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Legionella pneumophila Mutants That Are Defective for Iron Acquisition and Assimilation and Intracellular Infection

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***Legionella pneumophila*, a parasite of macrophages and protozoa, requires iron for optimal extracellular and intracellular growth. However, its mechanisms of iron acquisition remain uncharacterized. Using mini-Tn*10* mutagenesis, we isolated 17 unique *L. pneumophila* strains which appeared to be defective for iron acquisition and assimilation. Eleven of these mutants were both sensitive to the iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) and resistant to streptonigrin, an antibiotic whose lethal effect requires high levels of intracellular iron. Six mutants were also defective for the infection of macrophage-like U937 cells. Although none were altered in entry, mutants generally exhibited prolonged lag phases and in some cases replicated at slower rates. Overall, the reduced recoveries of mutants, relative to that of the wild type, ranged from 3- to 1,000-fold. Strain NU216, the mutant displaying the most severe lag phase and the slowest rate of replication, was studied further. Importantly, within U937 cells, NU216 was approximately 100-fold more sensitive than the wild type was to treatment with the Fe^{3+} chelator deferoxamine, indicating that it is defective for intracellular iron acquisition and assimilation. Furthermore, this strain was unable to mediate any cytopathic effect and was impaired for infectivity of an amoebal host. Taken together, the isolation of these mutants offers genetic proof that iron acquisition and assimilation are critical for intracellular infection by *L. pneumophila*.**

Legionella pneumophila, the bacterium responsible for the majority of cases of Legionnaires' disease, is a facultative intracellular parasite (9, 14, 59). In its natural aquatic environment as well as man-made water systems, *L. pneumophila* survives within a variety of protozoa (20, 51). Similarly, the pathogenesis of legionellosis is absolutely dependent on the ability of the microbe to replicate within alveolar macrophages (28). To date, research has identified only a handful of the factors required for optimal intracellular infection (14, 39). For example, the major outer membrane porin OmpS mediates attachment to CR1 and CR3 on monocytes (2, 27). In addition, whereas *mip* potentiates survival immediately after entry into macrophages and protozoa (10, 11), the *icm* and *dot* loci are essential for the replicative phase of intracellular infection (3, 5, 52). Finally, the *hel* locus promotes host cell killing (1).

Three lines of evidence signal that the ability of *L. pneumophila* to replicate within mammalian cells is dependent on iron. First, human peripheral blood monocytes treated with iron chelators, such as deferoxamine (DFX), apotransferrin, and apolactoferrin, do not support *Legionella* replication, a condition reversed by the addition of ferric iron (6, 7, 28). Second, gamma interferon inhibits *L. pneumophila* growth by reducing the amount of intracellular iron (6). Third, peritoneal macrophages from A/J mice become permissive for *Legionella* infection after the addition of iron (22). As determined on bacteriologic media, the ferric or ferrous iron requirement for *L. pneumophila* is 3 to 13 μM for minimal growth and >20 μM for optimal growth (30, 34, 41, 47, 48). It has been argued that one potential reason for this unusually high-level iron requirement is that the *L. pneumophila* cytoplasm may contain a high concentration of an Fe-containing aconitase (31, 34). Given these observations and the fact that some bacterial (i.e., *Salmonella*) phagosomes can contain as little as 0.1 μM iron (45), we suspect that the intracellular environment can be iron limiting for *L. pneumophila* and thus requires the expression of iron acquisition functions.

Little is understood about how *L. pneumophila* acquires and assimilates iron; however, it does not possess a number of functions described for other pathogenic bacteria. For example, it does not produce hydroxamate or phenolate siderophores when grown in iron-deficient media (47). Similarly, *L. pneumophila* does not bind transferrin and cannot use it as an iron source (30, 46). Finally, although lactoferrin binding has been detected, it does not promote bacterial growth (4). An iron binding activity has been detected in *L. pneumophila* supernatants; however, the basis for this activity remains unclear (24, 33). On the other hand, *L. pneumophila* does express ferric reductases within its cytoplasm and periplasm (30, 42), and a locus (*hbp*) that promotes hemin binding has been defined (37). In addition, *Legionella* spp. possess Fur, a transcriptional regulator which responds to iron levels (25). Recently, several iron- and Fur-regulated *L. pneumophila* genes were detected and a subset of these were implicated in macrophage infection (26).

We have adopted an alternative genetic approach to identify factors involved in both iron acquisition and intracellular infection. After mini-Tn*10* mutagenesis (43), we isolated 17 mutants that appeared to be defective for some stage of iron acquisition and assimilation. Importantly, six of these strains were also defective for macrophage infectivity.

(Portions of this work were previously presented [38, 44].)

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *L. pneumophila* 130b (Wadsworth), a clinical isolate and a member of serogroup 1, was previously described (18). To facilitate the identification of genes involved in iron acquisition and intracellular infection, we had previously mutagenized this strain with a kanamycin resistance (Km^r)-bearing mini-Tn*10* (43). After electroporation with the transposon delivery vector pCDP05, greater than 96% of the Km^r bacteria contained single, unique, and stable mini-Tn*10* insertions.

Legionellae were generally grown on buffered charcoal yeast extract (BCYE) agar without the ferric pyrophosphate supplement for 48 h at 37°C (16). How-

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ever, when mini-Tn*10*-containing strains were passaged, kanamycin was added to the medium at 25 μg/ml. The mutagenized *L. pneumophila* library was also plated on complete BCYE agar containing the antibiotic streptonigrin at 0.5 to 2.0 μg/ml. To render BCYE iron limiting, we added the ferric iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA; Sigma Chemical Co., St. Louis, Mo.). It has been demonstrated that EDDA limits *L. pneumophila* extracellular growth by scavenging Fe^{3+} (41). Prior to incorporating EDDA into media, it was deferrated in the manner described by Rogers (50). We did note, however, that the iron chelating efficiency of the deferrated stocks varied and decreased with storage. EDDA gradient plates consisted of a lower, sloped layer of 30 ml of BCYE agar containing either 150 or 175 μM chelator, which was then covered with 30 ml of plain BCYE. Finally, BCYE agar containing 100 mM NaCl was used to determine the salt sensitivities of *L. pneumophila* strains (52).

Plasmid pCDP05 was maintained in *Escherichia coli* HB101 grown on Luria-Bertani medium supplemented with 50 μg of kanamycin per ml (43). *mip*-encoding pSMJ31.42 (17), *hel*-bearing pJA2 (a gift from M. McClain and N. C. Engleberg), and *hbp*-containing pEH12 (37) were used as probes in Southern hybridizations. They were maintained in HB101 grown on Luria-Bertani agar with 50 μg of ampicillin per ml. The counterselectable vector pBOC20 was used for allelic-exchange mutagenesis (37). *E. coli* cells harboring this plasmid were maintained on media containing 30 μg of chloramphenicol per ml.

Southern hybridization analysis. Total DNA from *L. pneumophila* was purified as previously described (18). Plasmids were isolated from HB101 by standard alkaline lysis and radiolabeled with ^{32}P by using a random primer kit (Gibco-BRL, Gaithersburg, Md.). Southern hybridizations were performed under high-stringency conditions (10).

Allelic-exchange mutagenesis. The procedure for allelic exchange with ColE1 vectors containing the *sacB* counterselectable marker was previously described (12, 36, 37). Briefly, pBOC20 containing insertionally inactivated *L. pneumophila* DNA was electroporated into 130b, and transformants were selected on BCYE agar supplemented with kanamycin and 5% sucrose. The resultant colonies were replica plated onto BCYE containing 3 μg of chloramphenicol per ml. Kanamycin-resistant (Kan^r), sucrose-resistant (Suc^r), chloramphenicol-sensitive (Chl^s) colonies represent strains that have undergone allelic exchange.

Intracellular infection of U937 cells with *L. pneumophila*. U937 cells, a human macrophage-like cell line, have been routinely used for *L. pneumophila* infectivity studies (3, 10, 32, 40, 49). They were maintained as replicative, nonadherent, monocyte-like cells in RPMI medium–10% fetal calf serum (Gibco-BRL). After phorbol 12-myristate 13-acetate (Sigma) treatment, nonreplicative, adherent, macrophage-like cells were infected with bacteria as previously described (40). To assess the relative infectivities of *L. pneumophila* strains for U937 cells, 50% infective doses (ID_{50}) were determined after 24 h of incubation (10). To monitor intracellular growth rates, replicate monolayers were inoculated with approximately 10^6 bacteria and incubated for various times and then lysates were plated for CFU (10, 36). Finally, to assess the cytopathic effects of *L. pneumophila* strains, the viability of the U937 monolayer was determined as previously described (40). Briefly, replicate monolayers were infected, and at various intervals, the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl-tetrazolium bromide (MTT; Sigma) was added. The amount of reduced formazan within monolayers was assessed by reading the optical density at 540 nm. Iron limitation within U937 cells was accomplished by the addition of 5, 7, or 10 μM DFX (Sigma) to the medium for 24 h prior to infection with *L. pneumophila* and during the incubation period (7, 22). In agreement with earlier studies, the viability of U937 monolayers, as measured by MTT assay, was not affected by any of these DFX concentrations.

Infection of *Hartmannella vermiformis* with *L. pneumophila*. To assess growth in protozoa, we utilized *H. vermiformis* as the host. *Hartmannella* amoebae have been associated with *Legionella* spp. in water samples implicated in cases of Legionnaires' disease (20). Amoebae were maintained in Axenic Media 1034 and were infected essentially as described before (11, 32). First, amoebae were brought to 10^5 cells per ml, and then 2 ml of cell suspension was added to replicate wells on 24-well tissue culture plates. Next, bacteria were added at a final concentration of 10^3 CFU/ml, and cocultures were incubated for 5 days at 35°C. The numbers of bacteria within culture were enumerated at 0, 24, 42, 52, 70, 80, 96, and 120 h postinoculation by plating aliquots from each well onto BCYE agar.

RESULTS

Isolation of *L. pneumophila* mutants which are sensitive to an iron chelator and/or resistant to streptonigrin. As a first approach toward isolating iron acquisition mutants, we screened a mini-Tn*10*-mutagenized *L. pneumophila* population for strains exhibiting defective growth on iron-poor media. Specifically, 2,448 kanamycin-resistant mutants were replica plated with a toothpick onto BCYE agar supplemented with 140 μM Fe^{3+} chelator EDDA, a concentration at which the wild-type strain was not inhibited. With an affinity constant of 10^{34} (35), EDDA is routinely used to identify and characterize iron acquisition mutants in a variety of bacteria (8, 53, 54, 56). Twelve *L. pneumophila* isolates (NU205 through NU216) appeared to be defective for growth on EDDA-containing media. To determine whether each of these EDDA-sensitive ($EDDA^s$) strains was unique, we used Southern hybridization analysis to localize the mini-Tn*10* within each mutant. To more easily compare strains, genomic DNAs were digested with enzymes that have recognition sites within mini-Tn*10* and thus should yield two fragments hybridizing with a transposon probe (Fig. 1A). Analysis of *Ava*I-, *Cla*I-, and *Hin*dIII-digested DNA indicated that 11 of the EDDA-sensitive strains contained a single, unique mini-Tn*10* insertion. On the other hand, NU213 (Fig. 1A, lane i) appeared to contain two transposon insertions. Thus, in this first screen, we identified 12 potential $EDDA^s$ mutants.

Both to confirm the EDDA sensitivity of these strains and to assess the degree of that sensitivity, we spotted equivalent numbers of bacteria onto BCYE agar plates containing a gradient of this iron chelator. Although NU206 and NU209 appeared to be resistant then (data not shown), the other 10 strains remained sensitive. Two levels of impaired growth were seen (Fig. 2). The first group of strains was easily distinguished from 130b on gradients of 0 to 150 μM EDDA (Fig. 2A). Within this group, NU215 was only slightly inhibited, while NU208, NU210, NU214, and NU216 were much more sensitive to this chelator. Interestingly, NU214 consistently exhibited small colonies atop its spots, suggesting that some bacteria may be capable of reverting to a more resistant phenotype. The second group of five strains (NU205, NU207, NU211, NU212, and NU213) grew as well as the wild type did on the shallow gradient; however, they showed a range of inhibition on 0 to 175 μM EDDA gradients (Fig. 2B). To confirm that the poor growth on EDDA-containing media represents iron deficiency, we assessed colony formation on BCYE agar supplemented with equimolar amounts of ferric pyrophosphate and this chelator. Indeed, in all cases, iron supplementation restored growth to wild-type levels (data not shown). Thus, by using EDDA sensitivity as a screen, we identified 10 potential iron acquisition mutants.

As an alternative approach toward isolating iron acquisition mutants, we screened our mini-Tn*10*-mutagenized population for strains resistant to streptonigrin. As first demonstrated in *E. coli*, the toxicity of streptonigrin is highly dependent upon intracellular iron levels (58). Thus, in a variety of bacterial systems, antibiotic-resistant strains represent iron uptake mutants (15, 23, 60). Streptonigrin-resistant (Sng^r) legionellae were obtained at a frequency of 10^{-5}; we chose nine of them (designated strains NU217 through NU225) for further analysis. Although these mutants were isolated on BCYE agar supplemented with 0.5 μM streptonigrin, all grew comparably well on media containing 0.65 μM streptonigrin. However, only four, NU220, NU223, NU224, and NU225, multiplied in the presence of 0.8 μM streptonigrin. Interestingly, strains NU223 and NU224 grew on BCYE agar containing as much as 2.0 μM this antibiotic. Southern hybridization analysis indicated that among these nine were seven unique mutants (Fig. 1B).

Although the 10 confirmed $EDDA^s$ mutants were clearly distinct from the 7 Sng^r strains (Fig. 1), we sought to determine whether they shared iron-related phenotypes. Thus, the streptonigrin resistance of the $EDDA^s$ strains and the sensitivity of the Sng^r mutants to this iron chelator were determined. In the majority of cases (i.e., 11 of 17 strains), EDDA sensitivity correlated with resistance to streptonigrin (Table 1). On the other hand, three mutants isolated on the basis of EDDA phenotype were Sng^s and three of the original Sng^r mutants

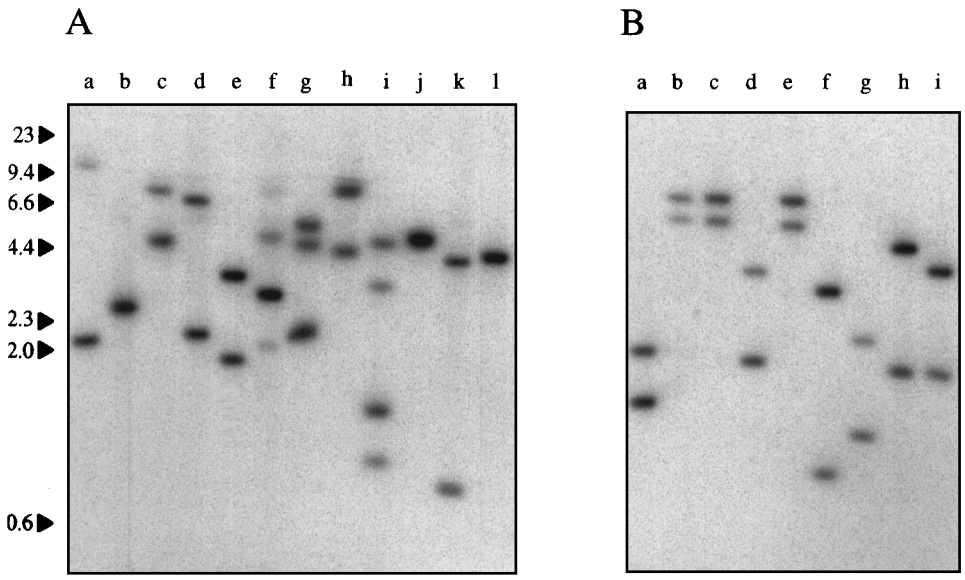

FIG. 1. Southern hybridization analysis of *L. pneumophila* mutants. Genomic DNAs were digested with *Ava*I and electrophoresed through 0.8% agarose. A Southern blot was made and hybridized with ^{32}P-labeled pCDP05. (A) Lanes: a, NU205; b, NU206; c, NU207; d, NU208; e, NU209; f, NU210; g, NU211; h, NU212; i, NU213; j, NU214; k, NU215; l, NU216. (B) Lanes: a, NU217; b, NU218; c, NU219; d, NU220; e, NU221; f, NU222; g, NU223; h, NU224; i, NU225. Analyses of *Cla*I and *Hin*dIII digests showed that the additional band in lanes f and g of panel A represents a partial digestion product. Although only one band is seen in lanes b, j, and l of panel A, the other restriction enzymes generated two hybridizing fragments. The migrations and sizes (in kilobases) of molecular markers are indicated.

grew as well as the wild type did on iron-depleted BCYE agar. Thus, by combining two distinct screening methods, we identified at least 11, and perhaps as many as 17, mutants that appeared to be defective for iron acquisition and assimilation. We employ the term iron acquisition and assimilation in a broad sense, denoting events from binding at the cell surface and transport through the cell wall to incorporation as a cofactor or stored nutrient.

Infectivity of *L. pneumophila* mutants for human macrophage-like cells. To focus attention on the mechanisms of intracellular iron acquisition and assimilation, we sought to determine which of our new mutants were defective for macrophage infection. Consequently, we assessed the abilities of the 17 unique mutants to grow within U937 cells by culturing inoculated monolayers 24 h after infection (Table 1). To highlight the mutants most defective in intracellular iron acquisition, we did not introduce any additional iron limitations into our systems. Thus, bacterial inocula were derived from standard BCYE cultures and U937 cells were not initially treated with any iron chelating agents. Nine of the EDDA^s strains showed a ≤twofold increase in ID_{50}, indicating no important defect in intracellular growth. However, the seven mutants isolated on the basis of their streptonigrin resistance averaged ID_{50} that were 3- to 14-fold higher than that of the wild type. Finally, NU216 was the most impaired, exhibiting an average increase in ID_{50} of approximately 200-fold.

To confirm and begin to define the infectivity defects of the eight potentially impaired mutants, we assessed their growth kinetics within U937 cells (Fig. 3). These mutants exhibited three types of growth defects. In the first case, NU217 initially grew as well as did 130b; however, between 18 and 30 h postinoculation, its growth rate declined slightly (Fig. 3A). The second type of defect was exhibited by four of the Sng^r mutants, which displayed prolonged lag phases but then grew at

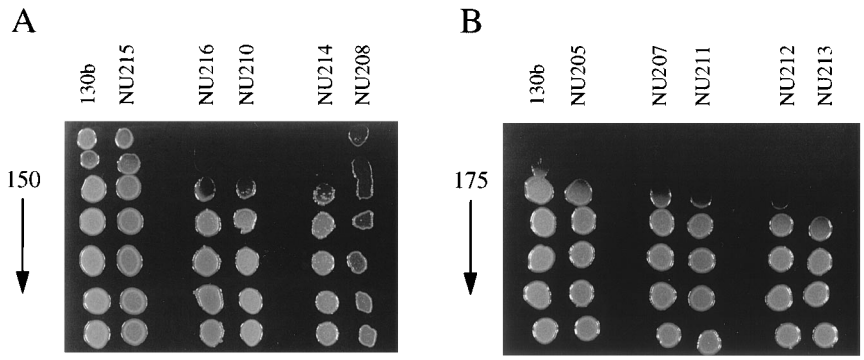

FIG. 2. EDDA sensitivity of *L. pneumophila* strains. Bacteria harvested from BCYE agar were suspended in sterile water, and then 5-μl aliquots containing approximately 5×10^4 CFU were spotted in a row on EDDA gradient plates. (A) Gradient (0 to 150 μM) plates after incubation for 72 h at 37°C; (B) gradient (0 to 175 μM) plates after 72 h at 37°C. The maximum chelator concentration is indicated to the left of each panel, and arrows indicate decreasing concentrations of EDDA. Although not readily apparent here, NU215 demonstrated slight EDDA sensitivity on several other occasions.

TABLE 1. Phenotypes of *L. pneumophila* strains

Strain	Phenotype[a]		ID_{50} ratio[b]
	EDDA[c]	Streptonigrin[d]	
130b	R	S	1, 1
NU205	S	S	2, 0.1
NU207	S	R	1, 3
NU208	S	S	0.1, 1
NU210	S	S	1, 1
NU211	S	R	1, 1
NU212	S	R	1, ND
NU213	S	R	1, 1
NU214	S	R	1, ND
NU215	S	R	1, ND
NU216	S	R	280, 141
NU217	R	R	3, 3
NU218	S	R	3, 3
NU220	S	R	4, 5
NU222	S	R	23, 5
NU223	R	R	15, 12
NU224	R	R	6, 2
NU225	S	R	6, 2

[a] R, resistance; S, sensitivity.
[b] ID_{50} ratios were calculated as the ID_{50} of the mutant divided by the ID_{50} of strain 130b and rounded to the nearest decimal. Strains were tested twice unless otherwise indicated (ND).
[c] Assessed on BCYE gradient plates with 0 to 150 or 175 μM EDDA.
[d] Generally determined on BCYE agar supplemented with 0.5 or 0.65 μM streptonigrin. For strains NU212 and NU216, Sng^r was manifest only on media that also contained EDDA at the subinhibitory concentration of 60 μM.

rates that were comparable to that of the wild type (Fig. 3B to D). However, the extent of that prolonged lag phase differed among strains, ranging from slight for strain NU220 (Fig. 3B) to quite pronounced for strain NU218 (Fig. 3D). In the third case, NU216, the mutant with the highest ID_{50}, exhibited an even more severe lag phase and replicated at a significantly slower rate (Fig. 3E). In addition, this mutant often showed a decrease in intracellular bacteria at 12 to 24 h postinoculation. None of these six mutants, however, showed reduced recoverability at 0 h, suggesting that they are not defective for attachment, entry, or resistance to immediate intracellular killing (Fig. 3). Overall, the reduced recoveries of mutants (relative to that of 130b at 48 h) ranged from 3- (NU217) and 10-fold (NU218) to 1,000-fold (NU216). In contrast, the growth kinetics of Sng^r NU222 and NU224 were identical to those of strain 130b (Fig. 3F and data not shown). This result was particularly curious for strain NU222 since it had, at one point, displayed an ID_{50} that was 23-fold greater than that of the wild type (Table 1). However, the ID_{50} of NU222 in its second trial was only fivefold higher than that of strain 130b, indicating that plate passage of this mutant may allow for phenotypic reversion of the infectivity defect. In summary, we have isolated 11 strains that appear to contain defects in extracellular iron acquisition and assimilation and, more importantly, 6 mutants that seem defective for both iron-related functions and macrophage infection. Several additional observations confirmed that these mutants were also distinct from other *L. pneumophila* mutants known to be defective for (heme) iron acquisition or macrophage infection. First, Southern hybridization analysis indicated that the mini-Tn*10* insertions within these strains were not in *hbp*, *mip*, or *hel* (data not shown). Second,

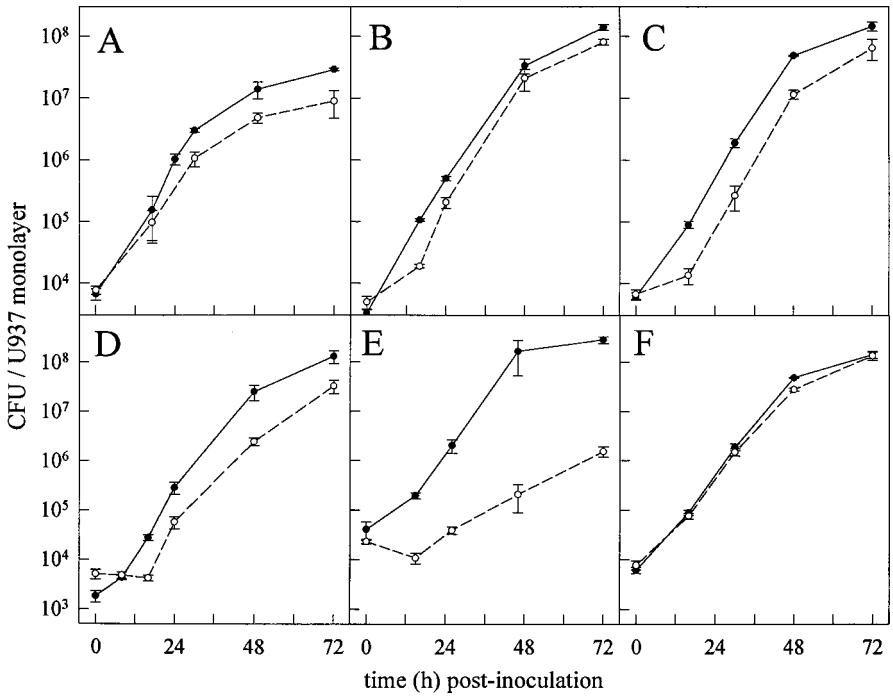

FIG. 3. Replication of *L. pneumophila* mutants within U937 cells. Monolayers ($n = 4$) were inoculated with approximately 5×10^5 CFU of either 130b (●) or a mutant (○), and after various incubation periods, the numbers of viable intracellular bacteria were determined. The mutants tested included NU217 (A), NU220 (B), NU223 (C), NU218 (D), NU216 (E), and NU222 (F). The intracellular replication of NU225 was similar to that of NU220 (data not shown). Since the inoculation of U937 cell monolayers includes a 2-h entry phase and several wash steps, the 0-h samples were actually obtained at about 3 h after bacterium-host cell interactions began. In panel D, the difference in CFU at 0 h is a reflection of unintentional differences in inoculum sizes. Each point represents the mean CFU recovered, and vertical bars indicate standard deviations. The differences in the recoveries of these strains were significant at all time points without overlapping error bars ($P < 0.005$; Student's *t* test).

unlike *icm* and other Mak$^-$ mutants, these strains did not grow on BCYE agar containing 100 mM NaCl (52).

Macrophage infectivity defects of *L. pneumophila* NU216. Given its severe infectivity defect, strain NU216 became the focus of our immediate attention. To support the notion that NU216 is defective for intracellular iron acquisition and assimilation, we assessed its relative ability to grow in DFX-treated U937 cells. The treatment of macrophages with DFX is well known to inhibit *L. pneumophila* replication by limiting intracellular iron availability (7, 22). In three trials, 7 μM DFX reduced the recovery of NU216 by an average of 94%, with little, if any, effect on the recovery of 130b (Table 2). A similar result was seen after the treatment of U937 cells with 5 μM DFX; however, the magnitude of growth inhibition for NU216 was smaller and the growth of 130b was never impaired (data not shown). At 10 μM DFX, however, the intracellular growth of both NU216 and 130b were inhibited. In conclusion, we believe that NU216 is indeed defective for intracellular iron acquisition and assimilation.

To determine if the delayed intracellular replication of NU216 (Fig. 3E) was due to the outgrowth of a revertant, we recovered bacteria from infected wells at 72 h postinoculation and assessed their kanamycin resistance. None of the 300 colonies tested had lost their Kan^r phenotype, indicating that if reversion occurs, it does so at a frequency of less than 10^{-3}. More importantly, NU216 recovered from infected wells was still impaired for macrophage infection. The ID_{50} of these bacteria was approximately 200-fold greater than that of the wild type, a value similar to that of plate-grown NU216 (Table 1). Thus, the mutant bacteria which survived and replicated within the monolayer were not revertants. During kinetic assays, we noticed that the U937 monolayers infected with NU216 were not as disrupted as those infected with 130b. As seen under an inverted microscope, wells infected with 130b contained few intact or normal-looking host cells; however, those infected with NU216 looked like the uninfected controls. To confirm this, cytopathicity was assessed by the standard MTT assay (Fig. 4). Whereas infection with 10^6 CFU of 130b caused maximal damage to the host monolayer within 48 h, NU216 did not produce any significant cytopathic effect even after being cultured for 96 h. Since NU216 can ultimately achieve 10^6 CFU per infected well, a level of growth that induces cytopathicity by the wild-type strain (Fig. 3 and 4), we next tested the possibility that this mutant is not able to lyse U937 cells. Since host cell lysis results in bacterial release into the supernatant, we simply enumerated extracellular bacteria at 72 h postinfection. For both strains, approximately 10% of the total number of bacteria in each well were in the supernatant, suggesting that NU216 is not defective for lysis of infected macrophages (data not shown). Taken together, these observations indicate that NU216 is defective for both intracellular replication and cytopathicity.

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

Expt	% Reduction in CFU from DFX-treated U937 cells[a]		
	130b	NU216	NU216R
1	0	85	55
2	0	98	98
3	22	98	98

[a] Within wells, CFU were determined at 72 h postinoculation. The bacterial titers from four replicate wells were averaged, and the percent reduction was calculated as the average CFU from untreated wells minus the average CFU from wells treated with 7 μM DFX divided by the average CFU from untreated wells. As expected (7, 20), DFX treatment did not reduce the uptake of either 130b or NU216 (data not shown).

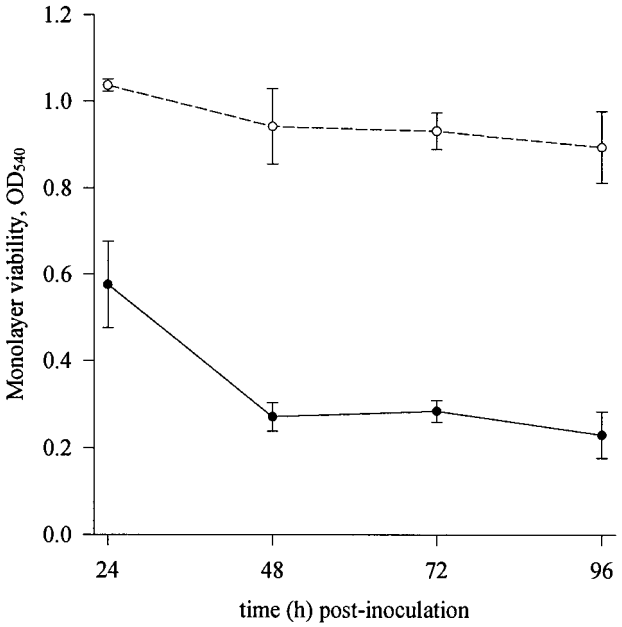

FIG. 4. Cytopathic effects of strains 130b and NU216 on U937 cells. Replicate monolayers (n = 10) were infected with ca. 10^6 CFU of either 130b (●) or NU216 (○). After various periods of incubation, the viability of host cells was measured by their ability to convert the vital dye MTT to formazan. Each point represents the mean optical density at 540 nm (OD_{540}), and vertical bars indicate standard deviations. The differences in cytopathicity between strains were significant at all time points ($P < 0.001$; Student's t test).

To establish whether the infectivity defect of NU216 was caused by the mini-Tn*10* insertion and not a spontaneous second-site mutation, we used allelic exchange to reintroduce the transposon mutation into 130b. First, an approximately 8-kb *Sal*I fragment containing mini-Tn*10* was isolated from NU216 and cloned into vector pBOC20. After electroporation of the resultant pCDP70 into 130b, four Kan^r, Suc^r, Chl^s colonies were examined by Southern blot analysis. Two of these strains had undergone the DNA rearrangement detected in NU216, i.e., a 3-kb, pCDP70-hybridizing *Eco*RI fragment was replaced by a 5-kb hybridizing fragment (data not shown). One of these confirmed mutants, NU216R, was tested for the defective phenotypes described for NU216. NU216R was sensitive to iron limitation, as was NU216, showing heightened growth inhibition on BCYE agar containing 80 μM EDDA as well as on 150 μM EDDA gradient plates (data not shown). The intracellular growth of NU216R was similar to that of the original mutant, exhibiting slight declines in the CFU recovered at time points prior to 24 h and achieving titers of only 10^6 CFU after 72 h of incubation (Fig. 5). Likewise, treatment of the U937 cell monolayer with 7 μM DFX resulted in greater growth inhibition for NU216R than for 130b (Table 2). Finally, as with NU216, the reconstructed mutant demonstrated a notable decrease in cytopathicity (data not shown). Thus, it is likely that the defective growth of NU216 in both iron-limited extracellular and intracellular environments is due to the mini-Tn*10* insertion mutation.

Intracellular growth of *L. pneumophila* NU216 within amoebae. Protozoa are a reservoir for *L. pneumophila* in freshwater and man-made water systems (11, 20, 32, 51). To assess the relative ability of NU216 to grow within a natural host, we studied bacterial replication within *H. vermiformis* cultures

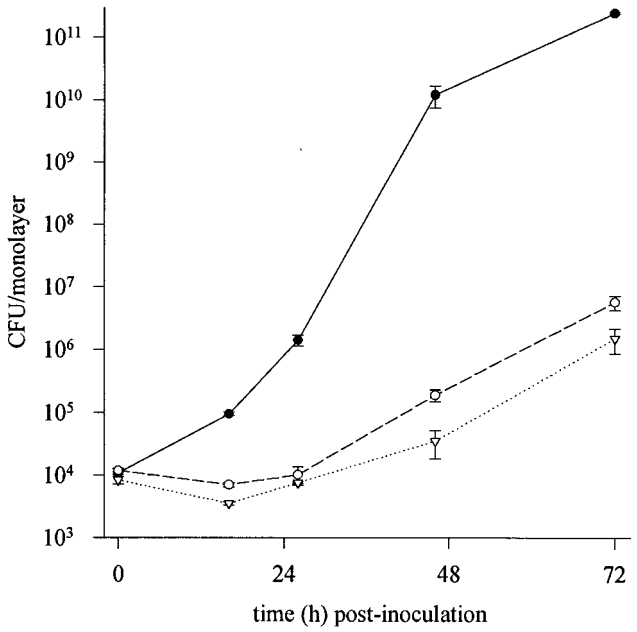

FIG. 5. Replication of NU216R within U937 cells. Monolayers ($n = 4$) were inoculated with ca. 5×10^5 CFU of either 130b (●), NU216 (○), or NU216R (▽), and after various incubation periods, the numbers of viable intracellular bacteria were determined. Each point represents the mean CFU recovered, and vertical bars indicate standard deviations. The differences between the recovery of 130b and those of NU216 and NU216R were significant at all time points without overlapping error bars ($P < 0.001$; Student's t test). The numbers of intracellular NU216 and NU216R did not differ significantly, except at the 72-h time point ($P < 0.05$).

(Fig. 6). The growth of this mutant in amoebae was not as robust as that of the wild type. NU216 displayed a lag between days 1 and 2, and the numbers ultimately reached were 10-fold less than those of strain 130b. The difference between strains was clearly not as great in this system as it was in U937 cells. Although there are a number of reasons why this might have occurred, the *Hartmannella* coculture system simply may not be as iron limiting as U937 cells are.

DISCUSSION

We have identified a set of 17 *L. pneumophila* mutants that appear to be defective for iron acquisition and assimilation. For 11 of these mutants (NU207, NU211, NU212, NU213, NU214, NU215, NU216, NU218, NU220, NU222, and NU225), the evidence provided by four lines of inquiry is strong. First, these strains were sensitive to EDDA, a chelator with a very high affinity for ferric iron and relatively low affinities for other metals (21, 35). Second, they grew as well as the wild type did on EDDA-containing media supplemented with an equimolar amount of iron. Third, they were resistant to streptonigrin, an antibiotic whose lethal effect is dependent on the presence of intracellular iron (58). Finally, at least one of these mutants (NU216) was sensitive to DFX during intracellular growth. Given these data, we hereafter refer to these 11 strains as *ira* (iron acquisition and assimilation) mutants. The remaining six mutants fell into two classes, and the argument that they bear iron-related defects is less compelling. Mutants in the first set (NU205, NU208, and NU210) were sensitive to EDDA but were not resistant to streptonigrin. These strains may have impaired iron uptake, but a linked inability to properly process or store iron might facilitate streptonigrin-mediated DNA or membrane damage (13). Alternatively, since iron helps to maintain cell wall structure (19), the EDDA sensitivity of these strains might reflect destabilization of the cell due to chelation at its surface. Finally, poor growth on EDDA-containing media might be caused by reductions in the internalization of metals other than iron. Mutants in the second set (NU217, NU223, and NU224) were resistant to streptonigrin but were not sensitive to EDDA. Several explanations for this observation are also possible. For example, alterations in intracellular processing and storage might preclude the interaction between this antibiotic and iron which normally results in DNA damage. Alternatively, streptonigrin resistance may not be linked to iron but to diminished antibiotic transport into cells or heightened transport out of cells. Finally, although iron is most effective in mediating the lethal effect of streptonigrin, several other metals, such as Cu^{2+}, can interact with streptonigrin and potentiate DNA strand breakage (55). Given the possibility that NU205, NU208, NU210, NU217, NU223, and NU224 contain iron acquisition and assimilation deficiencies, we hereafter tentatively call them *ira* mutants as well.

Six of the *ira* mutants (NU216, NU217, NU218, NU220, NU223, and NU225) exhibited defects in macrophage infection. A number of observations indicate that these defects are compatible with reduced intracellular iron acquisition and assimilation. First, none of these strains were completely inhibited in infectivity. Given that bacteria generally have multiple iron uptake systems and can utilize various iron sources (57), it seems unlikely that the elimination of one *ira* locus would abolish growth within unstressed macrophages. Indeed, among a set of 55 *L. pneumophila* (*icm*) mutants (selected from 4,536 Tn*903*-induced mutants) that exhibited greater than 16-fold reductions in macrophage infectivity, none were sensitive to iron chelators (52). Second, *ira* strains were not defective for entry or resistance to immediate bactericidal functions, phases

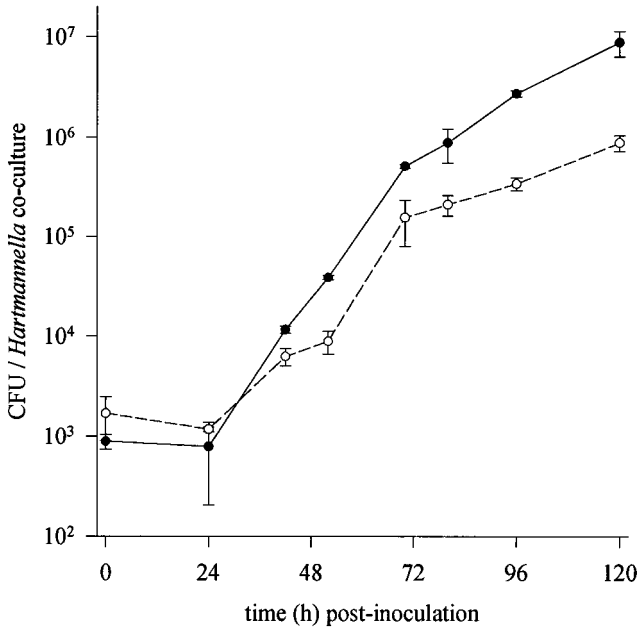

FIG. 6. Growth of *L. pneumophila* 130b and NU216 in *H. vermiformis*. Wells ($n = 4$) containing 10^5 amoebae were inoculated with approximately 10^3 CFU of either 130b (●) or NU216 (○) and cocultured for 5 days. Data are mean CFU, with vertical bars representing standard deviations at each time point. The differences in the recoveries of mutant and wild-type strains were significant at all time points without overlapping error bars ($P < 0.001$; Student's t test).

unlikely to require iron. Third, all strains had prolonged lag phases and some had slower rates of growth, indicating their reduced capacity to adjust to the intracellular environment. One possible reason for this behavior is that these *ira* mutants lack the preferred mechanism for iron acquisition and thus may shift to an alternative and, in some cases, inferior uptake system. As noted above, the growth defect of NU216 was indeed exacerbated by DFX-induced reductions in intracellular iron. Likewise, the relatively small infectivity defects seen in NU217, NU218, NU220, NU223, and NU225 might also become more profound in iron-depleted or activated macrophages. Taken together, the identification of *ira* infectivity mutants confirms that iron acquisition and assimilation are important components of *L. pneumophila* intracellular infection and that the bacterial phagosome is a low-iron environment.

We do not know the nature of the iron-related defects in *ira* mutants, except that they do not result from insertions within *hbp*. In other bacterial systems, EDDA sensitivity and streptonigrin resistance result from alterations in a variety of iron uptake systems, involving the loss of cytoplasmic and cell wall proteins. For example, in *Vibrio* spp. and *Pseudomonas aeruginosa*, alterations in siderophore production and transport are manifest as EDDA sensitivity (8, 53, 54). Likewise, a *Streptococcus pneumoniae* heme utilization mutant was identified by EDDA selection (56). Furthermore, Sng^r mutants of *Neisseria* spp. are defective in transferrin utilization (15, 23), while those in *E. coli* lack iron citrate transport (60). Further biochemical analysis of the *ira* mutants, such as radiolabeled iron uptake studies, will help determine at what point in the iron acquisition and assimilation pathway these strains are defective.

Aside from its apparent defect in intracellular iron acquisition and assimilation, several other aspects of strain NU216 made it particularly interesting. First, it displayed a severe infectivity defect, yielding greater than 1,000-fold fewer bacteria than the wild type from infected monolayers. In addition, it was noncytopathic, a phenotype which could not be entirely explained by its slower growth rate. There have been other instances in which *L. pneumophila* intracellular growth and cytopathicity did not completely correlate, e.g., an avirulent strain demonstrated cytopathicity in the absence of replication (29). Thus, the cytopathic effect of *L. pneumophila* may be a combination of intracellular growth and the elaboration of some cytotoxic factor. Regardless, the severe growth defect and the noncytopathicity of NU216 suggest that it, more so than any other mutant, has a mutation of pleiotropic consequence. Finally, NU216 showed impaired growth within *H. vermiformis*, signaling that iron acquisition and assimilation are important in natural protozoan hosts. This observation also supports the notion that similar genes promote *L. pneumophila* infection of both protozoan and human hosts (11).

The similar defects of NU216 and NU216R demonstrate that in these strains, the *ira* locus maps to the transposon insertion. For two reasons, we suspect that the other mutant phenotypes are also the result of transposon insertions and not spontaneous second-site mutations. First, $EDDA^s$ and Sng^r mutants were detected at frequencies well below those for spontaneous mutations. Second, Sng^r mutants were 100-fold less frequent for non mutagenized *L. pneumophila* than for mutagenized *L. pneumophila*. Since transposons can have polar effects, additional studies are needed to determine whether an *ira* gene maps precisely to the mini-Tn*10* insertion site or it represents a downstream gene. Furthermore, ongoing analysis will determine whether some strains bear mutations in the same gene or operon. We do know, however, that the *ira* locus does not include any other gene previously found to be involved in intracellular infection. Aside from the data ruling out *mip*, *icm*, and *hel*, the fact that *ira* mutants replicate within U937 cells distinguishes them from *dotA* mutants (3). Clearly, further genotypic and phenotypic analyses of these mutants will provide valuable insights into the mechanisms of *Legionella* iron acquisition. In addition, by focusing on those mutants impaired for macrophage infection, we will more quickly identify genes and mechanisms that contribute to virulence.

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