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Reciprocal Expression of Integration Host Factor and HU in the Developmental Cycle and Infectivity of *Legionella pneumophila*▽†

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Legionella pneumophila **is an intracellular parasite of protozoa that differentiates late in infection into metabolically dormant cysts that are highly infectious. Regulation of this process is poorly understood. Here we report that the small DNA binding regulatory proteins integration host factor (IHF) and HU are reciprocally expressed over the developmental cycle, with HU expressed during exponential phase and IHF expressed postexponentially. To assess the role of these regulatory proteins in development, chromosomal deletions were constructed. Single (*ihfA* or *ihfB*) and double deletion (Δ*ihf*) IHF mutants failed to grow in *Acanthamoeba castellanii* unless complemented in *trans* when expressed temporally from the *ihfA* promoter but not under P_{tac} (isopropyl-β-D-thiogalactopyranoside). In contrast, IHF mutants were infectious for HeLa cells, though electron microscopic examination revealed defects in late-stage cyst morphogenesis (thickened cell wall, intracytoplasmic membranes, and inclusions of poly-β-hydroxybutyrate), and were depressed for the developmental marker MagA. Green fluorescent protein promoter fusion assays indicated that IHF and the stationary-phase sigma factor RpoS were required for full postexponential expression of *magA*. Finally, defects in cyst morphogenesis noted for Δ*ihf* mutants in HeLa cells correlated with a loss of both detergent resistance and hyperinfectivity compared with results for wild-type cysts. These studies establish IHF and HU as markers of developmental stages and show that IHF function is required for both differentiation and full virulence of *L. pneumophila* in natural amoebic hosts.**

The gram-negative intracellular pathogen *Legionella pneumophila* is responsible for most cases of Legionnaires' disease, an atypical pneumonia that often develops following inhalation of *Legionella*-laden aerosols (17, 23). In natural environments, *L. pneumophila* has evolved as an intracellular parasite of protozoa, in which it progresses through stages of a distinct dimorphic developmental cycle, alternating between vegetative replicative forms and planktonic cyst forms, also referred to as MIFs (19, 26, 33). The characteristic features of the metabolically dormant cyst forms include the following: (i) thickened cell walls, (ii) laminations of intracytoplasmic membranes, and (iii) cytoplasmic inclusions of poly-β-hydroxybutyrate (PHBA). These forms are highly resistant to lysis by detergents and antibiotics and are hyperinfectious in cell-based infection models (14, 19, 26, 27). Cysts are not appreciably produced in U937 macrophages (26) or under in vitro conditions, since morphogenesis aborts in stationary phase. The developmental program is activated upon ingestion of vegetative bacteria by *Tetrahymena* species, a process by which the bacteria ablate digestion and in the absence of bacterial replication rapidly differentiate into cysts, which are excreted in fecal pellets (8, 20). The underlying regulatory cascades and environmental cues controlling this dimorphic life cycle are poorly understood.

A common feature of aquatic and soil microorganisms is their ability to form cysts or spores that promote survival during periods of desiccation, starvation, or other extreme conditions (52). In contrast to our knowledge of endospores, the regulatory control of cyst biogenesis has not received much attention. In *Azotobacter vinelandii*, cyst morphogenesis occurs postexponentially with synergistic synthesis of alginate and PHBA under the control of a regulatory cascade involving the stationary-phase sigma factor RpoS, the two-component regulatory system GacA/S, and the transcriptional regulator AlgR (13). Similarly, cyst formation by *L. pneumophila* in natural hosts occurs postexponentially and coordinately with the appearance of transmission phenotypes previously described for stationary-phase bacteria that include increased infectivity, motility, sodium sensitivity, osmotic resistance, and cytotoxicity (3, 12, 26, 61). Our studies have identified several developmentally regulated proteins, including MagA (*M*IF-*a*ssociated *g*ene of unknown function) and the alkyl hydroperoxide reductases AhpC1, AhpC2, and AphD, that serve as stage-specific markers (38, 41, 42). Genetic studies indicate that the postexponential transmission traits and MagA expression are partly controlled by a regulatory cas-

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cade involving, among other regulatory factors, RpoS and the two-component system LetA/S (an ortholog of GacA/S) (4, 5, 22, 25, 34, 45, 46, 47, 65). OxyR, a non-redox-active LysR variant, is induced in late exponential phase and directly represses *ahpC2D* (41). Tight regulation of developmental stages appears to be important, since *rpoS* and *letA* mutants show defects in both cyst morphogenesis and host range (20). Moreover, several studies have indicated that the major virulence system (Dot/Icm type IVB secretion system) is not developmentally regulated (20, 54).

Analysis of the promoter region of *magA* revealed a regulatory motif resembling the *Escherichia coli* consensus integration host factor (IHF) binding site (30). In the aquatic dimorphic organism *Caulobacter crescentus*, IHF is required for temporal activation of flagellar genes and promotes efficient chromosomal replication (29, 49, 50, 56). Likewise, IHF appears to be involved in transcription activation of developmentally regulated genes in *Chlamydia trachomatis* (63). IHF was originally identified and characterized for *E. coli* as a heterodimeric protein (α and β subunits, encoded by *himA* and *himD*, respectively) that recognizes a 13-bp core consensus DNA sequence which upon binding bends the DNA up to an angle of 180° (2, 15, 30, 32, 51). By bending the DNA, IHF acts as an accessory factor that influences regulatory processes such as replication and transcription (31). In addition, IHF has been shown to be involved in virulence gene expression in a wide range of bacterial pathogens (37, 43, 44, 56, 57), and loss-of-function single (*himA*) or double (*himA himD*) mutations in IHF subunits are often associated with a loss of virulence (43, 49, 58, 62).

Here we show that the IHF proteins of *L. pneumophila* are most abundant postexponentially and become concentrated in the transmissive cyst form. In contrast, HU, a heat-stable nucleoid protein paralogue of IHF (1, 16), is undetectable in the cyst and is one of the most abundant proteins, like AhpC2 and AhpD expressed by replicative forms. A loss of IHF function results in pleiotropic phenotypes, including depressed levels of MagA and of PHBA-containing inclusions and incomplete differentiation into the cyst form. Moreover, IHF function, while not required for infectivity of HeLa cells, is required for infection of its natural host, *Acanthamoeba castellanii*.

MATERIALS AND METHODS

Bacterial strains, oligonucleotides, media, and general methods. Bacterial strains and oligonucleotides used in this study are listed in Table 1 and in Table S1 in the supplemental material, respectively. All *L. pneumophila* strains were grown in BYE broth or BCYE agar as described previously (21), which when required was supplemented with thymidine (100 μg/ml). *E. coli* DH5α and *E. coli* BL21(DE3) CodonPlusRIL, used as host strains for cloning strategies and recombinant protein expression, respectively, were grown in LB medium. Antibiotics (streptomycin [100 μg/ml], kanamycin [40 μg/ml], gentamicin [10 μg/ml], ampicillin [100 μg/ml], metronidazole [20 μg/ml], and chloramphenicol [4 or 20 μg/ml]) were added for selection where appropriate. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), Invitrogen (Frederick, MD), or Qiagen (Mississauga, Ontario, Canada). All restriction enzymes were purchased from New England Biolabs. DNA manipulations followed general protocols (53). All protein concentrations were determined using Bradford's protein assay reagent (Bio-Rad).

Protein expression and purification of IHFα and IHFβ subunits and HU. *L. pneumophila* orthologs of the *E. coli* IHFα (*himA*) and -β (*himD*) subunit genes and the HU gene (*hupB*) were found via bioinformatic analyses of the *L. pneumophila* Philadelphia-1 genome sequence (http://genolist.pasteur.fr/LegioList/) and henceforth are referred to as *ihfA* (Lpg2709), *ihfB* (Lpg2955), and *hupB* (Lpg1858), respectively. *L. pneumophila ihfA* (primers IHFαNdeIF and IHFαXhoIR), *ihfB* (primers IHFβNdeIF and IHFβXhoIR), *hupB* (primers HUNdeIF and HUXhoIR), and *rpoS* (primers *rpoS*NdeIF and *rpoS*XhoIR) were PCR amplified, directionally cloned into NdeI and XhoI restriction sites to produce His_6-tagged fusion proteins in the expression vector pET16b, and overexpressed in *E. coli* BL21(DE3) CodonPlus RIL (Stratagene) by using 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) induction at an optical density at 660 nm (OD_{660}) of ~0.500 in 1 liter of Luria-Bertani medium for 60 min. Extraction and Ni interaction purification were performed as described previously (41). In order to attain the IHFαβ heterodimer, IHFα and IHFβ eluted protein preparations were mixed at a 1:1 ratio before dialysis to facilitate formation of heterodimers and verified by gel filtration (64).

Gel retardation assays. The binding reaction mix contained 1.5 mg/ml sheared fish sperm DNA (Sigma), 1 mg/ml bovine serum albumin (New England BioLabs), 5% glycerol, 40 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 M NaCl, and ~10,000 cpm of radiolabeled 245-bp P_{magA} promoter region DNA generated by PCR using the primers *magA*BamHIF and *magA*XbaIR. The radioisotope [α-^{32}P]dCTP (3,000 Ci/mmol) was obtained from Amersham BioSciences (Baie D'Urfé, Quebec, Canada) and used in PCR-based labeling as described previously (41). The purified recombinant proteins IHFαβ, *E. coli* IHFαβ ($IHF\alpha\beta_{Ec}$), and RpoS were added over a range of 0 to 14 μM. The total volume of the reaction mix was then adjusted to 20 μl with sterile distilled water. Protein was omitted from the control tube in order to determine the electrophoretic mobility of free probe DNA. All of the reaction mixtures were incubated for 30 min at room temperature, and 2 μl of loading buffer (0.3% glycerol and 0.01% bromophenol blue in 10× Tris-borate buffer) was added to each of the reaction mixtures, which were run in prerun (1 h at 20 mA) 6% 0.5× Tris-borate-EDTA nondenaturing polyacrylamide gel electrophoresis at 20 mA, dried, and exposed to Kodak BioMax MR autoradiographic film. Consistent results were obtained from three replicates.

Construction of *L. pneumophila* Lp02Δ*ihf* mutant strain. IHF chromosomal deletions were made by utilizing an in-house gene replacement suicide vector (pRDX) strategy (41). Briefly, ~500-bp sequences flanking *ihfA* were amplified by PCR (primer pairs IHFαP1 and IHFαP2 and IHFαP3 and IHFαP4) and ligated into pBluescript (pBS), creating pBS *ihfA* (pMGM001), after which the gentamicin cassette was inserted into the BamHI site within the insert, creating pBS *ihfA*::Gen^r (pMGM002). The *ihfA*::Gen^r construct was then excised from pMGM002 and ligated into the suicide vector pRDX, creating pRDX *ihfA*::Gen^r (pMGM003). Likewise, the same strategy was employed to create the pRDX *ihfB*::Kan^r (pMGM006) construct except that the cloning vector pUC18 (pMGM004) and a kanamycin resistance cassette (pMGM005) were utilized (see Table S1 in the supplemental material for primers). Approximately 10 μg of pMGM006 was electroporated into electrocompetent *L. pneumophila* Lp02, plated on BCYE medium supplemented with streptomycin, kanamycin, and thymidine, and incubated for 3 to 4 days. The resulting transformants were replica plated and screened for loss of metronidazole sensitivity on BCYE supplemented with 20 μg/ml of metronidazole (loss of the plasmid vector). The selected Lp02*ihfB* (Km^r) and Lp02*ihfA* (Gen^r) chromosomal deletion strains were confirmed by PCR amplification for the absence of *ihfB* and *ihfA*, respectively. To create the Lp02 *ihfA*::Gen^r *ihfB*::Kan^r (henceforth referred to as Lp02Δ*ihf*) chromosomal deletion double mutant strain, approximately 10 μg of pMGM003 was electroporated into Lp02*ihfB* cells and plated on BCYE supplemented with streptomycin, gentamicin, kanamycin, and thymidine. After replica plating and screening for metronidazole resistance, the Lp02Δ*ihf* chromosomal double-deletion mutant strain was confirmed for the absence of *ihfA* and *ihfB* by PCR amplification and by immunoblotting with rabbit polyclonal anti-*E. coli* IHF (IHF_{Ec}) (supplied by S. D. Goodman).

IHF complementation in Lp02Δ*ihf*. Complementing constructs were created by PCR, with *ihfB* positioned behind *ihfA* (containing its own Shine-Delgarno sequence) and under the control of both the *ihfA* and vector P_{tac} promoters (including a Shine-Delgarno sequence upstream of *ihfA*) in pMMB206. The resulting plasmid, pMMB206::*ihfA*$^+$ *ihfB*$^+$ (pMGM007), was electroporated into Lp02Δ*ihf* to create the complemented strain MGM004, and expression of both IHF subunits was confirmed by reverse transcription-PCR and immunoblotting with rabbit polyclonal anti-IHF_{Ec}. The overexpression strain MGM002 was created by electroporating the plasmid pMGM007 into Lp02. For negative controls, pMMB206 was electroporated into Lp02 and Lp02Δ*ihf* to create MGM001 and MGM003, respectively.

Immunoblots. Approximately 1 μg each of purified *L. pneumophila* IHFα, IHFβ, and HU proteins was loaded onto a 4-to-12%-gradient Bis-Tris NuPage gel (Invitrogen). *L. pneumophila* cells were harvested from 48-h BCYE plate growth, normalized to an OD_{620} of 0.20, pelleted, and resuspended in 50 μl of 50 mM Tris-Cl (pH 7.5). *L. pneumophila* SVir cyst forms were isolated from in-

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Description	Reference or source
L. pneumophila		
SVir	Spontaneous Str^r isolate of Philadelphia-1 strain	39
Lp02	Str^r, Thy$^-$, HsdR$^-$ derivative of Philadelphia-1 strain	7
JV918	Lp02 Δ*dotB* mutant strain	55
Lp02*ihfA*	Lp02 Str^r, Thy$^-$, HsdR$^-$ *ihfA*::Gen^r	This study
Lp02*ihfB*	Lp02 Str^r, Thy$^-$, HsdR$^-$ *ihfA*::Kan^r	This study
Lp02Δ*ihf*	Lp02 Str^r, Thy$^-$, HsdR$^-$ *ihfA*::*gen ihfB*::*kan*	This study
Lp02Δ*rpoS*	Lp02 Str^r, Thy$^-$, HsdR$^-$ *rpoS*::*kan*	3
MGM001	pMMB206 into Lp02	This study
MGM002	pMGM007 into Lp02	This study
MGM003	pMMB206 into Lp02Δ*ihf*	This study
MGM004	pMGM007 into Lp02Δ*ihf*	This study
KB120	pBH6119 into Lp02; Str^r, Thy$^+$	This study
KB123	pBH6119 into Lp02Δ*ihf*; Str^r Kan^r Gen^r Thy$^+$	This study
KB130	pKB127 into Lp02; Str^r, Thy$^+$	This study
KB131	pKB127 into Lp02Δ*rpoS*; Str^r Kan^r, Thy$^+$	This study
KB135	pBH6119 into Lp02 *rpoS*; Str^r Kan^r, Thy$^+$	This study
KB151	pKB127 into Lp02Δ*ihf*; Str^r Kan^r Gen^r, Thy$^+$	This study
E. coli		
DH5α	F′ *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ80d*lac*Δ(*lacZ*)M15)	New England Biolabs
BL21(DE3) CodonPlus RIL	B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU ileY leuW* Cam^r]	Stratagene
Plasmids		
pET16b	N-terminal 10-histidine-tagged fusion protein expression vector; Amp^r	Novagen
pBluescriptII KS(+)	Cloning vector; Amp^r	Stratagene
pUC18	Cloning vector; Amp^r	Invitrogen
pMMB206	Derivative of pRSF1010; P_{tac} promoter and IPTG-inducible *lacIQ* system; Amp^r Cam^r, Thy$^+$	Michele Swanson
pBH6119	Promoterless GFP vector; Amp^r, Thy$^+$	35
pKB127	pBH6119::*magA* 245-bp promoter region cloned into BamHI and XbaI sites	This study
pRDX	Dual suicide vector pBOC20 with *Bacillus subtilis sacB* and *Helicobacter pylori rdxA* (nitroreducase) as counterselectable markers; Cam^r	This study
pKB58	pET16b::*himA* (IHFα) into NdeI and XhoI sites; Amp^r	This study
pKB59	pET16b::*himD* (IHFβ) into NdeI and XhoI sites; Amp^r	This study
pKB129	pET16b::*hupB* (HU) into NdeI and XhoI sites; Amp^r	This study
pMGM001	pBS *ihfA*	This study
pMGM002	pBS *ihfA*::Gen^r	This study
pMGM003	pRDX *ihfA*::Gen^r	This study
pMGM004	pUC18 *ihfB*	This study
pMGM005	pUC18 *ihfB*::Km^r	This study
pMGM006	pRDX *ihfB*::Km^r	This study
pMGM007	pMMB206::*ihfA*$^+$ *ihfB*$^+$	This study

fected HeLa cells as described elsewhere (26). Pelleted cysts with an OD_{620} of 1.0 (1 ml) were resuspended in 5 ml of warmed BYE broth (no antibiotics) and incubated in a 37°C shaker. At various time points, cell samples at an OD_{620} of 0.20 were taken, pelleted, and resuspended in 50 μl of 50 mM Tris-Cl (pH 7.5). Samples were normalized for protein loading and run on a 10% sodium dodecyl sulfate-polyacrylamide gel or on a precast 4 to 12% NuPAGE Bis-Tris gel (Invitrogen), transferred to nitrocellulose (BioLynx) as per the laboratory protocol, and immunoblotted with anti-IHF_{Ec} antibody that was prepared as described elsewhere (30) at a 1:1,000 dilution.

Green fluorescent protein (GFP) promoter fusion assays. The *magA* 245-bp promoter region (P_{magA}*gfp*) was PCR amplified and ligated into the appropriate restriction sites in pBH6119 (35), creating pKB127. The control plasmid pBH6119 and the construct pKB127 were then electroporated into electrocompetent Lp02, Lp02Δ*ihf*, and Lp02Δ*rpoS* cells. Strains KB120, KB123, KB130, KB131, KB135, and KB151 were streaked on BCYE plates (supplemented with appropriate antibiotics) from frozen stock and incubated at 37°C for 48 h. Bacteria were suspended in BYE broth and normalized to a final OD_{620} of 0.01 to initiate growth. For fluorescence analysis at each time point, aliquots of cells were washed twice with 1× phosphate-buffered saline (PBS), resuspended in 1× PBS, and normalized to an OD_{620} of 0.1, and fluorescence was measured in 2-ml samples in a VeraFluor fluorometer (Bio-Rad) at excitation and emission wavelengths of 488 nm and 510 nm, respectively. Means and standard deviations were determined from triplicate assays, and data were analyzed by using a *t* test.

***Acanthamoeba castellani* infection.** *A. castellanii* (ATCC 30010) was maintained in ATCC medium 354 at 25°C. For the infection experiments, a 48-h culture of *A. castellanii* was washed and resuspended in Tris-buffered salt solution containing 2 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM Tris, with the pH adjusted to 6.8 to 7.2 (9). For infections with Lp02 strains, 100 μg/ml of thymidine was added to the medium. Approximately 10^5 cells per ml of *A. castellanii* were infected with ~10^3 CFU/ml of *L. pneumophila*. All experiments were performed in triplicate at 25°C, and at each time point (0, 24, 48, and 72 h postinfection), amoeba cells were disrupted by passage through a 27-gauge needle. Intracellular bacteria were enumerated following decimal dilution and plating on BCYE agar plates, and CFU per ml are reported as the means and standard deviations of triplicates.

HeLa cell infection. HeLa cells were grown in minimal essential medium (MEM) as described previously (28). HeLa cells were harvested, counted in a Neubauer hemocytometer, and resuspended in MEM without antibiotics to a concentration of 10^6 cells/ml. Cells from spinner bottles were dispensed into 12-well plates at 10^6 cells per well (1 ml) and left to adhere and spread for 1 to 2 h. Plate-grown *L. pneumophila* was harvested in double-distilled water, and the bacterial suspension was standardized to an OD_{600} of 1.0. One hundred microliters of the standardized bacterial suspensions were added to triplicate wells to a final inoculum of 10^8 bacteria/10^6 HeLa cells (28). Plates were centrifuged at 500 × *g* for 10 min at room temperature to maximize contact of bacteria with the HeLa cell monolayer and then incubated for 3 h to facilitate infection. For

attachment-and-invasion assays, plates were washed six times with PBS, the monolayers were lysed, and dilutions were plated for bacterial enumeration. For invasion assays, monolayers were washed three times with PBS and then treated for 1 h with MEM containing 100 μg/ml gentamicin and similarly enumerated for viable bacteria. For studies with the Lp02 strain, MEM was supplemented with thymidine and 2 mM IPTG. For cyst purification, monolayers were harvested using a cell scraper, resuspended in ddH_2O, and passaged through a 26-gauge needle to lyse HeLa cells as described previously (26).

Cell cycle experiments. Cyst germination studies were performed with BYE broth in which freshly isolated Lp02 cysts were suspended to an OD_{660} of 0.1 and allowed to grow over a period of 48 h. Samples were taken at early exponential phase (OD_{660} = 0.2 to 0.4), mid-exponential phase (OD_{660} = 0.6 to 0.8), late exponential phase (OD_{660} = 0.9 to 1.1), and post-exponential phase (OD_{660} = 1.2 to 1.5). Samples were normalized to an OD_{660} of 1.0 following suspension in sodium dodecyl sulfate sample buffer and subjected to electrophoresis and immunoblotting to track IHF and HU protein production as previously outlined. All experiments were performed in triplicate to ensure reproducibility.

Transmission electron microscopy (TEM). At 24, 48, and 72 h postinfection, light microscopic images of *Legionella*-infected HeLa cells were captured at magnification ×40 using an Olympus IX-71 (Olympus) inverted microscope equipped with a 12-bit Evolution QET Monochrome camera (Media Cybernetics) to confirm the stage of intracellular growth. Thin sections of infected HeLa cells were prepared from specimens fixed in glutaraldehyde and embedded in epoxy resin as reported previously (19). Multiple sections from each sample were photographed, and of 20 to 50 cells examined, one typical photograph was selected to represent the group. To ensure conformity, multiple replicates on different days were examined. Statistical analysis was generally unnecessary since photographs represent 90% or more of forms observed for each sample.

RESULTS

Analysis of upstream sequences of the developmentally regulated *magA* gene revealed a putative integration host factor binding consensus sequence (TATCCAAAAATAA), suggesting that IHF might participate in postexponential regulation. Potential IHF binding motifs are also found in upstream sequences of ~200 *L. pneumophila* genes (based on BLAST searches of the *L. pneumophila* genome on the Legiolist web server), which includes several regulators of postexponentially expressed genes (*letA*, *letE*, *fleQ*, *rpoS*, and *ihfA*). Since IHF proteins are highly conserved, we found that a polyclonal IHF_{Ec} antibody cross-reacted with purified IHFα and IHFβ subunits and with a paralogous protein, HU (see Fig. 1A). *L. pneumophila* HU shares 78% identity and 84% similarity with *E. coli* HU, as well as ~37% identity and 57% similarity to IHFα and IHFβ of *L. pneumophila* (data not shown). The IHF immunoblot of cellular extracts of *L. pneumophila* Lp02 cells revealed two bands close together in the 10- to 12-kDa range: the upper band corresponded to IHF subunits based on the absence of these bands in the Lp02Δ*ihf* mutant strain, and the lower band migrated similarly to purified HU (Fig. 1B). For verification purposes, *L. pneumophila* SVir, harvested at late and post-exponential phases, were compared with Lp02 and Lp02Δ*ihf* mutant strains to confirm these identities. Note that the IHFα and IHFβ subunits are not distinguishable in Fig. 1B and C due to a lower resolving gel percentage than that viewed in Fig. 1A. Similar allelic replacement strategies used to knock out the *ihfA* and *ihfB* genes were used to knock out *hupB*, but these proved unsuccessful and were not further pursued (data not presented).

To track IHF and HU protein levels during the in vitro growth cycle, bacterial growth was synchronized by utilizing purified cysts harvested from 48-h-infected HeLa cells. As seen in Fig. 1C, cysts are enriched for IHF proteins, whereas the HU protein is undetectable. Following germination of cysts into vegetative bacteria (~18 h) in BYE broth and proceeding through early-, mid-, and late-exponential phases (growth curve), IHF protein levels were found to decrease coordinately with increasing levels of HU. During late log phase, IHF levels were visible and increased to near cyst levels during stationary phase while HU levels decreased. The trajectory of IHF production followed those of other developmentally regulated protein markers, MagA and AhpC1 (data not presented).

Complementation of Lp02Δ*ihf*. To restore IHF production in Lp02Δ*ihf*, the coding sequences for $ihfA^+$ and $ihfB^+$ under the control of both the *ihfA* promoter and the P_{tac} promoter of pMMB206 and both subunits were verified by PCR (Fig. 2A) and by immunoblotting for MGM004 (Fig. 2B).

In vitro and in vivo growth. Since IHF may be involved in controlling the transcription of many genes, some of which might have growth-enfeebling effects, we next examined the in vitro growth characteristics of the Lp02Δ*ihf* strain. As is shown in Fig. 3A, the growth rates of Lp02 and Lp02Δ*ihf* were indistinguishable, indicating that the Lp02Δ*ihf* mutant was not defective for in vitro growth. To assess the role of IHF in the intracellular multiplication of *L. pneumophila*, infectivity studies with in vitro-grown bacteria were performed with *Acanthamoeba* and HeLa cells. As shown in Fig. 3B, single- and double-knockout mutants of *L. pneumophila* failed to grow in *A. castellani*, with both Lp02Δ*ihf* and Lp02*ihfA* mutants being cleared while Lp02*ihfB* mutants persisted but failed to grow. Complementation of the Lp02Δ*ihf* mutant with pMGM007 (the MGM004 strain) restored infectivity and intracellular growth, but only from the P_{ihfA} (endogenous) promoter. Constitutive expression from pMGM007 (IPTG) failed to restore infectivity (Fig. 3B), suggesting that inappropriate expression in early exponential phase likely results in dysregulation of developmental systems or factors required for infectivity of amoebae.

In contrast to the results with natural hosts, all of the mutants displayed comparable growth rates in HeLa cells (Fig. 3C). Microscopic examination revealed that there were extracellular bacteria present in the culture medium after 24 h, indicating no obvious defect in escape from the host cell (data not shown). To determine if constitutive overexpression of complementing IHF from pMGM007 (the MGM004 strain) also resulted in intracellular growth defects, infections were carried out in the presence of IPTG. Overexpression of both IHF subunits in the MGM004 strain resulted in no measurable differences from results for the Lp02 and MGM002 strains. In addition, we found no appreciable differences between Lp02Δ*ihf* and Lp02 in efficiency of attachment or invasiveness for HeLa cells (data not shown). Thus, IHF mutants show infectivity defects only in natural hosts, a phenotype also reported with *rpoS* mutants (34).

MagA expression. We have previously reported that MagA is a marker for postexponential development in *L. pneumophila* (38). To confirm that IHF function is associated with the expression of *magA*, P_{magA}*gfp* reporter constructs were introduced into Lp02Δ*ihf* and into an Lp02Δ*rpoS* mutant strain, and fluorescence intensity was tracked over the in vitro growth cycle. Figure 4A shows the upstream region of *magA*, including the putative IHF binding motif and inferred RpoS promoter sequences. Figure 4B shows the relative fluorescence levels for Lp02 P_{magA}::GFP (KB130), Lp02Δ*ihf* P_{magA}::GFP (KB151),

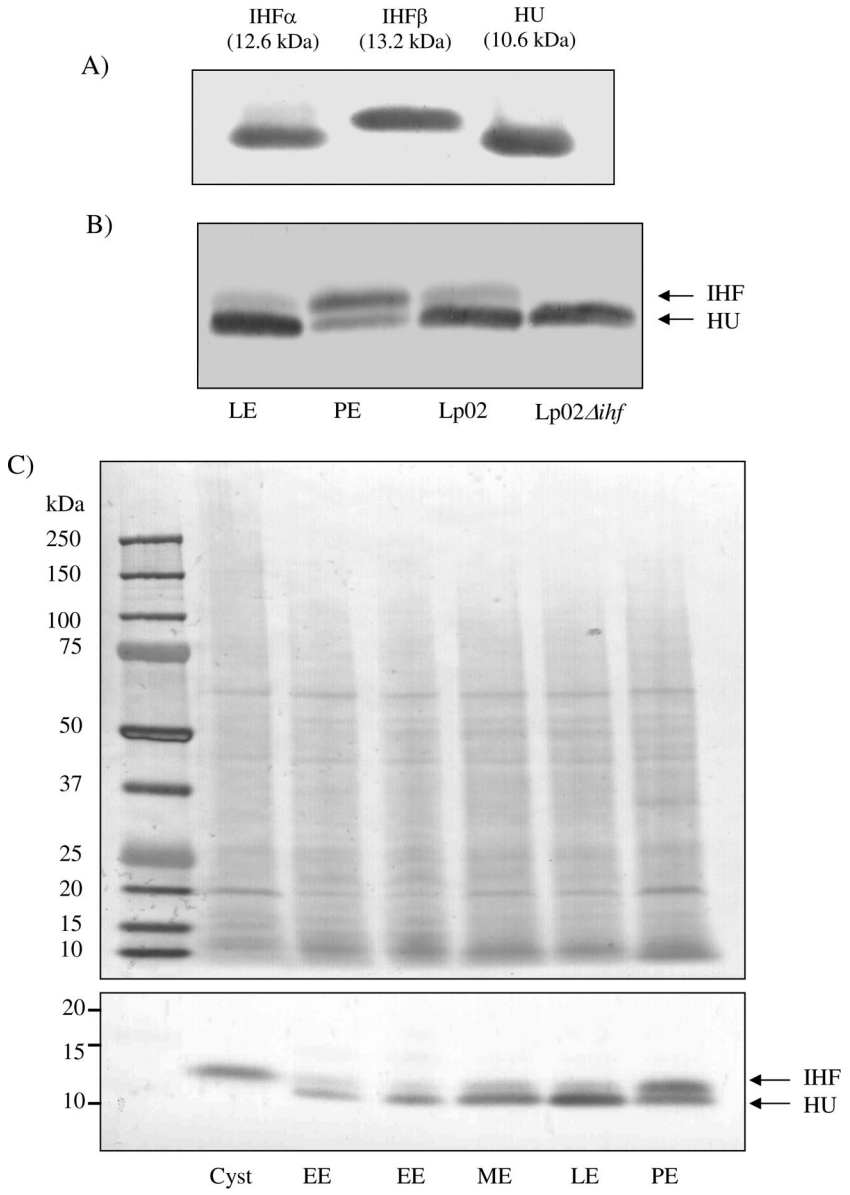

FIG. 1. Polyclonal IHF_{Ec} immunoblots. (A) Polyclonal $IHF\alpha\beta_{Ec}$ antibody detects purified histidine-tagged IHFα and IHFβ proteins and cross-reacts with histidine-tagged HU protein. Molecular masses of the proteins (including the His_{10} tag) are indicated. (B) Determination of IHF and HU bands in *L. pneumophila* SVir time point samples (LE and PE are identical to those samples used in panel C) and in strains Lp02 and Lp02Δ*ihf*. (C) IHF production varies throughout the *L. pneumophila* SVir cell cycle (bottom panel), and Ponceau Red staining of the immunoblot (top panel) indicates equal protein loading of all time point samples. Note that the LE and PE time point samples are identical to the samples used in panel B. Also note that the gel percentage is higher in panel A than in panels B and C, and hence there is no discrimination between the IHFα and IHFβ subunits in *L. pneumophila* cell samples in panels B and C. EE, early exponential phase; ME, mid-exponential phase; LE, late exponential phase; PE, post-exponential phase. Details of the phases of growth are presented in the text. Experiments depicted are typical of at least three replicates for two independent experiments.

and Lp02Δ*rpoS* P_{magA}::GFP (KB131), along with the vector controls Lp02 vector GFP (KB120), Lp02Δ*ihf* vector GFP (KB123), and Lp02Δ*rpoS* vector GFP (KB135) measured at mid-, late-, and post-exponential growth phases. No significant differences were observed in the GFP expression levels in Lp02, Lp02Δ*ihf*, and Lp02Δ*rpoS* at mid-exponential growth phase (Fig. 4B). However, statistically significant differences in GFP expression levels ($P = 0.001$ by *t* test) became evident throughout the late- and post-exponential growth phases in that the GFP expression levels in the Lp02Δ*ihf* and Lp02Δ*rpoS* strains were similar to one another and both were below the GFP expression level observed for Lp02. Thus, the observed decreased fluorescence suggested that RpoS and IHF contribute to the post-exponential expression of this developmental marker.

EMSA. To confirm that the IHF and RpoS proteins indeed bound the *magA* promoter region (direct regulatory role), we performed DNA binding electrophoretic mobility shift assay

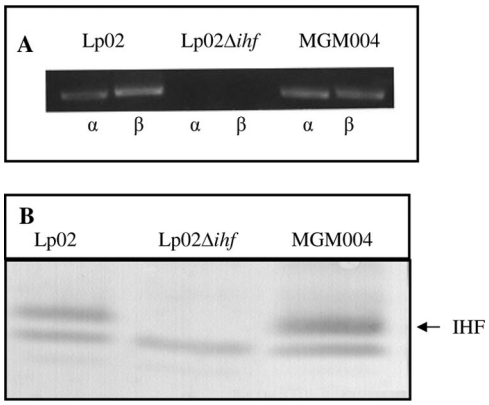

FIG. 2. Deletion, complementation, and expression of IHF. (A) Presence or absence of *ihfA* and *ihfB* subunit genes as determined by PCR amplification. (B) Expression of IHF determined by polyclonal IHF_{Ec} immunoblotting of in vitro growth (postexponential) of Lp02 (lane 1), Lp02Δ*ihf* (lane 2), or MGM004 (pMGM007; $ihfA^+$ $ihfB^+$) complement (lane 3).

(EMSA) studies. As shown in Fig. 4C, only the IHFαβ and $IHF\alpha\beta_{Ec}$ heterodimers produced a gel shift, and in a concentration-dependent manner. Since mixing of the IHFα and IHFβ subunits produces heterodimers, we cannot rule out the possibility that some of the remaining monomers might form dimers through interaction with the IHF DNA motif. Similar studies with RpoS provided some evidence for binding of RpoS to *magA* promoter sequences. Since sigma factors generally bind poorly to DNA in the absence of RNA polymerase, our findings are only suggestive of an interaction. These studies support a direct role for IHF and perhaps RpoS in the postexponential regulation of *magA* gene expression.

Lp02Δ*ihf* mutants display altered morphology in HeLa cells. As seen in Fig. 5, HeLa cell vacuoles containing Lp02 (panel A) and those containing Lp02Δ*ihf* (panel B) were similar at 48 h postinfection, as described previously (28). In contrast to Lp02, Lp02Δ*ihf* was conspicuous for an absence of PHBA-containing inclusions (Fig. 5B), suggesting that postexponential production of PHBA may be dependent on IHF function.

Attempts to restore PHBA inclusions through IHF complementation yielded mixed results, as has been reported for *letA* and *rpoS* complements (20). As seen in Fig. 5C and D, respectively, neither the complemented mutant strain MGM004 (IPTG induced) nor the mock-complemented mutant MGM003 contained PHBA inclusions. TEM examination of HeLa cells infected with the IPTG-induced strain MGM002 (Lp02 constitutively expressing $ihfA^+$ and $infB^+$) also revealed an absence of inclusions (Fig. 5E), while the mock-infected and IPTG-induced strain MGM001 was essentially wild type for PHBA-containing inclusions. Thus, it would appear that either the lack of IHF or its constitutive overexpression severely affects PHBA production within the infected HeLa cells.

Lp02Δ*ihf* mutant strain is defective in cyst morphogenesis. The observed defects in morphogenesis, as well as the apparent decrease in PHBA inclusions, led us to more closely examine the maturation process of Lp02Δ*ihf* to determine its ability to form cysts. The intracellular maturation process of *L. pneumophila* in HeLa cells and the characteristic features of cysts (i.e., multilayered, tightly associated internal membranes, cytoplasmic inclusions, and irregular shapes) have been described previously (19, 20, 26). For confirmatory purposes, the Lp02 strain (Fig. 6A) used in this study was determined to undergo differentiation similar to that described for the SVir strain (Fig. 6B). Ultrastructural analysis of Lp02Δ*ihf* within HeLa cells (panel C) revealed defects in the late stages of differentiation; Lp02Δ*ihf* typically did not possess the same level of internal membrane layering as was seen with the wild-type strains (Fig. 6A and B) but instead possessed more loosely associated bilayers and fewer cytoplasmic inclusions and exhibited a more typical gram-negative envelope organization than wild-type strains (Fig. 6A and B). These defects were only partly restored by *trans* complementation using strain MGM004 (Fig. 6D). An examination of five randomly selected fields revealed only 6 to 10% of bacteria contained inclusions or showed evidence of cyst maturation. The mock-complemented MGM003 strain was similar in ultrastructure to Lp02Δ*ihf* (data not shown). Ultrastructural analysis of the overexpressing MGM002 strain within HeLa cells also showed defects in maturation, particularly in the formation of cytoplasmic inclusions (Fig. 6E). Of the five randomly selected fields containing replicative vacuoles of the MGM002-infected HeLa cells, only 26% of the bacterial population contained any cytoplasmic inclusions whereas 6% contained inclusions comparable to those observed with wild-type strains. In comparison to HeLa cells infected with the mock overexpression strain MGM001, nearly 100% of the bacterial population within the replicative vacuoles contained cytoplasmic inclusions (Fig. 6F). Thus, the absence of or constitutive overexpression of IHF affects not only PHBA production but also the ultrastructure of the cyst cell wall.

Resilience properties of defective Lp02Δ*ihf* cysts. We previously demonstrated that cysts from Lp02 and Svir strains are highly resistant to the effects of detergents and antibiotics, and they were hyperinfectious in cell-based assays (26). Using these methods, we compared in vitro stationary-phase grown bacteria and the cyst forms harvested from HeLa cells. As seen in Table 2, there were no differences in detergent tolerance or in infectivity between the Lp02Δ*ihf* strain and Lp02 in stationary growth phase. However, the cyst forms of Lp02Δ*ihf* were much more susceptible to lysis by detergents than were Lp02 cysts. Moreover, reinfection efficiencies for the Lp02Δ*ihf* cysts were 100-fold lower than with Lp02 cysts. These findings are consistent with differences in cell wall morphology noted between these cyst forms by TEM.

DISCUSSION

Developmental cycles have been described for aquatic and soil bacteria originating from a variety of prokaryotic genera with the common element of formation of cyst-like progeny tailored for survival in nutrient-poor environments (48, 52). Accordingly, the transition from vegetative growth to terminally differentiated cysts (and germination) must be tightly controlled through developmental programs composed of stage-specific regulatory elements. Here we show that IHF and HU are reciprocally expressed across the developmental cycle of *L. pneumophila*, following trajectories similar to those previously reported for other developmentally regulated proteins,

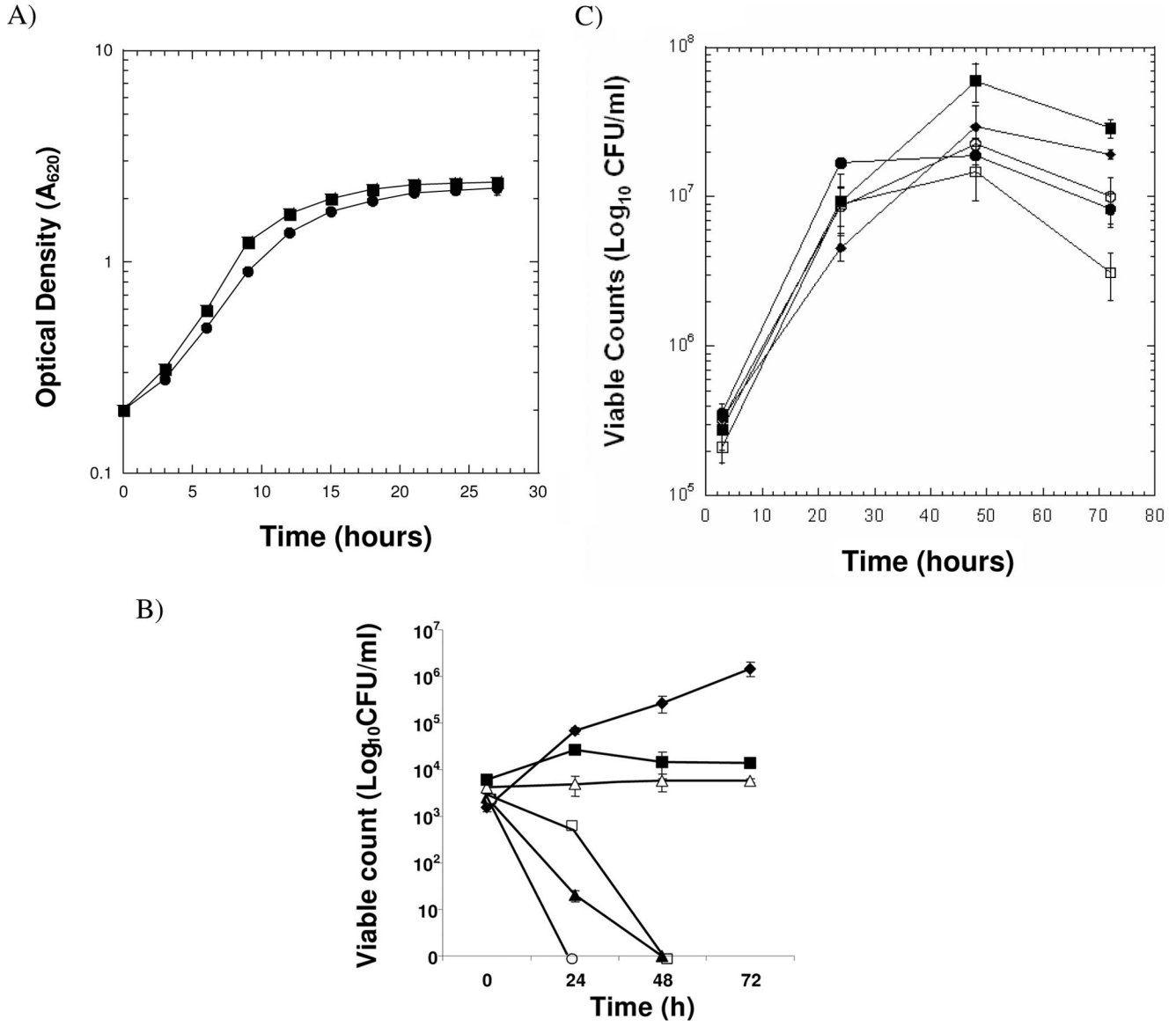

FIG. 3. Growth curves. (A) In vitro growth in BYE broth of Lp02 (■) or Lp02Δ*ihf* (●). (B) Intracellular growth in *A. castellanii* of Lp02 (◆), Lp02*ihfA* (▲), Lp02*ihfB* (△), MGM004 (Lp02Δ*ihf*/pMGM007 complement)(■), Lp02Δ*ihf* (○), or MGM004 plus IPTG (□). (C) Intracellular growth in HeLa cells of Lp02 (■), MGM002 (Lp02/pMGM007) with 2 mM IPTG (◆), MGM001 (Lp02/pMMB206 vector control) with 2 mM IPTG (□), Lp02Δ*ihf* (●), or MGM002 (Lp02/pMGM007) (○). All experiments were performed in triplicate, and mean and standard deviation are depicted.

including the alkyl hydroperoxide reductases AhpC2D and AhpC1 (42), MagA (38), and the developmental regulator OxyR (41). The nucleoid protein HU and AhpC2 and AhpD are most abundant during exponential phase, whereas IHF, AhpC1, and MagA are most abundant postexponentially and become concentrated in the developing cyst. The LysR regulator OxyR is expressed postexponentially and specifically represses the *ahpC2D* operon and possibly other genes associated with differentiation (41). As with LetA and RpoS, IHF function is required for infection of natural amoebic hosts but dispensable for infection of mammalian cells, and both are required for full expression of the developmentally regulated gene *magA*. EMSA studies confirmed that both IHF and to a lesser extent RpoS directly bind to the promoter region of *magA*.

While the role of HU appears to be essential for cell division, IHF is not essential, possibly being compensated for by other small DNA binding proteins, such as Fis (H-NS is absent in *Legionella* spp.). However, the absence of IHF function correlates with several postexponential phenotypes, including altered terminal vacuole morphology in HeLa cells and an absence of typical cyst morphology (e.g., absence of a thick cell wall, laminations of intracytoplasmic membranes, and PHBA inclusions). The latter phenotypes also correlate with increased susceptibility to detergents and a 100-fold decrease in infectiousness compared with results for cysts from wild-type strains

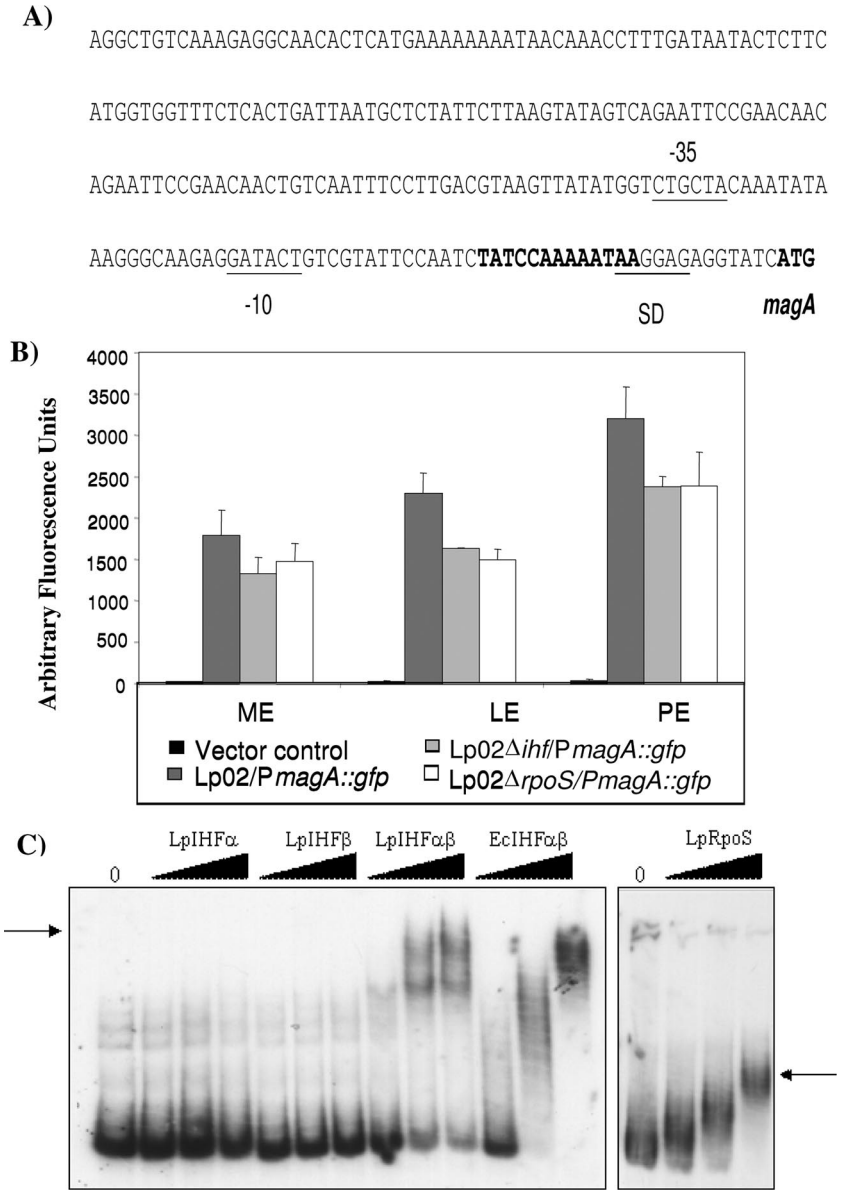

FIG. 4. Expression of *magA-gfp* fusions in mutants of *L. pneumophila*. (A) Promoter sequence of *magA*, depicting the −10 and −35 hexamers and the putative IHF and RpoS binding sites (bold). (B) MagA promoter GFP fusions were monitored at different points of in vitro growth (ME, midexponential; LE, late exponential; PE, postexponential). GFP vector control, Lp02/pBH6119::P_{magA}*gfp*, Lp02Δ*ihf*/pBH6119::P_{magA}*gfp*, and Lp02Δ*rpoS*/pBH6119::P_{magA}*gfp*) are analyzed. For simplicity, a single GFP vector control is depicted, which is representative of controls for Lp02, Lp02Δ*ihf*, and Lp02Δ*rpoS*. (C) Gel retardation analysis of the 245-bp P_{magA} promoter region with IHFαβ, IHFαβ_{Ec}, and RpoS. The radiolabeled 245-bp P_{magA} amplicon was incubated with increasing concentrations of IHFα (2.95, 8.95, and 14.9 μM), IHFβ (2.8, 8.5, and 14.2 μM), IHFαβ (1.5, 4.6, and 7.65 μM), the IHFαβ_{Ec} protein (1.7, 5.1, and 8.5 μM), or RpoS (7.4, 22.1, and 36.8 μM). The control lane (0) does not include the IHF or RpoS protein in the binding reaction mix. Arrows denote band shifts.

(26). These phenotypic differences have also been noted with *letA* and *rpoS* mutant strains in HeLa cells (20), suggesting a requirement for several independent signaling pathways in order to activate the developmental program. Allelic complementation of the IHF mutant from the *ihfA* endogenous promoter partially restored infectivity for *A. castellani*, but induction with IPTG (P_{tac} promoter) and constitutive expression resulted in a loss of infectivity. In the HeLa cell infection model, constitutive overexpression of IHF proteins did not reverse phenotypic changes noted for the Lp02Δ*ihf* strain and caused mutant phenotypic changes to strain Lp02, including the absence of PHBA-containing inclusions. Thus, timing of IHF production appears to be crucial for the proper initiation of the postexponential developmental program. Our studies are consistent with the general view of postexponential regulatory cascades (3, 4, 12, 34, 35, 36, 41, 60, 65), in which upregulation of IHFαβ postexponentially contributes broadly to regulation of as many as 200 genes, based on consensus IHF binding sequences within 300 bp upstream of the translational start sites. Accordingly, either overexpression or inappropriate

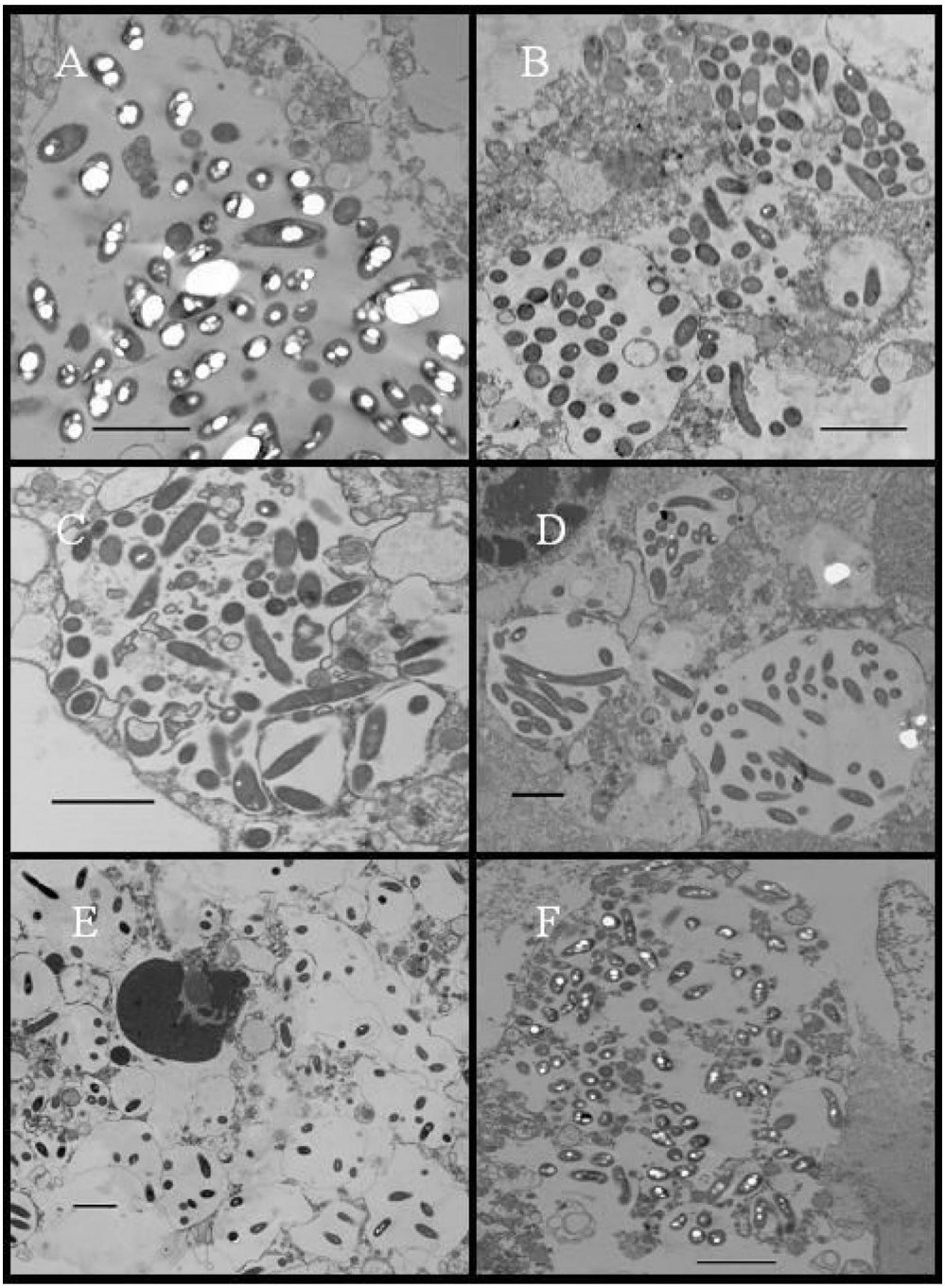

FIG. 5. Electron microscopic examination of altered vacuoles. Transmission electron microscopy of HeLa cells 48 h postinfection with Lp02 (A), Lp02Δ*ihf* (B), complemented MGM004 (Lp02Δ*ihf*/pMGM007) strain (C), mock-complemented MGM003 (lp02Δ*ihf*/pMMB206) strain (D), overexpression MGM002 (Lp02/pMGM007) strain (E), or mock-overexpression MGM001 (Lp02/pMMB206) strain (F). Scale bar = 2 μm for all images. Images are representative of some 25 fields examined.

expression of IHF appears to disrupt the developmental program, affecting cyst morphogenesis as well as infectivity for natural hosts.

In *E. coli*, IHF_{Ec} is recognized as a global regulatory factor associated with the transcription of many genes (2, 40). As in *E. coli* (2, 18), expression of IHF and HU in *L. pneumophila* is growth phase dependent, with maximal protein levels in postexponential and exponential growth phases, respectively. However, the basis for developmental regulation of IHF in *L. pneumophila* remains to be determined. Synthesis of IHF has been shown to be upregulated in other species, such as *C. crescentus* (29), *Shigella flexneri* (49), *Neisseria gonorrhoeae* (24), and *C. trachomatis* (63) upon entry into exponential phase. More importantly, the *C. trachomatis* IHF protein is also developmentally expressed and controls the expression of late genes associated with morphogenesis of the resilient in-

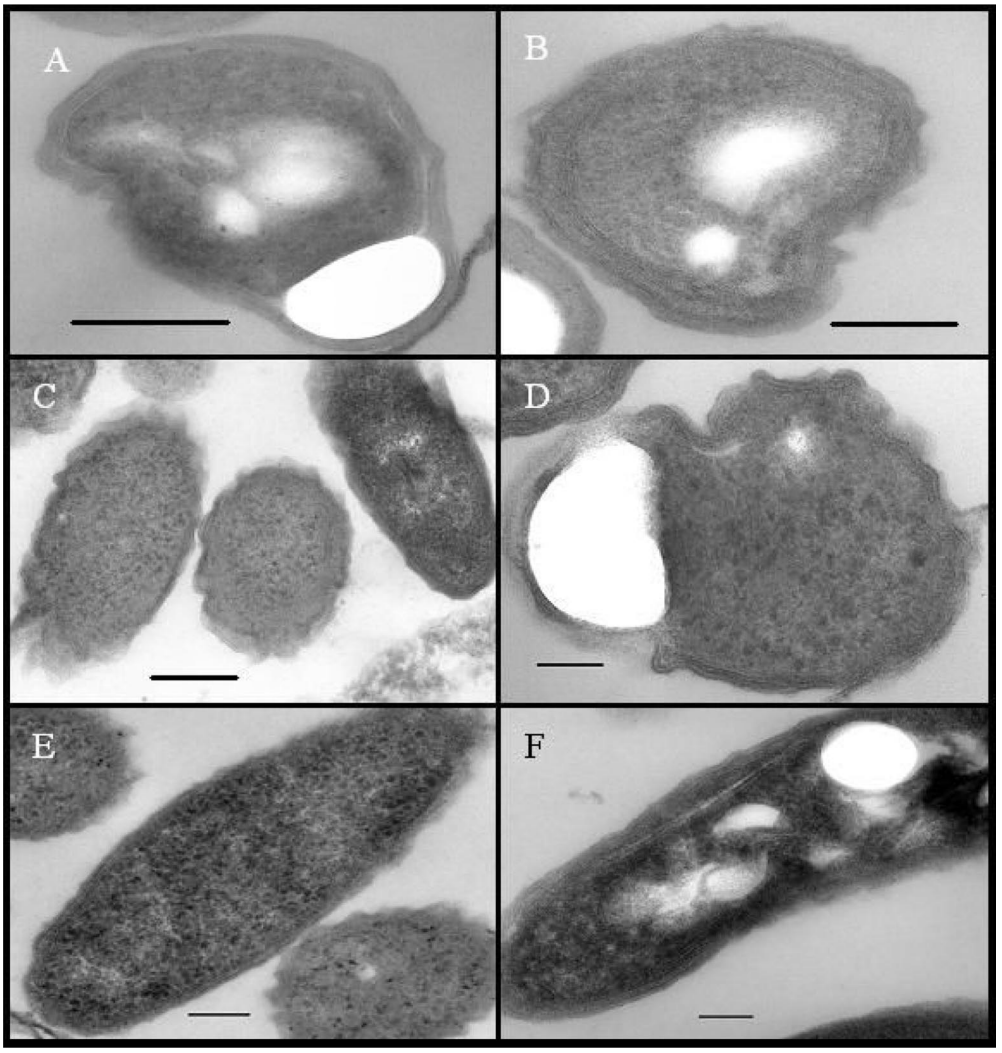

FIG. 6. MIF morphogenesis phenotypes. Transmission electron microscopy of HeLa cells 48 h postinfection with Lp02 (A), Svir (B), Lp02Δ*ihf* (C), complemented MGM004 (Lp02Δ*ihf*/pMGM007) (D) strain, overexpression MGM002 (Lp02/pMGM007) strain (E), or mock-overexpression MGM001 (Lp02/pMMB206) strain (F). Scale bar = 100 nm for all images. The images are representative of 30 fields examined for each strain.

fectious elementary bodies (63). The Lp02Δ*ihf* strain showed no obvious growth defects in vitro or in vivo in HeLa cells. However, IHF function seems to be more important late in intracellular infection as vegetative bacteria differentiate into resilient cysts. Electron micrographs of Lp02Δ*ihf* show early stages of cyst morphogenesis, including some intracytoplasmic membranes and cell wall modifications that correlate with some postexponential phenotypes (sodium sensitivity). The paucity of PHBA inclusions in the Lp02Δ*ihf* mutant suggests that IHF must regulate some step in the biosynthetic pathway, since none of the PHBA synthase genes contains obvious consensus IHF binding sites. Further study is required to identify additional genes or regulatory factors associated with late stages of cyst morphogenesis which are dependent on IHF function.

TABLE 2. Resilience properties of cyst forms[a]

Bacterial strain	Cell type	Time (min) to 50% turbidity with detergent		Reinfection efficiency (%)
		Sarkosyl	Triton X-100	
Lp02	SP	90	1,170	
	Cyst	∞	∞	0.256
Lp02Δ*ihf*	SP	90	1,170	
	Cyst	4,440	4,020	0.216×10^{-2}

[a] Cyst-like forms were harvested at 72 h postinfection from HeLa cells and compared with in vitro growth stationary-phase bacteria (SP).

The role of HU in controlling gene expression in *L. pneumophila* is not as apparent as for IHF. In enteric bacteria, HU is a heterodimer composed of the *hupA*- and *hupB*-encoded subunits (6, 10). In *L. pneumophila*, HU is a homodimer and the product of a single gene, *hupB*. HU, a structural homologue of IHF (1, 2), does not exhibit DNA binding sequence specificity (10, 15) and preferentially binds distorted DNA (i.e., single-stranded gaps, cruciforms, kinks, bends, etc.) to provide architectural assistance in various DNA transactions (59). Recently it has been shown for *E. coli* that the efficiency of stationary-phase sigma factor *rpoS* translation is reduced in

cells lacking HU; thus, *rpoS* transcription or RpoS stability is affected by HU (6). Furthermore, HU specifically bound an RNA fragment containing the translational initiation region of *rpoS* mRNA, strongly suggesting that HU regulates *rpoS* translation (6). In *L. pneumophila*, *rpoS* transcripts appear mainly during exponential phase, when HU levels are high, and are translated postexponentially as HU levels begin to diminish (5). While control of RpoS would not be considered an essential function (since RpoS is not essential), we speculate that HU essentiality may reflect functions unique to the exponential growth stage, much as LpOxyR is essential for postexponential transitions (41).

The postexponential role of RpoS in controlling stationary-phase events is well established for *L. pneumophila* (3, 34). As shown in this study, RpoS and IHF participate in regulating expression of the developmental stage marker MagA, either independently of each or in a cooperative manner. However, we cannot rule out the possibility of epistatic effects that might be caused by IHF modulation of *rpoS* expression (the *rpoS* promoter region contains an IHF consensus binding motif, as do those of the *ihfA* and *ihfB* genes). Clearly there are additional levels of regulation, since cyst morphogenesis is aborted in stationary phase in vitro and these bacteria express both IHF and RpoS (20, 26). In contrast, our studies indicate that IHF function is necessary for cyst maturation and perhaps activation of genes associated with PHBA production. The enrichment of IHF in mature cysts may also play an important role in germination, perhaps through selective activation of early genes whose function is required for subversion of host cells in order to produce a replication permissive endosome. In summary, HU and IHF are reciprocally expressed across the developmental cycle of *L. pneumophila* and appear to be uniquely adapted to the control of growth-stage-specific genes.

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