An Iron- and Fur-Repressed *Legionella pneumophila* Gene That Promotes Intracellular Infection and Encodes a Protein with Similarity to the *Escherichia coli* Aerobactin Synthetases

ERIN K. HICKEY AND NICHOLAS P. CIANCOTTO*

Department of Microbiology-Immunology, Northwestern University, Chicago, Illinois 60611

Received 26 August 1996/Returned for modification 15 October 1996/Accepted 31 October 1996

Legionella pneumophila, a parasite of alveolar macrophages, requires iron for intra- and extracellular growth. Although its mechanisms for iron assimilation are poorly understood, this bacterium produces Fur, a protein that can repress gene transcription in response to iron concentration. Because iron- and Fur-regulated genes are important for infection in other bacteria, the identification of similar genes in *L. pneumophila* was undertaken. A wild-type strain of *L. pneumophila* was randomly mutated with a mini-Tn10' *lacZ* transposon, and the resulting gene fusions were tested for iron regulation by assessing β -galactosidase production in the presence and absence of iron chelators. Of the initial six mutants with iron-repressed *lacZ* fusions, two strains, NU229 and NU232, possessed fusions that were stably iron regulated. To assay for Fur regulation, the levels of β -galactosidase were measured in strains no longer producing Fur. As in a number of pathogenic bacteria, *L. pneumophila* fur could not be insertionally inactivated, but spontaneous Fur⁻ derivatives were generated by selecting for manganese resistance. Strain NU229 contained a Fur-repressed fusion based on derepression of *lacZ* expression in its manganese-resistant derivative. Extracellular growth of NU229 in bacteriological media was similar to that of wild-type strain 130b. To assess the role of an iron- and Fur-regulated (*frgA*) gene in intracellular infection, the ability of NU229 to grow within U937 cell monolayers was tested. Quantitative infection assays demonstrated that intracellular growth of NU229 was impaired as much as 80-fold. Reconstruction of the mutant by allelic exchange proved that the infectivity defect in NU229 was due to the inactivation of *frgA* and not to a second-site mutation. Subsequently, complementation of the interrupted gene by an intact plasmid-encoded gene demonstrated that the infectivity defect was due to the loss of *frgA* and not to a polar effect. Nucleotide sequence analysis revealed that the 63-kDa FrgA protein has homology with the aerobactin synthetases IucA and IucC of *Escherichia coli*, raising the possibility that *L. pneumophila* encodes a siderophore which is required for optimal intracellular replication. Southern hybridization analysis determined that *frgA* is specific to *L. pneumophila*.

Legionella pneumophila, the agent of Legionnaires' disease, exists in many environments, such as human alveolar macrophages, freshwater protozoa, and biofilms (14, 22, 24, 39). To survive, the bacterium must obtain essential nutrients from its environment, and the capacity to sense the availability of a particular nutrient may be important for the regulation of its uptake. Iron is essential for *L. pneumophila* growth both extracellularly and intracellularly (11, 23, 41, 68, 71, 75), serving as a cofactor for an aconitase, a superoxide dismutase, and other enzymes (36, 56, 79). *L. pneumophila* requires ferric iron at a concentration of 3 to 13 μ M for extracellular growth, a level that is relatively high compared to that of other bacteria (36, 41, 43, 56, 68, 76, 77). However, iron within the macrophage host is either bound by transferrin, complexed as ferritin, or sequestered in the labile iron and heme pools (10, 19, 59). Thus, intraphagosomal *L. pneumophila* likely has evolved strategies for scavenging scarce free iron or iron bound to cellular chelators. How *L. pneumophila* obtains iron from its environment remains unknown. High-affinity siderophores that are commonly used for Fe³⁺ assimilation by other bacteria are reported to be absent from legionellae (49, 76). Also, *L. pneumophila* does not bind and utilize transferrin or lactoferrin (8, 11, 12, 43, 74). *L. pneumophila* does, however, express two internal

ferric reductases which likely process internalized Fe³⁺ (43, 69). In addition, it can bind and utilize hemin (62). Undoubtedly, the legionellae, like so many other pathogens, have multiple iron uptake systems (30, 65, 66, 87).

In the pursuit of new *L. pneumophila* virulence factors, we are currently using two approaches to discover intracellular iron assimilation pathways. In the first approach, we isolated mutants that were sensitive to iron chelators, resistant to streptonigrin, and defective for macrophage infection (71). In the second strategy, reported here, we sought genes whose expression is controlled by iron and whose loss diminishes infectivity. In other bacteria, the factors involved in iron uptake, such as siderophores and transferrin receptors, are regulated by the repressor protein Fur (4, 20, 30, 52, 84). In the presence of ferrous iron, the Fur dimer binds to promoter regions and represses the transcription of iron acquisition genes (4, 31). However, when the availability of iron is low, Fur does not bind and the genes are expressed. In addition to the controlling factors involved in iron uptake, Fur represses a variety of other virulence determinants, such as exotoxin A in *Pseudomonas aeruginosa* and a hemolysin in *Vibrio cholerae* (51, 73). Recently, we isolated the *L. pneumophila* fur gene and showed that its predicted amino acid sequence is 50 to 60% identical to that of other bacteria (35). Furthermore, the cloned fur encoded a protein able to repress *Escherichia coli* *fliC* expression, as well as to decrease enterobactin production. These data had suggested that *L. pneumophila* iron acquisition is regulated by

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-0385. Fax: (312) 503-1339. E-mail: n-cianciotto@nwu.edu.

Fur by using the concentration of iron in the environment as a signal for when to repress gene expression.

Using a new random mutagenesis protocol, we present the identification of *L. pneumophila* loci that are regulated by iron. One particular gene was also modulated by Fur and, importantly, was involved in infection of a human macrophage-like cell line. Sequence analysis revealed that this gene (*frgA*) has homology to genes in the *E. coli* aerobactin operon. Thus, *L. pneumophila* virulence factors may be identified based upon their mode of regulation.

(Portions of this work were previously presented [34]).

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The *L. pneumophila* *frg* genes were cloned from and inactivated within serogroup 1 strain 130b. Other *L. pneumophila* strains tested for the presence of *frgA* include serogroup 2 strain ATCC 33154, serogroup 3 strain ATCC 33155, serogroup 4 strain ATCC 33156, serogroup 7 strain ATCC 33823, serogroup 8 strain ATCC 35096, serogroup 13 strain B2A3105, serogroup 14 strain 1169-MN-H, as well as representatives of serogroups 9 to 12, which were obtained from the Michigan Department of Public Health (17). Other *Legionella* strains examined include *L. Birminghamensis* 1407-AL-H, *L. dumoffii* ATCC 33279, *L. erythra* SE-32A, *L. gormanii* ATCC 33297, *L. feeleii* WO-44C, *L. hackeliae* Lansing 2, *L. israelensis* Bercovier 4, *L. jamestownensis* JA-26, *L. longbeachae* ATCC 33462, *L. micdadei* Rivera, *L. oakridgensis* OR-10, *L. parisiensis* PF-209, *L. saintelhelens* Mt. St. Helen's 4, *L. santicus* SC-63, *L. spiritensis* MSH-9, and *L. tucsonensis* 1087-AZ-H (17).

All *Legionella* strains were grown at 37°C on buffered-charcoal-yeast-extract (BCYE) agar for 48 to 72 h or within buffered yeast extract (BYE) broth (17). When appropriate, 25 µg of kanamycin per ml, 3 µg of chloramphenicol per ml, 5% (wt/vol) sucrose, or 0.5 µg of streptomycin per ml was added to the medium. To select and maintain *L. pneumophila* Fur mutants, 0.4 to 0.6 mM manganese chloride was added to BCYE instead of the iron supplement. To generate low-iron BCYE, 50 to 60 µM of deferoxamine mesylate (DFX) or 50 to 80 µM of ethylenediamine di(o-hydroxyphenylacetic acid) (EDDA) was added in place of the standard 355 µM ferric pyrophosphate supplement. These levels of Fe³⁺ chelators did not significantly inhibit bacterial growth. Chemically defined medium (CDM) and acid-washed glassware were used for monitoring *L. pneumophila* growth kinetics in liquid (76). The indicated concentrations of iron within CDM were achieved by adding ferric pyrophosphate supplements. *E. coli* HB101 served as a host for recombinant plasmids and was grown on Luria-Bertani (LB) agar containing 50 µg of kanamycin per ml, 50 µg of ampicillin per ml, or 30 µg of chloramphenicol per ml (3). Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Random mutagenesis of *L. pneumophila* with mini-Tn10/*lacZ*. To identify *L. pneumophila* genes regulated by iron, promoterless *lacZ* (*'lacZ'*) genes were randomly inserted into the 130b chromosome. Plasmid pEH40, which was used to introduce mini-Tn10/*lacZ* into *L. pneumophila*, was constructed in two steps (Fig. 1). First, a *'lacZ'* cassette, isolated from *Bam*HI-digested pNK2804, was ligated to the *Bgl*II site in the mini-Tn10 of pGI6145 (47). Then, the resulting plasmid was digested with *Pst*I and ligated with the *sacB*-containing *Pst*I fragment from pUCD800+oriT (16).

To isolate mini-Tn10/*lacZ*-containing legionellae, pEH40 was electroporated into strain 130b, and transformants were selected on BCYE agar containing kanamycin and sucrose (18, 70). The frequency of kanamycin-resistant (Km^r), sucrose-resistant colonies was 6.4×10^{-6} , suggesting the transposition frequency of mini-Tn10/*lacZ* is comparable to that of mini-Tn10 without *'lacZ'* (70). To identify those mutants that produced β-galactosidase, the base plates were topped with 0.7% agar containing 0.6 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside puriss (XGAL) (58). Approximately 46% of Km^r, sucrose-resistant colonies exhibited blue pigmentation. To quantitate the degree of *lacZ* expression in *L. pneumophila*, β-galactosidase activity was measured as described by Miller (58). In preparation for the assay, strains were grown for 48 to 72 h on BCYE agar and then suspended in sterile water to an optical density at 660 nm (OD₆₆₀) of 0.4 to 0.7.

Directed mutagenesis and trans-complementation of *L. pneumophila*. To mutate specific genes in the *L. pneumophila* chromosome, the cloned genes, inactivated with Km^r inserts, were ligated into the counterselectable plasmid pEA75, and allelic exchange was performed as described previously (16, 63). For attempts at interrupting *L. pneumophila* *fur*, the cloned gene was obtained from the plasmid pEH22a (35). To complement a chromosomal mutation, the relevant *L. pneumophila* gene was cloned into the pACYC184 derivative pSU2719, transferred into the mutant by electroporation, and maintained with chloramphenicol selection (16, 54).

Isolation of *L. pneumophila* genomic DNA. Genomic DNA was isolated from *L. pneumophila* strains by using a protocol, adapted from that of Neumann et al. (60), that is significantly faster than our previous method (16). The bacteria were swabbed from a fresh agar plate and resuspended in 1 ml of sterile water. After a 2-min centrifugation (16,000 × g), the bacterial pellet was suspended in 0.5 ml

FIG. 1. Plasmid for mini-Tn10/*lacZ* mutagenesis of *L. pneumophila*. The ca. 10-kb pEH40 contains (i) a mini-Tn10 (see IS10 elements) which carries a promoterless *lacZ* (*'lacZ'*) and a kanamycin resistance gene (Km-r), (ii) a doubly mutated Tn10 transposase gene (*ats1ats2*) which promotes increased variability of the transposon insertions compared to the wild-type transposase (47), (iii) the counterselectable levanucrase gene of *Bacillus subtilis* (*sacB*) (16), and (iv) a ColE1 origin of replication (*oriV*). Key restriction sites are indicated.

of SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris [pH 7.5]) containing 1 mg of lysozyme per ml and 0.1 mg of RNase A per ml. The mixture was incubated for 30 min at 37°C before 0.1 volume of 10% sodium dodecyl sulfate (SDS) and 0.05 volume of a 20-mg/ml proteinase K solution were added. Following incubation at 55°C for 2 h, one-third volume of NaCl-saturated water was added to the tube. Next, 1 volume of 0.1 M Tris (pH 8)-equilibrated phenol was added, and the mixture was incubated at room temperature for 30 min with rotation. The phases were separated by a 5-min centrifugation (16,000 × g), and the aqueous phase was removed to a new tube. Two additional extractions were performed with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then 1 volume of chloroform-isoamyl alcohol (24:1). Finally, after the aqueous phase was removed to a new tube, the DNA was precipitated by adding 1 volume of isopropanol.

DNA hybridizations. For Southern blot hybridizations, genomic DNAs from *L. pneumophila* strains were digested with restriction enzymes according to the manufacturer's protocols (New England Biolabs, Beverly, Mass.). DNAs were then electrophoresed through 0.8% agarose and transferred to a nitrocellulose filter (3). Digoxigenin labeling of the indicated probes and high-stringency hybridizations (i.e., ca. 10% base pair mismatch) were performed by using Genius System v2.0 (Boehringer Mannheim, Indianapolis, Ind.). Two modifications were made to the Genius System protocols to achieve low-stringency conditions, i.e., ca. 30% base pair mismatch. First, the hybridization and wash temperatures were dropped to 49°C, and second, the SSC concentration in the wash buffer was raised to 5× (750 mM NaCl, 75 mM sodium citrate [pH 7.0]).

For colony blot hybridizations, a *L. pneumophila* 130 genomic library, consisting of 4- to 6-kb *Sau*3A1 fragments cloned into pBR322 (3), was plated on LB-ampicillin agar for isolated colonies. The colonies were transferred to nitrocellulose and lysed by incubation of the membranes on Whatman paper saturated with 10% SDS, followed by subsequent incubations on a denaturing solution (0.5 N NaOH, 1.5 M NaCl), a neutralizing solution (1 M Tris-HCl [pH 8.0], 1.5 M NaCl), and 2× SSC. All incubations were carried out at room temperature for 5 min. Digoxigenin labeling of probes and high-stringency hybridizations were performed as described above.

Western blotting. Whole-cell lysates were harvested and reacted with anti-serum as described previously (17). Antiserum against *E. coli* Fur was received from Michael Vasil and used at a 1/100 dilution (73). A horseradish peroxidase-conjugated secondary antibody was used at a 1/1000 dilution (Gibco BRL, Gaithersburg, Md.).

Intracellular infection of U937 cells. U937 is a human cell line that differentiates into macrophage-like cells after treatment with phorbol esters (67). U937 cell monolayers were prepared and infected as previously described (67). After inoculation, the monolayers were incubated for 2 h to permit bacterial uptake and then washed to remove unattached bacteria. The infected monolayers were incubated at 37°C in RPMI medium supplemented with 10% fetal bovine serum (Gibco BRL). To assess the relative infectivity of *L. pneumophila* strains, 50% infective doses (ID₅₀) were determined after a 3-day incubation (15, 63). To quantitate intracellular bacteria, replicate (*n* = 4) monolayers were inoculated

FIG. 2. Mini-Tn10'*lacZ* insertions in *L. pneumophila* NU229 through NU236 (lanes A to H, respectively). Genomic DNAs were digested with *Hind*III, electrophoresed, and hybridized with pEH40. Molecular weight markers (in kilobases) are indicated on the left. Since *Hind*III cuts asymmetrically within mini-Tn10'*lacZ* (Fig. 1), one hybridizing fragment is more intense than the other within each lane. The intensity of the bands varied between the lanes because all lanes did not contain identical amounts of DNA. Although the hybridizing bands in lane C do not appear, in this gel, to comigrate with those in lanes A and B, additional blots with *Hind*III- and *Eco*RI-digested DNAs illustrate that the corresponding strains do have identical insertions. Note that lane F has a faint second band at approximately 2.1 kb, while lane H has one at approximately 12 kb.

with approximately 10^6 CFU, incubated for 24 to 72 h, and then lysed (63). Ten-fold serial dilutions of the lysates were plated on BCYE agar, containing chloramphenicol where appropriate, and the resulting CFU were used to calculate the corresponding numbers of bacteria per monolayer.

DNA sequence analysis. Cloned *L. pneumophila* DNA was sequenced from double-stranded plasmids by the dideoxy chain termination method with 35 S-dATP (Amersham Life Science, Arlington Heights, Ill.) and Sequenase (United States Biochemical, Cleveland, Ohio) (3). Initially, M13-based primers (5'-CCC AGTCACGACGTTGTAAAACG and 5'-AGCGGATAACAATTTTCACAC AG) and an IS10-based primer (5'-CCTTAACCTAATGATTTTAC) were used to sequence DNAs cloned into pSU2719, which contains *lacZ* and the multicloning site from pUC19 (1). Twenty custom 18- to 20-mer oligonucleotide primers were used in subsequent reactions. The primers were prepared by the Northwestern University Biotechnology Center using the Applied Biosystems DNA synthesizer (Perkin-Elmer, Foster City, Calif.). Sequencing reactions were performed according to the manufacturer's protocols, and then the reaction mixtures were electrophoresed in Long-Ranger (AT Biochem, Malvern, Pa.) acrylamide gels. Both strands of DNA were sequenced and then analyzed by using PCGene (Intelligenetics, Mountain View, Calif.). Nucleotide and predicted amino acid sequences, as well as initial homology studies, were obtained from GenBank at the National Center for Biotechnology Information (NCBI), National Library of Medicine, National Institutes of Health. Sequence homologies on the complete predicted amino acids sequences were determined by using PCGene.

Nucleotide sequence accession number. The *L. pneumophila* *frgA* sequence is deposited in the GenBank database at the NCBI under accession no. U76559.

RESULTS

Identification of iron-repressed genes in *L. pneumophila*. To identify iron-repressed genes of *L. pneumophila*, we mutated 130b with mini-Tn10'*lacZ* and screened for strains that had the highest levels of β -galactosidase expression under low-iron growth conditions. Approximately 700 colonies, which appeared white to light blue on standard BYCE agar overlaid with XGAL, were replica plated onto BCYE agar containing 50 μ M DFX. The chelator DFX has its greatest affinity (i.e., 10^{31}) for Fe^{3+} , while its next highest affinity (i.e., 10^{14}) is for Cu^{2+} (45). Eight mutants, designated as strains NU229 through NU236, consistently expressed high levels of β -galactosidase activity in the low-iron medium, suggesting that they contained iron-repressed *lacZ* fusions. Southern blot analyses were performed to determine the number and location of the mini-Tn10'*lacZ* insertions in these strains. More specifically, genomic DNAs were digested with *Hind*III, an enzyme that cuts once within mini-Tn10'*lacZ* (Fig. 1), and hybridized with pEH40 (Fig. 2). The banding patterns revealed that each mu-

FIG. 3. β -galactosidase (β -gal) production in *L. pneumophila* *lacZ* fusion strains grown in the presence or absence of ferric iron chelators. Strains were grown for 48 h on BCYE agar with either the standard iron supplement (solid bars) or an iron chelator (hatched bars). (A) DFX (50 μ M) was used in the experiment; (B) 52 μ M EDDA was used. Panels A and B are representative of two and three separate trials, respectively. In each trial, all samples were tested in triplicate.

tant had only one transposon insertion but no additional pEH40 sequences. However, strains NU229, NU230, and NU231 contained identical insertions (see lanes A, B, and C). These results were confirmed by Southern hybridization with *Eco*RI-digested DNAs (data not shown). Thus, in this initial screening, we identified six unique *lacZ* fusions that appear to be iron regulated. Of course, some of these strains may bear mutations in the same gene or operon. Since the number of iron-repressed bacterial genes has been estimated to be 1% of the total number of genes (26, 53), the isolation of up to six iron-regulated fusions from a pool of 700 *L. pneumophila* mutants was not unexpected. Because our goal was to assess the role, if any, of iron-repressed genes in *L. pneumophila* intracellular infection, rather than an exhaustive search for the total number of iron-regulated genes, we initially concentrated on these six mutants.

To quantitate the repression of these gene fusions by iron, liquid β -galactosidase assays were performed with NU229, NU232, NU233, NU234, NU235, and NU236. In two initial trials, all strains, except NU233, produced less β -galactosidase when grown under high-iron conditions (Fig. 3A) ($P < 0.0001$, Student's *t* test). NU229 and NU232 showed a 17-fold repression, whereas NU234, NU235, and NU236 exhibited 2- to 3-fold drops in *lacZ* expression. Similar levels of repression

TABLE 1. Manganese resistance and alterations in Fur expression^a

Concn of MnCl ₂ (mM)	Frequency of Mn ^r CFU	No. of Mn ^r strains tested for Fur	No. of strains with:		
			Full-length Fur	Truncated Fur	No Fur
0.1	8.5×10^{-1}	0	ND ^b	ND	ND
0.2	5.0×10^{-4}	0	ND	ND	ND
0.4	1.2×10^{-6}	2	2	0	0
0.5	3.6×10^{-7}	10	3	1 ^c	6
0.6	3.6×10^{-7}	4	1 ^c	2 ^c	1

^a Bacteria were grown in BCYE media for 48 h and then resuspended in sterile water. Serial dilutions were then plated on BCYE agar containing MnCl₂ at concentrations ranging from 0 to 0.6 mM.

^b ND, not determined.

^c Cross-reactive band on the Western blot was barely detectable.

have been seen in other iron-regulated genes, e.g., *E. coli* *sodA* (2- to 3-fold) and *Vibrio anguillarum* *fatA* (14-fold) (61, 81). In subsequent trials in which increased amounts of DFX were used, a greater derepression was seen for NU229 and NU232. However, the phenotypes of NU234, NU235, and NU236 were different. In each case, the level of *lacZ* expression on standard BCYE agar appeared to increase (data not shown). Thus, we assessed β -galactosidase production in the presence of EDDA to confirm the nature of iron regulation within these strains (Fig. 3B). Like DFX, EDDA is best able to chelate Fe³⁺, having an affinity of 10^{34} (27, 57). In these experiments, the levels of β -galactosidase production in NU234, NU235, and NU236 remained unresponsive to iron. We do not know the reason for these changes but assume that compensatory mutations were sustained and maintained during passage and storage. However, the *lacZ* fusions in NU229 and NU232 again demonstrated significant derepression when grown on BCYE agar containing an iron chelator. Taken together, these experiments indicate that two mutants, NU229 and NU232, contain stable iron-repressed *lacZ* fusions. We have designated the inactivated genes as *frg* for iron (Fe)-repressed gene.

Fur repression of *L. pneumophila* *frg::lacZ* fusions. To begin to understand the mechanism of *frg* iron regulation, we assessed *lacZ* expression in *L. pneumophila* strains that differed only in Fur production. Western blot analysis confirmed our earlier prediction that strain 130b expresses a protein that is cross-reactive with and slightly smaller than *E. coli* Fur (data not shown) (35). Initially, we had hoped to use cloned *L. pneumophila* *fur* and allelic exchange to insertionally inactivate the chromosomal *fur* genes. However, all attempts with this method were unsuccessful in strain 130b (data not shown). The inability to interrupt *fur* also has been seen in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *P. aeruginosa*, and *Vibrio anguillarum* (7, 73, 80, 81). As an alternate approach for isolating Fur mutants, we sought spontaneous manganese-resistant (Mn^r) mutants. Since Mn²⁺ and Fe²⁺ can displace each other as cofactors, manganese resistance in *E. coli* is often achieved through *fur* mutations that permit increased intracellular iron levels (32). The feasibility of this approach was tested in wild-type *L. pneumophila*. Mn^r legionellae were isolated on BCYE agar containing 0.4 to 0.6 mM MnCl₂ and examined for the loss of Fur by Western blot analysis (Table 1). Whereas strains isolated on agar containing 0.4 mM manganese expressed full-sized Fur at normal levels, those obtained with higher concentrations of manganese expressed either a more rapidly migrating protein or an undetectable amount of Fur. The apparently truncated proteins were approximately 5 kDa smaller than Fur and appeared as faint bands on the immunoblot. Introduction of cloned *L. pneumophila* *fur* into Mn^r strains restored man-

TABLE 2. NU229 *frgA::lacZ* expression in the presence or absence of Fur

Trial	β -galactosidase activity (U) ^a	
	NU229 Fur ⁺	NU229 Fur ⁻
1	4.2 ± 0.4	160.2 ± 20.1
2	2.4 ± 1.5	320.5 ± 9.4
3	3.1 ± 0.3	583.4 ± 10.0

^a Fur⁺ (Mn^r) NU229 and Fur⁻ (Mn^r) NU229 cells were grown on standard BCYE agar for 48 h. For each trial, each strain was examined in triplicate. Results are the means $\pm$ standard deviations.

gane sensitivity (data not shown). Therefore, as in *P. aeruginosa*, *V. anguillarum*, and *V. cholerae*, it is possible to isolate *L. pneumophila* Fur mutants by selecting for MnCl₂ resistance (51, 73, 81).

Returning to our original goal of assessing Fur regulation of *frg*, Mn^r mutants of NU229 and NU232 were derived on 0.6 mM MnCl₂ and then tested for the loss of Fur by Western blotting. Although a Fur null mutant of NU229 was readily obtained, all seven Mn^r derivatives of NU232 tested still produced a full-size Fur protein (data not shown). To determine if the *frg::lacZ* fusion in NU229 is Fur-repressed, quantitative β -galactosidase assays were performed with lysates from Mn^s and Mn^r bacteria grown on standard (high-iron) BCYE agar (Table 2). In three trials, the Mn^r derivative displayed elevated enzyme activity ($P < 0.001$, Student's *t* test), indicating that the loss of Fur allows for *frg::lacZ* expression in the presence of high iron. The differences in β -galactosidase production within the Fur⁻ populations likely reflect reversion of the *fur* mutation since the bacteria used in the assays were necessarily grown in the absence of manganese. In contrast to Mn^r NU229, a representative Mn^r derivative of NU232 did not display derepression of *lacZ* when it was grown in high-iron media (data not shown), a result compatible with the presence of full-length Fur (see above). Strain NU229 became the focus of our immediate attention since it possessed a stable iron- and Fur-regulated *lacZ* fusion, designated *frgA*.

Extracellular growth of NU229. Because many iron- and Fur-repressed genes are involved in iron uptake, whether interruption of *frgA* affected extracellular growth, particularly under iron-stressed conditions, was determined. More specifically, 130b and NU229 were compared for their abilities to grow in CDM containing either a 0, 4, 8, or 16 μ M concentration of added iron (Fig. 4). As expected, no significant growth of 130b was seen in the absence of iron, and as the concentration of iron was increased, the duration of the lag phase decreased. On entering logarithmic growth, all 130b cultures reached stationary phase within 48 to 72 h. Although the final OD₆₆₀ value achieved by the NU229 cultures was slightly lower than that reached by 130b, the growth characteristics of the mutant were remarkably similar to those of the wild type (Fig. 3B to D). Indeed, the mutant entered logarithmic growth at the same time as the wild-type strain and had a comparable growth rate. Similar growth characteristics for 130b and NU229 were also seen when these strains were grown in BYE (data not shown). Therefore, it was concluded that the interruption of *frgA* had minimal effects on the extracellular growth capacity and extracellular iron acquisition/assimilation functions of *L. pneumophila*. To further demonstrate the latter point, we assessed the mutant's sensitivity to EDDA and its resistance to streptonigrin, an antibiotic that acts with internal iron sources to inhibit bacterial growth (86). Toward this end, 130b, NU229, and strains previously shown to be either strep-

FIG. 4. Extracellular growth of NU229 in CDM with increasing iron concentrations. Wild-type strain 130b (●) and mutant NU229 (○) were grown in CDM with increasing iron concentrations. (A to D) The growth in cultures containing added iron at concentrations of 0, 4, 8, and 16 μ M, respectively, is depicted. Bacteria used to inoculate the cultures had been grown overnight in BYE broth without the iron supplement and washed once with CDM (0 μ M added iron). Growth was estimated by measuring the OD₆₆₀ of the culture over a 7-day period. Panels A to D are representative of three separate trials, and each result is the average of two separate cultures. Growth in CDM containing a 20 μ M iron supplement was not different from growth in medium containing 16 μ M iron (data not shown).

tonigrin resistant or EDDA sensitive (71) were plated on BCYE containing either 0.5 μ g of the antibiotic per ml or a 50 to 80 μ M concentration of the chelator. Both 130b and NU229 failed to grow on the streptonigrin-containing medium and grew comparably in the presence of EDDA (data not shown), indicating that there was no change in iron assimilation or storage in the *frgA* mutant under standard extracellular growth conditions.

Intracellular infection by *L. pneumophila frg* mutants. Because intracellular growth is the focal point of *Legionella* pathogenesis, the next step was to determine if NU229 was impaired in infection of macrophages. Initially, ID₅₀ analyses were done with macrophage-like U937 cells. In four separate experiments, strain NU229 displayed a 3.2-fold $\pm$ 1.4-fold increase in ID₅₀. More importantly, the monolayers infected with the mutant appeared to contain fewer bacteria than those infected with the wild-type strain. Taken together, these preliminary data suggested that *frgA* has a role in infection in addition to being iron and Fur regulated. Incidentally, NU232, NU234, and NU235 showed no change in infectivity during these same ID₅₀ experiments, whereas NU236 exhibited an 11.2-fold $\pm$ 3.1-fold increase in ID₅₀ (95% confidence limits are defined by ID₅₀ ratios of 5.0 and 17.4). To confirm that the infectivity defect seen in NU229 was significant, U937 cell monolayers were infected with comparable numbers of 130b and NU229, and the levels of intracellular bacteria were assessed at 0, 6, 12, 24, 48, and 72 h postinoculation (Fig. 5A). At the first three timepoints, there were no differences in recovery between the wild type and the mutant, indicating that NU229 is not impaired in entry or resistance to immediate intracellular

killing. Beginning at 24 h postinoculation, however, significantly fewer bacteria were recovered from monolayers infected with NU229. Indeed, there were 9-, 80-, and 30-fold fewer bacteria at 24, 48, and 72 h postinoculation, respectively. Similar results were obtained in two subsequent experiments in which the number of bacteria per monolayer at 72 h postinoculation was determined (data not shown). Therefore, NU229 has a significant and reproducible defect in intracellular growth.

We next sought to confirm that the reduced infectivity of NU229 was due to the inactivation of *frgA* rather than to a spontaneous second-site mutation. Thus, the mutant was re-made by allelic exchange. More specifically, an approximately 12-kb *Pst*I fragment containing *frgA::lacZ* (and Km^r) was isolated from NU229 and cloned into the *Pst*I site in *sacB*-containing pEA75. Following electroporation of the recombinant plasmid into strain 130b, six Km^r, sucrose-resistant colonies were obtained. All of these clones, when tested by Southern hybridization, contained the same DNA insertion as NU229 (data not shown). In addition, all produced higher levels of β -galactosidase when grown under low-iron conditions, as did NU229. One strain, designated NU229R, was further tested in U937 cells. NU229R demonstrated no infectivity difference from the original NU229 on three separate occasions, one of which is represented in Fig. 5A. The similarities between NU229 and NU229R indicate that the *frgA* interruption, not a second-site mutation, caused the defect in intracellular growth.

Sequence analysis of *L. pneumophila frgA*. As the first step towards understanding the behavior of NU229, we determined the nucleotide sequence of *frgA*. Toward that end, the 12-kb

FIG. 5. Intracellular infection of U937 cells by *L. pneumophila* mutants. (A) The monolayers ($n = 4$) were inoculated with 10^6 CFU of either 130b (●), NU229 (○), or NU229R (△), and after various incubation periods, the numbers of bacteria were determined. (B) The U937 cells were inoculated with 10^6 CFU of either 130b(pSU2719) (●), NU229(pSU2719) (○), or NU229(pEH75) (△). All results represent the mean number of CFU recovered, and vertical bars indicate standard deviation. The differences in the recoveries of these strains were significant at all timepoints without overlapping error bars ($P < 0.01$, Student's t test).

*Pst*I fragment containing *frgA::lacZ* was cloned into pSU2719 to yield pEH44 (Fig. 6). To initiate sequence analysis using IS10 primers, we utilized the subclones pEH45 and pEH46 (Fig. 6). Subsequent sequencing reactions were performed with the pEH44 template DNA and a series of custom primers. To confirm the nucleotide sequence at the point of transposon insertion, a plasmid (pEH65) containing the uninterrupted 3' end of *frgA* was isolated from a genomic library by using probe A (Fig. 6) and then analyzed. Figure 7 contains the sequence of the *frgA* open reading frame (ORF), as well as flanking regions. The *frgA* ORF began at an ATG start codon and ended 1,725 bp downstream at a TAA stop codon. This ORF would encode a protein of 575 amino acids and with a size of 63.3 kDa. The *L. pneumophila frgA* ORF was preceded by a ribosome binding site within 10 bp of the start codon, as well as a -10 and a -35 region that closely matched the *E. coli* consensus sequence (78). Most promoter regions of genes regulated by Fur contain at least one iron box (13, 20), generally occurring within the

-35 region to the -10 region, that represents the binding site(s) for Fur-Fe²⁺ repressors (51). The *L. pneumophila frgA* promoter region contained two overlapping iron boxes that had four or five mismatches, compared to the *E. coli* consensus sequence, 5'-GATAATGATAATCATTATC (Fig. 7) (13, 20). This finding supports our earlier conclusion that *frgA* is iron and Fur regulated.

Database searches with the predicted amino acid sequence of *frgA* showed that it shares homology with two proteins, IucA and IucC, encoded by the first and third genes in the Fur-repressed *E. coli* aerobactin operon (Fig. 8) (55). In the third step of the siderophore's biosynthesis, IucA catalyzes the addition of *N*^ε-acetyl-*N*^ε-hydroxylysine to citric acid to yield the intermediate *N*^α-citryl-*N*^ε-acetyl-*N*^ε-hydroxylysine. Subsequently, IucC adds another *N*^ε-acetyl-*N*^ε-hydroxylysine moiety to the intermediate to form aerobactin (21). In a previous report by Martínez et al. (55), a 21% identity and a 47% similarity between IucA and IucC were recognized as significant. While

FIG. 6. Restriction map of *L. pneumophila* DNA containing the interrupted *frgA* gene. The cloned 12-kb region containing *L. pneumophila* DNA and the transposon insert is depicted in the top line. The transposon consists of the thin arrows, which represent the *'lacZ'* and *Km^r* genes, and the closed circles indicate the location of the *IS10* elements. The thick lines flanking the transposon represent the interrupted *frgA* gene. Probe B is *frgA* specific, containing only *frgA* DNA in addition to the *IS10* element. Restriction enzymes *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Xba*I (Xb), and *Xho*I (X) are indicated.

IucA and IucC catalyze similar reactions and have structural homology, they are not interchangeable (21). The overall identity and similarity between *L. pneumophila* FrgA and *E. coli* IucA were 15.7 and 26.1%, respectively. Furthermore, FrgA had an 18.3% identity and a 32.6% similarity to IucC. BLASTX results from the NCBI specified the three regions of greatest homology between FrgA and the *E. coli* proteins. The three boxes in Fig. 8 denote these regions for IucA, and the BLASTX identity and similarity results were 23 and 51%, 29 and 45%, and 31 and 46%, respectively. The three shaded areas denote these regions for IucC, with BLASTX identity and similarity results of 21 and 47%, 45 and 75%, and 32 and 51%, respectively. It is also worth noting that FrgA is comparable in size to both IucC and IucA; indeed, it is predicted to have the same number of amino acids as IucA (Fig. 8). Taken together, these data suggest *frgA* encodes an IucA- or IucC-like protein. Sequences upstream and downstream of *frgA*, however, did not reveal any ORFs that could be a part of an *iuc*-like operon (Fig. 7) (data not shown).

Complementation of *frgA* mutation. To determine if the infectivity defect in NU229 is due to the loss of FrgA or due to a polar effect (i.e., a block in transcription of a gene downstream of *frgA*), we sought to restore full infectivity to the mutant by introducing into it an intact plasmid-encoded *frgA*. First, with an *frgA*-specific probe (see probe B in Fig. 6), the complete gene was isolated on plasmid pEH74 from our genomic library. Next, *frgA* was subcloned on a 2,886-bp *Kpn*I-*Xho*I fragment into the vector pSU2719 to yield pEH75. Although pEH75 contains 1,093 bp upstream of the *frgA* start codon, it does not contain any ORFs that would be transcribed in the same direction as *frgA*. More importantly, pEH75 does not contain any downstream ORFs since the *Xho*I site is only 62 bp downstream from the *frgA* stop codon (Fig. 7). Finally, pEH75 was electroporated into NU229, and transformants were obtained on BCYE containing kanamycin and chloramphenicol. To have controls for the infectivity studies, we also isolated NU229 and 130b containing the *Cm^r* pSU2719 vector. In three separate experiments, the results of one of which is depicted in Fig. 5B, strain NU229(pEH75) exhibited intracellular growth characteristics comparable to those of 130b. This result demonstrates that the presence of *frgA* alone was sufficient to restoring infectivity to NU229 and that this gene is required for optimal intracellular infection.

Distribution of *frgA* among *L. pneumophila* strains and *Legionella* species. To determine if *frgA* is conserved among dif-

ferent *L. pneumophila* strains and *Legionella* species, Southern hybridization analyses were performed by using probe B (Fig. 6). The *frgA*-specific probe hybridized under high-stringency conditions to DNAs from the 11 strains of *L. pneumophila* that were tested (i.e., representatives of serogroups 1 to 4 and 6 to 14) (data not shown). However, the probe did not hybridize, even under low-stringency conditions, with DNAs from *L. birminghamensis*, *L. dumoffii*, *L. erythra*, *L. gormanii*, *L. feeleii*, *L. hackeliae*, *L. israelensis*, *L. jamestowniensis*, *L. longbeachae*, *L. micdadei*, *L. oakridgensis*, *L. parisiensis*, *L. saintelensi*, *L. santicusis*, *L. spiritensis*, and *L. tucsoniensis*. The strains tested represent both clinical and environmental isolates. These results suggest that *frgA* is conserved within and is specific to strains of *L. pneumophila*.

DISCUSSION

The identification of genes whose expression is repressed by Fur in response to iron availability may lead to the isolation of virulence determinants for two central reasons. First, genes encoding iron acquisition systems can themselves be essential for bacterial survival within mammalian hosts. Examples of these are the ferrous iron uptake system and the aerobactin operon of pathogenic *E. coli* (44, 66, 88). Second, factors not directly involved in iron assimilation can be repressed by Fur. Several examples of these are the Shiga-like toxin and superoxide dismutase from *E. coli*, a hemolysin and the outer membrane IrgA protein from *V. cholerae*, and exotoxin A from *P. aeruginosa* (13, 28, 51, 61, 73). The majority of information on iron- and Fur-repressed genes comes from studies on either extracellular pathogens or facultative intracellular parasites of the gastrointestinal tract (52). Consequently, we have continued our analysis of *L. pneumophila* Fur and begun characterization of its regulon.

Our data indicated that *L. pneumophila* Fur is structurally and antigenically similar to *E. coli* Fur, but the *Legionella fur* gene, unlike *Escherichia fur*, appears to be essential for bacterial viability, as seen by the inability to insertionally inactivate it. A complete loss of Fur in *L. pneumophila*, as well as in *N. meningitidis*, *N. gonorrhoeae*, *P. aeruginosa*, and *V. anguillarum*, probably increases the levels of iron to a point that is bactericidal under aerobic growth conditions (7, 73, 80–82). To study Fur-regulated genes, we relied on spontaneous *Mn^r* mutants. *Mn^r* strains of *V. anguillarum* and *V. cholerae* have been shown to bear point mutations in *fur*, generally causing changes in the

atttaactcagaatacagaactttttatccaaaatcaaaactctattgccaatataat 60
 -35 -10
 attttatttgcgccacctcttgcgaatgagaatgattatcatttatatttaattataac 120
 aaaaataatcctcagagagataaATGCCCTGGCGTACGGTAATTTTCATGAATCAGC 180
 M A L A Y G N F H E L S 12
 CATCAATTAGCGTTTACTATTGAAATGGCATAGGACTACCCAGAAATAGTGTGGAT 240
 H Q L R F L L F E I G I G L P Q N S V D 32
 TATTTTATACCTTAGCTCATAAAAATACCCTGAAGCTTTTCAATGGCGAAATCAGG 300
 Y F I T L A H K N T L K R L Q H A S I K 52
 GAAGGATTAAATCAATCAGCATTCGAAGTCACCATATCCATGATTTCATTGACCAATTG 360
 E G L I Q S A I A S H H I H D F I D Q L 72
 CAGATAAAATCAAAATCAATGCCGGAAGTAAGTTTTCATGGCGAAATCAGG 420
 Q I K L K N S M P E S K F F Q W R K I R 92
 GAAGCATTAGATGAATCGATTGCCAATGAGGCTTTGGCTTACGGCTACAGGCAAACTGG 480
 E A L D A E S I A N E A L A Y A Y R Q N W 112
 AACACCAATTAAGAAATGACCATCAATCAAGAGTCTGTGGACATGGATAAATAAT 540
 N T Q L R N E A M H Q Y K S L W T W I N N 132
 GAATATCTCGTATCAAGCTTATTATTTCGGAACATGGGCGATTGAGGCATCCC 600
 E L S P Y Q T L L F L E Q W G S L R H P 152
 TATCACCCAGCATTCAGCGCAAGCTGGTTTACGCGAAGAGTACTCCAACTCT 660
 Y H P A F S A K T A G N Q Y T R R E V L Q N S 172
 CCCGAATTCAGGCGCAAGCTGAGTATCATTTGGTGTGCATTAATAAAGCAAAATTCAG 720
 P E F Q A K V S V H W C A L N K T K I Q 192
 TCAATAAGCCCAAAATGATTATGCCAACCAATTTCTCAAGAATTTCCCAAGAAATAT 780
 S I S P K I D Y A N Q I S Q I S Q E F P K E Y 212
 TTTTATTTGGGCGAAAAATGTTTATTAGCCACATCAACCTGATGATTATTTCAAAAT 840
 F Y W R E K L L F S H I N P D D Y Y P I 232
 CTTGTTCCACCTTGGCAGTGAGGAATCAATACAAATGGCGTTTGCATCTTTAATGTAT 900
 P V H P F W Q M R A N Q F T S L D 252
 AATAAATCCCTCATCTTGTACTCATCAACCACTAATACCTCTTTATCAGCTGAT 960
 N K S L I L L P H H Q T L I P S L S P D 272
 ATTATGATGCCACACATCCACTCAATGTACATTAATCTGGCTACCACTTTAAGCACC 1020
 I M M P A K T S C T Q T L K L A T T L S T 292
 TCAATGGCTGGAAGAACTGATAATCTAATGATATGGTATTGCTTACCAGATGGATCGAT 1080
 S M A G K L D N S N D M V L L T R W I D 312
 TCCCTGTTAGCAAAACCAATATACCAAACTACCTTGTATATGAAACCTGGAG 1140
 S L L A K T N Y Y Q N T L F I C K N L E 332
 AGCATGAGCGCTATGATCAAACTCTCTGATGCAATCGAGTAAATATTGTTGGC 1200
 S M S A Y D Q T L S E C N R V K L L F G 352
 TTATATCAAAACCACTCCCAAAATAGACAGGATCAAGAGCAGTTCATTACCTGACC 1260
 L Y Q N P L H K A I R Q D Q R A V P L P A 372
 CTATTAAGTATTCCCTTGTAGCAATACACCATCTATTGAAATATTAAAGCTAGC 1320
 L L T D S P C S N T P L L I E I I K A S 392
 GGCTGCACCAACGACTTATTCTCACTGAATATTGTTATAAGATGTTATTGGACAATTG 1380
 G L H P T T Y F T E Y C Y K M L F G Q L 412
 CATCTATTGCTAAATATGGATTAGCAGTAGAGTGGAGCAACAAATTTTATGTCAT 1440
 H L L I G C L R A N E Y Q I P S R T L W G 512
 TTAGCTCGCAAGTCATGCAAACTGATTTTAAAGACTTATCCAAAGACATTGATCCGCGT 1740
 L A R Q V M Q T V F K D L S K D I D P R 532
 ATTCTAAGTTGGCAACACATCTATTGCTTCATGATACTGGGAGCATCAACCTGAATTG 1800
 I L S W Q Q H L L H D N W E H Q Q P E A L 552
 TTATTAAGTCTGCATTCCAAATCAATCGAATATTACAAATAAGGAATACAACCCATTA 1860
 L L S L H S K I N R N I T I K E Y N P L 572
 TCAGAGATCTAAGctctactggacttacgaaaaacccatcgcgctcttggccaatacta 1920
 S E I - 575
 XhoI
 aaatatgagaactctcagggcggtgatggaactatgagtaataatccaattga 1980

FIG. 7. DNA and predicted amino acid sequences of *L. pneumophila* *frgA*. The potential ribosome binding site is boxed. The -35 and -10 regions are shown in bold and labeled. Two overlapping iron boxes are underlined or double-underlined and have four or five mismatches, respectively. The target sequence that was duplicated as a result of mini-Tn10/*lacZ* insertion is indicated by a black box. Recognition sites for *EcoRI* and *XhoI* are labeled and italicized.

IucA MILPSEKSATDVAAQCFLNALIRETKDWQLAEY-----PPDELIIPLDE -44
 FrgA MALAYGNFHELHSHQLRFLLEFEGIGLPONSVDY---FITLAHKNLTKRLQ -47
 IucC M-----NKKDWDLVNRRLVAKMLSELEYEQVFHAESEQDDRYCIN -40
 IucA QKSLHFRVAYFSPTQHHRFAPFAHLVLTASGSYFVDFTTLSRLIIDKLRHQ -94
 FrgA HASIKEGLIQSAISHHIDFDLQIKLKNMSPESKFFQWRKIREALDE -97
 IucC LPGAQWRFAERGIWGLWIDAQTLRCADPEVLAQTLLMLQKQVLSMSDA -90
 IucA LFLPVLCEFTHQRVLESYAHTQQTIDARHDW---AILREKALNFGAEQ -141
 FrgA SIANEALAYAYRONWNTQLRNEAMHYKSLWTWINNELSPYQTLFLLEQWG -147
 IucC TVAEHM-----QDLYATLUGDLQLKARRGLSASDLINLNADRLQCLLS -134
 IucA ALLTCAHFPAPKSHPEFNRQEAERYLDMAPHFPLRWFSDKT----- -165
 FrgA SL--RFPYHPAFSAKTGFTBREVLONSSEFOAKVSWHWCALNKT----- -169
 IucC G---HPKFEVFNKGRGKGKEALERYAPEYANTFRLHLWLVKREHMIWRC -180
 IucA QIAGESLHNLQQLRLE-----FAAENAPQLNLSLNDQWLFPLRPWG -229
 FrgA KIQSISPKIDYANQISQEPFPEYFWEKLLFSHINPDDYXPLFVHPWQ -239
 IucC DNEMDIHQLLTAAMDPOEPARFSPQVQWENGL-----DHNWLFPLVHPWQ -225
 IucA EYLFQVQVQCQLFAKGLIRDLG---EAGTSWLTPTSSRLYCATSRDMIKF -277
 FrgA RNOLOMAFASLIDNKSLLLPH---HOT---LIPSLSPDIMPPTQSTQCTL -284
 IucC QEKIATDFIADFGGRMVS LGFEGDQ---WLQQLSLRLTNASRRGLDI -272
 IucA SLSVRLTNSVRLTSVKE---VERGMRLARLAOTDGWQMLQARFPTFRVMQ -324
 FrgA KLATLSTSMAGKLDNS---NDMVLLTRWIDSLAKTNYQNTLFIKCNL -331
 IucC KPLITTYNTSCYRGIPGRYIAAGFLASRLQVQVFTDATLVQSGAV--IL -320
 IucA EDDWTGLRDLNGNIMQESLFSPAWKTLLEQPSQSR-----TNVLVSL -366
 FrgA ESMSAYDQTLSECNRVKLLFGLYNPLHKIRQDQR-----AVPLPAL -373
 IucC GEPAAGYVSHEGYAAARAPYRYQEMLGVIWRENPCRWLKPFDESPFLMAT -370
 IucA TQAGPHGGDSLLVSAVKRLSDRLGITVQQAHHAVDAVCCQVQLKPLFTAE -416
 FrgA LTDSPCSNTPILLIEIK-----ASGLHPTTYTETCYKMLFGLHLL -415
 IucC LMENDENQPLAGAYD-----RSGDLAETWLTQVFRVVVFLYHLL -412
 IucA ADYGLVLLAHQONILVQMLGDI-PVGFIYRI-CQGSAMPHATEWLDITIDE -465
 FrgA LKYGLALEVEQHNILVIFDDNKPGIITKE-PNNLKLCHN--ELFKNVQK -462
 IucC CRYGVALLAHGQNTLAMKEGVFQVRVLLKDFQGDMLRVKE--EF----- -454
 IucA AQAENIFTRQQLRYFPYLLVNSTFAVTA--LGAAGLSEANLMAR-V -512
 FrgA PNAPDSLSIYTKDLNQVRLTFIQGTLLKNHL--HLIGCLRNEYQIPSRIL -510
 IucC -PEMDSLP---QEVROVTSRLSADYLIHLDLQGTGHEVTVLRFISPLMVL -499
 IucA RTLLAEVRDQVTHKTCNLNVLESFYWNVKGNFCYLDNHNENTIVDPSVI -562
 FrgA WGLARQVMQTVFKDLKSKDIPRLSWQHLLHNDNEHQPELLLSLHSLI -560
 IucC -----GVPERRFYQLLAUV-----LSOYMKKHQMSERFALFSLF -534
 IucA Y--FDEANPLQAQEV -575
 FrgA NRNITIKENPLSEI -575
 IucC RPQIRRVVLP -545

FIG. 8. Homology between *FrgA* of *L. pneumophila* and *IucA* and *IucC* of *E. coli*. The *E. coli* sequences were reported previously (55). Identical amino acids are indicated with vertical lines, while similar amino acids, as defined by PC Gene, are indicated with black dots. The three boxed and three shaded regions show the homologous sequences indicated in the BLASTX results from the NCBI. In these regions only, additional conserved amino acids, as defined by the BLAST program, are indicated with small, lowered dots. Note that these regions overlap the areas that are most similar between *IucA* and *IucC* (55).

amino-terminal half of Fur (48, 85). Since minimal levels of Fur activity are likely present in our Mn²⁺ mutants, we may be unable to identify genes whose expression is regulated only slightly by Fur. Nevertheless, the ability to generate *fur* mutants and effectively introduce *lacZ* into virulent legionellae allowed us to study the iron regulation of five *L. pneumophila* (*frg*) loci.

Stable iron regulation was noted in two of the five strains,

NU232 and NU229. The *frg* (*frgB*) in strain NU232 was repressed 17-fold in response to iron. However, its Fur regulation could not be assessed because an NU232 Fur⁻ derivative could not be obtained. An initial attempt to demonstrate Fur repression of *frgB* in *E. coli* also proved inconclusive; i.e., when cloned along with 3 kb of upstream DNA, *frgB-lacZ* was expressed to similar degrees in Fur⁺ and Fur⁻ strains (unpublished results). The basis for the lethality of the *fur-frgB* double mutation is unclear, but it likely involves toxic levels of intracellular iron associated with the *fur* mutation. This result may provide a clue to *frgB* function. For example, the inability of the *frgB* mutation to offset Fur derepression suggests that it is not involved in iron uptake. Instead, it may encode an iron-storage protein, such as bacterioferritin or the ferritin H-like protein, whose absence would facilitate iron overload (1, 40). Alternately, it may express a protein, such as a DNA repair enzyme, superoxide dismutase, or peroxidase, involved in the protection of DNA damage from iron-catalyzed oxygen radicals (82). The *frgA* gene in strain NU229 had a level of iron regulation similar to that of *frgB* but was repressed by Fur. Since we could isolate a *fur* mutation in NU229, *frgA* is more likely to be involved in iron uptake than is *frgB*. The level of derepression associated with the loss of Fur was greater than that achieved by growth under low-iron conditions. A likely explanation for this result is that some Fur dimers were still bound to promoter regions at the levels of iron chelator that were tested. We did attempt to assess *lacZ* expression under conditions of greater iron limitation. However, an increased concentration of the chelator caused an inhibition of growth by both the wild-type and mutant strains, prohibiting any further examination of specific gene expression. Regardless, the characterization of NU229 demonstrates that iron regulation in *L. pneumophila* is explained, at least in part, by Fur-mediated repression.

Although essentially unaltered in its extracellular growth capability, NU229 was significantly impaired in intracellular growth. Despite an apparently normal capacity to enter into host cells and to resist macrophage bacteriocidal elements, the mutant replicated in a manner that yielded significantly fewer progeny than the wild type in a 72-h period. A comparison of macrophage growth kinetics suggests that NU229 is distinct from all other *L. pneumophila* mutants (i.e., *mip*, *icm*, *dot*, and *ira* mutants) found to be impaired for intracellular replication (6, 9, 15, 71). Complementation analysis further demonstrated that the infectivity defect of NU229 was due directly to the inactivation of *frgA*. Thus, *frgA* can be added to the small but growing list of *L. pneumophila* infectivity genes. Furthermore, the demonstration that an iron- and Fur-regulated gene can promote intracellular growth supports the notion that the phagosomal compartment is a low-iron environment (71, 72).

The *L. pneumophila* FrgA protein was similar to IucA and IucC in the aerobactin iron uptake system of *E. coli*. A comparison of IucA and IucC sequences disclosed three hydrophilic domains with greater than 57% similarity, which are thought to contain the active centers of these enzymes (55). Interestingly, the regions of highest homology between FrgA and the Iuc proteins overlap these sequences. Thus, the 21 to 45% amino acid identities that exist between FrgA and IucA/C may reflect a common function. In a potentially analogous situation, the carboxyl termini of *L. pneumophila* Mip and the FK506-binding protein of *Neurospora crassa* share 39% of their sequences and still possess similar levels of peptidyl-prolyl *cis/trans*-isomerase activity (25, 83). The discovery that *frgA* has homology with genes intimately involved in siderophore biosynthesis was exciting but also quite unexpected for several reasons. First, the absence of an aerobactin-like siderophore in

L. pneumophila strains was demonstrated by the Csaky assay, which identifies hydroxamate siderophores by their chemical structure (76). Second, the chrome azurol S assay, a procedure which measures chelating activity independent of structure, failed to detect siderophores in culture supernatants of strain 130b (29, 49). Third, in a classic bioassay, concentrated supernatants from one iron-depleted *L. pneumophila* culture did not stimulate the growth of a second iron-starved culture (76). Finally, NU229 was not sensitive to iron limitation and was not resistant to streptonigrin when grown in bacteriological media. On the other hand, the importance of *frgA* for growth within the iron-limiting environment of a macrophage and its iron-dependent regulation by Fur suggest a role in iron acquisition. These data can be rationalized in one of two ways. Either FrgA catalyzes a reaction similar to the ones mediated by IucA and IucC but does not result in the production of a "classic" siderophore, or along with unlinked loci, *frgA* is involved in the production of an iron chelator prevalent in the intracellular environment. The absence of Fe³⁺-chelating activity in *L. pneumophila* supernatants, despite *frgA* expression in low-iron bacteriological media, suggests that the production of this putative siderophore is not solely responsive to iron and Fur fluctuations. Clearly, the role of *frgA* in iron acquisition is yet to be elucidated; however, its sequence has caused us to once again reexamine our view of *L. pneumophila* and siderophores.

The *frgA* gene was interesting not only for its role in macrophage infection and its sequence homologies but also for its unique distribution among legionellae. Southern hybridization analysis indicated that *frgA* sequences are limited to *L. pneumophila* strains. Nearly all previously identified *L. pneumophila* genes, including *flaA*, *fur*, *hip*, *lly*, *mip*, *ompS*, and *pplA* (*pal*), have had homologs in all, or virtually all, other *Legionella* species (5, 17, 33, 35, 37, 38, 64). We recently reported that the one exceptional gene, *hbp*, had analogs in only two of the eight species tested (62). The absence of *frgA* among the 16 *Legionella* species examined signals that the gene should be considered for use as a diagnostic reagent. The *mip* primers used for the PCR detection of *L. pneumophila* within water and clinical samples have, in several instances, amplified DNAs from other *Legionella* species (2, 18, 42, 46, 50). Thus, *frgA* primers, more so than *hbp* primers, should enhance the specificity of *L. pneumophila* detection protocols.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI34937 from the National Institutes of Health.

We thank Michael Vasil for *E. coli* Fur antibodies.

REFERENCES

- Andrews, S. C., P. M. Harrison, and J. R. Guest. 1989. Cloning, sequencing, and mapping of the bacterioferritin gene (*bfr*) of *Escherichia coli* K-12. *J. Bacteriol.* **171**:3940-3947.
- Atlas, R. M., J. F. Williams, and M. K. Huntington. 1995. *Legionella* contamination of dental-unit waters. *Appl. Environ. Microbiol.* **61**:1208-1213.
- Ausubel, F. M., R. B. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. *Current protocols in molecular biology*. John Wiley & Sons, New York.
- Bagg, A., and J. B. Neilands. 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a co-factor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**:5471-5477.
- Bender, L., M. Ott, A. Debes, U. Rdest, J. Heesemann, and J. Hacker. 1991. Distribution, expression, and long-range mapping of legiolsin gene (*lly*)-specific DNA sequences in legionellae. *Infect. Immun.* **59**:3333-3336.
- Berger, K. H., J. J. Merriam, and R. R. Isberg. 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila* *dotA* gene. *Mol. Microbiol.* **14**:809-822.
- Berish, S. A., S. Shambhavi, C. Chen, D. L. Trees, and S. A. Morse. 1993. Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae*. *Infect. Immun.* **61**:4599-4606.
- Bortner, C. A., R. R. Arnold, and R. D. Miller. 1989. Bacteriocidal effect of

- lactoferrin on *Legionella pneumophila*: effect of the physiological state of the organism. *Can. J. Microbiol.* **35**:1048–1051.
9. Brand, B. C., A. B. Sadosky, and H. A. Shuman. 1994. The *Legionella pneumophila* *icm* locus: a set of genes required for intracellular multiplication in human macrophages. *Mol. Microbiol.* **14**:797–808.
 10. Bridges, K. R. 1990. Modulation of the availability of intracellular iron, p. 297–314. In P. Ponka, H. M. Shulman, and R. C. Woodsworth (ed.), *Iron storage and transport*. CRC Press, Boca Raton, Fla.
 11. Byrd, T. F., and M. A. Horwitz. 1989. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J. Clin. Invest.* **83**:1457–1465.
 12. Byrd, T. F., and M. A. Horwitz. 1991. Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in nonactivated and interferon gamma-activated human monocytes depending on its degree of iron saturation. Iron-lactoferrin and nonphysiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. *J. Clin. Invest.* **88**:1103–1112.
 13. Calderwood, S. B., and J. J. Mekalanos. 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.* **169**:4759–4764.
 14. Cianciotto, N., B. I. Eisenstein, N. C. Engleberg, and H. Shuman. 1989. Genetics and molecular pathogenesis of *Legionella pneumophila*, an intracellular parasite of macrophages. *Mol. Biol. Med.* **6**:409–424.
 15. Cianciotto, N., B. I. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg. 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infect. Immun.* **57**:1255–1262.
 16. Cianciotto, N. P., R. Long, B. I. Eisenstein, and N. C. Engleberg. 1988. Site-specific mutagenesis in *Legionella pneumophila* by allelic exchange using counterselectable ColEI vectors. *FEMS Microbiol. Lett.* **56**:203–208.
 17. Cianciotto, N. P., J. M. Bangsberg, B. I. Eisenstein, and N. C. Engleberg. 1990. Identification of *mip*-like genes in the genus *Legionella*. *Infect. Immun.* **58**:2912–2918.
 18. Cianciotto, N. P., and B. S. Fields. 1992. *Legionella pneumophila* *mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc. Natl. Acad. Sci. USA* **89**:5188–5191.
 19. Crichton, R. R., and M. Charlotiaux-Wauters. 1987. Iron transport and storage. *Eur. J. Biochem.* **164**:485–506.
 20. de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. *J. Bacteriol.* **169**:2624–2630.
 21. de Lorenzo, V., and J. B. Neilands. 1986. Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*. *J. Bacteriol.* **167**:350–355.
 22. Dowling, J. N., A. K. Saha, and R. H. Glew. 1992. Virulence factors of the family *Legionellaceae*. *Microbiol. Rev.* **56**:32–60.
 23. Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith. 1978. Primary isolation media for Legionnaires disease bacterium. *J. Clin. Microbiol.* **8**:320–325.
 24. Fields, B. S. 1996. The molecular ecology of legionellae. *Trends Genet.* **4**:286–290.
 25. Fischer, G., H. Bang, B. Ludwig, K. Mann, and J. Hacker. 1992. *Mip* protein of *Legionella pneumophila* exhibits peptidyl-prolyl-cis/trans isomerase (PPIase) activity. *Mol. Microbiol.* **6**:1375–1383.
 26. Foster, J. W., and H. K. Hall. 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations in iron- and pH-regulated protein synthesis. *J. Bacteriol.* **174**:4317–4323.
 27. Freedman, H. H., A. E. Frost, S. J. Westerback, and A. E. Martell. 1957. Chelating tendencies of N, N'-ethylenebis-[2-(o-hydroxyphenyl)] glycine. *Nature* **179**:1020–1021.
 28. Goldberg, M. B., S. A. Boyko, and S. B. Calderwood. 1991. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **88**:1125–1129.
 29. Goldoni, P., P. Visca, M. C. Pastoris, P. Valenti, and N. Orsi. 1991. Growth of *Legionella* spp. under conditions of iron restriction. *J. Med. Microbiol.* **34**:113–118.
 30. Gueriot, M. L. 1994. Microbial iron transport. *Annu. Rev. Microbiol.* **48**:743–772.
 31. Hantke, K. 1981. Regulation of ferric iron transport in *Escherichia coli* K-12: isolation of a constitutive mutant. *Mol. Gen. Genet.* **182**:228–292.
 32. Hantke, K. 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K-12: *fur* not only affects iron metabolism. *Mol. Gen. Genet.* **210**:135–139.
 33. Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Luck, K. H. Mann, R. Marre, M. Ott, and J. Hacker. 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. *Infect. Immun.* **63**:2499–2507.
 34. Hickey, E., and N. P. Cianciotto. 1995. Identification of *Fur*-repressed genes from *Legionella pneumophila*, abstr. B-375, p. 230. In Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
 35. Hickey, E. K., and N. P. Cianciotto. 1994. Cloning and sequencing of the *Legionella pneumophila fur* gene. *Gene* **143**:117–121.
 36. Hoffman, P. 1984. Bacterial physiology, p. 61–67. In C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowsky (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
 37. Hoffman, P. S., C. A. Butler, and F. D. Quinn. 1989. Cloning and temperature-dependent expression in *Escherichia coli* of a *Legionella pneumophila* gene coding for a genus-common 60-kilodalton antigen. *Infect. Immun.* **57**:1731–1739.
 38. Hoffman, P. S., M. Ripley, and R. Weeratna. 1992. Cloning and nucleotide sequence of a gene (*ompS*) encoding the major outer membrane protein of *Legionella pneumophila*. *J. Bacteriol.* **174**:914–920.
 39. Horwitz, M. A. 1992. Interactions between macrophages and *Legionella pneumophila*. *Curr. Top. Microbiol. Immunol.* **181**:265–282.
 40. Izuhara, M., K. Takamune, and R. Takata. 1991. Cloning and sequencing of an *Escherichia coli* K12 gene which encodes a polypeptide having similarity to the human ferritin H subunit. *Mol. Gen. Genet.* **225**:510–513.
 41. James, B. W., W. S. Mauchline, R. B. Fitzgeorge, P. J. Dennis, and C. M. Keevil. 1995. Influence of iron-limited continuous culture on physiology and virulence of *L. pneumophila*. *Infect. Immun.* **63**:4224–4230.
 42. Jaulhac, B., M. Nowicki, N. Bornstein, O. Meunier, G. Prevost, Y. Piemont, J. Fleurette, and H. Monteil. 1992. Detection of *Legionella* spp. in bronchoalveolar lavage fluids by DNA amplification. *J. Clin. Microbiol.* **30**:920–924.
 43. Johnson, W., L. Varner, and M. Poch. 1991. Acquisition of iron by *Legionella pneumophila*: role of iron reductase. *Infect. Immun.* **59**:2376–2381.
 44. Kammler, M., C. Schön, and K. Hantke. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. *J. Bacteriol.* **175**:6212–6219.
 45. Keberle, H. 1964. The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann. N. Y. Acad. Sci.* **119**:758–768.
 46. Kessler, H. H., F. F. Reinthaler, A. Pschaid, K. Pierer, B. Kleinhappl, E. Eber, and E. Marth. 1993. Rapid detection of *Legionella* species in bronchoalveolar lavage fluids with the EnviroAmp *Legionella* PCR amplification and detection kit. *J. Clin. Microbiol.* **31**:3325–3328.
 47. Kleckner, N., J. Bender, and S. Gottesman. 1991. Uses of transposons with emphasis on *Tn10*. *Methods Enzymol.* **204**:139–180.
 48. Lam, M. S., C. M. Litwin, P. A. Carroll, and S. B. Calderwood. 1994. *Vibrio cholerae fur* mutations associated with loss of repressor activity: implications for the structural-functional relationships of *Fur*. *J. Bacteriol.* **176**:5108–5115.
 49. Liles, M. R., and N. P. Cianciotto. 1996. Absence of siderophore-like activity in *Legionella pneumophila* supernatants. *Infect. Immun.* **64**:1873–1875.
 50. Lindsay, D. S., W. H. Abraham, and R. J. Fallon. 1994. Detection of *mip* gene by PCR for diagnosis of Legionnaires' disease. *J. Clin. Microbiol.* **32**:3068–3069.
 51. Litwin, C. M., S. A. Boyko, and S. B. Calderwood. 1992. Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. *J. Bacteriol.* **174**:1897–1903.
 52. Litwin, C. M., and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**:137–149.
 53. Litwin, C. M., and S. B. Calderwood. 1994. Analysis of the complexity of gene regulation by *Fur* in *Vibrio cholerae*. *J. Bacteriol.* **176**:240–248.
 54. Martinez, E., B. Bartolomé, and F. de la Cruz. 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZa* reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **68**:159–162.
 55. Martínez, J. L., M. Herrero, and V. de Lorenzo. 1994. The organization of intergenic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the *iucABCD iutA* genes. *J. Mol. Biol.* **238**:288–293.
 56. Mengaud, J. M., and M. A. Horwitz. 1993. The major iron-containing protein of *Legionella pneumophila* is an aconitase homologous with the human iron-responsive element-binding protein. *J. Bacteriol.* **175**:5666–5676.
 57. Miles, A. A., and P. L. Khimji. 1975. Enterobacterial chelators of iron: their occurrence, detection, and relation to pathogenicity. *J. Med. Microbiol.* **8**:477–492.
 58. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 59. Muller-Eberhard, U., and H. Nikkila. 1989. Transport of tetrapyrroles. *Semin. Hematol.* **26**:86–104.
 60. Neumann, B., A. Pospiech, and H. U. Schairer. 1992. Rapid isolation of genomic DNA from gram-negative bacteria. *Trends Genet.* **8**:332–333.
 61. Niederhoffer, E. C., C. M. Naranjo, K. L. Bradley, and J. A. Fee. 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. *J. Bacteriol.* **172**:1930–1938.
 62. O'Connell, W. A., E. K. Hickey, and N. P. Cianciotto. 1996. A *Legionella pneumophila* gene that promotes hemin binding. *Infect. Immun.* **64**:842–848.
 63. O'Connell, W. A., J. M. Bangsberg, and N. P. Cianciotto. 1995. Characterization of a *Legionella micdadei* *mip* mutant. *Infect. Immun.* **63**:2840–2845.
 64. Ott, M., L. Bender, E. Chirinos, W. Ehret, and J. Hacker. 1991. Phenotype versus genotype of the 19 kD peptidoglycan associated protein of *Legionella*

- (PpIA), among legionellae and other gram-negative bacteria. Microb. Pathog. **11**:357–365.
65. **Otto, B. R., A. M. J. J. Verweij-van Vught, and D. M. MacLaren.** 1992. Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit. Rev. Microbiol. **18**:217–233.
 66. **Payne, S. M.** 1993. Iron acquisition in microbial pathogenesis. Trends Microbiol. **1**:66–69.
 67. **Pearlman, E., A. H. Jiwa, N. C. Engleberg, and B. I. Eisenstein.** 1988. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. Microb. Pathog. **5**:87–95.
 68. **Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell.** 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. J. Clin. Microbiol. **9**:615–626.
 69. **Poch, M. T., and W. Johnson.** 1993. Ferric reductases of *Legionella pneumophila*. Biometals **6**:107–114.
 70. **Pope, C. D., L. Dhand, and N. P. Cianciotto.** 1994. Random mutagenesis of *Legionella pneumophila* with mini-Tn10. FEMS Microbiol. Lett. **124**:107–112.
 71. **Pope, C. D., W. A. O'Connell, and N. P. Cianciotto.** 1996. *Legionella pneumophila* mutants that are defective for iron acquisition and assimilation and intracellular infection. Infect. Immun. **64**:629–636.
 72. **Portillo, F. G., J. W. Foster, M. E. Maguire, and B. B. Finlay.** 1992. Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. Mol. Microbiol. **6**:3289–3297.
 73. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175**:2589–2598.
 74. **Quinn, F. D., and E. D. Weisberg.** 1988. Killing of *Legionella pneumophila* by human serum and iron-binding agents. Curr. Microbiol. **17**:111–116.
 75. **Reeves, M. W., L. Pine, S. H. Hunter, J. R. George, and W. K. Harrell.** 1981. Metal requirements of *Legionella pneumophila*. J. Clin. Microbiol. **13**:688–695.
 76. **Reeves, M. W., L. Pine, J. B. Neilands, and A. Ballows.** 1983. Absence of siderophore activity in *Legionella* species grown in iron-deficient media. J. Bacteriol. **154**:324–329.
 77. **Ristroph, J. D., K. W. Hedlum, and S. Gowda.** 1981. Chemically defined medium for *Legionella pneumophila* growth. J. Clin. Microbiol. **13**:115–119.
 78. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
 79. **Steinman, H. M.** 1992. Construction of an *Escherichia coli* K-12 strain deleted for manganese and iron superoxide dismutase genes and its use in cloning the iron superoxide dismutase of *Legionella pneumophila*. Mol. Gen. Genet. **232**:427–430.
 80. **Thomas, C. E., and P. F. Sparling.** 1994. Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. Mol. Microbiol. **11**:725–737.
 81. **Tolmasky, M. E., A. M. Wertheimer, L. A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* gene: role in regulation of expression of the FatA outer membrane protein and catechols. J. Bacteriol. **176**:213–220.
 82. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despiéd.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δfur mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177**:2305–2314.
 83. **Tropschug, M., E. Wachter, S. Mayer, E. R. Schönbrunner, and F. X. Schmid.** 1990. Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyses protein folding. Nature **346**:674–677.
 84. **Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron.** 1995. Fur regulon of *Salmonella typhimurium*: identification of new iron-regulated genes. J. Bacteriol. **177**:4628–4637.
 85. **Wertheimer, A. M., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1994. Structural and functional analyses of mutant Fur proteins with impaired regulatory function. J. Bacteriol. **176**:5116–5122.
 86. **White, J. R., and H. N. Yeowell.** 1982. Iron enhances the bactericidal action of streptonigrin. Biochem. Biophys. Res. Commun. **106**:407–411.
 87. **Wooldridge, K. G., and P. H. Williams.** 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol. Rev. **12**:325–348.
 88. **Valvano, M. A., and J. H. Crosa.** 1984. Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of *Escherichia coli* K1. Infect. Immun. **46**:159–167.

Editor: P. J. Sansonetti