Legionella pneumophila feoAB Promotes Ferrous Iron Uptake and Intracellular Infection

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In order to determine the role of ferrous iron transport in Legionella pathogenesis, we identified and mutated the feoB gene in virulent Legionella pneumophila strain 130b. As it is in Escherichia coli, the L. pneumophila feoB gene was contained within a putative feoAB operon. L. pneumophila feoB insertion mutants exhibited decreased ferrous but not ferric iron uptake compared to the wild type. Growth on standard buffered charcoal yeast extract agar or buffered yeast extract broth was unaffected by the loss of L. pneumophila FeoB. However, the L. pneumophila feoB mutant had a reduced ability to grow on buffered charcoal yeast extract agar with a reduced amount of its usual iron supplementation, a phenotype that could be complemented by the addition of FeoB in trans. In unsupplemented buffered yeast extract broth, the feoB mutant also had a growth defect, which was further exacerbated by the addition of the ferrous iron chelator, 2,2'-dipyridyl. The feoB mutant was also 2.5 logs more resistant to streptomycin than wild-type 130b, confirming its decreased ability to acquire iron during extracellular growth. Decreased replication of the feoB mutant was noted within iron-depleted Hartmannella vermiformis amoebae and human U937 cell macrophages. The reduced intracellular infectivity of the feoB mutant was complemented by the introduction of a plasmid containing feoAB. The L. pneumophila feoB gene conferred a modest growth advantage for the wild type over the mutant in a competition assay within the lungs of A/J mice. Taken together, these results indicate that L. pneumophila FeoB is a ferrous iron transporter that is important for extracellular and intracellular growth, especially in iron-limited environments. These data represent the first evidence for the importance of ferrous iron transport for intracellular replication by a human pathogen.

Iron is essential for the growth of essentially all organisms, and its limitation plays an important role in host defense against infection by restricting bacterial replication (46, 60). L. pneumophila, the causative agent of Legionnaires’ disease, is a facultative intracellular parasite of human macrophages and freshwater amoebae (31, 41, 50). The ability of L. pneumophila to acquire host cell iron is pivotal for it to establish successful intracellular infection (8–10, 24, 25, 26, 29, 30, 42, 45, 58). For example, gamma interferon-activated macrophages are non-permissive for L. pneumophila growth due to decreased intracellular iron (40). Also, sequestration of intracellular iron by chelators inhibits the growth of L. pneumophila in human macrophages (24). Furthermore, a reduced ability of legionellae to establish successful intracellular infection is correlated with decreased levels of the receptor for transferrin, the key iron transport protein of the host cell (8).

In order to occupy its intracellular niche, L. pneumophila likely has developed multiple iron acquisition mechanisms. Factors that may be involved in L. pneumophila iron acquisition include heme utilization (42); the iraAB locus, which may encode a transporter of iron-loaded peptides (58); the cytochrome c maturation (ccm) genes (59); the iron-regulated frgA, whose product has homology to aerobactin synthetases (30); legiobactin, the first example of a Legiobacter siderophore (36); and two internal ferric reductases (44).

Iron exists in a dynamic equilibrium between the ferrous (Fe2⁺) and ferric (Fe3⁺) forms, depending on its environment (1). In aerobic conditions at neutral pH, the majority of iron is generally in the insoluble ferric form (1). However, at acid pH or in anaerobic conditions, the equilibrium shifts to the soluble ferrous form (1). Within host cells, the majority of iron is sequestered by the eukaryotic storage proteins ferritin and hemosiderin (46, 62). Reports have indicated that Fe2⁺ is the predominant form of iron in the cytosolic pools in mammalian cells (5, 19).

Within its host cells, L. pneumophila resides in a specialized phagosome, where its precise source of iron is unclear but presumably involves active access to the iron pool (8). Although the L. pneumophila phagosome does not fuse with endosomes and lysosomes and is at nearly neutral pH during the early stages of the intracellular life cycle, it appears to fuse with low-pH cellular compartments during later stages of the infection (61). If there were a concomitant decrease in phagosomal pH, then the amount of soluble ferrous iron available to the bacteria may increase (54). Taken together, these data suggest that L. pneumophila may access significant amounts of ferrous iron for its intracellular replication. Furthermore, previous reports suggested that L. pneumophila can use ferrous iron during replication in bacteriological media, as reduced growth by wild-type bacteria was observed in the presence of the specific ferrous iron chelator 2,2'-dipyridyl (DIP) (47). Because ferrous iron may be important to the bacterium during infection of host cells, we began to consider the means by which L. pneumophila acquires and/or utilizes this form of iron.

Ferrous iron transport has been an important area of re-

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search in both eukaryotic and prokaryotic systems. For example, several determinants involved in ferrous iron transport have been identified in eukaryotic cells, such as Nramp-2 in mammalian cells and the $FET3$ gene in Saccharomyces cerevisiae (3, 4). In bacteria, the $feoAB$ operon of Escherichia coli is the most-characterized ferrous iron transport system (33). $FeoB$ is an inner membrane protein, believed to comprise an ATP-driven transporter (33). Genes homologous to $feoB$ have been detected in the genomes of many bacterial species. In contrast, the function of $FeoA$ remains unclear, and the $feoB$ gene is not always present alongside $feoB$ (33).

In *E. coli*, loss of $feoA$ or $feoB$ results in decreased ferrous iron uptake (33). Furthermore, *E. coli* $feoB$ mutants exhibit decreased gut colonization in mice, suggesting a role for $feoB$ in pathogenesis (53). However, the importance of $feoB$ and $Fe^{2+}$ transport in intracellular parasitism remains to be demonstrated. Given our desire to understand both the specific mechanisms of *Legionella* pathogenesis and the general importance of ferrous iron transport in intracellular infection, we sought to identify and characterize the *L. pneumophila* genes involved in the acquisition and utilization of $Fe^{2+}$.

This study describes the characterization and mutational analysis of the *L. pneumophila* $feoB$ gene and suggests that the acquisition of ferrous iron is relevant for the growth of *L. pneumophila* both extracellularly in artificial media and intracellularly in macrophages and ameobae.

**MATERIALS AND METHODS**

**Bacterial strains, media, and extracellular growth conditions.** *L. pneumophila* strain 130b (ATCC BAA-74, also known as Wadsworth or AA100) was used for mutagenesis of the *Legionella* $feoB$ gene and subsequently served as the wild-type control (18). *L. pneumophila* strains were routinely grown on standard buffered charcoal yeast extract (BCYE) agar for 3 days at 37°C or in buffered yeast extract (BYE) broth at 37°C with shaking at 225 rpm (Lab-Line Instruments; Dubuque, Iowa; model 3525) (16, 35). For selection of allelic exchange mutants, BCYE agar was supplemented with 5% (wt/vol) sucrose (35). Standard BCYE agar and BYE broth contain an iron supplement consisting of 0.25 g of ferric pyrophosphate (Sigma, St. Louis, Mo.) per liter.

In order to monitor extracellular growth in liquid media, *L. pneumophila* grown on BCYE agar was used to inoculate 25 ml of BYE broth overnight. The culture was washed once with BYE broth without iron supplementation and then used as a inoculum in BYE broth, which was allowed to grow at 37°C with shaking at 225 rpm. Bacterial growth was monitored by determining the optical density of the culture at 660 nm (OD$_{660}$) Beckman, Fullerton, Calif.; spectrophotometer model DU500). In order to assess bacterial growth in iron-limiting BYE broth, iron supplementation was omitted and, where stated, DIP (Sigma) was added at various concentrations. To further control the amount of iron in BYE broth cultures, Milli-Q water and acid-washed glassware were used throughout (14).

To assess the growth and survival of *L. pneumophila* on iron-limited solid media, bacteria grown on BCYE agar were resuspended in distilled water to an OD$_{660}$ of 0.3 to 0.4, and then 100 µl of 10-fold serial dilutions was plated on BCYE agar containing either 32-fold less (0.0078 g per liter) iron supplementation and 0 µM FeCl$_3$, 0.01 µM FeCl$_3$, or 0 µM FeCl$_3$ plus 10 µM FeCl$_3$ (35).

*Escherichia coli* DH5α was used as a host for recombinant plasmids and was cultured in Luria-Bertani broth or agar (27). Where appropriate, media were supplemented with the following antibiotics at final concentrations suitable for *L. pneumophila* (*E. coli*): kanamycin, 25 µg/ml (50 µg/ml), and chloramphenicol, 6 µg/ml (30 µg/ml).

**Cloning and DNA sequence analysis.** The double-stranded sequence of the *Legionella feoAB* locus was obtained by using plasmid pMRJ. This plasmid was identified by colony hybridization of a genomic library that contained SallAI-digested fragments of strain 130b cloned into pBR322 (32). Hybridization was performed using a DNA probe prepared by PCR amplification of a 1.4-kb internal fragment of the *L. pneumophila* $feoB$ gene with primers FEOB-F (5'-CAT AGTATGCCACGCAAC-3') and FEOB-R (5'-AAAGCAGCTCAGTGATAGACTAG-3'). Sequencing of the *L. pneumophila* $feoB$, $feoA$, and upstream region was performed by the dyeoxy chain termination method with BigDye (ABI Prism, Applied Biosystems; Foster City, Calif.) with a series of primers devised from the DNA sequence of the $feoB$ region from the *L. pneumophila* strain Philadelphia-1 unfinished genome database (http://genome3.cpmc.columbia.edu /~legion).

DNA sequencing was performed by the Northwestern University Biotechnology Laboratory, and primers were obtained from Integrated DNA Technologies (Coralville, Iowa). Nucleotide sequences were analyzed with Seqman (DNASTar, Madison, Wis.), and Blast homology searches were conducted through GenBank at the National Center for Biotechnology Information. DNA motifs and structural analyses were conducted with the Prosite prediction model (http://us.expasy. org/prosite) and the SOSUI program (http://sosui.proteome.bio.tsut.ac.jp).

**Mutation and complementation analyses.** An internal fragment of the *L. pneumophila* strain 130b $feoB$ gene was amplified by PCR with primers FEOB2-F and FEOB2-R. The resultant 1.4-kb DNA fragment was then cloned into pGEMEasy (Promega, Madison, Wis.) to give plasmid pMR3. The cloned *L. pneumophila* $feoB$ was mutated by insertion of a 1.1-kb kanamycin resistance (Km') gene at the Klengow-treated KpnI 21 site of pMR3, resulting in pMR4. The Km'-EcorV fragment containing the Km' gene was obtained from plasmid PVK4, a derivative of PVK3 with the antibiotic resistance gene in the opposite orientation (58). The mutated $feoB$ gene was then cloned on a 2.6-kb NotI fragment into the counterselectable pBC20 to yield pMR5. The chloramphenicol-resistant pBOC20 is based on a ColE1 replicon and facilitates allelic exchange in *L. pneumophila* by virtue of its sacB gene (35).

Production of competent 130b cells and electroporation of pMR5 into *L. pneumophila* were achieved as previously described (35). Potential mutants were selected based on chloramphenicol sensitivity and kanamycin and sucrose resistance, indicative of introduction of the mutated gene into the 130b chromosome by homologous recombination. Verification of mutant genotypes was carried out by PCR and Southern hybridization with the same primers and DNA probe used to identify pMR1. Genomic DNA was isolated from *L. pneumophila* as described previously (17).

To facilitate complementation, a 2.4-kb PCR fragment containing only the $feoB$ gene was first amplified from pMRJ with primers FEOBSKpnI (5'-CGGG GTACCCCCGTGGATGCTCCATTAGG-3') and pBRREV (5'-CGCCCA GCAACCGACACCTGTC-3'). PCR fragments containing $feoB$ plus the upstream $feoA$ and promoter region and just the $feoB$ gene were generated with primers FEB5e (5'-TATCCTGAGCTCGATCCCGTG-3') and pBR-R or FEOBSKpnI and FEO9B (5'-CCTG ATG CTC GGC AAT ATC CA 3'), respectively. Next, the PCR fragments were cloned into pGEMT (Promega) to yield plasmids pMR17, pMR18, and pMR19, encoding $feoB$, $feoA$, and $feoA$, respectively. Primers used during amplification were designed against the known sequence of the *feoB* region from strain 130b. Next, the cloned $feoB$, $feoAB$, and $feoA$ genes were transferred on the kpnI- and XhoI-digested fragments of pMMB207, yielding pMR20, pMR21, and pMR22, respectively (39). Plasmid pMMB207 provides a promoter for the expression of cloned genes and has been used previously for trans complementation of *L. pneumophila* mutants, including those with infectivity defects (37, 51, 59). Plasmids were transformed into *L. pneumophila* by electroporation by standard methods (35).

**Iron uptake assays.** To investigate the ability of *L. pneumophila* strains to take up iron, studies were performed with $^{55}$FeCl$_3$ (Perkin Elmer, Boston, Mass.) (16.07 µCi/ml) based on the protocol of Kammler et al. (33). *L. pneumophila* was grown overnight in 25 ml of BYE broth without supplemental iron to the late logarithmic phase (i.e., corresponding to an OD$_{660}$ of 2) at 37°C with shaking. Bacteria were then subcultured into 25 ml of BYE broth without supplemental iron and allowed to grow again to the late logarithmic phase. The final cultures were centrifuged at 3,500 rpm for 15 min at 30°C, and the bacterial pellet was resuspended in 25 ml of MOPS transport buffer (40 mM morpholinepropanesulfonic acid [MOPS]-KOH [pH 7.0], 100 mM MgSO$_4$, 0.5 mM CaCl$_2$, and 0.2% glucose, warmed to 37°C) to 1000 CFU/ml, a concentration that was based on the OD$_{660}$ of the suspension and confirmed by plating aliquots on BCYE agar.

To assess ferrous iron uptake, 10 ml of bacterial suspensions was added to 0.5 µM $^{55}$Fe (by the addition of 10 µl of a 3 mM stock solution of $^{55}$FeCl$_3$ in 0.1 M HCl in the final suspension to 10 µl of a 1 M freshly prepared stock solution; Sigma). For monitoring ferric iron uptake, the bacteria and radiolabeled iron samples were mixed in the presence of 1 mM sodium citrate (10 µl of a 1 M freshly prepared stock solution; Sigma). At various times, 1 ml of the bacterial suspension was removed to a 0.45-µm nitrocellulose
L. pneumophila feoAB the standard and kanamycin-supplemented BCYE. Wild type to mutant were estimated by plating 10-fold serial dilutions on both followed by vigorous pipetting. The numbers of viable bacteria and the ratio of incubation of the tissue sample with 100 mesh grid into 10 ml of phosphate-buffered saline. Host cell lysis was achieved by infected monolayers were incubated at 37°C for 3 h, and and emission at 584 was assessed. fl washed twice and incubated at 37°C for 3 h, and flBlue was added (1/11th volume in RPMI 1640) to monolayers that had been previously described (2, 32, 48, 49). The fact that the predicted stop codon of feoA overlaps the predicted start codon of feoB suggests that L. pneumophila feoA and feoB form an operon, similar to E. coli feoAB (33).

Upstream of the L. pneumophila feoA coding region was a putative Fur binding site, which contained 14 of the 19 residues conserved in E. coli Fur-regulated promoters (15). This suggests that the L. pneumophila feoAB locus, like its E. coli counterpart, is subject to regulation by bacterial intracellular Fe^{2+} levels (15). Further upstream of L. pneumophila feoA was an open reading frame whose deduced protein product was homologous (41% identity, 60% similarity) to a hypothetical protein in Pseudomonas aerugiosa (accession number NP_253128). Analysis of the L. pneumophila genomic database indicates that downstream of feoAB there is an open reading frame that is transcribed in the opposite orientation and whose predicted product does not bear homology to any proteins in the GenBank database.

In order to assess the role of FeoB in L. pneumophila physiology and pathogenesis, a feoB mutation was generated in strain 130b by allelic exchange of the wild-type gene for a cloned copy that had been inactivated by a Km\(^{r}\) insertion. Mutant NU269, which contained a Km\(^{r}\) insertion at nucleotide 1600 of the 2,256-bp gene, was verified by PCR and Southern hybridization (data not shown).

Iron uptake by L. pneumophila strains. To investigate whether L. pneumophila FeoB was a functional homologue of E. coli FeoB, wild-type and mutant legionellae were compared in \(^{55}\)Fe iron uptake assays. To specifically monitor the uptake of ferrous iron, the procedure was performed in the presence of ascorbate, which is known to maintain iron in a reduced state over the necessarily short period of the assay (33). L. pneumophila 130b exhibited increasing ferrous iron accumulation over the assay period; i.e., there was 5.3-fold greater ra-
dilabel incorporation at 20 min versus 1 min (Fig. 1). In comparison, the *L. pneumophila* *feoB* mutant did not exhibit significant ferrous iron uptake upon incubation (Fig. 1). This severe defect in NU269 is consistent with a role for FeoB as a ferrous iron transporter. Indeed, the mutant’s defect in ferrous iron uptake could be complemented by the addition of *feoB* in *trans* (Table 1).

When the uptake assays were performed in the presence of citrate, which maintains the iron in the ferric form, the wild type and the *feoB* mutant exhibited similar levels of iron uptake (Table 1), indicating that *L. pneumophila* FeoB is not required for ferric iron uptake. In comparing the data in Fig. 1 and Table 1, less Fe$^{3+}$ than Fe$^{2+}$ was associated with the wild type, suggesting that, in *L. pneumophila*, ferrous iron uptake may be more efficient than ferric iron uptake. The *feoB* mutant was not defective for legiobactin siderophore activity, based on the CAS assay (data not shown). Based on our sequence data and the defect in Fe$^{2+}$ uptake exhibited by NU269, we conclude that *L. pneumophila* *feoB* encodes a factor that is required for ferrous iron transport and is the functional equivalent of *E. coli* FeoB.

**Extracellular growth characteristics of *L. pneumophila* strains.** To investigate the role of *feoB* and ferrous iron transport in the extracellular growth of *L. pneumophila*, we compared wild-type and mutant bacteria for their ability to grow on both standard solid and liquid media and media that had been depleted of iron. Growth on BCYE agar was assessed by plating suspensions of bacteria and then noting both the number of recovered CFU and the time to colony formation. Strains 130b and NU269 grew similarly on standard BCYE agar, which is routinely supplemented with 0.25 g of ferric pyrophosphate per liter.

To start to investigate the role of iron in growth, BCYE agar was prepared with only 0.0078 g of the ferric pyrophosphate supplement per liter. This 32-fold decrease in iron supplementation did not affect the growth of 130b. However, the *feoB* mutant now took 10 as opposed to 3 days to form colonies, although similar numbers of CFU were eventually attained. When pMR20, containing an intact copy of *feoB* alone, was introduced into NU269, the mutant formed colonies in 4 days on the BCYE agar that had a diminished iron concentration. Further increasing the iron limitation, by omission of the ferric pyrophosphate, did not alter the growth of the wild type. However, the *feoB* mutant now required 13 to 15 days for the generation of single colonies on unsupplemented BCYE agar. Taken together, these data, which were observed in at least three experiments, indicate that NU269 is defective for extracellular growth in iron-limited conditions and that this phenotype is not present in 130b.

### TABLE 1. Ferrous and ferric iron uptake by *L. pneumophila* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Iron state</th>
<th>Mean μM $^{55}$Fe ($\mu$/10$^6$ CFU/ml) ± SD$^a$ at:</th>
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<tr>
<td></td>
<td></td>
<td>1 min</td>
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<tr>
<td>130b (pMMB207)</td>
<td>Fe$^{2+}$</td>
<td>178 ± 86</td>
</tr>
<tr>
<td>NU269 (pMMB207)</td>
<td>101 ± 70</td>
<td>101 ± 70</td>
</tr>
<tr>
<td>NU269 (pMR20)</td>
<td>77 ± 8</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>130b</td>
<td>Fe$^{3+}$</td>
<td>62 ± 32</td>
</tr>
<tr>
<td>NU269</td>
<td>82 ± 29</td>
<td>82 ± 29</td>
</tr>
</tbody>
</table>

$^a$ $^{55}$Fe$^{2+}$ or $^{55}$Fe$^{3+}$ was supplied to wild-type 130b and NU269 mutant bacteria, and 1-ml samples (n = 3) were tested for radioactivity. Complementation was assessed by introducing pMR20, which contains the intact *feoB*, into NU269 and then comparing NU269(pMR20) iron uptake to that of the wild type and mutant bacteria containing the empty vector pMMB207.$^a$

$^b$ Significant differences were noted between NU269(pMMB207) and NU269 (pMR20) (Student’s t test, $P < 0.01$) and 130b(pMMB207) and NU269(pMMB207) (Student’s t test, $P < 0.05$).
specifically associated with the loss of feoB function. Interestingly, NU269 was able to form colonies in 4 to 5 days on unsupplemented BCYE agar when plated next to confluent lawns of either itself or wild-type bacteria.

To more accurately compare the growth of the feoB mutant and its wild-type parent, we investigated the strains’ behaviors in iron-replete and -depleted liquid media. NU269 grew similar to the wild type in BYE broth, which is routinely supplemented with 0.25g of ferric pyrophosphate per liter (Fig. 2A). In BYE broth with no iron supplementation, the wild type grew as it had in standard BYE broth, but the feoB mutant exhibited a small but reproducible defect in the early stages of growth compared to 130b (Fig. 2B). When available ferrous iron in unsupplemented BYE broth was reduced by the addition of 35, 40, or 45 μM of the Fe\(^{2+}\) chelator DIP, 130b grew similarly to how it had grown in BYE broth without iron supplementation (Fig. 2C and D and data not shown).

Upon addition of 60 μM DIP, the wild-type bacteria entered the stationary phase slightly earlier than usual (Fig. 2E). In contrast, the feoB mutant had a decreased growth rate in unsupplemented BYE broth containing 35 μM DIP, which increased as the concentration of DIP increased to 45 μM and then to 60 μM (Fig. 2C to E). It is of note that even in the presence of 60 μM DIP, the feoB mutant eventually attained wild-type levels of growth (Fig. 2E). As confirmation that the inhibitory effect of DIP on the mutant was due to decreased iron in the media, maximal growth of NU269 in unsupplemented BYE broth was restored when 80 μM but not 10, 20, or 40 μM ferric chloride was added to the 60 μM DIP-containing cultures (Fig. 2F and data not shown). Taken together, these data indicate that L. pneumophila FeoB is not absolutely required for growth in bacteriological media but that, as iron becomes restricted, Fe\(^{2+}\) transport is relevant for extracellular replication.

Streptonigrin resistance of L. pneumophila. To gain further support for the role of FeoB in L. pneumophila extracellular iron acquisition, we compared 130b and NU269 for their resistance to streptonigrin. Bacteria with low intracellular iron pools are more resistant to streptonigrin, as this antibiotic has a well-documented iron-dependent toxicity and has been used by others to actually select E. coli FeoB mutants (28). The survival of wild-type legionellae was diminished as the concentration of streptonigrin increased within the media from 0 to 1.5 μM, as in earlier studies (Table 2) (30, 45). Importantly, NU269 was 3-fold and 25-fold more resistant than 130b at 0.75 and 1.5 μM streptonigrin, respectively (Table 2). The increased resistance of NU269 suggests that L. pneumophila feoB mutants have less intracellular iron than the wild type, a situation that is compatible with an involvement of feoB in iron transport.

Intracellular growth of L. pneumophila in amoebae and macrophages. The role of FeoB in L. pneumophila intracellular infection of protozoa was assessed by using H. vermiformis (21). Within the amoeba cocultures, both 130b and the feoB mutant were recovered in similar numbers at 0, 24, 48, and 72 h postinoculation (Fig. 3 and data not shown). These results could indicate that FeoB does not have an essential role in L. pneumophila growth and/or survival in H. vermiformis. However, as we observed in BYE broth cultures, it remained possible that the importance of feoB would be manifest in iron-depleted conditions. Hence, cocultures were performed in the presence of DIP, with a concentration of the iron chelator that did not adversely affect the viability of H. vermiformis, >90% of the amoebae reduced AlamarBlue at 0 to 50 μM DIP.

Upon sampling at 48 h postinoculation, a ca. 10-fold reduction in recovery of wild-type bacteria was noted at 25 and 50 μM DIP (Fig. 3). NU269 grew similarly to the wild type in cocultures containing 0 or 25 μM DIP (Fig. 3). However, in 50 μM DIP cocultures, the feoB mutant exhibited a 150-fold reduction in CFU recovered (Fig. 3). The numbers of NU269 recovered at 48 h postinoculation from cocultures containing 50 μM DIP were less than those originally inoculated, suggesting death of the mutant bacteria. Strains 130b and NU269 survived equally well in the medium used to culture the amoebae (data not shown). Together, these results indicate that feoB is important for growth and/or survival of L. pneumophila within iron-restricted amoebae.

The role of the L. pneumophila FeoB in intracellular growth was next assessed in U937 cells, a widely used model for infection of human macrophages (43, 48, 49). Numbers of bacteria were monitored over time following infection of macrophages with either 130b or NU269. Equivalent numbers of bacteria were recovered from monolayers infected with 130b or NU269 at 0 h postinoculation (Fig. 4A), indicating that there was no obvious defect for NU269 in attachment and invasion of macrophages. However, the feoB mutant exhibited a 10-fold reduced recovery compared to the wild-type parent at 24 h, increasing to 15-fold at 48 and 72 h postinoculation (Fig. 4A). Strains 130b and NU269 survived equally well in the medium used to culture the macrophages (data not shown). Taken together, these data indicate that the feoB mutant has a reduced ability to replicate and/or survive in human macrophages.

Partial complementation of the growth defect of the feoB mutant was achieved with pMR20, which contains the feoB gene linked to a vector promoter (Fig. 4B). However, introduction of pMR21, which contains feoAB and its promoter region, produced greater complementation (Fig. 4B). The latter complementation event was due to the feoB gene, since introduction of pMR22, which contains only feoA and the promoter region, did not produce complementation in NU269 (data not shown). We strongly suspect that the increased complementation observed with pMR21 over pMR20 is associated with higher levels of expression of feoB from the native feoAB promoter. At the least, these data indicate that L. pneumophila feoAB is required for optimal intracellular infection of macrophages.

Further evidence of the role of L. pneumophila FeoB in infections of macrophages was provided by a cytopathicity assay, which monitors the reduction in host cell viability that is associated with Legionella infections (2). With the same MOI as during the kinetic studies (i.e., MOI = 1), the cytopathic effect of the wild type became apparent at 72 h postinoculation, when <5% of macrophages survived compared with uninfected monolayers (Fig. 5A). In contrast, the viability of macrophages infected with NU269 did not decrease significantly over the assay period (Fig. 5A). With an MOI of 10, a defect in cytopathicity was also observed; whereas 130b elicited 40% and <5% macrophage survival at 48 and 72 h, respectively, 50% of the NU269-infected cells remained viable after 72 h
FIG. 2. Growth of *L. pneumophila* in standard and iron-limited BYE broth. Wild-type 130b (solid squares) and *feoB* mutant NU269 (open squares) were grown in BYE broth (A) with standard iron supplementation, (B) without iron supplementation, (C) without iron supplementation in 35 μM DIP, (D) with 45 μM DIP, or (E) with 60 μM DIP. In panel F, NU269 was grown either in BYE broth that lacked its standard iron supplement (solid line) or in BYE broth that lacked its standard iron supplement but contained 60 μM DIP and 80 μM FeCl₃ (dotted line). Data represent the means and standard deviations from triplicate samples from a representative experiment. In panels B to E, significant differences were noted between the wild-type and the *feoB* mutant between 5 and 11 h (Student’s t test, *P* < 0.05). Experiments were repeated in triplicate with similar results.
Thus, loss of \textit{L. pneumophila} \textit{feoB} is associated with a decreased capacity for macrophage killing, substantiating the involvement of \textit{feoB} in intracellular infection.

To provide a direct correlation between iron levels in macrophages and the growth defect observed in the \textit{feoB} mutant, intracellular infection assays were repeated with U937 cells that had been depleted of iron by treatment with DIP. Preliminary experiments showed that the concentrations of DIP used did not cause any gross morphological changes in the macrophages and did not adversely affect their viability (data not shown). Wild-type bacteria were recovered in similar numbers independent of the iron-depleted state of the U937 cells (Fig. 6). In marked contrast, the 15-fold intracellular growth defect of the \textit{feoB} mutant in untreated U937 cells at 48 h postinoculation was increased to 103-fold and 105-fold by the presence of 25 and 50 \( \mu \text{M} \) DIP, respectively (Fig. 6). In monolayers treated with 50 \( \mu \text{M} \) DIP, fewer mutant bacteria were recovered than were originally inoculated, suggesting death of the infecting bacteria (Fig. 6). In sum, these results indicate that the \textit{feoB}

![Fig. 3. Coculture of \textit{L. pneumophila} with \textit{H. vermiformis} amoebae.](http://iai.asm.org/download/article/FIG3.jpg)

![Fig. 4. Intracellular growth of \textit{L. pneumophila} in U937 macrophages.](http://iai.asm.org/download/article/FIG4.jpg)

**TABLE 2.** Streptonigrin resistance of \textit{L. pneumophila} strains

<table>
<thead>
<tr>
<th>Streptonigrin (( \mu \text{M} ))</th>
<th>Mean growth (10^7 CFU/ml) ± SD</th>
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<tr>
<td>0</td>
<td>( 106.67 \pm 12.58 )</td>
</tr>
<tr>
<td>0.75</td>
<td>( 16.07 \pm 0.72 )</td>
</tr>
<tr>
<td>1.5</td>
<td>( 0.33 \pm 0.21 )</td>
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</table>

\( ^{a} \text{Bacteria were resuspended to 10^9 CFU/ml, and dilutions were plated on BCYE agar containing no iron supplementation and either 0, 0.75, or 1.5 \( \mu \text{M} \) streptonigrin. Data show the mean and standard deviation (n = 3). This experiment was performed in duplicate with similar results. Significant differences were noted between the wild type and the \textit{feoB} mutant when plated on agar containing 0.75 \( \mu \text{M} \) streptonigrin (Student’s \( t \) test, \( p < 0.01 \)).} \)
mutant is hypersensitive to iron depletion within U937 cells and that ferrous iron transport is important during L. pneumophila intracellular infection of macrophages.

**Virulence of L. pneumophila in A/J mice.** To investigate whether the L. pneumophila feoB mutant was defective for virulence, a competition assay was performed in the A/J mouse model as previously described (48). More specifically, bacteria were recovered from the lungs of mice following intratracheal inoculation of 130b and NU269 in a 1:1 ratio. Compared to NU269, increased numbers of 130b were recovered from infected mouse lungs at day 1; the average ratio of CFU of 130b to NU269 per lung was 2.25 (Fig. 7). By day 3, the average ratio

![Graph A](image1)

**FIG. 5.** Cytopathicity of L. pneumophila for U937 macrophages. Wild-type (solid squares) and NU269 (open squares) strains were used to infect U937 macrophages at an MOI of (A) 1 or (B) 10, and host cell viability was assessed at various times postinoculation. Cytopathicity data are presented as the percentage of surviving macrophages in infected monolayers compared to the numbers in uninfected monolayers and represent the mean and standard deviation from six wells. Experiments were performed in triplicate with similar results. Significant differences were observed between the wild-type and mutant strains at 48 and 72 h postinoculation (Student’s t test, P < 0.05).

![Graph B](image2)

**FIG. 6.** Intracellular growth of L. pneumophila in iron-depleted U937 cells. The numbers of wild-type (solid bars) and NU269 (open bars) cells were determined in U937 cell monolayers cultured in medium containing 0, 25, or 50 μM DIP. Data represent means and standard deviations recovered from triplicate wells. The experiment was performed in triplicate with similar results. Significant differences were observed between the wild-type and mutant strains at 25 and 50 μM DIP (Student’s t test, P < 0.05).

![Graph C](image3)

**FIG. 7.** Pulmonary infections of A/J mice with L. pneumophila. Wild-type 130b and mutant NU269 strains in a 1:1 ratio were introduced into the lungs of A/J mice by intratracheal inoculation. At days 1 and 3 postinoculation, the ratio of 130b to Km’ NU269 in infected lungs was determined. Data are representative of actual values obtained per mouse (n = 3 to 5), and a solid bar represents the mean value.
of 130b to NU269 had increased to 3.09 (Fig. 7), suggesting the involvement of FeoB in L. pneumophila virulence in A/J mice.

DISCUSSION

The requirement for iron by L. pneumophila for its replication both extracellularly and intracellularly in human macrophages and freshwater amoebae is well documented (8, 10, 11, 30, 59). Our laboratory has identified several determinants that may be involved in the acquisition and/or utilization of iron, likely in the ferric form (29, 30, 36, 42, 45, 58, 59). In this report, we addressed the importance of ferrous iron and its acquisition in L. pneumophila extracellular growth and intracellular infection. We identified and characterized L. pneumophila FeoAB, which bear homology to E. coli and Salmonella enterica serovar Typhimurium FeoAB (33, 56) and Helicobacter pylori FeoB, which lacks the accompanying FeoA (57). In those bacteria, FeoB has been shown to be a ferrous iron transporter. FeoA, although involved in ferrous iron uptake in some bacteria, is poorly understood, and hence our focus was the well-characterized FeoB-like protein in our studies (33, 56).

We strongly believe that L. pneumophila feoAB encodes a functional ferrous iron transporter for several reasons. First, L. pneumophila feoAB mutants have decreased ferrous iron accumulation compared to the wild type, a phenotype that was complemented by a copy of the feoB gene supplied in trans. Second, L. pneumophila feoB mutants are streptomycin resistant, a phenotype associated with iron acquisition mutants in general and feoB mutants in particular (28). Finally, there was strong similarity between the L. pneumophila feoAB predicted protein products and the E. coli and S. enterica FeoAB.

L. pneumophila feoB mutants grew similar to wild-type bacteria in standard BYE broth and BCYE agar, indicating that mutant phenotypes were not the result of a generalized growth defect. Growth in BYE broth without its usual iron supplementation resulted in a minor growth defect in NU269 compared to 130b, which was greatly exacerbated by the addition of the ferrous iron chelator DIP in a concentration-dependent manner. Growth of the feoB mutant could be restored by the addition of FeCl3 to the DIP-treated cultures. This is similar to the case with E. coli and H. pylori feoB mutants, which exhibit decreased extracellular growth in iron-depleted artificial media, which, in the case of the Helicobacter mutants, could be similarly restored by the presence of FeCl3 (53, 57). DIP-treated cultures of NU269 eventually attained wild-type levels of growth by stationary phase, which may indicate the ability of other iron acquisition systems to compensate for the defect in Fe2+ uptake due to the loss of FeoB.

Increased time to colony formation was noted in the feoB mutant compared to the wild type on BCYE agar containing 32-fold less of the usual iron supplementation or no supplementation at all. Although this phenomenon has not been described for other bacterial feoB mutants, it was clearly due to the loss of FeoB, as it was complemented by the introduction of a cloned copy of feoB into NU269. Thus, FeoB plays a key role in extracellular L. pneumophila growth under iron-limited conditions. Although aerobic conditions typically result in a predominance of ferric iron, our data suggest that ferrous iron is present within Legionella cultures grown in the presence of oxygen.

In vitro infection data demonstrate, for the first time, the importance of a feoB gene in intracellular replication. The L. pneumophila feoB mutant exhibited a 15-fold decreased intracellular growth and/or survival in human U937 cell macrophages, which could be increased to 103-fold with iron-starved, DIP-treated macrophages. Importantly, the defect in intracellular replication in macrophages could be complemented by the addition of FeoAB in trans. The only other feoB mutations in an intracellular parasite are in S. enterica serovar Typhimurium; however, the Salmonella mutants were not defective for growth in human epithelial Hep-2 or HeLa cells or in mouse J774 cell macrophages (56). However, S. enterica feoB mutants are defective for gut colonization, suggesting an inadequacy of cell models or a role for FeoB in extracellular growth in vivo.

In our own in vivo studies, the wild-type bacteria outcompeted the feoB mutant, indicating that ferrous iron is available during colonization of the lungs of mice by L. pneumophila and that feoB has a role in Fe2+ acquisition within a mammalian host. The role of L. pneumophila feoB in virulence in mice appears to be relatively modest, although it is of similar magnitude to the S. enterica feoB mutant at 3 days postinoculation; i.e., in S. enterica the ratio of CFU per milligram of feces of wild-type bacteria versus the feoB mutant was approximately 4, compared to the ratio of 3 in the CFU/lung of the L. pneumophila wild-type bacteria versus the feoB mutant (56).

A defect in recovery of the feoB mutant was also noted in iron-starved, DIP-treated H. vermiformis amoebae but not under standard iron-replete coculture conditions. The difference in the recovery of the feoB mutants in macrophages and amoebae under iron-replete conditions suggests that this gene is more important in macrophages than in amoebae. There are other examples of L. pneumophila mutants that are more defective in macrophages than in protozoa (23). Alternatively, it could indicate that more ferrous iron is present during amoebal cocultures or that L. pneumophila has greater access to Fe2+ during infection of H. vermiformis. That the feoB mutant is not completely defective for intracellular growth in macrophages or amoebae and exhibits only a modest virulence defect in mice implies that other uptake systems and/or iron sources can overcome the decreased ferrous iron uptake. The redundancy of determinants involved in using or acquiring iron underscores the key importance of this element for the growth of Legionella and other bacteria. For example, growth defects on iron-limiting agar are demonstrable in an E. coli feoB mutant only if that strain carries additional mutations in siderophore production and uptake determinants, i.e., an aroB tonB background (33, 56).

In summary, we have demonstrated the involvement of the L. pneumophila feoAB locus in ferrous iron transport, extracellular growth, and intracellular infection. The infectivity defect of the feoB mutant further suggests that ferrous iron is present and accessible in the L. pneumophila intracellular niche. Thus, we have shown, for the first time, the involvement of FeoB in bacterial growth in host cells and in the lungs of mice, findings that could have implications for other intracellular parasites and respiratory pathogens.

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