The *Legionella pneumophila iraAB* Locus Is Required for Iron Assimilation, Intracellular Infection, and Virulence

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***Legionella pneumophila*, a facultative intracellular parasite of human alveolar macrophages and protozoa, causes Legionnaires' disease. Using mini-Tn*10* mutagenesis, we previously isolated a *L. pneumophila* mutant that was hypersensitive to iron chelators. This mutant, NU216, and its allelic equivalent, NU216R, were also defective for intracellular infection, particularly in iron-deficient host cells. To determine whether NU216R was attenuated for virulence, we assessed its ability to cause disease in guinea pigs following intratracheal inoculation. NU216R-infected animals yielded 1,000-fold fewer bacteria from their lungs and spleen compared to wild-type-130b-infected animals that had received a 50-fold-lower dose. Moreover, NU216R-infected animals subsequently cleared the bacteria from these sites. While infection with 130b resulted in high fever, weight loss, and ruffled fur, inoculation with NU216R did not elicit any signs of disease. DNA sequence analysis revealed that the transposon insertion in NU216R lies in the first open reading frame of a two-gene operon. This open reading frame (*iraA*) encodes a 272-amino-acid protein that shows sequence similarity to methyltransferases. The second open reading frame (*iraB*) encodes a 501-amino-acid protein that is highly similar to di- and tripeptide transporters from both prokaryotes and eukaryotes. Southern hybridization analyses determined that the *iraAB* locus was largely limited to strains of *L. pneumophila*, the most pathogenic of the *Legionella* species. A newly derived mutant containing a targeted disruption of *iraB* showed reduced ability to grow under iron-depleted extracellular conditions, but it did not have an infectivity defect in the macrophage-like U937 cells. These data suggest that *iraA* is critical for virulence of *L. pneumophila* while *iraB* is involved in a novel method of iron acquisition which may utilize iron-loaded peptides.**

Legionella pneumophila is the causative agent of Legionnaires' disease, a form of community-acquired and nosocomial pneumonia. *L. pneumophila* is a gram-negative facultative intracellular parasite that infects protozoa and macrophages (1, 12, 23). The organism enters alveolar macrophages by coiling or conventional phagocytosis and replicates within a phagosome that appears not to fuse with the endosomes or lysosomes (16, 17, 38). Various factors that enable *L. pneumophila* to productively infect protozoa and macrophages have been reported. These include the outer membrane porin (27, 43), Mip (13, 25), type II and type IV secretion systems (4a, 46, 47), type IV pili (47, 71), flagella (61; C. Dietrich, K. Heuner, J. Hacker, and B. C. Brand, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. B/D-358, p. 99, 1999), a catalase-peroxidase (6), growth phase (11) and the products of the numerous *dot*, *icm*, *eml*, *mil*, and *pmi* loci (1, 66, 69, 73). While these factors are clearly implicated in infection, relatively few have been fully characterized at the mechanistic level and fewer still have actually been demonstrated to be critical for causing disease in animal models (13, 46, 49, 51).

Iron is a key requirement for *L. pneumophila* virulence and intracellular growth (22, 60, 64). Indeed, chelators that deplete the macrophage iron pool block *L. pneumophila* intracellular replication (8, 29, 60). Also, gamma interferon inhibits *L. pneumophila* growth within human monocytes by reducing the levels of intracellular iron (9, 10). The mechanisms by which *L. pneumophila* acquires iron, especially intracellular iron, are poorly understood. *L. pneumophila* can proteolytically degrade

transferrin and use the released iron in steady-state, iron-limited cultures (39). However, this indirect mode of iron acquisition is unlikely to be relevant for intracellular growth, since the *L. pneumophila* phagosome does not contain transferrin, and the bacterium itself does not bind transferrin (15, 40, 62). *L. pneumophila* can bind lactoferrin, but this does not lead to iron assimilation (7). While *L. pneumophila* binds hemin and can utilize it as the sole iron source for extracellular growth, disruption of a gene that promotes hemin binding does not impair replication in macrophages (56). Two internal ferric reductases are thought to be important for iron assimilation by *L. pneumophila*, although their roles in pathogenesis have not been delineated (40, 59). It had been reported that *L. pneumophila* does not produce siderophores when grown in iron-deficient media (39, 45, 65). However, we have recently demonstrated that *L. pneumophila* does elaborate a nonhydroxamate, nonphenolate siderophore (legiobactin), but only under specific growth conditions (M. R. Liles, T. A. Scheel, and N. P. Cianciotto, submitted for publication). Curiously, we have also demonstrated the presence of a *L. pneumophila* homolog of a hydroxamate biosynthetic gene (37). A targeted disruption of this gene results in reduced growth within macrophages, suggesting that *L. pneumophila* may produce and require an additional siderophore within host cells.

We earlier reported the isolation, by mini-Tn*10* mutagenesis, of various *L. pneumophila* (*ira*) mutants defective for iron acquisition and assimilation (60). Most of these mutants were hypersensitive to the iron chelator ethylenediaminediacetic acid (EDDA) but were resistant to streptonigrin, an antibiotic whose bactericidal activity is enhanced by high levels of intracellular iron (74). One of these mutants, NU216, was especially impaired for infection of macrophage-like U937 cells, showing a prolonged lag phase and a reduced growth rate (60). The impairment of NU216 in macrophages was exacerbated nearly

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TABLE 1. *Legionella* strains[a] used in this study

Species	Strain	Serogroup	Implicated in Disease	*iraA* and *iraB* present
L. pneumophila	130b (Wadsworth)	1	Yes	Yes
L. pneumophila	ATCC 33154	2	Yes	Yes
L. pneumophila	ATCC 33155	3	Yes	Yes
L. pneumophila	ATCC 33156	4	Yes	Yes
L. pneumophila	ATCC 33216	5	Yes	Yes
L. pneumophila	MDPH[b]	6	Yes	Yes
L. pneumophila	MDPH	7	Yes	Yes
L. pneumophila	MDPH	9	Yes	Yes
L. pneumophila	MDPH	11	Yes	Yes
L. pneumophila	MDPH	12	Yes	Yes
L. pneumophila	ATCC 43736	13	Yes	Yes
L. birminghamensis	ATCC 43702		Yes	No
L. erytha	SE-32A		No	No
L. feeleii	WO-44C		Yes	No
L. gormanii	ATCC33297		Yes	Yes
L. hackeliae	Lansing 2		Yes	No
L. israelensis	ATCC 43119		No	No
L. jamestowniensis	ATCC 35298		No	No
L. longbeachae	ATCC 33462		Yes	No
L. micdadei	ATCC 33204		Yes	No
L. oakridgensis	ATCC 33761		Yes	No
L. parisiensis	PF-209		Yes	No
L. sainthelensi	Mt. St. Helens 4		Yes	No
L. santicrucis	ATCC 35301		No	No
L. spiritensis	ATCC 35249		No	No

[a] For the origin of these strains and their disease association, see references 54 and 61 and the references therein.
[b] Obtained from the Michigan Department of Public Health.

100-fold in the presence of the iron chelator desferrioxamine (DFX), indicating that it included a defect in intracellular iron assimilation. As a first step toward identifying the genetic basis of the phenotype of the mutant, allelic exchange was used to reintroduce the mutation into the wild-type background to confirm that the defects were actually linked to the mini-Tn*10* disruption. The new strain, NU216R, was EDDA hypersensitive and showed the same phenotype in U937 cells as the original mutant. NU216 and NU216R were not impaired for extracellular growth in standard *Legionella* media (60).

In the present study, we further characterized the iron uptake and infectivity impairment of NU216R. First, we showed that the *ira* locus that was inactivated in this strain is critical for virulence in guinea pigs. Subsequent sequence analysis of the locus revealed the presence of a two-gene operon (*iraAB*). Additional mutant analysis indicated that *iraA* alone is required for intracellular infection while *iraB*, encoding a putative peptide transporter, may promote iron assimilation by a novel method.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The *L. pneumophila* serogroup 1 strain 130b (Wadsworth), its mini-Tn*10* derivative NU216, and the reengineered allelic equivalent NU216R have been described previously (60). Additional legionellae tested for the presence of the *iraAB* locus are listed in Table 1. *L. pneumophila* strains were routinely cultured on buffered charcoal yeast extract (BCYE) agar for 2 to 3 days at 37°C (19). Antibiotics (chloramphenicol, 3 μg/ml; kanamycin, 25 μg/ml) or sucrose (5%, wt/vol) were added to the medium when appropriate. Growth curve experiments were performed in liquid buffered yeast extract medium (BYE broth) with or without the standard iron supplementation (19). A *L. pneumophila* plasmid library containing *Sau*3 AI-digested 130b DNA cloned into the *Bam*HI site of pBR322 was maintained in *Escherichia coli* HB101 (36). *E. coli* strain NovaBlue (Novagen, Madison, Wis.) was routinely used for maintaining and propagating newly isolated plasmids. Plasmid pCDP70, containing the mutated *ira* locus from NU216, has been described previously (60).

Experimental infection of guinea pigs and U937 cells by *L. pneumophila*. To assess the virulence of *L. pneumophila* strains, we examined their ability to cause disease in guinea pigs following intratracheal injection (13, 20, 75). The procedure for infection was described previously (46). Briefly, BCYE agar-grown bacteria were transferred to BYE broth and grown at 37°C in a shaking incubator. The bacteria were then resuspended at the desired concentration and injected into surgically exposed trachea in 0.3-ml volumes. The delivered bacterial inoculum was determined by plating on BCYE agar plates. The study used Hartley male guinea pigs weighing around 300 g. The animals were observed for signs of infection for 7 days, with daily measurements of body weight and rectal temperature. The right lower lobes of the lungs and spleen were harvested from the respective groups each day, and ground homogenates were cultured quantitatively.

Intracellular infection of macrophage-like U937 cells generally was performed as described previously (60). However, to assess the effect of intracellular iron depletion, U937 cells were treated with DFX and intracellular legionellae were quantitated at various times postinoculation (10, 29, 60). The percent reduction in recovery was calculated as 100 × (average CFU from untreated cells − average CFU from DFX-treated cells)/(average CFU from untreated wells).

Sequence and Southern analysis of the *iraAB* locus. Initial sequence analysis of the *ira* locus mutated in NU216 was performed on subclones of pCDP70 with the transposon primer 5′-GTGACGACTGAATCCGGT. To facilitate further sequencing, we sought an additional *iraAB*-containing plasmid from our library of 130b DNA. To do this, we used labeled pCDP70 as a probe in colony blots that were performed with the Genius system kit (Boerhinger, Mannheim, Germany). The isolated plasmid, pCDP71, contained approximately 5 kb of *Legionella* DNA. The sequence of the pCDP71 insert was determined by primer walking. Sequence analysis was performed using either the DyeTerminator cycle-sequencing reaction mix or the BigDye terminator cycle-sequencing reaction mix from PE Applied Biosystems (Foster City, Calif.). Primers for sequencing and 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 using the programs based on the BLAST algorithm (3).

To determine the distribution of the *iraAB* locus among legionellae, Southern hybridization analysis was performed using *iraA*- and *iraB*-specific fluorescently labeled probes and the Genius system kit. The fragments to be labeled were generated by PCR using the primers shown below in Fig. 3. Chromosomal DNA for the blots was isolated from *Legionella* strains as described previously (60).

Transcomplementation and directed-mutagenesis studies. To facilitate complementation analyses of NU216R, we used pCDP71 to construct three new plasmids; pVK100 (carrying both *iraA* and *iraB*), pVK101 (carrying only *iraA*), and pVK102 (carrying only *iraB*). pVK100 was made by inserting the *Acc*I-*Bst*BI fragment from pCDP71 into the *Acc*I site of the Cm[r] vector pSU2719 (50). pVK101 was generated by eliminating the *iraB*-containing *Sac*I fragment from

pVK100. To obtain pVK102, the 4.2-kb *Pst*I-*Sph*I fragment from pVK100 was treated with Klenow enzyme to generate blunt ends and then circularized. To attempt to generate a *L. pneumophila* strain with an in-frame deletion in *iraA*, plasmid pVK107 was generated in two steps. First, the *Eco*RV-*Sal*I fragment from pCDP71 was ligated to *Nru*I-*Sal*I-digested pBOC20, a vector containing the counterselectable *sacB* (54). Subsequently, the 63-bp *Nsi*I-*Pst*I fragment in *iraA* was eliminated from pCDP74 to generate an in-frame deletion. Plasmids were introduced into *L. pneumophila* by electroporation (14).

To target a disruption in *iraB*, the *Pst*I-containing Km^r fragment from pMB2190 was cloned into the *Pst*I site of pBluescript II SK(+) (Stratagene, La Jolla, Calif.) to generate pVK3. The *aph* gene in pVK3 was determined to be in the same direction as the *lacZ* gene of the vector. The Km^r-containing region from pVK3 was amplified using the primer 5'-GGGAGCTCAGGTCTGCCTC GTGAAGAA and the M13 reverse primer. The *Sac*I-digested PCR product was cloned into the *Sac*I site of pCDP71 to generate pVK104 with a disrupted *iraB* gene. As the first step toward introducing the *iraB* mutation into *L. pneumophila*, the *Sal*I fragment from pVK104 containing the inactivated *iraB* gene was moved into the *Sal*I site of pBOC20. The resulting Km^r Cm^r plasmid, pVK105, was electroporated into wild-type *L. pneumophila*. Cm^r Km^r electrotransformants were then streaked onto plates containing karamycin and 5% sucrose (14). Km^r Cm^s sucrose-resistant colonies were selected as putative mutants containing a disruption in *iraB*. The identity of these mutants was confirmed by PCR analysis. PCR was performed in a 50-μl reaction mix, as recommended by the manufacturer, in the presence of 1 to 2 U of *Taq* polymerase (Life Technologies, Rockville, Md.). One of these *iraB* mutants, designated NU244, was chosen for further study.

Nucleotide sequence accession number. The *L. pneumophila iraAB* locus sequence is deposited in the GenBank database at the National Center for Biotechnology Information under accession no. AF167992.

RESULTS

Cell morphology of *L. pneumophila iraAB* mutants. It was observed that NU216 cells collected from 3-day-old BCYE agar plates and suspended in BYE broth did not show a correspondence between optical density at 660 nm (OD$_{660}$) and CFU per milliliter that was comparable to that of 130b. Thus, NU216 cells resuspended to the same OD$_{660}$ as 130b and plated onto BCYE agar plates resulted in only half the number of CFU. Gram staining revealed that the NU216 culture was far more filamentous than 130b (data not shown). To determine whether this altered cellular morphology was associated with the transposon insertion in the *ira* locus, we examined NU216R. The morphology of this strain was similar to that of 130b, indicating that the filamentous nature of NU216 was due to a spontaneous secondary mutation. Since NU216R possesses the iron uptake and infectivity defects of NU216 (60), we concluded that these defects are not due to any changes in cellular morphology. We therefore used NU216R for all of the studies detailed in this paper.

Pulmonary infection of guinea pigs by *L. pneumophila* strains. The decreased infection of U937 cells by NU216 and NU216R suggested that the *ira* locus disrupted in these strains was required for virulence (60). To test this hypothesis, we evaluated the relative virulence of NU216R for guinea pigs. An initial, qualitative assessment of virulence was obtained by observing the ability of various doses of bacteria to cause fatal disease following intratracheal inoculation. Guinea pigs infected by intratracheal injection of virulent *L. pneumophila* develop an acute pneumonia resembling human Legionnaires' disease (13, 20, 75). Indeed, we observed that three out of four animals infected with 6×10^6 CFU of 130b succumbed to the infection within 4 days. In contrast, none of the guinea pigs infected with 7.8×10^7 CFU of NU216R had weight loss or respiratory distress, and all were alive 10 days postinoculation. Thus, the *ira* mutant NU216R appears to have a 50% lethal dose at least 100-fold greater than that of the wild-type strain.

An alternative, quantitative assessment of virulence was obtained by inoculating guinea pigs with NU216R and 130b and then comparing the *L. pneumophila* concentrations in their lungs and the spleens on each day following infection. After the introduction of 10^6 CFU into the animals, the numbers of 130b increased over 100-fold in the lungs within 24 h (Fig. 1A). The numbers of wild-type bacteria in the lungs continued to increase on subsequent days, reaching a maximal value of 4.9×10^9 CFU on day 3 postinfection. In marked contrast, even when the animals were infected with a 50-fold-greater number of NU216R, this strain did not significantly increase in numbers within the lungs in the first 24 h (Fig. 1A). Furthermore, on subsequent days, the mutant bacteria steadily decreased in numbers. Thus, by day 3 postinfection, there were approximately 1,000-fold fewer CFU of NU216R than of 130b in the lungs of the infected animals. Taken together, these data indicate that this *ira* mutant is not able to replicate within a mammalian lung.

The wild-type bacterium disseminated to the spleen in significant numbers within the first day (Fig. 1B). Following this, the number of wild-type bacteria continued to increase for at least another day before dropping on subsequent days. This pattern of accumulation in the spleen has been observed previously (46). In contrast, 1,000-fold-less NU216R was observed in the spleen 1 day postinfection, and the number of bacteria increased only marginally on the following day and then decreased progressively. Interestingly, 1 and 2 days postinfection, the two strains showed a greater difference in numbers in the spleens compared with those in the lungs. By 10 days postinfection, the mutant bacteria were below the detectable limit (10^2 CFU) in both the lungs and the spleen, indicating clearance.

To further document the progression of the disease, the temperature and weight of the guinea pigs were monitored during the course of the clearance study. Animals in both experimental groups displayed an increase in body temperature 1 day postinfection (Fig. 2A). However, animals infected with the wild-type strain displayed higher body temperatures that continued to increase. The drastic drop in body temperature on day 5 was a reflection of their moribund state. In contrast, the temperature of NU216R-infected animals dropped at 2 days postinfection and resolved to baseline levels (Fig. 2A). As has been previously demonstrated (20, 46), animals infected with wild-type bacteria had an average weight loss of 20% by 4 days postinfection (Fig. 2B). NU216R-infected animals, on the other hand, gained weight by 12% in the same period. Taken together, the mutant's higher LD$_{50}$, inability to accumulate in the lungs or spleen, and failure to mediate any signs of disease indicated that this *ira* locus encodes a virulence determinant(s).

Sequence analysis of the *iraAB* locus. To characterize the genetic lesion responsible for the drastic attenuation in virulence of NU216R, we cloned and sequenced the *ira* locus from the *L. pneumophila* chromosome. To construct NU216R, we had earlier cloned an approximately 8-kb *Sal*I fragment containing the Km^r marker and flanking DNA from NU216 (60). Using subclones of this plasmid, pCDP70, as well as pCDP71, which contains the intact *ira*, we sequenced this locus and mapped the transposon insertion in NU216 and NU216R. In all, 3,763 bp was sequenced from both strands (Fig. 3). This represents 815 bp upstream and 2,967 bp downstream of the transposon insertion. The base composition of this region (G+C content = 38%) was typical for *L. pneumophila* (47). Sequence analysis revealed that the transposon disrupted the first open reading frame (ORF) in what appears to be a two-gene operon. This orf, designated *iraA*, is preceded by sequences with strong homology to prokaryotic promoter sequences and an appropriately positioned Shine-Dalgarno sequence. *iraA* is predicted to encode a soluble 272-amino-acid protein. Although it did not show overall homology to proteins in the database, IraA contained the *S*-adenosylmethionine (SAM)-
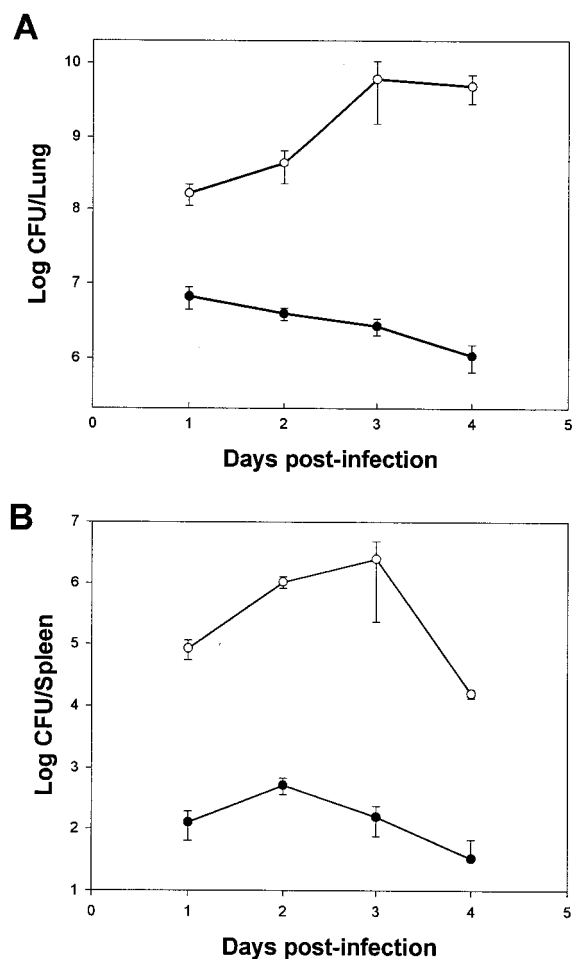
A

B

FIG. 1. Replication of *L. pneumophila* strains within guinea pig lung and spleen. Doses of 1.0×10^6 CFU of 130b (open circles) or 5.2×10^7 CFU of NU216R (solid circles) were introduced intratracheally into guinea pigs, and the total number of bacterial CFU per lung (A) and spleen (B) were quantified in three or four animals at various times after inoculation. Each data point represents the mean ± standard error ($n = 4$ for days 1 and 2, and $n = 3$ for days 3 and 4). At every time point, there was a statistically significant difference between the numbers of wild-type and mutant bacteria ($P < 0.05$; Student's t test).
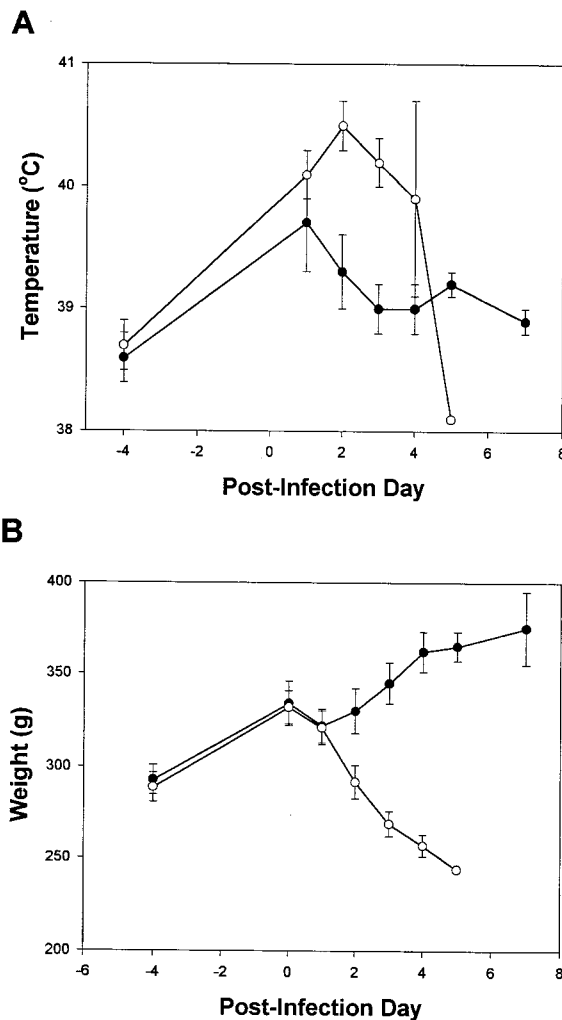
A

B

FIG. 2. Signs of *L. pneumophila* pulmonary infection. Rectal temperature (A) and body weight (B) of the 130b-infected (open circles) and NU216R-infected animals (solid circles) were monitored through the course of infection. Each point represents the mean ± standard error from three or more animals with the exception of one 130b-infected guinea pig on day 5. 130b-infected animals were moribund on day 5 postinfection and were sacrificed (temperature: $P < 0.05$ for days 2 to 4; weight: $P < 0.05$ for days 2 to 4 [Student's t test]).

binding motif present in several prokaryotic and eukaryotic small-molecule methyltransferases. Recently, a consensus sequence, oL(D/E)oGsGsG-$X_{16–21}$-(D/E)-$X_{28–94}$-oDso (o = hydrophobic amino acids; s = small amino acid), was generated from numerous eukaryotic methyltransferases (37, 46). The IraA sequence includes the stretch ILDLGVGNG-X_{18}-D-X_{87}-QDTI that fits the above consensus (Fig. 3). Interestingly, a comparison of the prokaryotic methyltransferases in this family revealed a slightly different and more extended conservation of sequences surrounding the initial o(L/D)oGsGsG sequence (Fig. 4).

The ORF designated *iraB* starts with a GTG codon 35 bp downstream of the *iraA* termination codon (Fig. 3). The use of GTG initiation codons in *L. pneumophila* and elsewhere has been reported previously (36). IraB is highly similar to prokaryotic and eukaryotic members of the PTR2 family of peptide transporters (70). These integral membrane proteins are involved in the proton-dependent uptake of di- and tripeptides. IraB is most closely related to a putative 500-amino-acid protein, YdgR, from *E. coli*, also predicted to be a member of this family. The *L. pneumophila* and *E. coli* ORFs align along

their entire length with an overall similarity of 51% and an identity of 29% (Fig. 5). The best-studied prokaryotic member of this class of proteins is the 463-amino-acid DtpT protein from *Lactococcus lactis* (Fig. 5) (34). Analysis using the SOSUI program predicts that IraB, like DtpT, is an integral membrane protein with 12 membrane-spanning regions (34; S. Mitaku, SOSUI, http://www.tuat.ac.jp/~mitaku/adv_sosui/). Other members of this family which show homology to IraB include the *Drosophila melanogaster* oligopeptide transporter 1, the *Lactobacillus helveticus* di- and tripeptide transport protein, and the rat kidney oligopeptide transporter (4, 52, 67).

A large ORF (predicted to encode >450 amino acids) whose product has no significant similarity to proteins in the database is present 159 bp upstream and divergent from *iraA* (Fig. 3). While the function of this ORF is unknown, it is highly unlikely, based on its position and orientation relative to the transposon, to be related to the iron and/or infectivity defects in NU216R. There were no large ORFs immediately downstream of *iraB* (Fig. 3). Further downstream of *iraB* was a

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  1 TTCCTATCCTGGCATTAGACATTTTTAAAAAACCGCAGTATACAATAAAAATTTCAGGTATTAAACAAAT
     G  I  R  A  N  S  M          AccI
                          ← Orf
 71 CCTGATTGAAGAACCAAAATATATAATTTAATAAATTTGTGTTTTCAATAGTCGCCAATTACCCCCTTTT
       -35              -10       IraA →
                                  M  S  L  K  A  M  Y  N  Q  I
141 AGGGATATACTGGATGAATCGAGTTAAAGTAGGAATTTTGCATGTCCTTGAAGGCCATGTATAACCAAAT
                          SD
     A  E  N  Y  A  T  A  N  R  F  G  S  I  S  E  S  H  H  V  A  I  E  Q
211 CGCAGAAAATTACGCGACAgctaatcgttttggttcaATTAGTGAAAGCCATCACGTTGCCATTGAGCAG

     M  R  K  F  H  L  G  L  K  P  H  Y  K [I  L  D  L  G  V  G  N  G] S
281 ATGAGGAAATTTCATTTGGGGTTGAAACCCCATTATAAAATCCTCGATCTGGGCGTTGGCAATGGCTCTT

     F  L  Q  K  L  H  H  L [M  P  M  A  N  F  T  G  I  D  V  S  S  E  M  L
351 TTTTGCAAAAATTGCATCACTTAATGCCAATGGCCAATTTCACAGGAATTGATGTTTCATCAGAAATGTT

     K  R  A] S  E  A  L  P  L  T  T  I  E  G  S  A  A  E  A  N  K  F  L
421 GAAACGAGCGAGCGAAGCCCTTCCTTTAACAACCATTGAAGGCAGTGCTGCAGAAGCCAACAAGTTTTTG
                                            PstI
     P  A  H  S  Q  D  L  V  L  A  H  F  I  N  A  Y  I  P  I  N  V  L  F
491 CCCGCACATAGCCAGGACTTGGTTCTGGCTCATTTTATTAATGCATATATCCCTATTAATGTTCTCTTTG
                               NsiI
     D  E  A  K  Y  L  T  R  A  N  G  H  F  S  L  I  T  T  T  Y  D  S  F  P
561 ATGAAGCCAAATACTTGACGCGTGCCAACGGTCACTTCTCTTTAATCACAACAACCTatgattctttttcc

     V  A  Q  Q  Q  L  A  N  F  I  A  Q  D  T  I  L  S  R  V  V  G  H  Y
631 tgtagcgcAACAACAACTGGCCAACTTCATTGCTCAGGATACTATTTTAAGCCGAGTTGTTGGTCACTAC

     Y  K  S  I  V  K  N  T  T  V  A  A  N  L  D  E  L  L  L  A  F  K  Q
701 TATAAATCCATAGTTAAAAATACGACTGTTGCTGCAAATCTCGACGAACTCTTGCTGGCATTTAAACAGC

     H  Q  F  N  I  L  D  H  Q  R  I  E  I  P  I  T  L  N  N  I  D  E  L  A
771 ATCAATTTAATATTTTGGATCACCAACGTATTGAAATACCCATTACCCTTAATAACATTGATGAATTGGC

     L  F  G  I  E  G  T  W  F  L  N  T  L  S  I  R  M  L  P  K  Y  F  L
841 ACTGTTTGGAATAGAGGGCACCTGGTTTCTTAACACTCTATCGATACGCATGCTGCCAAAATATTTTTA

     I  Q  R  L  K  R  L  F  S  K  I  F  T  F  P  Y  H  D  T  H  I  I  D
911 ATTCAGCGTCTTAAACGACTTTTCAGCAAAATCTTTACTTTTCCCTACCATGACACTCATATTATTGATG
                                                         IraB →
     V  V  L  A  R  K  *                                M  K  L  I
981 TAGTTCTTGCCAGGAAGTGATAATCTTTGGCGGTGGGTTAGAAAAAACGGAATGAGGTGTGAAATTGATT

     S  Q  A  V  K  N  Y  L  P  E  L  S  L  R  Q  K  Q  T  N  N  I  I  F
1051 TCTCAAGCAGTTAAAAATTATCTTCCAGAACTAAGTCTTCGCCAAAAACAAACCAATAATATTATTTTTA

     I  T  F  W  S  Q  F  S  V  Y  A  L  N  T  I  L  V  L  F  L  T  R  P  L
1121 TTACTTTTTGGAGCCAGTTTTCTGTCTATGCCCTGAATACAATTCTGGTTTTATTTTTAACTCGACCCTT

     I  A  Q  G  L  G  Y  S  Q  A  K  A  Y  A  F  I  G  V  T  Q  A  T  G
1191 GATTGCTCAGGGATTAGGCTACAGCCAAGCCAAAGCGTACGCTTTCATTGGTGTAACTCAAGCCACAGGC

     Y  L  M  P  I  L [G  G  Y  M  A  D  N  I  I  G  V  R  R  S  I  L  L
1261 TACCTGATGCCTATTTTAGGTGGGTATATGGCTGATAATATTATTGGTGTCAGACGATCCATTCTTTTGG

     G  S  I  M  L  A  C  A] Y  L  L  V  M  L  S  G  Y  T  I  S  S  L  G  D
1331 GTAGCATCATGCTGGCTTGTGCTTATTTACTGGTCATGTTAAGTGGGTACACCATCAGTTCTCTTGGGA

     Q  L  F  I  I  A  F  A  F  I  P  A  T  N  S  L  L  M  G  T  A  S  S
1401 TCAATTATTTATCATAGCTTTTGCCTTTATCCCTGCAACCAACTCGCTATTAATGGGAACCGCATCCAGT

     M  V  S  H  I  Y  S  D  D  A  I  K  A  K  S [A  M  T  I  Y  M  A  I
1471 ATGGTTTCTCATATTTATTCcgatgatgccattaaagcCAAATCCGCTATGACTATATATATGGTTATTA
```

```
     N  V  G  A  L] L  A  T  M  I  A  P  V  L  L  E  S  R  Y  G  P  L  S  V
1541 ATGTTGGAGCTCTGCTGGCAACCATGATCGCGCCAGTTCTACTAGAAAGTCGTTATGGTCCACTTTCAGT
          SacI
     L  T  L  T  F  I  G  K  S  I  A  A  L  N  F  A  K  R  Y  S  I  Y  D
1611 ACTCACCTTAACCTTTATTGGCAAATCGATTGCTGCCTTGAATTTTGCGAAACGTTACTCCATATATGAT

     S  V  I  W  G  M  D  K  S  K  F  S  N  Q  G  L  L  R  L  V  A  Y  I
1681 AGTGTCATCTGGGGTATGGATAAATCCAAATTTTCTAACCAAGGCCTATTGCGCCTTGTTGCTTATATAG

     A  A  I  Y  S  L  T  L  Y  A  Y  T  H  V  Y  I  A  S  T  L  I  G  A  G
1751 CGGCTATCTATTCTCTTACTTTATATGCTTATACTCATGTTTATATCGCCAGCACACTGATAGGCGCAGG

     C  A  I  G  I  L  W  F  L  I  K  T  I  M  L  K  G  E  T  R  S  K  Q
1821 ATGTGCTATAGGAATTCTCTGGTTTTTAATTAAAACCATTATGCTTAAAGGCGAAACTCGCAGTAAACAA

     M  I  A  V  L  L  I  I  E  A  I  V  F  F  I  I  Y  N  Q  M  N  S  T
1891 ATGATCGCTGTATTACTGATAATAGAAGCTATAGTATTTTTTTATCATATACAATCAAATGAACAGTACCT

     L  V  L  F  A  E  N  N  S  D  L  S  F  L  W  F  K  I  S  P  A  Q  Y  Q
1961 TGGTATTATTTGCAGAAAACAATTCTGATCTAAGTTTTCTTTGGTTTAAAATTTCACCCGCTCAATACCA

     M  L  N  P  L  L  I  L  L  I  G  S  Q  L  P  R  F  Y  R  F  F  S  R
2031 AATGCTAAATCCTTTGCTCATTTTACTGATTGGAAGTCAACTTCCTCGTTTCTACAGATTTTTTTCGCGC

     F  T  I  P  Y  Q  F  A  A  G  T  I  L  S  G  I  A  L  L  V  M  A  F
2101 TTTACGATCCCCTATCAATTTGCCGCAGGCACAATTCTCTCTGtatcgccctgctggttaTGGCTTTTG

     A  A  Q  N  A  I  N  G  L  V  N  G  N  Y  I  A  L  T  Y  I  L  I  S  I
2171 CAGCCCAAAATGCAATCAATGGATTAGTGAATGGTAACTACATTGCTTTGACCTACATTTTAATTTCAAT

     A  E  L  W  V  S  A  I  G  L  S  M  I  G  L  Y  C  D  A  R  A  I  A
2241 TGCCGAATTATGGGTTAGTGCCATTGGTCTTAGCATGATTGGACTGTATTGTGATGCACGTGCCGATTGCC

     F  A  M  G  V  W  Y  L  A  S  S  M  S  N  A  I  S  G  R  I  A  G  W
2311 TTTGCGATGGGCGTATGGTACCTGGCCAGTTCCATGTCCAATGCCATTTCAGGACGAATTGCAGGTTGGG

     V  A  I  P  E  N  I  N  S  A  L  E  S  L  P  Y  Y  K  N  Y  Y  L  I  M
2381 TCGCCATACCAGAGAATATTAATTCTGCTCTTGAAAGTTTACCCTATTATAAAAATTATTATTTGATTAT

     G  I  S  A  L  G  L  G  I  L  M  Y  F  V  A  Y  Y  L  H  K  I  M  K
2451 GGGCATTAGTGCTTTGGGGCTTGGTATTCTGATGTATTTTGTAGCTTATTATTTACACAAGATTATGAAA

     R  R  G  I  E  L  A  *
2521 CGACGAGGTATTGAGTTAGCATAACGTAACTGAACATTGTAATATTCGAATATGCTTCGAGACGGTGCTT
                                                BstBI
2591 CGCACCTCCTCAGCATGAACGGCCAGATGGGAAAATCCAATTTTGTAATCAGCCTTGGAGTTTTATGCAA

2661 ACCATCTTCCGATTATCCATAGGTTTTTCTTGTTAATTGGATAAATCCGTTATTATATTAAACCGTTCAT

2731 GCTGAGGAAGTGCGAAGCACCATCTTGAAGCAAAAACGGCCCGATGGGAAAATCCAATTTTGTAATCAGC

2801 CTTCGGATTTTAAGCATACCATTTTCCAATTATCCATAGATCTTAATTGGATAAATTAGTTATTATCTTA

2871 ATCCGTTCATGCTGAGGGGGTGCAAAGCACCGTCTCGAAGCATGCTTAAAAACATAGTATAAGGGATTGA
         Orf →
          M  F  W  V  Y  I  L  Q  C  S  D  K  S  Y  Y  T  G  Q  T  D  N  L
2941 CTAAATGTTTTGGGTTTATATTCTTCAATGCAGTGATAAGAGTTATTATACAGGGCAAACGGATAATTTG

     E  K  R  L  T  Q  H  Q  D  K  M  I  P  G  C  Y  T  S  T  R  L  P  I
3011 GAAAAACGCCTTACTCAACATCAAGATAAAATGATTCCGGGTTGTTATACAAGCACAAGATTACCAATTC

     Q  L  K  F  S  Q  E  F  M  S  R  E  E  A  L  S  A  E  R  Q  I  K  G  W
3081 AATTAAAAATTCAGCCAGGAATTTATGTCTCGTGAAGAAGCCTTGAGCGCTGAGAGACAAATCAAAGGATG

     S  R  R  K  K  E  A  L  I  N  G  D  W  Q  A  L  S  D  Y  S  K  R  K
3151 GAGTCGGAGAAAGAAAGAGGCTTTAATAAATGGAGACTGGCAAGCGTTATCAGACTATTCTAAACGTAAA

     N  *
3221 AATTAAGAGTGGGCTGTGTTTCATTCGAACATGCTTCGAGACGGTGCTCTGCACCTCCTCAGCATGAACG

3291 GTTTAATATAATAACTGATTTATCCAATTAACAAGAAAAATTTATGGATAATCGGAAGATGGTTTGCATA

3361 AAGCCCGAAGGCTGATTACAAAAAGTGGATTTTCCCATCGGGCCATTCTTGCTTCAAGATGGTGCTTCGC

3431 ACTTCCTCAGCATGAACGGTTTAATGGTTTAATAAAAAAATCTAATTTCTTTAACTAATAATAAAAATTATT

3501 TATAGCCAATTCGAAAAACACAAGCTGTTCACTAACTTCAAACATCAAGCCCTAAATTATCCCGTTTGAA
                                                  *  V  D  L  G  L  N  D  R  K  F
3571 CACTCGGTCAGCCCTATAGCTTGATCGAACTAATGGGCCTGAAGCCACTTCAAAAAAGCCTTTTTTTTAGA
     V  R  D  A  R  Y  S  S  R  V  L  P  G  S  A  V  E  F  F  G  K  K  L
3641 CCAATTTGCCTTAGTTCAGAAAAAGTTTCAGGAGTTACGTAACGTGCTATAGGTAAATGATTTTTAGTTG
     G  I  Q  R  L  E  S  F  T  E  P  T  V  Y  R  A  I  P  L  H  N  K  T  P
3711 GTTGCAAATATTGTCCCAAAGTCAAAAATATCCACGTGATGAGTTCGGAGATC
     Q  L  Y  Q  G  L  T  L  I  D  V  H  H  T  R  L  D
                                       ← LipA
```

FIG. 3. Nucleotide sequence of the *L. pneumophila iraAB* locus. The deduced amino acid sequences of the various ORFs and the termination codons (*) are indicated. Arrows indicate the directions of translation of the ORFs. Putative promoter sequences are indicated by the −10 and −35 designations upstream of *iraA*, and the Shine-Dalgarno sequence is indicated as SD. The target sequence for the miniTn*10* insertion in NU216 and NU216R is indicated in bold. The two underlined sequences represent a 250-bp IR. Restriction sites used for making the constructs described in this paper are indicated. The Km^r cassette insertion in NU244 is in the *Sac*I site. The IraA sequences corresponding to the SAM-binding consensus and the IraB sequences corresponding to the PTR consensus are enclosed within parentheses. PCR was performed using primers 5′-AGCTA ATCGTTTTGGTTCA and 5′-GCGCTACAGGAAAAGAATCAT to generate *iraA*-specific fragments and 5′CCGATGATGCCATTAAAGC and 5′TAACCA GCAGGGCGATAC to generate *iraB*-specific fragments for hybridization analyses. The primer-binding regions are indicated in lowercase type. For brevity, only the beginning of the upstream ORF is shown. Double-stranded sequence data were compiled with GeneRunner (Hastings Software, Inc., Hastings, N.Y.).

divergently transcribed ORF that was highly similar to the lipoic acid biogenesis gene *lipA* from *Pelobacter carbinolicus*, *E. coli*, and other organisms (57, 63). Lipoic acid is a coenzyme required for the activity of enzyme complexes such as pyruvate dehydrogenase (41). The 994-bp sequence between the ends of *iraB* and *lipA* contains an interesting feature, i.e., a 93-amino-acid-encoding ORF flanked by a 250-bp imperfect inverted repeat (IR) (Fig. 3). Two subrepeats of 82 bp are present within each IR, and an additional 82-bp subrepeat is present upstream and divergent from the first IR. The ORF itself is similar to sequences encoding various hypothetical "intergenic" proteins of approximately the same length (28, 44). The significance of these DNA sequences is unknown, although the IR could play a regulatory role. Thus, the sequence analyses indicated that this *ira* locus is bicistronic and that the impairments of NU216R were due to a loss of IraA and/or IraB.

Construction of an *L. pneumophila iraB* mutant. To determine whether the iron and/or infectivity defects in NU216R were due to the loss of *iraA* and/or a polar effect on the expression of *iraB*, we attempted to complement NU216R with plasmids containing either *iraA* alone (pVK101), *iraB* alone (pVK102), *iraA* and *iraB* (pVK100), or vector alone (pSU2719). Curiously, the transformation efficiency was significantly lower with the *ira*-containing plasmids than with vector (data not shown). Additionally, in three separate attempts, it was observed that the strains transformed with the *ira*-containing plasmids took at least an extra day to appear on BCYE agar plates than did vector-transformed bacteria. Furthermore, it was observed that the *iraAB*-containing plasmids were not consistently maintained in these strains. This was unexpected, since in several instances we have been successful in complementing other *Legionella* mutants with the appropriate fragment of DNA cloned into various vectors (14, 37, 46). This suggested that overexpression of the genes from this locus was detrimental to *Legionella* survival. As an alternate approach to determining the relative contributions of *iraA* and *iraB*, we sought a mutant containing a nonpolar mutation in *iraA*. Thus, pVK107 was transformed into NU216R in order to replace the mini-Tn*10*-disrupted *iraA* with an in-frame deletion in *iraA*. Unfortunately, no transformants were obtained in three attempts. We finally decided to determine if a targeted disruption of *iraB* itself would result in the same phenotype(s) as NU216R. Using the counterselectable pVK105 for allelic exchange, we readily obtained strain NU244, which contained a Km^r cassette inserted in the middle of *iraB* (Fig. 3). Importantly, NU244 grew in BYE broth and on BCYE agar as well as did 130b, and so it was suitable for phenotypic analysis (see below). Incidentally, the cell morphology of this strain was similar to that of 130b. We therefore evaluated NU244 for EDDA sensitivity and infectivity to begin to determine the contribution of *iraB* to iron acquisition and virulence.

Sensitivity of *L. pneumophila* strains to iron limitation. To determine if a mutation in *iraB* results in sensitivity to iron deprivation, strains 130b, NU216R, and NU244 were compared for their ability to grow in BYE broth containing different amounts of free iron. The three strains did not display significant differences for growth in standard BYE medium (Fig. 6A), confirming that the *iraAB* mutants do not have a generalized growth defect. Interestingly, however, NU244 was impaired for growth in BYE medium lacking iron supplementation, while 130b and NU216R grew as in the supplemented medium (Fig. 6B). The normal growth of 130b and NU216R in unsupplemented BYE medium is not surprising since it contains approximately 27 μM residual iron (31). The impaired growth of NU244 in unsupplemented BYE implies that it is more sensitive to iron starvation than is NU216R. To confirm

this notion, we further depleted the BYE medium of iron by including 20 μM EDDA (Fig. 6C). As expected, NU244 was highly sensitive to iron depletion. To our surprise, however, NU216R had lost its hypersensitivity to EDDA since our last report (60) (Fig. 6C). In separate experiments, we observed that the original mutant, NU216, had also lost its EDDA hypersensitivity (data not shown). While this loss was observed for NU216 and NU216R on three different occasions, both strains continued to grow on karamycin and the persistence of their transposon mutations was confirmed by PCR analysis (data not shown). The impaired growth of NU244 in media lacking added iron, combined with its hypersensitivity to EDDA, indicated that *iraB* is involved in the assimilation of iron in extracellular conditions. Thus, we suspect that the original EDDA hypersensitivity of NU216 and NU216R was partly, if not completely, a result of a polar effect on *iraB* expression.

Intracellular infection of U937 cells by *L. pneumophila* strains. To determine whether *iraB* also promotes intracellular infection, we evaluated the relative ability of NU244 to infect U937 cells. Given its change in EDDA sensitivity, we were also eager to retest the capacity of NU216R to infect the macrophage-like cells. In three separate experiments (one of which is shown in Fig. 7), NU244 growth in U937 cells was comparable to that of 130b. In contrast, NU216R was as highly impaired for intracellular replication (Fig. 7), as had been reported previously (60). In other experiments, it was also observed that the original mutant, NU216, continued to be impaired for infection in U937 cells (data not shown). Thus, it appears that lesions in the linked *ira* genes result in distinct infectivity phenotypes; i.e., while disruption of *iraA* results in stable impairment of replication in macrophages, disruption of *iraB* does not. Nevertheless, we felt compelled to evaluate if NU244, given its growth impairment in low-iron media, would be impaired in iron-stressed macrophages. Consequently, infections were performed as above, but with U937 cells continuously maintained in the presence of various concentrations of DFX (10, 29, 60). In an initial experiment, we observed that the intracellular growth of NU244, like that of 130b, was not significantly inhibited in the presence of 10 μM DFX (Table 2). We subsequently repeated the experiment, assessing the effect of a higher concentration of DFX (15 μM) on these strains (Table 2). Again, NU244 recovery was not significantly different from that of 130b, although both strains begin to show signs

```
Lp IraA    46  KILDLGVGNG -9-  MPMANFTGIDVSSEMLKRASE
Aa Pmt    101  DVLEVGVGTG -6-  RPEKQITGIDLSADMLAKARE
Tm UbiE    50  KVLDLATGTG -9-  APHLEITGLDSSSKMLEIAEK
Sg CrtT    51  RVLDLGCGTG -7-  APRARITGVDASAGMLDRATA
Cp Umt     49  SLLDLCAGTG -9-  HPQASVTLVDFSSAMLDIAKQ
Rs Pmt     51  RVLEVGVGTG -6-  SHRVAVTGIDFSHEMLARARE


Consensus      oLDogsGtG -X^{6-9}  p   oTgoD S  ML  A
                                   E
```

FIG. 4. Partial alignment of the *L. pneumophila* IraA protein sequence with prokaryotic homologs. The sequences are derived from *L. pneumophila* (*Lp*) IraA, *Acetobacter aceti* (*Aa*) phosphatidylethanolamine *N*-methyltransferase (Pmt) (Hanada et al., unpublished), *Thermotoga maritima* (*Tm*) ubiquinone/menaquinone biosynthesis methyltransferase (UbiE) (53), *Streptomyces griseus* (*Sg*) carotenoid biosynthetic gene (CrtT) (68), *Chlamydia pneumoniae* (*Cp*) UbiE (S. Kalman, W. Mitchell, R. Marathe, C. Lammel, J. Fan, J. Olinger, J. Grimwood, R. W. Dain S, and R. S. Stephens, GenBank accession no. AEOO-1636 [unpublished results]), and *Rhodobacter sphaeroides* (*Rs*) Pmt (5). Alignments were achieved using CLUSTALW (72). In the consensus sequence, invariant residues are indicated in capitals, highly conserved residues are indicated in lowercase type, o indicates any hydrophobic amino acid, and s indicates a small amino acid. Regions corresponding to the two signature sequences unique to this class of proteins are indicated in bold type.

```
IraB   1   MKLISQAVKNYLPELSLRQKQTNNIIF-ITFWSQFSVYALNTILVLFLTRPLIAQGLGYS
YdgR       MSTANQKPTESVSLNAFKQPKAFYLIFSIELWERFGYYGLQGIMAVYLVKQL-----GMS
DtpT       ------------------------------MRAILVYYLYALTTADNAGL-----GLP
                                       :       :.      *      *  .

IraB   60  QAKAYAFIGVTQATGYLMPILGGYMADNIIGVRRSILLGSIMLACAYLLVMLSGYTISSL
YdgR       EADSITLFSSFSALVYGLVAIGGWLGDKVLGTKRVIMLGAIVLAIGYALVAWSGHDAG--
DtpT       KAQAMAIVSIYGALVYLSTIVGGWVADRLLGASRTIFLGGILITLGHVALATPFGLSS--
           :*.:  ::..     *   *     :**::.*.::*. * *:**.*::: .:   :   .      .

IraB   120 GDQLFIIAFAFIPATNSLLMGTASSMVSHIYSDDAIKAKSAMTIYMAINVGALLATMIAP
YdgR       ---IVYMGMAAIAVGNGLFKANPSSLLSTCYEKNDPRLDGAFTMYYMSVNIGSFFSMIAT
DtpT       ----LFVALFLIILGTGMLKPNISNMVGHLYSKDDSRRDTGFNIFVVGINMGSLIAPLIV
               . :.:  *     ..::   . *.::.  *...:  :  . .:.::      . : : :

IraB   180 VLLESRYGPLSVLTLTFIGKSIAALNFAKRYSIYDSVIWGMDKSKFSNQGLLRLVAYIAA
YdgR       PWLAAKYGWSVAFALSVVGLLITIVNFAFCQRWVKQYGSKPDFEPINYRNLLLTIIGVVA
DtpT       GTVGQGVNYHLGFSLAAIGMIFALFAYWYGRLRHFPEIGREPSNPMDAKAKRNFIITLTI
                 :       .    ::*: :*  :: . :           . :. :      :  :.

IraB   240 IYSLTLYAYTHVYIAS------------TLIGAGCAIGILWFLIKTIMLKGETRSKQMIA
YdgR       LIAIATWLLHNQEVAR-----------MALGVVAFGIVVIFGKEAFAMKGAARRKMIVA
DtpT       VLIVALIGFFLIYQASPANFINNFINVLSIIGIVVPIIYFVMMFTSKKVESDERRKLTAY
           :  ::      *          :*      . :   :  : ::. * *

IraB   288 VLLIIEAIVFFIIYNQMNSTLVLFAENNSDLSFLWFK----ISPAQYQMLNPLLILLIGS
YdgR       FILMLEAIIFFVLYSQMPTSLNFFAIRNVEHSILGLA----VEPEQYQALNPFWIIIGSP
DtpT       IPLFLSAIVFWAIEEQSSTIIAVWGESRSNLNPTWFGFTFHIDPSWYQLLNPLFIVLLSP
           . *::.**:*: : .*  :  : .:.  .:  . :     :. *  ** ***: *:: ..

IraB   344 QLPRFYRFFSR--FTIPYQFAAGTILSGIALLVMAFAAQNAIN-GLVNGNYIALTYILIS
YdgR       ILAAIYNKMGDT-LPMPTKFAIGMVMCSGAFLILPLGAKFASDAGIVSVSWLVASYGLQS
DtpT       IFVRIWNKLGDRQPSTIVKFGLGLMLTGASYLIMTLPGLLNGTSGRASALWLVLMFAVQM
           :  ::. :.    .   :*. *  :: . : *::.: .       * .. ::.  : :

IraB   401 IAELWVSAIGLSMIGLYCDARAIAFAMGVWYLASSMSNAISGRIAGWVAIPENINSALES
YdgR       IGELMISGLGLAMVAQLVPQRLMGFIMGSWFLTTAGANLIGGYVAGMMAVPDNVTDPLMS
DtpT       AGELLVSPVGLSVSTKLAPVAFQSQMMAMWFLADSTSQAINAQITPIFKA----ATEV--
           .** :* :**::       . . *. *:*: : :: *.. ::  .          :

IraB   461 LPYYKNYYLIMGISALGLGILMYFVAYYLHKIMKRRGIELA------
YdgR       LEVYGRVFLQIGVATAVIAVLMLLTAPKLHRMTQDDAADKAAKAAVA
DtpT       H-----FFAITGIIGIIVGIILLIIKKPILKLMGDVR----------
           :    *:   :.::: :      : ::
```

FIG. 5. Complete alignment of *L. pneumophila* IraB with *E. coli* YdgR (GenBank accession no. 1742700) (2) and the *Lactococcus lactis* di- and tripeptide transporter, DtpT (GenBank accession no. 625863) (33). An asterisk indicates invariant residues, a colon indicates regions of high similarity, and a dot indicates regions of low similarity. Alignments were performed using Align Plus for Windows (Scientific and Educational Software, State Line, Pa.).

of inhibition at 15 μM DFX. In a third experiment, it was observed that NU244 and the wild type behaved identically to each other whether cultured at 24, 48, or 72 h postinfection (data not shown). In the course of assessing NU244, we reexamined the DFX sensitivity of NU216R. Consistent with our earlier observation (60), NU216R was hypersensitive to intracellular iron depletion (Table 2). Thus, the growth of NU216R was inhibited by ~73% in the presence of 10 μM and ~88% in the presence of 15 μM DFX ($P < 0.05$ by Student's *t* test). Two conclusions can be drawn from these experiments. First, NU244, despite being very sensitive to growth in low-iron media, does not demonstrate hypersensitivity to intracellular iron depletion, indicating that *iraB* is critical only for extracellular iron acquisition. Second, NU216R, despite losing its EDDA inhibition phenotype in broth cultures, continued to show chelator hypersensitivity in macrophages, indicating that *iraA* is critical only for intracellular iron acquisition. Since NU216R was routinely maintained and passaged on laboratory media, a reversion of the sensitivity to iron-limited media without a

concomitant reversion of the DFX sensitivity in macrophages is not unreasonable.

Distribution of *iraA* and *iraB* in various *Legionella* species and *L. pneumophila* serogroups. The *L. pneumophila* species has 14 serogroups, all of which have been associated with disease, while the *Legionella* genus currently includes 43 species, about half of which have been associated with human disease (48, 55). Given the drastic impairment of NU216R in guinea pigs, we proceeded to determine if there was a correlation between the distribution of *iraAB* among the various *L. pneumophila* serogroups and *Legionella* species and the disease association of the organism. To do this, the presence of *iraA* and *iraB* in various *Legionella* species and *L. pneumophila* serogroups was determined by Southern hybridizations using *iraA*- and *iraB*-specific probes (Fig. 3, Table 1). Hybridization analysis under conditions of high (10% mismatch) stringency revealed that these two ORFs were present in all 11 of the *L. pneumophila* serogroups tested. Of the 14 other species examined, hybridization was observed under low (30% mismatch)-
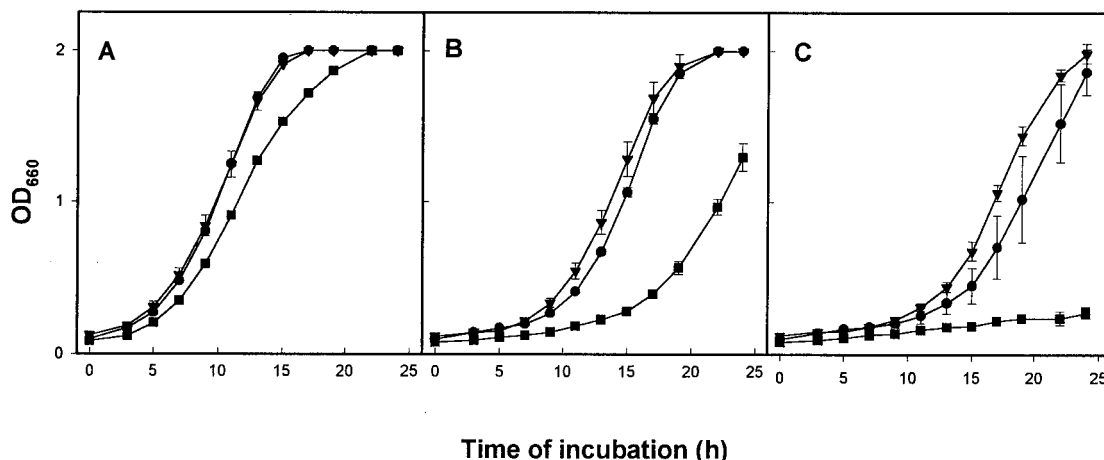
FIG. 6. Growth of *L. pneumophila* strains in BYE broth containing different amounts of free iron. Strains 130b (circles), NU216R (triangles), and NU244 (squares) were inoculated from 2-day-old BCYE agar plates at equivalent OD_{660} to BYE broth with the standard 335 μM ferric pyrophosphate supplementation (A), BYE without iron supplementation (B), and BYE with 20 μM EDDA in the absence of iron supplementation (C). OD_{660} was monitored for 24 h. Each point represents the mean ± standard error from three different cultures. In panels B and C, NU244 growth was significantly different from 130b growth after 12 and 15 h of incubation, respectively ($P < 0.05$; Student's *t* test). These data are representative of two different experiments. At 30 μM EDDA, all three strains were impaired for growth (data not shown).

stringency conditions only with DNA from *L. gormanii*. Thus, *iraAB* appears to be largely limited to *L. pneumophila*, the species that is mostly associated with disease.

DISCUSSION

In our earlier analysis (60), we had established that the *ira* mutant NU216 was defective for growth in macrophage-like U937 cells and that the infectivity defect was indeed linked to the transposon insertion. Here, we demonstrate that this mutant is also severely impaired in a guinea pig model of Legionnaires' disease. This is consistent with earlier reports showing that specific mutants defective for macrophage infection are less virulent (13, 46). Thus, while 130b established an infection in the animals, the mutant failed to elicit any signs of infection even when inoculated at 50-fold-higher levels than the wild-type strain. Consistent with this, a 1,000-fold-greater number of wild-type bacteria than of the mutant could be recovered from the animals, despite the difference in the initial inoculum. Interestingly, NU216R was more drastically impaired in the guinea pig lungs than in U937 cells, suggesting an additional role(s) for this locus in the extracellular context. Additionally, the greater difference on day 1 postinoculation between the mutant and the wild type in the spleen than in the lungs may suggest that the mutant is also defective for dissemination. The drastic impairment of NU216R following intratracheal inocu-

lation establishes *iraAB* as a significant contributor to virulence, and thus one of very few *L. pneumophila* loci whose importance has been confirmed and extended by animal models (13, 21, 46, 49, 51).

Based on the following observations, we propose that *iraAB* is a two-gene operon. First, the start codon for *iraB* is only 35 bp downstream of the *iraA* termination codon. Second, there are no other ORFs in the same direction adjacent to *iraA* and *iraB*. Third, a consensus promoter and Shine-Dalgarno sequence are present upstream of *iraA*. The absence of an appropriately positioned Shine-Dalgarno sequence upstream of *iraB* may be indicative of translational coupling between *iraA* and *iraB*. Fourth, preliminary experiments suggest that the two genes are coordinately regulated. Specifically, RNA dot blot analyses revealed that both *iraA* and *iraB* transcripts are detectable in wild-type *L. pneumophila* isolated form U937 cells while neither transcript could be detected in broth-grown cultures (V. K. Viswanathan, P. Edelstein, C. D. Pope, and N. P. Cianciotto, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998, abstr. 210, p. 90, 1998). Southern hybridization analyses suggested that the *iraAB* genes are present in all *L. pneumophila* serogroups tested, as well as *L. gormanii*. *L. gormanii* is a relatively rare isolate that has been associated with disease (54). This distribution is atypical, since most other genes examined (e.g., *mip*, *fur*, and *pilD*) are present in all legionellae

TABLE 2. Growth of *L. pneumophila* strains within DFX-treated U937 cells

Expt	Strain	10^5 CFU/monolayer recovered 48 h postinfection[a] (mean ± SE)		
		No DFX	10 μM DFX	15 μM DFX
I	130b	937 ± 55	813 ± 45 ($P = 0.16$)	ND
	NU244	907 ± 106	753 ± 74 ($P = 0.30$)	ND
II	130b	537 ± 77	420 ± 12 ($P = 0.21$)	211 ± 0.94 ($P = 0.06$)
	NU216R	6.3 ± 0.85	1.8 ± 0.50 ($P = 0.01$)	0.75 ± 0.26 ($P = 0.003$)
	NU244	583 ± 74	436 ± 23 ($P = 0.13$)	230 ± 67 ($P = 0.03$)

[a] U937 monolayers were incubated for 24 h in the presence of various concentrations of DFX. These cells were infected with equivalent numbers of the various *L. pneumophila* strains. As before (60) DFX treatment did not reduce the uptake of the legionellae (data not shown). The bacterial titers from three replicate wells were determined at 48 h. The *P* values (Student's *t* test) for the difference in recovery compared to untreated wells are indicated in parentheses.
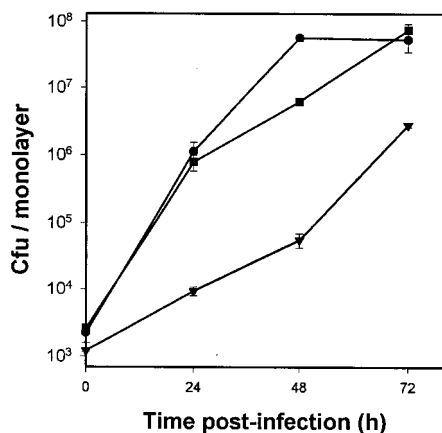
FIG. 7. Growth of *L. pneumophila* strains within U937 cells. Macrophage monolayers were infected in triplicate with 130b (circles), NU216R (triangles), and NU244 (squares) from 3-day-old BCYE agar plates. Bacterial CFU per monolayer were quantified at 0, 24, 48, and 72 h after inoculation. Each data point represents the mean ± standard error for three independent monolayers. The 48-h time point for NU244 was identical to that for 130b in two independent experiments and as such does not represent a significant difference from 130b. The difference at $t = 0$ between 130b and NU216R is consistent with the observation that the wild-type inoculum contained proportionally more bacteria. Significant differences in recovery were observed between 130b- and NU216R-infected macrophages at all times except $t = 0$ h ($P < 0.05$; Student's t test).

tested (36, 37, 47). Of the very few exceptions, *frgA* appears to be present only in *L. pneumophila* serogroups (37). The presence of *iraAB* in only two species of *Legionella* is reminiscent of the distribution of the *hbp* locus, which was found only in three *Legionella* species (56). It is likely that the *iraAB*-flanking genes *lipA* (Fig. 3) and *hisF* (located approximately 1.5 kb upstream of *iraA* [unpublished results]) are more extensively conserved among the various *Legionella* species.

Phenotypic analyses of NU216R and NU244 reveal a complex role for *iraAB* in *L. pneumophila* growth and pathogenesis. NU216R, but not NU244, is impaired for replication in macrophages, implying that IraA, but not IraB, is required for intracellular growth. The stable hypersensitivity of NU216R, but not NU244, to intracellular iron limitation indicates that the role of IraA in virulence is related to iron acquisition within macrophages. However, sequence analysis revealed that IraA is unlike any known promoter of intracellular infection or iron assimilation. Rather, IraA contains the consensus sequence shared between prokaryotic and eukaryotic small-molecule methyltransferases (35, 42). This consensus sequence is the only region of similarity among the various enzymes that use SAM as a donor to methylate a wide variety of acceptors. Indeed, mutation of the conserved residues in this region, which is thought to be the SAM-binding site, eliminates enzyme activity (35). As a group, the small-molecule methyltransferases catalyze reactions leading to the biosynthesis of compounds such as phosphatidylcholine (PC), ubiquinone, and carotenoids (5, 52, 69). Given that these compounds are involved in functions as diverse as osmoprotection, aerobic respiration, and pigmentation, respectively, it is not unreasonable to suspect that IraA could be involved in iron acquisition. The substrate for the putative IraA enzyme obviously remains to be determined. However, BLAST analyses revealed that the strongest overall match between IraA and the database is with the *Acetobacter aceti* phosphatidylethanolamine methyltransferase, an enzyme involved in the conversion of phosphatidylethanolamine to PC (T. Hanada, Y. Koizumi, S. Udaka, and F. Yanagida, GenBank accession no. BAA34057 [unpublished

results]). Interestingly, while very few bacteria produce PC, 10 different isolates of *L. pneumophila* were demonstrated to have unusually high PC content, and PC was the most abundant phospholipid in almost all of them (24). Taken together, our results suggest a novel role for IraA in iron acquisition and virulence. It remains to be established if these two phenotypes are directly linked or if IraA has a function that indirectly affects both iron acquisition and virulence. Although the importance of IraA for intracellular iron acquisition is clear, it is not obvious if IraA also plays a role in extracellular iron uptake. While both broth-grown NU216 and NU216R were initially hypersensitive to EDDA, they subsequently lost this phenotype without the concomitant loss of either their transposons or infectivity defects. An unrelated second-site mutation, possibly one that enhanced iron uptake only in broth cultures, may have resulted in a partial complementation of the *iraA* phenotype. Alternatively, the EDDA hypersensitivity of NU216 and NU216R may not be directly due to a loss of IraA but, rather, may have resulted from a polar effect on *iraB* expression (see below). Loss of polarity, possibly by enhanced expression of *iraB* from a cryptic promoter, could explain the loss of the EDDA hypersensitivity.

The hypersensitivity of NU244 to iron starvation indicates that IraB is required for *L. pneumophila* extracellular iron uptake. However, IraB, like IraA, appears unlike any other iron uptake system. Several lines of evidence suggest that IraB is a homolog of the PTR2 family of peptide transporters. First, IraB shows strong similarity to di- and tripeptide transporters from various prokaryotic and eukaryotic organisms. Second, the alignment with these proteins is along the entire length of the sequence. Third, the IraB sequence AMTIYMAINVGAL (Fig. 3) is very similar to the PTR family signature sequence F-(S/T/N/M)-X-F-(V/Y/F)-h-X-I-N-h-G-(S/A)-h (where h indicates a hydrophobic residue). A second region of consensus unique to this family of proteins is also present in IraB (GGY MADNIIGVRRSILLGSIMLACA) (Fig. 3) (34). Fourth, like the PTR2 homolog DtpT from *L. lactis*, IraB is predicted to have 12 membrane-spanning regions (32, 34). In most microorganisms, this family represents but one of several systems of peptide transport (58). DtpT has been demonstrated to transport di- and tripeptides via a proton motive force-dependent mechanism. While it primarily transports hydrophilic and neutral peptides such as Ala-Glu, Ala-Ala, Leu-Leu, Gly-Asp, Pro-Gly, Glu-Glu, and Asp-Glu (26, 33), this protein can also mediate the uptake of peptides with two negative charges, such as Glu-Glu and Asp-Glu. It has been suggested that DtpT is required for *Lactococcus* to use caseins as the sole source of nitrogen (26). The role of di- and tripeptide transporters in other prokaryotes is not known, but we hypothesize that *L. pneumophila* utilizes IraB to import iron-loaded peptides as a source of iron. Although peptides can be components of siderophores, there have been as yet no reports of free peptides involved in iron transport in microorganisms (18). However, iron acquisition through low-molecular-weight chelates such as glycyl-L-histidyl-L-lysine has been observed in human macrophages, and cysteine-containing N-terminal peptides enhance iron uptake in Caco-2 intestinal cells (30). This study, however, does not rule out an indirect relationship between peptide transport and iron uptake.

In summary, the juxtaposition of the genes for a putative methyltransferase and a peptide transporter represents a unique situation. This is the first report of a possible link between peptide transport, a small-molecule methyltransferase, and iron acquisition. Therefore, the *iraAB* locus may encode a novel mechanism of iron transport that is relevant in different environments, including the mammalian lung.

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