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A *Legionella pneumophila* Gene That Promotes Hemin Binding

WILLIAM A. O'CONNELL, ERIN K. HICKEY, AND NICHOLAS P. CIANCIOTTO*

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

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The ability to bind and utilize hemin is a trait common to many human pathogens. Nevertheless, the relationship between *Legionella pneumophila*, the agent of Legionnaires' disease, and hemin has received little attention. Thus, we explored the capacity of a virulent, serogroup 1 strain of *L. pneumophila* to bind hemin and use it as an iron source. Hemin, but not protoporphyrin IX, restored bacterial growth in iron-limiting media, indicating that it can serve as an iron source for *L. pneumophila*. In support of this idea, we observed that wild-type legionellae were able to bind 50 to 60% of added hemin, a binding capacity that was comparable to those of other pathogens. To begin to identify proteins involved in hemin acquisition, we identified a *Legionella* locus that conferred hemin binding upon *Escherichia coli*. Subcloning and nucleotide sequence analysis determined that a single open reading frame, which was designated *hbp* for hemin-binding promotion, was responsible for this binding activity. The *hbp* gene was predicted to encode a secreted, 15.5-kDa protein. To ascertain the importance of this gene in *L. pneumophila* biology, we used allelic exchange to construct an *hbp* mutant. Importantly, the mutant displayed a 42% reduction in hemin binding, confirming that *hbp* potentiates hemin acquisition by *L. pneumophila*. However, the strain was unaltered in its ability to grow within macrophage-like cells and freshwater amoebae, indicating that *hbp* is not required for intracellular infection. Despite this, Southern hybridization analysis and database searches demonstrated that *hbp* is nearly exclusive to the *L. pneumophila* species.

A number of pathogens, including both intra- and extracellular parasites, have evolved mechanisms for interacting with hemin and heme-containing compounds (52, 66). This interaction can serve several functions. First, and perhaps most often, it serves as a means for iron acquisition. Strains of *Aeromonas* spp., *Bacteroides fragilis*, *Bordetella pertussis*, *Campylobacter jejuni*, *Escherichia coli*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Neisseria gonorrheae*, *Neisseria meningitidis*, *Plesiomonas shigelloides*, *Porphyromonas gingivalis*, *Serratia marcescens*, *Shigella flexneri*, *Streptococcus pneumoniae*, *Vibrio cholerae*, *Vibrio vulnificus*, *Yersinia enterocolitica*, and *Yersinia pestis* scavenge the iron from hemin and related substances (15, 16, 27, 40, 41, 44, 52, 64, 66, 67). Second, in *H. influenzae* and *P. gingivalis*, heme structures are absolutely required as sources for porphyrin rings (8, 14). Third, hemin binding to a bacterial surface may facilitate infection of eukaryotic cells. For example, in *S. flexneri* and enteroinvasive *E. coli*, it enhances invasion of epithelial cells, whereas in *Aeromonas salmonicida*, hemin interaction with S-layer proteins promotes macrophage association (15, 25, 62). Fourth, hemin (heme) serves as a cofactor for intracellular cytochromes and enzymes; e.g., the heme-binding FixL of *Rhizobium meliloti* is an oxygen-sensing membrane kinase (46). Finally, *Y. pestis* has an extraordinary capacity to store hemin (54). In recent years, a number of investigators have turned their attention toward the molecular and genetic bases of hemin binding and utilization. This complex process generally involves the concerted effort of surface/outer membrane receptors and periplasmic/inner membrane transporters and has been characterized as a TonB-dependent uptake event (6, 14, 21, 28, 29, 42, 45, 61). In *S. marcescens*, heme acquisition is initiated by an extracellular heme-binding protein (41).

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

The relationship between *Legionella pneumophila*, the agent of Legionnaires' disease, and hemin has received very little attention. The gram-negative *L. pneumophila* is a facultative intracellular parasite of both freshwater protozoa and human alveolar macrophages (10, 20, 24, 34, 50, 60). Although ferric/ferrous iron clearly plays a critical role in extra- and intracellular *Legionella* growth (7, 23, 26, 57, 58), the role of hemin is unclear. Several early studies demonstrated bacterial growth on complex and semidefined media which contained hemin or hemoglobin supplements (23, 55). However, since the heme compounds were easily replaced with ferric salts, it was assumed that they were not required for *Legionella* growth (23). This idea was later confirmed by the development of defined media which completely lacked hemin but supported effective *L. pneumophila* replication (58, 59, 65). Nevertheless, hemin and hemoglobin did enhance *L. pneumophila* growth on several types of complex media (23, 36). In one study, the growth of seven *L. pneumophila* strains, representing six serogroups, was stimulated by ≥100-fold by the addition of hemin to a yeast extract phosphate (YP) medium (36). Taken together, these data suggest that hemin can serve as an accessory iron source. To better appreciate the role of hemin in *L. pneumophila* biology, we investigated further the growth-enhancing capacity of hemin, assessed the ability of virulent organisms to bind hemin, and sought the gene(s) involved in hemin binding.

MATERIALS AND METHODS

Bacterial strains and media. Virulent *L. pneumophila* serogroup 1 strains Wadsworth 130b and Philadelphia 1, serogroup 8 strain Concord 3, and serogroup 13 strain B2A3105 were previously described (9). Strains of *Legionella erthyra*, *L. feeleii*, *L. hackeliae*, *L. longbeachae*, *L. micdadei*, *L. moravica*, *L. sainticrusis*, and *L. spiritensis* were also included in the present study (9). Like *L. pneumophila*, the species *L. feeleii*, *L. hackeliae*, *L. longbeachae*, and *L. micdadei* have been associated with human disease. Generally, the legionellae were grown on standard buffered charcoal yeast extract (BCYE) agar plates for 48 h at 37°C, and, when appropriate, 3 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, or 5% (wt/vol) sucrose was added to the medium. For experiments that assessed hemin utilization and binding, we employed as our base medium a slightly modified form of the YP medium (1, 36). This medium contained as a supplement 250 mg of ferric PP_i and 400 mg of cysteine per liter. The chemically

defined medium (CDM) described by Reeves et al. was employed to further assess the ability of *Legionella* species to use hemin as an iron source (58). Buffered-yeast extract (BYE) broth cultures were washed in CDM and then inoculated into acid-washed, 125-ml flasks containing 25 ml of CDM. The resultant cell suspensions were incubated at 37°C with agitation.

E. coli HB101 served as the host for recombinant plasmids (3). It was maintained on Luria-Bertani agar medium containing either 30 μg of chloramphenicol, 50 μg of kanamycin, or 50 μg of ampicillin per ml (3). Recombinant *E. coli* cells were also grown on M9CA salts agar supplemented with either 100 μg of hemin or Congo red per ml (28).

Unless otherwise noted, key chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Plasmids and a genomic library. Plasmids pBR322 and pUC18 were used as cloning vehicles (3). Another ColE1 replicon, pBOC20, was used in our allelic exchange protocol. This plasmid represents the multicloning site from pHXK cloned into pEA75 (48). Importantly, it contains a selectable chloramphenicol resistance (Cm^r) marker and the counterselectable *sacB*. The vector pNK2794 served as the source for a 1.7-kb *Bam*HI fragment which contains a kanamycin resistance (Km^r) gene (48). Finally, the *mip*-containing plasmid pSMJ31.42 was used as a probe in Southern hybridizations (22). Plasmids were isolated from *E. coli* by the alkaline lysis procedure (3). The genomic library used in this study was derived from *L. pneumophila* 130b and consisted of 3- to 6-kb *Sau*3A fragments cloned into pBR322 (31).

Electroporation and allelic-exchange mutagenesis. Plasmids were introduced into *L. pneumophila* by electroporation (12). The procedure for allelic exchange with ColE1 vectors containing counterselectable markers has been previously described (13, 48). Using this protocol, we achieved insertional inactivation of *hbp* within a strain that had been passaged six times on BCYE agar plates.

Northern (RNA) and Southern hybridizations. Whole-cell RNAs and DNAs were extracted from *Legionella* strains as described previously (13, 22). Northern hybridizations were performed by standard protocols (3). RNAs were electrophoresed through a gel that contained 1% agarose and 2.2 M formaldehyde and that was bathed in 1× morpholinepropanesulfonic acid (MOPS) buffer. The sizes of the molecular weight standards that were used to estimate the lengths of mRNA species were 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb (Gibco-BRL, Gaithersburg, Md.). Southern hybridizations were performed under high- and low-stringency conditions which permit approximately 10 and 30% bp mismatching, respectively (3, 9). Probes consisted of both plasmids and gel-isolated restriction fragments which were radiolabeled with ^{32}P by using a random primer labeling kit (Gibco-BRL).

DNA sequence analysis. Cloned *L. pneumophila* DNA was sequenced from double-stranded plasmids by the dideoxy chain-termination method with ^{35}S-dATP and Sequenase (Amersham, Arlington Heights, Ill.) (3). Initially, M13-based primers were used to sequence DNAs cloned into pUC18; however, custom 20-bp oligodeoxyribonucleotide primers were used in subsequent reactions. The unique primers were prepared by the Northwestern University Biotechnology Center with an Applied Biosystems DNA synthesizer. Sequencing reactions were performed according to the manufacturer's protocols. Nucleotide sequences were analyzed with PCGENE (IntelliGenetics), and homology searches were conducted through GenBank at the National Center for Biotechnology Information.

Liquid hemin-binding assay. To quantitate the ability of *L. pneumophila* and *E. coli* strains to bind hemin, we employed the standard liquid hemin-binding assay (15, 17, 27, 28, 37). Prior to exposure to hemin, the legionellae were grown for 48 h on agar media and then subjected to a series of washes. First, the bacteria were harvested from plates into 40 ml of distilled water, achieving an optical density at 660 nm (OD_{660}) of approximately 1.5. Then, after centrifugation of the cell suspension for 10 min at 4,500 × *g*, the pellet was dissolved in 15 ml of 0.1 M Tris (pH 8.0). Subsequent to a second centrifugation step, the bacteria were resuspended for the last time in 15 ml of BYE broth. After removal of 0.1 ml from the final cell suspension for a CFU determination, 1-ml aliquots were placed into 1.5-ml Microfuge tubes and brought to concentrations of either 5, 10, 15, or 20 μg of hemin per ml by additions from a freshly prepared 1-mg/ml stock in BYE. The bacterium-hemin mixture was then rotated at 37°C. After a 1-h incubation, the cell suspension was centrifuged for 2 min at 8,000 × *g*. Finally, 0.75 ml of the supernatant was examined, along with the appropriate hemin-BYE control, for its A_{400}. As always, the extent of hemin binding was a reflection of the reduction in the OD_{400}. *E. coli* strains were grown and assayed in M9CA salts broth.

Intracellular infection of U937 cells and amoebae by *L. pneumophila*. U937 cells are a human cell line, which, when treated with phorbol esters, differentiate into macrophage-like cells (63). U937 cell monolayers were prepared and infected with *L. pneumophila* as previously described (11, 48, 53). Following inoculation, the monolayers were incubated for 2 h to permit bacterial uptake and were then vigorously washed to remove unattached bacteria. The infected monolayers were incubated at 37°C in RPMI medium supplemented with 10% fetal bovine serum. To assess the relative infectivity of strains for U937 cells, 50% infective doses were determined after 72 h of incubation (11). To monitor intracellular growth rates, replicate monolayers were inoculated with approximately 10^6 bacteria, incubated for various times, and then lysed (11, 48). Tenfold serial dilutions of the lysates were plated on BCYE agar, and the resulting CFU were used to calculate the corresponding numbers of bacteria per monolayer. In some experiments, the U937 cells were treated before and after infection with 7 μM deferoxamine. Deferoxamine inhibits *Legionella* replication within macrophages by reducing intracellular iron availability (7, 26, 56).

Intracellular infection of the freshwater amoeba *Hartmannella vermiformis* was performed as previously described (12, 39). Briefly, replicate *Hartmannella* cultures containing 10^5 amoebae were infected with 10^3 CFU, and after various incubation periods, the numbers of legionellae within the cocultures were determined by plating aliquots on BCYE medium.

Nucleotide sequence accession number. The *hbp* sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number U43385.

TABLE 1. Plating efficiency of *L. pneumophila* 130b on YP media

Agar medium[a]	Average no. of CFU/ml recovered[b]	
	Expt 1	Expt 2
BCYE	1.7×10^8	1.6×10^8
YP	5.5×10^6	ND
YP + hemin	4.7×10^7	ND
YP − Fe	$<10^2$[c]	$<10^1$[c]
YP − Fe + hemin	3.9×10^7	2.1×10^7
YP − Fe + PP	ND	$<10^1$[c]

[a] YP + hemin, YP supplemented with 30 μM hemin; YP − Fe, YP lacking its ferric PP_i supplement; YP − Fe + hemin YP lacking ferric PP_i but supplemented with 30 μM hemin; YP − Fe + PP, YP lacking ferric PP_i but supplemented with 30 μM protoporphyrin IX.

[b] Bacteria were grown on BCYE agar plates for 48 h, resuspended in distilled H_2O to an OD_{660} of approximately 0.3, and then plated in triplicate for determinations of the numbers of CFU on the indicated media. ND, not determined.

[c] No CFU recovered.

RESULTS

Hemin utilization and binding by *L. pneumophila*. Since the ability of hemin to enhance *Legionella* growth is most manifest on YP agar plates (36), we examined the behavior of wild-type strain 130b on this medium. Initially, we confirmed two key observations of the previous study. First, the number of CFU recoverable on YP agar was 3% of that recoverable on BCYE (Table 1). Second, the addition of hemin to the medium increased the CFU by nearly 10-fold (Table 1). However, to highlight more clearly the role of hemin in *Legionella* physiology, we examined the growth of strain 130b on YP media that were lacking Fe^{3+} supplements and that were thus nonpermissive (Table 1). In six separate experiments (the results of two of which appear in Table 1), the addition of 30 μM hemin completely restored the ability of strain 130b to form colonies on low-iron YP medium. Importantly, equimolar amounts of protoporphyrin IX could not substitute for hemin. Taken together, these data suggest that hemin can be an iron source for *L. pneumophila*. To support this idea, we examined the ability of hemin to replace ferric iron in a CDM (Fig. 1). The omission of ferric salts from the CDM prevented the growth of strain 130b, confirming that iron is essential for *L. pneumophila* replication. The addition of 6.25, 12.5, or 25 μM Fe^{3+} to the medium fully restored bacterial growth (Fig. 1 and data not shown). More importantly, growth of 130b was supported by Fe^{3+}-free CDM that had been supplemented with hemin. Whereas the addition of 6.25 μM hemin yielded 75 to 90% maximal growth, the addition of 12.5 or 20 μM hemin promoted full replication (Fig. 1 and data not shown). Since 6.25 μM Fe^{3+} supported better growth than did an equimolar amount of heme-iron, we suspect that ferric iron is the more effective iron source for the legionellae. Nevertheless, these data demonstrate that hemin can be the sole iron source for *L. pneumophila*.

To substantiate the idea that *L. pneumophila* can directly utilize heme compounds, we assayed strain 130b for its ability

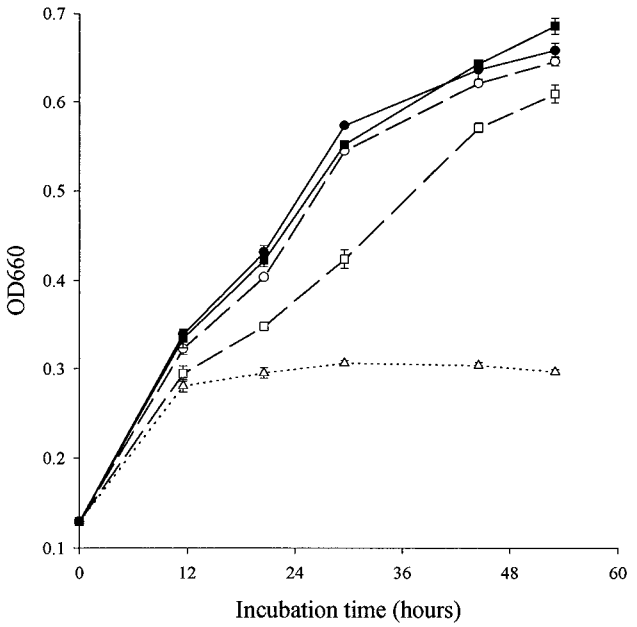

FIG. 1. Growth of *L. pneumophila* 130b within a CDM. Bacteria from overnight BYE cultures were inoculated into CDM that was lacking its ferric iron component, and, at various times, the extent of growth was assessed by measuring the OD_{660}. Each datum point represents the mean OD for three replicate cultures, and the vertical bars denote the standard deviations. Supplements to the CDM were as follows: no supplementation, i.e., no iron source in the medium (△); 6.25 μM Fe^{3+}, i.e., 1.56 μM ferric PP_i (○); 12.5 μM Fe^{3+}, i.e., 3.12 μM ferric PP_i (●); 6.25 μM hemin (□); or 12.5 μM hemin (■). In another experiment in which the inocula were derived from log-phase BYE cultures, there was absolutely no growth in the iron-lacking control.

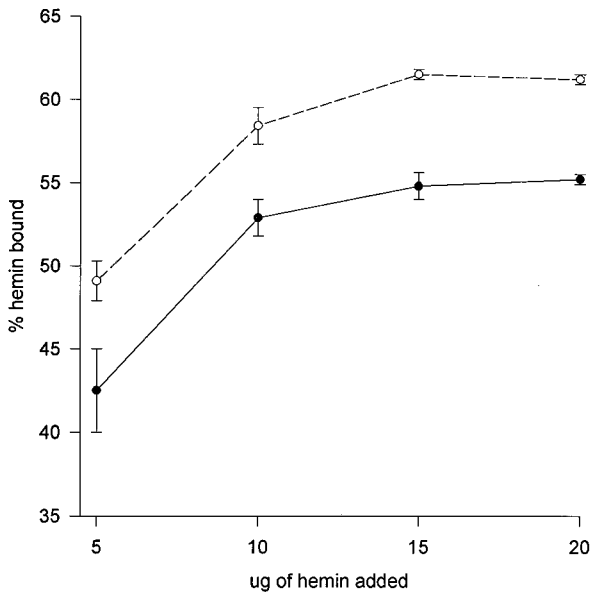

FIG. 2. Hemin binding by wild-type *L. pneumophila*. A total of 1.0×10^8 CFU of strain 130b obtained from YP-minus-Fe-plus-hemin agar (○) and 1.6×10^8 CFU harvested from BCYE agar (●) were assayed for their ability to remove hemin from the solution. Each datum point represents the mean percent hemin bound for three replicate cultures, and the vertical bars denote the standard deviations. The differences in binding between the YP-minus-Fe-plus-hemin and BCYE cultures were significant at each hemin concentration ($P = <0.001$ for all concentrations except 5 μg, for which $P = <0.01$ [Student's *t* test]).

to bind hemin. Following growth on YP-minus-Fe-plus-hemin (Table 1) media, *L. pneumophila* consistently bound 50 to 60% of the added hemin (Fig. 2). This level of hemin binding was 10-fold greater than that of *E. coli* HB101 (see below). Legionellae harvested from BCYE agar plates also bound appreciable amounts of hemin, indicating that *L. pneumophila* hemin binding was not a peculiarity of growth on YP media (Fig. 2). Interestingly, however, these bacteria adsorbed noticeably less of the compound that did those obtained from the hemin-containing YP agar. Taken together, our initial experiments predicted that *L. pneumophila* has surface structures (proteins) which promote hemin acquisition.

Identification of a *L. pneumophila* gene that promotes hemin binding. To identify *L. pneumophila* proteins involved in hemin acquisition, we screened a genomic library for a locus that could confer upon *E. coli* HB101 the ability to bind hemin. Using an approach which recently led to the characterization of a hemin-binding membrane protein of *H. influenzae* (28), we sought recombinant bacteria that appeared brown on M9CA salts agar plates containing 0.01% hemin. Since bacteria and proteins that specifically interact with hemin often bind Congo red (15, 17, 27, 37, 62), we screened for clones that were also colored on media containing that dye. Three pigmented recombinant strains were obtained. Importantly, the plasmids pEH1, pEH2, and pBOC3, which were isolated from these clones, conferred pigmentation upon retransformation into HB101. To confirm that HB101(pEH1), HB101(pEH2), and HB101(pBOC3) had enhanced hemin-binding activity, we assessed their abilities to remove hemin from solution. Indeed, all three clones bound ca. 60% more hemin than did HB101 (pBR322) (Fig. 3A). Restriction enzyme digestion analyses and Southern hybridizations indicated that pEH1, pEH2, and pBOC3 were overlapping and contained *L. pneumophila* 130b DNA (Fig. 4A).

Subcloning mapped the locus responsible for hemin binding to a 1.1-kb *Sac*I-*Afl*II fragment (Fig. 4A). DNA sequence analysis indicated that this region of the *L. pneumophila* chromosome contained one intact open reading frame (ORF). A Km^r insertion into the *Hin*cII site of pEH12 abolished pigmentation in recombinant *E. coli*, confirming that this ORF is required

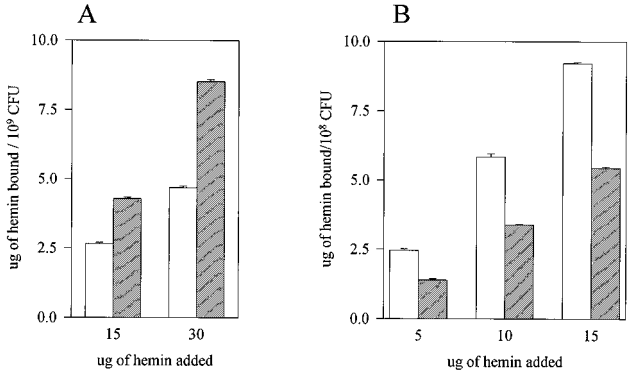

FIG. 3. Hemin binding by recombinant *E. coli* and mutant *L. pneumophila*. (A) *E. coli* HB101(pBR322) (open box) and HB101(pEH1) (shaded box) were grown to stationary phase in M9CA salts broth, and then ca. 10^9 CFU of each were assayed for their ability to remove hemin from solution. Each datum point represents the mean binding for three replicate cultures, and the vertical bars denote the standard deviations. The differences in binding between the two strains were significant at both hemin concentrations ($P = <0.001$ [Student's *t* test]). HB101(pEH2) and HB101(pBOC3) exhibited hemin-binding capacities that were comparable to that of HB101(pEH1) (data not shown). (B) *L. pneumophila* 130b (open box) and NU226 (shaded box) were grown on YP-minus-Fe-plus-hemin agar plates (see Table 1), and then ca. 10^8 CFU were assayed for hemin binding. Significant differences in binding were evident at all hemin concentrations, including the 20-μg/ml level not depicted here ($P = <0.001$ [Student's *t* test]).

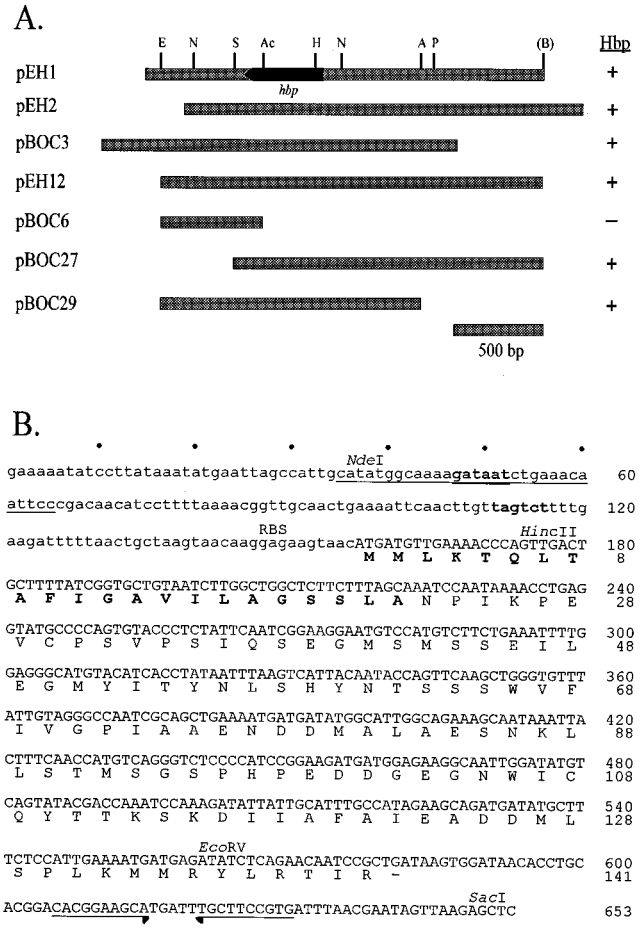

FIG. 4. Identification of the *L. pneumophila hbp* ORF. (A) Restriction maps and phenotypes for pEH1 and its subclones. All recombinant plasmids represent *Legionella* DNA cloned into pBR322. + and −, the HB101 transformant was or was not pigmented on agar media containing hemin or Congo red, respectively. Restriction enzyme recognition sites are indicated as follows: A, *Afl*II; Ac, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; N, *Nde*I; P, *Pst*I; and S, *Sac*I. (B) Nucleotide sequence of the *hbp* region and the predicted amino acid sequence of Hbp. Potential −10 regions for the *hbp* promoter are in boldface lowercase type, and the area containing possible iron boxes is underlined. The locations of key restriction enzyme recognition sites, the ribosome-binding site, and the transcriptional terminator are also clearly indicated. The putative signal sequence for Hbp is in boldface uppercase type.

for hemin binding (Fig. 4). We have designated the ORF as *hbp* for hemin-binding promotion. (Note that in a preliminary report we had referred to the ORF as *heb* for hemin binding [47]). The *hbp* ORF was predicted to encode a protein (Hbp) of 141 amino acids and 15.5 kDa. Interestingly, Hbp contained a 22-residue signal sequence (Fig. 4B), indicating that it may be secreted in *E. coli* and *L. pneumophila*. Database searches failed to reveal significant degrees of homology between Hbp and known proteins.

The *hbp* ORF was preceded by a ribosome-binding site within 13 bp of the initiation codon (Fig. 4B). Further upstream, there existed two potential −10 regions. The first of these putative promoter regions contained several sets of sequences that had homology with Fur-binding sites (31). The presence of ironboxes suggested that *hbp* is regulated by *L. pneumophila* Fur and intracellular iron levels (31). Finally, the presence of a potential transcription terminator shortly after the Hbp stop codon suggested that *hbp* is transcribed as a monocistronic message (Fig. 4B). To confirm this idea, we performed Northern blot analysis using RNA isolated from strain 130b and, as a probe, the *hbp*-containing *Nde*I-*Eco*RV fragment of pEH12 (Fig. 4). *L. pneumophila* expressed a single *hbp*-hybridizing transcript (data not shown). Importantly, the length of that mRNA was estimated to be 440 bases, a size which is compatible with that of the *hbp* ORF.

Construction and characterization of an *L. pneumophila hbp* mutant. To ultimately determine whether *hbp* promotes hemin acquisition by *L. pneumophila*, we isolated an *hbp* mutant. Specifically, we employed allelic exchange to insertionally inactivate the *hbp* gene within strain 130b. The plasmid used for this procedure, pBOC22, was constructed in two steps. First, a blunt-end 1.7-kb fragment containing Km^r was introduced into the *Hin*cII site of pEH12 to yield pBOC21. As noted above, this DNA insertion into *hbp* abolished hemin binding in recombinant *E. coli*. Second, the mutated gene was transferred on a *Sac*I-*Bam*HI fragment into the Cm^r and *sacB*-containing pBOC20. As the next step toward the construction of our mutant, pBOC22 was electroporated into strain 130b, and the transformation mixture was plated onto BCYE agar containing both kanamycin and sucrose. By simultaneously selecting for Km^r and counterselecting against *sacB*, strains in which the plasmid is lost and the chromosomal *hbp* is exchanged for its mutated allele can be isolated (Fig. 5A). To identify a strain that had undergone allelic exchange, we performed Southern hybridization analysis on a Km^r, sucrose-resistant clone which had lost its resistance to chloramphenicol (Fig. 5B). To simultaneously confirm that the strain contained a Km^r insertion while lacking other vector sequences, pBOC22 was used as the probe. As predicted, this strain, which was designated NU226, had a 2.5-kb *hbp*-containing *Nde*I fragment in place of a 0.8-kb hybridizing *Nde*I fragment (compare lanes c and d). Southern hybridization analysis of genomic DNAs digested with *Hin*cII, *Hin*dIII, *Pst*I, and *Pvu*I further confirmed that NU226 underwent allelic exchange (Fig. 5B, lanes a and b; and data not shown).

To ascertain whether *hbp* is associated with hemin binding in *L. pneumophila*, NU226 and 130b were grown on YP-minus-Fe-plus-hemin agar and then compared for their abilities to remove hemin from solution (Fig. 3B). The mutant strain displayed a 42% reduction in hemin binding, indicating that although *hbp* is not essential, it does enhance the acquisition of hemin by *Legionella* organisms.

To determine whether *hbp* is required for intracellular infection of macrophages by *L. pneumophila*, we assessed the relative ability of NU226 to infect U937 cells. Regardless of whether the inocula were prepared from YP-minus-Fe-plus-hemin or BCYE cultures, the mutant exhibited a 50% infective dose that was comparable to that of the wild-type (data not shown). Furthermore, the intracellular growth pattern of NU226 was nearly identical to that of strain 130b (Fig. 6). To explore the possibility that *hbp* is important for growth within iron-depleted host cells, we repeated the infections using U937 cells which had been treated with deferoxamine. However, in two experiments we still recovered comparable numbers of mutant and wild-type bacteria after 72 h of incubation (data not shown). Finally, NU226 was not impaired in its ability to infect a protozoan host, the amoeba *H. vermiformis* (data not shown). Taken together, these results indicate that although *hbp* is a promoter of hemin binding, it is not required for intracellular infection by *L. pneumophila.*

Distribution of *hbp* among *L. pneumophila* strains and other *Legionella* species. The *L. pneumophila* species consists of 14 serogroups, and the *Legionella* genus includes 39 species (18). To determine whether *hbp* is conserved among *L. pneumophila*

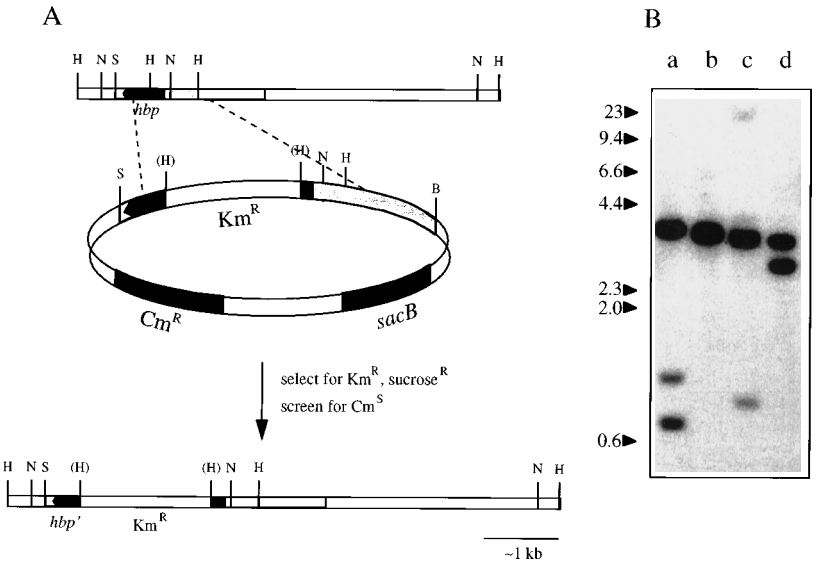

FIG. 5. Mutagenesis of the *hbp* gene of *L. pneumophila*. (A) Diagram of the mutagenesis procedure. The top half depicts a pBOC22-containing *L. pneumophila* transformant. The dashed line indicates a double-crossover event between the plasmid and the chromosome. The predicted result of this recombination, with the mutated *hbp* replacing the wild-type gene, is shown at the bottom. The locations of *Bam*HI, *Hin*cII, *Nde*I, and *Sac*I recognition sites are indicated. (H), the *Hin*cII site that was lost upon insertion of Km^r into *hbp*. (B) Demonstration of allelic exchange by Southern hybridization analysis. Genomic DNAs were digested with either *Hin*cII (lanes a and b) or *Nde*I (lanes c and d) and were probed with ^{32}P-labeled pBOC22. Strain 130b appears in lanes a and c, and strain NU226 is represented in lanes b and d. The migrations and sizes (in kilobases) of molecular markers are indicated.

strains and *Legionella* species, we performed Southern hybridization analysis using an *hbp*-specific probe. Under high-stringency conditions, the probe hybridized with DNAs from all *L. pneumophila* strains tested, suggesting that *hbp* is well conserved within the *L. pneumophila* species (Fig. 7A). In contrast, *hbp* was largely absent from other *Legionella* species. Not only did the probe fail to hybridize with DNAs from these species under high stringency (data not shown), in most cases, it did not even hybridize under reduced-stringency conditions (Fig. 7B). Curiously, *L. moravica*, an environmental isolate, and *L. hackeliae*, a clinical isolate, were the only species that reacted with the *hbp* probe. Furthermore, these hybridizations were quite weak compared with those of *L. pneumophila* (Fig. 7B, lanes a, e, and g). In a control Southern blot, DNAs from all of the *Legionella* spp. hybridized, as expected, with a *mip* probe under low-stringency conditions (data not shown) (9). Taken together, these experiments indicate that although *hbp* is not required for intracellular infection, it is nearly exclusive to the most pathogenic of *Legionella* species.

DISCUSSION

With the results presented here, *L. pneumophila* now shares with a number of other pathogens the ability to bind and utilize hemin as an iron source. Furthermore, the capacity of hemin to replace ferric iron in a CDM indicates that it can be the sole source of iron. This experience with hemin also suggests that *L. pneumophila* can bind and use a variety of heme-containing compounds (23, 52, 66). Since cultures supplemented with hemin did not achieve the same degree of growth as those supplemented with ferric PP_i, ferric salts should nevertheless remain the preferred iron component of *Legionella* media. The level of hemin binding associated with the virulent strain 130b (i.e., 50 to 60% of added hemin) is quite comparable to those of other pathogens (15, 17, 27, 28). In other systems, hemin binding is followed by the internalization of the heme moiety, with the extraction of iron occurring within an intracellular compartment (16, 45, 61). However, a recent report on *N. gonorrhoeae* indicates that, in some cases, iron is extracted from hemin at the cell surface (19). Clearly, transport studies with radiolabeled hemin are needed to ascertain which heme iron assimilation pathway is operative in the legionellae. However, the inability of hemin to restore growth to strain 130b cultures containing the ferric iron chelator desferal (unpub-

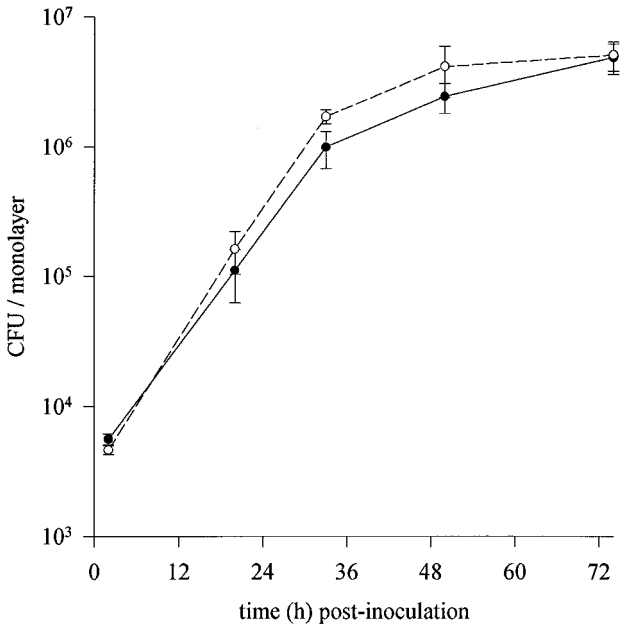

FIG. 6. Intracellular infection by strains of *L. pneumophila*. (A) U937 cell monolayers (n = 4) were inoculated with 10^5 CFU of either strain 130b (●) or strain NU226 (○), and after various incubation periods, the numbers of viable intracellular bacteria were determined. Since 2 h were allowed for bacterial attachment and entry, the first sample that was collected is presented as a 2-h datum point. Each point represents the mean CFU recovered, and the vertical bars indicate the standard deviations.

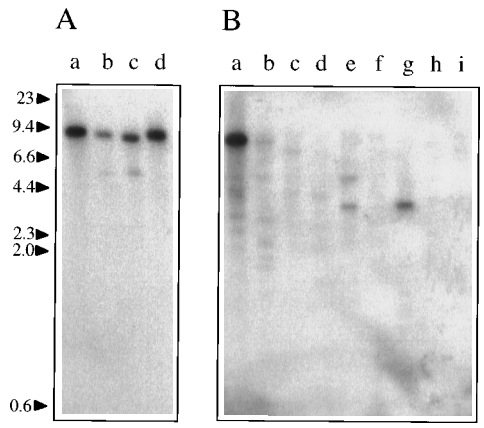

FIG. 7. Hybridization of DNAs from *Legionella* species with an *hbp* probe. DNAs were digested with *Eco*RI and electrophoresed through 0.8% agarose. A Southern blot was then hybridized with the *hbp*-containing *Nde*I-*Eco*RV fragment of pEH12 (see Fig. 4) under high (A)- and low (B)-stringency conditions. (A) Lanes: a and b, *L. pneumophila* serogroup 1 strains 130b and Philadelphia 1, respectively; c and d, *L. pneumophila* serogroups 8 and 13, respectively. (B) Lanes: a, *L. pneumophila* 130b; b, *L. micdadei*; c, *L. erthyra*; d, *L. feeleii*; e, *L. hackeliae*; f, *L. longbeachae*; g, *L. moravica*; h, *L. santicrusis*; and i, *L. spiritensis*. The migrations and sizes (in kilobases) of molecular weight markers are indicated.

lished results) suggests that *L. pneumophila* is akin to *N. gonorrhoeae*. The observation that legionellae harvested from YP-minus-Fe-plus-hemin media exhibited a binding activity level greater than those of legionellae harvested from BCYE media warrants additional comment. Two explanations for this observation can be offered. First, it is possible that in *Legionella* spp., as in other microbes, the presence of hemin (in the YP medium) induces the expression of hemin acquisition functions (8, 27). Alternately, the presence of a high level of iron (in the BCYE medium) represses hemin-binding activity (29, 54, 67). The latter possibility is supported by the placement of an iron box-like sequence upstream of *hbp*.

In *L. pneumophila*, as in other microbes, hemin binding and utilization undoubtedly require the action of numerous genes. For five reasons, we strongly suspect that the newly identified *hbp* is one of those genes. First, when cloned into *E. coli*, it promoted hemin binding both on plates and in liquid. Second, the extent of that binding was comparable to what is observed with genes cloned from other bacteria (28). Third, the cloned *hbp* also conferred Congo red binding, which is a trait often associated with hemin binding (15, 17, 27, 37, 62). Fourth, the predicted product of *hbp* appears to be secreted, a characteristic that is common to proteins which are specifically associated with hemin acquisition (6, 14, 21, 28, 29, 42, 45, 61). Fifth, and most importantly, an *L. pneumophila* strain containing a DNA insertion within the monocistronic *hbp* displayed a 43% reduction in hemin binding. It is not clear how *hbp* (Hbp) potentiates hemin acquisition in *L. pneumophila* or *E. coli*. In one scenario, Hbp is the surface protein that directly binds hemin. In a second scenario, it facilitates the expression or activity of another, possibly conserved, component of the gram-negative cell wall. The fact that the *Legionella hbp* mutant still exhibited some hemin binding would tend to support the second scenario. The absence of a second membrane-spanning domain within Hbp is most compatible with a periplasmic rather than outer membrane location for the protein. A hemin-binding protein of *H. influenzae* which was identified by screening a genomic library on hemin plates is believed to be located, at least in part, within the periplasm (28).

In three different assays, the *L. pneumophila hbp* mutant was not impaired in its ability to infect eukaryotic hosts, clearly indicating that *hbp* is not required for intracellular infection. This result diminishes significantly but does not eliminate the possibility that *hbp* encodes a virulence factor; i.e., only experimental animal infections can ascertain whether *hbp* promotes (extracellular) survival and/or growth in vivo. For two reasons, this result also does not necessarily mean that hemin acquisition and utilization are not critical for intracellular *L. pneumophila* growth. On the one hand, the residual hemin binding displayed by the mutant may suffice inside host cells. On the other hand, intracellular legionellae might express other genes which can restore hemin binding to its full capacity. Clearly, additional genes involved in *L. pneumophila* hemin assimilation need to be identified and characterized.

Southern hybridization analysis indicated that *hbp* sequences are nearly limited to the *L. pneumophila* species. This type of gene distribution is rather peculiar. When assessed, all previously identified *L. pneumophila* genes, including *flaA*, *fur*, *htp*, *lly*, *mip*, *ompS*, and *pplA* (*pal*), have had homologs in all or virtually all other *Legionella* species (5, 9, 30–33, 51). Although the biological significance of this observation is unclear, the limited distribution of *hbp* among the legionellae warrants its consideration as a diagnostic reagent. Currently, *mip* primers are used for the PCR detection of *L. pneumophila* within water and clinical samples (2, 4, 35, 38, 43, 49). However, in several instances, these primers amplify DNAs (i.e., *mip*-like genes) from other *Legionella* species (2, 9, 35, 49). Thus, *hbp* primers might enhance the specificity of *L. pneumophila* PCR detection protocols.

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