Biometals (2007) 20:323–331
DOI 10.1007/s10534-006-9057-4

Iron Acquisition by *Legionella pneumophila*

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Received: 18 July 2006 / Accepted: 28 November 2006 / Published online: 16 December 2006

Abstract For nearly 20 years, it was believed that *Legionella pneumophila* does not produce siderophores. Yet, we have now determined that *L. pneumophila* secretes a siderophore (legiobactin) that is detectable by the CAS assay. We have optimized conditions for legiobactin expression, shown its biological activity, and found genes (*lbtAB*) involved in its production and secretion. LbtA is homologous with siderophore synthetases from *E. coli* (aerobactin), *Sinorhizobium* (rhizobactin), and *Bordetella* (alcaligin), while LbtB is a member of the major facilitator superfamily of multidrug efflux pumps. Mutants lacking *lbtAB* produce 40–70% less CAS reactivity. The *lbtA* mutant is also defective for growth in deferrated media containing citrate, indicating that legiobactin is required in conditions of severe iron limitation. *lbtAB* mutants grow normally in macrophages and amoebae host cells as well as within the lungs of mice. *L. pneumophila* does express *lbtA* in macrophages, suggesting that legiobactin has a dispensable role in infection. Legiobactin is iron repressed and does not react in the Csáky and Arnow assays. Anion-exchange HPLC has been used to purify legiobactin, and thus far, structural analysis suggests that the molecule is similar but not identical to rhizobactin, rhizoferrin, and alcaligin. The residual CAS reactivity present in supernatants of the *lbtAB* mutants suggests that *L. pneumophila* might produce a second siderophore. Besides siderophores, we have determined that ferrous iron transport, encoded by *feoB*, is critical for *L. pneumophila* growth in low-iron conditions, in host cells, and in the mammalian lung. Some of our other studies have discovered a critical, yet undefined, role for the *L. pneumophila* cytochrome *c* maturation locus in low-iron growth, intracellular infection, and virulence.

Keywords Legionnaires' disease · Siderophores · Legiobactin · FeoB · Cytochrome *c* maturation

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Introduction

In 1976, an outbreak of respiratory illness occurred among attendees of a bicentennial celebration of the American Legion in Philadelphia. The analysis of patient specimens led to the description of a new bacterial genus, *Legionella*. The pneumonia associated with *Legionella* has become known as Legionnaires' disease. The *Legionella* genus presently includes 49 species, comprising 70 serogroups. It is also now appreciated that the legionellae are common causes of

community-acquired and nosocomial pneumonia. *L. pneumophila*, the species first isolated, is responsible for 85–90% of Legionnaires' disease cases. About 20 other *Legionella* species are also known agents of human disease. *Legionella* species also elicit a flu-like illness called Pontiac Fever.

The legionellae are gram-negative organisms belonging to the γ-proteobacteria. They exist in aquatic habitats, surviving free, in biofilms, and as parasites of protozoa. *L. pneumophila* has been isolated from lakes, rivers, and wet soil and may be present in 60% of large building plumbing systems. Iron and other metals promote the bacterium's persistence in these systems and may heighten resistance to biocides. Protozoan hosts for *L. pneumophila* include amoebae and ciliates, such as *Acanthamoeba*, *Hartmannella*, and *Tetrahymena*. The legionellae are catalase-positive, motile rods that exist at 4–63°C and within a pH range of 5.4–8.1. They undergo aerobic respiration and obtain carbon and energy from amino acids. In the laboratory, they are cultured on buffered charcoal yeast extract (BCYE) agar, which contains L-cysteine and an iron supplement. The complete genome sequence has been reported for three serogroup 1 strains of *L. pneumophila*.

L. pneumophila infection occurs after the inhalation of contaminated aerosols generated by air-conditioners, cooling towers, and other devices. In the lung, the bacterium grows in the resident alveolar macrophages. Effective clearance requires the Th1 T-cell response and cytokines such as γ-interferon, which activates macrophages, rendering them non-permissive for the legionellae. Hence, legionellosis is largely a disease of immunocompromised individuals. Other risk factors for contracting Legionnaires' disease infections include smoking, the male sex, and advanced age. The ability of *L. pneumophila* to grow within macrophages is central to pathogenesis. It is widely believed that the adaptation of *Legionella* to protozoan niches in nature engendered it with the ability to infect mammalian phagocytes. *L. pneumophila* enters the macrophage by conventional or coiling phagocytosis. After entry, legionellae reside in a phagosome that does not fuse with endosomes or lysosomes, thereby avoiding acidification and degradative enzymes. The phagosome then associates with material derived from the rough endoplasmic reticulum, and replication of the bacteria ensues. Ultimately, the *L. pneumophila* phagosome fills the host cell, and macrophage death occurs. Processes in addition to macrophage infection likely contribute to disease by *L. pneumophila*; e.g. the bacterium may replicate or, at a minimum, must survive extracellularly in the alveoli. A variety of virulence determinants have been identified in *L. pneumophila*. Notable among these are type II and type IV secretion systems that elaborate a large number of protein effectors that contribute to intracellular infection and tissue destruction. For further background information on *Legionella* and Legionnaires' disease, the reader is referred to any number of fine review articles (Fields et al. 2002).

Iron and *Legionella pneumophila*

Iron is a key requirement for *L. pneumophila* growth (Reeves et al. 1981). Originally, the iron requirement was thought to be 3–13 μM for minimal growth and >20 μM for optimal growth (Reeves et al. 1983; Johnson et al. 1991; Mengaud and Horwitz 1993). But, studies using defined media indicate that the requirement is <1 μM (James et al. 1995; Liles et al. 2000). In standard BCYE agar, iron is added as ferric pyrophosphate, though ferric chloride, ferric nitrate, and ferrous sulfate can be used. Upon incubation with $^{55}FeCl_3$, *L. pneumophila* uptakes labeled iron in an energy-dependent, protease-resistant process (Johnson et al. 1991). *L. pneumophila* also binds and uses hemin as a source of iron (O'Connell et al. 1996). As in other bacteria, iron serves as a cofactor in enzymes, such as an aconitase (Mengaud & Horwitz 1993). But, for *L. pneumophila*, iron also catalyzes the formation of homogentisic acid melanin, a brownish pigment made in stationary phase (unpublished results).

Iron is very relevant for *Legionella* infection. Much evidence signals that the ability of *L. pneumophila* to replicate in the mammalian host is dependent upon iron. First, human monocytes and macrophages treated with iron chelators

(e.g., desferrioxamine [DFX]) do not support *Legionella* growth, and interferon-γ inhibits growth by reducing available iron (Byrd and Horwitz 2000; Viswanathan et al. 2000). Second, mouse macrophages can become permissive for *Legionella* infection following the addition of iron (Gebran et al. 1994). Third, a reduced ability of legionellae to establish intracellular infection is correlated with decreased levels of transferrin receptor (Byrd and Horwitz 2000). Fourth, introduction of iron into animals increases their susceptibility to infection. Fifth, legionellae grown under iron-deplete conditions exhibit reduced pathogenicity (James et al. 1995). Finally, patients with iron overload as well as smokers whose lungs also contain elevated iron are at increased risk for Legionnaires' disease (O'Brien-Ladner et al. 2000; Fields et al. 2002; Vikram and Bia 2002). The source of intracellular iron for *L. pneumophila* likely involves the cytosolic, labile iron pool (Byrd and Horwitz 2000).

The first genetic data on the role of iron in *Legionella* was the identification of the gene for the transcriptional repressor Fur (Hickey and Cianciotto 1994). *L. pneumophila* Fur is comparable in size and cross-reactive with *Escherichia coli* Fur. Furthermore, it has an amino-acid identity of >54% and a similarity of >72% to Fur from *E. coli, Pseudomonas aeruginosa*, and others (Hickey and Cianciotto 1994). Its repressive activity is, not surprisingly, highest in legionellae grown in iron-rich media (Hickey and Cianciotto 1994, 1997). The promoter region of *L. pneumophila fur* contains putative Fur-binding sites, suggesting that this *fur* is auto-regulated. Other *Legionella* species examined contain *fur*. Since *L. pneumophila fur* could not be insertionally inactivated, Fur is likely essential for *Legionella* aerobic growth. The importance of *L. pneumophila fur* is evident from the identification of multiple iron- and Fur-regulated genes (Hickey and Cianciotto 1997).

Although the importance of iron for *Legionella* has always been clear, the mechanisms used by the bacterium to acquire and transport iron have been rather elusive. Traditional biochemical methods nicely determined that *L. pneumophila* encodes a cytoplasmic and a periplasmic ferric reductase (Johnson et al. 1991; Poch and Johnson 1993; James et al. 1997), but, as will be described momentarily, proof of the existence of a siderophore was 25 years in waiting. Other components of *Legionella* iron acquisition and assimilation have been uncovered through the examination of the *L. pneumophila* genome database or the characterization of mutants defective for growth under low-iron conditions.

Legionella siderophores

In 1983, it was reported that *L. pneumophila* does not make siderophores (Reeves et al. 1983). This conclusion was based largely on results from Arnow and Csáky assays, which detect catecholate and hydroxamate structures, respectively. Eight years later, the issue of *Legionella* siderophores was revisited using the CAS assay, which detects iron chelators independently of structure (Goldoni et al. 1991). CAS reactivity was detected in statically grown cultures, suggesting the existence of a non-catecholate, -hydroxamate siderophore. However, a later study determined that the CAS reactivity was due to the cysteine in the growth medium (Liles and Cianciotto 1996). When supernatants where derived from cultures made with cysteine-free media, no siderophore was detected using the CAS-, Arnow-, or Csáky assays (Liles and Cianciotto 1996; James et al. 1997).

In 2000, the status of *Legionella* siderophores changed, when we showed that *L. pneumophila* could excrete a high-affinity iron-chelator (Liles et al. 2000). Indeed, when grown with shaking at 37°C in a low-iron, chemically defined medium (CDM), *L. pneumophila* strains secrete a substance that is highly reactive in the CAS assay. Importantly, the siderophore activity is only seen when cultures are inoculated with bacteria that had been grown to log or early-stationary phase. Inocula derived from late-stationary phase cultures, despite growing in the CDM, do not elaborate CAS reactivity. The basis for this unusual form of regulation remains unknown. *L. pneumophila* CAS reactivity was observed for multiple virulent serogroup 1 strains as well as clinical and environmental isolates representing all nine of the other *L. pneumophila* serogroups

tested (Liles et al. 2000). The nature of the CAS reactivity has been studied further in *L. pneumophila* strain 130b, and all data indicate that it represents a *bona fide* siderophore (Liles et al. 2000). First, the presence of CAS reactivity correlates with enhanced aerobic growth in an iron-deplete defined medium. Second, the chelating activity is subject to iron-repression; i.e., the addition of iron to the CDM decreases reactivity, whereas the removal of additional iron from the CDM cultures increases CAS reactivity. Third, the CAS reactivity is less than 1 kDa and is resistant to heat and proteases. Fourth, CAS reactions are rapid and intense. Fifth, CAS-positive supernatants facilitate the growth of wild-type legionellae in BCYE agar containing otherwise inhibitory concentrations of the iron chelator 2,2′-dipyridyl (DIP) (Allard et al 2006). The iron chelating activity in *L. pneumophila* supernatants is known as legiobactin (Liles et al. 2000). CAS-reactive supernatants remain negative in the Arnow and Csáky assays, implying that legiobactin is not a typical catecholate or hydroxamate. In support of this notion, solvents used to extract those types of siderophores do not extract the *Legionella* siderophore (Liles et al. 2000). Representatives of most other *Legionella* species tested also secrete CAS reactivity (Liles et al. 2000; Starkenburg et al. 2004). Although it cannot be concluded these species are producing legiobactin, their supernatants, like that of the *L. pneumophila* strains, are negative in the Arnow and Csáky assays.

We recently identified two linked genes (*lbtAB*) that are involved in the expression of legiobactin (Allard et al. 2006). *lbtA* encodes a protein with significant homology to siderophore synthetases, including enzymes involved in the biosynthesis of aerobactin in *E. coli* and *Shigella* species, alcaligin in *Bordetella* species, and rhizobactin 1021 in *Sinorhizobium meliloti* (Allard et al. 2006). Based upon this homology, we predicted that LbtA is involved in the biosynthesis of legiobactin. LbtA is also highly related to FrgA, an iron-regulated *L. pneumophila* protein that we had previously uncovered in the course of our Fur studies but is not required for siderophore expression (Hickey et al 1997; Liles et al. 2000; Allard et al. 2006). Very recent studies have also uncovered an LbtA-like gene in *Francisella tularensis* (Deng et al. 2006; Sullivan et al. 2006). In contrast to *lbtA*, the *lbtB* gene encodes a protein that is homologous with members of the major facilitator superfamily (MFS) of multidrug efflux pumps. MFS members have recently been implicated in siderophore export, including proteins required for the export of enterobactin in *E. coli*, protochelin in *Azotobacter vinelandii*, achromabactin in *E. chrysanthemi*, and alcaligin in *Bordetella* species (Furrer et al. 2002; Brickman and Armstrong 2005; Franza et al. 2005). Thus, we predicted that LbtB is involved in legiobactin export. Mutants inactivated for *lbtA* or *lbtB* are defective for legiobactin expression, producing 40–70% less CAS reactivity in deferrated CDM (Allard et al. 2006). In bioassays, mutant CDM culture supernatants, unlike those of wildtype, did not support growth of iron-limited wild type bacteria in DIP-containing BCYE agar and a ferrous iron transport mutant (see below) on BCYE agar without added iron. The *lbtA* mutant was modestly defective for growth in deferrated CDM containing the iron chelator citrate, indicating that legiobactin is required in conditions of severe iron limitation. Since the mutant and wildtype were similarly sensitive to DFX, we suspect that the iron (III) K_s for legiobactin is between 10^{11} and 10^{31}, the K_s for citrate and DFX, respectively (Neilands 1981; Allard et al. 2006). This is compatible with legiobactin being a siderophore, since the iron (III) stability constants of most siderophores range from $K_s = 10^{22}$–10^{50} (Ratledge and Dover 2000). Complementation of the *lbt* mutants restored both siderophore expression as measured by the CAS assay and bioassays and bacterial growth in deferrated, citrate-containing media (Allard et al. 2006). Several Fur boxes precede *lbtAB*, suggesting that Fur mediates the iron-regulation associated with legiobactin production. *lbtA* is widely distributed among *Legionella* strains, a result compatible with the fact that many species produce CAS reactivity (Allard et al. 2006).

We have now purified legiobactin and obtained an initial indication of its structure (unpublished results). The *Legionella* siderophore was purified by an initial DEAE Sephadex A-50 anion exchange column followed by anion exchange

HPLC in a water-NaCl gradient. Since it has no visible absorbance, legiobactin was monitored at 220 nm. It eluted in approximately 180 mM NaCl and was then deferrated by 8-hydroxyquinoline treatment. The legiobactin peak was defined as that portion of purified *L. pneumophila* supernatants that promotes growth of iron-starved legionellae and is absent in the *lbtA* mutant supernatants. For structural analysis, we have thus far performed NMR and MALDI. A preliminary MALDI experiment showed legiobactin to be ca. 350 amu in size. The siderophore was found to contain 13 carbons; three are carbonyl carbons and ten are aliphatic carbons with various shielding effects from either N or O functional groups. Interestingly, one of the carbonyl carbons may be an aldehyde. At this point, the identity of LbtA homologs is helpful in hypothesizing the structure of legiobactin. LbtA is most homologous to FslA, a *F. tularensis* enzyme that links putrescine to citrate with an amide bond to form rhizoferrin (Sullivan et al. 2006). LbtA is also related to AcsC, RhbF, SbnF and AlcC, enzymes that form amide linkages between the amino group in diverse substrates with the monoamide or monoester derivative of citrate or succinate to form achromabactin, rhizobactin 1021, staphylobactin and alcaligin, respectively (Challis 2005). Based on our spectroscopic results and the homology of LbtA with other siderophore enzymes, we expect that legiobactin will be similar but not identical to the non-peptide siderophores rhizoferrin, rhizobactin, and alcaligin. Recently obtained crystallized legiobactin and elemental analysis will confirm the structure.

Bacteria generally have large siderophore operons encoding multiple biosynthetic enzymes and a ferrisiderophore receptor gene. Indeed, this is the case for those bacteria encoding potential relatives of legiobactin; e.g., there appear to be six biosynthetic genes for rhizobactin, and three to five for alcaligin (Challis 2005). The *L. pneumophila lbt* system seems different. There is a downstream gene (*lbtC*) linked to *lbtAB*, but LbtC is predicted to be another MFS member and mutations in *lbtC* do not alter CAS reactivity (Allard et al. 2006). Additionally, other than the existence of *frgA*, we have not identified other obvious candidate legiobactin genes unlinked to *lbtABC*. Thus, the biosynthesis of legiobactin may be uniquely simple, perhaps involving LbtA and one or two precursor molecules. Alternately, other legiobactin genes may exist, but they would appear to be unusual in content and location. The gene upstream of *lbtABC* is predicted to encode an outer membrane protein that, because of an iron box, would be Fur-regulated; we speculate that this protein is a receptor for ferrilegiobactin (Allard et al. 2006). Regardless of whether that is true, the uptake of legiobactin would appear to be mediated by a unique process because the *L. pneumophila* genome does not reveal a TonB protein (Koebnik 2005).

To start to gauge the role of siderophores in *Legionella* infection and environmental persistence, *lbtAB* mutants were tested for their ability to infect human macrophages, *Hartmannella*, and the lungs of mice. Although *lbtA* is expressed intracellularly, the mutants were unimpaired in macrophages, amoebae, and the lung, suggesting that legiobactin is relevant but not required for infection (Allard et al. 2006). These data do not, however, indicate that siderophores are not needed intracellularly; e.g., another siderophore could compensate for the loss of legiobactin. Interestingly, mutants lacking FrgA are defective in macrophages (Hickey and Cianciotto 1997), supporting the notion that *L. pneumophila* has a second siderophore that, unlike legiobactin, is necessary for intracellular infection. Since *frgA* is absent from non-*pneumophila* strains (Hickey and Cianciotto 1997), such a siderophore might be specific to *L. pneumophila*. Mutants lacking *lbtA* and *frgA* were no more defective than *frgA* mutants (Allard et al. 2006).

The fact that mutations in *lbtAB* do not completely abolish CAS reactivity suggests that there is more than one *L. pneumophila* iron chelator produced in low iron environments. The residual activity was found to be temperature-regulated, since both wildtype and *lbtA* mutants give an increase in CAS activity at room temperature (Allard et al. 2006). The activity was also not FrgA-dependent, since it was retained by an *lbtAfrgA* double mutant (Allard et al. 2006). This residual activity might represent one or multiple molecules, including a legiobactin precursor(s),

other low affinity siderophore(s), or non-siderophore CAS-reactive specie(s). If the residual activity indeed represents a new siderophore, it would be a non-traditional siderophore not detected in the Arnow and Csáky assays. The growth-promoting activity of this residual CAS activity is unclear, since *lbtA* mutant supernatants are not active in our current bioassays (Allard et al. 2006).

With the discovery of legiobactin, we now, after years of disbelief, have evidence for the existence of a *Legionella* siderophore. It is probable that the novel influence of the bacterial inoculum on siderophore production is the main reason that legiobactin was not detected earlier. It is not surprising that *L. pneumophila* produces a siderophore, since many other aquatic bacteria produce these iron scavengers. Based upon the behavior of *lbtA* mutants, legiobactin is most critical for extracellular, rather than intracellular, growth. Thus, it is conceivable that the siderophore is vital for growth in aquatic environments such as biofilms. Whereas legiobactin is linked to extracellular growth, a second siderophore might be critical for intracellular infection. That *L. pneumophila* uses multiple siderophores in order to flourish in different niches is quite reasonable

Ferrous iron transport

Since Fe^{2+} is present in the eukaryotic iron pool, presumably available to intracellular legionellae, we addressed the means by which *L. pneumophila* acquires ferrous iron. Thus, the *feoB* gene was identified and mutated in *L. pneumophila* strain 130b (Robey and Cianciotto 2002). *L. pneumophila* FeoB shares 44% identity and 61% similarity with *E. coli* FeoB. Like its *E. coli* counterpart, *Legionella* FeoB has 10 transmembrane domains, consistent with an inner membrane location. The gene's promoter region has a putative Fur binding site, suggesting *L. pneumophila feoB* is iron-regulated. FeoB mutants exhibit decreased ferrous, but not ferric, radiolabeled iron uptake and show impaired growth on low-iron BCYE agar (Robey and Cianciotto 2002). The mutant shows accelerated growth on the low-iron agar when plated around wells containing ferric (but not ferrous) iron salts or legiobactin. The *feoB* mutant also has a growth defect in low-iron BYE broth. This defect is exacerbated by the addition of DIP to the media, a result that can be reversed by additional supplementation with ferric chloride (Robey and Cianciotto 2002). These data indicate that *L. pneumophila* FeoB and Fe^{2+} transport are required for extracellular growth in low-iron conditions. Although aerobic conditions can result in a predominance of ferric iron, ferrous iron is present within *Legionella* cultures.

The role of FeoB in infection was assessed using U937 cells and *H. vermiformis* amoebae (Robey and Cianciotto 2002). In the macrophage cell line, the *feoB* mutant exhibits a 10–15-fold reduced recovery. Further evidence of the role of *L. pneumophila* FeoB in macrophage infection was seen using a cytopathicity assay. Whereas >95% of the host cells are destroyed 72 h after inoculation with wildtype bacteria, the viability of macrophages infected with FeoB-deficient bacteria does not decrease significantly over the assay period. A correlation between iron levels in macrophages and the growth defect was obtained from infection assays using U937 cells that were depleted of iron by treatment with DIP. Indeed, the growth defect increases to 10^5-fold in the presence of DIP. When amoebal cultures were done in the presence of DIP, the mutant had a 150-fold reduction in growth. Together, these results indicate that *feoB* and ferrous transport are important for the growth of *L. pneumophila* in macrophages and amoebae (Robey and Cianciotto 2002). The role of *feoB* in disease was examined using a competition assay and the A/J mouse model of pneumonia (Robey and Cianciotto 2002). Bacteria were recovered from the lungs of mice following intratracheal inoculation with equal numbers of wildtype and mutant. At 3 days post-inoculation, the wildtype had outgrown the mutant by 3-fold, indicating that FeoB promotes in vivo growth. In summary, *L. pneumophila* FeoB is important for extracellular and intracellular growth. Our data also represent the first evidence for the importance of ferrous iron transport for intracellular replication by a human pathogen. That the *feoB* mutant is not completely defective for intracellular and in vivo growth implies that other

uptake systems overcome decreased ferrous iron uptake. We suspect that FeoB and LbtAB are components of two critical pathways, since simultaneous inactivation of *feoB* and *lbtA* was incompatible with growth under standard conditions (Allard et al. 2006).

Cytochrome *C* maturation and iron assimilation

We have observed that mutations within the cytochrome *c* maturation (*ccm*) locus greatly diminish the ability of *L. pneumophila* to grow on low-iron media; e.g., *ccmB, ccmC*, and *ccmF* mutants have a greatly reduced efficiency of plating on BCYE agar lacking added iron (Viswanathan et al. 2002; Naylor and Cianciotto 2004). Ccm mutants are also dramatically impaired for replication in macrophages, amoebae, and the lungs of mice (Viswanathan et al. 2002; Naylor and Cianciotto 2004). The mutants' infectivity defects are exacerbated by treatment of the macrophages with DFX but ameliorated somewhat by the addition of iron, indicating that *L. pneumophila ccm* is required for optimal intracellular iron assimilation (Viswanathan et al. 2002; Naylor and Cianciotto 2004). Ccm systems are generally believed to mediate the transfer of heme into the periplasm and then the attachment of heme to apocytochrome *c* (Cianciotto et al. 2005). Thus, *L. pneumophila ccm* mutants lack cytochrome *c* (Viswanathan et al. 2002). However, our observations are the fourth example of a linkage between *ccm* genes and bacterial iron assimilation, complementing studies with *Paracoccus*, *Pseudomonas*, and *Rhizobium* species (Cianciotto et al. 2005). The manner in which *ccm* promotes iron assimilation is unclear, although, in the case of the other bacteria, *ccm* mutants have changes in siderophore expression. Since the *L. pneumophila ccm* mutants produce normal levels of CAS reactivity (Viswanathan et al. 2002), it is possible that the *Legionella ccm* locus facilitates growth in low-iron conditions by a novel mechanism. The infectivity defects of the *L. pneumophila ccm* mutants indicate, for the first time, that *ccm* can be necessary for growth within an intracellular niche.

There are a variety of hypotheses for the unexplained phenotypes that are associated with *ccm* mutations (Cianciotto 2005). One is a situation in which the loss of Ccm proteins results in the loss of a yet-to-be-defined *c*-type cytochrome(s); notably, *c*-type cytochromes have been shown to act as extracellular/periplasmic ferric reductases in *Geobacter sulfurreducens* (Seeliger et al. 1998). Another suggests that periplasmic heme is also used for purposes other than the maturation of a cytochrome. A third postulates that the Ccm system exports and/or processes a molecule other than heme, and that this substrate is required for iron acquisition (Daltrop et al. 2002; Stevens et al. 2004). Of course, the unexplained iron phenotypes might be the result of a combination of these and other events (Cianciotto et al. 2005).

Other possible modes of iron acquistion

Following a screen for mutants that are hypersensitive to EDDA and/or resistant to streptonigrin, we identified the *iraAB* (iron acquisition/assimilation) locus (Pope et al. 1996). Insertions in *iraA* result in a severe lag in intracellular growth and a slowed rate of replication such that macrophages infected with *iraA* mutants yield 1000-fold fewer bacteria (Pope et al. 1996; Viswanathan et al. 2000). This defect is worse when host cells are treated with DFX, indicating that the mutants are defective for intracellular iron acquisition (Pope et al. 1996; Viswanathan et al. 2000). *iraA* encodes a 272-aa protein that shows sequence similarity to methyltransferases that use *S*-adenosylmethionine as a donor. *iraB* encodes a 501-aa protein that is highly similar to a family of di/tripeptide transporters and predicted to be an inner membrane protein with 12 membrane spanning domains. A mutant lacking *iraB* consistently shows reduced growth in iron-depleted liquid broth (a phenotype eventually lost by *iraA* mutants) but does not have a defect in macrophages (Viswanathan et al. 2000). Thus, *iraA* is critical for intracellular infection, although it is not clear how IraA promotes infection or serves as a dispensable facilitator of iron assimilation. In contrast, *iraB* is critical for

growth under low-iron extracellular conditions, and IraB may be involved in a pathway that imports iron-loaded peptides.

Concluding comments

L. pneumophila uses multiple pathways for iron acquisition. It may produce several siderophores; i.e., there is direct biochemical and genetic evidence for legiobactin, and indirect data for the existence of a second, secreted ferric iron chelator. Once internalized by a Ton-B independent process, the *Legionella* ferrisiderophore(s) may be acted upon by the periplasmic ferric reductase, yielding ferrous iron that would be transported across the inner membrane by FeoB. Alternatively, a yet-to-be described transporter might deliver the ferrisiderophore across the inner membrane, whereupon the cytoplasmic reductase would act. Based upon observations with other bacteria, it is still possible that Ccm proteins somehow influence *Legionella* siderophores. *L. pneumophila* environments contain significant amounts of ferrous iron and thus, a Fe^{2+}-uptake pathway may also feed into FeoB. As yet another pathway, the *L. pneumophila iraAB* locus may encode an inner-membrane transporter that imports iron-loaded peptides. Finally, we have also found that *L. pneumophila* can bind and use hemin in addition to ferric and ferrous iron. The existence of multiple iron uptake systems in *L. pneumophila* is entirely in keeping with its varied intra- and extracellular lifestyles. Based upon our data thus far, we suspect that some of these systems are critical for infection of the mammalian host, whereas others play a more significant role in the environment. Although some aspects of *L. pneumophila* iron acquisition have parallels in other bacteria, other attributes are offering potentially new ideas about iron acquisition.

Acknowledgements The author acknowledges the many contributions of both past and present lab members. Kim Allard, in particular, is acknowledged for her genetic and biochemical work on legiobactin. Dom Castignetti, Prakash Sanjeevaiah, and David Crumrine at Loyola University, Chicago are thanked for their assistance with purifying legiobactin and characterizing its structure. This work is supported by NIH grant AI34937.

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