The major facilitator superfamily-type protein LbtC promotes the utilization of the legiobactin siderophore by *Legionella pneumophila*

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The Gram-negative bacterium *Legionella pneumophila* elaborates the siderophore legiobactin. We previously showed that cytoplasmic LbtA helps mediate legiobactin synthesis, inner-membrane LbtB promotes export of legiobactin, and outer-membrane LbtU acts as the ferrisiderophore receptor. RT-PCR analyses now identified lbtC as an iron-repressed gene that is the final gene in an operon containing lbtA and lbtB. *In silico* analysis predicted that LbtC is an inner-membrane protein that belongs to the major facilitator superfamily (MFS). Although capable of normal growth in standard media, lbtC mutants were defective for growth on iron-depleted agar media. While producing normal levels of legiobactin, lbtC mutants were unable to utilize supplied legiobactin to stimulate growth on iron-depleted media and displayed an impaired ability to take up radiolabelled iron. All lbtC mutant phenotypes were complemented by reintroduction of an intact copy of lbtC. When a cloned copy of both lbtC and lbtU was introduced into a heterologous bacterium (*Legionella longbeachae*), the organism acquired the ability to utilize legiobactin to grow better on low-iron media. Together, these data indicate that LbtC is involved in the uptake of legiobactin, and based upon its predicted location is most likely the mediator of ferrilegiobactin transport across the inner membrane. The data are also a unique documentation of how an MFS protein can promote bacterial iron-siderophore import, standing in contrast to the vast majority of studies which have defined ABC-type permeases as the mediators of siderophore import across the Gram-negative inner membrane or the Gram-positive cytoplasmic membrane.

**INTRODUCTION**

*Legionella pneumophila* is the agent of Legionnaires’ disease (Diederren, 2008; Edelstein & Cianciotto, 2010). In water, this Gram-negative bacterium survives planktonically, in biofilms, and as an intracellular parasite of protozoa (Declerck, 2010; Lau & Ashbolt, 2009; Taylor et al., 2009). Disease occurs after the inhalation of contaminated droplets that derive from aerosol-generating devices (Craun et al., 2010; Pagnier et al., 2009). *L. pneumophila* grows in the lungs by infecting alveolar macrophages, and intrapulmonary survival likely also involves growth in epithelia and extracellular survival (Allard et al., 2009; McCoy-Simandle et al., 2011; Newton et al., 2010). The acquisition of iron is a critical aspect of *L. pneumophila* ecology, intracellular infection and virulence (Cianciotto, 2007, 2008a, b). The mediators of Fe\(^{2+}\) assimilation include a secreted pigment that has Fe\(^{3+}\) reductase activity and the inner-membrane (IM) Fe\(^{2+}\) transporter FeoB (Chatfield & Cianciotto, 2007; Robey & Cianciotto, 2002). The key mediator of Fe\(^{3+}\) assimilation is legiobactin. This siderophore is detected when *L. pneumophila* is grown in low-iron, chemically defined medium (CDM) and supernatants are tested in the chrome azurol S (CAS) assay (Allard et al., 2006; Liles et al., 2000). Legiobactin is also identified in a bioassay whereby culture supernatants or purified material are able to stimulate the growth of iron-starved legionellae (Allard et al., 2006, 2009). Mutants specifically defective for siderophore expression are impaired for infection of the murine lung, demonstrating a role for legiobactin in *L. pneumophila* virulence (Allard et al., 2009). Many but not all other *Legionella* species appear to express legiobactin (Allard et al., 2006; Starkenburg et al., 2004).

Given the relevance of legiobactin, we have sought to identify the factors involved in the synthesis, secretion, uptake and utilization/assimilation of the siderophore. Initially, we identified two linked genes, lbtA and lbtU, that are required for the production of legiobactin (Allard et al.,...
Bacteriological media and extracellular growth experiments. L. pneumophila strains were routinely cultured at 37 °C on buffered charcoal yeast extract (BCYE) agar, which has an iron supplement consisting of 0.25 g ferric pyrophosphate l⁻¹ (Allard et al., 2006, 2009; Chatfield et al., 2011). When appropriate, the agar was supplemented with chloramphenicol at 6 μg ml⁻¹, kanamycin at 25 μg ml⁻¹ or gentamicin at 2.5 μg ml⁻¹. To monitor the basal extracellular growth capacity of L. pneumophila, bacteria grown on BCYE agar were inoculated into buffered yeast extract (BYE) or CDM broth, and then the optical density of the cultures was determined at 660 nm (Allard et al., 2006, 2009; Chatfield et al., 2011). BYE and CDM broths also typically contain the iron supplement. E. coli were grown in Luria–Bertani media, containing kanamycin (50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹) or ampicillin (100 μg ml⁻¹). To assess the extracellular growth of L. pneumophila in iron-limiting conditions, strains were inoculated in deferrated CDM and growth was monitored spectrophotometrically (Allard et al., 2006). To judge growth on iron-limited solid media, legionellae were tested for their efficiency of plating on BCYE agar that lacked its iron supplement (Allard et al., 2006; Chatfield et al., 2011; Robey & Cianciotto, 2002; Viswanathan et al., 2002). Also, strains were tested on non-iron-supplemented BCYE agar that had been made even more iron-limited by the inclusion of 10–14 μM deferoxamine mesylate (DFX) (Allard et al., 2006; Chatfield et al., 2011). In each case, bacteria were pre-cultured for 3 days on standard BCYE agar, suspended in PBS containing 1 mM IPTG at 1 × 10⁶ c.f.u. ml⁻¹, and then 10 μl aliquots taken from 10-fold serial dilutions in PBS containing 1 mM IPTG were spotted on the assay media. Growth was recorded after 5 days of incubation at 37 °C.

Siderophore and iron uptake assays. Preparations of legiobactin were obtained as described before (Allard et al., 2006, 2009; Chatfield et al., 2011). L. pneumophila strains were grown in deferrated CDM at 37 °C, and then at 24 h post-inoculation, cell-free supernatants were concentrated threefold by vacuum centrifugation and filter-sterilized. Siderophore activity in the supernatants was confirmed using the CAS assay, with DFX serving as the standard (Allard et al., 2006, 2009; Chatfield et al., 2011; Liles et al., 2000; Starkenburg et al., 2004). Supernatants were assessed for legiobactin bioactivity by examining their ability to rescue the growth of an L. pneumophila ferrous transport (feoB) mutant (Table 1) on non-iron-supplemented BCYE agar, as previously described (Allard et al., 2006, 2009; Chatfield et al., 2011). In order to judge L. pneumophila strains for their ability to use legiobactin, we used previously described methodology (Allard et al., 2006; Chatfield et al., 2011). Briefly, bacteria were pre-cultured for 3 days on BCYE agar, suspended in PBS containing 1 mM IPTG, and then 1 × 10⁵ c.f.u. were spread onto non-iron-supplemented BCYE agar containing 12–14 μM DFX or 400–500 μM 2,2-dipyrisdyl (DIP). Small wells cut in the centre of the agar were filled with 100 μl of supernatants containing legiobactin obtained from wild-type cultures grown in deferrated CDM. Negative-control wells contained equal volumes of supernatants lacking legiobactin obtained from lbtA mutant cultures grown in deferrated CDM. Positive-control wells contained 5 μM Fe³⁺ pyrophosphate or 20 μM Fe³⁺ ammonium sulfate. Growth around the wells was then assessed. The uptake of radiolabelled ferrilegiobactin was determined as described previously (Chatfield et al., 2011). Briefly, L. pneumophila strains were cultured for 18 h in deferrated CDM in order to produce legiobactin, and then ³⁵FeCl₃ (Perkin Elmer) was added to the cultures at a final concentration of 0.04 mCi ml⁻¹ (1.48 MBq ml⁻¹). After 60–90 min of incubation, during which time radiolabelled ferrilegiobactin forms and becomes available for uptake, 1 ml of the culture (n=3) was filtered through nitrocellulose and then washed with 5 ml 0.5 % thioglycolate to remove external iron salts. c.p.m. of radioactivity associated with the bacteria were then measured using a Beckman LS6500 scintillation counter and reported as the mean of the c.p.m. recorded over a 5 min period.

PCR, inverse PCR, and RT-PCR analysis. DNA was isolated from L. pneumophila using Lyse-and-Go reagent (Thermo Pierce Scientific) according to the manufacturer’s instructions. Primers were obtained from Integrated DNA Technologies. In order to detect the lbt genes in different strains of bacteria, PCR using recombinant Taq (Invitrogen) was performed over 30 cycles using primers LbtB INT F and LbtU INT R for the detection of lbtU, LbtA INT F and LbtA INT R for detecting lbtA, LbtB INT F and LbtB INT R for lbtB, and LbtC INT F and LbtC INT R for lbtC (Table 2). Reaction products were separated by agarose-gel electrophoresis and detected by ethidium bromide staining. The location of the mini-Tn10 insertion in L. pneumophila strain L. pneumophila was ascertained by inverse PCR and subsequent sequence determination, as previously described.
Table 1. L. pneumophila strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type strains</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130b (BAA-74)</td>
<td>Clinical isolate, serogroup 1</td>
<td>Cianciotto et al. (1990)</td>
</tr>
<tr>
<td>Togus-1 (33154)</td>
<td>Clinical isolate, serogroup 2</td>
<td>McKinney et al. (1979)</td>
</tr>
<tr>
<td>Bloomington-2 (33155)</td>
<td>Clinical isolate, serogroup 3</td>
<td>McKinney et al. (1979)</td>
</tr>
<tr>
<td>Dallas-1E (33216)</td>
<td>Clinical isolate, serogroup 5</td>
<td>Garrity et al. (1982)</td>
</tr>
<tr>
<td>Chicago-8 (33823)</td>
<td>Clinical isolate, serogroup 7</td>
<td>Bibb et al. (1983)</td>
</tr>
<tr>
<td>Concord-3 (35096)</td>
<td>Clinical isolate, serogroup 8</td>
<td>Bissett et al. (1983)</td>
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<td>82A3105 (43736)</td>
<td>Clinical isolate, serogroup 13</td>
<td>Lindquist et al. (1988)</td>
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<tr>
<td>1169-MN-H (43703)</td>
<td>Clinical isolate, serogroup 14</td>
<td>Benson et al. (1988)</td>
</tr>
<tr>
<td><strong>Mutant strains</strong></td>
<td></td>
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</tr>
<tr>
<td>NU302</td>
<td>lptw_13341 (lbtA) mutant of 130b</td>
<td>Allard et al. (2006)</td>
</tr>
<tr>
<td>NU269</td>
<td>lptw_29101 (fxbB) mutant of 130b</td>
<td>Robey &amp; Cianciotto (2002)</td>
</tr>
<tr>
<td>NU383</td>
<td>lptw_13361(lbtU) mutant of 130b</td>
<td>Chattfield et al. (2011)</td>
</tr>
<tr>
<td>NU305, NU306</td>
<td>lptw_13321 (lbtC) mutants of 130b</td>
<td>Allard et al. (2006)</td>
</tr>
<tr>
<td>NU404, NU405</td>
<td>lbtU/lbtC double mutants of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU205</td>
<td>lptw_10081 (ald) mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU207</td>
<td>lptw_15831 (kefA) mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU208</td>
<td>lptw_09401 (ccmC) mutant of 130b</td>
<td>Viswanathan et al. (2002)</td>
</tr>
<tr>
<td>NU210</td>
<td>lptw_26201 (lpf) mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU211</td>
<td>lptw_03861 mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU212</td>
<td>lptw_22731 (trbl) mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU213</td>
<td>lptw_03271(gbsA) mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU214</td>
<td>Undefined mini-Tn10 insertion</td>
<td>Pope et al. (1996)</td>
</tr>
<tr>
<td>NU215</td>
<td>lptw_26471 (mdcC) of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU216</td>
<td>lptw_08281 (iraA) mutant of 130b</td>
<td>Viswanathan et al. (2000)</td>
</tr>
<tr>
<td>NU217</td>
<td>Undefined mini-Tn10 insertion</td>
<td>Pope et al. (1996)</td>
</tr>
<tr>
<td>NU220</td>
<td>Undefined mini-Tn10 insertion</td>
<td>Pope et al. (1996)</td>
</tr>
<tr>
<td>NU223</td>
<td>Undefined mini-Tn10 insertion</td>
<td>Pope et al. (1996)</td>
</tr>
<tr>
<td>NU224</td>
<td>Undefined mini-Tn10 insertion</td>
<td>Pope et al. (1996)</td>
</tr>
<tr>
<td>NU225</td>
<td>lptw_07571 mutant of 130b</td>
<td>This study</td>
</tr>
<tr>
<td>NU244</td>
<td>lptw_08291 (iraB) mutant of 130b</td>
<td>Viswanathan et al. (2000)</td>
</tr>
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*Wild-type strains are listed with their original designation, followed in parentheses by the corresponding ATCC designation.

(Chattfield & Cianciotto, 2007; Liles et al., 1998; Naylor & Cianciotto, 2004; Viswanathan et al., 2002). To monitor L. pneumophila transcription, RT-PCR was performed as previously described (Allard et al., 2006; Chattfield et al., 2011; Liles et al., 1998). RNA was isolated from 18 h CDM cultures of L. pneumophila using RNA STAT-60 reagent (Tel-Test) according to the manufacturer’s instructions. In order to isolate bacterial RNA from infected human macrophages, U937 cells were infected for 24 h as previously described (see below). After removal of the tissue culture medium, the cell monolayer was lysed with 50% RNAprotect (Qiagen)/1% saponin, and RNA was extracted using RNA STAT-60. Total RNA from the CDM cultures or the infected U937 cells was treated with DNase I (Ambion), and cDNA was synthesized using random primers and Superscript III reverse transcriptase (Invitrogen). Primers Mip F and Mip R were used for amplifying mip as described previously (Table 2) (Allard et al., 2006; Chattfield et al., 2011). Primers LbtC RT F and LbtC RT R (Table 2) were used to detect LbtC transcripts. To assess co-transcription of lbtA and lbtB, primers LbtA RT F and LbtB RT2 R were employed, and to monitor co-transcription of lbtB and lbtC, primers LbtB RT3 F and LbtC RT R were used (Table 2). Control experiments in which the reverse transcriptase was omitted from the reaction were done to rule out contributions from contaminating DNA in the DNAse-treated samples. Relative endpoint PCRs were separated by agarose-gel electrophoresis and detected with ethidium bromide staining (Allard et al., 2006; Chattfield et al., 2011; Liles et al., 1998).

DNA and protein sequence analysis. The arrangement and sequence of the lbtU–lbtABC locus in L. pneumophila strains were determined by accessing the databases at http://genolist.pasteur.fr/LegioList/, http://www.ncbi.nlm.nih.gov/bioproject/48801, and http://www.ebi.ac.uk/ena/data/view/FR687201. Transmembrane helices in LbtC were predicted using the TMHMM 2.0 server (www.cbs.dtu.dk/services/TMHMM-2.0/). Transmembrane data were visualized using the TMRPRED2D program (http://biophysics.biol.uoa.gr/TMRPRED2D/index.jsp). BLAST homology searches were done through the Transporter Classification Database (http://www.tcdb.org/), GenBank at the National Center for Biotechnology Information, and the L. pneumophila database at http://genolist.pasteur.fr/LegioList/. CLUSTAL analysis and sequence alignments were done via BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) and the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/). 3D modelling was done using the I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/) modelling servers (Bennett-Lovsey et al., 2008; Glekas et al., 2010; Zhang, 2008). Only models within the published threshold were considered; i.e. the LbtC model.
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’) (restriction sites underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LbtU INT F</td>
<td>AAAATTGACCTACGATGCCC</td>
</tr>
<tr>
<td>LbtU INT R</td>
<td>CAGTCGCCAGTCTTTGTTCCCGTATTCC</td>
</tr>
<tr>
<td>LbtA INT F</td>
<td>CATCGGCCACAGAAATGTCTTGGAT</td>
</tr>
<tr>
<td>LbtA INT R</td>
<td>TGCCCTAAGCCAACTGGAAAAAAGGA</td>
</tr>
<tr>
<td>LbtB INT F</td>
<td>CATATTGACGCCACAAAAGCA</td>
</tr>
<tr>
<td>LbtB INT R</td>
<td>ATGACCATGAGGATGCTCCGAAAT</td>
</tr>
<tr>
<td>LbtC INT F</td>
<td>TAATGCAAGGCCATTTGCTGAGG</td>
</tr>
<tr>
<td>LbtC INT R</td>
<td>GGGATTATTTCCAGAGTTATCGG</td>
</tr>
<tr>
<td>LbtA RT F</td>
<td>CAGTTATATGCAGTTATC</td>
</tr>
<tr>
<td>LbtB RT2 R</td>
<td>GATGCGAACGGATAATTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>LbtB RT3 F</td>
<td>GGCCAGATGACAGATTATTTTCTAAATTC</td>
</tr>
<tr>
<td>LbtC RT R</td>
<td>CCCCATAGCTGGAAAGAGGACC</td>
</tr>
<tr>
<td>LbtC RTF</td>
<td>TCAAGGTAAAATCAAGGCGCTCT</td>
</tr>
<tr>
<td>LbtC RTR</td>
<td>TAATGCAAGGCCATTTGCTGAGG</td>
</tr>
<tr>
<td>Mip F</td>
<td>AAGGCGATGCAAGGCTAA</td>
</tr>
<tr>
<td>Mip R</td>
<td>GTATCGGAATTTTCTCCGGT</td>
</tr>
<tr>
<td>OR77</td>
<td>TCGGCTGTCATTAATGTGTGG</td>
</tr>
<tr>
<td>OR78</td>
<td>ACCGGTTCTGCTGTGATT</td>
</tr>
<tr>
<td>LbtC EXT F</td>
<td>GAGCTTGTTAAATCACGGCGAGAT</td>
</tr>
<tr>
<td>LbtC EXT1 R</td>
<td>CATATGCTTCTTCTAGACGAAATACACGCGAAACT</td>
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<tr>
<td>CCO5</td>
<td>GCTATTTTCTAGATCTACATAAAGGATA</td>
</tr>
<tr>
<td>LbtB EXT R</td>
<td>CTCATGAAACATACGCGAAATCAGGTAGG</td>
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<tr>
<td>LbtA 9-F</td>
<td>ATACTGCCATAGCATCGGG</td>
</tr>
<tr>
<td>LbtU EXT F</td>
<td>TGCCAGATTATTTGCTAGTGCGCTGCTGCTG</td>
</tr>
<tr>
<td>LbtC EXT2 R</td>
<td>AGTGACGCCATAGCATCGGCA</td>
</tr>
</tbody>
</table>

generated by the Phyre server exhibited 95% confidence, and the model produced by the I-TASSER server gave a C score of 1.65 and a TM score of 0.95±0.05.

Genetic complementation of *L. pneumophila* mutants. For standard trans-complementation analysis of *L. pneumophila* lbtC mutants, the plasmid pLbtC was constructed as follows. The lbtC gene was PCR-amplified from 130b DNA using primers LbtC EXT F and LbtC EXT1 R (Table 2), and then the resulting fragment was cloned into pGEM-T Easy (Promega) to yield pGMlbtC. The SacI/XbaI fragment of pGMLbtC that contained only the lbtC coding region was then ligated into SacI/XbaI-digested pMMB2002 (Rossier et al., 2004), placing lbtC under the control of the IPTG-inducible P* tac* promoter. This final construct, pLbtC, was then electrooporated into NU305 and NU306, the two previously described mutants that contain a gentamicin-resistance cassette inserted into lbtC (Table 1). Chloramphenicol-resistant (Cm') clones were confirmed as carrying pLbtC by PCR with primers OR77 (Chatfield et al., 2011) and OR78 (Table 2). In order to determine the effect of the other lbt genes on the lbtC mutants, two additional plasmids (i.e. pLtbtUABC, pLbtUAB) were made as follows. In order to construct pLbtUABC, the region of *L. pneumophila* chromosome encoding lbtU and lbtABC was amplified from 130b DNA using primers LbtU EXT F and LbtC EXT2 R (Table 2), and then the resulting 4.5 kb PCR product was cloned into pGEM-T Easy, yielding pGEM-UABC. The XbaI/SacI fragment of pGEM-UABC that contained lbtU–lbtABC was then subcloned into XbaI/SphI-digested pMMB2002, producing pLbtUABC. The plasmids pLbtUABC and pLbtUAB, the cloned genes were placed under the control of the vector’s P* tac* promoter. These plasmids were electroporated into the lbtC mutants NU305 and NU306, and then Cm' clones were confirmed by PCR.

**L. pneumophila** mutant constructions. Double mutants of *L. pneumophila* 130b lacking both lbtC and lbtU were obtained by transforming pUCB6UK with its kanamycin-resistance (Km')-inactivated lbtU gene (Chatfield et al., 2011) into the previously described gentamicin-resistant lbtC mutant NU305 (Table 1), and then selecting for the acquisition of kanamycin resistance. The Km' insertion into the chromosomal lbtU gene was confirmed by PCR using primers LbtU INT F and LbtU INT R (Table 2).

Analysis of *L. pneumophila* genes in *L. longbeachae*. In order to determine the effect of cloned lbt genes on *L. longbeachae*, the plasmids pLtbtUABC and pLbtUAB described above were introduced into *L. longbeachae* ATCC 33462 by electroporation (Chatfield et al., 2011) and Cm' clones were confirmed by PCR. A third plasmid, pLtbtABC, was also introduced in a similar fashion. To make pLtbtABC, the lbtABC operon was amplified with primers LbtA 9-F and LbtC EXT1 R (Table 2), and then the 4.5 kb PCR product was cloned into pGEM-T Easy, yielding pGEM-ABC. The XbaI/SacI fragment of pGEM-ABC that contained lbtABC was then subcloned into XbaI/SacI-digested pMMB2002, producing pLbtABC.

Intracellular infection assays. To examine the ability of *L. pneumophila* strains to grow intracellularly, *Hartmannella vermiformis* amoebae, *Acanthamoeba castellanii* amoebae, and U937 macrophages were infected as previously described (Allard et al., 2006, 2009; Pearce & Gianciotto, 2009; Rossier et al., 2008).
RESULTS

Characterization of the lbtC gene

Past sequence analysis of the *L. pneumophila* 130b chromosome had identified the gene immediately downstream of *lbtA* and *lbtB*, and based upon the 4 bp overlap between the beginning of that gene and the end of *lbtB*, we had hypothesized that the gene is transcriptionally linked to *lbtA* and *lbtB*, and dubbed it *lbtC* (Allard et al., 2006). To begin the present study, the operon structure of *lbtABC* was confirmed by RT-PCR analysis, which identified transcripts spanning the two intergenic regions (Fig. 1a). As was previously shown for *lbtA* and *lbtB* (Allard et al., 2006; Chatfield et al., 2011), RT-PCR analysis indicated that the levels of *lbtC* mRNA were greater in *L. pneumophila* grown in deferrated CDM than in bacteria cultured in iron-supplemented CDM (Fig. 1b). These data are consistent with the presence of a putative Fur box upstream of *lbtA* (Fig. 1a). Compatible with these findings, further RT-PCR analysis detected *lbtC* and *lbtB* mRNA in *L. pneumophila* growing in macrophages (Fig. 1c), as had been the case for *lbtA* transcripts (Allard et al., 2006). In the recently completed genome sequence of strain 130b (Schroeder et al., 2010), *lbtC* is denoted as lpw_13321. Examination of the other *L. pneumophila* databases indicated that *lbtC* is present in the five other sequenced strains of *L. pneumophila*, being denoted as lpp1278, lpg1323, lpl1277, lpc0738 and lpa01952 in serogroup-1 strains Paris, Philadelphia-1, Lens, Corby and Alcoy, respectively (Cazalet et al., 2004; Chien et al., 2004; D’Auria et al., 2010; Glockner et al., 2008). PCR analysis determined that *lbtC*, as well as *lbtA*, *lbtB* and *lbtU*, is present in all other seven strains of *L. pneumophila* tested (Table 1), including representatives of serogroups 2, 3, 5, 7, 8, 13 and 14 (data not shown). Taken together, these experiments confirmed that *lbtC* is iron-regulated, conserved in *L. pneumophila*, and transcriptionally linked to genes involved in legiobactin production and secretion. Thus, we hypothesized that *lbtC* has a function related to the *L. pneumophila* siderophore.

Characterization of the LbtC protein

Upon examination of the 130b genome database, *lbtC* was predicted to encode a 386 aa protein (LbtC). Protein sequence comparisons indicated that the LbtC protein of 130b is 85–86 % identical to the LbtC proteins encoded by the five other sequenced strains of *L. pneumophila*, demonstrating a strong level of LbtC conservation within the species *pneumophila*. Bioinformatics analysis predicted that LbtC has 12 transmembrane, 8-helical domains (and no β-barrel structure), and several extended loops on each side of the membrane (Fig. 2a). These attributes placed LbtC into the MFS, a large class of membrane transporters, which, in Gram-negative bacteria, includes many types of IM permeases (Law et al., 2008; Paulsen et al., 1996; Saier et al., 1999). Some MFS transporters transport substrate into the cytoplasm, others efflux molecules out, and still others transport in both directions (Law et al., 2008; Pao et al., 1998). When LbtC was compared with all known MFS transporters in the Transporter Classification Database, it exhibited the greatest similarity to IM multi-drug efflux proteins in the drug: H⁺ antiporter (12-spanner) family.

![Fig. 1. Location and iron regulation of lbtC. (a) Depiction of the region of the L. pneumophila chromosome that contains lbtC. The white horizontal arrows denote the relative size and orientation of the lbt genes. The two hatched boxes denote the location of two putative Fur boxes, with the actual nucleotide sequences shown below. The vertical black arrowhead marks the approximate location of the insertion mutations in the lbtC mutants NU305 and NU306. The thinner horizontal lines below the gene map signify the approximate size and location of the two intergenic transcripts identified by RT-PCR analysis. (b) Extracellular expression of lbtC transcripts. Wild-type 130b was grown in deferrated CDM (CDM – Fe) or deferrated CDM that was supplemented with 5 μM ferric pyrophosphate (CDM + Fe), and then RNA was analysed by RT-PCR utilizing primers specific to lbtC (top row) or mip, a gene that is not subject to iron regulation (bottom row) (Allard et al., 2006; Chatfield et al., 2011). (c) Intracellular expression of lbtC transcripts. U937 cells were infected with wild-type 130b for 24 h, and then RT-PCR was done using primers that amplify the lbtB and lbtC-specific transcript. That the PCR products obtained in (b) and (c) resulted from mRNA templates was confirmed by the lack of product obtained when the PCR did not incorporate reverse transcriptase (–RT lanes). Additional PCR products obtained from genomic DNA appear in the left-most lanes, indicating that the mRNAs observed are full length. The results presented are representative of at least three independent experiments.](http://mic.sgmjournals.org)
For example, LbtC shared 24% identity and 41% similarity over a span of 333 residues (E=6 x 10^-20) with E. coli EmrD, and 20% identity and 41% similarity over 356 residues (E=6 x 10^-16) with E. coli Bcr (Pao et al., 1998; Paulsen et al., 1996) (Supplementary Fig. S1). From this same BLASTP analysis, LbtC showed relatedness, albeit at a lower level, to IM MFS transporters implicated in siderophore export (Miethke et al., 2008), i.e. 26% identity and 43% similarity over 134 residues (E=4 x 10^-9) with AlcS of Bordetella pertussis and 22% identity and 37% similarity over 346 residues (E=4 x 10^-8) with LbtB of L. pneumophila (Allard et al., 2006; Brickman & Armstrong, 2005) (Supplementary Fig. S2). But because lbtC mutants of strain 130b (i.e. NU305 and NU306) produce culture supernatants that contain wild-type levels of legiobactin (Allard et al., 2006), LbtC would appear not to be involved in legiobactin export (or synthesis). When we performed BLASTP analysis using the entire NCBI database (i.e. considered similarity to uncharacterized proteins), LbtC revealed its greatest level of similarity (57% similarity and 39% identity, with E=9 x 10^-62) to a Francisella tularensis protein known as FslD in F. tularensis subsp. tularensis and FigD in F. tularensis subsp. novicida (Deng et al., 2006; Sullivan et al., 2006) (Fig. 3). This higher level of similarity was also evident when we compared the predicted secondary structures (Fig. 2b). Interestingly, the fslD/figD gene is part of an iron-regulated operon in which one of the other genes encodes a siderophore synthetase (FslA/FigA) that is related to LbtA and another gene encodes a putative efflux pump protein (FslB/FigB) that is akin to LbtB (Deng et al., 2006; Sullivan et al., 2006). As we had observed for our lbtC mutants (Allard et al., 2006), F. tularensis figD mutants produce wild-type levels of siderophore (Kiss et al., 2008). From these various data, we hypothesized that LbtC might represent a new type of MFS protein that acts during ferrisiderophore assimilation to transport siderophore across the IM and into the cytoplasm.

**LbtC is required for the utilization of legiobactin by L. pneumophila**

To discern whether LbtC is required for the assimilation of legiobactin, we compared wild-type 130b and our two lbtC
mutants, NU305 and NU306 (Fig. 1a), for their abilities to use legiobactin to stimulate growth on low-iron media (Fig. 4). As expected (Chatfield et al., 2011), the wild-type was stimulated to grow on non-iron-supplemented BCYE agar containing the iron chelator DFX when supplied with supernatants containing legiobactin, but growth did not occur when the bacteria were supplied with supernatants lacking legiobactin. In marked contrast, both of the lbtC mutants were unable to use the legiobactin sample. When an intact copy of lbtC alone was introduced into mutant NU305 on a multicopy plasmid (i.e. pLbtC), legiobactin utilization was restored (Fig. 4). A similar result was obtained when lbtC was introduced into NU305 on a plasmid that also contained lbtU, lbtA and lbtB (i.e. pLbtUABC) (Fig. 4). These data indicate that LbtC is needed for the ability of L. pneumophila to utilize legiobactin for growth under low-iron conditions. Because the lbtC mutants, like the wild-type and the complemented mutant, grew well on iron-limited BCYE agar when it was supplemented with ferric or ferrous iron salts (Fig. 4), LbtC is not required for the assimilation of all forms of iron and may be specific for legiobactin usage. In these assays, the phenotype of the lbtC mutant was similar to that of an lbtU mutant (Fig. 4), suggesting that LbtC and LbtU are equally important in legiobactin assimilation.

That LbtC and LbtU are functionally distinct, however, was apparent when plasmids containing lbtU but not lbtC (e.g. pLbtUAB) failed to rescue the growth of the lbtC mutant (Fig. 4).

To obtain additional evidence for the role of LbtC in iron-siderophore uptake, we grew the wild-type and an lbtC mutant in deferrated CDM leading to the production of legiobactin, and then determined the abilities of the different cultures to take up added radiolabelled Fe\(^{3+}\). As observed previously (Chatfield et al., 2011), the wild-type displayed substantial iron uptake after 60 min of incubation in the presence of siderophore and radiolabel (Fig. 5). The lbtC mutants NU305 and NU306 incorporated label at a level that was significantly below that of the wild-type after 60 (Fig. 5a) and 90 min (data not shown) of incubation. Importantly, complemented lbtC mutant NU305 incorporated radiolabelled Fe\(^{3+}\) at wild-type levels (Fig. 5a). A similar result was obtained when lbtC mutant NU306 complemented with pLbtC was examined on three occasions (data not shown). These data confirmed that the lack of uptake shown by the lbtC mutant was due to the loss of LbtC. Again, the lbtC mutant showed a phenotype that was akin to that of an lbtU mutant (Fig. 5b). Considering the results from these two phenotypic assays

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**Fig. 3.** Amino acid sequence alignment of L. pneumophila LbtC with F. tularensis FslD and FigD. Below the alignment is a consensus sequence showing the many positions that have similar (.) or identical (*) residues. Above the alignment are indicated the 12 predicted transmembrane (TM) domains of LbtC, which are similarly reflected in the FslD and FigD proteins.

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and the cellular location of proteins, we hypothesize that ferrilegibactin enters *L. pneumophila* via the LbtU OM receptor and then LbtC mediates the transport of the ferrisiderophore across the IM.

**L. pneumophila* **ira** mutants are not impaired for legiobactin utilization**

With the demonstration that LbtC and LbtU are involved in legiobactin utilization, we posited that there might be genes outside the *lbtU–lbtABC* locus that aid in legiobactin assimilation. To begin to address this hypothesis, we examined a set of 16 putative iron-acquisition (**ira**) mutants of 130b that had been previously generated by mini-Tn10 mutagenesis (Pope et al., 1996). These mutants (listed in Table 1 by numbers ranging from NU205 to NU244) had been initially identified based upon their hypersensitivity to the iron chelator EDDA in their growth media and/or their resistance to streptonigrin, an antibiotic whose toxicity requires significant levels of intracellular iron (data summarized in Table 3). Subsequent studies found that none of these mutants is impaired for the production of legiobactin (summarized in Table 3), with the exception of NU208, which had proved to contain an insertion in the cytochrome *c* maturation locus (Table 1) (Allard et al., 2006; Yip et al., 2011). Continuing the genetic analysis that had begun with NU208, NU216 and NU244 (Viswanathan et al., 2000, 2002), we performed inverse PCR and DNA sequence analysis and defined the site of the mini-Tn10 insertion in most of the **ira** mutants, i.e. NU205, NU207, NU210, NU211, NU212, NU213, NU215 and NU225 (Table 1). The inability to obtain an inverse PCR product precluded further definition of the mini-Tn10 insertion in **ira** mutants NU214, NU217, NU220, NU223 and NU224 (Table 1). Although some of these mutants were sensitive to the addition of DFX to the BCYE agar in a manner similar to the *lbtC* and *lbtU* mutants, none displayed an impaired ability to utilize added legiobactin for growth stimulation on low-iron media (Table 3).

**LbtC is required for *L. pneumophila* growth on low-iron media**

In the past, we determined that *lbtA* and *lbtU* mutants are not impaired for growth on BCYE agar that lacked its iron supplement or on unsupplemented BCYE agar that contained up to 400 μM of the iron chelator DIP (Allard et al., 2006; Chatfield et al., 2011). However, the mutants are impaired for growth on unsupplemented BCYE agar that contains the more powerful ferric iron chelator DFX (Chatfield et al., 2011). Compatible with these data, mutation of *lbtC* did not impair *L. pneumophila* growth on non-iron-supplemented BCYE agar with or without DIP, but did reduce efficiency of plating on non-iron-supplemented media containing DFX (Fig. 6a, c for NU305, Fig. 6b for NU306, and data not shown). The effect of the *lbtC* mutation was similar to that resulting from mutation of *lbtA* or *lbtU* (Fig. 6a). Double mutants lacking *lbtC* and *lbtU* (i.e. NU404 and NU405) behaved similarly to the *lbtC* mutant and the *lbtU* mutant (Fig. 6b), further indicating that LbtC and LbtU operate within the

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**Fig. 4.** Legiobactin utilization by *L. pneumophila* wild-type and *lbtC* mutants. (a) We plated wild-type (WT) 130b (column 1), *lbtU* mutant NU383 (column 2), *lbtC* mutants NU305 and NU306 (columns 3 and 4), mutant NU305 complemented with pLbtC (column 5), mutant NU305 complemented with pLbtUABC (column 6) or mutant NU305 carrying pLbtUAB (column 7) onto non-iron-supplemented BCYE agar containing DFX, and wells cut in the agar were filled with legiobactin-containing supernatant from wild-type (*Lbt*+) (top row), legiobactin-deficient supernatant from the *lbtA* mutant NU302 (*Lbt*–) (second row), ferric pyrophosphate (*Fe*<sup>3+</sup>) (third row) or ferrous ammonium sulfate (*Fe*<sup>2+</sup>) (bottom row). After 3 days of incubation at 37 °C, growth was recorded. The results shown are representative of at least three independent trials.
same pathway, namely, the uptake of ferrilegiobactin. When lbtC was reintroduced into lbtC mutant NU305 on a multi-copy plasmid, a wild-type level of growth was observed (Fig. 6c). A similar result was obtained when lbtC mutant NU306 complemented with pLbtC was examined on three occasions (data not shown). Together, these data indicate that LbtC, like LbtA, LbtB and LbtU, is required for L. pneumophila growth under conditions of severe iron depletion.

Previously, we observed that mutants lacking legiobactin (e.g. lbtA mutants) are not impaired for infection of H. vermiformis or A. castellanii, even when the amoebal growth medium contained lowered levels of iron (Allard et al., 2006; Chatfield et al., 2011). Past studies also found that lbtA mutants are not defective for in vitro infection of macrophages and lung epithelia, suggesting that the demonstrated importance of legiobactin in lung infection is due to processes beyond intracellular growth in resident lung cells (Allard et al., 2006, 2009). Compatible with these data, we found that the lbtC mutants grew as the wild-type did within both types of amoebae as well as U937 cell macrophages (Fig. 7, and data not shown). Taken together, these data indicate that lbtC, though expressed within host cells (Fig. 1c), is not required for intracellular growth by L. pneumophila.

**lbtU–lbtABC can confer legiobactin utilization upon another species of Legionella**

*L. longbeachae* is one of only two other species of Legionella whose genome has been completely sequenced (Cazalet et al., 2010; Kozak et al., 2010). Previously, we had examined the *L. longbeachae* database and found that the bacterium does not contain the lbtU–lbtABC locus (Chatfield et al., 2011). However, we had previously shown that introduction of pLbtU into *L. longbeachae* 33462 conferred the ability to bind legiobactin, suggesting that *L. pneumophila* proteins can be expressed and properly localized in *L. longbeachae* (Chatfield et al., 2011). Therefore, we introduced pLbtUABC into *L. longbeachae* and then assayed the new strain for its ability to utilize legiobactin for growth stimulation on low-iron BCYE agar (Fig. 8). *L. longbeachae* (pLbtUABC), unlike *L. longbeachae* carrying vector pMMB2002 alone, was able to grow on the low-iron medium when supplied with 130b supernatants containing legiobactin. The strain did not grow when supplied with lbtA mutant supernatants lacking legiobactin. Because plasmids missing just LbtC (i.e. pLbtUAB) or just LbtU (i.e. pLbtABC) were not able to promote legiobactin utilization (Fig. 8), we conclude that both LbtC and LbtU are necessary to confer legiobactin utilization upon *L. longbeachae*, a scenario consistent with our study of *L. pneumophila*.

**DISCUSSION**

Multiple experimental results support the hypothesis that LbtC functions specifically in legiobactin utilization in *L. pneumophila*. First, lbtC was the third and last gene in an operon that also included lbtA and lbtB, genes known to be required for legiobactin biosynthesis and secretion, respectively (Allard et al., 2006, 2009). Second, lbtC transcription was regulated by iron levels, with high-iron growth conditions leading to gene repression in a process that likely involves *L. pneumophila* Fur (Hickey & Cianciotto, 1994, 1997) binding to a putative Fur box located upstream of lbtA (Allard et al., 2006). Third, multiple, independently derived lbtC mutants were defective in
their capacity to grow on DFX-containing BCYE agar but not on iron-sufficient media, and such behaviour had previously been displayed by both \( \text{lbtA} \) mutants and \( \text{lbtU} \) mutants, which lack the OM receptor for legiobactin (Chatfield et al., 2011). Fourth, \( \text{lbtC} \) mutants could not be stimulated to grow on low-iron media by addition of legiobactin-containing supernatants; \( \text{lbtU} \) mutants that lack the OM receptor for legiobactin behaved similarly (Chatfield et al., 2011). Fifth, \( \text{lbtC} \) mutants were able to grow normally on the low-iron media when they were given ferric or ferrous iron salts. Sixth, like \( \text{lbtU} \) mutants (Chatfield et al., 2011), \( \text{lbtC} \) mutants were impaired for uptake of radiolabelled \( \text{Fe}^{3+} \) combined with legiobactin. Seventh, all defective phenotypes that were displayed by the \( \text{lbtC} \) mutants could be complemented by the introduction of a cloned copy of \( \text{lbtC} \) on a plasmid vector. Eighth, a plasmid expressing both \( \text{lbtC} \) and \( \text{lbtU} \) was able to confer upon a heterologous bacterium (\( \text{L. longbeachae} \)) the ability to use legiobactin to stimulate growth on low-iron media.

The identification of LbtC as a mediator of legiobactin import across the IM represents an important addition to our knowledge of siderophore transport, and arguably the first full documentation of an MFS protein being involved in bacterial siderophore import. In the vast majority of earlier studies, ATP-binding cassette (ABC)-type permeases have been implicated as the mediator of ferrisiderophore import across the Gram-negative IM or the Gram-positive cytoplasmic membrane (Cuı´v et al., 2008; Koster, 2005; Krewulak & Vogel, 2008; Rees et al., 2009; Vajrala et al., 2010). ABC-type permeases function as part of multi-protein complexes, whereby the IM permease interacts with both a periplasmic chaperone that delivers the ferrisiderophore to the IM and a cytoplasmic ATPase that provides the energy for moving the molecule through the permease. To our knowledge, there are only a handful of earlier reports implicating a non-ABC-type permease in siderophore import across a bacterial membrane; i.e. RhtX of \( \text{Sinorhizobium meliloti} \) and the homologous FptX protein of \( \text{Pseudomonas aeruginosa} \), which promote the utilization of rhizobactin 1021 and pyochelin, respectively; and FoxB and FiuB of \( \text{P. aeruginosa} \) and FegB of \( \text{Bradyrhizobium japonicum} \), which facilitate the use of ferrichrome (Benson et al., 2005; Cuı´v et al., 2004; Hannauer et al., 2010; Michel

### Table 3. Legiobactin utilization by \( \text{L. pneumophila} \) \( \text{ira} \) mutants

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<th>Legiobactin utilization§</th>
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*Data previously reported (Pope et al., 1996).
‡Relative growth on non-iron-supplemented BCYE agar containing 10–14 \( \mu \text{M DFX} \).
§Ability to utilize legiobactin-containing supernatants for growth stimulation on non-iron-supplemented BCYE agar containing 400 \( \mu \text{M DIP} \) at 25 °C.
||Unexplained variable phenotype.
et al., 2007). Although YbtX of *Yersinia pestis* has sequence similarity to RhtX and FptX, the loss of ybtX does not diminish siderophore usage by *Y. pestis* (Fetherston et al., 2010). Since none of these past studies examined their IM permeases for placement in the MFS, we did BLASTP analysis of RhtX (GenBank accession no. AAK65915.1), FptX (NP_252901.1), FoxB (NP_251155.1), FiuB (NP_249167.1) and FegB (AAR12904.1) using the Transporter Classification Database. As we had observed for LbtC, RhtX and FptX were placed in the MFS, albeit in a family (‘2.A.1.5’) different from that of LbtC. Based upon the earlier comparison of LbtC with the Fsd/FigD protein of *F. tularensis* (Figs 2 and 3), we suspect that homologues of LbtC exist in other types of bacteria. From these observations, we posit that there are at least two types of MFS IM transporters involved in siderophore import in bacteria, namely, the LbtC-like proteins and the RhtX/FptX-like proteins. Although the significance of MFS transporters and other non-ABC-type transporters for bacterial siderophore utilization can still be described as an emerging story, the involvement of MFS proteins in fungal siderophore import has been broadly appreciated for some time (Saier et al., 1999); the so-called siderophore-iron transporter (SIT) family includes Arn3/Sit1, Arn1p and Enb1p of *Saccharomyces cerevisiae*, and MirA and MirB of *Aspergillus nidulans* (Haas et al., 2003; Heymann et al., 2000; Lesuisse et al., 1998; Moore et al., 2003; Yun et al., 2000).

Beyond the results obtained from mutant analysis coupled with bioinformatics, there are several additional observations compatible with the view that members of the MFS, as exemplified by LbtC and RhtX, can be involved in siderophore import in bacteria. First, because LbtC (in combination with the OM LbtU) conferred legiobactin utilization upon *L. longbeachae* and because RhtX can functionally replace an ABC-transporter consisting of FhuCDB (Cuívé et al., 2004), a single MFS protein can provide siderophore import across the IM. This is consistent with the mechanism of action of MFS transporters that act as single-polypeptide carriers for other types of molecules (Pao et al., 1998). Second, known MFS transporters act as proton antiporters or symporters in response to chemiosmotic gradients (Law et al., 2008; Pao et al., 1998; Paulsen et al., 1996), and we have observed that ferric iron import into *L. pneumophila* requires the bacterial proton motive force (Chatfield et al., 2011). Third, members of the MFS have already been demonstrated to mediate siderophore export across the Gram-negative IM or the Gram-positive cytoplasmic membrane, and examples include

**Fig. 6.** Growth of *L. pneumophila* wild-type and lbtC mutants on media containing various amounts of iron. (a) We spotted dilutions of wild-type (WT) 130b, lbtA mutant NU302, lbtU mutant NU383 and lbtC mutant NU305 onto standard BCYE agar (left panel) or BCYE lacking its usual iron supplement and containing 12 μM DFX (right panel). After 5 days of growth at 37 °C, growth was recorded. Each strain (i.e. each column of growth) was spotted on its own plate, to prevent siderophore made by some strains from stimulating the growth of others nearby and thereby confounding mutant analysis. (b) Wild-type 130b, lbtC mutant NU306 and the lbtC lbtU double mutant NU404 were assayed as indicated above. (c) Wild-type 130b, lbtC mutant NU305 and mutant NU305 complemented with pLbtC were assayed for their growth on standard BCYE agar or BCYE lacking its usual iron supplement and containing 14 μM DFX. The reduced growth of the lbtC mutants relative to wild-type and the complemented mutant (as depicted in the right-hand panels) was observed in at least three [for (a) and (b)] and two [for (c)] independent experiments.
AlcS of *B. pertussis* and *Bordetella bronchiseptica*, CshX of *Azotobacter vinelandii*, EntS of *E. coli*, LbtB of *L. pneumophila*, PvsC of *Vibrio paraohaemolyticus*, YhcA of *Erwinia chrysanthemi* and YnfE of *Bacillus subtilis* (Allard et al., 2006; Brickman & Armstrong, 2005; Franz et al., 2005; Furrer et al., 2002; Miethke et al., 2008; Page et al., 2003; Tanabe et al., 2006). Fourth, based upon modelling using the I-TASSER and Phyre servers, a 3D structural prediction suggests that LbtC is capable of providing a pore through the IM (Supplementary Fig. S3).

With the current characterization of LbtC and our previous identification of LbtU, the picture of siderophore uptake by *L. pneumophila* is coming into clearer focus. As a first step, we believe that the ferrilegiobactin binds to LbtU residues on the bacterial cell surface and then moves through the OM via an LbtU pore (Chatfield et al., 2011). As a last step, we can now argue that ferrilegiobactin moves across the IM by interacting with LbtC. However, many questions remain. For example, we do not know how LbtU and LbtC conjoin to facilitate legiobactin transport across the cell envelope. It is plausible that a periplasmic protein(s) and/or a periplasmic-spanning, membrane-anchored protein(s) helps to facilitate their interaction. Given that the expression of LbtU and LbtC confers legiobactin utilization on *L. longbeachae*, we would predict that this third factor is not *L. pneumophila*- or legiobactin-specific. Because *L. pneumophila* does not possess TonB-ExbBD (Chatfield et al., 2011), another key question that also remains is how energy is transduced to the OM in order to mediate the initial entrance of legiobactin into the bacterial cell. Thus, we believe that the continued study of *L. pneumophila* has the potential to uncover a new paradigm for iron uptake by bacteria.

![Fig. 7. Intracellular growth of *L. pneumophila* wild-type and lbtC mutants. (a) *A. castellanii* and (b) U937 cells were infected with wild-type 130b (■) and lbtC mutant NU305 (▲), and then at the indicated times, the c.f.u. in the infected monolayers was determined by plating. Data are the mean and SD from four (a) or three (b) infected wells, and are representative of at least three independent experiments.](image)

![Fig. 8. Legiobactin utilization by *L. longbeachae* containing cloned lbtC and lbtU. We plated *L. longbeachae* strain 33462 containing the vector pMMB2002 (column 1), pLbtUABC expressing *L. pneumophila* LbtU, LbtA, LbtB and LbtC (column 2), pLbtUAB expressing LbtU, LbtA and LbtB (column 3) or pLbtABC expressing LbtA, LbtB and LbtC (column 4) onto non-iron-supplemented BCYE agar containing DFX, and wells cut in the agar were filled with legiobactin-containing supernatant from wild-type *L. pneumophila* 130b (Lbt+) (top row), legiobactin-deficient supernatant from the *L. pneumophila* lbtA mutant NU302 (Lbt−) (second row), ferric pyrophosphate (Fe$^{3+}$) (third row) or ferrous ammonium sulfate (Fe$^{2+}$) (bottom row). After 3 days of incubation at 37 °C, growth was recorded. The results presented are representative of at least three independent experiments.](image)
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