiting in concentration. Based upon these results, we hypothesize that LimB is a lipoprotein that serves as a receptor for a divalent metal cation(s) at the surface of the pathogen during active replication within host cells. Considering that bio-reactive metal acquisition is often key to bacterial replication (particularly if the ligand is limiting in concentration). Based upon these results, we hypothesize that LimB is a lipoprotein that serves as a receptor for a divalent metal cation(s) at the surface of the pathogen during active replication within host cells. Considering that bio-reactive metal acquisition is often key to bacterial replication (especially if the ligand is limiting in concentration). Based upon these results, we hypothesize that LimB is a lipoprotein that serves as a receptor for a divalent metal cation(s) at the surface of the pathogen during active replication within host cells.

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