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Cytochrome c_4 is required for siderophore expression by *Legionella pneumophila*, whereas cytochromes c_1 and c_5 promote intracellular infection

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A panel of cytochrome *c* maturation (*ccm*) mutants of *Legionella pneumophila* displayed a loss of siderophore (legiobactin) expression, as measured by both the chrome azurol S assay and a *Legionella*-specific bioassay. These data, coupled with the finding that *ccm* transcripts are expressed by wild-type bacteria grown in deferrated medium, indicate that the Ccm system promotes siderophore expression by *L. pneumophila*. To determine the basis of this newfound role for Ccm, we constructed and tested a set of mutants specifically lacking individual *c*-type cytochromes. Whereas ubiquinol-cytochrome *c* reductase (*petC*) mutants specifically lacking cytochrome c_1 and *cycB* mutants lacking cytochrome c_5 had normal siderophore expression, *cyc4* mutants defective for cytochrome c_4 completely lacked legiobactin. These data, along with the expression pattern of *cyc4* mRNA, indicate that cytochrome c_4 in particular promotes siderophore expression. In intracellular infection assays, *petC* mutants and *cycB* mutants, but not *cyc4* mutants, had a reduced ability to infect both amoebae and macrophage hosts. Like *ccm* mutants, the *cycB* mutants were completely unable to grow in amoebae, highlighting a major role for cytochrome c_5 in intracellular infection. To our knowledge, these data represent both the first direct documentation of the importance of a *c*-type cytochrome in expression of a biologically active siderophore and the first insight into the relative importance of *c*-type cytochromes in intracellular infection events.

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INTRODUCTION

Legionella pneumophila is an aerobic, Gram-negative bacterium that is best known as the agent of Legionnaires' disease, a potentially fatal form of pneumonia (Diederen, 2008). In its aquatic habitat, *L. pneumophila* survives planktonically, within biofilms, and as an intracellular parasite of protozoa (Taylor *et al.*, 2009). Human infection occurs after inhalation of contaminated droplets that originate from a variety of aerosol-generating devices. In the lung, *L. pneumophila* grows in macrophages, and bacterial persistence may also involve growth in epithelia and extracellular survival (Allard *et al.*, 2009; Newton *et al.*, 2010). Iron acquisition is a key component of *L. pneumophila* growth, intracellular infection and virulence (Cianciotto, 2007; Cianciotto, 2008a, b). Factors involved in *Legionella* Fe^{2+} assimilation include an inner-membrane Fe^{2+} transport (FeoB) system and a secreted pyomelanin pigment that has Fe^{3+} reductase activity (Chatfield & Cianciotto, 2007; Robey & Cianciotto, 2002). *L. pneumophila feoB* mutants are impaired in lung infection, confirming the importance of Fe^{2+} assimilation for pathogenesis (Robey & Cianciotto, 2002). The principal aspect of *L. pneumophila* Fe^{3+} uptake is legiobactin. When *L. pneumophila* strains are grown in a low-iron, chemically defined medium (CDM), the siderophore is detected by the chrome azurol S (CAS) assay (Allard *et al.*, 2006; Liles *et al.*, 2000). Legiobactin is also detected in a bioassay, in which CDM culture supernatants or purified siderophore stimulate the growth of iron-starved legionellae (Allard *et al.*, 2006, 2009). Some but not all other *Legionella* species appear to make legiobactin (Allard *et al.*, 2006; Starkenburg *et al.*, 2004). Two linked genes, *lbtA* and *lbtB*, have been implicated in the production of legiobactin. LbtA, required for the synthesis of siderophore, has sequence similarity to several other siderophore synthetases (Allard *et al.*, 2006). LbtB is believed to be an inner-membrane transporter that promotes the secretion of legiobactin (Allard *et al.*, 2006). Importantly, *lbtA* mutants, but not their complemented derivatives, are defective for infection of the murine lung, documenting a role for legiobactin in *L. pneumophila* virulence (Allard *et al.*, 2009).

In addition to characterizing FeoB, ferric reductase and legiobactin, we previously determined that the *ccm* locus promotes *L. pneumophila* growth in low-iron conditions,

Abbreviations: CAS, chrome azurol S; Ccm, cytochrome *c* maturation.

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suggesting that the cytochrome *c* maturation system has a role in iron acquisition (Naylor & Cianciotto, 2004; Viswanathan *et al.*, 2002). In *L. pneumophila* and a variety of other bacteria, the *ccm* locus is an eight-gene operon (*ccmA* through *ccmH*) that encodes a multi-protein complex which transports haem across the inner membrane and then attaches it to apocytochromes in the periplasm as the final step in the maturation of *c*-type cytochromes (Cianciotto *et al.*, 2005; Kranz *et al.*, 2009; Sanders *et al.*, 2010). Also important for the maturation of *c*-type cytochromes are the Sec translocon, which delivers (reduced) apocytochromes across the inner membrane, and the extracytoplasmic DsbA/DsbB pathway, which converts the reduced apocytochromes to the oxidized forms that are acted on by the Ccm system (Sanders *et al.*, 2010). Initially, we found that mutations in *ccmC* reduced the plating efficiency of *L. pneumophila* on low-iron buffered charcoal yeast extract (BCYE) agar (Pope *et al.*, 1996; Viswanathan *et al.*, 2002). The *ccmC* mutants were also impaired for infection of *Hartmannella vermiformis* amoebae, human macrophage-like cells (U937 and THP-1 lines) and the murine lung (Naylor & Cianciotto, 2004; Viswanathan *et al.*, 2002). The infectivity defect was exacerbated when the host cells were treated with the Fe^{3+} chelator desferrioxamine but ameliorated when supplementary iron was added, suggesting that the *ccmC* mutants are impaired for both extracellular and intracellular iron acquisition (Naylor & Cianciotto, 2004; Viswanathan *et al.*, 2002). Complementation analysis confirmed that *ccmC* is required for *L. pneumophila* growth on low-iron media and during infection (Viswanathan *et al.*, 2002). By characterizing additional *ccmB*, *ccmC* and *ccmF* mutants, we confirmed that the entire Ccm system is required for *L. pneumophila* growth in low-iron conditions (Naylor & Cianciotto, 2004). We now report that *L. pneumophila* Ccm and more specifically cytochrome c_4 are required for the expression of legiobactin.

METHODS

Bacterial strains. *L. pneumophila* 130b (ATCC strain BAA-74, also known as AA100 or Wadsworth) served as our wild-type (Allard *et al.*, 2009). This serogroup 1 strain is a virulent clinical isolate. Previously described mutants of 130b used in this study were as follows: NU257 and NU295 are *ccmC* mutants, NU292 and NU293 *ccmB* mutants, NU296 and NU297 *ccmF* mutants, NU269 an *feoB* mutant, and NU302 an *lbtA* mutant (Allard *et al.*, 2006; Naylor & Cianciotto, 2004; Robey & Cianciotto, 2002; Viswanathan *et al.*, 2002). *Escherichia coli* DH5α (Invitrogen) was the host for recombinant plasmids.

Bacteriological media and extracellular growth experiments. *L. pneumophila* strains were routinely cultured at 37 °C on BCYE agar, which has an iron supplement consisting of 0.25 g of ferric pyrophosphate per litre (Allard *et al.*, 2006). 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 judge the basic extracellular growth capacity of *L. pneumophila*, bacteria grown on BCYE agar were inoculated into buffered yeast extract (BYE) broth, and then the optical density of the cultures was determined at 660 nm (OD_{660}) (Hickey & Cianciotto, 1997; Liles *et al.*, 2000; Viswanathan *et al.*, 2000). 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 medium, legionellae were tested for their ability to form colonies on BCYE agar that lacked its iron supplement (Allard *et al.*, 2006; Robey & Cianciotto, 2002; Viswanathan *et al.*, 2002). Bacteria were pre-cultured for 3 days on standard BCYE agar, suspended in PBS at 1×10^9 c.f.u. ml^{-1}, and then 10 μl aliquots taken from 10-fold serial dilutions in PBS were spotted on the assay medium. Growth was recorded after 4 days of incubation at 37 °C. *E. coli* was grown in Luria–Bertani medium, containing kanamycin (50 μg ml^{-1}), gentamicin (2.5 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}) or ampicillin (100 μg ml^{-1}).

Siderophore assays. Legiobactin production, secretion and utilization were examined as described previously (Allard *et al.*, 2006, 2009). Briefly, *L. pneumophila* strains were grown in BYE to an OD_{660} of 1.0, inoculated into deferrated CDM to an OD_{660} of 0.3, and then incubated at 37 °C. At 24 h post-inoculation, siderophore activity in supernatants was quantified using the CAS assay as previously done, with desferrioxamine serving as the standard (Allard *et al.*, 2006, 2009; Liles *et al.*, 2000; Starkenburg *et al.*, 2004). Supernatants were tested for siderophore biological activity by examining their ability to promote the growth of the NU269 *feoB* mutant on non-iron-supplemented BCYE agar (Allard *et al.*, 2006, 2009). NU269 lacks an inner-membrane Fe^{2+} permease and thus is defective for uptake of Fe^{2+} but not Fe^{3+} (Robey & Cianciotto, 2002). To compare wild-type and mutant *L. pneumophila* for their ability to use legiobactin, bacteria were pre-cultured for 3 days on BCYE agar, suspended in PBS, and then 1×10^4 c.f.u. were spread onto non-iron-supplemented BCYE agar containing 400 μM 2,2′-dipyridyl (Allard *et al.*, 2006). Small wells cut in the centre of the agar were filled with 75 μl of supernatants obtained from deferrated CDM cultures. Control wells contained deferrated CDM, 5 μM Fe^{3+} pyrophosphate or 20 μM Fe^{2+} ammonium sulfate. Growth around the wells was assessed after incubation at 25 °C for 8–10 days.

DNA, RNA and protein analysis. DNA was isolated from *L. pneumophila* as before (Cianciotto & Fields, 1992). DNA sequencing was done at the Northwestern University Biotech Lab, with primers from Integrated DNA Tech. Reverse transcription (RT)-PCR was done as previously described (Allard *et al.*, 2006; Liles *et al.*, 1998). RNA was isolated from 18 h CDM or BYE cultures of *L. pneumophila* using RNA STAT-60 according to the manufacturer's instructions (TEL-TEST B, Inc.). Total cDNA was amplified with random hexamers (Invitrogen) and detected using standard PCR. Primer pairs used for amplifying the *ccm*, *petC* (encoding cytochrome c_1 of ubiquinol-cytochrome *c* reductase), *cyc4* (cytochrome c_4), *cycB* (cytochrome c_5), *lbtA* and *dsbA* genes are listed in Table 1. Control experiments lacking reverse transcriptase were done to rule out contributions from contaminating DNA in the DNase-treated samples. PCR products obtained from genomic DNA confirmed that the mRNAs observed were of the appropriate length. PCR products were separated by agarose-gel electrophoresis and detected with ethidium bromide (Allard *et al.*, 2006; Liles *et al.*, 1998). Homology searches were done through the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/ and *L. pneumophila* databases at http://genolist.pasteur.fr/LegioList/, http://www.ebi.ac.uk/ena/data/view/FR687201 and http://www.ncbi.nlm.nih.gov/genomeprj/48801.

Mutant constructions. To obtain a cytochrome c_1 mutant, the *petC* gene was amplified from the genomic DNA of strain 130b by PCR using primers c1-F and c1-R (Table 1), and the resulting 1.6 kb fragment was cloned into pGEM-T Easy (Promega). The resultant plasmid, pGpetC, was digested with *Ssp*I and ligated to a fragment of pMB2190 that carries a kanamycin-resistance gene (Km^r) (Rossier *et al.*, 2004). This final construct, pGpetC::Km^r, was then introduced

Table 1. Primers used in this study

Primer	Sequence (5′–3′)
ccmB-F (orf1-5—F)	TATTAATCCAGGTGCGGCAA
ccmB-R (ccmB5′-R)	GGATATCCACACTAATCCGGGAGC
ccmC-F (orf2-1—F)	TGATGCGTGGTAGAATCCTT
ccmC-R (ccmC5′-R)	GCATCCCCTTGCTGGATATCTG
ccmD-F (ccmD3′-F)	CGTTGGTTTAAGAGATAGGATATCTG
ccmD-R (ccmC-R)	ACCTCCAACTCGGATGTGGTGTTT
ccmF-F (ccmF12-F)	ATGAAGGTTCCATGCTGTTA
ccmF-R (ccmE-R)	AAAGCCAGGATCTTGCAGCAATGG
ccmG-F (ccmH-F)	TCCTCATCATTTGCCTTCAGCTCG
ccmG-R (ccmF-R)	TCTCGCGTGCTCTGGATTGTCTTT
ccmH-F (ccmG3′-F)	TCCGCTACAGGGATATCGGGATA
ccmH-R (ccmH5′-R)	TCTGCGATATCCTGATTTTGGCAT
petC-F (c1-F)	GCCCGCTAATCCAATGGTG
petC-R (c1-R)	CTTCCGCACGGGCTACTG
cyc4-F (c4-F)	GGTGTTTTGCTGGTTCTTGC
cyc4-R (c4-R)	CGATTGTTTCGTTGCCAGTG
cycB-F (c5-F)	GCTCCAGCCTGATTGAGTG
cycB-R (c5-R)	GTTCATTCGCAGAAACGGC
lbtA-F	CATTTGATCGATGGCCTCTT
lbtA-R	GCGCGGAAATTAGGATGATA
dsbA-F	GCCAAAGCCTATTACACAGC
dsbA-R	CTCCCTGCCATTTGTAAGTC

into 130b by transformation (Allard *et al.*, 2006; Rossier *et al.*, 2004; Stewart *et al.*, 2009), and transformants were selected on antibiotic-containing BCYE agar. Mutation of *petC* was confirmed by PCR using c1-F and c1-R. Two independently derived *petC* mutants were designated NU375 and NU376. To make a cytochrome c_4 mutant, the *cyc4* gene was PCR-amplified from 130b DNA using primers c4-F and c4-R (Table 1), and the resultant 1.2 kb fragment was cloned into pGEM-T Easy to yield pGcyc4. This plasmid was digested with *Nae*I, and Km^r was cloned into *cyc4*. The resulting pGcyc4::Km^r was digested with *Sph*I/*Spe*I, filled in, and subcloned into pRE112. Electroporation was used to introduce the plasmid containing the mutated gene into 130b. Two independently derived *cyc4* mutants, NU379 and NU380, were obtained after antibiotic selection and PCR confirmation. To make a cytochrome c_5 mutant, the *cycB* gene was amplified by PCR using primers c5-F and c5-R (Table 1). The 1.6 kb product was cloned into pGEM-T Easy, yielding pGcycB. The new plasmid was then digested with *Eco*RI, and Km^r was inserted into *cycB*. The resulting plasmid, pGcycB::Km^r, was introduced into 130b by transformation, and mutants NU381 and NU382 were obtained.

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

RESULTS

L. pneumophila ccm promotes the expression of legiobactin

Since our initial studies on *ccm* mutants (Naylor & Cianciotto, 2004; Viswanathan *et al.*, 2002), we have improved our ability to produce and detect *L. pneumophila* siderophore (Allard *et al.*, 2006, 2009). Therefore, as a next step toward understanding the mechanistic connection between Ccm and *Legionella* growth in low-iron conditions, we examined anew six of our *ccm* mutants for their expression of legiobactin. Two mutants (NU292, NU293) contained an inactivated *ccmB*, two (NU257, NU295) had mutations in *ccmC*, and two (NU296, NU297) were defective for *ccmF* (Fig. 1a). The mutants were grown in deferrated CDM, and then cell-free culture supernatants were tested in the CAS assay. All of these *ccm* mutants displayed a significant reduction in CAS reactivity when compared to their wild-type parent, strain 130b (Fig. 1b). Over the course of multiple experiments, the siderophore activity of the mutants ranged from 20 to 50 % of that of wild-type. Further trials confirmed that the reduction in CAS reactivity exhibited by the *ccm* mutants was comparable to that of an *lbtA* mutant (Allard *et al.*, 2006) that is known to not make legiobactin (Fig. 1c). To confirm the impact of the *ccm* mutations on siderophore production, we tested the supernatants from these mutants in a legiobactin-specific bioassay. Like the samples derived from the *lbtA* mutant, supernatants obtained from the *ccm* mutants were unable to stimulate the growth of iron-starved legionellae (Fig. 1d). Ccm mutants were, however, capable of using supplied legiobactin obtained from wild-type supernatants in order to stimulate their growth on iron-deplete media (data not shown). Taken together, these data indicated that an intact *ccm* locus is required for the production but not utilization of legiobactin. In support of this conclusion, RT-PCR analysis confirmed that genes within the *ccm* locus are expressed when wild-type 130b is grown in deferrated CDM (Fig. 2a). This analysis additionally indicated that *ccm* gene transcripts also occur when 130b is grown in BYE broth (Fig. 2a), indicating that *ccm* expression occurs in both minimal, iron-depleted conditions and rich, iron-replete medium, compatible with its having significance for multiple facets of bacterial growth besides siderophore expression. When RT-PCR analysis was extended to include *lbtA*, we found that the legiobactin synthesis gene was expressed in the *ccm* mutants (Fig. 3a, left panel), implying that the absence of a Ccm system (in *ccm* mutants) did not trigger some sort of feedback that shut down *lbtA* and legiobactin synthesis. Based on these data and coupled with the fact that Ccm operates within the inner membrane and periplasm, we posited that Ccm is promoting the maturation or secretion of legiobactin.

Cytochrome c_4 is required for the expression of legiobactin

Since the Ccm system is best known for its role in the maturation of *c*-type cytochromes (Sanders *et al.*, 2010), we sought to determine the importance of individual *c*-type cytochromes for legiobactin expression. After examining the genomes of all five sequenced strains of *L. pneumophila* (strains Philadelphia-1, Paris, Lens, Corby, Alcoy and 130b) (Cazalet *et al.*, 2004; Chien *et al.*, 2004; D'Auria *et al.*, 2010; Glöckner *et al.*, 2008; Schroeder *et al.*, 2010), we

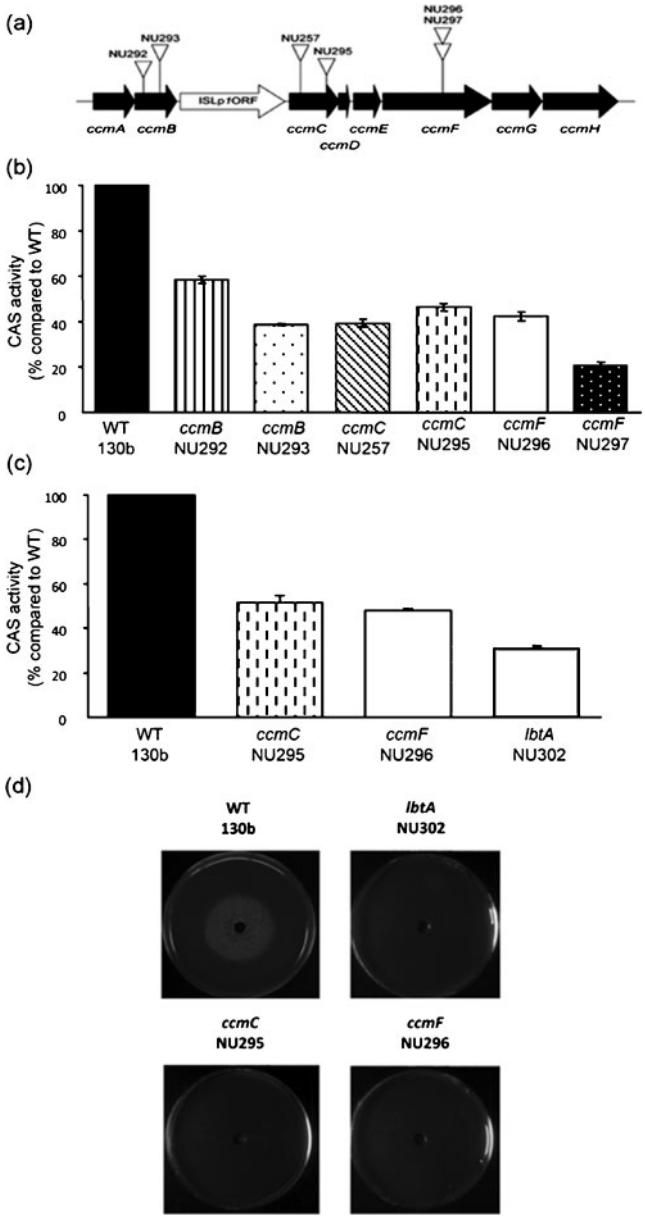

Fig. 1. Legiobactin production by *L. pneumophila* wild-type and *ccm* mutants. (a) The region of the *L. pneumophila* chromosome containing the *ccm* locus. Horizontal black arrows depict the relative sizes and orientation of the eight *ccm* genes (i.e. *ccmA*–*ccmH*) in strain 130b. The white horizontal arrow denotes the insertion sequence element between *ccmB* and *ccmC* (Viswanathan *et al.*, 2002). The vertical arrowheads point to the locations of the Km^R insertion in six *ccm* mutants. (b) CAS activity of the *ccmB*, *ccmC* and *ccmF* mutants compared to wild-type 130b. The CAS values are the means and standard deviations from duplicate cultures. The results presented are representative of at least three independent experiments. (c) CAS activity of the *ccm* mutants compared to an *lbtA* mutant. In (b) and (c), the CAS activities of the various *ccm* mutants were significantly less than that of wild-type (Student's *t*-test, $P<0.05$). In (c), the CAS activities for the *ccm* mutants were not significantly different from that of the *lbtA* mutant ($P>0.05$). (d) Siderophore biological activity of wild-type and mutants. We plated approx. 10^5 c.f.u. of *feoB* mutant legionellae onto non-iron-supplemented BCYE agar and a centre well was filled with a supernatant sample obtained from deferrated CDM cultures of wild-type 130b, *lbtA* mutant NU302, *ccmC* mutant NU295 and *ccmF* mutant NU296, as indicated. After 5 days, the growth of the bacteria was recorded. The results shown are representative of at least three experiments. Although not shown here for the sake of space, *ccmB* mutants NU292 and NU293, *ccmC* mutant NU257 and *ccmF* mutant NU297 also lacked siderophore biological activity.

targeted the three genes encoding *c*-type cytochromes. The first gene (*petC*) encodes cytochrome c_1, which is 28 kDa in size and possesses one haem-attachment site (the motif CXXCH) (Nomenclature Committee of the International Union of Biochemistry, 1992; Sanders *et al.*, 2010). As is typical in other bacteria (Davidson & Daldal, 1987; Thöny-Meyer *et al.*, 1991), *L. pneumophila petC* is the last gene in an operon with the other two genes (*petA* and *petB*) encoding the Rieske iron–sulfur protein and cytochrome *b* (Rossier & Cianciotto, 2005). The second gene (*cyc4*) that we targeted encodes cytochrome c_4, which is 21 kDa in size and a di-haem (i.e. with two CXXCH motifs) protein (Chang *et al.*, 2010; Nomenclature Committee of the International Union of Biochemistry, 1992; Deeudom *et al.*, 2008; Giudici-Orticoni *et al.*, 2000). The third gene (*cycB*) gene encodes a 15 kDa, mono-haem cytochrome c_5 (Chang *et al.*, 2010; Nomenclature Committee of the International Union of Biochemistry, 1992; Klarskov *et al.*, 1998; Li *et al.*, 2010). Both *cyc4* and *cycB* are in a two-gene operon, with the gene downstream of *cyc4* being *dsbA*, and the gene downstream of *cycB* being *dsbB*. As noted earlier, DsbA and DsbB mediate the oxidative folding of apocytochrome *c* molecules prior to their interaction with the Ccm system (Heras *et al.*, 2009; Sanders *et al.*, 2010). In the strain 130b database, *petC, cyc4* and *cycB* are also denoted by the ORF designations lpw_29591, lpw_01241 and lpw_29881, respectively (Schroeder *et al.*, 2010). Using allelic exchange, as we have done many times to make other *L. pneumophila* mutants of strain 130b (Allard *et al.*, 2006; Pearce & Cianciotto, 2009; Stewart *et al.*, 2009), we constructed multiple mutants inactivated for either *petC* (NU375, NU376), *cyc4* (NU379, NU380) or *cycB* (NU381, NU382) and then tested them in the legiobactin assays. Whereas the *petC* mutants and *cycB* mutants behaved as the wild-type did, the *cyc4* mutants displayed a reduction in CAS activity that was comparable to that of the *ccm* mutants (Fig. 4a). In a similar vein, supernatants from the *cyc4* mutants were unable to stimulate the growth of iron-starved legionellae, whereas supernatants from the *petC* mutants and *cycB* mutants did stimulate growth (Fig. 4b). Because multiple independently derived *cyc4* mutants had the same phenotype, the loss of siderophore activity in these strains was due to the *cyc4* mutation rather than a spontaneous second-site mutation. Furthermore, because the *cyc4*

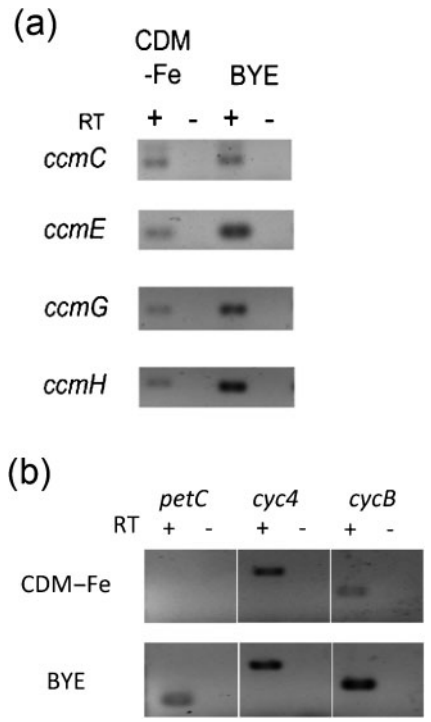

Fig. 2. Transcription of the *ccm*, *petC*, *cyc4* and *cycB* genes. (a) Expression of *ccm* transcripts. Wild-type 130b was grown in deferrated CDM (CDM–Fe) or standard BYE broth (BYE), and then RNA was analysed by RT-PCR utilizing primers specific to *ccmC*, *ccmE*, *ccmG* or *ccmH*. That the PCR products obtained resulted from mRNA templates was confirmed by the lack of product obtained when the reaction did not incorporate RT (–RT lanes). In additional experiments, *ccmB* and *ccmF* transcripts were detected in 130b growing in CDM–Fe (data not shown). (b) Expression of the *c*-type cytochrome genes. RNA from the wild-type was analysed by RT-PCR utilizing primers specific to *petC*, *cyc4* or *cycB*. The results presented are representative of three independent experiments.

mutants continued to express *dsbA* transcripts (Fig. 3b), this mutant phenotype was not due to polarity on the downstream *dsbA*. The fact that Ccm$^-$ mutants were also impaired for legiobactin also strongly argues that this mutant phenotype involves the loss of cytochrome c_4 as opposed to being only due to a possible polar effect on *dsbA* and the loss of activities of DsbA that are unrelated to cytochrome maturation. Compatible with a role for cytochrome c_4 in siderophore expression, *cyc4* transcripts were detected in *L. pneumophila* grown in deferrated CDM (Fig. 2b). Like the *ccm* genes, this cytochrome gene was also expressed when bacteria were grown in BYE broth (Fig. 2b). The *cycB* transcripts were also detected in bacteria grown in either medium, whereas *petC* mRNA was only evident in bacteria cultured in BYE (Fig. 2b). Together, these data indicate that cytochrome c_4, but not cytochromes c_1 and c_5, is required for legiobactin expression, and the importance of Ccm for siderophore expression is tied to its role in producing a certain *c*-type cytochrome. Given the cellular location of *c*-type cytochromes as well as the fact that *lbtA* expression is evident in the *cyc4* mutants (Fig. 3a, right panel), we posit that cytochrome c_4 promotes the maturation or secretion of the *Legionella* siderophore.

Effect of *c*-type cytochromes on *L. pneumophila* extracellular growth and intracellular infection

None of the newly made cytochrome mutants displayed a growth defect in standard BYE broth (data not shown) or on standard BCYE agar (Fig. 5a), indicating that they do not have a generalized growth defect. As noted above, *L. pneumophila ccm* mutants show a reduced ability to grow on BCYE agar that lacks an iron supplement (Naylor & Cianciotto, 2004; Viswanathan *et al.*, 2002). However, none of the new mutants showed this defect (Fig. 5b), indicating that the growth defect of the *ccm* mutants on non-iron-supplemented media is not due to the loss of one of the *c*-type cytochromes. This is compatible with the fact that *lbtA* mutants grow normally on non-iron-supplemented BCYE agar (Allard *et al.*, 2006) (Fig. 5b). Turning to intracellular growth assessments, the *petC* mutants and *cycB* mutants, but not the *cyc4* mutants, exhibited a reduced ability to grow in amoebal hosts (Fig. 6a, b). That *cyc4* mutants grew like the wild-type did was not at odds with their lack of siderophore and impaired growth on iron-depleted media, because as we previously determined, legiobactin is not needed for intracellular infection under standard conditions (Allard *et al.*, 2006). The defects of the *petC* mutants and *cycB* mutants were noted in *H. vermiformis* and *A. castellanii* (Fig. 6a, b). Whereas the *petC* mutants displayed a relatively modest infectivity defect of 10- to 100-fold, depending upon the time post-inoculation, the *cycB* mutants appeared completely unable to infect the protozoa. Indeed, the *cycB* mutants were as defective as the *ccmC* mutants were (Fig. 6a, b), implying that the importance of Ccm for *L. pneumophila* infection of amoebae is due largely to a need for cytochrome c_5. Mirroring the results obtained from the amoebal assays, the *petC* mutants and *cycB* mutants, but not the *cyc4* mutants, were impaired for growth in macrophages (Fig. 6c). One difference was the fact that the *cycB* mutant was not nearly as defective as a *ccm* mutant was, suggesting that the importance of Ccm has a more complex basis in macrophages than it does in amoebae. Because multiple independently derived *petC* mutants had impaired growth in host cells and because there is no transcriptionally linked gene downstream of *petC*, the loss of infectivity by the *petC* mutants was due to the loss of PetC rather than any second-site mutation or polarity. Given that multiple *cycB* mutants showed impaired intracellular infection, the loss of infectivity in these mutants was due to the mutation in *cycB* as opposed to a second-site mutation. Since *cycB* mutants and the *ccm* mutants (which lack *c*-type cytochromes because of a mutation in a distinct chromosomal locus) had similar infectivity defects, we further

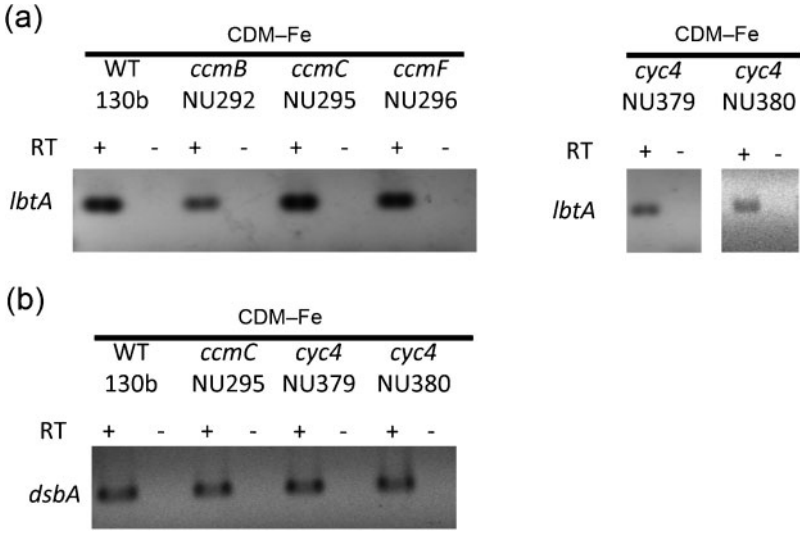

Fig. 3. Expression of *lbtA* and *dsbA* by *ccm* and *cyc4* mutants and wild-type *L. pneumophila*. (a) Wild-type 130b, *ccmB* mutant NU292, *ccmC* mutant NU295, *ccmF* mutant NU296, and *cyc4* mutants NU379 and NU380 were inoculated into deferrated CDM (CDM–Fe), and then RNA was analysed by RT-PCR utilizing primers specific to *lbtA*. (b) Wild-type 130b, *ccmC* mutant NU295, and *cyc4* mutants NU379 and NU380 were inoculated into CDM–Fe, and then RNA was analysed by RT-PCR utilizing primers specific to *dsbA*. Data are representative of three independent experiments.

conclude that these mutant phenotypes resulted from the loss of cytochrome c_5 as opposed to being only due to a possible polar effect on *dsbB* and the loss of DsbB activities that are unrelated to cytochromes. In sum, these data indicate that cytochromes c_1 and c_5 are required for the optimal intracellular growth of *L. pneumophila*.

DISCUSSION

For multiple reasons, we conclude that the Ccm system is required for full expression of legiobactin. First, a variety of independent *ccm* mutants of a virulent strain of *L. pneumophila* lack siderophore expression. Second, multiple, independently derived *cyc4* mutants lacking a particular *c*-type cytochrome exhibit a similar lack of siderophore. Third, the loss of siderophore was documented by both the CAS assay and a legiobactin-specific bioassay. Fourth, transcription of the *ccm* and *cyc4* genes occurs in legionellae growing in deferrated media. Our data bring to four the number of cases in which a Ccm system is linked to siderophore. Past examples include pyoverdine production by *Pseudomonas aeruginosa* (Baert *et al.*, 2008), pyoverdine and thioquinolobactin synthesis by *Pseudomonas fluorescens* (Baysse *et al.*, 2002, 2003; Gaballa *et al.*, 1996; Matthijs *et al.*, 2007) and siderophore expression by *Paracoccus denitrificans* and *Rhizobium leguminosarum* (Pearce *et al.*, 1998; Yeoman *et al.*, 1997). The fact that *Legionella*, *Pseudomonas*, *Paracoccus* and *Rhizobium* are quite distinct from each other, as are the structures of their siderophores (Allard *et al.*, 2009), suggests that the connection between Ccm and siderophores likely also exists in a variety of other bacteria, including both environmental and pathogenic strains.

The molecular basis for the role of Ccm in siderophore expression has been the subject of speculation. We and others had theorized that Ccm might be facilitating siderophore production through its role in the maturation of *c*-type cytochromes, the delivery of haem into the periplasm for purposes other than its ligation to apocytochromes, or the export of a molecule besides haem (Cianciotto *et al.*, 2005). An early report had suggested the involvement of a *cyc4*-like gene (*pvcD*) in the production of pyoverdine chromophore by *P. aeruginosa* (Baysse *et al.*, 2001; Stintzi *et al.*, 1999); however, it was later determined that the *pvc* locus aids in the production of isonitrile-functionalized coumarin and pseudoverdine, which do not have siderophore biological activity (Clarke-Pearson & Brady, 2008; Stintzi *et al.*, 1996). By specifically targeting individual *c*-type cytochromes for mutation and using both chemical and biological assays for detection of legiobactin, we can now conclude that the role of Ccm in siderophore production by *L. pneumophila* is linked to *c*-type cytochromes, i.e. cytochrome c_4. One hypothesis to explain the newfound importance for the *c*-type cytochrome is that the biosynthesis of legiobactin requires an electron-transfer step within the periplasm, e.g. shuttling electrons, possibly from an electron-transport chain, to a substrate or enzyme that is needed for legiobactin synthesis and/or secretion. In support of this hypothesis, periplasmic enzymes have been shown to be necessary for the completion of siderophore synthesis in some other bacteria (Yeterian *et al.*, 2010). It does remain formally possible however that cytochrome c_4 indirectly promotes the processing of legiobactin by helping to maintain a certain redox homeostasis in the periplasm or acting as a signalling molecule. Regardless, the fact that cytochrome c_4, but not cytochromes c_1 and c_5, is critical for legiobactin expression suggests that there is specificity to the interaction between the siderophore and cytochrome pathways.

To our knowledge, the current study represents the first investigation into the relative importance of *c*-type cytochromes for *L. pneumophila* growth. Since all of our *ccm* mutants grew normally on standard media, *c*-type

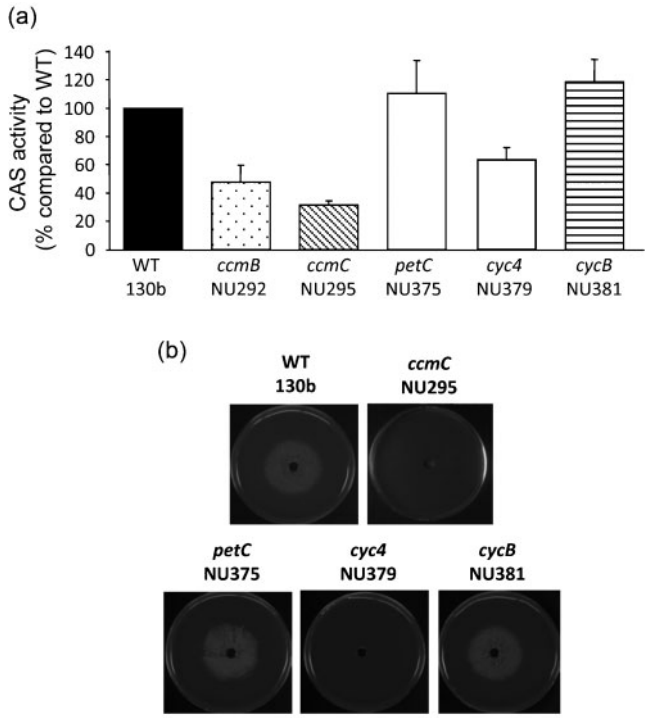

Fig. 4. Legiobactin production by *L. pneumophila* wild-type and mutants lacking *c*-type cytochromes. (a) CAS activity of *petC*, *cyc4* and *cycB* mutants compared to that of wild-type 130b and the *ccmB* and *ccmC* mutants. The CAS values are the means and standard deviations from duplicate cultures, and the results presented are representative of at least three independent experiments. The levels of CAS activity displayed by the *cyc4* mutants were significantly less than that of wild-type (Student's *t*-test, $P<0.05$). They were not, however, different from that of the *ccm* mutants, nor were the levels of CAS activity displayed by the *petC* and *cycB* mutants different from that of wild-type ($P>0.05$). (b) We plated approx. 10^5 c.f.u. of the *feoB* mutant onto non-iron-supplemented BCYE agar and a centre well was filled, as indicated, with supernatant obtained from deferrated CDM cultures of wild-type 130b, *ccmC* mutant NU295, *petC* mutant NU375, *cyc4* mutant NU379 or *cycB* mutant NU381. After 5 days, the growth of the bacteria was recorded. The results shown are representative of at least three experiments. Although not shown here for the sake of space, *cyc4* mutant NU380 also lacked siderophore activity in its culture supernatants, whereas *petC* mutant NU376 and *cycB* mutant NU382 behaved like the wild-type in this regard.

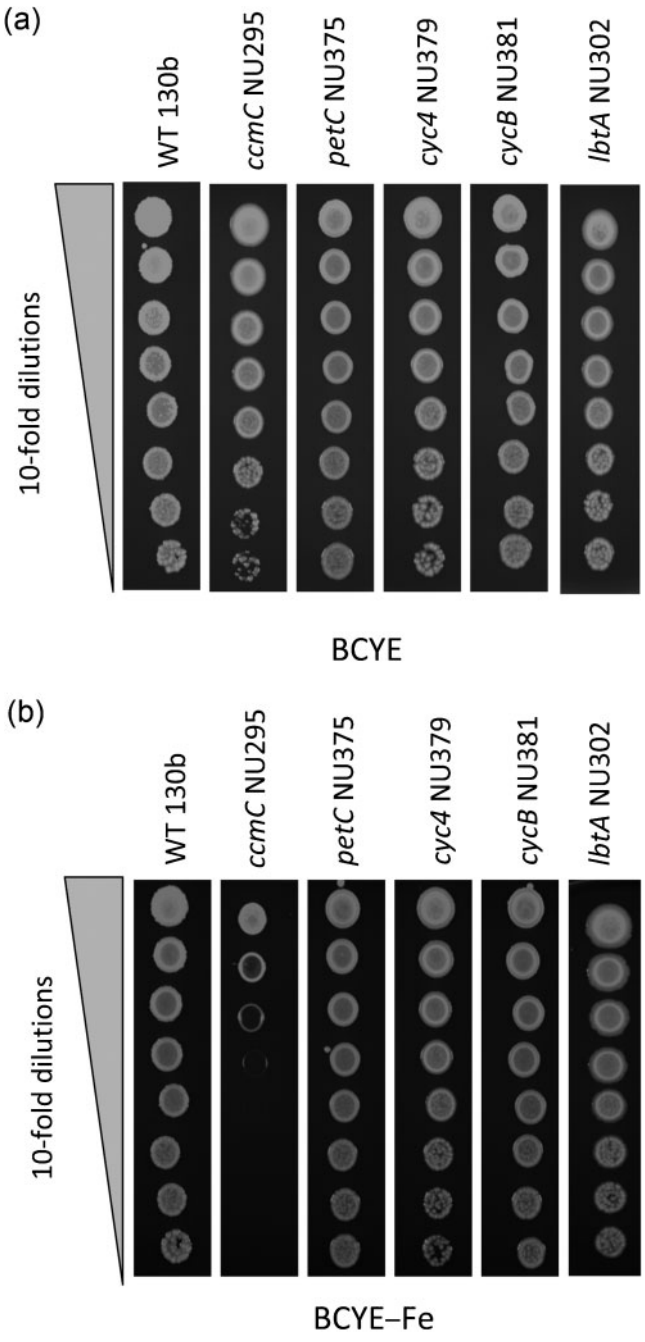

Fig. 5. Growth of *L. pneumophila* wild-type and *c*-type cytochrome mutants on BCYE agar. We spotted 10 μl aliquots from 10-fold serial dilutions of wild-type 130b, *ccmC* mutant NU295, *petC* mutant NU375, *cyc4* mutant NU379, *cycB* mutant NU381 and *lbtA* mutant NU302 onto standard BCYE agar (a) or BCYE lacking its usual iron supplement (b). After 4 days, growth was recorded. Each strain (i.e. each column of growth depicted here) was spotted on its own plate, to prevent diffusible factors produced by some strains from stimulating the growth of others nearby and thereby confounding mutant analysis. The results shown are representative of three experiments.

cytochromes are not essential for *L. pneumophila* extracellular growth. That the mutants lacking *petC*, *cyc4* or *cycB* also grew normally on BCYE agar and in BYE broth supports this conclusion. These data are compatible with the fact that *L. pneumophila* also has *a*-, *b*- and *d*-type cytochromes, with at least *d*-type cytochromes supporting respiration via a quinol-oxidizing branch that is independent of cytochrome *c* (Cazalet *et al.*, 2004; Hoffman & Pine, 1982; Miller & Hammel, 1985; Thöny-Meyer, 1997). In

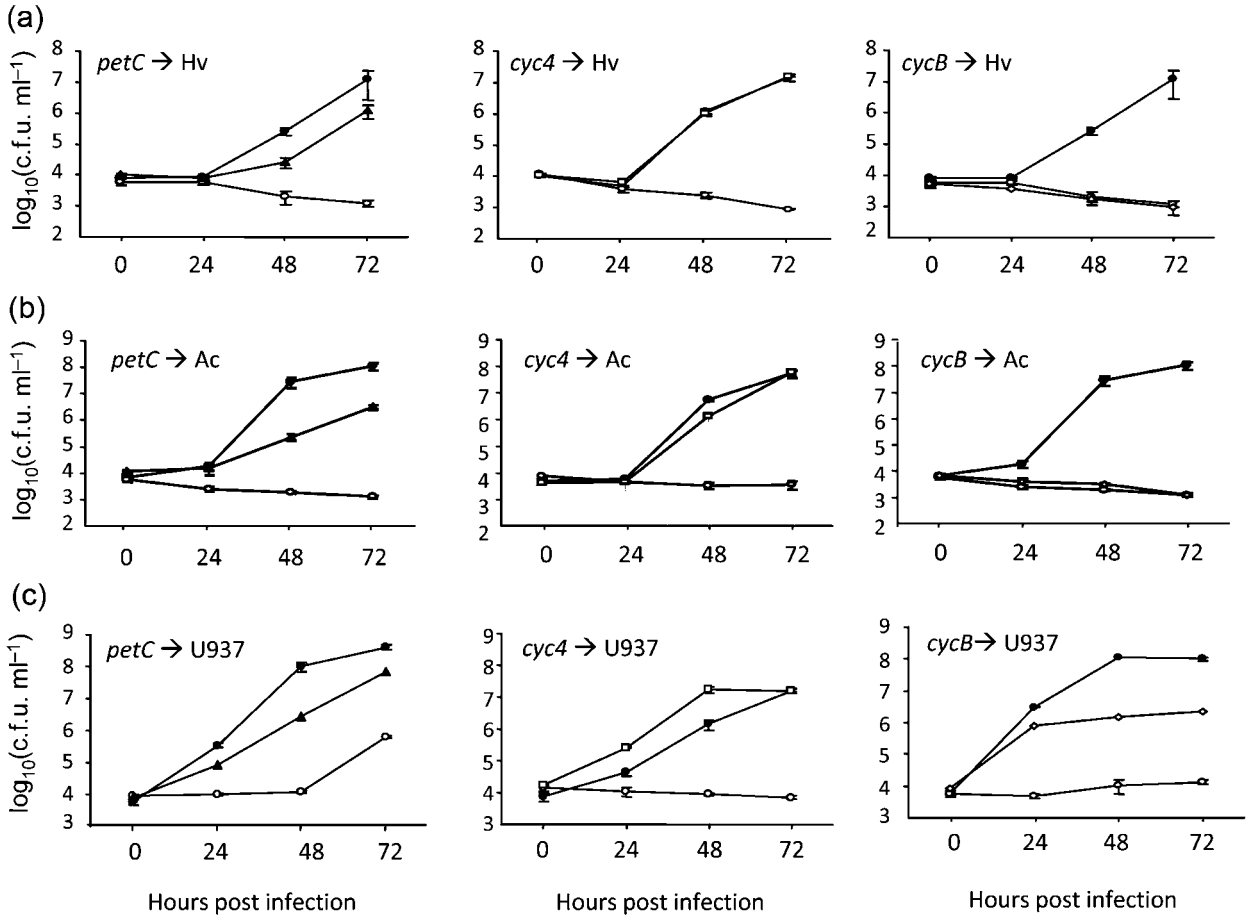

Fig. 6. Intracellular infection by *L. pneumophila* wild-type and c-type cytochrome mutants. *H. vermiformis* (Hv, a), *A. castellanii* (Ac, b), and U937 cell macrophages (c) were infected with wild-type 130b (●) and *ccmC* mutant NU295 (○) (all panels in a–c) as well as the *petC* mutant NU375 (▲) (left panels), *cyc4* mutant NU379 (□) (centre panels), and *cycB* mutant NU381 (◇) (right panels), and then at the indicated times, the c.f.u. in the infected wells were determined by plating. Data are the mean and standard deviations from four infected wells (error bars not shown where smaller than symbols). In (a) and (b), the recovery of the *petC* and *cycB* mutants was significantly less than that of wild-type at 48 and 72 h (Student's *t*-test, $P<0.05$). In (c), the recovery of the *petC* and *cycB* mutants was less than that of wild-type at 24, 48, and 72 h (Student's *t*-test, $P<0.05$). Each of the cytochrome mutants was tested on at least two occasions, with the results obtained being comparable to those depicted here. Although not shown here for the sake of space, additional experiments using NU376 and NU382 confirmed the infectivity defects of the *petC* mutant and *cycB* mutant.

contrast, under conditions of moderate iron limitation (i.e. non-iron-supplemented BCYE agar without iron chelator), *ccm* mutants exhibited a growth defect that was not recapitulated by the cytochrome mutants nor ascribed to lack of siderophore. Three basic scenarios can be envisioned to explain these data. In the first case, there is a functional redundancy among cytochromes c_1, c_4 and c_5, such that impaired growth under these conditions requires the absence of more than one of the cytochromes. In the second scenario, there is an additional *c*-type cytochrome(s) expressed by *L. pneumophila*. In support of this possibility, when examining the database, we did find several ORFs that might encode cytochrome *c*-like proteins (unpublished results). As to how *c*-type cytochromes (be they c_1, c_4, c_5 or encoded by an uncharacterized ORF) might facilitate growth in moderately low-iron conditions, it is possible that they promote Fe^{3+} reduction as has been documented for some of the *c*-type cytochromes produced by species of *Geobacter* and *Shewanella* (Dale *et al.*, 2007; Londer *et al.*, 2002; Mehta *et al.*, 2005; Shi *et al.*, 2007). That *L. pneumophila* can utilize Fe^{2+} transport to grow on low-iron media (Robey & Cianciotto, 2002) gives support to this possibility. In the final case, the importance of Ccm under extracellular conditions of moderate iron-depletion is independent of its role in cytochrome maturation and may involve an alternate use of Ccm-exported haem.

As to the role of cytochromes in intracellular growth, cytochromes c_1 and c_5 vs cytochrome c_4 proved to be the most important. Remarkably, the *cycB* mutants, like the

ccm mutants, were completely unable to grow in amoebae, indicating a critical role for this cytochrome c_5. We believe that the current study is the first to discern the relative importance of different *c*-type cytochromes during an intracellular infection event. Since our experiments testing the *cycB* and *petC* mutants utilized host cells that were not iron-stressed, and since mutants lacking Fe^{3+} (legiobactin) or Fe^{2+} (FeoB) uptake do not have this level of impairment, the key function of these *c*-type cytochromes during infection may involve their roles in respiration. On the other hand, there is a growing list of cases where Ccm or a cytochrome is linked to processes that are distinct from respiration and iron acquisition (Cianciotto *et al.*, 2005; El-Naggar *et al.*, 2010; Yurgel *et al.*, 2007). In light of the importance of Ccm in lung infection by *L. pneumophila* (Naylor & Cianciotto, 2004), particularly intriguing is a recent report demonstrating that two *c*-type cytochromes regulate virulence factor (toxin) gene regulation in *Bacillus anthracis* (Wilson *et al.*, 2009). Thus, studies on *L. pneumophila* Ccm and its *c*-type cytochromes should provide new insights into bacterial iron acquisition, intracellular infection and virulence.

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