Purification of Legiobactin and Importance of This Siderophore in Lung Infection by *Legionella pneumophila* ▼

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When cultured in a low-iron medium, *Legionella pneumophila* secretes a siderophore (legiobactin) that is both reactive in the chrome azurol S (CAS) assay and capable of stimulating the growth of iron-starved legionellae. Using anion-exchange high-pressure liquid chromatography (HPLC), we purified legiobactin from culture supernatants of a virulent strain of *L. pneumophila*. In the process, we detected the ferrated form of legiobactin as well as other CAS-reactive substances. Purified legiobactin had a yellow-gold color and absorbed primarily from 220 nm and below. In accordance, nuclear magnetic resonance spectroscopy revealed that legiobactin lacks aromatic carbons, and among the 13 aliphatics present, there were 3 carbonyls. When examined by HPLC, supernatants from *L. pneumophila* mutants inactivated for *lbtA* and *lbtB* completely lacked legiobactin, indicating that the LbtA and LbtB proteins are absolutely required for siderophore activity. Independently derived *lbtA* mutants, but not a complemented derivative, displayed a reduced ability to infect the lungs of A/J mice after intratracheal inoculation, indicating that legiobactin is required for optimal intrapulmonary survival by *L. pneumophila*. This defect, however, was not evident when the *lbtA* mutant and its parental strain were coinoculated into the lung, indicating that legiobactin secreted by the wild type can promote growth of the mutant in *trans*. Legiobactin mutants grew normally in murine lung macrophages and alveolar epithelial cells, suggesting that legiobactin promotes something other than intracellular infection of resident lung cells. Overall, these data represent the first documentation of a role for siderophore expression in the virulence of *L. pneumophila*.

The gram-negative bacterium *Legionella pneumophila* is the principal etiologic agent of Legionnaires’ disease, a common and serious form of pneumonia that often affects immunocompromised individuals (33, 38). Human infection occurs after the inhalation of *Legionella*-contaminated water droplets that can originate from a wide variety of aerosol-generating devices. Within the lung, *L. pneumophila* replicates primarily within the resident macrophages that line the alveolus. Protein secretion systems are well known to contribute greatly to the organism’s facility to grow within that intracellular niche (49, 88). Iron acquisition is another key requirement for *L. pneumophila* replication, intracellular infection, and virulence (21–23). Previously, we determined that when *L. pneumophila* is grown in a low-iron, chemically defined medium (CDM) it secretes a low-molecular-weight, siderophore activity that is detected by chrome azurol S (CAS), a reagent that identifies high-affinity ferric iron chelators independently of structure (55, 87). Supernatants containing this CAS-reactive material stimulate the growth of iron-starved *L. pneumophila*, including the wild type and a mutant lacking ferrous iron transport (*feoB*) function (1). We have named the secreted CAS-reactive material that stimulates bacterial growth legiobactin. Other *Legionella* species also appear to express legiobactin (1, 89). To determine the role of legiobactin in infection, we have sought to test *L. pneumophila* mutants specifically lacking the siderophore in the murine model of Legionnaires’ disease. Previously, we had identified two linked genes, *lbtA* and *lbtB*, that appeared to be required for the expression of legiobactin, i.e., supernatants from mutants inactivated for *lbtA* or *lbtB* showed both a 40 to 70% loss in CAS reactivity and a complete inability to stimulate the growth of iron-starved legionellae (1). LbtA has homology to siderophore synthetases of *Bordetella* spp., *Escherichia coli*, *Erwinia chrysanthemi*, *Francisella tularensis*, *Vibrio parahaemolyticus*, and others (1, 27, 30, 40, 44, 57, 58, 90, 93). LtbB is akin to inner membrane siderophore exporters of *Azotobacter* sp., *Bordetella* sp., *Escherichia* sp., and others (1, 10, 26, 40, 42, 62, 72, 94). Thus, we believe that cytoplasmic LbtA is involved in the synthesis of legiobactin whereas LtbB promotes transit across the inner membrane prior to final export. As a necessary prelude to assessing *lbtA* or *lbtB* mutants in disease models, we now report the purification of legiobactin and the demonstration of these mutants specifically and completely lacking this molecule. Legiobactin mutants, but not their complemented derivatives, were then found to be defective for infection of the mammalian lung, indicating, for the first time, the importance of a siderophore in *L. pneumophila* virulence.

MATERIALS AND METHODS

Bacterial strains, growth media, and chemicals. *L. pneumophila* strain 130b (American Type Culture Collection [ATCC] strain BAA-74, also known as AA100) was our wild-type strain (1, 55, 84). Mutants of 130b used in this study were NU300, which has a kanamycin resistance cassette inserted into *lbtA*; NU302, which has an unmarked deletion mutation in *lbtA*; NU303, which has a gentamicin resistance cassette inserted into *lbtB*; and NU269, which has a kana-
mycin resistance cassette inserted into foeb (1, 78). Complemented derivatives that contain lbtA or lbtB cloned into pMMB202 (i.e., pBBa or pBbB) have also been described previously (1). Legionellae were routinely grown at 37°C on buffered charcoal yeast extract (BCYE) agar as previously described (1, 78). NU269 lacks an inner membrane and therefore is impaired for uptake of ferrous but not ferric iron (78). The feob mutant also has a reduced ability to grow when cultured on low-iron BCYE agar or in low-iron buffered yeast extract broth (1, 78), correlating with appreciable levels of ferrous iron in the cultures as a result of l-cysteine that is standardly added to the yeast extract base (37). This mutant growth deficit can be reversed by the addition of ferric iron salts or supernatants containing legiobactin, a mediator of ferric iron uptake (1).

Purification of legiobactin. Supernatants were prepared as described above but scaled up to yield more material for purification. In preliminary experiments, we tested the binding of the CAS reactivity in L. pneumophila supernatants to hydrophobic and charged resins. Whereas 73% of the CAS reactivity bound to hydrophobic resin XAD-16, 98% of the CAS reactivity was removed from supernatants upon incubation with the cation-exchange resin CM Sephadex C-25 or the anion-exchange resin DEAE Sephadex A-50. There was no binding to Sephadex G-25 and G-50 beads, indicating that the high level of binding to the cation and anion exchangers was specific. The A-50 resin was chosen for the next step in legiobactin purification. Five liters of CAS-reactive supernatant was harvested from strain 130b cultures, and 500-ml batches were loaded onto DEAE Sephadex A-50 (Pharmacia, Piscataway, NJ). Supernatant expressing bound CAS activity was washed with 2 column volumes of MOPS (morpholinepropanesulfonic acid) buffer and then eluted after exposure to increasing concentrations of NaCl. All CAS-reactive supernatant eluted in 300 to 400 mM NaCl. CAS-reactive A-50 fractions (500 ml) were concentrated to 10 ml by rotary evaporation, filtered, and desalted by filtration through Sephadex G-10 columns (Amersham). Fractions were concentrated down to 4 ml prior to high-pressure liquid chromatography (HPLC) analysis. Legiobactin was finally purified by HPLC using a water-400 mM NaCl gradient method. An anion-exchange column (TSK-GEI-DEAE-25W, 5 mm, 4.6 mm by 250 mm; Tosoh Bioscience, Montgomeryville, PA) was connected to a Waters 1525 binary HPLC pump system equipped with a Waters 717 Plus auto sampler, a 2996 photodiode array detector, and a Fraction Collector II (Waters Corporation, Milford, MA). Waters columns were set to a 300-nm detection wavelength, an absorbance at 300 nm, an absorbing wavelength at 220 nm. The CAS-reactive supernatant was concentrated to 1 ml using an Amicon Ultra 15 filter unit. The concentrated, semipurified supernatant (500 to 1000 µl) was mixed with 2.5% 8-hydroxyquinoline in 20 ml dichloromethane and incubated at 4°C. After 2 h of incubation, the characteristic green color of ferriquinoline was obvious. Deferriegiobactin in ddH2O was then mixed with 20 ml dichloromethane three times to remove the remaining 8-hydroxyquinoline. Logiobactin was lyophilized and kept at −80°C.

NMR spectroscopy. Proton nuclear magnetic resonance (NMR) and carbon-13 NMR were performed with purified legiobactin dissolved in D2O at 4°C on a Varian spectrophotometer (Varian, Inc., Palo Alto, CA) operating at 300-MHz1H frequency and 75-MHz13C frequency. Chemical shifts are reported in parts per million, using dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as a reference. DSS was placed externally to relate the chemical shifts of legiobactin and to avoid contaminating the sample.

Analysis of gene expression. Reverse transcription-PCR was done as described before (1). L. pneumophila DNA was isolated using RNA STAT-60 (Tel-Test B, Friendswood, TX). The primers (Integrated DNA Tech., Coralville, IA) used were lbtA-F1RT (5′-CATTGTAGCTGATTCGCTT-3′) and lbtA-R2RT (5′-GGATCCATGGAAGATGACATT-3′) to amplify a 226-bp internal fragment of lbtA and lbtB-F1RT (5′-GGATCCTGCTAAAACAAATTGCAA-3′) and lbtB-R2RT (5′-CACCAGTGTTACTGTTG) to amplify a 324-bp fragment encompassing the 3′ end of lbtA and the 5′ end of lbtB. Controls in which reverse transcriptase was omitted from the PCR were done to rule out contributions of contaminating DNA in the DNase-treated RNA samples.

Pulmonary infection of A/J mice with L. pneumophila. Female, 6- to 8-week-old A/J mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized and then inoculated intratracheally with legionellae (13, 79). As we have done before (28, 83), to determine the relative abilities of strains to replicate and survive in mouse lungs, groups of mice (n = 5) were infected separately with 106 CFU of wild-type and mutant bacteria, and at various hours postinoculation, the bacterial CFU in the lungs were determined by plating on BCYE agar. Competition assays were done as described previously (28) and inoculated with 106 CFU of a ca. 1:1 ratio of wild-type and mutant bacteria, and then 1 and 3 days later, the ratio of wild type to mutant in lung homogenates were determined by plating. Animal experiments were approved by the Animal Care and Use Committee of Northwestern University.

Intracellular infection with L. pneumophila. Bone marrow-derived macrophages and explanted alveolar macrophages were obtained from A/J mice and infected with L. pneumophila as previously described (29, 41, 91). Briefly, monolayers consisting of 1 × 105 to 2.5 × 106 macrophages were infected with bacteria at a multiplicity of infection equal to 1 (for bone marrow-derived cells) or 5 (for alveolar cells), incubated for 2 h to allow bacterial entry, and then washed three times with media to remove unincorporated bacteria. At various times postinoculation, infected monolayers were lysed and serial dilutions were plated on BCYE agar in order to determine the numbers of legionellae. The AS49 alveolar epithelial cell line (ATCC CCL-185) was maintained and infected with legionellae at a multiplicity of infection equal to 10, as previously described (43).

RESULTS

Purification of legiobactin and detection of ferrilegiobactin. Concentrated CAS-reactive supernatants of strain 130b were subjected to anion-exchange HPLC analysis (Fig. 1). Three CAS-reactive peaks were detected, with each displaying absorbance at 220 nm. The first two CAS-reactive peaks were not readily separated from each other and, over the course of our experiments (for reasons noted above), eluted at different times within the 55- to 75-min range. The third CAS-reactive peak eluted in 180 mM NaCl. In the HPLC run depicted in Fig. 1, this corresponded to an elution time of 102 min; however, over the course of later trials (for reasons noted above), this peak appeared at or near 90 min (e.g., see Fig. 2). These data confirmed that L. pneumophila supernatants contain more than one CAS-reactive substance (1). Importantly, however, the only CAS-positive peak that supported the growth of the feob mutant on low-iron medium was that which eluted in 180 mM NaCl and at 90 to 102 min (Fig. 1). Thus, we concluded that the last CAS-positive peak represents the siderophore legiobactin. During HPLC analysis, we consistently observed a peak that eluted just before legiobactin and was CAS negative but bioassay positive (Fig. 1). We hypothesized that this peak was iron-loaded legiobactin, i.e., ferrilegiobactin. To investi-
mammalian cell cultures (2). To evaluate this possibility, we utilized a mammalian cell line that highly expresses 53-16-1 (3). Strain 130b was cultured in CDM culture supernatants. Concentrated supernatants obtained from defer-
rated CDM cultures of wild-type strain 130b were injected onto a TSK-GEL DEAE-2SW anion-exchange column and then subjected to NaCl elution over a 120-min period. Fractions obtained were analyzed at A230, and tested for their reactivity (positive [+] or negative [−]) in the CAS assay and the feoB bioassay. Images showing the ability or inability of a supernatant fraction to stimulate the growth of the feoB mutant on low-iron BCYE plates are inserted over the A230 scan. The results presented are representative of at least four independent experiments. AU, arbitrary units.

FIG. 1. Anion-exchange HPLC analysis of L. pneumophila CDM culture supernatants. Concentrated supernatants obtained from defer-
rated CDM cultures of wild-type strain 130b were injected onto a TSK-GEL DEAE-2SW anion-exchange column and then subjected to NaCl elution over a 120-min period. Fractions obtained were analyzed at A230, and tested for their reactivity (positive [+] or negative [−]) in the CAS assay and the feoB bioassay. Images showing the ability or inability of a supernatant fraction to stimulate the growth of the feoB mutant on low-iron BCYE plates are inserted over the A230 scan. The results presented are representative of at least four independent experiments. AU, arbitrary units.

FIG. 2. Anion-exchange HPLC analysis of legiobactin samples treated with iron. HPLC fractions containing legiobactin (i.e., both CAS-positive and bioassay-positive material) were pooled and rein-
jected into the HPLC following no addition of iron (left), addition of 0.67 mM FeCl3 (middle), or addition of 1 mM FeCl3 (right). Fractions were eluted with NaCl over a 120-min period. Shown here are those peaks eluted between 80 and 90 min, as detected at A230 (top line) and A254 (bottom line). The results presented are representative of at least two independent experiments. AU, arbitrary units.

gate this, we pooled legiobactin fractions obtained from five HPLC runs and reinjected that material either following no further treatment or after ferration. When the untreated, pooled legiobactin peaks were reinjected, we saw not only the elution of the CAS-positive, bioassay-positive (legiobactin) peak at nearly 90 min but also the emergence of the earlier CAS-negative peak eluting nearer to 80 min (Fig. 2). This suggested that legiobactin was acquiring iron upon isolation through the HPLC lines and/or during the subsequent HPLC, resulting in a downshift in elution time and an increased ab-
sorbance at 254 nm for ferrilegiobactin. More dramatically, when pooled legiobactin samples were treated with 0.67 mM or 1 mM FeCl3, and then subjected to another round of HPLC, we observed a large diminution or complete loss of the CAS-positive peak coincident with a large increase in the preceding peak (Fig. 2A, center and right). When the material in the earlier peaks was treated with the iron-binding reagent 8-hy-
droxyquinoline, there was a restoration of CAS reactivity. Thus, upon exposure to ferric iron, legiobactin undergoes a reversible shift in elution and a loss of CAS reactivity that are compatible with the creation of ferrilegiobactin. As expected, ferrilegiobactin preparations were very capable of rescuing the growth of the feoB mutant on low-iron media (data not shown).

Absorption and NMR analysis of legiobactin. Purified legiobactin had a yellow-gold color in ddH2O, a trait common to carboxylate siderophores (102). The Legionella siderophore absorbed only in deep UV, from 220 nm and below, with a small shoulder at 325 to 350 nm, correlating with the visible yellow color it emits (data not shown). In accordance, 300-
as opposed to simply having altered levels of expression. Thus, the residual CAS reactivity that is present in mutant supernatants is most likely due to a nonspecific (nonsiderophore) CAS-reactive species or another siderophore that is not detected by the bioassays.

**Importance of legiobactin in *L. pneumophila* lung infection.** Having validated the nature of our *lbtA* and *lbtB* mutants, we next used an *lbtA* mutant in order to determine the importance of legiobactin in pathogenesis. Thus, we monitored the replication and persistence of wild-type 130b versus those of *lbtA* mutant NU300 following intratracheal inoculation into separate groups of A/J mice (Fig. 6A). As we and others have seen before (13, 28), the number of wild-type bacteria in the lungs increased ca. 10-fold during the first 24 h and then gradually

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**FIG. 3.** Proton NMR spectrum of legiobactin. Purified legiobactin in deuterium oxide was subjected to 300-MHz proton NMR. The protons of legiobactin were detected from 1.1 to 4.2 ppm. A large DOH peak was present at 4.8 ppm due to the deuterium picking up a hydrogen atom, and water suppression experiments showed that no legiobactin-related peaks were hidden under the D₂O peak. The peak near 10 ppm was not observed in repeat experiments. The results presented are representative of three independent experiments.

**FIG. 4.** Proton-decoupled C-13 NMR spectrum of legiobactin. Purified legiobactin in deuterium oxide revealed 13 aliphatic carbons (0 to 80 ppm), with 3 of those being carbonyls (170 to 181 ppm). The results presented are representative of two independent experiments.
declined over time. At 24, 48, and 72 h postinoculation, however, the \textit{lbtA} mutant exhibited a statistically significant three- to fourfold decrease in CFU relative to the level for the wild type. In a previous study, we had found that the \textit{L. pneumophila feoB} mutant has a reduced ability to grow in the murine lung (78). The magnitude of the defect exhibited by the \textit{lbtA} mutant was nearly identical to that of the ferrous iron transport mutant (Fig. 6A). In a follow-up experiment, the independently derived \textit{lbtA} deletion mutant NU302 behaved similarly to NU300 (Fig. 6B), indicating that the reduced CFU observed in vivo was due to the mutation in \textit{lbtA}, versus a second-site mutation. As confirmation, the complemented derivative of NU302 containing an intact copy of \textit{lbtA} on a plasmid behaved as the wild type did in the mouse lung (Fig. 6B). In these two experiments, the \textit{lbtA} mutants’ defects were evident at 24 h and the size of the difference in recovery between the mutant and the wild type did not increase much over the next 48 h. In a third experiment, however, the magnitude of the defect of mutant NU302 did increase over time, going from ca. threefold at 24 h to ninefold at 72 h (Fig. 6C). In a final trial that compared 130b to NU302, the size of the defect went from 5-fold at 24 h to 13-fold at 48 h (data not shown). Although we do not know the reason for the modest variation in size of the mutant defect, the defect appeared greatest in those trials in which the attainment of peak growth by the wild type was delayed (e.g., compare Fig. 6A and B to C). Together, these data demonstrate that \textit{lbtA} is required for optimal lung infection by \textit{L. pneumophila} and that mutants lacking legiobactin display an in vivo defect that ranges from 3-fold to 13-fold. From the initial ratio of 1:1. In light of the findings just described, we repeated the in vivo competition assay on the chance that NU300 might have changed phenotype. However, the mutant once again did not show a competitive disadvan-
vantage (data not shown). These data do not question the new-
found importance of lbtA in L. pneumophila infection but sim-
ply indicate that legiobactin secreted by the wild type can
promote in trans the growth of an lbtA mutant when the two
are coinfecting the lung.

Lack of a required role for legiobactin in L. pneumophila
intracellular infection of lung macrophages and epithelia. As
a first step toward identifying the reason(s) for the reduced
ability of legiobactin mutants to infect the lung, we assessed
the capacity of an lbtA mutant to infect A/J mouse macrophages.
However, mutant NU302 grew like the wild type did in both
bone marrow-derived macrophages (not shown) and explanted
alveolar macrophages (Fig. 7). Previously, we found that lbtA
mutants also grow normally in the human macrophage-like
alveolar macrophages (Fig. 7). Previously, we found that
lbtA mutants grew normally in lung macrophages and epithelial cells, the impor-
tance of legiobactin appears to most significantly involve a
process other than intracellular growth in resident lung cells.
On the one hand, legiobactin could promote growth and/or
survival of a subset of legionellae that reside in the extracel-
lar milieu. That extracellular growth and survival are com-
ponents of L. pneumophila infection has been suggested be-
fore, when various other mutants were found to be more
defective in vivo than during intracellular-infection assays (28,
35, 65, 79, 83). On the other hand, legiobactin might be critical
for promoting intracellular growth after the innate immune
system has been triggered. For example, gamma interferon-
activated macrophages contain reduced levels of iron for L.
pneumophila growth (7, 50, 51, 66). That fewer numbers of lbtA
mutant bacteria were recovered during the first 24 h postin-
oculation is compatible with legiobactin promoting growth in
the lung in extracellular compartments and/or in an immune-
activated intracellular niche (13, 14). Since the in vivo defect
increased in magnitude over time in some of our experiments,
legiobactin may also be needed in later stages of persistence.
The infectious role of siderophores has been examined for
several other pathogens that inhabit the lung or are facultative
intracellular parasites. That a siderophore can promote extra-
cellular growth and/or survival in lungs is evident from studies
of B. bronchiseptica, B. pertussis, B. cenocepacia, K. pneu-
moniae, and P. aeruginosa (11, 53, 77, 92, 98). However, sid-
erophores have also been shown to be necessary for optimal
growth in (non-immune-activated) macrophages, in the case of
B. anthracis, B. abortus, Mycobacterium tuberculosis, and S.
tuber (17, 32, 45, 73). In a situation perhaps reminiscent of
ours, S. flexneri siderophore mutants are not defective for in-
tracellular infection of host cells but are nonetheless defective
when examined in an in vivo model of infection (67, 74).
Finally, the fungus Aspergillus fumigatus produces different sid-
erophores that operate during the different extra- and intra-
cellular stages of infection (86). Thus, although the impor-
tance of legiobactin for pathogenesis is clear, the most critical site
of action for the siderophore is yet to be defined. But, in light of
the field’s major emphasis on studying L. pneumophila macro-
phage infection, our data implicating a role for legiobactin in
extracellular survival or growth in immune-activated host cells
should lead to an increased understanding of an understudied
aspect of Legionella pneumonia.

The magnitude of the in vivo defect exhibited by lbtA mu-
tants is entirely compatible with the current understanding of
bacterial iron acquisition during mammalian infection. Indeed,
it is well known that pathogens have multiple pathways for iron
assimilation and that the elimination of a single pathway gen-
erally does not completely abolish in vivo growth and virulence
(39, 85). L. pneumophila is no exception, as we have found that
the organism has, among other things, a secreted pyomelanin

**DISCUSSION**

Based on the behavior of legiobactin null mutants in a mu-
rine model of Legionnaires’ disease, we have documented that
a siderophore is required for optimal infection of the lung by L.
pneumophila. That legiobactin promotes legionellosis is in
keeping with our understanding of the role of siderophores in
infection. The importance of siderophores in mammalian in-
festation has been shown for many bacteria, including Bacillus
anthracis, Bordetella bronchiseptica, Bordetella pertussis, Bruc-
cella abortus, Burkholderia cenocepacia, Escherichia coli, Fran-
cisella tularensis, Klebsiella pneumoniae, Pseudomonas aerugi-
nosa, Salmonella enterica, Shigella flexneri, Staphylococcus
aureus, Vibrio vulnificus, Yersinia enterocolitica, and Yersinia
pestis (4, 6, 9, 11, 12, 17, 26, 27, 31, 53, 56, 60, 63, 67, 76, 77, 92,
96, 98, 100, 101). Because L. pneumophila lbtA mutants grew
normally in lung macrophages and epithelial cells, the impor-
tance of legiobactin appears to most significantly involve a
process other than intracellular growth in resident lung cells.
On the one hand, legiobactin could promote growth and/or
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the organism has, among other things, a secreted pyomelanin

![FIG. 7. Intracellular infection of murine alveolar macrophages by wild-type and lbtA mutant L. pneumophila. Explanted alveolar macrophages from A/J mice were infected with wild-type strain 130b (●) and lbtA mutant NU302 (○), and then at various time points, the CFU in infected monolayers were determined by plating. Data are the means and standard deviations (error bars) obtained from four infected wells. No significant differences were obtained between the CFU recovered from cells infected with the wild type and those infected with the mutants at 0, 24, 48, and 72 h postinoculation (Student’s t test, P > 0.05). The results presented are representative of two independent experiments.](image)
that has ferric reductase activity, a ferrous iron (feoB) transport system, heme-binding capability, and a possible iron peptide (iraAB) transporter (19, 24, 48, 68, 71, 75, 78, 81, 99).

Based on our HPLC detection of additional CAS-reactive substances in wild-type supernatants as well as the presence of an lbtA-like gene (frgA) in the L. pneumophila genome (1, 46), it is also conceivable that the organism secretes another siderophore. In keeping with the "modest" defect observed for the lbtA mutant, L. pneumophila feoB, iraAB, and frgA mutants are only partly defective when assayed for intracellular infection or lung infection (46, 78, 99).

The preceding discussion has highlighted the role of legiobactin as a direct mediator of ferric iron acquisition, a logical supposition based upon the typical role of siderophores in many other bacteria (61); however, there are other potential ways in which a siderophore, like legiobactin, might promote virulence. For example, the P. aeruginosa siderophore pyoverdine, in addition to its role as an iron scavenger, acts as a signaling molecule, regulating the expression of other virulence factors, including exotoxin A and a protease (5, 52). Also, pyoverdine is implicated as having a role in biofilm formation and surface motility (59, 97), and another P. aeruginosa secreted factor, PQS, can function as both an iron chelator and a quorum-sensing molecule (8). Furthermore, P. aeruginosa pyochelin is implicated, by virtue of being a catalyst for generating a hydroxyl radical, as a mediator of tissue damage (15). Finally, several different siderophores are capable of directly altering, at least in vitro, the viability and function of cells of the immune system, including T cells and macrophages (2, 3, 20, 47, 54, 95). Thus, future examination of the role of legiobactin in infection needs to consider the variety of ways that siderophores can act.

Our lung infection data highlight the importance of using multiple assays when judging the role of a secreted factor in pathogenesis. Indeed, uncovering the lbtA mutant defect required that the mutant be inoculated apart from the wild type. Clearly, coinoculation, i.e., a competition assay, was not effective in discerning the importance of legiobactin. In contrast, the feoB mutant, due to its lack of a membrane transporter, showed a defect whether it was tested in separate animals, as was done here, or by coinoculation, as we had done in the past (78). However, for L. pneumophila studies, this, interestingly, has not always been the case. For example, when we tested mutants lacking in type II protein secretion, we observed the mutant defect in both in vivo assays (28, 83). Thus, in order for the wild type to assist the lbtA mutant, but not the type II mutant, it would appear that the secreted siderophore is made in a larger amount, is more stable or more diffusible, or is able to exert an effect over a larger distance.

The purification of legiobactin was a critical first step in allowing us to document the importance of legiobactin in L. pneumophila lung infection, i.e., by purifying the wild-type siderophore and then comparing the HPLC profiles of the lbtA and lbtB mutants to that of the wild type, we were able to discern the true lack of siderophore in our mutants and thereby use those strains to assess the role of legiobactin in infection. However, the purification scheme developed here can also be used in future studies aimed at determining the structure of legiobactin. Based upon the inability of CAS-reactive supernatants to give a positive reaction in the Arnow and Csaky assays (55), legiobactin appears not to be a typical catecholate or hydroxamate. The biochemical analyses of purified legiobactin presented here further indicate that the Legionella siderophore has the traits of a (noncatecholate, nonhydroxamate) carboxylate siderophore, e.g., a yellow color, a weak absorption near 335 nm, the absence of aromatic carbons, and the presence of multiple carbonyls (102). Another clue to structure is the fact that LbtA has sequence similarity to amide bond-forming siderophore enzymes that are associated with the production of carboxylates, including acharmobactin of E. chrysanthemi, rhizoferrin of F. tularensis, and viribioferrin of V. parahaemolyticus (1, 30, 40, 93, 102). Some nonclassical siderophores contain diaminos that serve as carriers for iron-chelating substructures (34). In F. tularensis, the LbtA homolog FslA/FigA is believed to form an amide bond between putrescine and citric acid to create a siderophore similar to rhizoferrin, whereas in V. para- haemolyticus, the LbtA homolog PvsB or PvsD links 2-oxoglutaric acid to l-alanine through an amine bond in viribioferrin (30, 90, 93, 102). However, based upon the number of carbons detected, legiobactin appears to be unique from the other carboxylates that are synthesized via LbtA-like proteins, i.e., whereas legiobactin has 13 carbons, acharmobactin has 22, rhizoferrin 16, and viribioferrin 15 (40, 90, 93, 102). In a similar vein, legiobactin appears distinct from other carboxylates, such as rhizobactin DM4 and the staphyloferrins, which contain amide linkages (102). Ultimately, knowledge of the structure of legiobactin might help us to better understand the way(s) in which this siderophore promotes infection and lead to the generation of siderophore inhibitors that control bacterial growth (61).

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REFERENCES


