Characterization of the *icmH* and *icmF* Genes Required for *Legionella pneumophila* Intracellular Growth, Genes That Are Present in Many Bacteria Associated with Eukaryotic Cells

Tal Zusman, Michal Feldman, Einat Halperin, and Gil Segal*

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences,
Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

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Legionella pneumophila, the causative agent of Legionnaires' disease, replicates intracellularly within a specialized phagosome of mammalian and protozoan host cells, and the Icm/Dot type IV secretion system has been shown to be essential for this process. Unlike all the other known Icm/Dot proteins, the IcmF protein, which was described before, and the IcmH protein, which is characterized here, have homologous proteins in many bacteria (such as *Yersinia pestis*, *Salmonella enterica*, *Rhizobium leguminosarum*, and *Vibrio cholerae*), all of which associate with eukaryotic cells. Here, we have characterized the *L. pneumophila icmH* and *icmF* genes and found that both genes are present in 16 different *Legionella* species examined. The *icmH* and *icmF* genes were found to be absolutely required for intracellular multiplication in *Acanthamoeba castellanii* and partially required for intracellular growth in HL-60-derived human macrophages, for immediate cytotoxicity, and for salt sensitivity. Mutagenesis of the predicted ATP/GTP binding site of IcmF revealed that the site is partially required for intracellular growth in *A. castellanii*. Analysis of the regulatory region of the *icmH* and *icmF* genes, which were found to be cotranscribed, revealed that it contains at least two regulatory elements. In addition, an *icmH::lacZ* fusion was shown to be activated during stationary phase in a LetA- and RelA-dependent manner. Our results indicate that although the *icmH* and *icmF* genes probably have a different evolutionary origin than the rest of the *icm/dot* genes, they are part of the *icm/dot* system and are required for *L. pneumophila* pathogenesis.

Bacterial pathogens, as well as bacteria that live in close contact with eukaryotic cells, have developed many mechanisms to subvert their host cells and grow in intimate association with them. Many bacterial pathogens, such as *Yersinia* spp., *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Escherichia coli* O157, use the type III secretion system as part of their pathogenesis determinants (14). Other bacteria such as *Agrobacterium tumefaciens*, *Bordetella pertussis*, and *Legionella pneumophila* use type IV secretion systems, which are functionally homologous to type III secretion systems but are evolutionarily related to bacterial conjugation systems, as opposed to the type III secretion systems, which are evolutionarily related to the bacterial flagellar basal body (9, 13).

L. pneumophila, the causative agent of Legionnaires' disease, is a facultatively intracellular pathogen that is able to infect, multiply within, and kill human macrophages, as well as free-living amoebae (32, 48). Two regions of *icm/dot* genes that constitute the *L. pneumophila icm/dot* type IV secretion system have been discovered (reviewed in references 53 and 64). Region I contains 7 genes (*icmV*, *-W*, and *-X* and *dotA*, *-B*, *-C*, and *-D*) (3, 6, 39, 63), and region II has been shown to contain 17 genes (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-L*, *-K*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, and *-F*) (1, 46, 50, 52, 63). The *icm/dot* genes were shown to participate in many aspects of *L. pneumophila* pathogenesis, such as phagocytosis (29, 66), immediate cytotoxicity (36, 71), inhibition of phagosome-lysosome fusion at early times during

infection (11, 60, 61, 67), association of the phagosome with the rough endoplasmic reticulum (35, 44), apoptosis (70), and exit from the phagosome (42). As a consequence of all these features, the *icm/dot* genes were found to be essential for intracellular multiplication in all of the hosts examined: HL-60- and U937-derived human macrophages (50, 67), murine bone marrow-derived macrophages (63), and the protozoan hosts *Acanthamoeba castellanii* (55) and *Dictyostelium discoideum* (57). Thus far, the *icm/dot* type IV system has been shown to translocate two effector proteins (RalF and LidA) into the host cell during infection (12, 44), and additional effectors for the system are expected to be found.

Eighteen proteins encoded by the *icm/dot* genes (IcmT, -P, -O, -M, -L, -K, -E, -G, -C, -D, -J, -B, -V, and -X and DotA, -B, -C, and -D) contain significant sequence homology to conjugation-related proteins from the IncI plasmid R64 (37, 56). The origins of the six *icm/dot* genes (*icmS*, *icmW*, *icmR*, *icmQ*, *icmN*, and *icmF*) that do not have homologues on the IncI plasmids are unknown. Among the products of these six genes, the IcmS protein was found to interact with IcmW and the IcmR protein was shown to interact with IcmQ (10, 73). The *icmN* gene is predicted to encode a lipoprotein that belongs to the OmpA protein family, which contains homologous proteins found in many gram-negative bacteria (50).

In this study, the *icmF* transcriptional unit was analyzed, and it was found to contain a new *icm* gene, which was named *icmH*. Homologues of the *L. pneumophila* IcmH and IcmF proteins were found in several bacteria that live in intimate contact with eukaryotic cells (such as *Yersinia pestis*, *E. coli* O157, *Vibrio cholerae*, *Rhizobium leguminosarum*, and *A. tumefaciens*), which is a unique property in comparison to the other

* Corresponding author. Mailing address: Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel. Phone: 972-3-6405287. Fax: 972-3-6409407. E-mail: GilS@tauex.tau.ac.il.

TABLE 1. Bacterial strains

Strain	Genotype and features	Reference or Source
<i>L. pneumophila</i>		
25D	Icm ⁻ avirulent mutant	31
GS3011	JR32 <i>icmT3011::Km</i>	54
GS3015	JR32 <i>icmF3015::Km</i>	This study
GS3016	JR32 <i>icmH3016::Km</i>	This study
GS-RelA	JR32 <i>relA::Km</i>	72
JR32	Homogeneous salt-sensitive isolate of AM511	49
LELA3118	JR32 <i>dotA3118::Tn903dIIlacZ</i>	49
LM1376	JR32 <i>rpoS::Km</i>	25
MW627	JR32 <i>tpaA::Km</i>	46
OG2001	JR32 <i>letA::Km</i>	23
OG2002	JR32 <i>cpxR::Km</i>	22
OG2003	JR32 <i>rpoE::Km</i>	22
OG2004	JR32 <i>cpxA::Km</i>	22
<i>E. coli</i>		
MC1022	<i>araD139 Δ(ara leu)7697 Δ(lacZ)M15 galU galK strA</i>	8
MC1061	<i>araD139 Δ(ara leu)7697 ΔlacX74 galU galK strA</i>	8

Icm/Dot proteins. Our results indicate that the IcmF and IcmH proteins are required for *L. pneumophila* intracellular multiplication, but a partially functional Icm/Dot complex is probably present in the bacteria even in their absence. Our results indicate a possible role for these proteins in the interaction of *L. pneumophila* and other bacteria with eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The *L. pneumophila* and *E. coli* strains used in this work are listed in Table 1. The plasmids and primers used in this work are described in Table 2 and Table 3, respectively. Bacterial media, plates, and antibiotic concentrations were used as described before (52).

Plasmid construction for complementation. The plasmid pGS-Lc-55-14 was used to construct new complementing plasmids for the *icmH-icmF-tpaA* operon. The plasmid pGS-Lc-55-14 was digested with XhoI, SacII, and a 7,109-bp fragment that contains the *icmH-icmF-tpaA* operon, as well as part of an open reading frame located upstream from *icmH*, was filled in and cloned into the SmaI site of the vector pMMB207ab-km-14 to generate pGS-Lc-70-14. The plasmid pGS-Lc-70-14 was digested with KpnI, and a 3,182-bp fragment was deleted from the plasmid after self-ligation to generate pGS-Lc-76-14. The resulting plasmid contained the *icmH* gene by itself (the *icmF* and *tpaA* genes were deleted due to the KpnI digestion). To generate a complementing plasmid that contained a nonpolar in-frame deletion in *icmH*, the plasmid pGS-Lc-70-14 was digested with SphI and XbaI, and the resulting 1,081-bp fragment was cloned into the corresponding sites in pUC-18 to generate pGS-Lp-71. An in-frame deletion in *icmH* was constructed by PCR, as described before (33), using the *icmH-F* and *icmH-R* primers (Table 3). The first 3 nucleotides of both primers were changed from the original sequence to form an EcoRV site after self-ligation (each primer contains half of an EcoRV site at its 5' end). The PCR conditions were 30 cycles at 95°C for 1 min, 60°C for 0.5 min, and 75°C for 5 min, performed in 100-μl reaction mixtures using the buffer supplied with the enzyme, 200 mM each nucleotide, ~0.1 μg of pGS-Lp-71 plasmid DNA, 50 pmol of each primer, and 2 U of Vent DNA polymerase (New England BioLabs). The PCR product was gel purified and self-ligated. After transformation, the plasmids prepared were examined for the presence of the EcoRV site expected to be generated by the ends of the two primers. The insert of the plasmid harboring the in-frame deletion in *icmH* was sequenced and named pGS-Lp-74. The plasmid pGS-Lp-74 was digested with XbaI and SphI, and the insert containing the in-frame deletion in *icmH* was cloned into pGS-Lc-70-14 to generate pGS-Lc-77-14. The plasmid pGS-Lc-77-14 contains an in-frame deletion in the *icmH* gene with the *icmF-tpaA* part of the operon unchanged.

Allelic exchange. To construct *icmF* and *icmH* knockout strains, plasmids containing parts of the *icmH* and *icmF* genes were constructed using the sequence information available from the *L. pneumophila* genome sequence database (<http://genome3.cpmc.columbia.edu/~legion/index.html>). For the construction of the *icmF* knockout strain, the plasmid pGS-Lc-55-14 was digested with EcoRI and XbaI, and a 3,797-bp fragment was cloned into the corresponding sites in pUC-18 to generate pGS-Lp-72. Then, the kanamycin resistance cassette (Pharmacia) was cloned into it instead of an internal 1,872-bp EcoRV-Eco47III fragment to generate pGS-Lp-72-Km. The plasmid pGS-Lp-72-Km was digested with PvuII, and the insert was cloned into the EcoRV site of the allelic-exchange vector pLAW344 to form pGS-Lp-72-Km-GR. This plasmid was used for allelic exchange, as described previously (52). The resulting strain (GS3015) contained the first 139 amino acids of the IcmF protein, as well as its last 175 amino acids. The internal 625 amino acids were deleted during the construction, and the kanamycin resistance cassette was cloned instead. Several isolates were analyzed by PCR to confirm that the right change occurred on the chromosome (data not shown). For the construction of the *icmH* knockout strain, the plasmid pGS-Lc-55-14 was digested with PstI, and a 4,417-bp fragment generated was cloned into the corresponding site in pHG-165 to generate pGS-Lp-75. Then, an in-frame deletion was constructed in the *icmH* gene with the same primers and by using the same method described for the construction of pGS-Lp-74 to generate pGS-Lp-78. The kanamycin resistance cassette was cloned into the EcoRV site generated by PCR to generate pGS-Lp-78-Km. The plasmid pGS-Lp-78-Km was digested with PvuII, and the insert was cloned into the EcoRV site of the allelic-exchange vector pLAW344 to form pGS-Lp-78-Km-GR. This plasmid was used for allelic exchange, as described previously (52). The resulting strain (GS3016) contained the first 19 amino acids of the IcmH protein, as well as its last 36 amino acids. The internal 207 amino acids were deleted during the construction, and the kanamycin resistance cassette was cloned instead. Several isolates were analyzed by PCR to confirm that the right change occurred on the chromosome (data not shown).

Construction of mutations in the predicted ATP/GTP binding site of IcmF. The plasmid pGS-Lp-71 (described above) was used as a template in site-directed mutagenesis using the overlap extension PCR method (30). For each mutation, two primers that contained the mutation and overlapped one another by 20 bp were designed (F-G2S-F, F-G2S-R, F-K2A-F, and F-K2A-R [Table 3]). The PCR mutagenesis includes two steps. In the first step, two PCR fragments were generated using the following primers: (i) a primer located on the vector upstream from the regulatory region (one of the primers containing the mutation) and (ii) a primer located on the vector (the second primer containing the mutation, on the complementary strand). The resulting two fragments were gel purified and used as templates in the second step, which included a third PCR using the two primers located on the vector. The resulting PCR product was digested with XbaI and SphI and cloned into the same sites in pGS-Lc-70-14 to generate pGS-Lc-70-G2S-14 and pGS-Lc-70-14-K2A-14. The mutations were confirmed by sequencing the whole region amplified during the PCR.

Construction of lacZ fusions. The promoterless *lacZ* vector pGS-lac-02 was used for cloning different fragments originating from the regulatory region of the *icmH-icmF-tpaA* operon. The fragments cloned were generated by PCR using the F-lac primer (Table 3) and a second primer located at different distances (Table 2) from the IcmH first methionine (primers T-F4 to T-F16 [Table 3]). The fragments generated were digested with BamHI and EcoRI and subsequently cloned into the same sites in pGS-lac-02. The plasmids generated were sequenced to confirm that no changes occurred in the regulatory region and were named according to the primer (e.g., the plasmid resulting from the PCR performed with F-lac and T-F4 was named pGS-reg-F4).

The site-directed mutagenesis was performed by the overlap extension PCR method (30), using the primers F-M2-F and F-M2-R (Table 3). The same mutation was constructed in the plasmids pGS-reg-F4 and pGS-reg-F5 to generate pGS-reg-F4-M2 and pGS-reg-F5-M2, respectively.

Intracellular growth in *A. castellanii*. Intracellular-growth assays were performed similarly to that described previously (55). *A. castellanii* (ATCC 30234) (1.5×10^5 organisms) in PYG (Proteose Peptone-yeast extract-glucose) was added to wells of a 24-well microtiter plate, and the amoebae were incubated for 1 h at 37°C to let the amoebae adhere. Then, the PYG was aspirated, and the wells were washed once with 0.5 ml of warm (37°C) *Acanthamoeba* buffer (Ac buffer), and 0.5 ml of warm Ac buffer was added to the wells. Then, *L. pneumophila* in Ac buffer was added to the wells at a multiplicity of infection (MOI) of ~0.1. The plate was incubated for 30 min at 37°C, the Ac buffer was aspirated, the wells were washed three times with 0.5 ml of warm Ac buffer, and 0.6 ml of warm Ac buffer was added to the wells. The supernatant of each well was sampled at intervals of ~12 or 24 h, and the numbers of CFU were determined

TABLE 2. Plasmids used in this study

Plasmid	Feature(s)	Reference or Source
pGS-lac-02	pAB-1 with a promoterless <i>lacZ</i> gene	24
pGS-Lc-55-14	The <i>icmH-icmF-tphA</i> operon in pMMB207 α b-Km-14	55
pGS-Lc-70-14	The <i>icmH-icmF-tphA</i> operon in pMMB207 α b-Km-14	This study
pGS-Lc-70-G2S-14	pGS-Lc-70-14 with a mutation in the <i>icmF</i> ATP-binding site	This study
pGS-Lc-70-K2A-14	pGS-Lc-70-14 with a mutation in the <i>icmF</i> ATP-binding site	This study
pGS-Lc-76-14	<i>icmH</i> in pMMB207 α b-Km-14	This study
pGS-Lc-77-14	pGS-Lc-70-14 with an in-frame deletion in <i>icmH</i>	This study
pGS-Lp-71	The <i>icmH</i> gene in pUC-18	This study
pGS-Lp-72	Part of the <i>icmH-icmF-tphA</i> operon in pUC-18	This study
pGS-Lp-74	pGS-Lp-71 with an in-frame deletion in <i>icmH</i>	This study
pGS-Lp-75	<i>icmH</i> and part of <i>icmF</i> in pHG-165	This study
pGS-Lp-72-Km	pGS-Lp-72 with kanamycin cassette in <i>icmF</i>	This study
pGS-Lp-72-Km-GR	Insert of pGS-Lp-72-Km cloned in pLAW344	This study
pGS-Lp-78	pGS-Lp-75 with an in-frame deletion in <i>icmH</i>	This study
pGS-Lp-78-Km	pGS-Lp-78 with kanamycin cassette in <i>icmH</i>	This study
pGS-Lp-78-Km-GR	Insert of pGS-Lp-78-Km cloned in pLAW344	This study
pGS-reg-F4	151 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F4-M2	pGS-reg-F4 with a mutation in the regulatory region	This study
pGS-reg-F5	278 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F5-M2	pGS-reg-F5 with a mutation in the regulatory region	This study
pGS-reg-F6	251 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F7	228 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F8	217 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F9	178 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F10	116 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F11	102 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F12	240 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F13	137 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pGS-reg-F14	126 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pHG165	<i>oriR</i> (colEI) MCS Ap ^r	59
pLAW344	<i>oriR</i> (colEI) <i>sacB</i> MCS <i>oriT</i> (RK2) Cm ^r Ap ^r	68
pMMB207 α b-Km-14	<i>oriV</i> (RSF1010) MCS <i>lacI</i> ^q Cm ^r <i>mobA::Km</i>	54
pUC18	<i>oriR</i> (colEI) MCS Ap ^r	69
pZT-reg-F15	91 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study
pZT-reg-F16	81 bp of the regulatory region of <i>icmH</i> in pGS-lac-02	This study

by plating samples on ABCYE {ACES[*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal-yeast extract} plates.

Intracellular growth in HL-60-derived human macrophages. Intracellular-growth assays were performed similarly to those previously described (54). Wells of a 24-well plate containing 2×10^6 differentiated HL-60-derived human macrophages were used for infection. *L. pneumophila* was added to the wells at an MOI of ~ 0.1 , and the infected HL-60-derived macrophages were incubated for 1 h at 37°C under CO₂ (5%). Then, the wells were washed three times, and 0.6 ml of RPMI containing 2 mM Gln and 10% normal human serum was added to the wells. The supernatant of each well was sampled at intervals of ~ 24 h, and the numbers of CFU were determined by plating samples on ABCYE plates.

In the experiments in which the numbers of intracellular bacteria were determined, at each time point a well containing infected cells was lysed by adding sterile double-distilled water to the well, and the numbers of bacteria inside the cells were determined by plating samples on ABCYE plates.

When entry of the bacteria into HL-60-derived human macrophages was determined, the infection was performed at an MOI of 1, followed by centrifugation and incubation with gentamicin for 1 h. Then, the cells were washed three times and the number of intracellular bacteria was determined as described above.

Immediate cytotoxicity to HL-60-derived human macrophages. The immediate-cytotoxicity assay was preordered as described before (7) with several modifications. Wells of a 96-well plate containing 4×10^5 differentiated HL-60-derived human macrophages were infected with twofold serial dilutions of *L. pneumophila* in RPMI, starting from $\sim 10^8$ bacteria/well. Then, the plate was centrifuged for 2 min at $880 \times g$ and incubated for 1 h at 37°C under CO₂ (5%). Later, the plate was washed three times, and 0.1 ml of RPMI containing 2 mM Gln, 10% normal human serum, and 10% Alamar-Blue (Biosource) was added to the wells. After incubation at 37°C under CO₂ (5%) for 4 h, the fluorescence

intensity was measured at 590 nm (excitation at 530 nm) to determine the extent of macrophage killing.

Sodium sensitivity. The sodium sensitivity assay was performed essentially as described before (7). A wild-type *L. pneumophila* strain (JR32) and several mutants were grown for 72 h on an ABCYE plate, scraped off the plate, and calibrated to an optical density at 600 nm (OD₆₀₀) of 4. Then, eight 10-fold serial dilutions were plated on ABCYE plates containing or lacking 100 mM NaCl. The sodium sensitivity was determined by comparing the numbers of bacteria growing on the plates, and it is presented as the percentage of bacteria that grew on the ABCYE plates containing NaCl in relation to the standard ABCYE plates.

β -Galactosidase assays. β -Galactosidase assays were performed as described elsewhere (41). *L. pneumophila* strains were grown for 48 h on ABCYE plates containing chloramphenicol. The bacteria were scraped off the plate and suspended in ACES-yeast extract broth, and the bacterial OD₆₀₀ was calibrated to 0.1 in ACES-yeast extract broth. The resulting cultures were grown on a roller drum for ~ 18 h until they reached an OD₆₀₀ of ~ 3.8 (stationary phase). To test the levels of expression at exponential phase, the cultures described were diluted to an OD₆₀₀ of 0.1 and grown for an additional 6 to 7 h until they reached an OD₆₀₀ of ~ 0.7 (exponential phase). The assays were done for 20, 50, or 100 μ l of culture, and the substrate for *lacZ* hydrolysis was *o*-nitrophenyl- β -D-galactopyranoside.

RNA manipulations. RNA was prepared as described before (22). To determine the transcription start site of the *icmH-icmF-tphA* operon, 5' rapid amplification of cDNA ends (RACE) (Invitrogen) was performed as described by the manufacturer. The RACE-GSP primer (Table 3) was used for generating the cDNA; this primer, together with the AA (abridged-anchor) primer supplied with the kit, was used for the first PCR, and the AUA (abridged universal amplification) primer supplied with the kit and RACE-F2 (Table 3) were used for the nested PCR. The resulting fragment from the second PCR was subse-

TABLE 3. Primers used in this study

Primer name	Sequence (5'-3')
<i>icmH</i> -F	ATCGCTGTAGGTATTGTAATTCTAGC
<i>icmH</i> -R	ATCAGGCTCTGTGATTGCAAGACG
F-G2S-F	ACGCCAATCTAAATCGGCACTGTAAAGCA
F-G2S-R	TGCCGATTTAGATTGGGCGTTTTTCCGGTGA
F-K2A-F	CCCAAGGCGCATCGGCACTGTAAAGCAAAG
F-K2A-R	CAGTGCCGATGCGCCTTGGGCGTTTTTCCGG
F-lac	CGGGGGATCCCCGTATTGCTCAGTTGTCATTATAT
F-M2-F	TTTACCTAGAGAATAAAATAAGAAATTATAGGAATAC
F-M2-R	CTTATTTATTCTCTAGGTAAAATCTGCTTCTAG
RT-F-2	TCCTTGAAATTGGCGGCACT
RT-F-GSP	GTGCTGGTTCGGCGATCAGT
RT-H-2	GAATTACGCGCCTTTCACAG
RT-Tp-1	AAGCCCACCAATTGGACGCA
RT-Tp-GSP	GCCGTCCTACATGATCAGCA
T-F4	GCCGGAATTGCAACAAGGAGCAAGTATTTTC
T-F5	GCCGGAATTCGCATGTATTAATTCTAAGCTTGAC
T-F6	GCCGGAATTCCTTGAACAAAGAGTCGTATATAAC
T-F7	GCCGGAATTCCTGATCATCTCTTTTAAATTGAG
T-F8	GCCGGAATTCCTTTTGTATTAGCATAAATGGACC
T-F9	GCCGGAATTCCTTTCTGTAGGACTGATGGACTTG
T-F10	GCCGGAATTCCTTACCTATAGAATAAATAAGAAATTATAGG
T-F11	GCCGGAATTCATAAGAATTATAGGAATACTTACTAATC
T-F12	GCCGGAATTCAGTCGTATATAACGTATCATCTTC
T-F13	GCCGGAATTCAGTATTTCTAGAAGCAGATTTTAC
T-F14	GCCGGAATTCGAAGCAGATTTTACCTATAGAATA
T-F15	GCCGGAATTCAGGAATACTTACTAATCAACTAGC
T-F16	GCCGGAATTCATAATCAACTAGCAATCACG
RACE-GSP	TTTACTGTGAAAGGCGCGTAA
RACE-F2	CGGGGGATCCGTGAGGACGGGATTTGCTCAGTTGTC

quently cloned, and seven different clones were sequenced to determine the transcription start site of the mRNA.

To determine if the *icmH*, *icmF*, and *tphA* genes are located on one transcriptional unit, a reverse-transcription (RT) reaction was performed using the primers RT-F-GSP and RT-Tp-GFP and avian myeloblastosis virus reverse transcriptase (Invitrogen). The cDNA product was analyzed by PCR using the primers RT-Tp-1 and RT-F-2 (Table 3) to discover whether *icmF* and *tphA* are located on one transcriptional unit, and the primers RT-F-GSP, RT-H-2, and RT-F-2 (Table 3) were used to discover if *icmF* and *icmH* are located on the same transcriptional unit.

Southern hybridization. *Legionella* chromosomal DNA was prepared as previously described (51). The *icmF* probe was purified from agarose gel and radiolabeled with [α - 32 P]dCTP by the random prime labeling kit (Roche). Hybridization was performed, using a nitrocellulose membrane, at 42°C for ~16 h in a solution containing 5× SSPE (0.18 M sodium chloride, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA), 2.5× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.25% sodium dodecyl sulfate (SDS), 150 mg of denatured herring sperm DNA per ml, and 20% formamide. After the hybridization, the filter was washed three times (briefly) in 2× SSPE-0.1% SDS at room temperature and two times with 2× SSPE-0.1% SDS at 42°C for 20 min each time. Then, the membrane was air dried and exposed to X-ray film (Fuji).

RESULTS

Previously, it was reported that the *L. pneumophila icm/dot* region II contains 17 genes (50). This region was shown to contain 16 *icm* genes (*icmT*, -*S*, -*R*, -*Q*, -*P*, -*O*, -*N*, -*M*, -*L*, -*K*, -*E*, -*G*, -*C*, -*D*, -*J*, and -*B*) organized in the same direction (in the order written) and an additional *icm* gene (*icmF*) located at the 5' end of this region and pointing in the opposite direction (50). It was proposed that the *icmF* gene is organized in the same transcriptional unit with another gene (*tphA*) located downstream of it that was shown to be dispensable for *L. pneumophila* intracellular growth (46, 55). Later, signature-

tagged mutagenesis performed with *L. pneumophila* identified an intracellular-growth-defective mutant containing an insertion in a gene located upstream from *icmF*, but it was not determined if the phenotype was due to polarity on *icmF* or to the lack of the gene product of the mutated gene (the mutant identified was designated 47:8g) (20).

Proteins homologous to the *L. pneumophila* IcmF protein are present in several bacteria. Of the 17 genes characterized in region II (*icmT*, -*S*, -*R*, -*Q*, -*P*, -*O*, -*N*, -*M*, -*L*, -*K*, -*E*, -*G*, -*C*, -*D*, -*J*, -*B*, and -*F*), 12 (*icmT*, -*P*, -*O*, -*M*, -*L*, -*K*, -*E*, -*G*, -*C*, -*D*, -*J*, and -*B*) have significant degrees of homology to genes involved in conjugation and are evolutionarily related to the IncI conjugative system present in plasmids such as R64 (37, 56). The five remaining genes (*icmS*, -*R*, -*Q*, -*N*, and -*F*) have no homologues on the R64 plasmid. Very recently, in silico analysis of the *V. cholerae icmF* homologue revealed that the gene is found in many proteobacteria, all of which associate with eukaryotic cells. The analysis indicated that *icmF* is usually found as one of a large number of genes (between 10 and 15) that have a conserved sequence and organization (17). We found that only one of these genes is present in *L. pneumophila* and named it *icmH* (see below). Examination of the gene region that surrounds the *icmF*- and *icmH*-homologous genes in the other bacteria revealed that there are several types of organizations for the two genes, and in several bacteria they are found in more than one copy and in more than one form of organization (Fig. 1). The functions of the proteins encoded by these genes in the different bacteria are not known, but one of them was identified previously during a transposon mutagenesis performed on *R. leguminosarum*. This gene (*impJ*) (Fig. 1)

FIG. 1. Schematic representations of several gene regions containing *icmH* and *icmF* homologues from different bacteria. Genes are represented as boxes with arrowheads indicating their orientations. Homologous genes are shown with the same pattern; open reading frames that have no homologues in the other regions presented are represented by open boxes. Where applicable, only the part of the gene encoding a protein homologous to IcmH is shaded. The gene regions from *R. leguminosarum* and *S. enterica* were described before (21, 47), and these genes are identified by their original names (*imp* and *sci*, respectively). In *Y. pestis*, two additional gene regions that contain homologues to *icmH* and *icmF* were found (21). In *P. aeruginosa*, one additional region that contains only the *icmF* gene was found (21). In *E. coli* O157, the two genes located upstream from the *icmF* homologue (wavy-line boxes) are homologous to one gene found in the same location in both *V. cholerae* and *Y. pestis*.

was shown to be located on the *sym* plasmid and to be required for nodulation (4, 47). In addition, this gene region was also found in *S. enterica* subspecies I in centisome 7. The genes in this region (*sci* genes) (Fig. 1) were found to affect the ability of the bacteria to enter eukaryotic cells (21). Moreover, the expression of an *icmF*-homologous gene in *V. cholerae* was found to be induced during infection (15, 16). It is important to mention that in several cases a gene region containing homologues to the *L. pneumophila icmF* and *icmH* genes was found in one or two species that belong to the same genus, and the genes were always found in the more pathogenic species. For example, in *Y. pestis* there are four gene regions that contain *icmF*- and *icmH*-homologous genes (21) (two of which are presented in Fig. 1), while in the less pathogenic *Yersinia enterocolitica* (http://www.sanger.ac.uk/Projects/Y_enterocolitica/) and *Yersinia pseudotuberculosis* (http://bbrp.llnl.gov/bbrp/bin/y.pseudotuberculosis_blast) there are no homologues of these genes. In *E. coli* K-12, there are no genes homologous to the *icmF* and *icmH* genes (5), while in the pathogenic *E. coli* O157 these genes are present in the two complete genomes available (28, 45) (Fig. 1). In addition, in *S. enterica* it was found, using DNA hybridization, that the *sci* genes are present only in subspecies I (out of seven subspecies), which is the only subspecies that was shown to be associated with warm-blooded organisms (21). All this information indicates that the functions of these genes are probably related to the intimate association of these bacteria with eukaryotic cells.

The *icmF* and *icmH* genes are present in many *Legionella* species. As described above, the gene regions containing the

icmF and *icmH* genes were found in one of several species that belong to the same genus or, in the case of *S. enterica*, in only one subspecies. To examine whether the *icmF* and *icmH* genes are also present in other *Legionella* species besides *L. pneumophila* (as was shown before for several *icm/dot* genes [*icmD*, *icmE*, *icmG*, *icmX*, *dotA*, and *dotB*] in *Legionella micdadei* and for *icmX* in several *Legionella* species [34, 40]), we used low-stringency Southern hybridization with the genes. The hybridizations performed with the *icmF* (Fig. 2) and *icmH* (data not shown) genes clearly indicate that homologues of the two genes are found in all the *Legionella* species examined. This result indicates that even though it seems that the genes were incorporated into the *icm/dot* system from another evolutionary source, in contrast to most of the other *icm/dot* genes (which probably originated from an IncI plasmid), they are found in all the *Legionella* species examined. This might indicate the importance of these genes for *Legionella* intracellular multiplication and fits in with the hypothesis that in nature all the *Legionella* species are intracellular parasites of amoebae and protozoa, and therefore all of them contain these genes.

***icmH* is required for *L. pneumophila* intracellular multiplication in *A. castellanii*.** Previously, it was shown that the *icmF* gene product is required for intracellular growth of *L. pneumophila* in the protozoan host *A. castellanii* and partially required for intracellular growth in HL-60-derived human macrophages (55). To determine whether *icmH* is required for intracellular multiplication as well, a deletion substitution was constructed in it (GS3016). An additional strain that was constructed contains a deletion substitution in the *icmF* gene

FIG. 2. *icmF* homologues are present in other *Legionella* species. Low-stringency Southern hybridization using the *L. pneumophila icmF* gene as a probe was performed as described in Materials and Methods. The probe was hybridized to EcoRI-digested chromosomal DNA that was prepared from each of the following *Legionella* species (indicated above the lanes): *L. pneumophila* serogroup 1 (JR32), *L. pneumophila* serogroup 3 (ATCC 33155), *L. cincinnatiensis* (ATCC 43753), *L. tucsonensis* (ATCC 49180), *L. sainthelensi* (ATCC 49322), *L. gormanii* (ATCC 43769), *L. birminghamensis* (ATCC 43702), *L. feeleii* (ATCC 35849), *L. dumoffii* (ATCC 35850), *L. dumoffii* (ATCC 33343), *L. bozemanii* (ATCC 33217), *L. longbeachae* (ATCC 33462), *L. longbeachae* (ATCC 33484), *L. hackeliae* (ATCC 35250), *L. gratiana* (ATCC 49413), *L. oakridgensis* (ATCC 700515), *L. micdadei* (ATCC 33204), and *L. micdadei* (ATCC 33218). The hybridizations performed with the *icmH* gene gave similar results, and both genes were always located on the same EcoRI fragment (data not shown).

(GS3015). This strain was constructed instead of the previously characterized transposon insertion in the *icmF* gene (LELA1718) because the latter strain was only partially complemented for intracellular growth (46). The deletion substitution mutants in the *icmF* and *icmH* genes (GS3015 and GS3016, respectively), were unable to multiply in the protozoan host *A. castellanii* (Fig. 3A and B). Both of these mutants were complemented to wild-type levels of intracellular growth with a plasmid containing the *icmF* and *icmH* genes (pGS-Lc-70-14). A plasmid containing the *icmH* gene by itself (pGS-Lc-76-14) did not complement the mutant strain in this gene (GS3016), indicating that the deletion substitution in *icmH* has a polar effect on *icmF*. However, a plasmid containing an in-frame nonpolar deletion in *icmH* (pGS-Lc-77-14) was able to complement the deletion substitution in the *icmF* gene but not the mutant containing the deletion substitution in the *icmH* gene. These results clearly indicate that *icmH* is required for intracellular multiplication and that the lack of growth observed with strain GS3016 did not occur due to polarity on *icmF* alone.

Analysis of the *icmH* and *icmF* mutants in HL-60-derived human macrophages. As was previously shown for the *icmF* gene (55), the *icmH* gene was found to be only partially required for intracellular multiplication in HL-60-derived human macrophages (Fig. 3C and D). The lack of both the *icmH* and *icmF* genes did not have an additive effect in comparison to the lack of one of them, as the phenotypes observed for the *icmF* insertion, the *icmH* insertion expressing the *icmF* gene, and the *icmF* insertion containing the vector were the same. These results might indicate that *icmF* and *icmH* perform their functions together, as was expected from the bioinformatics analysis.

The partial intracellular-growth phenotype observed with the *icmH* and *icmF* mutants in HL-60-derived human macrophages might occur due to several possible defects. As the

growth rates observed in the wild-type and the *icmH* and *icmF* mutants were similar (Fig. 3C and D), it might be that the 48-h delay in the appearance of the bacteria outside the cells occurred due to a defect in entry, intracellular growth, or exit of the bacteria from the cells (since in the assays performed, bacteria were sampled from the media surrounding the cells). To distinguish these three possibilities, we examined the frequency of entry of the *icmH* mutant strain into HL-60-derived human macrophages and compared the intracellular and extracellular bacterial numbers during infection, and the results are presented in Fig. 4. As can be seen in Fig. 4A, the *icmH* mutant had an entry defect, and only 20% of the bacteria entered the cells in comparison to the wild-type strain and the complemented mutant strain. A similar entry phenotype was described before for all the *icm/dot* mutant strains examined (29). However, this fivefold reduction in entry cannot account for the 2-log-unit difference in the numbers of CFU observed 68 and 92 h postinfection (Fig. 3C and D). One other possibility was that the *icmH* and *icmF* mutants were defective in exit from the cells, and due to that, the bacterial numbers in the medium outside the cells were lower than those of the wild-type strain. If this was the situation, the numbers of an *icmH* mutant inside the cells should have been similar to those of the wild-type strain. However, as can be seen clearly in Fig. 4B, the numbers of the *icmH* mutant inside the cells 44, 68, and 92 h postinfection were significantly lower (~2 log units) than those of the wild-type strain and the complemented mutant strain, indicating that the intracellular-growth phenotype observed in HL-60-derived human macrophages did not occur due to a defect in exit from the cells. Therefore, it is most likely that the defect observed with the *icmH* and *icmF* mutant strains occurs mainly due to a defect in intracellular growth. However, unlike most of the other *icm/dot* mutants, the *icmH*

FIG. 3. The *L. pneumophila icmH* and *icmF* genes are required for intracellular growth. Intracellular-growth experiments in the protozoan host *A. castellanii* (A and B) and in HL-60-derived human macrophages (C and D) were performed as described in Materials and Methods. Shown are *icmF* mutant GS3015 (A and C) and *icmH* mutant GS3016 (B and D) containing the following plasmids: pMMB207αb-Km-14 (solid box), *icmH-icmF-tphA* operon (pGS-Lc-70-14) (solid triangle), *icmH* gene (pGS-Lc-76-14) (solid circle), and *icmF* and *tphA* genes (pGS-Lc-77-14) (open triangle). Solid diamond, wild-type *L. pneumophila* (JR32); open circle, 25D intracellular defective mutant. The experiments were performed at least three times, and similar results were obtained.

FIG. 4. *icmF* and *icmH* are partially required for intracellular growth in HL-60-derived human macrophages. (A) Entry experiments were performed as described in Materials and Methods. The bacteria examined were wild-type *L. pneumophila* JR32 (W.T.), the *icmH* mutant GS3016 containing the vector pMMB207αb-Km-14 (*icmH*) and containing the *icmH-icmF-tphA* operon (*icmH+icmHF*). (B) Analysis of intracellular bacteria in HL-60-derived macrophages 44 (open bars), 68 (shaded bars), and 92 (solid bars) h postinfection. The bacterial strains examined are the same as in panel A. The experiments were performed three times, and similar results were obtained. The intracellular growth of the *icmH* mutant strain was found to be significantly different ($P > 0.005$) from the intracellular growth of the wild-type strain or the complemented *icmH* mutant, as determined by the standard *t* test. The error bars indicate standard deviations.

FIG. 5. *icmF* and *icmH* are partially required for immediate cytotoxicity and salt sensitivity. (A) Immediate cytotoxicity to HL-60-derived human macrophages was determined as described in Materials and Methods. Solid diamonds, wild-type *L. pneumophila* (JR32); open triangles, *icmT* insertion mutant (GS3011); solid triangles, *dotA* transposon insertion (LELA3118); ×, *tphA* insertion mutant (MW627); open squares, *icmF* mutant GS3015 containing the vector pMMB207αb-Km-14; solid squares, *icmH-icmF-tphA* operon (pGS-Lc-70-14); open circles, *icmH* mutant GS3016 containing the vector pMMB207αb-Km-14; solid circles, *icmH-icmF-tphA* operon (pGS-Lc-70-14). (B) The strains indicated in part A were also analyzed for salt sensitivity as described in Materials and Methods. The error bars indicate standard deviations.

and *icmF* mutants were able to grow intracellularly to some extent.

IcmH and IcmF are partially required for immediate cytotoxicity and salt sensitivity. Two additional phenotypes that were shown to be associated with mutations in the *icm/dot* genes are immediate cytotoxicity (36) and salt resistance (49). It was previously shown that *L. pneumophila*, at a high MOI, rapidly kills host cells in an *icm/dot*-dependent manner, a phenomenon called immediate cytotoxicity (36). On the other hand, it has been known for a long time that wild-type *L. pneumophila* is salt sensitive while *icm/dot* mutants become salt resistant (49). As the *icmH* and *icmF* genes seem to have a different evolutionary origin than most of the other *icm/dot* genes, the deletion substitutions in the *icmH* and *icmF* genes were examined for these two phenotypes, as shown in Fig. 5.

The *icmH* and *icmF* genes were found to be partially required for immediate cytotoxicity, in comparison to the wild-type strain (JR32) and insertion mutations in the *icmT* and *dotA* genes (GS3011 and LELA3118, respectively) (Fig. 5A).

FIG. 6. The IcmF ATP/GTP binding site is partially required for intracellular growth. (A) Predicted ATP/GTP binding sites in IcmF-homologous proteins. The bacteria, the sequences of the predicted ATP/GTP binding sites, and the amino acid positions are indicated. The conserved amino acids ([A/G]XXXXGK[T/S]) are in boldface. (B) Predicted ATP/GTP binding sites of *L. pneumophila* Icm/Dot proteins, presented as in panel A. (C) Mutations constructed in the *L. pneumophila* IcmF predicted ATP/GTP binding site; the changes constructed are underlined. (D) Intracellular-growth experiments in the protozoan host *A. castellanii* were performed as described in Materials and Methods. Solid diamonds, wild-type *L. pneumophila* (JR32); asterisks, *icmF* mutant GS3015 containing the vector pMMB207 α b-Km-14; solid squares, wild-type *icmF* gene (pGS-Lc-70-14); open circles, *icmF* gene containing the K-to-A mutation (pGS-Lc-70K2A-14); open triangles, *icmF* gene containing the G-to-S mutation (pGS-Lc-70G2S-14). (E) Analysis of intracellular growth of the strains shown in panel D. Solid bars, wild-type *L. pneumophila* (JR32); stippled bars, *icmF* mutant GS3015 containing the wild-type *icmH-icmF-tpa* operon (pGS-Lc-70-14); shaded bars, same plasmid containing the K-to-A mutation in IcmF (pGS-Lc-70K2A-14); open bars, same plasmid containing the G-to-S mutation in IcmF (pGS-Lc-70G2S-14). The experiment was performed at different MOIs, and the results obtained 48 h postinfection are presented. Similar differences were obtained 60 h postinfection (data not shown). The error bars indicate standard deviations. The intracellular growth of the *icmF* mutant complemented with the mutated ATP/GTP binding site was found to be significantly different ($P > 0.0001$) from the intracellular growth of the wild-type strain or the *icmF* mutant complemented with the wild-type genes at all the MOIs examined, as determined by the standard *t* test.

When the same strains were analyzed for salt sensitivity, similar results were obtained (Fig. 5B). The *icmH* and *icmF* insertion mutants (GS3015 and GS3016, respectively) were found to be partially resistant to sodium, a phenotype that was clearly distinguishable from the degree of sensitivity of the wild-type strain (JR32) and the degree of resistance of the *icmT* and *dotA* mutants (Fig. 5B). Both the immediate-cytotoxicity and salt resistance phenotypes were completely complemented when the *icmH* and *icmF* genes were supplied on a plasmid (Fig. 5). These results indicate that, similar to what was observed for intracellular growth in HL-60-derived human macrophages, the *icmH* and *icmF* genes are partially required for two additional phenotypes related to the *icm/dot* system.

Analysis of the predicted ATP/GTP binding site of the *L. pneumophila* IcmF protein. The N-terminal parts of all the IcmF homologous proteins contain a putative ATP/GTP binding motif (Fig. 6A) that corresponds to the Walker box A nucleotide-binding site ([A/G]XXXXGK[T/S]) found in many ATP-binding proteins (65). In the *icm/dot* system, four proteins were found to contain this motif (Fig. 6B). To determine the

importance of this site for the function of the *L. pneumophila* IcmF protein, two mutations were constructed in it. The changes were made in 2 amino acids (glycine to serine and lysine to alanine) (Fig. 6C) that are the most conserved amino acids identified in this motif. In addition, the mutations constructed were chosen based on previous reports indicating that such changes result in inactivation of the site (38, 58, 62). The two mutations described were constructed on the complementing plasmid that contains both *icmH* and *icmF* (pGS-Lc-70-14), and the plasmids containing the changes (pGS-Lc-70-G2S-14 and pGS-Lc-70-K2A-14) were examined for the ability to complement the *icmF* deletion substitution mutant (GS3015) for intracellular growth. As can be seen in Fig. 6D, the *icmF* mutant strain (GS3015) harboring the plasmids containing the mutations was partially attenuated in intracellular growth. The major defect was observed between 48 and 60 h postinfection, when up to 1-log-unit reduction in the number of bacterial CFU in comparison to the wild-type strain (JR32) or the *icmF* deletion substitution mutant (GS3015) containing the complementing plasmid without the mutations (pGS-Lc-70-14) was

FIG. 7. Truncation analysis of the *icmH-icmF-tphA* operon regulatory region. (A) Sequences of the *icmH-icmF-tphA* regulatory region and the truncations constructed. The first nucleotide of each truncation is marked with an asterisk, and the truncation number is indicated above it. The transcription start site (TSS) is in boldface and underlined, the first ATG codon of the IcmH protein and the predicted promoter sequence are in boldface, and the single nucleotide that was mutated (T to G) is underlined (Fig. 8A). (B and C) β -Galactosidase activities of different truncations (the length of the regulatory region of each truncation is given in Table 2) in *L. pneumophila* during exponential growth. β -Galactosidase activity was measured as described in Materials and Methods. The results are the averages $\pm$ standard deviations of at least three different experiments. The β -galactosidase activities of the F14 and F11 truncations were found to be significantly different ($P > 0.0001$), as determined by the standard *t* test.

observed. These results were reproducible, and they were also observed at different MOIs and always in the same period postinfection (Fig. 6E and data not shown). When the same mutants were examined in HL-60-derived human macrophages, they fully complemented the *icmF* mutation (data not shown).

mRNA analysis of the *icmH-icmF-tphA* transcriptional unit. The finding that in *V. cholerae* the *icmF* gene was activated during infection (16) led us to examine the *icmH* regulatory region carefully. Using RT analysis, we found that *icmH*, *icmF*, and *tphA* are located on the same transcriptional unit (data not shown), as was expected from the complementation experiments and the gene organizations. In addition, using the RACE system, the transcription start site of the *icmH-icmF-tphA* transcriptional unit was determined and found to be located 52 bp upstream from the first ATG of the *icmH* gene (Fig. 7A). This result indicates that the -10 promoter element of this transcriptional unit is unique among the *icm* genes, as it does not contain the conserved TATACT consensus sequence that was identified in the other *icm* gene regulatory regions (24).

Analysis of the regulatory region of the *icmH-icmF-tphA* transcriptional unit. To obtain additional information about the regulation of the *icmH-icmF-tphA* transcriptional unit,

truncation analysis was performed (Fig. 7A). As can be seen clearly from the results presented in Fig. 7B and C, there were three points where the level of expression of the *icmH::lacZ* fusions dropped dramatically. (i) Between truncations F6 and F12, the deletion of 11 nucleotides (from 251 to 240 bp upstream) (Fig. 7A) reduced the level of expression from $\sim 12,000$ to $\sim 2,500$ Miller units (MU). (ii) Between truncations F13 and F14, the deletion of 11 nucleotides (from 137 to 126 bp upstream) (Fig. 7A) reduced the level of expression from $\sim 2,500$ to ~ 70 MU. (iii) Between truncations F10 and F11, the deletion of 14 nucleotides (from 116 to 102 bp upstream) (Fig. 7A) reduced the level of expression to ~ 30 MU (Fig. 7C), and it got very close to the promoter region (Fig. 7A) and to the level of expression of the vector control (data not shown). These results indicate that there are at least two additional regulatory elements located in the *icmH* regulatory region besides the promoter element.

In a previous study, a regulatory element (CTATAGAAT) located between the F10 and F11 truncations was identified, and one nucleotide substitution in this site (constructed on the F4 *icmH::lacZ* fusion) completely abolished the expression of the fusion (24). To determine the relationship between this site and the potential regulatory element located between the F6 and F12

FIG. 8. Regulatory elements and factors affecting expression of the *icmH::lacZ* fusion. (A) Effect of a single-base-pair substitution (Fig. 7A) on the levels of expression of the F4 and F5 truncations. Expression from the F5 and F4 truncations (open bars) was compared to the expression of the fusions containing the mutation indicated in Fig. 7A (shaded bars). The β -galactosidase activities of the F5 truncation and the mutated F5 truncation were found to be significantly different ($P > 0.0001$), as determined by the standard t test. (B) Effects of growth phase and regulators related to stationary phase on the level of expression of the F5 truncation. The level of expression of truncation F5 was examined at exponential (open bars) and stationary (shaded bars) phases in the wild-type (WT) strain, the *relA* mutant strain GS-*RelA* (*RelA*), the *rpoS* mutant strain LM1376 (*RpoS*), and the *letA* mutant strain OG2001 (*LetA*). β -Galactosidase activity was measured as described in Materials and Methods. The results are the averages $\pm$ standard deviations of at least three different experiments. The β -galactosidase activities of the F5 truncation at exponential and stationary phases were found to be significantly different ($P > 0.0001$), as determined by the standard t test.

truncations, the same mutation was introduced into the F5 *icmH::lacZ* fusion. As can be seen in Fig. 8A, the mutation constructed completely abolished the expression from the F4 *icmH::lacZ* fusion, but it reduced the level of expression from the F5 *icmH::lacZ* fusion by $\sim 2,500$ MU. This result indicates that the upstream regulatory element (probably located between the F6 and F12 *icmH::lacZ* fusions) is probably independent of the site mutated (located between the F10 and F11 *icmH::lacZ* fusions), since the level of expression of the F5 *icmH::lacZ* fusion was reduced only by the portion contributed by the downstream regulatory element. Comparison of the level of expression of the F5 *icmH::lacZ* fusion to the level of expression of the other *icm::lacZ* fusion (24) clearly shows that the *icmH-icmF-tphA* transcriptional unit has the highest level of expression of all the *icm* genes and operons examined.

The expression of the *icmH-icmF-tphA* operon is influenced by *RelA* and *LetA*. Because the regulation of genes required for intracellular growth was postulated to be activated during stationary phase (2, 26, 27, 43), we examined the level of expression of the F5 *icmH::lacZ* fusion during stationary phase in comparison to exponential growth phase. As can be seen in Fig. 8B, the level of expression of this fusion increased at stationary phase (from $\sim 12,000$ MU at exponential phase to $\sim 15,000$ MU at stationary phase). This result led us to examine whether regulators related to gene expression during stationary phase are required for this phenomenon. When the *icmH::lacZ* fusion was examined in *relA* and *letA* insertion mutants (GS-*RelA* and OG2001, respectively), no increase was observed at stationary phase, while the same increase was observed when the fusion was examined in an *rpoS* insertion mutant (LM1376) (Fig. 8B). These results clearly indicate that the *icmH-icmF-tphA* transcriptional unit is regulated by *RelA*

and *LetA*, which were shown previously to be part of one regulatory circuit in *L. pneumophila* (43). When additional regulators (*CpxR*, *CpxA*, and *RpoE*) were examined to see if they were involved in the regulation of the *icmH-icmF-tphA* transcriptional unit, no effect was observed (data not shown).

DISCUSSION

Thus far, 25 *icm/dot* genes required for intracellular multiplication have been described and characterized in *L. pneumophila*. The majority of the *Icm/Dot* proteins (18 proteins) encoded by the *icm/dot* genes are homologous to proteins involved in conjugation encoded by *InC* plasmids (37, 56). The finding that most of the *icm/dot* genes probably constitute part of the secretion complex directed most of the research to the *icm/dot* genes that have no homologues in conjugative systems (*icmN*, *icmS*, *icmW*, *icmQ*, *icmR*, *icmF*, and *icmH*). Besides the *icmN* gene product, which has many homologous proteins in other bacteria, the six other proteins can be divided into pairs.

The *IcmS* and *IcmW* proteins were found to interact with one another, and this property was also found to be conserved in the *IcmS* and *IcmW* homologues from *Coxiella burnetii* (10, 73). These two proteins were found not to be required for pore formation (10), and null mutants in each of them, as well as in both of them together, can still replicate to some extent in HL-60-derived human macrophages (10, 52, 71, 73). The specific functions of these two genes and their relationship to the rest of the *Icm/Dot* system are not known.

The *IcmQ* and *IcmR* proteins were also found to interact with one another. The *IcmQ* protein was found to form homopolymers, and the *IcmR* protein was shown to possess chaperon activity for *IcmQ* and to prevent its polymerization (10, 18). In addition, an *icmR* insertion mutant was found to retain some small ability to multiply inside host cells and to kill HL-60-derived human macrophages (10, 52). Very recently, it was reported that the *IcmQ* protein is exposed on the surfaces of bacteria after contact with macrophages and that it forms pores in lipid membranes, and the pore formation was shown to be inhibited by *IcmR* (19). Surprisingly, the exposure of the *IcmQ* protein on the surfaces of bacterial cells was found to be independent of the other *icm/dot* components (19). However, mutants in the *icmR* and *icmQ* genes become salt resistant, indicating that they are connected to the *icm/dot* system (49, 52).

The *IcmF* and *IcmH* proteins are the subjects of this report, and our results indicate that *icmH* and *icmF* probably perform their function together, because the phenotype of a mutant lacking both genes was similar to that of a mutant that lacked either gene (possible interactions between *IcmH* and *IcmF* were examined using a bacterial two-hybrid system, but no interactions could be obtained, perhaps due to their expected membrane location [T. Zusman, G. Segal, unpublished results]). In addition, the information obtained from the analysis of immediate cytotoxicity indicates that bacteria lacking these genes are still able to translocate a pore into host cells, but less efficiently than the wild-type strain. Moreover, the partial phenotype for salt resistance gives further strength to the assumption that a functional *icm/dot* system is present in these bacteria, as salt resistance is a property thought to be associated with a defective *icm/dot* secretion system (64). Both these results and the partial phenotype for intracellular multiplication in

HL-60-derived human macrophages indicate that IcmH and IcmF are probably not integral parts of the *icm/dot* system, because a partially functional *icm/dot* system is present in bacteria that lack both proteins. Additional support for this assumption comes from analysis of the *icmH-icmF-tphA* regulatory region, which was found to contain a different promoter sequence than the other *icm/dot* genes and operons. These genes were shown to contain a conserved -10 promoter element (24), which is missing in the *icmH* regulatory region.

As indicated above, the evolutionary origins of the majority of the Icm/Dot proteins are probably proteins involved in conjugation of IncI plasmids, and these Icm/Dot proteins are most likely the major components of the Icm/Dot type IV secretion complex. One interesting question concerning the seven Icm/Dot proteins that have no homologues on IncI plasmids is from where do they originate. In comparison to the other Icm/Dot proteins, the IcmH and IcmF proteins are unique in the sense that they have homologous proteins in several bacteria that live in close contact with eukaryotic cells (Fig. 1). This finding is in contrast to the other Icm/Dot proteins which do not contain homologous proteins encoded by IncI plasmids. Proteins homologous to IcmS, IcmW, and IcmQ were found only in *C. burnetii*, which contains all the Icm/Dot proteins except IcmR, which has no known homologue (73). In the other bacteria in which proteins homologous to IcmF and IcmH were found, these proteins were encoded by genes in a large region that contained between 10 and 15 genes; however, *L. pneumophila* does not contain homologues to the proteins other than IcmH and IcmF. In three cases, this large gene region was shown to participate in the interaction of the bacteria with eukaryotic cells (4, 15, 16, 21, 47). This information might indicate that the Icm/Dot proteins probably originated from at least three different evolutionary sources: (i) 18 Icm/Dot proteins probably originated from an IncI conjugative plasmid; (ii) IcmH and IcmF probably originated from a common ancestral system, from which *L. pneumophila* and the other bacteria obtained their *icmH* and *icmF* homologues; (iii) IcmWS and IcmRQ have unknown evolutionary origins, but since there are no homologues of these proteins in the other two systems described, it is most likely that these proteins have a third (or third and fourth) evolutionary origin.

Among the bacteria that contain proteins homologous to the IcmH and IcmF proteins, there are bacteria that use type III secretion systems (*E. coli* O157 and *S. enterica*) or type IV secretion systems (*L. pneumophila* and *B. pertussis*) for pathogenesis and there are human and animal pathogens (*Y. pestis* and *V. cholerae*) and plant pathogens (*A. tumefaciens* and *Pseudomonas fluorescens*), as well as plant symbionts (*R. leguminosarum* and *Mesorhizobium loti*). Deeper analysis of the *L. pneumophila icmH* and *icmF* genes, as well as of homologous genes in the other bacteria, might reveal their importance for the association of these bacteria with eukaryotic cells and the relationship of these genes to the functions of the main systems that participate in the interactions of the bacteria with eukaryotic cells.

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