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.

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INTRODUCTION

Legionella pneumophila is the agent of Legionnaires' disease (Diederer, 2008; Edelstein & Cianciotto, 2010). In water, this Gram-negative bacterium survives planktonically, in biofilms, and as an intracellular parasite of protozoa (Declercq, 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 *lbtB*, that are required for the production of legiobactin (Allard *et al.*,

Abbreviations: CAS, chrome azurol S; CDM, chemically defined medium; DFX, deferoxamine mesylate; DIP, 2,2-dipyridyl; IM, inner membrane; MFS, major facilitator superfamily; OM, outer membrane.

Three supplementary figures are available with the online version of this paper.

2006, 2009). Supernatants from mutants inactivated for *lbtA* or *lbtB* lack both CAS reactivity and the ability to stimulate growth of iron-starved legionellae. Cytoplasmic LbtA is homologous to siderophore synthetases and is undoubtedly involved in the synthesis of legiobactin. LbtB, a member of the major facilitator superfamily (MFS) of transporters, is akin to IM siderophore exporters and most likely promotes the transport of legiobactin across the IM prior to its final export. More recently, we identified and characterized an outer-membrane (OM) protein (LbtU) that acts as the receptor for ferrilegiobactin (Chatfield *et al.*, 2011). The *lbtU* gene is directly upstream of *lbtA* and *lbtB* and, like those genes, is transcriptionally repressed by high iron. Although mutants lacking LbtU grow normally in standard media, they, like legiobactin-synthesis mutants, are impaired for growth on iron-depleted media. Although able to secrete normal levels of siderophore, *lbtU* mutants are unable to use legiobactin to stimulate growth on iron-depleted media and to take up radiolabelled iron. *In silico* analysis predicts that LbtU consists of a 16-stranded transmembrane β -barrel, multiple extracellular domains, and short periplasmic tails. The sequence and predicted structure of LbtU are distinct from those of known siderophore receptors, which generally have a 22-stranded β -barrel and an N terminus that binds TonB in order to transduce energy from the IM. This observation, coupled with the fact that *L. pneumophila* does not encode TonB, suggests that *L. pneumophila* uses a mode of ferrisiderophore uptake that is mechanistically distinct from existing paradigms (Chatfield *et al.*, 2011). In support of this hypothesis, we now report the characterization of an IM protein (LbtC) that is also required for the ability of *L. pneumophila* to utilize ferrilegiobactin and is unlike previously characterized IM permeases in the MFS.

METHODS

Bacterial strains. *L. pneumophila* 130b [American Type Culture Collection (ATCC) strain BAA-74, also known as AA100] served as our wild-type (Chatfield *et al.*, 2011). Strain 130b is a virulent clinical isolate that belongs to serogroup 1. Other wild-type *L. pneumophila* and mutants of 130b that were examined for siderophore-related function are listed in Table 1. The *Legionella longbeachae* strain (ATCC 33462) used is a clinical isolate that has been described elsewhere (McKinney *et al.*, 1981). *Escherichia coli* DH5 α (Invitrogen) was the host for recombinant plasmids.

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 μ g ml⁻¹, kanamycin at 25 μ g ml⁻¹ or gentamicin at 2.5 μ g 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 μ g ml⁻¹), chloramphenicol

(30 μ g ml⁻¹) or ampicillin (100 μ g 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 μ M 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 1×10^9 c.f.u. ml⁻¹, and then 10 μ l 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 1×10^4 c.f.u. were spread onto non-iron-supplemented BCYE agar containing 12–14 μ M DFX or 400–500 μ M 2,2-dipyridyl (DIP). Small wells cut in the centre of the agar were filled with 100 μ l 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 μ M Fe³⁺ pyrophosphate or 20 μ M Fe²⁺ 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 ⁵⁵FeCl₃ (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 LbtU 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* *ira* mutants (Pope *et al.*, 1996) was ascertained by inverse PCR and subsequent sequence determination, as previously described

Table 1. *L. pneumophila* strains used in this study

Strain	Description	Reference or source
Wild-type strains*		
130b (BAA-74)	Clinical isolate, serogroup 1	Cianciotto <i>et al.</i> (1990)
Togus-1 (33154)	Clinical isolate, serogroup 2	McKinney <i>et al.</i> (1979)
Bloomington-2 (33155)	Clinical isolate, serogroup 3	McKinney <i>et al.</i> (1979)
Dallas-1E (33216)	Clinical isolate, serogroup 5	Garrity <i>et al.</i> (1982)
Chicago-8 (33823)	Clinical isolate, serogroup 7	Bibb <i>et al.</i> (1983)
Concord-3 (35096)	Clinical isolate, serogroup 8	Bissett <i>et al.</i> (1983)
82A3105 (43736)	Clinical isolate, serogroup 13	Lindquist <i>et al.</i> (1988)
1169-MN-H (43703)	Clinical isolate, serogroup 14	Benson <i>et al.</i> (1988)
Mutant strains		
NU302	lpw_13341 (<i>lbtA</i>) mutant of 130b	Allard <i>et al.</i> (2006)
NU269	lpw_29101 (<i>feoB</i>) mutant of 130b	Robey & Cianciotto (2002)
NU383	lpw_13361 (<i>lbtU</i>) mutant of 130b	Chatfield <i>et al.</i> (2011)
NU305, NU306	lpw_13321 (<i>lbtC</i>) mutants of 130b	Allard <i>et al.</i> (2006)
NU404, NU405	<i>lbtU lbtC</i> double mutants of 130b	This study
NU205	lpw_10081 (<i>ald</i>) mutant of 130b	This study
NU207	lpw_15831 (<i>kefA</i>) mutant of 130b	This study
NU208	lpw_09401 (<i>ccmC</i>) mutant of 130b	Viswanathan <i>et al.</i> (2002)
NU210	lpw_26201 (<i>lptJ</i>) mutant of 130b	This study
NU211	lpw_03861 mutant of 130b	This study
NU212	lpw_22731 (<i>trbL</i>) mutant of 130b	This study
NU213	lpw_03271 (<i>gbsA</i>) mutant of 130b	This study
NU214	Undefined mini-Tn10 insertion	Pope <i>et al.</i> (1996)
NU215	lpw_26471 (<i>mdcC</i>) of 130b	This study
NU216	lpw_08281 (<i>iraA</i>) mutant of 130b	Viswanathan <i>et al.</i> (2000)
NU217	Undefined mini-Tn10 insertion	Pope <i>et al.</i> (1996)
NU220	Undefined mini-Tn10 insertion	Pope <i>et al.</i> (1996)
NU223	Undefined mini-Tn10 insertion	Pope <i>et al.</i> (1996)
NU224	Undefined mini-Tn10 insertion	Pope <i>et al.</i> (1996)
NU225	lpw_07571 mutant of 130b	This study
NU244	lpw_08291 (<i>iraB</i>) mutant of 130b	Viswanathan <i>et al.</i> (2000)

*Wild-type strains are listed with their original designation, followed in parentheses by the corresponding ATCC designation.

(Chatfield & 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; Chatfield *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 % RNAlprotect (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; Chatfield *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 end-point PCRs were separated by agarose-gel electrophoresis and

detected with ethidium bromide staining (Allard *et al.*, 2006; Chatfield *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 and 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/TMRPres2D/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

Primer	Sequence (5'–3') (restriction sites underlined)
LbtU INT F	AAAATTGACCTACGATGGGC
LbtU INT R	CAGTCCCAGTCTTTGTTTCGGTATTCC
LbtA INT F	CATCGCGCACCAGCAATGTTTAGAT
LbtA INT R	TGCCTAAGCCAACTGGAAAAGAGGA
LbtB INT F	CACTATTGATCGCGACCAAAGCA
LbtB INT R	ATGACCATGAGGATGGCTCCGAAT
LbtC INT F	TAATGCAAGGGCCATTGCTGAGG
LbtC INT R	GGGTATTATTTCCGAACGTTATGGG
LbtA RT F	CAGTTTATGTCATGTTATC
LbtB RT2 R	GATTGCAAGCGAGATAATTGTGTGCTTTTG
LbtB RT3 F	GGCAGATGACAGATTTTCTAATTCT
LbtC RT R	CCCATAACGTTCCGAAAGAAGACCC
LbtC RTF	TCAGCGTTTAATACCAGCGCCTCT
LbtC RTR	TAATGCAAGGGCCATTGCTGAGG
Mip F	AAAGGCATGCAAGACGCTAA
Mip R	GTATCCGATTTTCCGGGTTT
OR77	TCGGCTCGTATAATGTGTGG
OR78	ACCGCTTCTGCGTTCTGATT
LbtC EXT F	<u>GAGCTCGGCTAAATGTCAGGCAGAT</u>
LbtC EXT1 R	<u>CATAGTCTTCTCTAGACAGAAATACACGCAAAC</u>
CC05	GCTGATTTTCTAGAGTATCCATAAAGCATA
LbtB EXT R	CCCATAAAATCATACTGCGGGCTTT
LbtA 9-F	ATACTGCCATAGCATCGGG
LbtU EXT F	TGACCGATTATCTAGATGCCGCTG
LbtC EXT2 R	AGTGAACCCTAGCATGCTTAGCAA

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 pGMLbC. The *SacI/XbaI* fragment of pGMLbC 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 *Ptac* promoter. This final construct, pLbtC, was then electroporated into NU305 and NU306, the two previously described mutants that contain a gentamicin-resistance cassette inserted into *lbtC* (Table 1). Chloramphenicol-resistant (Cm^r) 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. pLbtUABC, pLbtUAB) were made as follows. In order to construct pLbtUABC, the region of the *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 6.5 kb PCR product was cloned into pGEM-T Easy, yielding pGEM-UABC. The *XbaI/SphI* fragment of pGEM-UABC that contained *lbtU*–*lbtABC* was then subcloned into *XbaI/SphI*-digested pMMB2002, producing pLbtUABC. In order to make pLbtUAB, the *lbtU*, *lbtA* and *lbtB* genes were PCR-amplified from 130b DNA using primers CC05 (Chatfield *et al.*, 2011) and LbtB EXT R (Table 2), and then the resulting 4.4 kb fragment was cloned into pGEM-T Easy, yielding pGEM-UAB. The *XbaI/SphI* fragment of pGEM-UAB that contained *lbtU*–*lbtAB* was then subcloned into

XbaI/SphI-digested pMMB2002, producing pLbtUAB. In pLbtUABC and pLbtUAB, the cloned genes were placed under the control of the vector's *Ptac* promoter. These plasmids were electroporated into the *lbtC* mutants NU305 and NU306, and then Cm^r clones were confirmed by PCR.

***L. pneumophila* mutant constructions.** Double mutants of *L. pneumophila* 130b lacking both *lbtC* and *lbtU* were obtained by transforming pUClbtUK with its kanamycin-resistance (Km^r)-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^r 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 pLbtUABC and pLbtUAB described above were introduced into *L. longbeachae* ATCC 33462 by electroporation (Chatfield *et al.*, 2011) and Cm^r clones were confirmed by PCR. A third plasmid, pLbtABC, was also introduced in a similar fashion. To make pLbtABC, 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 & Cianciotto, 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; Glöckner *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, α -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

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 μ M 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.

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. 2. Predicted IM location and 2D structure of LbtC and its *F. tularensis* homologue. (a) LbtC of *L. pneumophila* strain 130b and (b) FslD of *F. tularensis* subsp. *tularensis* strain Schu S4 (GenBank accession no. YP_169102.1) both display 12 membrane-spanning domains with α -helical structure as well as several extra-membrane loops. The predicted secondary structure of the FigD protein of *F. tularensis* subsp. *novicida* strain U112 (accession no. YP_899296.1) is essentially identical to that of FslD (data not shown).

(Pao *et al.*, 1998; Saier *et al.*, 1999). For example, LbtC shared 24 % identity and 41 % similarity over a span of 333 residues ($E=6 \times 10^{-20}$) with *E. coli* EmrD, and 20 % identity and 41 % similarity over 356 residues ($E=6 \times 10^{-16}$) 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 ($E=4 \times 10^{-9}$) with AlcS of *Bordetella pertussis* and 22 % identity and 37 % similarity over 346 residues ($E=4 \times 10^{-8}$) 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 $E=9 \times 10^{-62}$) 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*

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.

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 (Fe³⁺) (third row) or ferrous ammonium sulfate (Fe²⁺) (bottom row). After 3 days of incubation at 37 °C, growth was recorded. The results shown are representative of at least three independent trials.

and the cellular location of proteins, we hypothesize that ferrilegiobactin 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

Fig. 5. Iron uptake by *L. pneumophila* wild-type and *lbtC* mutants. (a) Wild-type 130b, *lbtC* mutants NU305 and NU306, and mutant NU305 complemented with pLbtC were grown in deferrated CDM, incubated with legiobactin and ⁵⁵FeCl₃ for 60 min, and then assayed for their incorporation of radiolabelled iron. (b) Wild-type 130b, *lbtC* mutant NU305 and *lbtU* mutant NU383 were assayed for iron uptake as indicated above. In (a) and (b), the data are the mean and SD obtained from three replicate cultures. As denoted by the asterisks, the amounts of label incorporated into the mutant samples were always significantly less than those of the wild-type and complemented mutant ($P < 0.05$, Student's *t* test). The reduced uptake exhibited by the mutant strains relative to wild-type was observed in at least three independent experiments. The behaviour of the complemented NU305 mutant was tested on two independent occasions with similar results.

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

Table 3. Legiobactin utilization by *L. pneumophila* *ira* mutants

Strain	Sensitivity (S)/resistance (R) to:			Legiobactin production‡	Legiobactin utilization§
	EDDA*	Streptonigrin*	DFX†		
130b	R	S	R	+	+
NU205	S	S	R	+	+
NU207	S	R	R	+	+
NU208	S	S	S	—	+
NU210	S	S	R	+	+
NU211	S	R	R	+	+
NU212	S	R	R	+	+
NU213	S	R	S	+	+
NU214	S	R	R	+	+
NU215	S	R	R	+	+
NU216	S	R	R	+	+
NU217	R	R	R	+	+
NU220	S	R	S	+	+
NU223	R	R	S	+	+
NU224	R	R	R/SII	+	+
NU225	S	R	S	+	+
NU244	S	R	R	+	+

*Data previously reported (Pope *et al.*, 1996).

†Relative growth on non-iron-supplemented BCYE agar containing 10–14 µM DFX.

‡Data previously reported (Allard *et al.*, 2006; Yip *et al.*, 2011).

§+, Ability to utilize legiobactin-containing supernatants for growth stimulation on non-iron-supplemented BCYE agar containing 400 µM DIP at 25 °C.

||Unexplained variable phenotype.

their capacity to grow on DFX-containing BCYE agar but not on iron-sufficient media, and such behaviour had previously been displayed by both *lbtA* mutants and *lbtU* mutants, which lack the OM receptor for legiobactin (Chatfield *et al.*, 2011). Fourth, *lbtC* mutants could not be stimulated to grow on low-iron media by addition of legiobactin-containing supernatants; *lbtU* mutants that lack the OM receptor for legiobactin behaved similarly (Chatfield *et al.*, 2011). Fifth, *lbtC* mutants were able to grow normally on the low-iron media when they were given ferric or ferrous iron salts. Sixth, like *lbtU* mutants (Chatfield *et al.*, 2011), *lbtC* mutants were impaired for uptake of radiolabelled Fe³⁺ combined with legiobactin. Seventh, all defective phenotypes that were displayed by the *lbtC* mutants could be complemented by the introduction of a cloned copy of *lbtC* on a plasmid vector. Eighth, a plasmid expressing both *lbtC* and *lbtU* was able to confer upon a heterologous bacterium (*L. longbeachae*) the ability to use legiobactin to stimulate growth on low-iron media. Lastly, LbtC is a member of the DHA-12 subfamily in the MFS that, in Gram-negative bacteria, is involved in small-molecule transport across the IM. From these data, we reason that LbtC helps to mediate the transport of ferrilegiobactin across the IM of *L. pneumophila*. The most simple and logical scenario to explain our data is that LbtC is the actual IM transporter for ferrilegiobactin, although it is possible that LbtC indirectly promotes iron assimilation

by facilitating the action of another protein(s) that is the membrane transporter.

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 (Cuív *et al.*, 2008; Koster, 2005; Krewulak & Vogel, 2008; Rees *et al.*, 2009; Vajjala *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 *Sinorhizobium meliloti* and the homologous FptX protein of *Pseudomonas aeruginosa*, which promote the utilization of rhizobactin 1021 and pyochelin, respectively; and FoxB and FiuB of *P. aeruginosa* and FegB of *Bradyrhizobium japonicum*, which facilitate the use of ferrichrome (Benson *et al.*, 2005; Cuív *et al.*, 2004; Hannauer *et al.*, 2010; Michel

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 μ M DFX (right panel). After 5 days of growth at 37 $^{\circ}$ 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 μ M 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.

placed in the MFS, albeit in a family ('2.A.1.57') different from that of LbtC. Based upon the earlier comparison of LbtC with the FslD/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 (Cuív *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

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

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.

AlcS of *B. pertussis* and *Bordetella bronchiseptica*, CsbX of *Azotobacter vinelandii*, EntS of *E. coli*, LbtB of *L. pneumophila*, PvsC of *Vibrio parahaemolyticus*, YhcA of *Erwinia chrysanthemi* and YmfE of *Bacillus subtilis* (Allard *et al.*, 2006; Brickman & Armstrong, 2005; Franza *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. 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 (Fe³⁺) (third row) or ferrous ammonium sulfate (Fe²⁺) (bottom row). After 3 days of incubation at 37 °C, growth was recorded. The results presented are representative of at least three independent experiments.

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