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A Specific Genomic Location within the *icm*/*dot* Pathogenesis Region of Different *Legionella* Species Encodes Functionally Similar but Nonhomologous Virulence Proteins

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***Legionella pneumophila*, the major causative agent of Legionnaires' disease, is a facultative intracellular pathogen that grows within human macrophages and amoebae. Intracellular growth involves the formation of a replicative phagosome that requires the Icm/Dot type IV secretion system. Part of the *icm*/*dot* region in *L. pneumophila* contains the *icmTSRQPO* genes. The proteins encoded by the *icmR* and *icmQ* genes were shown to exhibit a chaperone-substrate relationship. Analysis of this region from other pathogenic *Legionella* species, i.e., *L. micdadei* and *L. longbeachae*, indicated that the overall organization of this region is highly conserved, but it was found to contain a favorable site for gene variation. In the place where the *icmR* gene was expected to be located, other open reading frames that are nonhomologous to each other or to any entry in the GenBank database were found (*migAB* in *L. micdadei* and *ligB* in *L. longbeachae*). Examination of these unique genes revealed an outstanding phenomenon; by use of interspecies complementation, the *icmR*, *migB*, and *ligB* gene products were found to be functionally similar. In addition, the function of these proteins was usually dependent on the presence of the corresponding IcmQ proteins. Moreover, each of these proteins (IcmR, LigB, and MigB) was found to interact with the corresponding IcmQ proteins, and the genes encoding these proteins were found to be regulated by CpxR. This study reveals new evidence of gene variation occurring in the same genomic location within the *icm*/*dot* locus in various *Legionella* species. The genes found at this site were shown to be similarly regulated and to encode species-specific, nonhomologous, but functionally similar proteins.**

Legionella pneumophila, the major causative agent of Legionnaires' disease, is a facultative intracellular pathogen that multiplies within and kills human macrophages and amoebae (14). Besides *L. pneumophila*, several other *Legionella* species have been found to be capable of causing pneumonia. *L. pneumophila* accounts for the vast majority of cases in most of the world, with *L. micdadei* ranking second and *L. longbeachae* ranking third (1, 15).

Twenty-five genes required for intracellular multiplication within human macrophages were identified for *L. pneumophila* and named the *icm*/*dot* genes. They were shown to be organized in two separate regions on the genome: region I contains 7 genes (*dotDCB*, *icmWX*, and *icmV*/*dotA*), and region II contains 18 genes (*icmTSRQPONMLKEGCDJB* and *icmHF*) (21, 28). Eighteen of the 25 *icm*/*dot* genes encode proteins that contain significant sequence homology to conjugation-related proteins encoded from IncI plasmid R64 (26). Therefore, the Icm/Dot system is believed to form a type IV secretion apparatus through which proteins are translocated across the macrophage plasma membrane (7, 17, 18). Of the seven proteins that are not homologous to proteins that are involved in conjugation, the IcmR and IcmQ proteins (encoded by adjacent genes located in region IIa) were shown to interact with one another (6, 9), and it was proposed that IcmR functions as a chaperone for IcmQ by preventing its aggregation and regulating its pore-forming activity (9, 10).

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The whole Icm/Dot system was also found in the Q fever agent *Coxiella burnetii*, except for the *icmR* gene (30, 31). In the place where the *icmR* gene was expected to be located according to the genomic organization of *L. pneumophila*, five open reading frames (ORFs) were found in *C. burnetii*.

We were interested in examining whether the *icmR* locus in the *icm*/*dot* region of *L. micdadei* and *L. longbeachae* is different from that in *L. pneumophila*, as in *C. burnetii*. Here we present new evidence of functionally similar proteins that share no sequence homology to any other proteins (including themselves) and that are all encoded by genes located in the same position in the *icm*/*dot* region and regulated by the same response regulator. A similar example of gene variation has not been described before for any type IV secretion system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *Legionella* species and strains used were as follows: *L. pneumophila* JR32 (wild-type strain), LELA3463 (*icmQ*), LELA3473 (*icmR*) (20), GS3001 (*icmS*) (23), and OG2002 (*cpxR*) (12); *L. longbeachae* serogroup 1 ATCC 33462 and *L. longbeachae* serogroup 2 ATCC 33484 (both clinical isolates); and MF323, MF385, and MF395 (ATCC 33462 *icmS*, *ligB*, and *icmQ*, respectively). The following *Legionella* species were all clinical isolates, with the exception of *L. gratiana*: *L. micdadei* ATCC 33218 and ATCC 33204, *L. birminghamensis* ATCC 43702, *L. bozemanii* ATCC 33217, *L. cinicinnatiensis* ATCC 43753, *L. dumoffii* ATCC 33343 and ATCC 35850, *L. feeleii* ATCC 35849, *L. gormanii* ATCC 43769, *L. gratiana* ATCC 48413, *L. hackeliae* ATCC 35250, *L. oakridgensis* ATCC 700515, *L. pneumophila* serogroup 3 ATCC 33155, *L. sainthelensi* ATCC 49322, and *L. tucsonensis* ATCC 49180. The *Escherichia coli* strains used were IO7012D (4) and MC1022 (5). Bacterial media, plates, and antibiotic concentrations were used as previously described (23). The plasmids used in this study are listed in Table 1.

Cloning of region IIa from *L. longbeachae* and *L. micdadei*. To clone the *icmTSRQPO* region from *L. longbeachae* and *L. micdadei*, a fragment containing

TABLE 1. Plasmids used in this study

Plasmid name	Description[a]	Reference or source
pBluescript SK(+)	*oriR* (ColE1) MCS *oriR* (M13) Ap^r	27
pGS-lac-02	pAB-1 with promoterless *lacZ*	13
pLAW344	*sacB* MCS *oriT* (RK2) Cm^r *loxP oriR* (ColE1) Ap^r *loxP*	29
pMMB207αB-Km14	RSF1010 derivative; IncQ $lacI^q$ Cm^r P_{tac} *oriT* MCS containing α complementation with *mobA*::Km	24
pT18	*oriR* (ColEI) T18 fragment of CyaA (amino acids 225–339) Ap^r	16
pT25	*oriR* (p15A) T25 fragment of CyaA (amino acids 1–224) Cm^r	16
pGS-Lc-35-14	$icmQ_{Lp}$ gene in pMMB207αB-Km14	31
pGS-Lc-36-14	*icmR* gene in pMMB207αB-Km14	24
pGS-Lc-37-14	*L. pneumophila icmTS* genes in pMMB207αB-Km14	24
pKP-T18R	*icmR* gene in pT18	This study
pKP-T25R	*icmR* gene in pT25	This study
pKP-T18Q	$icmQ_{Lp}$ gene in pT18	This study
pKP-T25Q	$icmQ_{Lp}$ gene in pT25	This study
pMF-SK-long110	pBluescript SK(+) containing *L. longbeachae icmT* to $icmQ_{Ll}$	This study
pMF-long110	*L. longbeachae icmT* to $icmQ_{Ll}$ in pMMB207αB-Km14	This study
pMF-long111	*ligB* gene in pMMB207αB-Km14	This study
pMF-long113	*ligB* and $icmQ_{Ll}$ genes in pMMB207αB-Km14	This study
pMF-long114	$icmQ_{Ll}$ gene in pMMB207αB-Km14	This study
pMF-ligB-lacZ	*ligB* regulatory region in pGS-lac-02	This study
pMF-ligB::Km	pMF-SK-long110 containing the Km^r cassette inside *ligB*	This study
pMF-longQ::Km	pMF-SK-long110 containing the Km^r cassette inside $icmQ_{Ll}$	This study
pMF-longS::Km	pMF-SK-long110 containing the Km^r cassette inside *L. longbeachae icmS*	This study
pMF-ligB::Km-GR	pLAW344 containing the insert of pMF-ligB::Km	This study
pMF-longQ::Km-GR	pLAW344 containing the insert of pMF-longQ::Km	This study
pMF-longS::Km-GR	pLAW344 containing the insert of pMF-longS::Km	This study
pMF-T18-ligB	*ligB* gene in pT18	This study
pMF-T25-ligB	*ligB* gene in pT25	This study
pMF-T18-longQ	$icmQ_{Ll}$ gene in pT18	This study
pMF-T25-longQ	$icmQ_{Ll}$ gene in pT25	This study
pMF-mic47	pBluescript SK(+) containing *L. micdadei icmT* to *icmP*	This study
pMF-mic10	*L. micdadei icmT* to $icmQ_{Lm}$ in pMMB207αB-Km14	This study
pMF-mic18	$icmQ_{Lm}$ gene in pMMB207αB-Km14	This study
pMF-mic19	*migB* gene in pMMB207αB-Km14	This study
pMF-mic21	*migB* and $icmQ_{Lm}$ genes in pMMB207αB-Km14	This study
pMF-T18-migA	*migA* gene in pT18	This study
pMF-T25-migA	*migA* gene in pT25	This study
pMF-T18-migB	*migB* gene in pT18	This study
pMF-T25-migB	*migB* gene in pT25	This study
pMF-T18-micQ	$icmQ_{Lm}$ gene in pT18	This study
pMF-T25-micQ	$icmQ_{Lm}$ gene in pT25	This study

[a] MCS, multiple cloning site.

part of their *icmP* and *icmO* genes was amplified by PCR with degenerate primers. The resulting fragment was used as a probe for Southern hybridization (22) with *L. longbeachae* or *L. micdadei* genomic DNA, and the desired fragment was cloned into pBluescript SK(+) (27). Clones harboring a 4-kb EcoRI-XhoI fragment containing the *L. longbeachae icmTS*, *ligB*, and *icmQ* genes (GenBank accession number AY512558) and a 3.5-kb XhoI-BglII fragment containing the *L. micdadei icmTS*, *migAB*, and *icmQ* genes (GenBank accession number AY512559) were named pMF-SK-long110 and pMF-mic47, respectively. These plasmids were sequenced and used for further analysis.

Plasmid construction for complementation with *L. longbeachae* genes. A 4-kb EcoRI-XhoI fragment from pMF-SK-long110 was filled in and cloned into pMMB207αB-Km14 (24) that had been partially digested with SmaI to generate pMF-long110 (containing the *icmTS*, *ligB*, and *icmQ* genes). pMF-long111 (containing the *ligB* gene) was generated by cloning a 1-kb XmnI-HincII fragment from pMF-SK-long110 into the SmaI site. pMF-long113 (containing the *ligB* and *icmQ* genes) was generated by cloning a 2-kb XhoI-NruI fragment from pMF-SK-long110 (that was filled in) into the SmaI site. pMF-long114 (containing the *icmQ* gene) was generated by cloning a 1.5-kb BalI fragment from pMF-SK-long110 into the same SmaI site.

Plasmid construction for complementation with *L. micdadei* genes. A 3.1-kb SmaI fragment from pMF-mic47 was cloned into the SmaI site of pMMB207αB-Km14 to generate pMF-mic10 (containing the *icmTS*, *migAB*, and *icmQ* genes). pMF-mic18 (containing the *icmQ* gene) was generated by cloning a 1.5-kb SnaI-SmaI fragment from pMF-mic47 into the same SmaI site. pMF-mic19 (containing the *migB* gene) was generated by cloning a 1-kb BalI-SspI fragment from pMF-mic47 into the SmaI site. To generate pMF-mic21 (containing the *migB* and *icmQ* genes), a 1.5-kb BalI-SwaI fragment from pMF-mic47 was cloned into the same SmaI site.

Plasmid construction for allelic exchange. In order to generate insertions in the *L. longbeachae* chromosome, a kanamycin resistance cassette digested with PstI was cloned into pMF-SK-long110 that had been partially digested with PstI to generate an insertion in the *ligB* gene. A kanamycin resistance cassette that had been digested with EcoRV was cloned into pMF-SK-long110 that had been digested with NruI to generate an insertion in the *icmS* gene or with SwaI to generate an insertion in the *icmQ* gene. The three resulting plasmids were digested with PvuII, and the inserts were cloned into pLAW344 (29) that had been digested with EcoRV to generate pMF-ligB::Km-GR, pMF-longS::Km-GR, and pMF-longQ::Km-GR. The allelic exchange procedure was performed as previously described (25).

Plasmid construction for the two-hybrid analysis. To clone the *L. pneumophila icmR* and *icmQ* genes, the *L. longbeachae ligB* and *icmQ* genes, and the *L. micdadei migA*, *migB*, and *icmQ* genes into the pT18 and pT25 vectors of the *Bordetella pertussis cyaA* two-hybrid system (16), all of these genes were amplified by PCR with primers designed to form in-frame fusions with the *cyaA* gene product. Each of the seven genes was amplified with two sets of primers: one PCR product was cloned into pT18 to generate pKP-pT18R, pKP-pT18Q, pMF-T18-ligB, pMF-T18-longQ, pMF-T18-migA, pMF-T18-migB, and pMF-T18-micQ, and the second PCR product was cloned into pT25 to generate pKP-pT25R, pKP-pT25Q, pMF-T25-ligB, pMF-T25-longQ, pMF-T25-migA, pMF-T25-migB, and pMF-T25-micQ. All of these plasmids were sequenced and then introduced as pairs into *E. coli* IO7012D (Δ*cyaA*), and *lacZ* expression levels were measured by using the β-galactosidase assay as previously described (31).

Construction of a *lacZ* translational fusion. To generate a *ligB*::*lacZ* translational fusion, the regulatory region of the *ligB* gene was amplified by PCR, digested with BamHI-EcoRI, cloned into pGS-lac-02 (13), and sequenced. Ex-

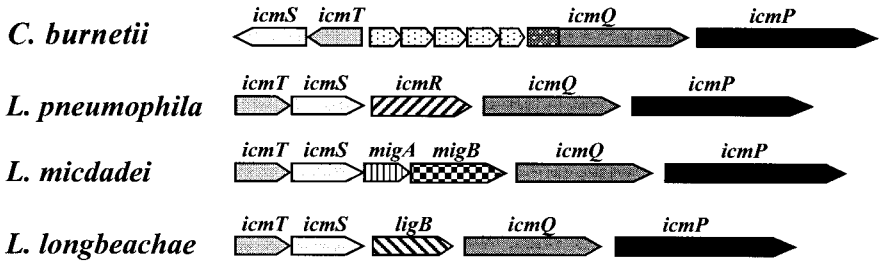

FIG. 1. Schematic drawing of region IIa in *C. burnetii*, *L. pneumophila*, *L. micdadei*, and *L. longbeachae*. Homologous genes are indicated by different colors or patterns, and the gene names are indicated above the arrows. *C. burnetii icmQ* contains an N-terminal region that is not present in the other *icmQ* homologues. The map is not drawn to scale.

pression levels were measured by using the β-galactosidase assay as previously described (13).

Intracellular growth in *Acanthamoeba castellanii* and HL-60-derived human macrophages. Intracellular assays for the growth of *L. pneumophila*, *L. longbeachae*, and their derived strains in *A. castellanii* and HL-60-derived human macrophages were performed as previously described (25).

Low-stringency Southern hybridization. The Southern hybridization procedure was performed as described previously (22) with 20% formamide.

RESULTS

Region IIa differs among *Legionella* species. When region IIa of *L. pneumophila* was compared with that of *C. burnetii* in the place where the *icmR* gene is located in *L. pneumophila*, five ORFs were found in *C. burnetii* (an *icmR* homologue is not present in the *C. burnetii* genome); four of these ORFs had no homology to any entry in the GenBank database, and the fifth ORF was homologous to a transposase gene. Cloning and sequencing of region IIa from *L. micdadei* and *L. longbeachae* (see Materials and Methods) revealed that in the place where the *icmR* gene was expected to be located (between *icmS* and *icmQ*), new ORFs were found; in *L. micdadei*, two new ORFs were found and named *migA* and *migB* (*L. micdadei icm* gene) (Fig. 1), and in *L. longbeachae*, one ORF was found in the same location and named *ligB* (*L. longbeachae icm* gene) (the *ligA* gene that was previously described for *L. pneumophila* [11] is not related to the *ligB* gene) (Fig. 1). These three genes (*migA*, *migB*, and *ligB*) showed no homology to any entry in the GenBank database, while the *icmT*, *icmS*, *icmQ*, and *icmP* gene products from the three *Legionella* species showed a very high degree of homology to one another (the identity between the IcmTSP homologous proteins was 74 to 92%, and that between the IcmQ homologous proteins was 57 to 68%).

For the sake of convenience, the different *icmQ* genes were named $icmQ_{Lp}$, for the *L. pneumophila icmQ* gene, $icmQ_{Ll}$, for the *L. longbeachae icmQ* gene, and $icmQ_{Lm}$, for the *L. micdadei icmQ* gene.

Distributions of newly discovered genes among different *Legionella* species. As shown in Fig. 1, the *icmR*, *ligB*, *migA*, and *migB* genes were located in the same position (between the *icmS* and *icmQ* genes), sharing no sequence homology to one another or to any entry in GenBank, implying that these genes are species specific. In order to verify this hypothesis, we performed low-stringency Southern hybridization with genomic DNA from 18 different *Legionella* species and strains (described in Materials and Methods) and six different probes—for the *L. pneumophila icmS*, *icmR* and $icmQ_{Lp}$ genes, for the *L. longbeachae ligB* gene, and for the *L. micdadei migA* and *migB* genes. The *icmS* gene was found in all species examined (Fig. 2A); the same result was obtained for the $icmQ_{Lp}$ gene (data not shown), indicating the presence of the *icm*/*dot* system in all of the species examined. The *migB* gene was found to be present only in the two *L. micdadei* strains examined (Fig. 2B), as was the *migA* gene (data not shown). The *icmR* gene was found to be present only in the two *L. pneumophila* strains examined (Fig. 2C). These results indicate that the *migA*, *migB*, and *icmR* genes are indeed species-specific genes (at least in the species examined). Surprisingly, the *ligB* gene was found in three additional *Legionella* species besides the two *L. longbeachae* strains examined (Fig. 2D). This gene was found in *L. cincinnatiensis*, *L. sainthelensi*, and *L. gratiana*, all of which are very closely related to one another (19); these results indicate that the *ligB* gene is not a species-specific gene. This information suggests that the *icmR*, *ligB*, and *migAB* genes entered the *Legionella* evolutionary tree through separate branches, while

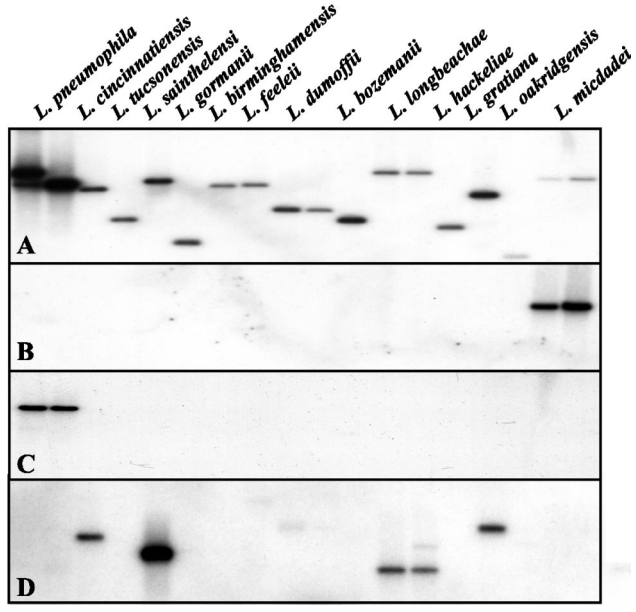

FIG. 2. Analysis of the distributions of *icm* genes in different *Legionella* species. The *icmS* gene is present in all *Legionella* species examined (A), *migB* is present in only two *L. micdadei* strains (B), *icmR* is present in only two *L. pneumophila* strains (C), and *ligB* is present in two *L. longbeachae* strains and in three additional closely related species (D). Hybridization was performed with chromosomal DNA digested with EcoRI as described in Materials and Methods.

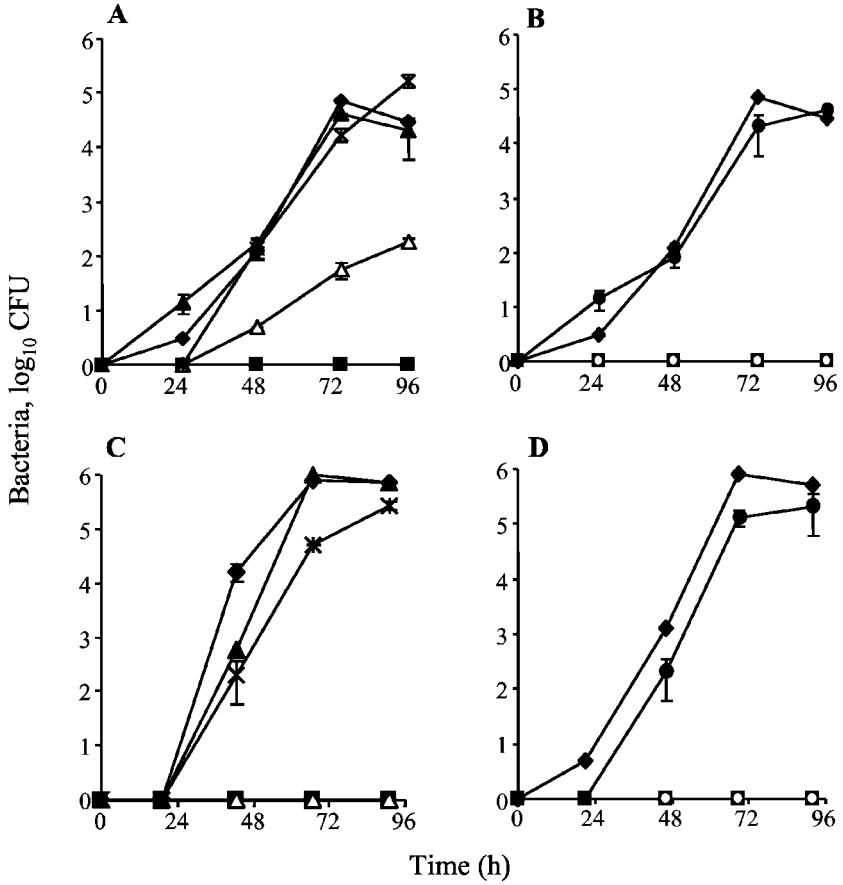

FIG. 3. Interspecies complementation of the *L. pneumophila icmR* insertion mutant. Experiments were performed with HL-60-derived human macrophages (A and B) or *A. castellanii* (C and D). Symbols: ◆, wild-type *L. pneumophila* JR32; ■, *L. pneumophila icmR* mutant (LELA3473) containing the vector; ∗, *L. pneumophila icmR* gene; △, *L. longbeachae ligB* gene; ▲, *L. longbeachae ligB* and $icmQ_{Ll}$ genes; ○, *L. micdadei migB* gene; ●, *L. micdadei migB* and $icmQ_{Lm}$ genes. The experiments were performed at least three times, and similar results were obtained. The error bars indicate standard deviations.

the *icmS* and *icmQ* genes entered earlier, as they are also present in *C. burnetii*. Since seven of the species examined did not contain any of the genes described above, it is possible that each of them has a distinctive gene located in the same position.

Interspecies complementation. Due to the high degree of homology among the majority of genes in region IIa (see above), we tested whether the *L. longbeachae* and *L. micdadei* region IIa gene products function in a manner similar to that of the *L. pneumophila* region IIa gene products. Therefore, we performed interspecies complementation assays with *L. pneumophila icmS*, *icmR* and $icmQ_{Lp}$ mutants and complemented each of them with plasmids containing the *L. longbeachae* and *L. micdadei* region IIa genes. As expected, full complementation of the *L. pneumophila icmS* and $icmQ_{Lp}$ mutants by the *L. longbeachae* and *L. micdadei* genes was observed in HL-60-derived macrophages as well as in *A. castellanii* (data not shown). However, to our surprise, complementation was also obtained for the *L. pneumophila icmR* mutant, although no *icmR* homologues were present on either of the complementing plasmids (data not shown). This unexpected result was studied further in order to determine which of the *L. longbeachae* and *L. micdadei* region IIa genes was responsible for complementation of the *icmR* mutant.

IcmR, MigB, and LigB are functionally homologous. The interspecies complementation assays described above marked the *L. longbeachae ligB* and *L. micdadei migB* genes as possible candidates for complementation of the *icmR* mutant due to their genomic location. To examine whether functional homology exists among the *icmR*, *ligB*, and *migB* gene products, we performed interspecies complementation assays with an *L. pneumophila icmR* insertion mutant and complemented it with plasmids containing the relevant genes from *L. micdadei* and *L. longbeachae*. The strain containing the insertion in the *L. pneumophila icmR* gene was fully complemented by the plasmid containing the *icmR* gene alone (Fig. 3A). However, the *L. longbeachae ligB* gene only partially complemented the *L. pneumophila icmR* insertion mutant, and the *L. micdadei migB* gene did not complement it at all (Fig. 3A and B, respectively). Nevertheless, plasmids containing a combination of the *L. longbeachae ligB* and $icmQ_{Ll}$ genes or the *L. micdadei migB* and $icmQ_{Lm}$ genes completely restored the ability of the *icmR* insertion mutant to grow intracellularly in HL-60-derived macrophages (Fig. 3A and B, respectively), although no *icmR* homologue was present on these plasmids. Similarly, in *A. castellanii*, neither the *ligB* gene nor the *migB* gene complemented the *icmR* insertion mutant, but a combination of the *L. longbeachae ligB* and $icmQ_{Ll}$ genes or the *L. micdadei migB* and

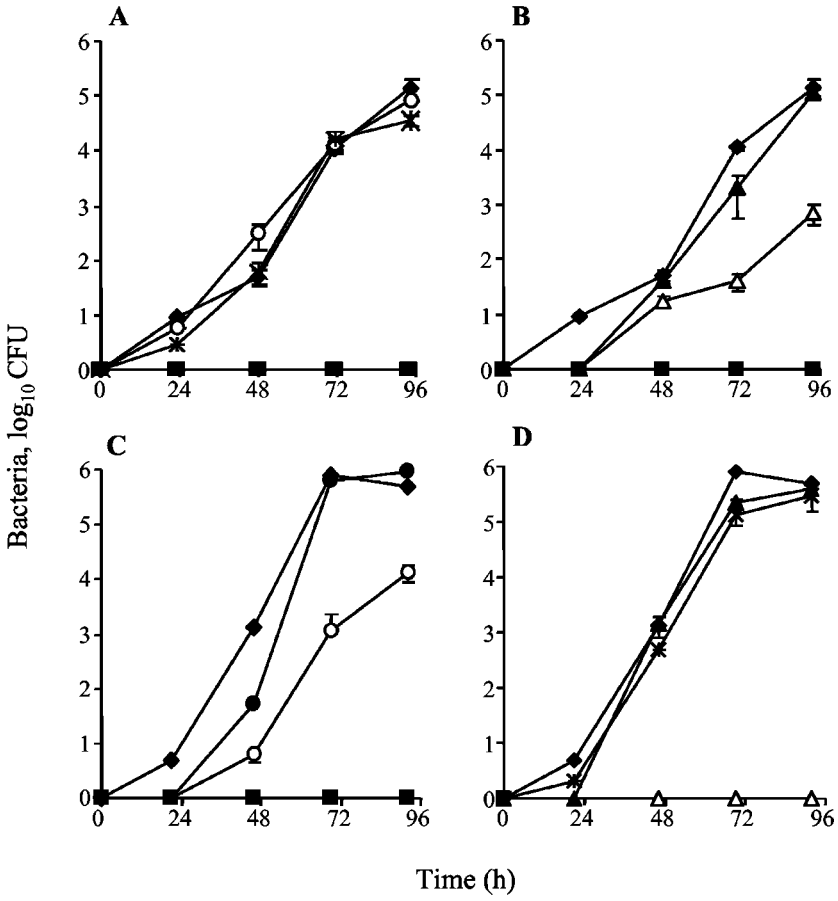

FIG. 4. Interspecies complementation of the $icmQ_{Lp}$ insertion mutant. The experiments were performed with HL-60-derived human macrophages (A and B) or *A. castellanii* (C and D). Symbols: ◆, wild-type *L. pneumophila* JR32; ■, $icmQ_{Lp}$ mutant (LELA3463) containing the vector; ∗, $icmQ_{Lp}$ gene; ○, $icmQ_{Ll}$ gene; ●, *L. longbeachae ligB* and $icmQ_{Ll}$ genes; △, $icmQ_{Lm}$ gene; ▲, *L. micdadei migB* and $icmQ_{Lm}$ genes. The experiments were performed at least three times, and similar results were obtained. The error bars indicate standard deviations.

$icmQ_{Lm}$ genes fully complemented the same insertion mutant (Fig. 3C and D, respectively). The data presented in Fig. 3 indicate that the *ligB*, *migB*, and *icmR* genes are functionally similar and that the presence of the *L. longbeachae* LigB and $IcmQ_{Ll}$ proteins together or the *L. micdadei* MigB and $IcmQ_{Lm}$ proteins together may have a significant role in pathogenesis which is similar to the role of the *L. pneumophila* IcmR and $IcmQ_{Lp}$ proteins. These results clearly indicate that although no sequence homology exists among the *icmR*, *migB*, and *ligB* genes, they seem to function similarly and their participation in the virulence pathway is related to the corresponding *icmQ* genes.

Analysis of IcmQ proteins from three *Legionella* species. The finding that *L. longbeachae* region IIa and *L. micdadei* region IIa complemented the $icmQ_{Lp}$ insertion mutant predicted that the $icmQ_{Ll}$ and $icmQ_{Lm}$ genes, respectively, were responsible for these results. However, the complementation results for the *L. pneumophila icmR* mutant predicted that the $IcmQ_{Ll}$ and $IcmQ_{Lm}$ proteins function together with the protein encoded by the gene located upstream from their genes. These two observations led us to examine whether the $icmQ_{Ll}$ and $icmQ_{Lm}$ genes by themselves can complement the $icmQ_{Lp}$ mutant. When examined in *A. castellanii*, the $icmQ_{Ll}$ gene only partially complemented the $icmQ_{Lp}$ mutant (Fig. 4C), while the $icmQ_{Lm}$ gene did not complement it at all (Fig. 4D). However, plasmids containing a combination of the *L. longbeachae ligB* and $icmQ_{Ll}$ genes or the *L. micdadei migB* and $icmQ_{Lm}$ genes together fully complemented the $icmQ_{Lp}$ insertion mutant in *A. castellanii* (Fig. 4C and D, respectively). When examined in HL-60-derived macrophages, the $icmQ_{Ll}$ gene fully complemented the ability of the $icmQ_{Lp}$ mutant to grow intracellularly, whereas the $icmQ_{Lm}$ gene only partially complemented this mutant (Fig. 4A and B, respectively). In this situation, too, a plasmid containing the *L. micdadei migB* and $icmQ_{Lm}$ genes was found to fully complement the $icmQ_{Lp}$ insertion mutant in HL-60-derived macrophages (Fig. 4B), implying that a combination of the *L. micdadei migB* and $icmQ_{Lm}$ genes was required for the complementation to occur. These results indicate that the $icmQ_{Ll}$ and $icmQ_{Lm}$ genes require the presence of the relevant gene located upstream from them for complete complementation, even though they are both homologous to the $icmQ_{Lp}$ gene.

The *L. longbeachae icm* genes are required for intracellular growth. Since the involvement of the *icm* genes in *L. longbeachae* pathogenesis has not been examined before, three insertion mutations in the *L. longbeachae icmS*, *ligB*, and $icmQ_{Ll}$ genes were constructed. All of the intracellular growth experiments with *L. longbeachae* strains were performed with

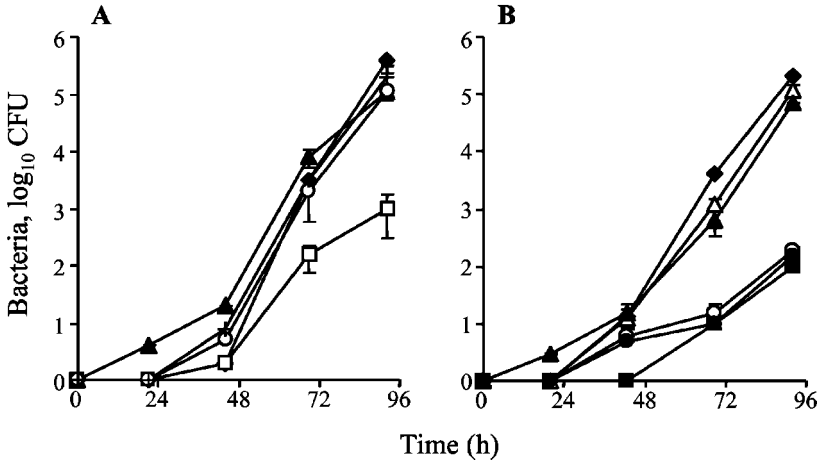

FIG. 5. Interspecies complementation of *L. longbeachae* mutants. (A and B) Complementation analysis of the $icmQ_{Ll}$ (A) and *ligB* (B) mutants with HL-60-derived human macrophages. Symbols for panel A: ◆, wild-type *L. longbeachae* ATCC 33462; □, $icmQ_{Ll}$ mutant (MF395) containing the vector; +, $icmQ_{Ll}$ gene; ▲, $icmQ_{Lp}$ gene; ○, $icmQ_{Lm}$ gene. Symbols for panel B: ◆, wild-type *L. longbeachae* ATCC 33462; ■, *L. longbeachae ligB* mutant (MF385) containing the vector; ▲, *L. longbeachae ligB* gene; △, *L. pneumophila icmR* gene; ○, *L. micdadei migB* gene; ●, *L. micdadei migB* and $icmQ_{Lm}$ genes. The experiments were performed at least three times, and similar results were obtained. The error bars indicate standard deviations.

HL-60-derived human macrophages, since *L. longbeachae* does not grow in *A. castellanii* (data not shown). The *L. longbeachae icmS* gene was found to be required for intracellular growth, and it was complemented by the *L. longbeachae*, *L. pneumophila*, and *L. micdadei icmS* genes (data not shown). The $icmQ_{Ll}$ insertion mutant showed a partial intracellular growth defect, which was fully complemented by the $icmQ_{Lp}$, $icmQ_{Ll}$, and $icmQ_{Lm}$ genes (Fig. 5A). The *ligB* insertion mutant also showed a partial intracellular growth phenotype, which was completely restored by complementing plasmids containing the *ligB* or the *icmR* gene (Fig. 5B) but not the *migB* gene or a combination of the *migB* and $icmQ_{Lm}$ genes (Fig. 5B). These results show that the *L. longbeachae icmS*, *ligB*, and $icmQ_{Ll}$ genes are required for intracellular growth, that the *ligB* and *icmR* genes are functionally similar, but that the *migB* gene functions in a manner similar to that of the *icmR* gene (Fig. 2B) but not that of the *ligB* gene (Fig. 5B).

IcmQ interacts with IcmR, MigB, and LigB. The functional homology among the three nonhomologous proteins (IcmR, LigB, and MigB), in addition to previous reports showing that the IcmR and $IcmQ_{Lp}$ proteins interact with one another (6, 9), led us to analyze whether each of the IcmR, MigB, and LigB proteins interacts with the corresponding IcmQ protein. For this analysis, a bacterial two-hybrid system that is based on the CyaA toxin of *B. pertussis* was used (16). The *L. pneumophila icmR* and $icmQ_{Lp}$ genes, the *L. longbeachae ligB* and $icmQ_{Ll}$ genes, and the *L. micdadei migA*, *migB*, and $icmQ_{Lm}$ genes were fused to the T18 (N-terminal fusion) and T25 (C-terminal fusion) fragments, and interactions among them were examined. In this system, an interaction between two proteins results in cyclic AMP production, which is determined by measuring the levels of expression of the *lacZ* gene product.

As shown in Fig. 6A to C, each of the three proteins interacts with the corresponding IcmQ protein (the MigA protein does not interact either with the $IcmQ_{Lm}$ protein or with the MigB protein) (Fig. 6C), strongly supporting the notion that the IcmR, LigB, and MigB proteins play similar roles and that their functions involve interactions with the corresponding IcmQ protein. Moreover, the interaction between each of the three proteins and the corresponding IcmQ protein was very strong when the IcmQ protein was fused to the T18 fragment and the when the IcmR/LigB/MigB proteins were fused to the T25 fragment, while the interaction in the opposite direction was significantly weaker. These results indicate that the interactions of the proteins examined probably occur between the C-terminal domain of the IcmR/LigB/MigB proteins and the N-terminal domain of the corresponding IcmQ protein.

In order to reinforce these observations, we performed interspecies interaction assays to examine all of the possible combinations of the IcmR/LigB/MigB proteins and the $IcmQ_{Lp}$, $IcmQ_{Ll}$, and $IcmQ_{Lm}$ proteins. Interactions were observed for all of the protein combinations (Fig. 6D to F), with the exception of MigB, which did not interact with either $IcmQ_{Lp}$ (Fig. 6D) or $IcmQ_{Ll}$ (Fig. 6E). This finding correlates with our results showing that the *migB* gene by itself did not complement the *icmR* or the *ligB* mutant (Fig. 3B and 5B) and can be explained by the fact that *L. micdadei* is evolutionarily more distant from *L. longbeachae* than *L. pneumophila* (19). All of the interactions observed occurred in the same orientation as that described above (Fig. 6D to F) and were significantly weaker in the opposite orientation (data not shown), indicating that indeed a specific orientation of the IcmR/LigB/MigB proteins is required for their interaction with the corresponding IcmQ protein. Very recently, it was shown that $IcmQ_{Lp}$ binds to IcmR via its N-terminal domain (10), a finding that strongly supports our results.

***icmR*, *ligB*, and *migB* are regulated by CpxR.** Although no sequence homology exists among *icmR*, *ligB*, and *migB*, the finding that they function similarly led us to wonder whether their expression is regulated in the same manner. It has been shown that the *L. pneumophila icmR* gene is positively regulated by the CpxRA two-component system through a conserved CpxR binding site (12). Careful examination of the regulatory region of the *ligB* and *migB* genes revealed that a

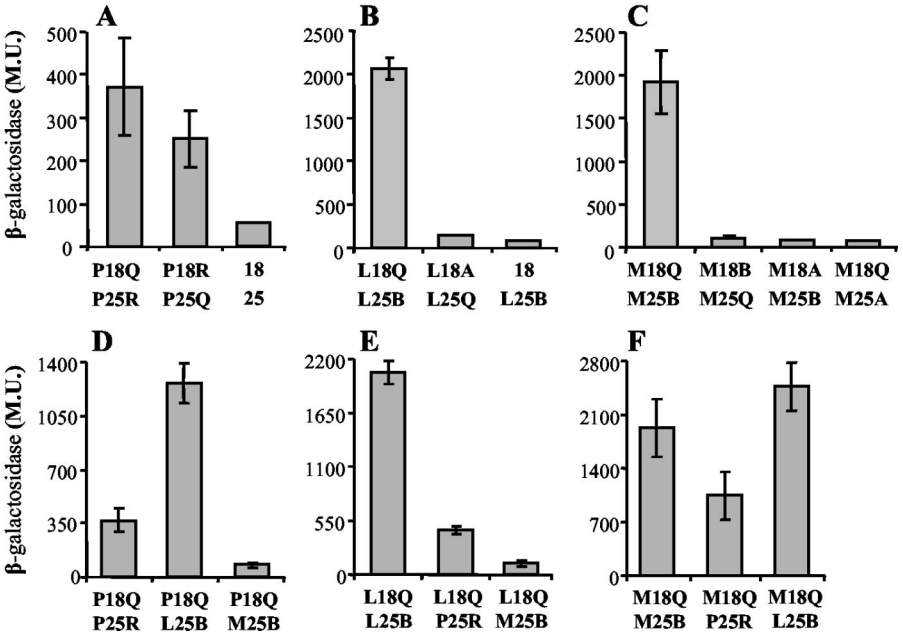

FIG. 6. Two-hybrid analysis of the interactions between the *L. pneumophila*, *L. longbeachae*, and *L. micdadei* protein pairs. Interactions were examined for the *L. pneumophila* IcmR and $IcmQ_{Lp}$ proteins (A), the *L. longbeachae* LigB and $IcmQ_{Ll}$ proteins (B), and the *L. micdadei* MigB and $IcmQ_{Lm}$ proteins (C); interspecies interactions were examined for the $IcmQ_{Lp}$ protein (D), the $IcmQ_{Ll}$ protein (E), and the $IcmQ_{Lm}$ protein (F). β-Galactosidase activity was measured in Miller units [M.U.] at stationary phase. The names of the genes used in this assay are marked with a code composed of two letters and a number between them. The letter on the left represents the species: P, *L. pneumophila*; L, *L. longbeachae*; M, *L. micdadei*. The number indicates to which *cyaA* fragment the examined gene was fused: 18, T18; 25, T25. The letter on the right represents the gene examined: R, *icmR*; A, *migA*; B, *ligB* (when the left letter is L) or *migB* (when the left letter is M); Q, *icmQ*. The results are the averages and standard deviations of at least three independent experiments.

putative CpxR binding site is also found in the *ligB* and *migB* regulatory regions (Fig. 7A). It also seems, from a comparison of the sequences shown in Fig. 7A, that the *Legionella* consensus binding site for the CpxR response regulator ($GTAAAn_6GAAA$) is slightly different from the one identified in *E. coli*: $GTAAAn_5GTAAA$ (8). In order to test whether these genes are indeed regulated by the same response regulator, we constructed a translational fusion between the *L. longbeachae ligB* regulatory region and the *lacZ* reporter gene and introduced the resulting plasmid into *L. longbeachae*, *L. pneumophila*, and an *L. pneumophila* strain containing an insertion mutation in the *cpxR* gene. β-Galactosidase activity was measured at stationary phase, and the results obtained strongly indicated that the *cpxR* mutant drastically reduced the level of expression of *ligB* (Fig. 7B). These results suggest that the *icmR*/*ligB*/*migB* genes are probably regulated similarly, a fact that correlates with the functional homology described above for the three proteins encoded by these genes.

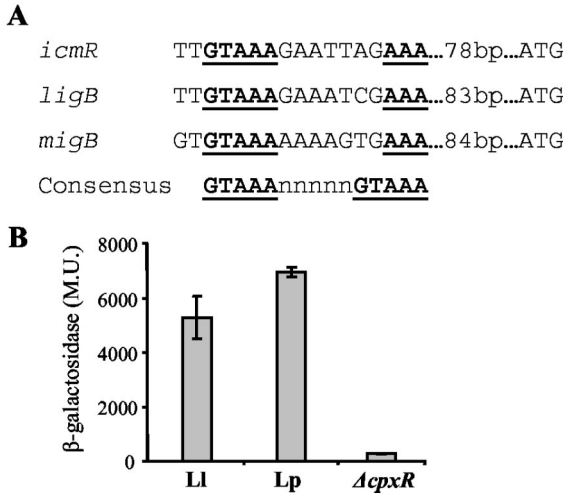

FIG. 7. The *icmR*, *ligB*, and *migB* genes are probably regulated by CpxR. (A) Part of the regulatory region of the *L. pneumophila icmR*, *L. longbeachae ligB*, and *L. micdadei migB* genes. The consensus recognition sequence of CpxR identified in *E. coli* is shown at the bottom. CpxR recognition sequences are shown in bold type and are underlined, and the distances to the first ATG are indicated. (B) Expression of a *ligB*::*lacZ* translational fusion in *L. longbeachae* (Ll), *L. pneumophila* (Lp), and an *L. pneumophila cpxR* gene insertion mutant (Δ*cpxR*) at stationary phase. The results, reported in Miller units (M.U.), are the averages and standard deviations of at least three independent experiments.

DISCUSSION

L. pneumophila grows intracellularly and kills the host cell due to the activity of the Icm/Dot type IV secretion system, which is believed to form a protein complex that translocates effector proteins from the bacterial cytoplasm into the host cell. The *icm*/*dot* system was recently discovered also in *C. burnetii* and included all of the *icm*/*dot* genes except for *icmR* (30, 31). The *icmR* gene, together with $icmQ_{Lp}$, is located in region IIa; their gene products are predicted to be found in the

bacterial cytoplasm and are not homologous to conjugation-related proteins encoded by plasmid R64. This information, together with the finding that *C. burnetii* contains the entire *icm*/*dot* system except for *icmR*, led us to focus on region IIa as a potential cause for differences between bacteria that contain the Icm/Dot virulence system.

Besides *L. pneumophila*, several pathogenic *Legionella* species have been described, and we chose to study two of them: *L. micdadei* which is the second most common agent of Legionnaires' disease in the world, and *L. longbeachae*, which is the third most common etiological agent of Legionnaires' disease in the world but the dominant agent in Australia (1, 15). Cloning and sequencing of region IIa from *L. longbeachae* and *L. micdadei* revealed an interesting discovery: the *icmR* gene was found to be missing in the region between *icmS* and *icmQ* in both species, and other ORFs were found in its place. In *L. longbeachae*, one ORF (*ligB*) was found in the place where *icmR* was expected to be located, and in *L. micdadei*, two ORFs (*migA* and *migB*) were found in the same position. In order to investigate the functions of these newly discovered gene products, a homology search was performed, but no homology was detected among the IcmR, LigB, and MigB proteins or to any other entry in GenBank.

These proteins seem to share a few properties in common with type III virulence-related chaperones. (i) The IcmR, MigB, and LigB proteins were all predicted by the PSORT program (http://psort.ims.u-tokyo.ac.jp) to be located in the bacterial cytoplasm. They were also predicted to have acidic pIs (4.75, 5.3, and 5.8, respectively), and they are all small proteins (88 to 120 amino acids). These properties are considered common features of type III secretion chaperones (2). (ii) Through interspecies complementation experiments, the IcmR, LigB, and MigB proteins were found to function similarly, although no sequence homology exists among them. Bioinformatics programs predicted that these three proteins contain an α-helical structure at their C-terminal domain, like that found for type III chaperones, which also do not share any sequence homology but which are structurally homologous (3). (iii) As was shown before for the *L. pneumophila* IcmR and $IcmQ_{Lp}$ proteins (6, 9), analysis of protein interactions showed that the *L. longbeachae* LigB and *L. micdadei* MigB proteins also interact with their corresponding IcmQ proteins and that the interactions occur via the C-terminal domain of the IcmR, LigB, or MigB protein and the N-terminal domain of the relevant IcmQ protein. It was shown before that type III virulence-related chaperones interact with their substrates in a specific orientation similar to the one described above (2). (iv) Type III chaperones are encoded by genes adjacent to the genes encoding their substrates, like IcmR, LigB, and MigB, which were shown to interact with the protein encoded by the gene located downstream from their genes (IcmQ).

All of the information described above strongly implies that the IcmR, LigB, and MigB proteins function similarly, probably as chaperones of the relevant IcmQ proteins. Even though the IcmR, LigB, and MigB proteins seem to share several properties in common with type III chaperones, other characteristics make these proteins quite distinct. First, all of these features of type III chaperones were found here in a type IV secretion system. Second, in a type III secretion system, each chaperone interacts with a specific substrate and not with similar substrates, as was found here. Another unique feature of these proteins is the specific location of their genes on the chromosomes of different *Legionella* species, a property that has not been described for type III chaperones.

As mentioned above, the *C. burnetii* genome contains the entire *icm*/*dot* system except for the *icmR* gene. However, the five ORFs that are located in its place encode large nonacidic proteins; therefore, it is highly unlikely that one of these proteins plays a role similar to that of IcmR, LigB, or MigB. In addition, it was proposed before that the *C. burnetii* IcmQ protein, which was shown not to complement the $icmQ_{Lp}$ mutant (30, 31), functions as its own chaperone, as it contains an extension of 47 amino acids at its N-terminal domain (Fig. 1).

Our results reveal new evidence of gene variation at a specific position in the pathogenicity region of different *Legionella* species; this finding has not been shown before for any type IV secretion system. The genes located at this site encode proteins that share no sequence homology but seem to function similarly and to be controlled by the same regulator. These findings lead to two main questions. (i) What is the mechanism that causes this gene variation? (ii) Why does such gene variation exist? As far as the first question, the evolutionary process that led to this intriguing phenomenon is quite mysterious due to the lack of evidence for horizontal gene transfer; this region does not contain any differences in its GC content, and no insertion sequence elements have been found in this region in the three *Legionella* species examined. The possible answer to the second question might be related to a recent study showing that $IcmQ_{Lp}$ functions as a pore-forming protein (10). This information leads to the hypothesis that the $IcmQ_{Lp}$, $IcmQ_{Ll}$, and $IcmQ_{Lm}$ proteins may be adapted to insert pores into the membrane of their specific protozoan host, a function that is mediated by the IcmR, LigB, and MigB proteins, respectively. It is possible that these proteins are part of a machinery that evolved to adapt a specific *Legionella* species to a certain amoeba host, thus functioning as host-specific determinants. These differences in the *icm*/*dot* systems of the three *Legionella* species examined may be directly or indirectly related to the fact that of these three species, *L. pneumophila* is the most common causative agent of Legionnaires' disease in the world.

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