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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 afflicts 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). LbtB 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 LbtB 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-

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mycin resistance cassette inserted into *feoB* (1, 78). Complemented derivatives that contain *lbtA* or *lbtB* cloned into pMMB2002 (i.e., plbtA or plbtB) have also been described previously (1). Legionellae were routinely grown at 37°C on buffered charcoal yeast extract (BCYE) agar or in buffered yeast extract broth (1, 55). In order to obtain siderophore-containing supernatants, strains were grown in a deferrated version of CDM (1). Unless otherwise noted, chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Siderophore assays. *L. pneumophila* supernatant samples were prepared and tested for siderophore chelating activity using the CAS assay as previously described (1, 55, 87, 89). However, to facilitate the testing of fractions obtained during purification (see below), the assay was also done in a 96-well-plate format using 50 μl of CAS solution and 100 μl of test material along with 50 μl of 50 mM K_2HPO_4 (pH 7) added to speed the reaction. Supernatants and fractions were also tested for siderophore biological activity by examination of their ability to promote the growth of the NU269 *feoB* mutant on non-iron-supplemented BCYE agar as previously described (1, 78). NU269 lacks an inner membrane ferrous iron permease 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 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). 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-GEL DEAE-2SW, 5 μm, 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). Wavelengths were set to include 220 nm, an absorbance for detecting compounds eluting from the column. All solvents were degassed by a Waters in-line degasser after being filtered through 0.45-μm filters (Gelman Sciences, Ann Arbor, MI). The concentrated, semipurified supernatant (500 to 900 μl) was injected during each run. No CAS reactivity was detected in the solvent front, suggesting that legiobactin bound quite well to the column and was not being lost due to overloading. The elution gradient used consisted of a combined flow rate of 0.7 ml min^{-1} of 100% A (double-distilled water [ddH_2O], pH 5.8) for 5 min, which then decreased to 40% A while B (400 mM NaCl in ddH_2O, pH 5.8) increased to 60% for the following 120 min. Over the next 2 min (min 125 to 127), the percentage of A declined to 0 and the percentage of B increased to 100, where it remained for 10 min (min 127 to 137). From min 137 to 140, the percent A increased from 0 to 100 while the percent B declined from 100 to 0. During the last 10 min (min 140 to 150), these percentages were maintained prior to injection of another sample. The CAS- and bioassay-reactive legiobactin eluted at or near 90 min, the time 45% B (45% of 400 mM NaCl) had been obtained. Over the course of our experiments, the column had to be replaced due to unexplained pressure increases; there appeared to be a component in supernatants that irreversibly bound to the resin and eventually caused clogging. This resulted in a small variation in the times that CAS-reactive material and legiobactin eluted in the different experiments. After being concentrated and desalted as described above, legiobactin-containing fractions were deferrated with 8-hydroxyquinoline (16). Briefly, siderophore in ddH_20 (5 to 7 ml) 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. Deferrated legiobactin in ddH_2O was then mixed with 20 ml dichloromethane three times to remove the remaining 8-hydroxyquinoline. Legiobactin 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 D_2O at 4°C on a Varian NMR spectrophotometer (Varian, Inc., Palo Alto, CA) operating at 300-MHz ^1H frequency and 75-MHz ^{13}C 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* RNA was isolated using RNA STAT-60 (Tel-Test B, Friendswood, TX). The primers (Integrated DNA Tech., Coralville, IA) used were lbtAF1RT (5′-CATTTGATCGATGGCCTCTT) and lbtAR1RT (5′-GCG CGGAAATTAGGATGATA) to amplify a 226-bp internal fragment of *lbtA* and lbtAF4RT (5′-GGATCCTGCTAAAACAAATTGCA) and lbtBR2RT (5′-CAC TACCGATGGTACTGGTG) 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 10^6 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 also performed as we have described previously (28, 78, 79, 82, 83); mice were inoculated with 10^5 CFU of a ca. 1:1 ratio of wild-type and mutant bacteria, and then 1 and 3 days later, the ratios 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 1.0×10^5 to 2.5×10^5 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 A549 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-

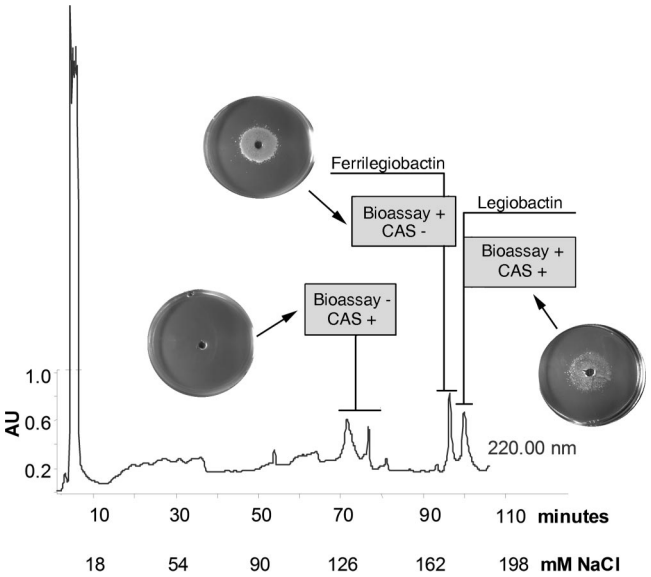

FIG. 1. Anion-exchange HPLC analysis of *L. pneumophila* CDM culture supernatants. Concentrated supernatants obtained from deferrated CDM cultures of wild-type strain 130b were injected onto a TSK-GEL DEAE-2SW anion-exchange column and then subjected to NaCl elution over a 120-min period. Fractions obtained were analyzed at A_{220} 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 A_{220} scan. The results presented are representative of at least four independent experiments. AU, arbitrary units.

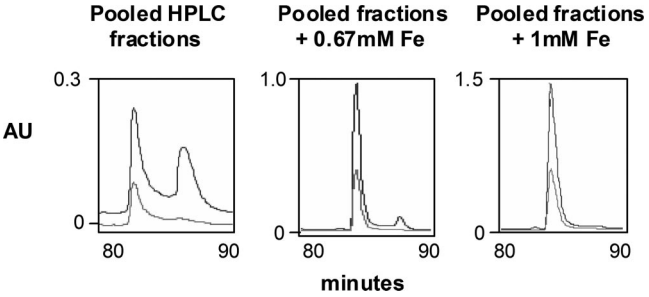

FIG. 2. Anion-exchange HPLC analysis of legiobactin samples treated with iron. HPLC fractions containing legiobactin (i.e., both CAS-positive and bioassay-positive material) were pooled and reinjected into the HPLC following no addition of iron (left), addition of 0.67 mM $FeCl_3$ (middle), or addition of 1 mM $FeCl_3$ (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 A_{220} (top line) and A_{254} (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, left). 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 absorbance at 254 nm for ferrilegiobactin. More dramatically, when pooled legiobactin samples were treated with 0.67 mM or 1 mM $FeCl_3$ 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-hydroxyquinoline, 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 ddH_2O, 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-MHz proton NMR (Fig. 3) indicated that the protons in legiobactin are not associated with any aromatic carbons, which would have given absorbance in the visible range. The protons were instead associated with aliphatic carbons, as evidenced by their detection from 0 to 4.2 ppm (Fig. 3). Based upon the peaks observed from 1.1 to 2.5 ppm, there are likely methyl, methylene, and methine protons, with some of these shifted downfield to 2.5 to 4 ppm due to a carbonyl carbon in close proximity. A hydrogen atom(s) associated with a nitrogen atom(s) would also be localized to the 2.5- to 4-ppm range. Thus, legiobactin appeared composed of mainly methylene, methine, and amine hydrogens. Proton-decoupled ^{13}C NMR was done to determine what types of carbons compose legiobactin (Fig. 4). The C-13 scan showed the presence of only aliphatic carbons, as seen from 0 to 80 ppm, confirming the proton NMR results. There appeared to be 13 carbons, with 3 of them being carbonyls, as seen from 170 to 181 ppm. These data suggest that legiobactin's iron-binding capacity comes from carbonyl oxygens, like those found in carboxylates, as in many other polycarboxylate siderophores (102).

Absence of legiobactin and ferrilegiobactin in *lbtA* and *lbtB* mutant supernatants. Previously, we had identified two neighboring genes, *lbtA* and *lbtB*, that appeared to be required for the expression of legiobactin (1). Using reverse transcription-PCR, we confirmed that *lbtA* and *lbtB* are cotranscribed in strain 130b (data not shown). To clarify the role of *lbtA* and *lbtB* in legiobactin expression, the supernatants of *lbtA* mutant NU300 and *lbtB* mutant NU303 were analyzed by HPLC, as described for parent 130b. The legiobactin and ferrilegiobactin peaks were completely absent from the supernatants of both mutants, and reintroduction of *lbtA* or *lbtB* into the corresponding mutant on pMMB2002 restored the siderophore peaks (Fig. 5). LbtA and LbtB mutants containing only the vector did not show restoration of siderophore activity. Whereas legiobactin peaks from wild-type and complemented mutant samples promoted *feoB* mutant growth, fractions from the mutants lacked bioactivity (data not shown). Together, these data confirm that *lbtA* and *lbtB* are absolutely required for the expression of legiobactin and that *lbtA* and *lbtB* mutants of strain 130b completely lack legiobactin and ferrilegiobactin,

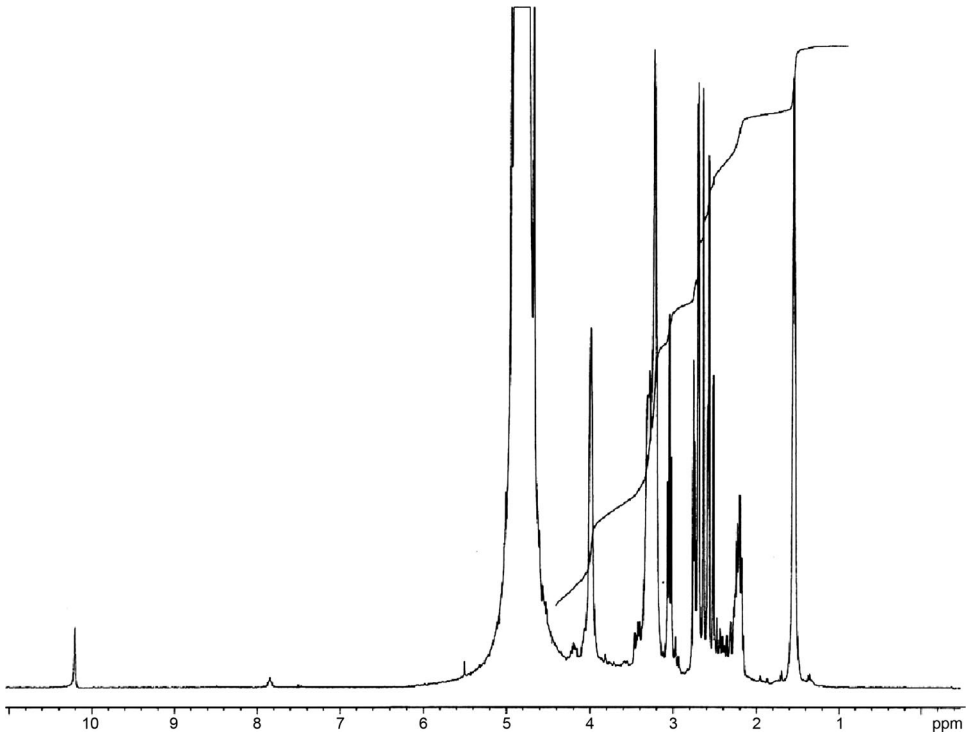

FIG. 3. Proton NMR spectrum of legiobactin. Purified legiobactin in deuterium oxide was subjected to 300-MHz proton NMR. The protons of legiobactin were detected from 1.1 to 4.2 ppm. A large DOH peak was present at 4.8 ppm due to the deuterium picking up a hydrogen atom, and water suppression experiments showed that no legiobactin-related peaks were hidden under the D_2O peak. The peak near 10 ppm was not observed in repeat experiments. The results presented are representative of three independent experiments.

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

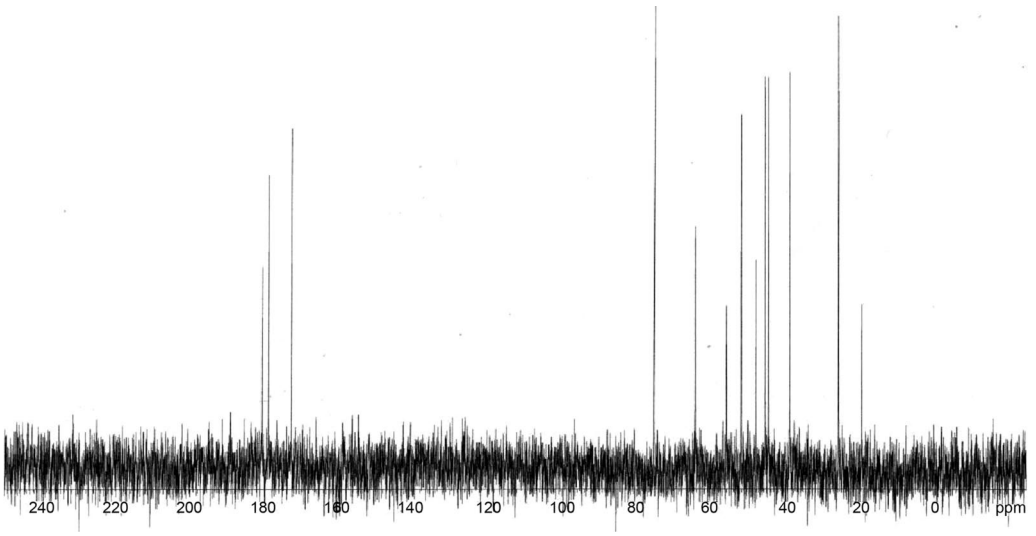

FIG. 4. Proton-decoupled C-13 NMR spectrum of legiobactin. Purified legiobactin in deuterium oxide revealed 13 aliphatic carbons (0 to 80 ppm), with 3 of those being carbonyls (170 to 181 ppm). The results presented are representative of two independent experiments.

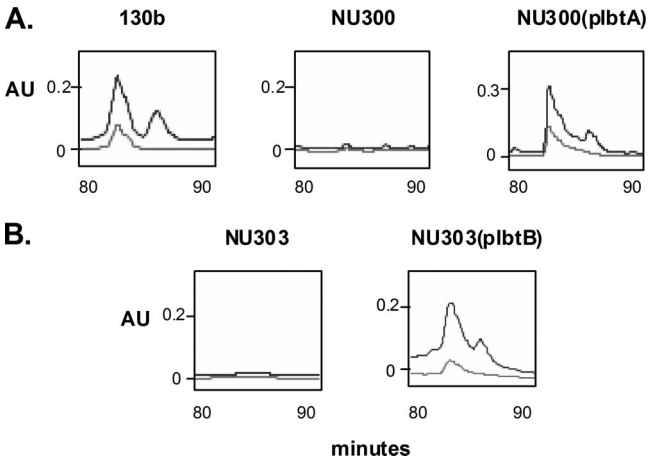

FIG. 5. HPLC analysis of CDM culture supernatants from *lbtA* and *lbtB* mutants. Concentrated supernatants obtained from deferrated CDM cultures of wild-type 130b, *lbtA* mutant NU300, and complemented mutant NU300(plbtA) (A) as well as *lbtB* mutant NU303 and complemented mutant NU303(plbtB) (B) were injected onto a TSK-GEL DEAE-2SW anion-exchange column and then subjected to NaCl elution over a 120-min period. Shown here are those peaks eluted between 80 and 90 min, as detected at A_{220} (top line) and A_{254} (bottom line). The results presented are representative of at least three independent experiments. AU, arbitrary units.

declined over time. At 24, 48, and 72 h postinoculation, however, the *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 *L. pneumophila feoB* mutant has a reduced ability to grow in the murine lung (78). The magnitude of the defect exhibited by the *lbtA* mutant was nearly identical to that of the ferrous iron transport mutant (Fig. 6A). In a follow-up experiment, the independently derived *lbtA* deletion mutant NU302 behaved similarly to NU300 (Fig. 6B), indicating that the reduced CFU observed in vivo was due to the mutation in *lbtA*, versus a second-site mutation. As confirmation, the complemented derivative of NU302 containing an intact copy of *lbtA* on a plasmid behaved as the wild type did in the mouse lung (Fig. 6B). In these two experiments, the *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 NU300, 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 *lbtA* is required for optimal lung infection by *L. pneumophila* and that mutants lacking legiobactin display an in vivo defect that ranges from 3-fold to 13-fold. Previously, in a preliminary examination of the role of *lbtA* in vivo, we examined the ability of *lbtA* mutant NU300 to grow in the A/J mouse lung when coinoculated with parental 130b (1). In that in vivo competition, the ratio of wild type to mutant at day 1 and day 3 postinoculation did not deviate significantly

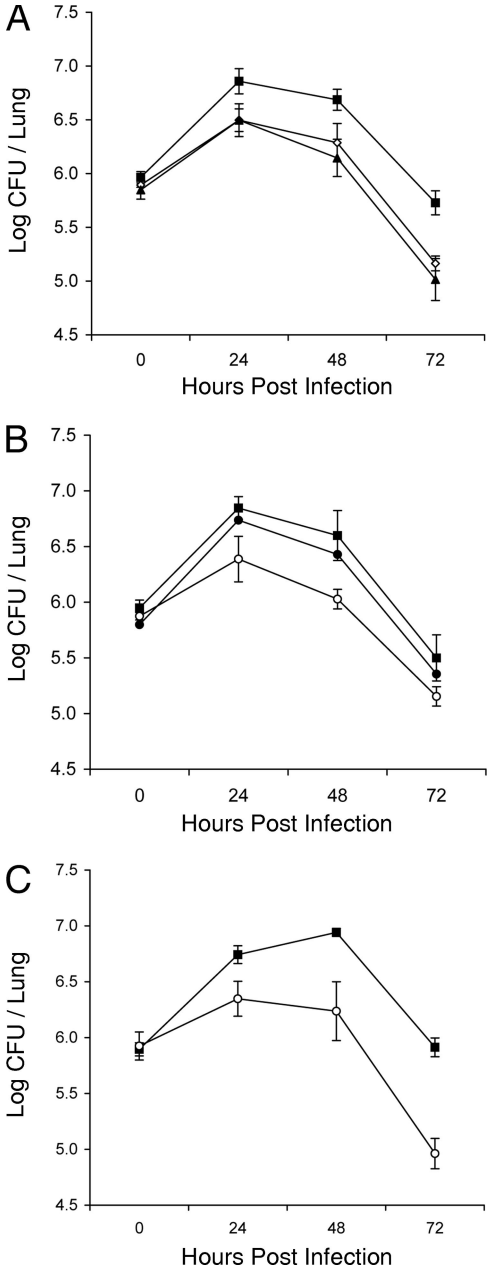

FIG. 6. Growth and survival of wild-type and *lbtA* mutant *L. pneumophila* in the lungs of infected mice. A/J mice were intratracheally inoculated with equal numbers of wild-type and mutant bacteria, and then at various time points, the CFU in infected lungs were determined by plating. (A) Infections with wild-type strain 130b (■), *lbtA* mutant NU300 (◇), and *feoB* mutant NU269 (▲). (B) Infections with 130b (■), *lbtA* mutant NU302 (○), and complemented mutant NU302(plbtA) (●). (C) Another infection with wild-type 130b (■) versus *lbtA* mutant NU302 (○). Data are the means and standard deviations (error bars) obtained from five infected animals. Significant differences were obtained between the CFU recovered from mice infected with 130b or the complemented mutants and those infected with NU300, NU302, or NU269 at 24, 48, and 72 h postinoculation (Student's *t* test, $P < 0.05$).

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-

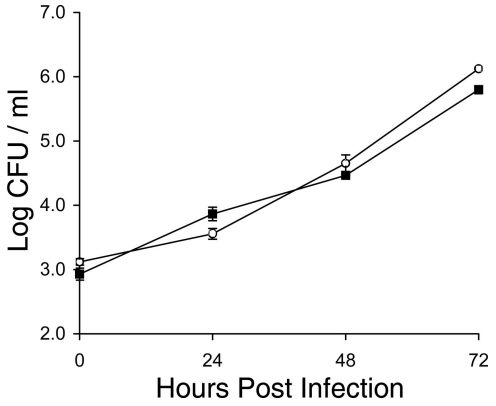

FIG. 7. Intracellular infection of murine alveolar macrophages by wild-type and *lbtA* mutant *L. pneumophila*. Explanted alveolar macrophages from A/J mice were infected with wild-type strain 130b (■) and *lbtA* mutant NU302 (○), and then at various time points, the CFU in infected monolayers were determined 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.

tage (data not shown). These data do not question the new-found importance of *lbtA* in *L. pneumophila* infection but simply 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 U937 cell line, even though *lbtA* is expressed by the wild type when growing in those host cells (1). In addition to infecting macrophages, *L. pneumophila* is able to invade and grow within alveolar epithelial cells (25, 43, 64, 80). Therefore, we next determined the relative ability of an *lbtA* mutant to infect A549 cells, an alveolar epithelial line that is known to support *L. pneumophila* infection (18, 36, 43, 69, 70, 103). However, the mutant infected these cells as well as parental 130b did (not shown). These data indicate that *lbtA* is not needed for multiple forms of intracellular infection and that the importance of legiobactin in the mammalian lung involves something other than intracellular growth in resident alveolar macrophages or lung epithelia.

DISCUSSION

Based on the behavior of legiobactin null mutants in a murine 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 infection has been shown for many bacteria, including *Bacillus anthracis*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Brucella abortus*, *Burkholderia cenocepacia*, *Escherichia coli*, *Francisella tularensis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *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 importance 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 extracellular milieu. That extracellular growth and survival are components of *L. pneumophila* infection has been suggested before, 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 postinoculation 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 extracellular growth and/or survival in lungs is evident from studies of *B. bronchiseptica*, *B. pertussis*, *B. cenocepacia*, *K. pneumoniae*, and *P. aeruginosa* (11, 53, 77, 92, 98). However, siderophores 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. enterica* (17, 32, 45, 73). In a situation perhaps reminiscent of ours, *S. flexneri* siderophore mutants are not defective for intracellular 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 siderophores that operate during the different extra- and intracellular stages of infection (86). Thus, although the importance 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* macrophage 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* mutants 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 generally 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

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 achromobactin of *E. chrysanthemi*, rhizoferrin of *F. tularensis*, and vibrioferrin of *V. parahaemolyticus* (1, 30, 40, 90, 93, 102). Some nonclassical siderophores contain diamines 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. parahaemolyticus*, the LbtA homolog PvsB or PvsD links 2-oxoglutaric acid to L-alanine through an amine bond in vibrioferrin (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, achromobactin has 22, rhizoferrin 16, and vibrioferrin 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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