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Characterization of a New Region Required for Macrophage Killing by *Legionella pneumophila*

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In a previous study, a collection of 55 *Legionella pneumophila* mutants defective for macrophage killing was isolated by transposon mutagenesis. In this study, nine of these mutants that belong to the same DNA hybridization group (group 3) were characterized. A wild-type DNA fragment that covers this DNA hybridization group was cloned and sequenced. This region was found to contain six new genes (designated *icmT*, *icmS*, *icmR*, *icmQ*, *icmP*, and *icmO*), five of which contain at least one transposon insertion. No transposon insertion was found in *icmS*. However, this gene was found to be required for macrophage killing, since a kanamycin resistance cassette introduced into *icmS* by gene replacement resulted in a mutant that was attenuated for macrophage killing. A plasmid containing the DNA fragment that covers this region complements all the mutants for macrophage killing, although various levels of complementation were observed for mutants in different genes. Complementation tests were also performed with plasmids containing one or two of these genes, as well as with plasmids containing nonpolar in-frame deletions. The results from these complementation tests indicated that all six genes located in this region are needed for macrophage killing and that they are probably arranged as two transcriptional units (*icmTS* and *icmPO*) and two genes (*icmR* and *icmQ*). A region upstream of the coding sequence of several *icm* genes may contain a potential promoter and/or regulatory site. Homology searches show that *icmP* and *icmO* bear significant homology to the *trbA* and *trbC* genes from the *Salmonella* R64 plasmid, respectively. The sequences of the other four genes do not show significant homology with any entries in sequence databases.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultatively intracellular pathogen. The bacteria are able to infect, multiply within, and kill human macrophages, as well as free-living amoebae (15, 26). The pathway for a successful *L. pneumophila* infection of human macrophages begins with attachment of the bacterium to the host cells via the complement receptors CR1 and CR3 (4, 35). Attachment is followed by an uptake mechanism known as coiling phagocytosis (24). The bacterium is then found in a specialized phagosome that does not fuse with lysosomes (23). This phagosome is sequentially associated with smooth vesicles, mitochondria, and the rough endoplasmic reticulum (22, 40). Then the bacteria multiply within the phagosome, and the cell eventually lyses, releasing *L. pneumophila* organisms that can start new rounds of infection.

To date, only a few well-defined virulence factors of *L. pneumophila* are known. A region reported to contain the *icmWXYZ* genes was shown to be required for intracellular replication (8). The *dotA* gene (transcribed divergently from *icmWXYZ*) was shown to be required for intracellular replication and organelle recruitment (5, 6), and recently it was shown to be located in the bacterial inner membrane (13). The Mip protein was shown to be responsible for efficient initiation of intracellular infection of human macrophages, and a *mip* mutant was shown to be less infective for human macrophages, as well as for *Acanthamoeba castellanii* (10, 11). The Mip protein exhibits peptidyl-prolyl *cis/trans* isomerase activity, but this activity was shown not to be required for intracellular survival (43). In addition, strains with mutations in the *eml* locus were found to have reduced cytopathicity for U937 cells and for *Hartmannella veriformis* (1). Although these genes (*icmWXYZ*, *dotA*, *mip*, and *eml*) were shown to be required for intracellular infection by *L. pneumophila*, their function remains unknown. The major outer membrane protein, a porin encoded by the *ompS* gene (18, 19), is another candidate for being a virulence factor. This protein plays a role during infection, as it binds the complement components C3b and C3bi, which bind to the CR1 and CR3 receptors (4); this binding results in the internalization of the bacteria. However, the *ompS* gene has not been shown to be required for macrophage killing.

In a previous report (36), a collection of 55 *L. pneumophila* transposon insertion mutants defective for macrophage killing was identified. The mutants were assigned to 16 DNA hybridization groups, one of which has been already characterized as the *icmWXYZ-dotA* locus (8).

The aim of this study was to characterize additional genes that are required for macrophage killing by *L. pneumophila*. The region described here was found to contain six new genes (*icmTSRQPO*), all of which were found to be required for macrophage killing. Complementation analysis indicates that these genes are probably organized as two operons (*icmTS* and *icmPO*) and two genes (*icmR* and *icmQ*).

MATERIALS AND METHODS

Media and reagents. *L. pneumophila* was grown in AYE broth (25) or on ABCYE agar plates (14). *Escherichia coli* was grown in Luria-Bertani (LB) broth or on LB agar plates (32). All reagents and chemicals were purchased from Fisher. For cell culture, RPMI 1640 was obtained from JRH Biosciences, fetal bovine serum (FBS) was obtained from Sigma, and L-glutamine (Gln) was obtained from Cellgro. Normal human serum (NHS) was obtained from healthy volunteers. Enzymes were supplied by New England Biolabs and Boehringer Mannheim Biochemicals. The concentrations of antibiotics for *L. pneumophila* were as follows: kanamycin (KM), 50 μg/ml; chloramphenicol (CM), 5 μg/ml. Antibiotics for *E. coli* were as follows: KM, 50 μg/ml; CM, 25 μg/ml; ampicillin, 100 μg/ml.

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TABLE 1. Bacterial strains

Strain	Genotype and description	Refrence or source
L. pneumophila		
25D	Icm$^-$ avirulent mutant	21
GS3001	JR32 *icmS3001*::Kan	This study
GS3002	JR32 *icmP3002*::Kan	This study
GS3003	JR32 *icmO3003*::Kan	This study
JR32	Homogeneous salt-sensitive isolate of AM511	36
LELA3037	JR32 *icmP3037*::Tn*903*dII*lacZ*	36
LELA3278	JR32 *icmR3278*::Tn*903*dII*lacZ*	36
LELA3352	JR32 *icmP3352*::Tn*903*dII*lacZ*	36
LELA3451	JR32 *icmP3451*::Tn*903*dII*lacZ*	36
LELA3463	JR32 *icmQ3463*::Tn*903*dII*lacZ*	36
LELA3473	JR32 *icmR3473*::Tn*903*dII*lacZ*	36
LELA4032	JR32 *icmO4032*::Tn*903*dII*lacZ*	36
LELA4086	JR32 *icmT4086*::Tn*903*dII*lacZ*	36
LELA4378	JR32 *icmQ4378*::Tn*903*dII*lacZ*	36
E. coli MC1022	*araD139* Δ(*ara leu*)*7697* Δ(*lacZ*)M15 *galU galK strA*	9

Bacterial strains, plasmids, and DNA manipulations. Bacterial strains and plasmids used in this work are described in Tables 1 and 2, respectively. *E. coli* MC1022 was used for all DNA manipulations. Preparation of plasmid DNA and DNA cloning procedures were performed as described elsewhere (30, 37). Colony hybridization, Southern hybridization, and chromosomal DNA preparation were performed as described elsewhere (38). Bacterial mating was carried out as described previously (36).

Construction of plasmids for complementation. The cloning vectors pMMB207 (33) and pHG-165 (39) were used to construct a new *L. pneumophila* cloning vector—pMMB207αb. pMMB207 was digested with *Xmn*I and *Eco*RI; after the two sites were filled in, the *Hpa*I-*Hpa*I fragment containing the *lacZ′* α complementation cassette from pHG-165 was cloned into it to generate pMMB207αb. This vector was used in this study.

First, a wild-type DNA fragment that covers the region of hybridization group 3 (36) was cloned. The *L. pneumophila* pLAFR1 library (41) was screened by using a 0.3-kb *Bss*HII fragment (bases 3683 to 4011 in the sequence) generated from the plasmid pAB-16 (36). Several positive cosmids were identified, and the same 8.8-kb *Eco*RI fragment was present in all of them (and some other *Eco*RI fragments as well). The 8.8-kb *Eco*RI fragment from one of these cosmids (pGS-cos-1) was cloned into the *Eco*RI site of pMMB207αb to generate pGS-Lc-32. All the subclones described below were constructed from pGS-Lc-32 and were cloned into the *Hin*cII site of pUC-18 in both orientations (the letter "b" at the end of the plasmid name indicates an orientation opposite to that of the vector promoter). A 1,142-bp fragment containing *icmT* and *icmS* was subcloned by using the *Bsa*BI and *Dra*III sites (bases 1 to 1142 in the sequence) to generate pGS-Lp-37 and pGS-Lp-37b. A 644-bp fragment containing *icmR* was subcloned by using the *Apa*I and *Stu*I sites (bases 942 to 1586 in the sequence) to generate pGS-Lp-36 and pGS-Lp-36b. A 1,037-bp fragment containing *icmQ* was subcloned by using the *Sac*I and *Nar*I sites (bases 1309 to 2346 in the sequence) to generate pGS-Lp-35 and pGS-Lp-35b. A 4,470-bp fragment containing *icmP* and *icmO* was subcloned by using the *Eco*RV and *Eco*47III sites (bases 1957 to 6432 in the sequence) to generate pGS-Lp-34 and pGS-Lp-34b. The inserts of the eight plasmids described above were cloned into pMMB207αb by using the *Eco*RI and *Hin*dIII sites of the polylinker. The internal letters of the plasmid name were changed from "Lp" to "Lc" to indicate the different vector; the plasmids are shown in Fig. 1.

Construction of in-frame deletions. In-frame deletions in *icmT* and *icmS* were constructed by PCR as described by Imai et al. (28). The plasmid pGS-Lp-37 was used for the PCR mutagenesis. The primers that were used to construct the in-frame deletion in *icmT* were 5′-TCCGCTTCTCGCTGAGTCACG-3′ (bases 435 to 417 in the sequence) and 5′-TCCCCGGTTTTTTTACGCTGG-3′ (bases 573 to 591 in the sequence); the first two bases of both primers were changed from the original sequence to form a *Bam*HI site after self-ligation (each primer contains half of a *Bam*HI site at its 5′ end). The primers that were used to construct the in-frame deletion in *icmS* were 5′-TCCCGCATTCATGCTTGCT GC-3′ (bases 699 to 682 in the sequence) and 5′-TCCAGCCGTGATTATACT CCC-3′ (bases 949 to 966 in the sequence); the first three bases of both primers were changed from the original sequence to form a *Bam*HI site. The PCR conditions were as follows: 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 by using the buffer supplied with the enzyme, 200 mM each nucleotide, 0.1 μg of plasmid DNA, 50 pmol of each primer, and 2 U of Vent DNA polymerase. The PCR products were gel purified and self-ligated. After transformation, the plasmids prepared were examined for the presence of the *Bam*HI site expected to be generated by the ends of the two primers. The plasmid harboring the in-frame deletion in *icmT* was named pGS-Lp-37-D1, and the plasmid harboring the in-frame deletion in *icmS* was named pGS-Lp-37-D2.

The in-frame deletion in *icmP* was constructed by using two *Nco*I sites located in the *icmP* coding region (bases 2342 and 2786 in the sequence). The plasmid pGS-Lp-34 was digested with *Nco*I and self-ligated to form pGS-Lp-34-D1, which contains an in-frame deletion in *icmP*. The inserts of the three plasmids described above were cloned into pMMB207αb by using the *Eco*RI and *Hin*dIII sites of the polylinker. The internal letters of the plasmid name were changed from "Lp" to "Lc" to indicate the different vector.

Construction of plasmids for allelic exchange. The plasmids pAB-16 and pAB-17 (36) were used to reconstruct the wild-type *Eco*RI fragment of hybridization group 3. The *Eco*RI-*Sal*I fragment from pAB-17 (containing the *Eco*RI fragment with the transposon from LELA3451) was joined to the *Sal*I-*Eco*RI fragment from pAB-16 (containing the *Eco*RI fragment with the transposon from LELA3037), and both were cloned together into the *Eco*RI site of pUC-18 to generate pGS-Lp-14. The plasmid pGS-Lp-14 contains the whole 8.8-kb *Eco*RI fragment of DNA hybridization group 3. A 4,470-bp fragment containing *icmP* and *icmO* was subcloned from pGS-Lp-14, by using the *Eco*RV and *Eco*47III sites (bases 1957 to 6432 in the sequence), into the *Hin*cII site of pUC-18 to generate pGS-Lp-30. To knock out the *icmP* gene, an in-frame

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Reference or source
pAB-16	*icmP*::*lacZ* from LELA3037 in pBSK	36
pAB-17	*icmP*::*lacZ* from LELA3451 in pBSK	36
pGS-cos-1	*icmTSRQPO* in pLAFR1	This study
pGS-Lc-14	Insert from pGS-Lp-14 in pMMB207αb	This study
pGS-Lc-32	*icmTSRQPO* from pGS-cos-1 in pMMB207αb	This study
pGS-Lc-34	Insert from pGS-Lp-34 in pMMB207αb	This study
pGS-Lc-34b	Insert from pGS-Lp-34b in pMMB207αb	This study
pGS-Lc-34-D1	Insert from pGS-Lp-34-D1 in pMMB207αb	This study
pGS-Lc-35	Insert from pGS-Lp-35 in pMMB207αb	This study
pGS-Lc-35b	Insert from pGS-Lp-35b in pMMB207αb	This study
pGS-Lc-36	Insert from pGS-Lp-36 in pMMB207αb	This study
pGS-Lc-36b	Insert from pGS-Lp-36b in pMMB207αb	This study
pGS-Lc-37	Insert from pGS-Lp-37 in pMMB207αb	This study
pGS-Lc-37b	Insert from pGS-Lp-37b in pMMB207αb	This study
pGS-Lc-37-D1	Insert from pGS-Lp-37-D1 in pMMB207αb	This study
pGS-Lc-37-D2	Insert from pGS-Lp-37-D2 in pMMB207αb	This study
pGS-Le-30-Ba-Km	Insert from pGS-Lp-30-Ba-Km in pLAW344	This study
pGS-Le-30-D1-Km	Insert from pGS-Lp-30-D1-Km in pLAW344	This study
pGS-Le-33-D2-Km	Insert from pGS-Lp-33-D2-Km in pLAW344	This study
pGS-Lp-14	*icmTSRQPO* from pAB-16 and pAB-17 in pUC-18	This study
pGS-Lp-30	*icmPO* from pGS-Lp-14 in pUC-18	This study
pGS-Lp-30-Ba-Km	pGS-Lp-30 with *icmO*::Km	This study
pGS-Lp-30-D1	pGS-Lp-30 with an in-frame deletion in *icmP*	This study
pGS-Lp-30-D1-Km	pGS-Lp-30-D1 with *icmP*::Km	This study
pGS-Lp-33	*icmTSR* from pGS-Lc-32 in pUC-18	This study
pGS-Lp-33-D2	pGS-Lp-33 with an in-frame deletion in *icmS*	This study
pGS-Lp-33-D2-Km	pGS-Lp-33-D2 with *icmS*::Km	This study
pGS-Lp-34	*icmPO* from pGS-Lc-32 in pUC-18	This study
pGS-Lp-34b	pGS-Lp-34 with insert in opposite orientation	This study
pGS-Lp-34-D1	pGS-Lp-34 with an in-frame deletion in *icmP*	This study
pGS-Lp-35	*icmQ* from pGS-Lc-32 in pUC-18	This study
pGS-Lp-35b	pGS-Lp-35 with insert in opposite orientation	This study
pGS-Lp-36	*icmR* from pGS-Lc-32 in pUC-18	This study
pGS-Lp-36b	pGS-Lp-36 with insert in opposite orientation	This study
pGS-Lp-37	*icmTS* from pGS-Lc-32 in pUC-18	This study
pGS-Lp-37b	pGS-Lp-37 with insert in opposite orientation	This study
pGS-Lp-37-D1	pGS-Lp-37 with an in-frame deletion in *icmT*	This study
pGS-Lp-37-D2	pGS-Lp-37 with an in-frame deletion in *icmS*	This study
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *loxP oriR*(ColE1) Ap^r *loxP*	42
pMMB207	RSF1010 derivative; *IncQ lacI*^{q} Cm^r P*tac oriT*	33
pMMB207αb	pMMB207 containing MCS (α complementation)	This study
pUC-18	*oriR*(ColE1) MCS Ap^r	44

[a] MCS, multiple cloning site.

deletion was constructed in the manner described for the construction of pGS-Lp-34-D1, generating pGS-Lp-30-D1. This plasmid was digested with *Nco*I, and after it was filled in, the kanamycin resistance cassette (Pharmacia Biotech) was cloned into it to form pGS-Lp-30-D1-Km. To knock out the *icmO* gene, the kanamycin resistance cassette was cloned into the *Bam*HI site (base 4352 in the sequence) located in the middle of the *icmO* gene, forming pGS-Lp-30-Ba-Km. The plasmids pGS-Lp-30-D1-Km and pGS-Lp-30-Ba-Km were digested with *Pvu*II, and the inserts were cloned into the *Eco*RV site of the allelic exchange vector pLAW344, to form pGS-Le-30-D1-Km and pGS-Le-30-Ba-Km, respectively.

In this step we found out that pGS-Lc-14 (which contains the insert of pGS-Lp-14 in pMMB207αb) contains a mutation in *icmR*, as it failed to complement the *icmR* mutants. This finding led us to clone the wild-type DNA of hybridization group 3 from the pLAFR1 library, as described above. The inserts of both plasmids, pGS-Lc-14 and pGS-Lc-32 (both of which contain the 8.8-kb *Eco*RI fragment of DNA hybridization group 3), were sequenced from the *Bsa*BI site to the *Eco*47III site (Fig. 1). The mutation in *icmR* was the only difference found between the two plasmids. Because no changes were found in the *icmPO* region, the two plasmids described above were used to construct the gene replacements in *icmP* and *icmO.*

The plasmid constructed to make a knockout of *icmS* was subcloned from pGS-Lc-32, described above. A 1,586-bp fragment containing *icmT*, *icmS*, and *icmR* was subcloned, by using the *Bsa*BI and *Stu*I sites (bases 1 to 1586 in the sequence), into the *Hin*cII site of pUC-18 to generate pGS-Lp-33. An in-frame deletion was constructed in *icmS*, with the same primers and by the same method described for the construction of pGS-Lp-37-D2, to generate pGS-Lp-33-D2. The kanamycin resistance cassette was cloned into the *Bam*HI site generated by the deletion to form pGS-Lp-33-D2-Km. The insert of this plasmid was cloned into pLAW344 in the manner described for pGS-Lp-30-D1-Km, to form pGS-Le-33-D2-Km.

Allelic exchange. Allelic exchange was performed as previously described (42). Briefly, the vector pLAW344, which contains a Cm^r marker and the counterselectable *sacB* gene from *Bacillus subtilis*, was used to mediate allelic exchange. The plasmids pGS-Le-33-D2-Km, pGS-Le-30-D1-Km, and pGS-Le-30-Ba-km (described above) were introduced into *L. pneumophila* JR32 by electroporation, grown in AYE for 5 h, and plated on ABCYE containing KM and CM. Transformants were patched on ABCYE plates containing KM and were then streaked on ABCYE plates containing KM and 2% (wt/vol) sucrose in order to select for cells that no longer contained pLAW344 sequences. Km^r Suc^r single isolates were patched on ABCYE plates containing KM and ABCYE plates containing CM. Km^r Cm^s isolates were tested by Southern hybridization for the correct change; at least four independent isolates were tested for each allelic exchange.

HL-60 cell culture. The human leukemia cell line HL-60 (12) was maintained in RPMI supplemented with 2 mM Gln and 10% FBS at 37°C under CO_2 (5%). HL-60 cells were differentiated into macrophages by incubation for 48 h with 10 ng of phorbol 12-myristate 13-acetate/ml in RPMI containing 2 mM Gln and 10% NHS. Adherent cells were washed three times with RPMI containing 2 mM Gln and were incubated with RPMI containing 2 mM Gln and 10% NHS before infection.

Cytotoxicity assay. The cytotoxicity assay was carried out as described previously (31). Briefly, wells of a 96-well microtiter dish containing 4×10^5 differentiated HL-60-derived macrophages were infected with 10-fold serial dilutions of *L. pneumophila* in RPMI, starting with about 10^8 bacteria/well. After 6 days of incubation at 37°C, the dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was added to each well at a concentration of 0.5 mg/ml. After incubation at 37°C for 4 h, the culture medium was removed and the remaining reduced formazan dye was suspended in 100 μl of isopropanol containing 0.04 M HCl and 1% sodium dodecyl sulfate. The A_{570} values of three wells containing the same multiplicity of infection (MOI) were averaged to determine the extent of macrophage killing.

Intracellular growth assay. Wells of a 96-well microtiter dish containing 4×10^5 differentiated HL-60-derived macrophages were used for infection. *L. pneumophila* was added to the wells at an MOI of approximately 0.01 and was centrifuged for 5 min at $800 \times g$. The infected HL-60 cells were incubated at 37°C under CO_2 (5%), and bacterial CFU were determined 0, 24, 48, and 72 h after infection. The number of CFU in a whole well was determined by removing the culture medium, adding 100 μl of deionized H_2O to the cell monolayer, combining the two fractions, and plating samples on ABCYE plates. Each infection was performed in triplicate.

DNA sequencing and sequence analysis. All the sequences were generated by the DNA Synthesis and Sequencing Facility of the Comprehensive Cancer Center, College of Physicians and Surgeons of Columbia University. DNA sequences and amino acid sequences of potential open reading frames (ORFs) were compared to sequences in the GenBank, EMBL, and SwissProt databases by using the programs BLAST and FASTA. Motif searches were carried out by using the Prosite program (3), and the locations of proteins in the cell were predicted by using the Psort program (34). The number of transmembrane domains was determined by the TMpred program (20), the isoelectric points and molecular weights of the proteins were calculated with the Compute *p*I/Mw program (7), and sequence alignment was carried out by using the SIM program (27).

Nucleotide sequence accession number. Sequence data for the complete *icmTSRQPO* locus have been assigned EMBL data accession no. Y12705.

RESULTS

Cloning of the wild-type DNA fragment. In a previous report, a collection of 55 *L. pneumophila* mutants defective for macrophage killing was described. Nine of these mutants were assigned to the same DNA hybridization group (group 3). In addition, the *Eco*RI fragment containing the transposon was cloned from three of these mutants (36).

To characterize this DNA hybridization group, the wild-type *Eco*RI fragment that covers this region was cloned from an *L. pneumophila* cosmid library. Several positive cosmids were identified, all of which contain the same 8.8-kb *Eco*RI fragment and some other *Eco*RI fragments as well. To get a first estimate of the locations of the transposons on this 8.8-kb *Eco*RI fragment, Southern hybridization was performed with chromosomal DNA derived from the nine mutants. The transposons were located on a 3.2-kb *Bss*HII fragment (internal to the 8.8-kb *Eco*RI fragment) in eight mutants and on a 0.3-kb *Bss*HII fragment in one of them (LELA4032) (data not shown). The nucleotide sequence was determined for the region covered by the transposons, and after potential ORFs were identified, additional sequencing was performed to cover all the ORFs and potential regulatory regions.

Genes and proteins found in the region. The region that was sequenced (6,432 bp, from the *Bsa*BI site to the *Eco*47III site [Fig. 1]) was found to contain six genes, designated *icmT*, *icmS*, *icmR*, *icmQ*, *icmP*, and *icmO*. The locations of the transposon insertions were determined by sequencing, and five of the genes (*icmTRQPO*) were found to contain at least one transposon insertion. The organization of the genes and the locations of the transposons are presented in Fig. 1 and Table 3. Sequence homology searches against the EMBL, GenBank, and SwissProt databases were conducted with these genes and the corresponding proteins. *icmP* and *icmO* were found to have significant homology with the *trbA* and *trbC* genes from *Salmonella* plasmid R64 (17), respectively. IcmP and TrbA were found to be 23% identical and 35% similar over 376 amino acids. IcmO and TrbC were found to be 24% identical and 36% similar over 783 amino acids. *trbA* and *trbC* were shown to be part of an operon containing the *trbB* gene between them (17); we found that *icmP* and *icmO* form an operon as well (see below). The other four genes found in this region are novel genes. Several properties of the six proteins are presented in Table 4.

Complementation of the mutants. The cytotoxicities for HL-60-derived macrophages of strains containing the vector (pMMB207αb) were compared with those of strains carrying pGS-Lc-32. The introduction of the vector had no effect on the strains' ability to kill HL-60-derived macrophages (data not shown). On the other hand, pGS-Lc-32 (Fig. 1), which contains the 8.8-kb *Eco*RI fragment that covers the whole group 3 region, complemented the mutants (Fig. 2). As can be seen in Fig. 2C and D, the mutants that contain a transposon in *icmR* (LELA3278 and LELA3473) or *icmQ* (LELA3463 and LELA 4378) retain some ability to kill HL-60-derived macrophages, in contrast to the avirulent mutant 25D (21). These four strains were complemented completely with pGS-Lc-32. The strains carrying mutations in *icmT* (LELA4086), *icmP* (LELA3352, LELA3037, and LELA3451), or *icmO* (LELA4032) were completely defective in their ability to kill HL-60-derived macrophages, and they were only partially complemented with pGS-Lc-32 (Fig. 2A, E, and F). As mentioned above, no transposon insertion was found in *icmS*. To test if *icmS* is needed for macrophage killing, a kanamycin resistance cassette was introduced into it. The resulting mutant (GS3001) (Fig. 1) was

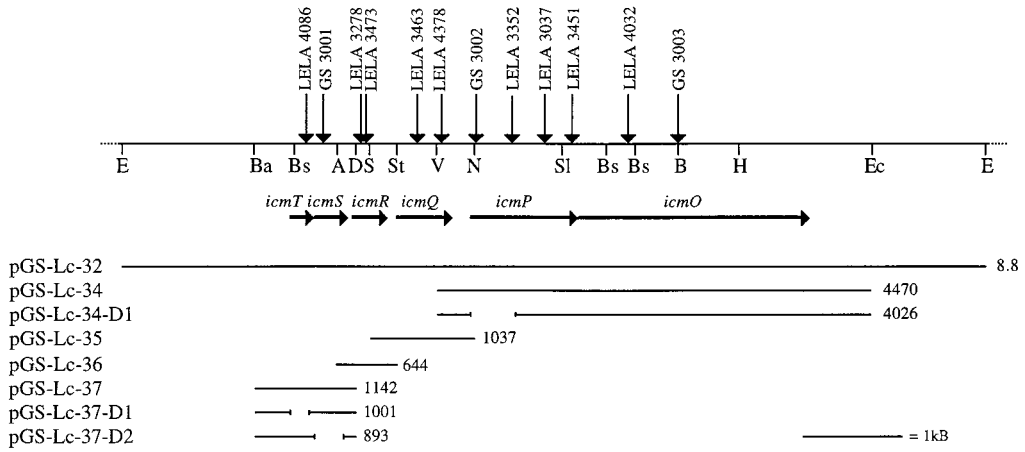

FIG. 1. Restriction map of the *icmTSRQPO* locus, and plasmids used for complementation studies. Coding regions are indicated by bold arrows. The sites of Tn*903*dIIlacZ insertions (LELA strains) and of kanamycin cassette insertions (GS strains) are indicated. The sizes of inserts are given on the right. Restriction enzymes: A, *Apa*I; B, *Bam*HI; Ba, *Bsa*BI; Bs, *Bss*HII; D, *Dra*III; E, *Eco*RI; Ec, *Eco*47III; H, *Hin*dIII; N, *Nar*I; S, *Sac*I; Sl, *Sal*I; St, *Stu*I; V, *Eco*RV (only relevant sites are marked).

found to be attenuated for macrophage killing, and it was partially complemented with pGS-Lc-32 (Fig. 2B).

The *icmT* and *icmS* genes. Plasmids pGS-Lc-37 and pGS-Lc-37b, which contain the *icmS* and *icmT* genes (Fig. 1), partially complemented LELA4086 and GS3001 for macrophage killing (Fig. 3). This result indicates that the insertions located in these two genes are not polar on the downstream *icm* genes (*icmRQPO*).

The mutants that we studied were generated by insertion mutagenesis, and they may act by polarity to decrease expression of downstream genes within the same transcriptional unit. GS3001, which contains an insertion in *icmS*, was only partially attenuated for macrophage killing (Fig. 2B); therefore, it is not possible that the transposon in *icmT* acts only by polarity on *icmS*. On the other hand, it is possible that both genes (*icmT* and *icmS*) are only partially required for macrophage killing and that the absence of both gene products (due to polarity of the transposon in *icmT*) results in complete attenuation for macrophage killing. To distinguish between these two possibilities, nonpolar in-frame deletions were generated in the complementing plasmid pGS-Lc-37. The plasmid pGS-Lc-37-D1 contains an in-frame deletion in *icmT*, and as can be seen in Fig. 3, it cannot complement LELA4086 but it does complement GS3001. The plasmid pGS-Lc-37-D2 contains an in-frame deletion in *icmS*. This plasmid cannot complement GS3001, but it restores the ability of LELA4086 for macrophage killing to a level similar to that observed for GS3001 (Fig. 3). These results indicate that the transposon located in *icmT* (LELA4086) is polar on *icmS* but that the lack of the *icmT* gene product by itself results in complete attenuation for macrophage killing. These complementation results also indicate that *icmT* and *icmS* probably form a single transcriptional unit.

The *icmR* and *icmQ* genes. Both *icmR* mutants (LELA3278 and LELA3473) were complemented by pGS-Lc-36 and pGS-Lc-36b (Fig. 4A), which contain the *icmR* gene on a 644-bp (Fig. 1) fragment (in opposite orientations). The same result was obtained for both *icmQ* mutants (LELA3463 and LELA 4378) complemented by pGS-Lc-35 and pGS-Lc-35b (Fig. 4B), which contain the *icmQ* gene on a 1,037-bp (Fig. 1) fragment (in opposite orientations).

These results indicate that the transposon insertions in *icmR* and *icmQ* are not polar on downstream *icm* genes and that

TABLE 3. Genes and transposons found

Gene	Sequence location[a]	Strain	Insertion location[b]	Frame[c]
icmT	385–645	LELA4086	593	+
icmS	646–990	GS3001	699	
icmR	1090–1452	LELA3278	1205	+
		LELA3473	1274	+
icmQ	1543–2118	LELA3463	1796	+
		LELA4378	2073	−
icmP	2305–3435	GS3002	2341	
		LELA3352	2750	+
		LELA3037	3076	−
		LELA3451	3382	−
icmO	3432–5783	LELA4032	3960	−
		GS3003	4352	

[a] In EMBL accession no. Y12705.
[b] For LELA strains, the insertion location is the first base of the transposon. For GS strains, the insertion location is the first base of the kanamycin resistance cassette.
[c] Indicates if the *lacZ* gene located on the transposon is in frame with the *icm* gene.

TABLE 4. Protein properties

Protein	Molecular size (kDa)	pI[a]	Predicted location[b]	TM[c]	Motif[d]	Homology[e]
IcmT	9.9	12	IM	1		
IcmS	12.6	4.4	Cyt			
IcmR	12.9	4.7	Cyt			
IcmQ	21.5	9.6	Cyt			
IcmP	43.2	8.3	IM	2	RGD	TrbA
IcmO	87.5	5.3	IM	2	A/G	TrbC

[a] Calculated by using the Compute *p*I/Mw program (7).
[b] Determined by the Psort program (34). IM, inner membrane; Cyt, cytoplasm.
[c] Number of predicted transmembrane domains (TM), determined by the TMpred program (20).
[d] Motif searches were carried out with the Prosite program (3). A/G, ATP- or GTP-binding site.
[e] Homology searches were carried out with the FASTA and BLAST programs.

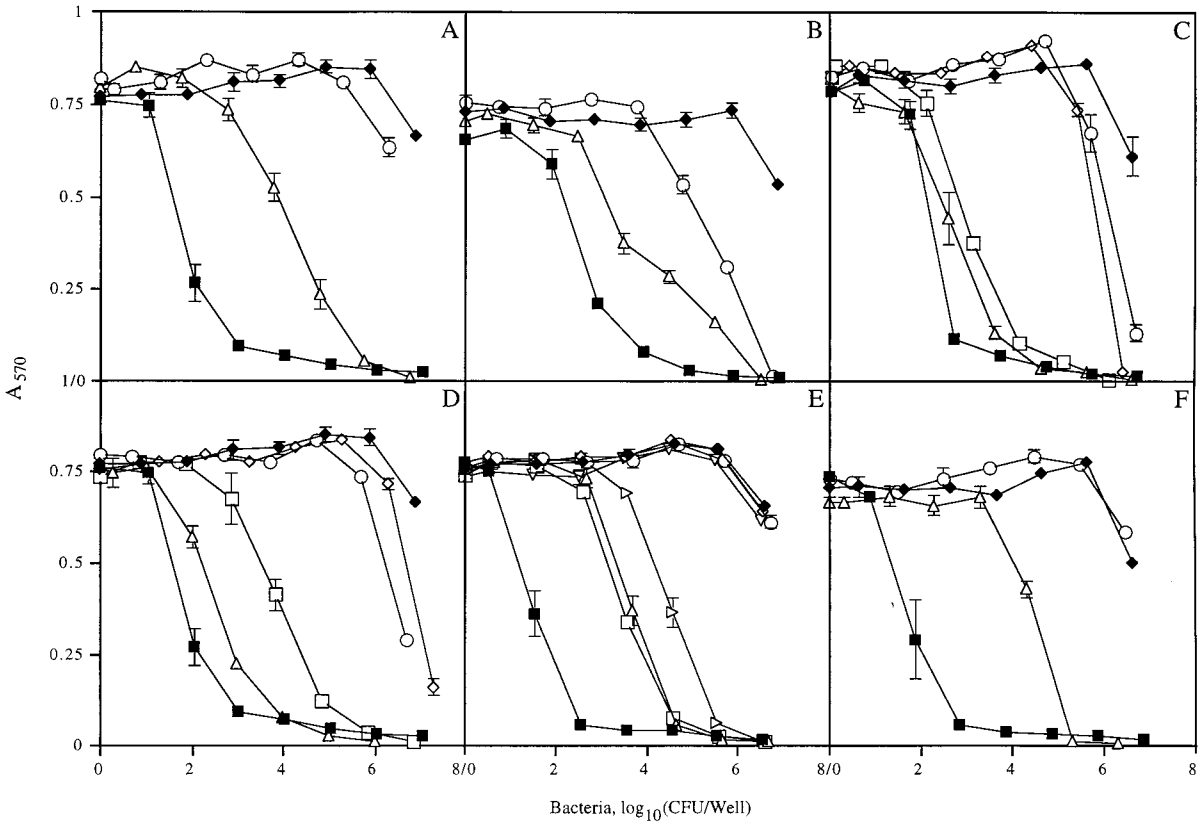

FIG. 2. Cytotoxicities of *L. pneumophila* strains for HL-60-derived macrophages. HL-60 cells were differentiated and infected in microtiter wells as described in Materials and Methods. Strains JR-32 (■) and 25D (◆) were used in all experiments. (A) *icmT* mutant LELA4086 containing pMMB207αb (○) or pGS-Lc-32 (△). (B) *icmS* mutant GS3001 containing pMMB207αb (○) or pGS-Lc-32 (△). (C) *icmR* mutants LELA3278 containing pMMB207αb (◇) or pGS-Lc-32 (□) and LELA 3473 containing pMMB207αb (○) or pGS-Lc-32 (△). (D) *icmQ* mutants LELA3463 containing pMMB207αb (○) or pGS-Lc-32 (△) and LELA4378 containing pMMB207αb (◇) or pGS-Lc-32 (□). (E) *icmP* mutants LELA3352 containing pMMB207αb (▽) or pGS-Lc-32 (▷), LELA3037 containing pMMB207αb (○) or pGS-Lc-32 (△), and LELA3451 containing pMMB207αb (◇) or pGS-Lc-32 (□). (F) *icmO* mutant LELA4032 containing pMMB207αb (○) or pGS-Lc-32 (△).

these two genes are probably transcribed as monocistronic mRNAs.

The *icmP* and *icmO* genes. The four strains with insertion mutations in *icmP* and *icmO* (*icmP* mutants LELA3352, LELA 3451, and LELA3037, and *icmO* mutant LELA4032) were partially complemented by pGS-Lc-32 (Fig. 2E and F). The three *icmP* mutants were also complemented, to the same level, by pGS-Lc-34, which contains the *icmP* and *icmO* genes (Fig. 1), but the *icmO* mutant was not complemented by this plasmid (data not shown). Because of the complementation problems described above, new mutants with insertions in *icmP* and *icmO* were constructed (see Materials and Methods).

The new insertion mutants, the *icmP* mutant GS3002 and the *icmO* mutant GS3003, were partially complemented by pGS-Lc-32, as well as by pGS-Lc-34 and pGS-Lc-34b (Fig. 5). The observation that the new *icmO* mutant (GS3003) was complemented by pGS-Lc-34, whereas the original mutant (LELA4032) was not, may indicate that LELA4032 contains an additional mutation in one or more of the other four genes located in this region (*icmTSRQ*). This assumption is based on the fact that both LELA4032 and GS3003 were complemented by pGS-Lc-32 (Fig. 2F and 5B).

To test if *icmP* by itself is required for macrophage killing or if the insertions located in it act by polarity on *icmO*, a nonpolar in-frame deletion was constructed in *icmP*. The plasmid pGS-Lc-34-D1, which contains the in-frame deletion in *icmP*, was tested for its ability to complement GS3002 and GS3003 (Fig. 5). As expected, this plasmid was able to complement the *icmO* mutant (GS3003) but failed to complement the *icmP* mutant (GS3002). A plasmid containing only *icmP* failed to complement insertions in *icmP* (data not shown). These results indicate that both *icmP* and *icmO* are required for macrophage killing and that they are probably transcribed as a single transcriptional unit.

The homology found between *icmP* and *trbA*, as well as that between *icmO* and *trbC*, led us to test whether *L. pneumophila* strains are able to conjugate small plasmids between one another. We first tested if the IncQ plasmid pMMB207αb can be conjugated from the wild-type strain JR32 to a rifampin-resistant JR32 recipient strain. We found that this plasmid was conjugated at a frequency of about 10^{-4}, which is 4 log units higher than the frequency at which spontaneous rifampin resistance strains were found. To determine if *icmP* and *icmO* are required in order to conjugate pMMB207αb, we tested whether conjugation still occurs with GS3002, which contains an insertion in *icmP* (in this strain, neither *icmP* nor *icmO* is expressed). When this mutant was used as a donor, the conjugation frequency was reduced 30-fold over that with the wild-type strain. The conjugation frequency was restored to the wild-type level when conjugation was carried out with this mutant containing the complementing plasmid pGS-Lc-34.

The mutants are defective in intracellular multiplication. The abilities of strains carrying mutations in the six genes described in this report to multiply inside HL-60-derived mac-

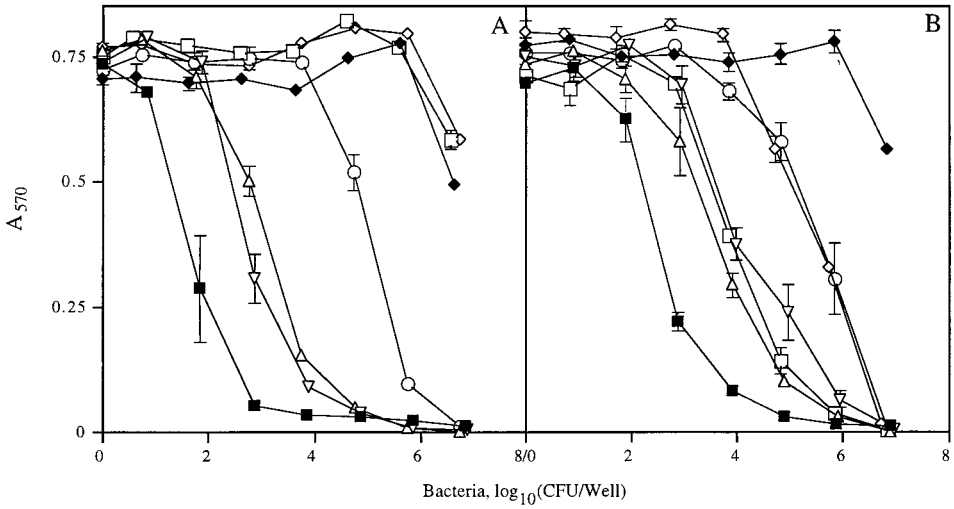

FIG. 3. Cytotoxicities for HL-60-derived macrophages of the *icmT* mutant LELA4086 (A) and the *icmS* mutant GS3001 (B) containing various plasmids and of strains JR-32 (■) and 25D (◆). HL-60 cells were differentiated and infected in microtiter wells as described in Materials and Methods. Plasmids: pMMB207αb (◇), pGS-Lc-37 (▽), pGS-Lc-37b (△), pGS-Lc-37-D1 (□), and pGS-Lc-37-D2 (○).

rophages were determined by assaying the changes in bacterial CFU during infection. One mutant was assayed for each gene. In the cases of mutants that contain an insertion in a monocistronic transcriptional unit (*icmQ* and *icmR*) or in the second gene of an operon (*icmS* and *icmO*), a comparison was performed between a mutant containing the vector and a mutant containing a complementing plasmid (Table 5). In the cases of mutants that contain an insertion in the first gene of an operon (*icmT* and *icmP*), the ability to multiply was compared between a strain containing a plasmid with an in-frame deletion in the first gene of the operon and a mutant containing a complementing plasmid (Table 5). In the latter case, the comparison was done in this way in order to determine the requirement of the first gene of the operon and not of the whole operon.

As can be seen in Table 5, mutants containing insertions in *icmT*, *icmR*, *icmQ*, *icmP*, and *icmO* were unable to multiply within HL-60-derived macrophages, and they were killed by the cells. Mutants containing insertions in *icmT*, *icmP*, and *icmO* were killed more efficiently than mutants containing insertions in *icmR* and *icmQ*. All these mutants regained their ability to grow inside HL-60-derived macrophages when a complementing plasmid was introduced into them, but different levels of complementation were observed with different mutants. The *icmS* mutant (GS3001) was able to grow inside HL-60-derived macrophages, but its multiplication was slower than the multiplication observed for this strain with a complementing plasmid. The results obtained from the intracellular multiplication experiments agree with the results of the cytotoxicity assays, described above.

DISCUSSION

L. pneumophila is a facultatively intracellular pathogen that overcomes many natural host defense mechanisms, enabling it

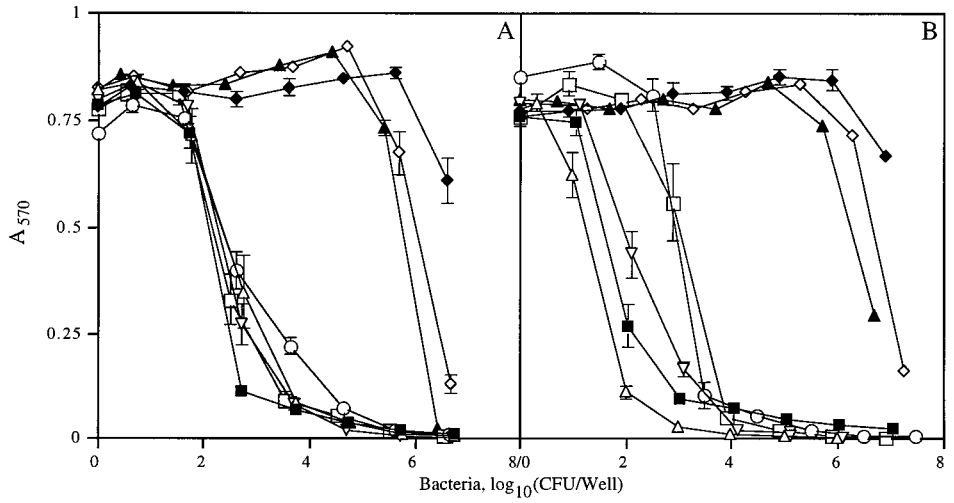

FIG. 4. Cytotoxicities for HL-60-derived macrophages of *icmR* and *icmQ* mutants and of strains JR-32 (■) and 25D (◆). HL-60 cells were differentiated and infected in microtiter wells as described in Materials and Methods. (A) *icmR* mutants LELA3278 containing pMMB207αb (▲), pGS-Lc-36 (△), or pGS-Lc-36b (▽) and LELA 3473 containing pMMB207αb (◇), pGS-Lc-36 (○), or pGS-Lc-36b (□). (B) *icmQ* mutants LELA3463 containing pMMB207αb (▲), pGS-Lc-35 (△), or pGS-Lc-35b (▽) and LELA4378 containing pMMB207αb (◇), pGS-Lc-35 (○), or pGS-Lc-35b (□).

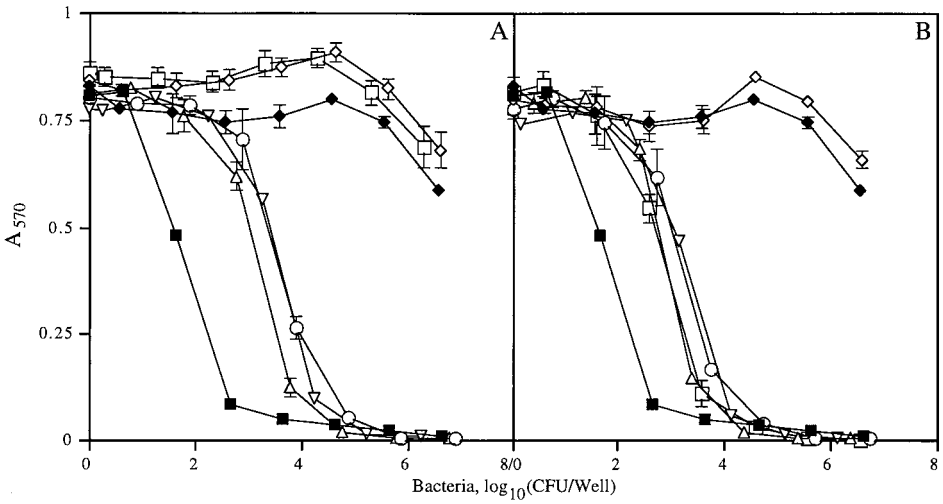

FIG. 5. Cytotoxicities for HL-60-derived macrophages of *icmP* and *icmO* mutants and of strains JR-32 (■) and 25D (◆). HL-60 cells were differentiated and infected in microtiter wells as described in Materials and Methods. (A) *icmP* mutant GS3002 containing pMMB207αb (◇), pGS-Lc-32 (○), pGS-Lc-34 (△), pGS-Lc-34b (▽), or pGS-Lc-34-D1 (□). (B) *icmO* mutant GS3003 containing pMMB207αb (◇), pGS-Lc-32 (○), pGS-Lc-34 (△), pGS-Lc-34b (▽), or pGS-Lc-34-D1 (□).

to cause disease in humans. Like *Mycobacterium tuberculosis* (2), *Chlamydia psittaci* (16), and *Toxoplasma gondii* (29), *L. pneumophila* infects human cells, survives, and multiplies within a specialized vacuole that does not fuse with secondary lysosomes (23). Although it has been known for some time that *L. pneumophila* grows within a specialized vacuole and changes the ability of the vacuole to fuse with lysosomes, it has not yet been established which genes directly control the ability of the organism to prevent phagosome-lysosome fusion.

In a previous study, a collection of 55 insertion mutants that showed a partial or complete block to their ability to kill HL-60-derived macrophages was isolated. Forty-nine of these mutants were assigned to 16 DNA hybridization groups (36). One of these groups (group 1) had already been characterized and was reported to contain the *icmWXYZ* and *dotA* genes. The *icm* genes were shown to be required for intracellular multiplication (8), and *dotA* mutants were found to have defects in organelle trafficking and were unable to grow within human macrophages (6).

In the present study, we characterized an additional locus containing six new genes, all of which were shown to be required for macrophage killing. The differences between mutations in different genes were quantitated in a cytotoxicity assay and in an intracellular growth experiment. These six genes can be divided into three groups: (i) *icmT*, *icmP*, and *icmO*, (ii) *icmR* and *icmQ*, and (iii) *icmS*. Mutants containing an insertion in the first group of genes (*icmT*, *icmP*, and *icmO*) completely lost their ability to kill HL-60 cells, and they were killed by the cells. Mutants containing an insertion in the second group of genes (*icmR* and *icmQ*) were found to have a moderate reduction in their ability to kill HL-60 cells, as killing was still observed at a high MOI. Mutants containing insertions in these genes were killed by the cells, but less efficiently than the mutants of the first group. The mutant containing an insertion in *icmS* was found to have a slight reduction in its ability to kill HL-60 cells, and it was able to multiply intracellularly, but less efficiently than the wild-type strain.

All the mutants were complemented for both the ability to kill and the ability to multiply within HL-60-derived macrophages, although different levels of complementation were observed for different genes. Mutants containing insertions in *icmT*, *icmS*, *icmP*, and *icmO* were partially complemented for macrophage killing, and mutants containing insertions in *icmR* and *icmQ* were complemented completely. On the other hand, *icmS*, *icmQ*, *icmP*, and *icmO* mutants were complemented better for growth inside HL-60 cells than *icmT* and *icmR* mutants. In the case of the *icmR* mutant, complete complementation for cell killing was observed in the cytotoxicity assay (Fig. 4A), but it was only partially complemented for growth inside HL-60 cells (Table 5). The variability in the degree of complementation may result from different expression of the genes from the plasmid and the chromosome.

Four of the proteins described in this paper (IcmT, IcmS, IcmR, and IcmQ) bear no homology to known prokaryotic or

TABLE 5. Intracellular multiplication of the *icm* mutants

Strain (mutation)	Plasmid	CFU/whole well			
		T_0[a]	T_{24}/T_0	T_{48}/T_0	T_{72}/T_0
JR32		4.0×10^3	12.0	1,050.0	725.0
25D		6.0×10^3	0.9	1.0	1.0
LELA4086 (*icmTS*)	pGS-Lc-37-D1 (*icmS*$^+$)	7.0×10^3	0.6	0.4	0.3
	pGS-Lc-37 (*icmTS*$^+$)	4.9×10^3	4.1	36.7	46.9
GS3001 (*icmS*)	pMMB207αb	6.0×10^3	0.6	13.8	76.7
	pGS-Lc-37 (*icmTS*$^+$)	6.0×10^3	25.0	183.3	166.7
LELA3278 (*icmR*)	pMMB207αb	4.5×10^3	0.8	0.8	0.8
	pGS-Lc-36 (*icmR*$^+$)	6.1×10^3	1.0	16.4	7.7
LELA3463 (*icmQ*)	pMMB207αb	8.0×10^3	0.8	0.6	0.5
	pGS-Lc-35 (*icmQ*$^+$)	3.9×10^3	12.3	187.2	189.7
GS3002 (*icmPO*)	pGS-Lc-34-D1 (*icmO*$^+$)	4.0×10^3	0.2	0.1	0.1
	pGS-Lc-34 (*icmPO*$^+$)	4.1×10^3	9.5	197.6	102.4
GS3003 (*icmO*)	pMMB207αb	3.5×10^3	0.1	0.2	0.2
	pGS-Lc-34 (*icmPO*$^+$)	4.0×10^3	9.2	175.0	145.0

[a] T_0, T_{24}, T_{48}, and T_{72}, 0, 24, 48, and 72 h after infection, respectively.

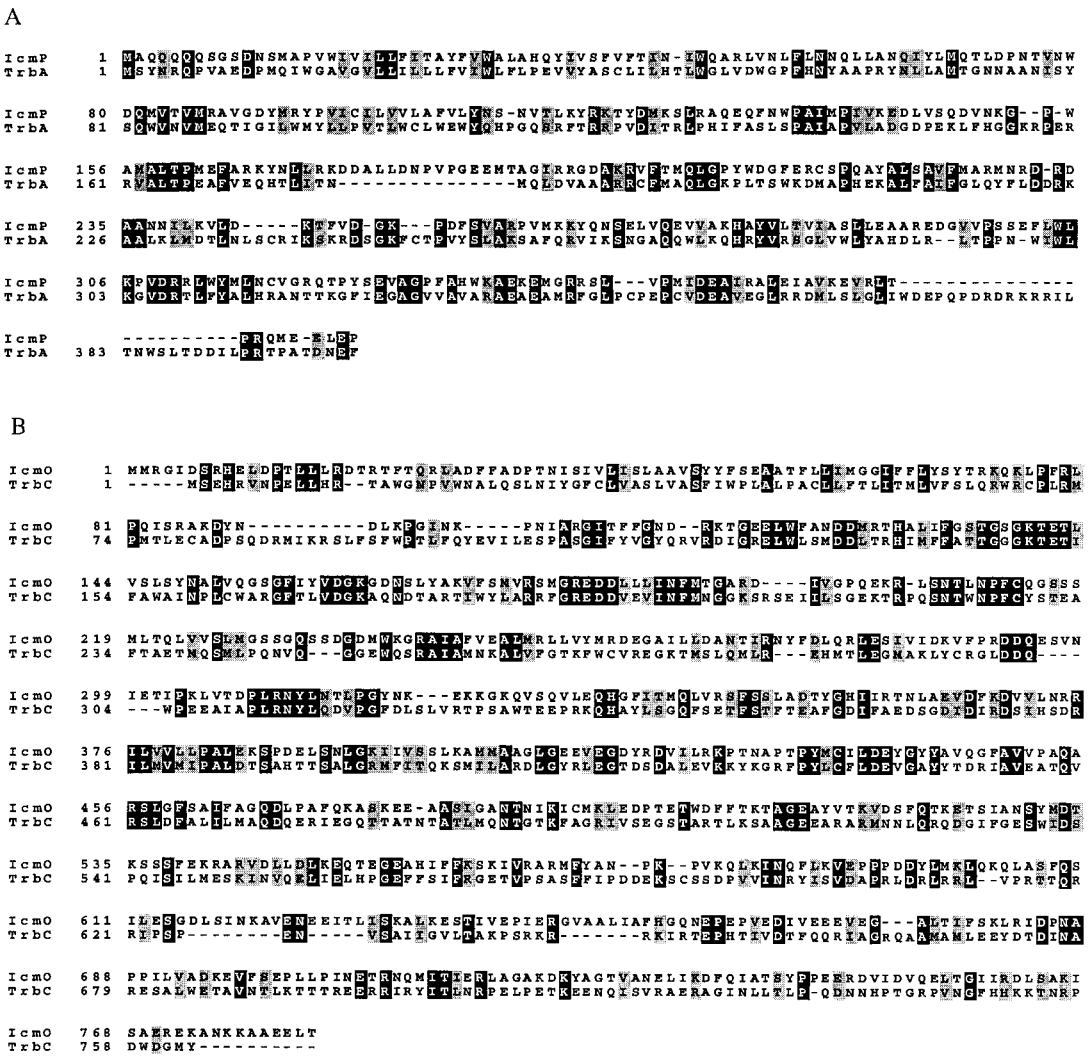

FIG. 6. Sequence alignments of IcmP and TrbA (A) and of IcmO and TrbC (B). The homology between IcmP and TrbA was found to be 23% identity and 35% similarity over 376 amino acids. IcmO and TrbC were found to be 24% identical and 36% similar over 783 amino acids. Identical amino acids are shown on a solid background; similar amino acids (shaded) are I, L, V, and M; H, K, and R; D and E; T and S; and Q and N.

eukaryotic proteins. The same result was also found for the proteins that were described in the *icmWXYZ-dotA* locus. On the other hand, IcmP and IcmO show significant degrees of homology to the TrbA and TrbC proteins from *Salmonella* plasmid R64 (Fig. 6), respectively. Both IcmO and TrbC are predicted to form two transmembrane domains at the N-terminal end, and each contains an ATP- or GTP-binding site at the same distance from the predicted transmembrane domains.

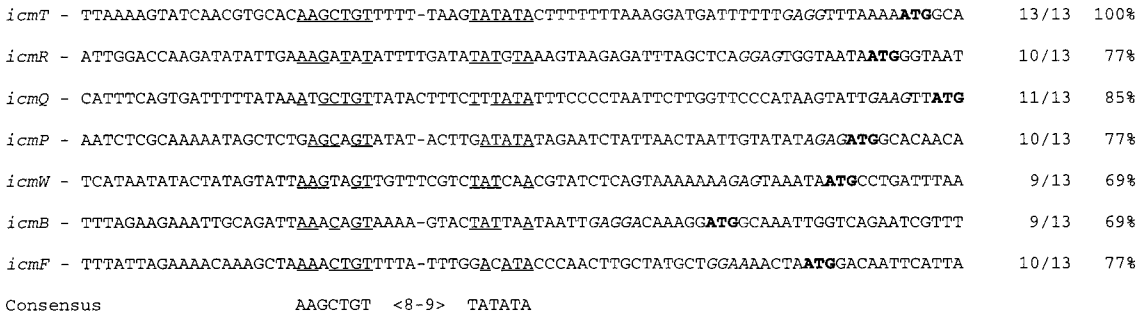

FIG. 7. Comparison among the *icmTS*, *icmR*, *icmQ*, *icmPO*, *icmW*, *icmB*, and *icmF* upstream regulatory regions. The sequence of *icmTSRQPO* is from EMBL accession no. Y12705, the sequence of *icmW* is from EMBL accession no. U07354, and the sequences of *icmB* and *icmF* are from reference 35a. The first ATG codon of each gene is in boldface, the putative ribosomal binding sites are in italics, and the bases that match the putative consensus promoter sequence are underlined. The number of matches and the percent identity with the consensus sequence are indicated to the right.

Moreover, the two proteins show 24% identity and 36% similarity over 783 amino acids (Fig. 6B). IcmP, which forms an operon with IcmO, was found to contain a significant degree of homology (23% identity and 35% similarity over 376 amino acids) with TrbA, which forms an operon with TrbC (Fig. 6A). The *trbA* and *trbC* genes were shown to be part of an operon containing three genes—*trbABC* (17). This operon was shown to be located within the transfer region of the IncI1 plasmid R64, adjacent to the *oriT* operon, in the opposite orientation. The *trbA* and *trbC* genes were shown to be indispensable for R64 transfer, while residual transfer was detected in a *trbB* deletion mutant. The connection between the ability of *L. pneumophila* to conjugate small plasmids, which was significantly reduced in an *icmPO* mutant, and its ability to kill human macrophages, which was completely abolished in this mutant, is currently not known.

Although several genes involved in *L. pneumophila's* ability to kill macrophages were cloned and sequenced, there is no information regarding promoters or transcription factors that regulate these genes. The complementation results presented in this paper indicate that the genes described are probably arranged as two operons (*icmTS* and *icmPO*) and two genes (*icmR* and *icmQ*). Careful analysis of the upstream regulatory regions of these genes and operons revealed a conserved sequence that might serve as a promoter or a recognition site for a transcription factor (Fig. 7). This sequence was also found upstream of *icmW* (8), as well as upstream of *icmB* and *icmF* (35a).

Two (of sixteen) DNA hybridization groups (groups 1 and 3) have been characterized so far. These two groups were found to contain 11 genes (*icmWXYZ*, *dotA*, and *icmTSRQPO*), all of which, except for *icmO* and *icmP*, show no homology to other known genes or proteins. This finding may indicate that *L. pneumophila* has developed a previously unrecognized system for survival and replication inside human macrophages.

ACKNOWLEDGMENTS

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