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The Cytochrome *c* Maturation Locus of *Legionella pneumophila* Promotes Iron Assimilation and Intracellular Infection and Contains a Strain-Specific Insertion Sequence Element

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Previously, we obtained a *Legionella pneumophila* mutant, NU208, that is hypersensitive to iron chelators when grown on standard *Legionella* media. Here, we demonstrate that NU208 is also impaired for growth in media that simply lack their iron supplement. The mutant was not, however, impaired for the production of legiobactin, the only known *L. pneumophila* siderophore. Importantly, NU208 was also highly defective for intracellular growth in human U937 cell macrophages and *Hartmannella* and *Acanthamoeba* amoebae. The growth defect within macrophages was exacerbated by treatment of the host cells with an iron chelator. Sequence analysis demonstrated that the transposon disruption in NU208 lies within an open reading frame that is highly similar to the cytochrome *c* maturation gene, *ccmC*. CcmC is generally recognized for its role in the heme export step of cytochrome biogenesis. Indeed, NU208 lacked cytochrome *c*. Phenotypic analysis of two additional, independently derived *ccmC* mutants confirmed that the growth defect in low-iron medium and impaired infectivity were associated with the transposon insertion and not an entirely spontaneous second-site mutation. *trans*-complementation analysis of NU208 confirmed that *L. pneumophila ccmC* is required for cytochrome *c* production, growth under low-iron growth conditions, and at least some forms of intracellular infection. Although *ccm* genes have recently been implicated in iron assimilation, our data indicate, for the first time, that a *ccm* gene can be required for bacterial growth in an intracellular niche. Complete sequence analysis of the *ccm* locus from strain 130b identified the genes *ccmA-H*. Interestingly, however, we also observed that a 1.8-kb insertion sequence element was positioned between *ccmB* and *ccmC*. Southern hybridizations indicated that the open reading frame within this element (ISLp *1*) was present in multiple copies in some strains of *L. pneumophila* but was absent from others. These findings represent the first evidence for a transposable element in *Legionella* and the first identification of an *L. pneumophila* strain-specific gene.

Legionella pneumophila is a ubiquitous inhabitant of natural and artificial aquatic environments, surviving in biofilms and as an intracellular parasite of protozoa (4, 29, 38, 50). Yet this aerobic, gram-negative bacterium is best known as the etiologic agent of Legionnaires' disease, a potentially fatal form of pneumonia (11, 85). Within the human respiratory tract, *L. pneumophila* flourishes as an intracellular parasite of alveolar macrophages (1, 23, 74, 78, 80, 84). Numerous studies indicate that iron is critical for *L. pneumophila* extracellular replication, intracellular infection, and virulence (14, 16, 35, 36, 40, 45, 69, 71, 72, 82). Indeed, the ability of gamma interferon to inhibit *L. pneumophila* growth in host cells involves reductions in intracellular iron (15).

In recent years, progress has been made toward understanding the mechanisms by which *L. pneumophila* acquires iron, including intracellular iron. For example, it has been shown that the organism possesses two internal ferric reductases but is unable to bind and utilize transferrin or lactoferrin (10, 44, 46, 67). We have demonstrated that *L. pneumophila* is capable of both binding and utilizing hemin and elaborating a nonhydroxamate, nonphenolate siderophore (legiobactin) whose expression is subject to a form of growth phase regulation (52, 63). We also identified homologues of hydroxamate siderophore biosynthetic genes and showed that at least one of these is a Fur-regulated gene which promotes infection (40, 82). Finally, using mini-Tn*10* mutagenesis, we isolated *L. pneumophila* (*ira*) mutants that are hypersensitive to the iron chelator ethylene diamine diacetate and, in some cases, resistant to streptonigrin, an antibiotic whose bactericidal activity is enhanced by high intracellular iron concentrations (69). A detailed characterization of the *ira* mutant most impaired for macrophage infection indicated, for the first time, that a bacterial peptide transporter could facilitate growth under low-iron conditions (83). Thus, the investigation of *L. pneumophila* is yielding new insights into bacterial iron acquisition and pathogenesis.

In the present study, we further characterized a second *ira* mutant (i.e., NU208), which previously had not been adequately examined for a potential infectivity defect (69). Based upon a detailed assessment of intracellular CFU, strain NU208 proved to be significantly impaired for growth within macrophage monolayers and amoeba cocultures. Genotypic analysis of NU208 as well as a set of independently derived infectivity

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TABLE 1. *L. pneumophila* strains examined

Strain	Serogroup	Source[a]	ATCC no.[b]	Reference
130b[c]	1	C	BAA-74	27
Philadelphia-1	1	C	33217	11
Togus-1	2	C	33154	11
Bloomington-2	3	E	33155	11
Los Angeles-1	4	C	33156	58
Dallas-1E	5	E	33216	26
Chicago-2	6	C	33215	59
Chicago-8	7	E	33823	7
Concord-3	8	C	35096	9
1425-CA-H	13	C	43736	55
1169-MN-H	14	C	43703	5

[a] C and E, clinical and environmental isolates, respectively.
[b] ATCC, American Type Culture Collection.
[c] Strain 130b is also known as AA100 and the Wadsworth strain.

mutants indicated that a bacterial *ccmC* locus could promote both iron acquisition and intracellular infection. In addition, this study has uncovered an insertion sequence (IS) element which is present within the *ccm* locus of strain 130b but absent from a number of other strains of *L. pneumophila*, including the well-studied Philadelphia-1 strain.

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MATERIALS AND METHODS

Bacterial strains. The *L. pneumophila* serogroup 1 strain 130b, which served as a wild-type control, was previously described (27). The sources of additional wild-type *L. pneumophila* that were tested for the presence of the *ccm* locus are listed in Table 1. NU208, the first *ccmC* mutant characterized, was originally isolated following mini-Tn*10* mutagenesis of strain 130b (69). GE193, another 130b derivative that also proved to have a *ccmC* mutation, was obtained in a previous mini-Tn*10* mutagenesis study (33). The NU257 mutant was constructed by allelic exchange in the following manner. Based on sequences obtained from an inverse PCR product (below), primers ccm6 (5′-GAACCGAACTGCCATG ATT-3′) and ccm7 (5′-GCCCAAAACCACGCAAACCA-3′) were used to amplify a fragment containing the entire *ccmC* gene from the 130b chromosome. This fragment was then cloned into pGEM-T (Promega, Madison, Wis.) to obtain pSM1. A *Sph*I-*Sac*I fragment that contained *ccmC* was then taken from pSM1 and cloned into *Sph*I-*Sac*I-digested pBOC20 (62), a *sacB*-counterselectable vector, to obtain pSM2. Next, a *Pst*I fragment containing the kanamycin resistance (Km^r) gene cassette from pVK3 (83) was ligated into the *Nsi*I site in the *ccmC* gene of pSM2 to obtain pSM3. Finally, pSM3 was electroporated into competent 130b, and then chloramphenicol-resistant, kanamycin-resistant electrotransformants were streaked onto plates containing kanamycin and sucrose (21, 62). PCR analysis (83) using *ccm* and kanamycin resistance gene primers identified Km^r, Cm^s, sucrose-resistant colonies that contained the disruption in *ccmC*. A remake of GE193 was also constructed by allelic exchange. More specifically, the *Eco*RI fragment containing the kanamycin resistance marker was cloned into pBC, and then the recombinant plasmid was introduced into strain 130b by natural transformation (79). *Escherichia coli* NovaBlue (Novagen, Madison, Wis.) was routinely used for propagating newly isolated plasmids.

Legionella **media and extracellular growth experiments.** *L. pneumophila* strains were routinely cultured on buffered charcoal yeast extract (BCYE) agar for 3 days at 37°C, with chloramphenicol (3 μg/ml), kanamycin (25 μg/ml), and sucrose (5%, wt/vol) added when appropriate (18, 25). To monitor the general growth capacity of the various strains, bacteria were inoculated into buffered yeast extract (BYE) broth, and replication was assessed by measuring the optical density of the cultures at 600 nm over the following 24 h (3, 25, 53). To assess bacterial growth under low-iron conditions, the legionellae were plated on BCYE agar that lacked its usual ferric pyrophosphate supplement. Finally, in order to examine legionellae for siderophore production, bacteria were grown in an iron-deplete chemically defined medium (CDM), and then culture supernatants were tested for reactivity in a chrome azurol S (CAS) assay (52). Briefly, CDM ordinarily consists of the 20 amino acids, nine trace metals in addition to iron, pyruvate, glutathione, α-ketoglutarate, morpholinepropanesulfonic acid buffer, KH_2PO_4, and NaCl (72)

Intracellular infection of U937 cells and freshwater amoebae by *L. pneumophila*. U937, a human cell line that differentiates into macrophage-like cells after treatment with phorbol esters, served as a host for in vitro infection by *L. pneumophila* (20). The cell line was maintained and infected as previously described (20, 33, 53). To quantitate intracellular growth, monolayers containing 10^5 macrophages were inoculated with approximately 10^5 CFU, incubated for 0 to 72 h, and then lysed. Serial dilutions of the lysates were plated on BCYE agar to determine the corresponding numbers of bacteria per monolayer. To assess the effect of intracellular iron depletion on bacterial growth, U937 cells were treated with desferrioxamine (DFX) (Sigma Chemical Co., St. Louis, Mo.) as described previously (83). DFX enters cultured macrophages, where it chelates iron present in the cytosolic, labile iron pool, and this chelation is believed to result in iron limitation for intracellular legionellae (15). To ascertain the cytopathic effect of *L. pneumophila* on U937 cells, infected monolayers were treated with Alamar blue (Biosource International, Vacaville, Calif.) as previously described (3, 33). Finally, to examine the ability of legionellae to grow within a protozoan host, *Hartmannella vermiformis* and *Acanthamoeba polyphaga* were infected and characterized as before (21, 33, 53). Thus, about 10^3 or 10^5 CFU were added to wells containing 10^5 amoebae, and at various times postinoculation, the numbers of bacteria within the cocultures were determined by plating.

Heme staining of *L. pneumophila* lysates. *L. pneumophila* strains were grown overnight in 5 ml of BYE broth. After centrifugation of the cultures, the resultant cell pellets were washed once in phosphate-buffered saline and then resuspended in lysis buffer containing 0.1% Triton X-100 and 0.2 mg of lysozyme per ml (3). After a 10-min incubation on ice, the cell suspension was sonicated four times with 30-s pulses. Aliquots of the cell lysates containing equivalent amounts of protein were boiled in the presence of dithiothreitol and then electrophoresed through a denaturing sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (30). The separated proteins were then stained for heme, as described previously (30). Under the denaturing conditions described above, cytochrome *c* is the only bacterial cytochrome detected by this method, since it alone retains its covalently bound heme. To reveal the other proteins present in the cell lysates, the gel was subsequently stained with Coomassie blue.

Sequence analysis of the *L. pneumophila ccm* locus. Initial nucleotide sequence analysis of the *ccm* locus was carried out with an inverse-PCR product obtained from NU208. Inverse PCR, which utilizes mini-Tn*10*-specific primers, was previously described (54). The entire *ccmC* gene was subsequently sequenced on both strands by primer walking on PCR templates that had been directly amplified from 130b chromosomal DNA (83). To facilitate further sequencing, we sought an additional *ccm*-containing plasmid from a genomic library of 130b DNA (39). To do this, a labeled *ccmC* fragment served as a probe in colony blots that were performed using the Genius system kit (Boehringer Mannheim, Mannheim, Germany). The isolated plasmid, pVK117, contained approximately 3 kb of *Legionella* DNA, which included *ccmCDE* and the ISLp *1* element (see below). Finally, plasmid pE193, which was obtained by cloning the mini-Tn*10* Km^r marker on an *Eco*RI fragment from mutant GE193, was used to obtain sequence data for the remainder of the *ccm* operon and its flanking DNA. The sequencing of the various cloned DNAs was facilitated by primer walking. Sequence analysis was performed using either a DyeTerminator cycle sequencing reaction mix or a BigDye terminator cycle sequencing reaction mix from PE Applied Biosystems (Foster City, Calif.). Primers for sequencing, as well as PCR, were obtained from the Biotech Facility at Northwestern University Medical School, Chicago, Ill. Automated sequence analysis was performed at the Biotech Facility on an ABI Prism 373 DNA sequencer (Applied Biosystems). Sequence database searches were performed with programs based on the BLAST algorithm.

Complementation analysis of *L. pneumophila* mutants. For complementation studies, two plasmids which contain *ccmC* as their only intact *Legionella* gene were constructed. The first, pSK35, was derived by cloning the 3.1-kb, *ccmC*-containing *Eco*RI/*Sal*I fragment from pVK117 (see above) into Cm^r pMMB207 (61). The second, pTA1, was obtained by transferring the 1.1-kb, *ccmC*-containing *Sph*I/*Sac*I fragment from pSM1 (see above) into Cm^r pSU2719 (19). Both pMMB207 and pSU2719 replicons have been successfully used as vectors for *trans*-complementation of *L. pneumophila* mutants, including those with infectivity defects (57, 83). Complementing plasmids, as well as vector controls, were introduced into wild-type and mutant legionellae by electroporation and maintained on chloramphenicol-containing BCYE agar (21, 62).

Southern hybridization analyses. To determine the distribution of *ccm* and ISLp1 genes in *L. pneumophila*, Southern hybridization analysis was performed with fluorescent-labeled probes and the Genius system kit (83). The fragments to be labeled were generated by PCR with primers ccm7 (see above) and ccm8 (5′-GATGCGTGGTAGAATCCTTC-3′) for the ccmCD probe and ccm4 (5′-GTGATATGCGTCATGGTCG-3′) and ccm11 (5′-AGACCCTAGAGCGCCGAATG-3′) for the ISLp1 probe. Chromosomal DNA for the blots as well as for PCR was isolated from *Legionella* strains as described earlier (69).

RT-PCR analysis of *L. pneumophila* gene transcription. To determine the expression pattern of ccm genes, RT-PCR was performed (54). *Legionella* RNA was isolated with the Trizol reagent (Gibco-BRL, Gaithersburg, Md.). Primers ccm8 (above) and ccm10 (5′-GTCGCACCTTGGTGTAAAGT-3′) were used to amplify sequences specific to *ccmC*, whereas primers ccm17 (5′-CTCTCTATGTTTGGCCTGC-3′) and ccm20 (5′-TTGCTTCCACCATACCTCCA-3′) were used to identify *ccmE* transcripts, and ccm3 (5′-GTGGTTGATTGTGCTGCTC-3′) and ccm18 (5′-AACTCTGTGGTGCTTGTCC-3′) were employed for detecting expression of the ISLp1 open reading frame (ORF). Control experiments in which reverse transcriptase was omitted were performed in order to eliminate from consideration the contribution of any DNA that might have been present in the DNase-treated RNA preparations.

Nucleotide sequence accession number. The National Center for Biotechnology Information (NCBI)/GenBank accession number for the *L. pneumophila ccm* locus is AF386079.

RESULTS

Extracellular growth and macrophage infection by NU208, an *L. pneumophila ccmC* mutant. This study was instigated by the fact that strain NU208 has a reduced ability to grow in the presence of the iron chelator ethylene diamine diacetate (69). To further test whether this mutant has alterations in iron acquisition and/or assimilation, we assessed its relative ability to grow on an agar medium deficient in iron. Toward that end, bacteria were grown for 3 days on standard BCYE agar, an iron-replete medium which is supplemented with 340 μM ferric pyrophosphate, and then washed and spread onto BCYE plates with or without added iron. For the parental strain 130b, a comparable number of colonies were consistently observed on the BCYE+Fe and BCYE−Fe plates (Table 2), indicating that wild-type strains of *L. pneumophila* can assimilate and store enough iron to permit subsequent growth on low-iron media. In contrast, NU208 was dramatically impaired for growth on subsequent passage to plates lacking the iron supplement; i.e., it produced at least 1,000-fold fewer colonies on plates lacking added iron compared to fully iron-replete plates (Table 2). This reduction in plating efficiency was not due to the loss of pyrophosphate in the medium, since NU208 grew as did the wild type when tested on media in which ferric citrate was exchanged for the ferric pyrophosphate supplement (data not shown). In a recent study, we discovered that *L. pneumophila* strain 130b, as well as a number of other legionellae, produces a siderophore (legiobactin) which is readily detected with the CAS assay (52). To determine whether a reduced ability to grow under low-iron conditions was due to altered legiobactin production, NU208 was tested for the production of a CAS-reactive substance when it was grown in low-iron CDM. On three occasions, the mutant produced a level of legiobactin that was comparable to that of strain 130b (data not shown). The reduced ability of NU208 to grow under low-iron conditions indicates a defect in a stage(s) of iron acquisition or assimilation.

To determine whether mutant NU208 is altered in its ability to infect human macrophages, U937 cells were infected with comparable numbers of wild-type and mutant bacteria and

TABLE 2. Plating efficiencies of *L. pneumophila* strains on low-iron BCYE agar[a]

Strain	Expt I			Expt II			Expt III		
	No. of CFU on:		EOP (%)	No. of CFU on:		EOP (%)	No. of CFU on:		EOP (%)
	BCYE−Fe	BCYE+Fe		BCYE−Fe	BCYE+Fe		BCYE−Fe	BCYE+Fe	
130b	$(6.1 \pm 0.3) \times 10^8$	$(5.9 \pm 0.4) \times 10^8$	103	$(3.7 \pm 0.03) \times 10^8$	$(3.2 \pm 0.05) \times 10^8$	116	$(2.9 \pm 0.08) \times 10^8$	$(3.1 \pm 0.4) \times 10^8$	94
NU208	$(3.5 \pm 0.7) \times 10^3$	$(6.1 \pm 0.6) \times 10^7$	0.006	$(3.5 \pm 0.7) \times 10^4$	$(8.5 \pm 3.0) \times 10^7$	0.04	$(8.5 \pm 0.9) \times 10^4$	$(7.5 \pm 1.3) \times 10^7$	0.10
NU257	$(7.0 \pm 0) \times 10^3$	$(1.8 \pm 0.3) \times 10^8$	0.004	ND	ND		ND	ND	
GE193	$(7.2 \pm 0.9) \times 10^6$	$(2.5 \pm 0.06) \times 10^8$	2.9	ND	ND		ND	ND	
NU208(pTA1)	ND	ND		$(5.5 \pm 2.1) \times 10^5$	$(4.9 \pm 0.07) \times 10^5$	112	$(2.2 \pm 0.6) \times 10^7$	$(2.1 \pm 0.9) \times 10^7$	105

[a] After growing for 3 days on BCYE agar, bacteria were suspended in water to a comparable optical density at 660 nm and then replated in duplicate on BCYE agar and BCYE agar lacking its iron supplement. The number of CFU on the two types of agar were compared after 3 days. The efficiencies of plating (EOP) of NU208 and NU208(pTA1), which contains *L. pneumophila ccmC*, were seen in three and two additional experiments, respectively. ND, not done.

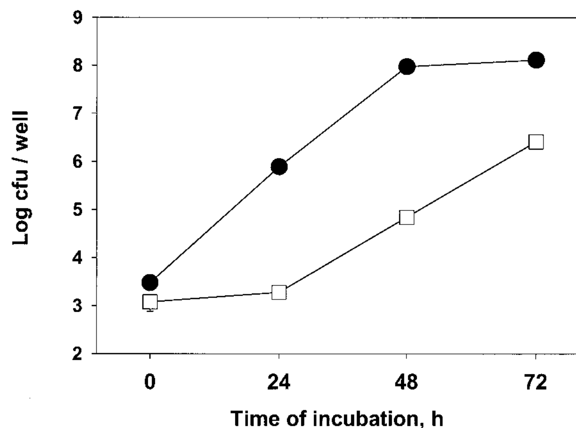

FIG. 1. Macrophage infection by *L. pneumophila* wild type and NU208. U937 cell monolayers were infected with 2.5×10^5 CFU of strain 130b (●) and 6.3×10^4 CFU of NU208 (□). CFU per well were quantitated at 0, 24, 48, and 72 h. Each data point is the mean for three monolayers (error bar, standard deviation). Significant differences in recovery between 130b and its mutant derivative were evident at 24 h and beyond ($P < 0.02$, Student's t test). These differences were seen in three additional experiments and were evident whether the inocula were obtained from standard BCYE agar or from BCYE agar lacking its iron supplement (data not shown).

TABLE 3. Growth of *L. pneumophila* strains within DFX-treated U937 cells[a]

[DFX] (μM)	10^5 CFU/monolayer (% reduction)	
	130b	NU208
0	710 ± 100	37 ± 3.5
5	2,300 ± 350 (<0)	25 ± 6.6 (32.4)
10	680 ± 170 (4.2)	0.210 ± 0.14 (99.4)
15	590 ± 140 (16.9)	0.084 ± 0.006 (99.9)

[a] U937 cell monolayers were incubated for 24 h in the presence of various concentrations of DFX. These cells were then infected with equivalent numbers of wild-type and mutant bacteria. As before (69, 83), DFX treatment did not reduce the uptake of legionellae (data not shown). The bacterial titers were determined at 72 h postinoculation from three replicate monolayers, except for the mutant 10 μM DFX result, which was derived from two wells. Reduction is reported relative to CFU in untreated wells. All reductions in NU208 CFU that were associated with DFX treatment were significant ($P < 0.01$, Student's t test). In a second experiment, 5 and 10 μM DFX reduced mutant CFU by 52 and 91%, respectively.

then, at various times, the bacteria within the monolayers were quantitated. In four separate experiments, NU208 exhibited a significant reduction in macrophage infectivity (Fig. 1). Following an apparently normal uptake period, as evidenced by no alteration in CFU at the earliest time point, the numbers of mutant bacteria did not increase for at least 24 h. Ultimately, NU208 replicated, but at an apparently lower rate, such that its monolayers yielded 1,000-fold fewer CFU at 48 h than did those of strain 130b (Fig. 1). In contrast to its intracellular growth defect, NU208 grew as well as the wild type did in standard BYE broth (data not shown). To explore whether the infectivity defect exhibited by NU208 was associated with reduced intracellular iron acquisition and/or assimilation, infections were performed as described above but with U937 cells continuously maintained in the presence of the iron chelator DFX (16, 35, 69, 83). DFX chelates the cytosolic, labile iron pool of macrophages, resulting in iron limitation for intracellular bacteria (15). In two trials, 5 and 10 μM DFX reduced the recovery of NU208 by an average of 42 and 95%, respectively, while having no negative effect on the recovery of wild-type 130b (Table 3). In addition, treatment of the U937 cells with 15 μM DFX diminished mutant recoverability by 99.9% (Table 3). Taken together, these data indicate that strain NU208 is defective for both macrophage infection and extra- and intracellular iron acquisition and/or assimilation.

To begin characterizing the genetic lesion in NU208, we performed inverse PCR analyses on the strain. Sequence analysis of the PCR product revealed that the transposon was inserted into a gene showing homology to the cytochrome *c* maturation gene, *ccmC* (Fig. 2). The CcmC protein is best known for being part of a multiprotein system which is encoded by an eight-gene operon in *E. coli* and which incorporates a heme moiety into apocytochrome *c* (51, 75, 81). Further sequence analysis indicated that additional *ccm*-like genes were located downstream of *L. pneumophila ccmC*, i.e., *ccmD* followed by *ccmEF* (Fig. 2). Early studies on the electron transport system of *L. pneumophila* revealed the presence of a *c*-type cytochrome (i.e., cytochrome c_{552}), as well as *a*-, *b*-, and *d*-type cytochromes and an *o*-type terminal oxidase (41). To validate the sequence data, we sought to determine whether the mutation in NU208 resulted in the loss of a *c*-type cytochrome. Since cytochrome *c*, unlike the other cytochromes, contains a covalently bound heme moiety (81), we compared wild-type and mutant cell lysates for proteins that retain heme staining upon denaturing SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Whereas a single protein was detected in the wild type (Fig. 3, lane 1), no heme-containing proteins were seen in the lysate of NU208 (lane 2). RT-PCR analysis demonstrated that the insertion mutation in NU208 eliminated complete *ccmC* transcription but did not abolish transcription of downstream genes; i.e., whereas primers ccm8 and ccm10 did not yield the 600-bp product associated with an intact *ccmC*, primers ccm 17 and ccm20 readily amplified a 400-bp *ccmE*-specific product (data not shown).

Independent isolation and phenotypic analysis of additional *ccmC* mutants. In order to establish whether the mini-Tn*10* insertion in *ccmC* or an entirely spontaneous second site mutation(s) was responsible for the defects seen with NU208, we examined the behavior of two more, independently derived *ccmC* mutants. The first, NU257, was constructed by allelic exchange and contained a kanamycin resistance cassette inserted 101 bp from the point into which the minitransposon had been inserted in NU208 (Fig. 2). The second, GE193, was isolated several years ago during a screen for 130b (*pmi*) mutants that had a reduced cytopathic effect in U937 cells and amoebae (33). Current sequence analysis revealed that GE193 contained a mini-Tn*10* insertion in *ccmC*, just upstream of the mutation in NU208 (Fig. 2). Like NU208, the new *ccmC* mutants were defective for cytochrome *c* production and growth on low-iron BCYE agar (Fig. 3 and Table 2).

To determine whether NU257 and GE193 had growth kinetics in macrophages that were comparable to that of NU208, we repeated the U937 cell infection assay. When these experiments began, which was about 6 months after the initial U937 cell studies were performed, we observed that the intracellular

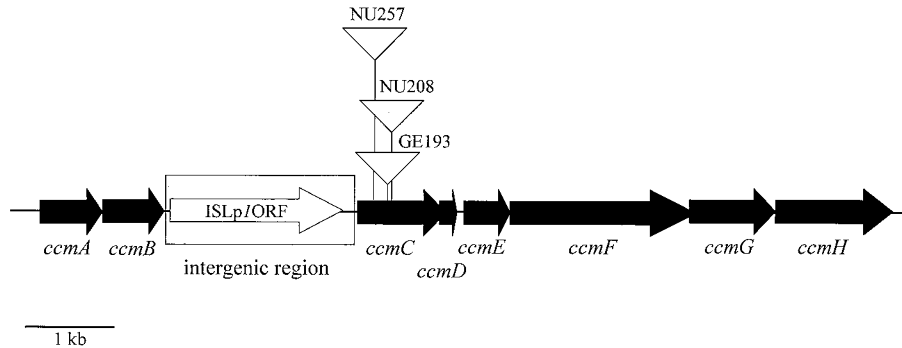

FIG. 2. *ccm* locus of *L. pneumophila* serogroup 1 strain 130b. The horizontal black arrows indicate the relative sizes and predicted transcriptional orientation of the eight *ccm* genes. The vertical white arrowheads show the point of mini-Tn*10* insertion in NU208 and GE193, as well as the position of the Km^r gene cassette in NU257. The horizontal white box denotes the ca. 1.8-kb stretch of non-*ccm* sequences present between *ccmB* and *ccmC* that is hypothesized to contain ISLp*1*. The white arrow within that box depicts the position of an ORF in the IS element. The figure is a representation of the 9,281 bp that were sequenced during this study.

infectivity defect of NU208 had become more pronounced. On four occasions, U937 cell monolayers inoculated with NU208 failed to exhibit any increases in bacterial CFU (Fig. 4). Most importantly, NU257 and GE193 behaved as NU208 did (Fig. 4), indicating that *ccmC* mutations are associated with reductions in macrophage infectivity. Supporting this notion, the *ccmC* mutants failed to elicit a cytopathic effect on U937 cells, even after extended incubation (Fig. 5). On the other hand, the *ccm* mutants were not deficient in the pore-mediated lysis (contact cytotoxicity) that occurs following inoculation with high multiplicities of infection (data not shown) (2, 43, 48). Taken together, these experiments indicate that insertion mutations in *ccmC* are associated with reduction in cytochrome *c* production, growth in low-iron media, and intracellular infection.

Infection of freshwater protozoa by *L. pneumophila ccmC* mutants. In nature, protozoa are a reservoir for *L. pneumophila* and are believed to be the natural hosts for this organism (4, 29, 38). Indeed, it has been argued that human macrophages are accidental hosts for this organism. While several loci are required for establishment of infection in both of these distinct hosts, some genes appear to be host specific (12, 21, 28, 33, 34, 68, 73, 77). To begin to determine if the *ccmC* locus is required for establishing an infection in protozoa, we assessed the relative ability of our mutants to grow in coculture with two types of amoebae. The three *ccmC* mutants exhibited reduced proliferation when cultured in the presence of either *H. vermiformis* or *A. polyphaga* (Fig. 6 and data not shown), suggesting that the *ccmC* locus of *L. pneumophila* is required for intracellular infection of both human macrophages and aquatic amoebae.

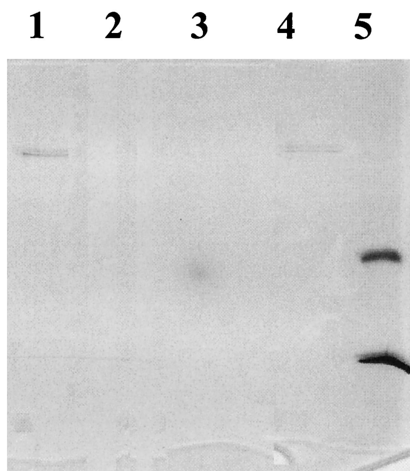

FIG. 3. Cytochrome *c* production by strains of *L. pneumophila*. Cell lysates from strain 130b (lane 1), NU208 (lane 2), NU257 (lane 3), and NU208(pTA1, containing *L. pneumophila ccmC*) (lane 4) as well as beef heart cytochrome *c* (Sigma Chemical Co.) (lane 5) were subjected to SDS–12% polyacrylamide gel electrophoresis and then stained for heme (lanes 1 to 5) and total proteins (data not shown).

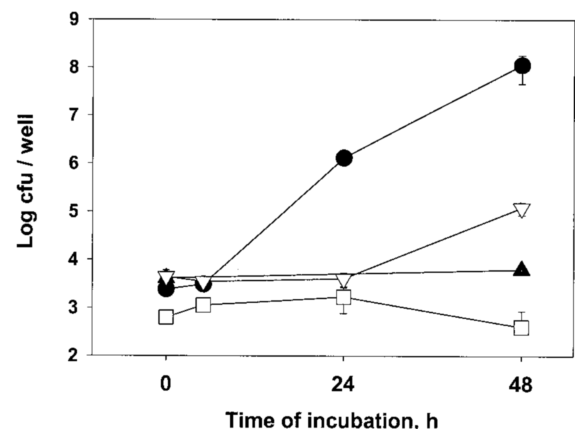

FIG. 4. Replication of *L. pneumophila* NU257 and GE193 within U937 cells. Monolayers were infected with 4.1×10^5 CFU of strain 130b (●), 2.8×10^4 CFU of NU208 (□), 1.7×10^5 CFU of NU257 (▲), and 4.3×10^5 CFU of GE193 (▽). CFU per well were quantitated at 0, 6, 24, and 48 h. Each data point represents the mean for three monolayers (error bars, standard deviation). Significant differences in recovery between 130b and its mutant derivatives were evident by 48 h ($P < 0.04$, Student's t test). These differences, which also reflected no increases in mutant CFU at 72 h postinoculation, were seen in three additional experiments (data not shown). The elevated GE193 counts at 48 h shown here were not observed in the other trials.

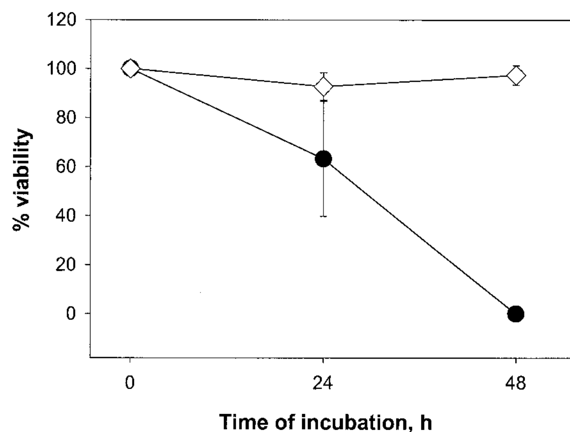

FIG. 5. Cytopathic effect of *L. pneumophila* strains on U937 cells. Replicate monolayers ($n = 6$) were infected with strains 130b (●) and GE193 (◇) at a multiplicity of infection equal to 0.5. After various periods of incubation, the viability of the infected monolayers was determined by Alamar blue staining. Viability is a percentage of that obtained for uninfected monolayers (error bars, standard deviations. Differences in cytopathic effect between 130b and its mutant derivative were significant at 48 h after inoculation ($P < 0.001$, Student's t test). Similar results were obtained in three additional experiments (data not shown).

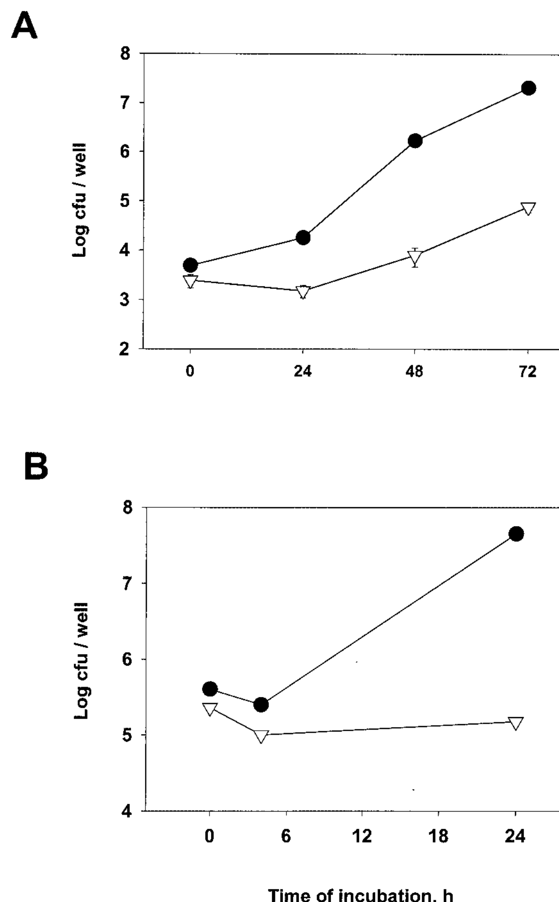

FIG. 6. Infection of freshwater amoebae by *L. pneumophila* strains. (A) A culture of *H. vermiformis* was infected with 5.0×10^3 CFU of strain 130b (●) and 2.1×10^3 CFU of strain GE193 (▽). (B) The same strains, given at a dose of 5.1×10^5 and 2.5×10^5 CFU, respectively, were allowed to infect cultures of *A. polyphaga*. Bacterial CFU per coculture were quantitated at 0, 6, 24, 48, or 72 h after inoculation. Each data point represents the mean for three wells (error bars, standard deviations). Significant differences in recovery between 130b and GE193 were evident after 24 h of incubation ($P < 0.002$, Student's t test). The *Hartmannella* coculture experiment was performed on an additional occasion, whereas the *Acanthamoeba* infections were done three other times. These experiments yielded results that were comparable to the findings presented here (data not shown).

Complementation analysis of *L. pneumophila ccmC* mutants. Phenotypic analysis of three independently derived *ccmC* mutants indicated that alterations in cytochrome *c* production, iron acquisition and/or assimilation, and intracellular infection can be associated with insertion mutations in *L. pneumophila ccmC*. To clearly define the role of *ccmC*, we performed *trans*-complementation, using plasmids that contained *ccmC* as their only intact *Legionella* gene. With primers ccm8 and ccm10, RT-PCR analysis confirmed that these plasmids (i.e., pTA1 and pSK35) expressed *ccmC* transcripts when introduced into *L. pneumophila* (data not shown). We next observed that pTA1 restored cytochrome *c* production to NU208 (Fig. 3), confirming that CcmC is required for cytochrome production in *L. pneumophila*. Since it is highly likely, based upon precedent, that the *ccm* genes downstream of *ccmC* are also required for cytochrome *c* maturation, these data further indicate that the insertion mutation in *ccmC* in NU208 does not abolish the expression of the downstream *ccm* genes. When tested for their ability to grow on low-iron medium, both NU208(pTA1) and NU208(pSK35) behaved like the wild type (Table 2 and data not shown), indicating that *ccmC* is indeed required for extracellular growth under low-iron conditions. Importantly, an intact *ccmC* restored the ability of NU208 to grow within *H. vermiformis* (Fig. 7), demonstrating conclusively that *ccmC* is also essential for intracellular infection of protozoa. However, the cloned *ccmC*, whether carried on the pSU2719-based pTA1 or the pMMB207-based pSK35, failed to enhance the growth of NU208 in U937 cells (data not shown). Thus, the reduced macrophage infectivity of the *ccmC* mutants is not simply due to the loss of CcmC. Regardless, these experiments, taken together, confirm that the *L. pneumophila ccmC* gene is required for optimal cytochrome *c* production, extracellular growth in a low-iron medium, and at least some forms of intracellular infection.

Complete sequence analysis of the *L. pneumophila ccm* locus. Given the newfound importance of *ccmC* to *L. pneumophila* growth in low-iron media and in protozoan hosts, we determined the sequence of the entire *ccm* locus in strain 130b (NCBI accession number AF386079). Double-stranded sequence analysis of nearly 9.3 kb of *Legionella* DNA revealed the presence of the customary eight *ccm* genes, i.e., *ccmA-H* (Fig. 2) (81). There was evidence for a promoter immediately upstream of *ccmA*, and with the exception of the *ccmB-ccmC* intragenic region (see below), there were few nucleotides (i.e., 0 to 5 bases) between the *ccm* genes. Immediately downstream of *ccmH*, there was a predicted transcriptional termination signal and no additional ORFs with predicted functions relevant to cytochrome maturation. Taken together, these data indicate that this *Legionella ccm* locus has many of the char-

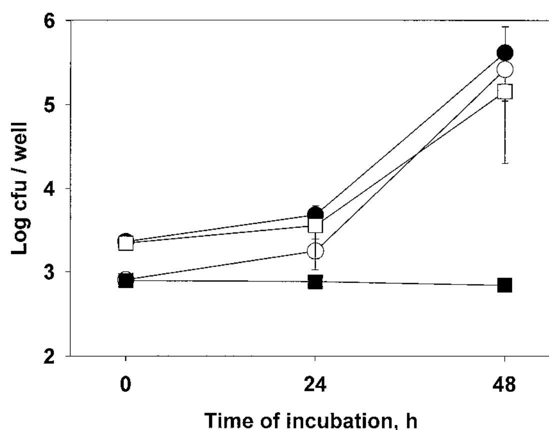

FIG. 7. *trans*-complementation of an *L. pneumophila ccmC* mutant. *H. vermiformis* amoebae were infected with 2.1×10^3 CFU of strain 130b(pSK35) (○), 6.2×10^3 CFU of strain 130b(pMMB207) (●), 5.5×10^3 CFU of strain NU208(pSK35) (□), and 2.5×10^3 CFU of strain NU208(pMMB207) (■). Cm^r bacterial CFU per coculture were quantitated at 0, 24, and 48 h after inoculation. Each data point represents the mean for three wells (error bars, standard deviations). Significant differences in recovery were not evident at 24 or 48 h postinoculation.

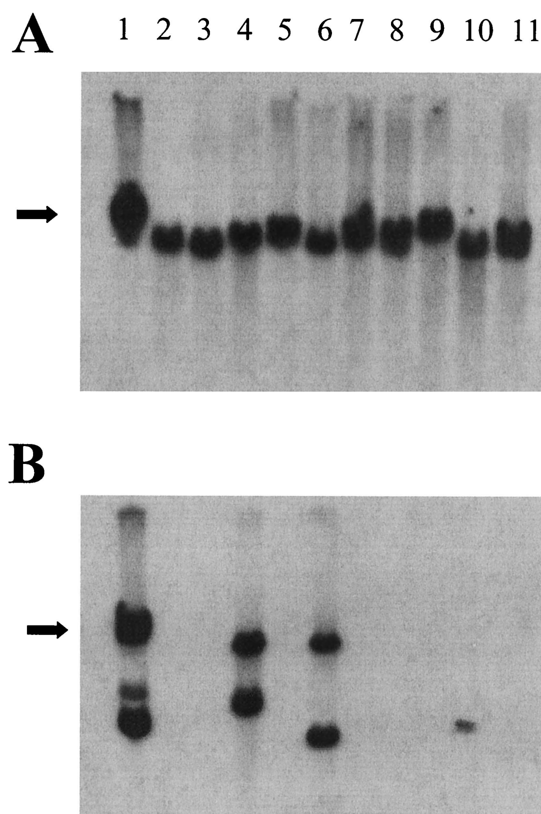

FIG. 8. Distribution of *ccm* genes and ISLp*1* in strains of *L. pneumophila*. Genomic DNA was digested with *Eco*RI, an enzyme not expected to cut within *ccmCD* or ISLp*1*, and then electrophoresed through 0.8% agarose. Southern blots were made and hybridized, under high-stringency conditions, with a *ccmCD*-specific probe (A) and an ISLp*1*-specific probe (B). Lanes: 1, strain 130b; 2, Philadelphia-1; 3, Togus-1; 4, Bloomington-2; 5, Los Angeles-1; 6, Dallas-1E; 7, Chicago-2; 8, Chicago-8; 9, Concord-3; 10, 1425-CA-H; 11, 1169-MN-H. Arrows show the positions of the hybridizing bands that are common to panels A and B. A third Southern hybridization, which utilized a probe containing *ccmB* sequences and a stripped version of the blot in panel B, further confirmed the conservation of *ccm* genes among *L. pneumophila* strains (data not shown).

acteristics of an operon. Incidentally, upstream of the *ccmA* promoter region, there was a gene, transcribed in the opposite direction from *ccm*, that is predicted to have weak homology to periplasmic serine proteases. The average GC content for the eight *ccm* genes was 39.5%, which is in agreement with the 39% GC content ascribed to the overall *L. pneumophila* genome (11). Southern hybridization analysis indicated that *ccm* genes are well conserved among strains of *L. pneumophila* (Fig. 8A). The predicted sizes and homologies of *L. pneumophila* CcmA, -B, -C, -D, -E, -F, -G, and -H are summarized in Table 4. The *Legionella* Ccm proteins and their homologues in other gamma *Proteobacteria* were of identical or near-identical size and generally shared 58 to 69% amino acid (aa) similarity and 38 to 48% aa identity. However, the *Legionella* CcmB and CcmH proteins exhibited reduced relatedness, showing 35 to 45% aa similarity and 23 to 28% aa identity to other CcmB and CcmH molecules. The CcmH-related sequences in *Legionella* also appeared to span two ORFs that overlapped by 7 bp; the first ORF encodes a 133-aa protein, and the second encodes a 228-residue protein.

Identification of a strain-specific IS element of *L. pneumophila*. As indicated above, the *ccmB-ccmC* intergenic region was notable for its size. Indeed, sequence analysis indicated that there were 1,903 bp between the end of *ccmB* and the beginning of *ccmC* (Fig. 2). ORF analysis of this intervening sequence revealed the presence of a single ORF that would be transcribed in the same direction as the *ccm* genes and is predicted to encode a 486-aa protein. The protein contained a 75-aa domain that had 44 to 50% similarity and 29 to 33% identity to a domain shared between transposases from *Xanthomonas campestris* (NCBI number AF051092) and *Neisseria meningitidis* (NCBI number AE002488). It also had significant homology (i.e., 23 to 24% overall identity and 38 to 43% overall similarity) to related hypothetical proteins from *Agrobacterium tumefaciens* (NCBI AB016260) and *Pseudomonas aeruginosa* (NCBI AE004566). The *Agrobacterium* and *Pseudomonas* proteins also have strong homology to transposases. Together, these data suggest that the region between *ccmB* and *ccmC* contains an IS element. In support of this notion, multiple copies of the element (i.e., its ORF) were present in 130b and two other strains of *L. pneumophila* (Fig. 8B). A fourth strain appeared to have a single copy of the putative IS element. The Southern hybridization analysis also indicated that a number of other *L. pneumophila* strains, although containing *ccmBCD*, did not possess the IS-like element (Fig. 8). The distribution of the element did not correlate with serogroup classification or strain source (Table 1). In keeping with recent convention (56), we have tentatively designated the newly discovered IS-like element ISLp*1*, in recognition of its being both distinct from known IS elements and the first IS-like element to be found in a strain of *L. pneumophila*. Since the GC content of the ORF within ISLp*1* is 38.6%, it appears that the IS element is not a relatively recent acquisition.

Given that ISLp*1* was not present in strain Philadelphia-1

TABLE 4. *L. pneumophila* Ccm proteins

Protein	No. of aa	Relatedness to other Ccm proteins[a]			
		% Similarity	% Identity	Organism	NCBI accession no. (reference)
CcmA	200	61	38	*Pantoea citrea*	AF103874 (70)
		58	41	*Vibrio cholerae*	AE004279
CcmB	226	38	28	*Pseudomonas putida*	AJ131925 (42)
		35	23	*Escherichia coli*	AA000309 (81)
CcmC	251	64	45	*Pseudomonas aeruginosa*	AE004577
		63	47	*Pantoea citrea*	AF103874 (70)
CcmD	73	58	38	*Pseudomonas fluorescens*	AAC44225
		64	45	*Vibrio cholerae*	AE004279
CcmE	143	59	48	*Pseudomonas fluorescens*	AAC44225
		53	41	*Vibrio cholerae*	AE004279
CcmF	650	59	43	*Vibrio cholerae*	AE004279
		60	47	*Escherichia coli*	AA000309 (81)
CcmG	177	66	46	*Pseudomonas aeruginosa*	AE004577
		69	48	*Pseudomonas fluorescens*	AAC44225
CcmH	361	45	26	*Escherichia coli*	AA000309 (81)
		44	26	*Haemophilus influenzae*	AAC22592

[a] Information is provided only for the two Ccm proteins that had the greatest homology with the *Legionella* proteins. Related proteins also exist within *Bacillus subtilis*, *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, and *Paracoccus denitrificans*, among others.

(Fig. 8B), we examined the portions of the *ccm* locus that are available as part of the *L. pneumophila* Philadelphia-1 genome sequencing project (http://genome3.cpmc.columbia.edu/′legion/index). That examination confirmed the presence of the *ccm* operon and the absence of ISLp*1* in the Philadelphia-1 strain. The latter observations strongly suggest that in strain 130b and perhaps in two other isolates (Fig. 8, lanes 1, 4, and 6), ISLp*1* had been inserted between the 3′ end of *ccmB* and the 5′ end of *ccmC*. Figure 2 presents the gross structure of ISLp*1* and the element's position within the *ccm* locus of strain 130b. The left end of the element undoubtedly exists somewhere in the 86 bp between the conserved end of *ccmB* and the beginning of the ISLp*1* ORF. The right end likely exists in the 302 bp between the end of the ISLp*1* ORF and the 55-bp *ccmB-ccmC* intragenic region that is conserved between strains 130b and Philadelphia-1. An examination of the two end regions failed to reveal any strict inverted repeats or obvious directly repeated target sequence, a situation that has been documented for several IS elements (56). Thus, the size of ISLp*1* is approximated at 1,848 bp. In sum, the characterization of the *L. pneumophila ccm* locus has led to the identification of a new type of IS element.

Transcription of genes within the *L. pneumophila ccm*-ISLp1 locus. The presence of cytochrome *c* in strain 130b (Fig. 3) as well as the fact that mini-Tn*10* insertions in *ccmC* lead to dramatic alterations in phenotype suggest that ISLp*1* does not abolish the expression of the (downstream) *ccm* genes. To confirm this notion, we further assessed the nature of *ccmC* transcripts using RT-PCR analysis. Primers that are internal to the gene's coding region (i.e., ccm10 and ccm8) readily detected the expected 600-bp PCR product in strain 130b. Interestingly, when the RT-PCR was repeated with ccm10 and a primer that was specific to the ORF within ISLp*1* (i.e., ccm3 or ccm18), a product was also obtained. The combination of ccm10 and ccm3 yielded a 1,850-bp product, whereas ccm10 plus ccm18 gave a 1,100-bp product (data not shown). Since the sizes of the latter two PCR products were compatible with the distances between ccm10 and the ISLp*1* primers, these data indicate that *ccmC* expression is transcriptionally linked to the expression of the gene contained within ISLp*1*.

DISCUSSION

Mutations within *L. pneumophila ccmC* resulted in three distinct changes in phenotype; i.e., the loss of cytochrome *c*, an impaired ability to grow extracellularly under low-iron conditions, and a reduction in the ability to infect macrophages and protozoa. Given our general understanding of cytochrome *c* biogenesis (81), the loss of cytochrome *c* in our mutants was entirely expected. *c*-type cytochromes are electron carriers that are widely distributed among prokaryotes and uniquely possess a covalently attached prosthetic heme group (81). In gram-negative organisms, the maturation of cytochrome *c* occurs in three stages (51, 81). First, apocytochrome *c* is translocated across the inner membrane by the general protein secretion machinery. Next, while it is in the periplasm, certain of its cysteine residues are oxidized by the DsbA/DsbB system. Finally, the apoprotein acquires its heme moiety through the action of the *ccm* gene products. CcmABC, perhaps along with CcmD, is believed to be an ABC transporter that exports the heme into the periplasm. CcmEFGH are thought to then promote the attachment of the heme to the apocytochrome. Thus, in various bacterial species, *ccm* mutants uniformly lack cytochrome *c* and may excrete large amounts of porphyrin (8, 24, 51, 65). Our complementation analysis confirmed that *ccmC* is required for *Legionella* cytochrome *c* production.

The reduced ability of the *L. pneumophila ccmC* mutant to grow in low iron was a more surprising, yet not entirely unexpected, result. In addition to their involvement in heme export and ligation, Ccm proteins are implicated in iron acquisition by three types of bacteria. In *Pseudomonas fluorescens*, mutations in *ccmC* impaired both the production of the siderophore pyoverdine and bacterial growth in the presence of iron chelators (32). In *Rhizobium leguminosarum*, mutations in either *ccmH* or *ccmF* reduced siderophore production and/or export (87). Finally, a *ccmF* mutant of *Paracoccus denitrificans* exhib-

ited diminished siderophore production (66). Thus, our observations in *Legionella* indicate that the linkage between the Ccm proteins and iron acquisition may be of broad significance. Complementation analysis confirmed that *ccmC* is required for growth on low-iron by *L. pneumophila*. However, it did not, given the expression of downstream genes in our *ccmC* mutants, eliminate a possible role for other *ccm* genes in *Legionella* iron metabolism. Indeed, preliminary analysis of several 130b mutants containing insertions in *ccmF* indicate that one or more of the final three *ccm* genes also promotes growth in low-iron (22, 33). Thus, the total number of Ccm proteins involved in these various bacterial systems and their actual roles remain to be determined. However, it does appear that the iron assimilation defects are not simply due to the loss of cytochrome *c*; e.g., different residues in the periplasmic domain of *Pseudomonas* CcmC proved to be critical for cytochrome *c* biogenesis and pyoverdine-mediated iron uptake (31). Unlike the *Pseudomonas*, *Rhizobium*, and *Paracoccus* studies, the present study was unable to demonstrate a link between a *ccm* mutation and an alteration in siderophore production. However, it is quite possible that *ccmC*, while not influencing the elaboration of legiobactin, facilitates the production of another *L. pneumophila* siderophore. For example, we have genetic evidence for the existence of an aerobactin-like hydroxamate scavenger, and the *L. pneumophila* genome database suggests the existence of a pyoverdine-like siderophore (40, 82). Alternately, the *L. pneumophila ccmC* gene and/or the *ccm* locus could promote iron acquisition by a siderophore-independent mechanism. A recent study with *Geobacter sulfurreducens* suggests that *c*-type cytochromes can act as periplasmic and extracellular ferric reductases (76). Our continued characterization of the *ccm* mutants as well as our other iron acquisition mutants should provide a means of resolving this question.

Complementation analysis confirmed that *ccmC* is required for *L. pneumophila* infection of *Hartmannella* amoebae. The demonstration that a *ccmC* gene promotes intracellular infection is an observation without precedent. Indeed, all previous *ccm* mutations that were suitably reconstructed and/or subjected to *trans*-complementation have been within bacteria that are associated with extracellular replication and are not pathogenic for humans (32, 66, 87). The question of how *Legionella* CcmC fosters growth within an intracellular niche is therefore of immediate importance. In one scenario, it is possible that the role of *ccmC* in intracellular infection derives from an involvement in iron acquisition. In a second scenario, it is conceivable that intracellular, unlike extracellular, multiplication requires cytochrome *c*-mediated respiration. Finally, it is possible that *L. pneumophila* Ccm proteins are involved in a variety of other processes that are vital to intracellular proliferation. That *ccm* mutations have pleiotropic effects is becoming increasingly apparent. In the extracellular bacterial systems, *ccm* has been implicated in manganese oxidation (17, 24), gluconate and 2-ketogluconate oxidation (70), methylamine oxidation (64), nicotinic acid hydroxylation (49), copper resistance (86), and the *cis-trans* isomerization of unsaturated fatty acids (42). Thus, future characterization of our infectivity mutants should provide new insights into *ccm* and cytochrome *c* function in particular and bacterial physiology and intracellular infection in general.

The relative role of the *ccm* locus in macrophage infection remains somewhat unclear. Mutants with multiple, independently isolated mutations mapping to *ccmC* were impaired for growth in U937 cells, suggesting that the *ccm* locus is important for macrophage infection. However, our inability to complement the mutants with a *ccmC*-containing plasmid prevents us from making definitive conclusions regarding the role of this locus in macrophage infection. This also underscores the differences between intra-amoebic and intramacrophage environments; a number of previous *Legionella* investigations have also found that the requirements for macrophage infection can vary from those of protozoan infection (34, 37, 68, 73). The inability to complement NU208 with a *ccmC*-containing plasmid may suggest that this strain does not express optimal amounts of the downstream *ccm* genes. Indeed, preliminary genetic analysis of several infectivity mutants containing insertions within *L. pneumophila* 130b *ccmF* indicates that one or more of the final three *ccm* genes contribute to intracellular growth within macrophages and protozoa (22, 33, 68).

The second set of novel observations reported here revolves around the discovery of ISLp*1*. For several reasons, we strongly suspect that ISLp*1* is a bona fide IS element, the first in the *Legionella* genus to be described. First, it contained an ORF whose predicted product has relatedness to transposases. Second, like known IS elements, it existed in multiple copies within the bacterial genome. Third, like other IS elements, it was not uniformly distributed within members of the bacterial species. Finally, its size (i.e., 1.8 kb) is comparable to that of a number of other elements. The only common feature of IS elements that was not evident in ISLp*1* was the presence of strict inverted repeats at its ends (56). However, at least three families of prokaryotic IS elements lack this attribute; i.e., the IS *91*, IS *110*, and IS *200*/IS *605* families found in *Escherichia*, *Streptomyces*, *Salmonella*, and *Helicobacter* (6, 13, 47, 60). Thus, ISLp*1* appears to be the first member of a new family of bacterial IS elements. The identification of ISLp*1* was notable for two additional reasons. First, in at least strain 130b, it appeared to have been inserted between *ccmB* and *ccmC* without eliminating the production of cytochrome *c*. In fact, RT-PCR analysis suggested that the expression of at least *ccmC* was coupled to the transcription of the IS element's ORF. Thus, it is conceivable that the regulation of *ccm* genes in 130b is different from that in other strains. Second, the ISLp*1* ORF represents the first *L. pneumophila* gene to exhibit a strain-specific distribution; i.e., to our knowledge, all previously identified *L. pneumophila* genes are conserved within all strains examined. Thus, ISLp*1* probes may provide a method for classifying *L. pneumophila* strains, for epidemiological as well as phylogenetic studies.

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