**lbA and lbB Are Required for Production of the Legionella pneumophila Siderophore Legiobactin**

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Under iron stress, *Legionella pneumophila* secretes legiobactin, a nonclassical siderophore that is reactive in the chrome azurol S (CAS) assay. Here, we have optimized conditions for legiobactin expression, shown its biological activity, and identified two genes, *lbA* and *lbB*, which are involved in legiobactin production. *lbA* appears to be iron repressed and encodes a protein that has significant homology with siderophore synthetases, and FrgA, a previously described iron-regulated protein of *L. pneumophila*. *lbB* encodes a protein homologous with members of the major facilitator superfamily of multidrug efflux pumps. Mutants lacking *lbA* or *lbB* were defective for legiobactin, producing 40 to 70% less CAS reactivity in deferrated chemically defined medium (CDM). In bioassays, mutant CDM culture supernatants, unlike those of the wild type, did not support growth of iron-limited wild-type bacteria in 2',2'-dipyridyl-containing buffered charcoal yeast extract (BCYE) agar and a ferrous iron transport mutant on BCYE agar without added iron. The *lbA* 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. Complementation of the *lb* mutants restored both siderophore expression, as measured by the CAS assay and bioassays, and bacterial growth in deferrated, citrate-containing media. The *lbA* mutant replicated as the wild type did in macrophages, amoebae, and the lungs of mice. However, *L. pneumophila* expresses *lbA* in the macrophage, suggesting that legiobactin, though not required, may play a dispensable role in intracellular growth. The discovery of *lbAB* represents the first identification of genes required for *L. pneumophila* siderophore expression.

The gram-negative organism *Legionella pneumophila* is an inhabitant of natural and man-made aquatic environments (24). Importantly, it is also the primary agent of Legionnaires’ disease, a serious form of pneumonia that often afflicts the immunocompromised (10, 24). In aquatic habitats, *L. pneumophila* survives free, in biofilms, and as an intracellular parasite of protozoa such as amoebae (32, 46, 71, 74, 79). In the lung, the organism replicates within alveolar macrophages (1, 66, 79, 83).

Iron has long been recognized as a key requirement for *L. pneumophila* replication, intracellular infection, and virulence (6, 13, 28, 34, 37, 45, 61). For many years, it was believed that *L. pneumophila* was an intracellular parasite of protozoa such as amoebae (32, 46, 71, 74, 79). In the lung, the organism replicates within alveolar macrophages (1, 66, 79, 83).

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ton motive force-dependent membrane efflux pumps. Mutational analysis revealed that \( \text{lbtA} \) and \( \text{lbtB} \) are required for optimal legiobactin production and as such represent our first insight into the genetics of \( \text{Legionella} \) siderophore expression.

**MATERIALS AND METHODS**

**Bacterial strains.** \( \text{L. pneumophila} \) strain 130b (American Type Culture Collection strain BAA-74, also known as AA100) was used for mutagenesis of \( \text{lbtA} \), \( \text{lbtB} \), \( \text{lbtC} \), \( \text{pvCB} \), and \( \text{pvCB} \) and served as a wild-type control (22, 35). Other \( \text{L. pneumophila} \) strains and \( \text{Legionella} \) species tested for the presence of \( \text{lbtA} \), \( \text{frgA} \), and siderophore activity are listed in Table 1. The \( \text{L. pneumophila} \) \( \text{frgA} \) mutant NU229 as well as NU232 were previously isolated during a screen for iron-regulated genes (35). That NU232 has a mini-Tn5 insertion in \( \text{nu229} \) as well as \( \text{nu232} \) were previously isolated during a screen for iron-regulated \( \text{nu229} \) as well as \( \text{nu232} \) were previously isolated during a screen for iron-regulated genes (35).

**TABLE 1.** \( \text{lbta} \), \( \text{frgA} \), CAS reactivity, and \( \text{fedoB} \) bioassay activity in \( \text{Legionella} \) strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>( \text{lbta}^b )</th>
<th>( \text{frgA}^b )</th>
<th>Growth in CDM lacking Fe</th>
<th>CAS reactivity</th>
<th>Bioassay activity</th>
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<tbody>
<tr>
<td>( \text{L. pneumophila} )</td>
<td>Various(^c)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>( \text{L. adelaidensis} )</td>
<td>49625</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>-</td>
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<tr>
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<td>+</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
</tr>
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<td>-</td>
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<td>+</td>
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<td>( \text{L. grahamii} )</td>
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<td>+</td>
<td>-</td>
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<td>ND</td>
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<td>-</td>
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<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>-</td>
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<td>+</td>
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<td>-</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>( \text{L. spiritiens} )</td>
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<td>+</td>
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<td>33877</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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</tbody>
</table>

\(^a\) For the origin of strains and the association of a strain with disease, see reference 75 and the references therein. The strain designations represent American Type Culture Collection strain numbers.

\(^b\) ++++, hybridization to \( \text{lbtA} \) or \( \text{frgA} \) probe under high-stringency conditions; ++, moderate; +, weak; --, no hybridization to the probe under low-stringency conditions.

\(^c\) \( \text{L. pneumophila} \) strains examined include strains BAA-74 (130b), 33217 (Philadelphia-1), 33154, 33155, 33156, 33216, 33823, 35096, 43736, and 43703.

\(^d\) The growth and/or CAS reactivity of this strain varied, for unknown reasons.

In order to assess siderophore production, legionellae were typically grown in CDM that lacked its iron component (39). The iron-deplete CDM was made using water that had been deferrated by passage through a Chelex-100 (Bio-Rad, Laboratories, Hercules, Calif.) column (15). The deferration of media was confirmed by the ferrozine assay (data not shown) (78). To further control the amount of iron in media, acid-washed glassware was used (15). To monitor the general extracellular growth capacity of \( \text{L. pneumophila} \) strains, bacteria grown on BCYE agar were inoculated into BYE broth, and the optical density of the resulting cultures was determined at 600 nm (OD\(_{600}\)) (35, 63, 81). To assess extracellular growth under iron-limiting conditions, \( \text{L. pneumophila} \) was inoculated into either BYE broth that lacked its iron supplement, Chelex-treated BYE broth, deferrated CDM, or deferrated CDM with 10 \( \mu \text{M} \) to 2 mM citrate, 5 to 30 \( \mu \text{M} \) deferoxamine mesylate (DFX) or 5 to 30 \( \mu \text{M} \) ethylenediamine \( \theta\text{(ii)-hydroxy-}

**Bioassays for \( \text{L. pneumophila} \) siderophores.** CAS-positive supernatants were tested for their ability to promote the growth of either wild-type 130b in non-supplemented BCYE agar that had been made even more iron limited by the inclusion of 100 to 400 \( \mu \text{M} \) 2,2’-dipiridyl (DIP).

**Siderophore assays.** \( \text{Legionella} \) culture supernatants were tested for siderophore activity using the CAS assay as previously described (39, 56, 75). Supernatants were also tested for catecholate and hydroxamate structures by the Arnow and Csáky assays (3, 13, 39, 56, 70). DFX was the standard for the CAS and Csáky assays, while 2,3-dihydroxybenzoic acid served that role for the Arnow procedure (39, 56). Low-molecular-weight fractions were obtained by passage of supernatants through Centric filters (Millipore, Bedford, Mass.) having a 3-kDa size limit. The heat and protease susceptibility of the \( \text{Legionella} \) CAS reactivity were determined by either boiling for 5 min or incubation with proteinase K at 1 mg/ml for 3 h at 37°C (39).
Iron-supplemented BCYE agar containing 100 to 600 μM of the iron chelator DIP or feoB mutant bacteria on non-iron-supplemented BCYE agar. To obtain the CAS-positive samples, strain 130b was grown in BYE to log phase (i.e., OD660 of 1.0), washed, and then inoculated into 20 ml of deferrated CDM to an OD660 of 0.25. After 20 h of incubation, a period of time sufficient to yield significant CAS reactivity (39), the culture was centrifuged at 3,000 rpm in a Beckman J2-21 for 10 min, and the supernatants were collected and passed through a 0.2-μm filter (Millipore, Bedford, Mass.). To obtain a low-molecular-weight fragment, 2 ml of the sterile supernatant was passed through a 3-kDa-cutoff Centrifor filter. To finally assess growth-stimulating activity, 50 μl of the sample was placed into a well cut out of the center of either the DIP-containing BCYE seeded with 107 CFU of wild type or the non-iron-supplemented BCYE agar unto which had been spread 105 CFU of the feoB mutant. As a positive control, we tested the stimulatory activity of 50 μl of 10 mM FeCl3 (in water). For negative controls, we monitored growth around wells containing 50 μl of either deferrated CDM or a <3-kDa fraction obtained from a CAS-negative supernatant of strain 130b.

To obtain the CAS-negative fraction, 130b bacteria were grown in BYE to stationary phase (OD660 of 2.1), inoculated into deferrated CDM to an OD660 of 0.25, and then incubated for 2 h (39). The growth of the wild-type bacteria was observed upon incubation at room temperature, whereas the feoB mutant plates were examined at 37°C. To assess the direct growth-stimulating ability of L. pneumophila strains and other Legionella species, legionellae were grown on non-iron-supplemented BCYE agar at 37°C for 2 to 3 days and spotted with a sterile stick onto non-iron-supplemented BCYE agar with or without 2 mM isopropylthiogalactoside (IPTG) onto which had been spread 107 CFU of the feoB mutant. The plates were then incubated and examined.

**DNA isolation and DNA sequencing.** DNA isolation was performed using a DNA isolation kit (QIAGEN, Valencia, Calif.). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Nucleotide sequences were analyzed with Seqman (DNASTar; Madison, WI), and BLAST homology searches were conducted through GenBank at the National Center for Biotechnology Information. Protein alignments were performed by the BCM Search Launcher: Multiple Sequence Alignments (http://searchlauncher.bcm.tmc.edu/multi-align.htm; last accessed June 27, 2017) and ClustalW (http://www.ch.embnet.org/software/BOX_form.html). DNA motifs and structural analyses were conducted using the Prosite prediction model (http://us.expasy.org/prosite) and SOSUI program (http://sosui.proteome.bio.tuat.ac.jp).

**RT-PCR analysis of L. pneumophila gene expression.** Reverse transcription (RT)-PCR was performed as before (42, 82). Legionella RNA was isolated from CDM-cultured bacteria or L. pneumophila-infected macrophages using RNA STAT-60 (TEL-TEST B, Inc., Friendswood, TX). Primers MIP1-F (5′-GATCGATGGCCTCTT) and PVCB6-R (5′-GTCATCTCGACAGGATGG) were used to amplify a 260-bp internal fragment of mip (11) and LTBA-F (5′-CATTTGGATCGATGGCCTCTT) and LTBA-R (5′-GGCGGAAATATATGAATGTTGCGTA) were used to amplify a 111-bp fragment of LTBA. Control experiments in which the reverse transcriptionase complex omitted from the reaction mixtures were used to confirm the rule out contamination of competing DNA in the DNase-treated RNA samples. These controls were performed with IBT primers described above.

**Mutation and complementation analysis.** To obtain a mutated ibtB gene with a kanamycin resistance (Km) marker, an internal fragment of the L. pneumophila strain 130b ibtB gene was amplified by PCR using primers LTBA-F (5′-ATAGCTGCTACATGACGGG) and LTBA-R (5′-TGTATTCAAACAGCTGTCG). The resultant 2-kb DNA fragment was then cloned into pGemTeasy (Promega, Madison, WI) to give plasmid pVK120. The cloned L. pneumophila ibtB was mutated by deletion of a 51-bp fragment at the two MfeI sites, the first located 563 bp from the gene's start site, resulting in pKA9. The mutated ibtB gene was amplified by PCR using primers USKanBam2 (5′-CTCAT) at the first BglII-digested site of pJSA1 after a 34-bp deletion, to give pVK121 or a Gm r gene PCR amplified from pJSD6, respectively (25, 77). The ibtB Cmr gene was generated by insertion of the PCR-amplified Cmr gene into the Kmr r gene of the plasmid pJSA1 using the Kmr r and Gm r markers. Multiple pVC and pVCB mutants were obtained by natural transformation of 130b with pSS3 and pSB6, respectively (25, 77). The IbtB Cmr double mutant was constructed in the Kmr r/lbtB r background by introducing pKA11 with lbtB::Cmr.

Production of competent 130b, NU229, lbtA, lbtB, and pvcB mutant cells and electroporation of plasmid pKA12, and pKA17, and pKA9 were carried out as described (41).

**To examine the ability of L. pneumophila to grow intracellularly, Hartmannella vermiformis amoebae and human U937 cells were infected as previously described (12, 63, 65).** Production of infection was monitored by measuring the Kmr r and Gm r mutant chromosome by homologous recombination. Verification of the Km r/lbtB r mutant genotype was carried out by PCR and Southern hybridization, using the same primers and DNA probe used to identify ibtB on pVK122, pKA11, and pKA17. The IbtB and IbtB Cmr mutant phenotypes were verified by PCR using the same primers to identify ibtB on pVK122, pKA11, and pKA17. The IbtB and IbtB Cmr mutant phenotypes were verified by PCR using primers LBTB-F and LBTB1-R (5′-ACTAATGATGCCAAGGCTG) (IbtB) and LBTB-F and LBTB1-R (IbtB). The pvcA and pvcB mutants were verified by PCR and Southern hybridization with the same primers used to identify pvcA and pvcB.

To facilitate complementation, a 2.1-kb KpnI-XbaI-digested fragment containing only the IbtB gene with its endogenous promoter was obtained from pKA2 and cloned under control of the tac promoter in pMMB2002 (65) to yield plBTA. For complementation of the IbtB mutants, a 1.6-kb fragment containing only the IbtB gene was amplified by PCR from L. pneumophila 130b DNA using primers LBTB-F and LBTB1-R and cloned into pGemTeasy (Promega; Madison, WI) to yield pKA14. Finally, the wild-type IbtB gene was cloned on a 1.7-kb SacI-SphI fragment from pKA14 into pMMB2002 under the control of the tac promoter to yield plBTA. Complementation with plBTA was obtained when IbtB was under the control of the tac promoter and induced with 2 mM IPTG (68). Plasmids were electroporated into L. pneumophila strains as previously described (41).

**Infection assays.** To examine the ability of L. pneumophila to grow intracellularly, Hartmannella vermiformis amoebae and human U937 cells were infected as previously described (2, 12, 63, 65). Infection of iron-depleted macrophages and amoebae was accomplished by the addition of 10 to 40 μM DFX to the medium for 24 h prior to infection with L. pneumophila and/or during the incubation period (7, 28, 59, 63, 81, 82). We observed that as much as 50% of the iron chelator DIP in U937 macrophages had no effect on the growth of 130b (data not shown). To assess the virulence of bacteria, competition assays were done following intratracheal inoculation of A/J mice, as described previously (63–65).

**Southern hybridization analysis.** Southern blots were carried out using EcoRI-restricted DNA from strains representing several L. pneumophila serogroups and a variety of Legionella spp. A digoxigenin nonradioactive labeling and
low-stringency washes (30% base pair mismatch) were used for hybridization and then inoculated into deferrated CDM to an OD 660 of 0.2. Over the next day, the growth of the cultures was monitored spectrophotometrically (left y axis), and the CAS reactivity of culture supernatants, reported as net DFX equivalents, was examined (right y axis). The values presented are the means and standard deviations from triplicate cultures. The CAS reactivity of the cultures was significantly above the medium control at all times of incubation (P < 0.05; Student’s t test). The results are characteristic of three independent experiments.

Optimization and kinetics of siderophore expression by *L. pneumophila* 130b. By acid-washing glassware and by deferrating the water used to make the CDM, we obtained culture supernatants that, based upon the CAS assay, consistently yielded ≥900 μM net DFX equivalents, with the highest values approaching 1,500 μM equivalents. The ability of strain 130b to elaborate CAS reactivity that was <3 kDa and heat- and protease-resistant still required the use of log-phase inocula. The increased level of siderophore activity observed in deferrated cultures was not associated with the expression of an Arnow- or Csáky-reactive material, suggesting that it is not due to turn-on of a “typical” catecholate or hydroxamate siderophore.

Mixtures of supernatants with siderophores DFX and DHB retained positivity in the structural assays, indicating *L. pneumophila* is not elaborating a substance that interferes with siderophore recognition. Since cysteine is reactive in the CAS assay (40), we optimized the detection of legiobactin by replacing the cysteine in deferrated CDM with cystine, a substance that is not CAS reactive (40). Supernatants obtained from cystine-containing cultures consistently displayed ≥900 μM DFX equivalents while having no background reactivity (data not shown). Thus, all subsequent legiobactin determinations were made using cystine-containing deferrated CDM and are presented as net DFX equivalents.

To better understand the kinetics of legiobactin production, we examined strain 130b cultures for CAS reactivity at multiple early time points (Fig. 1). Legiobactin expression was detectable as early as mid-log phase, and the level of siderophore increased within the culture until stationary phase was established and then it declined. The decline in CAS reactivity appeared to be due to the action of the bacteria; i.e., filter-sterilized supernatants obtained at 24 h of incubation maintained full CAS reactivity even when stored 37°C (data not shown). This suggests that stationary-phase *L. pneumophila* may degrade and/or not recycle legiobactin. Given the clear coincidence of CAS reactivity with the most active stage of bacterial growth, legiobactin is likely an enhancer of *L. pneumophila* replication in low-iron conditions.

**RESULTS**

**Effect of temperature on *L. pneumophila* siderophore expression.** Since *L. pneumophila* survives and replicates at temperatures ranging from 4 to 63°C (33, 42, 73) and since siderophore expression is elevated at lower temperatures in some other aquatic bacteria (14), strain 130b was grown in deferrated CDM at 25°C and 37°C, and supernatants were tested in the CAS assay (Fig. 2). As before, the legionellae grew more slowly at room temperature than they did at 37°C (73), but the peak optical density for the 25°C cultures was always comparable to that seen with 37°C cultures. CAS reactivity was readily apparent in 25°C cultures, with the level of siderophore activity increasing along with the progression toward stationary phase and then declining afterward (Fig. 2, and data not shown). The maximal amount of CAS-reactive mate-
rial in 25°C cultures was 2 to 3 times greater than in 37°C cultures. However, as was the case at 37°C, the CAS reactivity at 25°C was Arnow- and Csa´ky-negative and required that the CDM be inoculated with log-phase legionellae. The elevated levels of CAS reactivity at 25°C may be due to increased expression of legiobactin or another siderophore. Alternatively, it may mean that degradation of legiobactin is greater at higher temperatures.

**Biological activities associated with legiobactin.** Next, we sought to develop a siderophore bioassay by testing CAS-positive supernatants for their ability to facilitate the growth of iron-starved wild-type legionellae. Toward that end, we first determined that wild-type 130b would not form colonies within 25°C BCYE agar that lacked its iron supplement and contained the ferrous iron chelator DIP at a concentration of \[100 \mu M\] (Fig. 3A). As expected, bacteria did grow in the DIP-containing medium, if a solution of 10 mM ferric chloride was placed into a well cut out of the agar (Fig. 3A).

To ascertain the effect of legiobactin, sterile supernatants were obtained from 130b cultures that had been inoculated with either log-phase or stationary phase bacteria, and then the <3-kDa fraction from the resulting CAS-positive and CAS-negative samples were compared for their ability to promote colony formation in the DIP-BCYE agar. The CAS-positive supernatant fraction alone, even when diluted 5-fold, facilitated the growth of wild-type legionellae (Fig. 3A), suggesting that legiobactin has biological activity.

**Identification of an L. pneumophila gene, lbtA, that is required for legiobactin production.** Since our frgA and ira mutants (59) continued to display wild-type levels of growth and CAS reactivity when cultured in deferrated, cystine-containing CDM (data not shown), it appeared that previously unrecognized genes encode legiobactin. While performing inverse PCR (42, 82) to characterize the nature of the mini-Tn10 insertions in the ira mutants, a primer (i.e., 5'-GGCTCACGA TGGCACTTG-3') that had been designed to facilitate the analysis of strain NU223 amplified, without the assistance of a second primer, a 2-kb DNA fragment, predicted to contain the entire open reading frame, was amplified from strain 130b. Sequence analysis of the PCR fragment identified an incomplete open reading frame whose predicted product appeared to have homology with the carboxyl end of FrgA. Subsequent examination of what was, at the time, the unfinished genome database of L. pneumophila revealed the presence of a similar open reading frame within strain Philadelphia-1. Using PCR primers based upon sequences in the database, a ~2-kb DNA fragment, predicted to contain the entire open reading frame, was amplified from strain 130b. Complete sequence analysis of the amplified fragment confirmed the existence of an L. pneumophila 1.74-kb gene, whose...

![Fig. 3. Biological activities associated with legiobactin. (A)](http://jb.asm.org/)

- **BCYE – Fe**
  - CDM
  - FeCl<sub>3</sub>
  - CAS<sup>−</sup> filtrate
  - CAS<sup>+</sup> filtrate

- **BCYE – Fe with DIP**
  - CDM
  - FeCl<sub>3</sub>
  - CAS<sup>−</sup> filtrate
  - CAS<sup>+</sup> filtrate

- **BCYE + Fe**
  - CDM
  - FeCl<sub>3</sub>
  - CAS<sup>−</sup> filtrate
  - CAS<sup>+</sup> filtrate

- **BCYE – Fe**
  - CDM
  - FeCl<sub>3</sub>
  - CAS<sup>−</sup> filtrate
  - CAS<sup>+</sup> filtrate

The results presented for each type of bioassay are representative of at least three independent experiments.
In order to determine if \( lbtA \) plays a role in siderophore expression, we constructed a nonpolar \( lbtA \) deletion mutant, NU302, which had a 626-bp fragment excised from \( lbtA \). NU302 supernatants displayed 40 to 70% less CAS-reactive material than the wild type and did not promote the growth of the \( feoB \) mutant in iron-limiting BCYE agar (Fig. 5A). trans-

Complementation with a plasmid (pbltA) containing \( lbtA \) as its only \( L.\ pneumophila \) gene restored both the CAS reactivity and bioactivity of NU302 to wild-type levels (Fig. 5A), indicating a role for \( lbtA \) in legiobactin production. By introducing pbltA into 130b, the amount of CAS reactivity was increased two- to threefold (Fig. 5A), suggesting that legiobactin production is increased if excess LbtA is available.

In support of the conclusion that \( lbtA \) is involved in siderophore expression, mutants NU300 and NU301 containing a kanamycin resistance (Km') cassette insertion in \( lbtA \) also displayed 40 to 70% less CAS-reactive material than the wild type and had no activity in the two siderophore bioassays (data not shown). The mutants showed a comparable reduction in siderophore expression when grown at room temperature (data not shown), indicating that the increased CAS activity at room temperature is \( lbtA \) independent. The \( lbtA \) mutants grew normally on standard BCYE agar and in standard BYE broth (data not shown), indicating that \( lbtA \) is not generally required for extracellular growth. Based upon the \( lbtA \) mutant phenotypes, we believe that LbtA is involved in the expression of legiobactin. The homology of LbtA with siderophore synthetases and the protein's predicted cellular location suggests that LbtA is involved in the biosynthesis of the \( L.\ pneumophila \) siderophore.

Identification of a second gene, \( lbtB \), involved in legiobactin expression. According to the completed genomes of \( L.\ pneumophila \) strains Philadelphia-1, Paris, and Lens (8, 9), \( lbtA \) is the first gene in a three-gene operon. The two genes downstream of \( lbtA \) are predicted to encode members of the MFS class of proton motive force-dependent membrane efflux pumps. The second gene in the operon, which we now designate \( lbtB \), is a 1.2-kb gene that encodes a 44.4-kDa protein with 12 transmembrane (TMS) domains that is 23% identical and 44% similar to the \( E.\ coli \) bicyclomycin resistance protein Bcr and 21% identical and 39% similar to the \( E.\ coli \) tetracycline efflux pump TetA.

The homology between LbtB and these proteins is greatest in five of the amino acid motifs conserved among MFS transporters, i.e., motifs A, B, C, D, and G (Fig. 6). Recently, members of the MFS family have been shown to include transporters involved in the export of bacterial siderophores, such as the \( E.\ coli \) enterobactin exporter EntS (27). The last gene in the \( lbt \) operon, \( lbtC \), encodes a 42.5-kDa protein with 12 predicted transmembrane domains that is related to LbtB and Bcr (data not shown). Given these data, we suspected that \( lbtB \) and \( lbtC \) are involved in legiobactin export.

In order to assess the role of \( lbtB \) in legiobactin production, we constructed two \( lbtB \) insertion mutants, NU303 and NU304. Both mutants grew normally on standard BCYE agar and in standard BYE broth (data not shown), indicating that, like \( lbtA \), \( lbtB \) is not generally required for extracellular growth. However, the \( lbtB \) mutants consistently produced 40 to 70% less CAS-reactive material than the wild type (Fig. 5B; NU303 with or without the vector pMMB2002 gave similar results, as
did NU304). In addition, NU303 supernatants did not promote the growth of the feoB mutant in BCYE agar lacking the iron supplement (Fig. 5B). Thus, the lbtB mutant was defective for legiobactin production. Importantly, trans-complementation with lbtB under the control of the tac promoter in plasmid plbtB restored both siderophore expression of the mutant to wild-type levels and the ability of NU303 supernatants to promote growth of the lbtB mutant on iron-limited BCYE agar (Fig. 5B).

Providing lbtB in trans to the wild type results in an almost twofold reduction in the amount of CAS reactivity detected in CDM supernatants. This does not occur in the lbtB mutant; reintroducing lbtB in this background only restores siderophore expression to normal wild-type levels. Since the lbtB mutant bears an insertion that may have downstream effects on lbtC expression, “excess” LbtB in the presence of “normal levels” of LbtC may have deleterious effects on siderophore excretion. Overall, these data confirm that LbtB is required for legiobactin expression.

Next, a role for lbtC in legiobactin production was assessed by introducing a mutation into the gene. Two mutants, NU305 and NU306, containing a 1.1-kb Gm\^R insertion in lbtC were obtained (data not shown). Both mutants grew normally on standard BCYE agar and in standard BYE broth (data not shown), indicating that lbtC is not generally required for extracellular growth. However, the lbtC mutants produced wild-type levels of legiobactin (data not shown), ruling out a required role for lbtC in legiobactin expression.

Since supernatants from lbtA and lbtB mutants had similar reductions in CAS reactivity and bioactivity, we believe that both lbtA and lbtB are required for optimal siderophore production by strain 130b. However, phenotypic differences between lbtA mutant NU300 and lbtB mutant NU303 were seen when the bacteria, as opposed to supernatants, were assessed in the feoB mutant bioassay (Fig. 7). When spotted atop the feoB mutant, 130b bacteria containing either vector pMMB2002, plbtA, or plbtB supported the growth of the iron-starved mutant. In keeping with the results obtained with supernatants, the lbtA mutant NU300 only stimulated growth if it contained plbtA (Fig. 7). In contrast, NU303 retained an ability to promote growth of the mutant regardless of recombinant plasmid content, suggesting that, unlike the lbtA mutant, the lbtB mutant still produces legiobactin. Indeed, the homology of lbtB with MFS exporters implies a role for LbtB in legiobactin export and not biosynthesis. We suspect that when the lbtB mutant is grown on the low-iron agar media, some cellular lysis occurs and released legiobactin stimulates the feoB mutant to grow, and the inability of NU303 supernatants to likewise
promote growth is likely due to a dilution and/or breakdown of siderophore in the broth.

Influence of *L. pneumophila* pvc-like genes and *frgA* on siderophore expression in the wild type and an *lbtA* mutant. The fact that supernatants from the *lbtA* and *lbtB* mutants were not completely lacking CAS-reactive material suggested, for the first time, that the CAS reactivity produced by *L. pneumophila* is the result of multiple CAS-reactive substances and that strain 130b might secrete a second siderophore. An investigation of the *L. pneumophila* genome database revealed a gene (i.e., lpg0174 in Philadelphia-1, lpp0236 in Paris, and lpl0236 in Lens) that was homologous with the *Pseudomonas aeruginosa* pyoverdine biosynthetic gene *pvcA* as well as an adjacent gene homologous with *pvcB* (76). However, *L. pneumophila* pvcA mutants (i.e., NU307 and NU308) and pvcB mutants (i.e., NU309 and NU310) produced wild-type levels of CAS reac-

![FIG. 6. Amino acid sequence alignments of *L. pneumophila* LbtB with *E. coli* Bcr and TetA. The consensus sequences for conserved motifs A, B, C, D, G are labeled above the boxed-in areas; x is any amino acid, upper case is a highly conserved amino acid, and lower case is a conserved, but variable amino acid. Motif A, conserved in both 12- and 14-TMS families, is located in the cytoplasmic loop between TMS 2 and TMS 3 and may be involved in substrate binding as well as opening and closing of the channel (54). Motif B, located in TMS 4, is predicted to be involved in proton transfer (55). Motif C, located in TMS 5, is implicated in the direction of transport and is only found in those transport proteins with efflux capacity (30, 55). The function of motif D, located in TMS 6 in 12- and 14-TMS families, has not been investigated. Motif G, located in TMS 11, is found only in 12-TMS families, although its function is unknown (55).](http://jb.asm.org/)

![FIG. 7. Phenotypes of *lbt* mutant bacteria in the *feoB* bioassay.](http://jb.asm.org/)
tivity when grown in deferrated CDM (data not shown), indicating that \textit{pvcAB}, like \textit{frgA}, is a siderophore-like gene that is dispensable for normal CAS reactivity in strain 130b.

However, it remained possible that increases in CAS reactivity due to legiobactin could have been masking a loss of a siderophore activity associated with FrgA or PvcAB. Thus, we constructed and characterized \textit{lbtA} \textit{frgA} double mutants (NU311 and NU312) and \textit{lbtA} \textit{pvcA} double mutants (NU313 and NU314). Both types of double mutants were identical to the \textit{lbtA} single mutants in the CAS assay and the \textit{feoB} bioassay (data not shown), showing that \textit{frgA} and \textit{pvcAB} are not required for wild-type CAS reactivity or the residual CAS activity of \textit{lbtA} mutants. Further BlastP searches of the \textit{L. pneumophila} genome using known siderophore biosynthetic, transport, and receptor proteins, as well as an examination of the annotation of the \textit{L. pneumophila} genome did not reveal any other candidate siderophore genes. Given these various data, we focused on determining the importance of \textit{lbtA} for \textit{L. pneumophila} growth.

Role of \textit{lbtA} in \textit{L. pneumophila} extracellular growth. \textit{lbt} mutants NU300, NU302, and NU303 grew in deferrated CDM as well as did the wild type (data not shown), suggesting that \textit{lbtA} and \textit{lbtB}, though necessary for full siderophore expression, may not be required for extracellular growth in iron-deplete conditions, even though \textit{lbtA} is expressed in low-iron CDM. Mutant NU302 also grew normally in BYE broth or on BCYE agar that lacked their iron supplements, on unsupplemented BCYE agar that contained 100 to 400 \(\mu\)M DIP, and in deferrated CDM with 5 to 30 \(\mu\)M EDDA or DFX (data not shown). These data suggested that the affinity of legiobactin for iron might be less than \(10^{-31}\), as the stability constants \((K_s)\) for DFX-iron(III) and EDDA-iron(III) are \(10^{31}\) and \(10^{34}\), respectively (48, 72).

Therefore, to identify a requirement for legiobactin, we investigated bacterial growth in deferrated CDM containing citrate, a chelator with a notably lower affinity for iron(III), \(10^{-31}\) (72). Indeed, when grown in deferrated CDM supplemented with 1 mM citrate, NU302 showed modestly reduced growth at 8 to 44 h postinoculation, and this defect was fully complemented by reintroducing \textit{lbtA} into the mutant on plbtA (Fig. 8A). When the \textit{lbtA} mutant was grown in the presence of citrate, including chelator levels that were not inhibitory to bacterial growth, it produced increasing amounts of CAS reactivity, eventually achieving a degree of reactivity that rivaled the wild-type level (Fig. 8B). This could represent the upregulation of the residual CAS activity found in \textit{lbtA} supernatants or the production of a new CAS reactive substance. However, the mutant supernatants continued to be inactive in the \textit{feoB} bioassay and negative in the Csáky and Arnow assays, indicating that the heightened CAS reactivity was not due to the turning on of a typical hydroxamate or catecholate. In summary, \textit{L. pneumophila} can replicate in a variety of low-iron conditions in the absence of \textit{lbtA}, but legiobactin is beneficial for growth under conditions of severe iron limitation when residual iron is not sequestered by a chelator(s) of extraordinarily high affinity.

Role of \textit{lbtA} in intracellular infection and virulence. We next determined the ability of an \textit{lbtA} mutant to infect human U937 cell macrophages and \textit{H. vermiformis} amoebae. Under standard conditions, NU300 behaved as 130b did in both host cells (data not shown). Likewise, NU300 did not differ from 130b when grown in amoebal cultures treated with 0, 25, and 50 \(\mu\)M DIP or in U937 cultures treated with either 10 to 15 \(\mu\)M DFX or 20 to 40 \(\mu\)M DIP. NU300 behaved like the wild type did whether the inoculum was derived from BCYE agar or log-phase BYE cultures (data not shown). Together, these data indicate that \textit{lbtA} and legiobactin are not required for optimal intracellular infection. The \textit{lbtA} \textit{frgA} double mutant NU311 was no more defective in U937 cells than the single \textit{frgA} mutant, and \textit{pvcA} and \textit{pvcB} single mutants and an \textit{lbtA} \textit{pvcA} double mutant all grew normally in the macrophage cell line (data not shown), again indicating that \textit{lbtA} is not necessary for intracellular growth.

However, RT-PCR experiments demonstrated that \textit{lbtA} is expressed by \textit{L. pneumophila} when growing within U937 cells (Fig. 4B). We were unable to obtain an \textit{lbtA} \textit{feoB} double mutant. Furthermore, the residual iron is not sequestered by a chelator(s) of extraordinary high affinity, indicating that the heightened CAS reactivity was not due to the turning on of a typical hydroxamate or catecholate. In summary, \textit{L. pneumophila} can replicate in a variety of low-iron conditions in the absence of \textit{lbtA}, but legiobactin is beneficial for growth under conditions of severe iron limitation when residual iron is not sequestered by a chelator(s) of extraordinarily high affinity.
mutant of strain 130b, whose isolation might have uncovered an intracellular role for a siderophore as it has in studies of Shigella (67). Next, a competition assay was performed in A/J mice (5, 63–65). However, the ratio of wild type to mutant in the mouse lung did not change significantly during the 3-day course of the experiment (data not shown), suggesting that lbtA and legiobactin are not required for L. pneumophila growth in the lungs of A/J mice.

Distribution of lbtA in L. pneumophila serogroups and in other Legionella species. The L. pneumophila species consists of 15 serogroups (24). Our analysis of strain 130b as well as the data contained within the Philadelphia-1, Paris, and Lens genomic databases indicates that lbtA is present within L. pneumophila serogroup 1. Southern hybridization analysis determined that lbtA is also in strains representing serogroups 2 to 5, 7, 8, 13, and 14 (Table 1). All strains that were found to contain lbtA produce CAS reactivity when grown in iron-deplete CDM (39), supporting a correlation between the presence of lbtA and legiobactin production in L. pneumophila.

The Legionella genus contains 49 species, in addition to L. pneumophila (24, 53). DNAs from most (i.e., 20 out of 27) species tested hybridized with the lbtA probe (Table 1). In agreement with the results of our earlier study (35), frgA was nearly absent from Legionella species other than L. pneumophila (Table 1). Thus, lbtA, unlike frgA, appears to be present within most species of Legionella, including strains isolated from clinical and environmental sources. Most Legionella species tested secrete a siderophore-like activity (75) (Table 1). Fifteen of these 18 CAS-positive species contained lbtA, suggesting that they may produce legiobactin or a related siderophore (Table 1). However, only L. adelaidensis, L. anisa, L. erythra, L. feelei, L. moravica, L. rubrilucens, and L. sanitcensis stimulated L. pneumophila fecB mutant growth when tested in the bioassay, suggesting that they, more so than the others, express legiobactin.

Among the CAS-positive species, L. birminghamensis, L. londiniensis, and L. quinlivanii did not contain lbtA and were negative in the fecB bioassay (Table 1), indicating that lbtA-dependent legiobactin is not the only Legionella siderophore. Although not previously believed to have a siderophore activity (75), L. oakridgensis rescued fecB mutant growth in the bioassay (Table 1).

In summary, lbtA sequences were broadly distributed within the L. pneumophila genus. In all L. pneumophila strains and most other Legionella species tested, the presence of lbtA correlated with CAS reactivity. However, there were examples of both siderophore activity in the absence of lbtA and lack of siderophore expression despite the presence of lbtA.

**DISCUSSION**

To begin, we optimized the conditions for siderophore production by virulent L. pneumophila strain 130b and demonstrated that CAS reactive supernatants can stimulate growth of iron-starved legionellae. Thus, the CAS reactive material elaborated by L. pneumophila behaves as a bona fide siderophore. It is not surprising that the legionellae produce a siderophore(s), since many other aquatic bacteria, when examined, are found to produce this type of iron scavenger. The other main feature of this study is the first identification of genes (lbtA and lbtB) involved in L. pneumophila siderophore production. The behavior of the lbtA mutant in defined media containing chelators has given an initial impression of the affinity of legiobactin for iron. Since the lbtA mutant and wild type were similarly sensitive to DFX, we suspect that the iron(III) Ks for legiobactin is roughly between 10^11 and 10^13, the Ks for citrate and DFX, respectively (48, 72). Such a situation would not be incompatible with legiobactin being a siderophore, since the iron(III) stability constants of most siderophores range from 10^22 to 10^50 (60). In the case of a pathogen like L. pneumophila, such a siderophore is sufficiently strong to chelate iron away from host transferrin and lactoferrin, both with a Ks of ~10^30 (60).

Formally, the reduction in CAS reactivity displayed by the lbtA mutants could be due to alterations in siderophore biosynthesis or secretion. However, since LbtA is related to biosynthetic enzymes and does not contain any transmembrane domains or secretion signals, we suspect that LbtA is involved in the biosynthesis of legiobactin rather than siderophore export. The reactions catalyzed by the LbtA-related enzymes can give clues to the possible LbtA-mediated reaction. In E. coli, IucA catalyzes the addition of N^4-acetyl-N^4-hydroxylysine to citrate by formation of an amide bond to yield the intermediate N^4-citryl-‘-N^4-hydroxylysine, and subsequently, IucC adds another N^4-acetyl-N^4-hydroxylysine moiety to the intermediate to form aerobactin (19).

In S. meliloti, it is believed that RrhB catalyzes the addition of N^4-acetyl-N^4-hydroxy-1-amino propane to citrate to yield an intermediate to which RrhB then adds N^4-acetyl-N^4-hydroxy-1-aminopropan e to form the immediate precursor to rhizobact in 1021 (43). Although these enzymes catalyze the formation of hydroxamate siderophores, and L. pneumophila is negative in the Csáky assay that detects hydroxamates, recent work in bacteria that produce polyhydroxy carboxylate siderophores has elucidated biosynthesis genes homologous with aerobactin iuc genes (18, 80). For example, although the structure of staphylactin is unknown, the SbeE protein, like LbtA, is essential for siderophore production and is homologous with IucA (17). Similarly, vibrioferrin proteins PvsB and PvsD are homologous with IucC and IucA; these enzymes catalyze the formation of the two amide bonds contained in vibrioferrin (80). Given the relatedness of LbtA with synthetases of diverse siderophores, a simplest hypothesis is that legiobactin assembly involves an LbtA-catalyzed amide bond formation between precursors.

Despite the extensive understanding of siderophore import, only recently have determinants of siderophore export been identified (4, 26, 27, 38, 50, 58, 85). For example, ExiT, an ATP-binding cassette-like transporter, has been found in Mycobacterium smegmatis, and in Pseudomonas aeruginosa the RND efflux pump, OprM, is implicated in siderophore export (38, 58, 85). However, most of the identified exporters are members of the MFS family, a group of proteins historically viewed as transporting small solutes, such as antibiotics (51). The known MFS members involved in siderophore export include proteins that are required for export of enterobactin in E. coli (EntS), protocellin in Azotobacter vinelandii (CbsX), achromobactin in E. chrysanthemi (YhcA), and alicyclic in Bordetella species (AlcS) (4, 26, 27, 50). These siderophore export proteins constitute the inner membrane channels that facilitate export of...
the siderophore out of the cytoplasm and into the periplasm. Following the example of antibiotic export in gram-negative bacteria (84), it is likely that the MFS siderophore exporters recruit outer membrane protein channels to excrete the siderophore out of the cell (4, 26, 27, 50). Due to the relatedness of LbtB with MFS permeases, its predicted inner membrane localization, and the partial growth-promoting ability of lbtB mutants, we suspect LbtB to be the latest MFS protein involved in siderophore export.

While many bacteria organize their siderophore-encoding genes into large operons containing multiple biosynthetic genes and often a ferrisiderophore receptor gene, the lbt system may encode only three genes, and perhaps only one required biosynthetic and one required transport gene. The wild-type phenotype of the L. pneumophila mutants lacking lbtC, the last gene in the lbt operon, is similar to that of Yersinia pestis ybtX mutants that secrete siderophore despite their loss of a gene encoding an MFS family member (23). Given that we have thus far not identified other candidate legiobactin genes linked or unlinked to lbtABC, the biosynthesis of legiobactin may be uniquely simple, perhaps involving only LbtA and one or two precursor molecules. Alternatively, other L. pneumophila legiobactin genes exist but they would appear to be unusual in content and location. Interestingly, the gene directly upstream of the lbt locus is predicted to encode a 40-kDa outer membrane protein that, because of an iron box, seems to be Fur regulated. Thus, it is tempting to speculate that this protein is a receptor for ferrilegiobactin.

The fact that mutations in lbtAB do not completely abolish CAS reactivity suggests that there may be more than one L. pneumophila iron chelator produced in low iron environments. The residual activity was found to be the temperature-regulated component of the CAS reactivity in CDM supernatants, since the lbtA mutants, like the wild type, showed an increase in CAS activity at room temperature. This activity might represent one or multiple molecules, including a legiobactin precursor(s), other low affinity siderophore(s), or nonsiderophore CAS-reactive specie(s). If the residual activity represents a new siderophore, it is still a member of the complexone class as it is not detected in the Arnow and Csáky assays.

The biological (i.e., growth-promoting) activity of this residual CAS activity is presently unclear. On the one hand, it was not active in the bioassays used in this study. On the other hand, since the lbtA and lbtB mutants grew normally in deferated CDM, this residual CAS activity may promote bacterial growth so long as an additional iron chelator, such as citrate, is not present. When we investigated candidate siderophore genes, we found that neither frgA nor the pvc locus was involved in production of the activity, or legiobactin for that matter.

Numerous assays and the use of single and double mutants indicate that lbtA and legiobactin are not required for optimal intracellular infection or virulence. These data, however, do not demonstrate that legiobactin has no relevance for intracellular growth or in vivo persistence. Indeed, lbtA is expressed by L. pneumophila within the macrophage, suggesting a dispensable role for legiobactin in this intracellular environment that can be compensated for by another siderophore or another iron uptake system. It is also conceivable that lbtAB and legiobactin are only expressed and required for extracellular growth or persistence in aquatic environments. Further, legiobactin may be made during the log phase of growth but stored and only utilized during the planktonic phase and perhaps within biofilms.

In contrast to lbtAB, frgA is required for optimal intracellular growth in macrophages (35). These data and the sequence homology of FrgA raise the possibility that L. pneumophila encodes a siderophore that, unlike legiobactin, is necessary for optimal intracellular replication. Presently, there are few data concerning the role of siderophores in macrophage or amoeba intracellular infection, although mycobactin of Mycobacterium tuberculosis and 2,5-dihydroxybenzoic acid of Brucella abortus have been shown to promote infection of macrophages (20, 52). The hypothesis that L. pneumophila has evolved multiple siderophores in order to flourish in distinct intra- and extracellular niches is reasonable and worthy of future investigation.

Our understanding of L. pneumophila iron acquisition is still, relatively speaking, in its infancy. But LbtAB now join FrgA, FeoB, IraAB, Ccm proteins, Fur, and ferric reductases as L. pneumophila proteins required for growth in low iron and presumably in iron acquisition (34, 35, 47, 59, 63, 81, 82). It remains to be determined whether these factors operate in common or distinct iron uptake pathways. However, we suspect that FeoB and LbtA are components of two critical pathways in iron uptake since simultaneous inactivation of feoB and lbtA was incompatible with growth under standard conditions. By utilizing various types of lbtAB mutations and mutants, we can design new genetic screens for identifying other components of L. pneumophila iron uptake.

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