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.

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$^{3+}$ 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,
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 hydroxide 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 BYE 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$^7$ 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; 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$^{3+}$ permease and thus is defective for uptake of Fe$^{3+}$ but not Fe$^{2+}$ (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$^6$ 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), *cycB* (cytochrome *c*$_3$), *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.ncbi.nlm.nih.gov/ and http://www.ncbi.nlm.nih.gov/genome/pri/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 *Sst*I and ligated to a fragment of pMB2190 that carries a kanamycin-resistance gene (*Km*) (Rossier et al., 2004). This final construct, pGpetC::Km$, was then introduced

http://mic.sgmjournals.org
Table 1. Primers used in this study

| Primer      | Sequence (5’–3’)                        |
|-------------|-----------------------------------------|
| ccmB-F (orf1-5–F) | TATTTATCCAGTGGCGGCAA                     |
| ccmB-R (ccmB5’–R) | GGTATCCACAGTATCGGGGAC                     |
| ccmF-F (orf2-1–F) | GATGCGTGTGATGATATCTTC                     |
| ccmC-R (ccmC5’–R) | GCATCCCCCTGCTGATATCTCG                     |
| ccmD-F (ccmD3’–F) | GTGTGGTTAAGGATAGGATATCTCG                    |
| ccmR-R (ccmC-R) | ACCCTCAACTCGGATGTTGTTT                      |
| ccmF (ccmF12–F) | ATAGAAAGTCCTACGTTA                           |
| ccmF (ccmE-R) | AAGCCGAGATCTGGAGCAATG                         |
| ccmG-F (ccmH-F) | TCCTCATGATTGTCGTATCTC                       |
| ccmG-R (ccmF-R) | TCTCGGCTGCTGATTGCTTJT                        |
| ccmH-F (ccmG3’–F) | TCGCTACAGGATATCGGATA                      |
| ccmH-R (ccmH5’–R) | TCTGCGGATATCGGATTTTGGGAT                    |
| petC-F (c1-F) | GCGCGCTAATCCGATCGT                         |
| petC-R (c1-R) | CTTGGCGATGCTGATTTTGGGAT                    |
| cyc4-F (c4-F) | GGTGTTTCTGCTGTTCTG                         |
| cyc4-R (c4-R) | CGATTGTTGCTGCTGCTG                         |
| cycB-F (c5-F) | GCTCCAGGCTGATTGCTG                         |
| cycB-R (c5-R) | GTTCACTCGGACAGAAGCG                         |
| lbtA-F | CATTGGCGATGCTGCTG                          |
| lbtA-R | GCGCGGAATTAGGATGATA                         |
| dabA-F | GCAAAAGGCTATTACAGACG                       |
| dabA-R | CTCCGCTGCACTATCGT                           |

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 side-

...pher (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 deferred 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. As 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 c₄ is required for the expression of legiobactin

Since the Ccm system is best known for its role in the maturation of c₄-type cytochromes (Sanders et al, 2010), we sought to determine the importance of individual c₄-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; Glückner et al., 2008; Schroeder et al., 2010), we
targeted the three genes encoding c-type cytochromes. The first gene (petC) encodes cytochrome c1, 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 c4, 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; Deudom et al., 2008; Giudici-Orticoni et al., 2000). The third gene (cycB) gene encodes a 15 kDa, mono-haem cytochrome c5 (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
Expression of the gene expressed in 130b growing in CDM–Fe (data not shown). (b) The type was analysed by RT-PCR utilizing primers specific to cyc4 or lanes. In additional experiments, product obtained when the reaction did not incorporate RT (–RT) resulted from mRNA templates was confirmed by the lack of signal. Then RNA was analysed by RT-PCR utilizing primers specific to deferrated CDM (CDM–Fe) or standard BYE broth (BYE), and (a) Expression of petC grown in either medium, whereas cycB2b). The gene expressed when bacteria were grown in BYE broth (Fig. 2b). 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 L. pneumophila 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 c5. 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 ccmC 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. 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 addition, 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 c4 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 c4 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 c4, but not cytochromes c1 and c5, 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 c4 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).
conclude that these mutant phenotypes resulted from the loss of cytochrome c2 as opposed to being only due to a possible polar effect on dsbB and the loss of DsbB activities that are unrelated to siderophores. In sum, these data indicate that cytochromes c1 and c2 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* (Bayssé 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 (Cianciootto 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* (Bayssé 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 pseudooverdine, 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 c2. 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 c5 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 c5, but not cytochromes c1 and c2, 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
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 α-, β- 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

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**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. 10⁵ 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.

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**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.
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 \textit{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 \textit{Geobacter} and \textit{Shewanella} (Dale et al., 2007; Londer et al., 2002; Mehta et al., 2005; Shi et al., 2007). That \textit{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 c₅. 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 cyb and petC mutants utilized host cells that were not iron-stressed, and since mutants lacking Fe³⁺ (legiobactin) or Fe²⁺ (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.

ACKNOWLEDGEMENTS

We thank present and past members of the Cianciotto lab for their help and support, including Christa Chatfield and Brendan Mulhern for assisting with siderophore bioassays. E.S.Y. and D.M.B. were partly supported by NIH training grant T32 AI007476. This study was funded by NIH grant AI034937 awarded to N.P.C.

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Edited by: H. Hilbi