JOURNAL OF BACTERIOLOGY, May 2007, p. 3382–3391 Vol. 189, No. 9
0021-9193/07/$08.00+0 doi:10.1128/JB.01742-06

A Pair of Highly Conserved Two-Component Systems Participates in the Regulation of the Hypervariable FIR Proteins in Different *Legionella* Species▽†

Michal Feldman and Gil Segal*

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

Received 13 November 2006/Accepted 15 February 2007

***Legionella pneumophila* and other pathogenic *Legionella* species multiply inside protozoa and human macrophages by using the Icm/Dot type IV secretion system. The IcmQ protein, which possesses pore-forming activity, and IcmR, which functions as its chaperone, are two essential components of this system. It was previously shown that in 29 *Legionella* species, a large hypervariable-gene family (*fir* genes) is located upstream from a conserved *icmQ* gene, but although nonhomologous, the FIR proteins were found to function similarly together with their corresponding IcmQ proteins. Alignment of the regulatory regions of 29 *fir* genes revealed that they can be divided into three regulatory groups; the first group contains a binding site for the CpxR response regulator, which was previously shown to regulate the *L. pneumophila fir* gene (*icmR*); the second group, which includes most of the *fir* genes, contains the CpxR binding site and an additional regulatory element that was identified here as a PmrA binding site; and the third group contains only the PmrA binding site. Analysis of the regulatory region of two *fir* genes, which included substitutions in the CpxR and PmrA consensus sequences, a controlled expression system, as well as examination of direct binding with mobility shift assays, revealed that both CpxR and PmrA positively regulate the expression of the *fir* genes that contain both regulatory elements. The change in the regulation of the *fir* genes that occurred during the course of evolution might be required for the adaptation of the different *Legionella* species to their specific environmental hosts.**

Legionella pneumophila is the most common causative agent of Legionnaires' disease, and it was shown to be able to grow within and kill human macrophages, as well as free-living amoebae (22, 42). The genome of *L. pneumophila* was shown to contain 25 genes, named the *icm*/*dot* genes, which form a type IV secretion complex (44, 45, 51, 52), through which effector proteins are translocated into infected host cells (4, 7, 27, 28, 34–36, 49). Two of the *icm*/*dot* genes encode the IcmR and IcmQ proteins, which were previously shown to interact with one another (6, 10), and IcmR was shown to function as a chaperone of IcmQ, thus regulating its pore-forming activity (10, 11). In addition, it was shown before that in various *Legionella* species, in the exact genomic location of the *icmR* gene, which is immediately upstream from the *icmQ* gene and downstream from the *icmS* gene, completely different genes were found. These highly variable (in sequence and length) genes were named *fir* genes and, although different in sequence, were found to encode proteins that function similarly to the corresponding IcmQ proteins, with which they were also shown to interact (12, 13). These findings, together with additional information, led to the hypothesis that the FIR and IcmQ proteins coevolved with one another (13).

The *L. pneumophila fir* gene (*icmR*) has been previously shown to be directly regulated by the two-component response regulator CpxR (16). The CpxR response regulator is part of a two-component system which includes its cognate CpxA inner-membrane sensor histidine kinase (9, 38). It has been found that this two-component system is activated in *Escherichia coli* by periplasmic stress, such as accumulation of misfolded proteins in the bacterial periplasm (37). Although CpxR was found to directly regulate the expression of *icmR* and to influence the expression of other *icm*/*dot* genes (16), the signal that activates the CpxAR two-component system in *L. pneumophila* is as yet unrevealed. In addition, the consensus regulatory element of CpxR was found to be slightly different in *Legionella* than in other bacteria; in *E. coli*, the CpxR binding site was shown to be GTAAAnnnnnGTAAA (8), whereas in *Legionella* species, it was shown to be GTAAAnnnnnnGAAAG (12). This finding correlates with previous evidence that *E. coli* CpxR does not recognize the *L. pneumophila icmR* regulatory region (16). The CpxR response regulator has been shown to belong to the OmpR winged helix-turn-helix protein family, the members of which all contain a characteristic helix before the wing domain, which serves as the DNA binding motif (1). Another response regulator that belongs to the same family is the PmrA response regulator, which is a part of the PmrAB two-component system. The PmrAB system has also been found to be present in different pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium (18), *Pseudomonas aeruginosa* (32), *Erwinia carotovora* (23), and *E. coli* (19). This system was shown in *S. enterica* to be responsible for the induction of genes

* Corresponding author. Mailing address: Department of Molecular Microbiology & 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.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 2 March 2007.

that encode enzymes that are involved in modification of bacterial lipopolysaccharide as a response to specific cues from the environment, such as extracytoplasmic Fe^{3+} and low pH, thus gaining resistance to host antimicrobial peptides (50). Although the CpxR and PmrA regulators have characteristics in common and were both found to regulate the expression of genes involved in pathogenesis, they were never shown to directly regulate the expression of the same gene.

In the presented study we show, by using bioinformatic, genetic, and biochemical tools, evidence that the CpxR and/or the PmrA response regulators directly bind to the regulatory region of the *fir* genes and positively regulate their expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *L. pneumophila* strains used in this study were *L. pneumophila* JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which is a wild-type strain in terms of intracellular growth (43); OG2002, a *cpxR* mutant (16); HK-PQ1, a *pmrA* mutant (57); and EA-CRPA, a *cpxR pmrA* double mutant (this study). Additional *Legionella* species used in this study were *L. erythra* ATCC 35303, *L. feeleii* ATCC 35849, *L. longbeachae* ATCC 33462, *L. micdadei* ATCC 33218, and *L. rubrilucens* ATCC 35304. The *E. coli* strains used were MC1022, MC1061 (3), and BL21 (Novagen). Bacterial media, plates, and antibiotic concentrations were used as described previously (47). For the plasmids and primers used in this study, see Tables S1 and S2 in the supplemental material, respectively.

Low-stringency Southern hybridizations. The genomic DNAs of the six *Legionella* species indicated above were extracted, digested with EcoRI, and separated by gel electrophoresis. The gel was then transferred to a nitrocellulose membrane and fixed with a UV cross-linker. Two such membranes were hybridized, one with a *cpxR* probe and the second with a *pmrA* probe. Both probes were prepared by PCR amplification of the *L. pneumophila* genome with the cpxR-pET-F and cpxR-pET-R primers for the *cpxR* probe and the PmrA-F and PmrA-R primers for the *pmrA* probe (see Table S2 in the supplemental material). The resulting fragments were then labeled with [α-^{32}P]dCTP and used for low-stringency hybridization with 20% formamide as previously described (46).

Cloning of the *L. micdadei* and *L. feeleii cpxR* and *pmrA* genes. The *cpxR* and *pmrA* genes from *L. pneumophila* were amplified by PCR (with the same primers mentioned above), and the DNA fragments were used as probes for low-stringency hybridization with genomic DNA of *L. micdadei* and *L. feeleii* that was digested with XbaI and PstI, respectively. Fragments of approximately 4 kb were then cloned into pUC-18 digested appropriately. Two hundred colonies from each ligation were stabbed onto a new plate, and these colonies were then transferred to a nitrocellulose membrane, which was positioned on a new plate and grown overnight. The colonies grown on the membrane were carefully lysed as previously described (46), and the membranes were used for low-stringency hybridization with the *L. pneumophila cpxR* or *pmrA* probe as mentioned above. Positive colonies were picked from the original plate, and the plasmids were extracted from them and sequenced. The pMF-mic21-cpxR and pMF-mic39-pmrA plasmids contained the *L. micdadei cpxR* (GenBank accession number EF094475) and *pmrA* (GenBank accession number EF094474) genes, respectively. The pMF-feel67-cpxR and pMF-feel43-pmrA plasmids contained the *L. feeleii cpxR* (GenBank accession number EF094473) and *pmrA* (GenBank accession number EF094472) genes, respectively (see Table S1 in the supplemental material).

Purification of six-His-tagged proteins. The *L. micdadei* and *L. feeleii* CpxR and PmrA proteins were fused to a six-histidine tag at their N termini by PCR amplification with the primers mic-CpxR-His-Nde and mic-CpxR-His-Bam for *L. micdadei* CpxR, mic-PmrA-His-Nde and mic-PmrA-His-Bam for *L. micdadei* PmrA, feel-CpxR-His-Nde and feel-CpxR-His-Bam for *L. feeleii* CpxR, and feel-PmrA-His-Nde and feel-PmrA-His-Bam for *L. feeleii* PmrA (see Table S2 in the supplemental material). The PCR products were then digested with BamHI and NdeI and cloned into the pET-15b vector to generate the pMF-mic-His-cpxR, pMF-mic-His-pmrA, pMF-feel-His-cpxR, and pMF-feel-His-pmrA plasmids (see Table S1 in the supplemental material). All four proteins were purified from *E. coli* BL21 containing the pRep4 plasmid with nickel bead columns (QIAGEN) according to the manufacturer's instructions. After purification, the fractions containing the protein were dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol for 2 h and with the same buffer containing 30% glycerol overnight. The proteins were then stored at −20°C.

Gel mobility shift assays. Gel mobility shift assays were performed as previously described (20), with few modifications. The regulatory regions of the *migB* and *figA* genes, with or without the substitutions (~180 bp), were amplified by PCR with the primers migB-Eco and migB-Bam for the *migB* gene and the primers figA-Eco and figA-Bam for the *figA* gene (see Table S2 in the supplemental material) and 3′ end labeled with digoxigenin (DIG) by using DIG-11-ddUTP (Roche). Increasing amounts of the purified proteins were mixed with 150 pg of the *migB*-labeled probe or 30 pg of the *figA*-labeled probe in buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 μg/ml poly(dI-dC), 5% glycerol, and 10 ng/ml herring sperm DNA. For samples containing unlabeled probe, 200 ng of the probe was allowed to bind the appropriate protein for 15 min before the addition of the DIG-labeled probe. A binding reaction was carried out for 30 min at room temperature, and samples were then loaded onto 6% polyacrylamide–0.25× Tris-acetate-EDTA gels in 0.5× Tris-acetate-EDTA running buffer. Following electrophoresis, the gels were transferred to nylon membranes and fixed by UV cross-linking. Detection of the DIG-labeled DNA fragments was performed according to the manufacturer's instructions.

Construction of *lacZ* translational fusions. To generate the *migB*::*lacZ* and *figA*::*lacZ* translational fusions, the regulatory regions of the *migB* and *figA* genes were amplified by PCR with the primers migB-Eco and migB-Bam for the *migB* gene and the primers figA-Eco and figA-Bam for the *figA* gene (see Table S2 in the supplemental material). The PCR products were then digested with BamHI and EcoRI, cloned into pGS-lac-02, and sequenced to generate the pMF-migB::lacZ and pMF-figA::lacZ plasmids, respectively (see Table S1 in the supplemental material). The levels of expression from these plasmids were measured by a β-galactosidase assay as described below.

Construction of substitutions in the CpxR and PmrA binding sites. To generate substitutions in the CpxR and PmrA binding sites in the *migB* and *figA* regulatory regions, site-directed mutagenesis was performed on the consensus sequences by the PCR overlap extension approach (21). The upstream part of the CpxR binding site was changed from GTAAA to AGCCC, the upstream part of the PmrA binding site was changed from CTTAAG into CGGCCA, or both sequences were mutated simultaneously in the regulatory region of the *migB* gene. The primers used for the mutagenesis were migB-cpx-mut-F and migB-cpx-mut-R for the mutagenesis of the CpxR site of *migB*, migB-pmrA-mut-F and migB-pmrA-mut-R for the mutagenesis of the PmrA site of *migB*, figA-cpx-mut-F and figA-cpx-mut-R for the mutagenesis of the CpxR site of *figA*, and figA-pmrA-mut-F and figA-pmrA-mut-R for the mutagenesis of the PmrA site of *figA* (see Table S2 in the supplemental material). The resulting fragments were digested with BamHI and EcoRI, cloned into pGS-lac-02, and sequenced, resulting in the pMF-MB-cpxR-mut, pMF-MB-pmrA-mut, and pMF-MB-cpxR-pmrA-mut plasmids containing the substitutions in the *migB* regulatory region and plasmids pMF-FA-cpxR-mut and pMF-FA-pmrA-mut containing the substitutions in the *figA* regulatory region (see Table S1 in the supplemental material). The plasmids containing the substitutions in the *migB* regulatory region were introduced into *L. micdadei* by electroporation with the setup used for *L. pneumophila* electroporation, and their levels of expression were determined. The plasmids containing the substitutions in the *figA* regulatory region were used for cloning the *L. feeleii cpxR* or *pmrA* gene under the control of the P_{tac} promoter as described below.

Construction of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *cpxR* and *pmrA*. The *L. feeleii cpxR* and *pmrA* genes were amplified by PCR with the primers feel-CpxR-EcoRI and feel-CpxR-His-Bam for the *cpxR* gene and feel-PmrA-EcoRI and feel-PmrA-His-Bam for the *pmrA* gene (see Table S2 in the supplemental material). The PCR products were then digested with EcoRI and BamHI and cloned into pMMB207 downstream from the P_{tac} promoter to generate the pMF-feel-cpxR-207 and pMF-feel-pmrA-207 plasmids. The resulting plasmids were then digested with XbaI and EheI, and the resulting fragments, containing the P_{tac}-*cpxR* or P_{tac}-*pmrA* gene together with the *lacI* gene, were cloned into the plasmid containing the regulatory region of the *figA* gene, as well as the plasmids containing the mutations in the CpxR or PmrA binding site described above, that were digested with XbaI and XmnI, to generate the pMF-FAC, pMF-FAP, pMF-CDC, pMF-CDP, pMF-PDC, and pMF-PDP plasmids (see Table S1 in the supplemental material).

Construction of the *L. pneumophila cpxR pmrA* double mutant. To generate an *L. pneumophila cpxR pmrA* double-mutant strain, the gentamicin resistance cassette digested with EcoRV was cloned into pOG-cpxR-1 digested with EcoRV to generate pEA-cpxR-Gm, containing an insertion in the *cpxR* gene, which was then digested with SmaI and cloned into pLAW344 (54) digested with EcoRV to generate pEA-cpxR-Gm-GR. This plasmid was used for an allelic-exchange

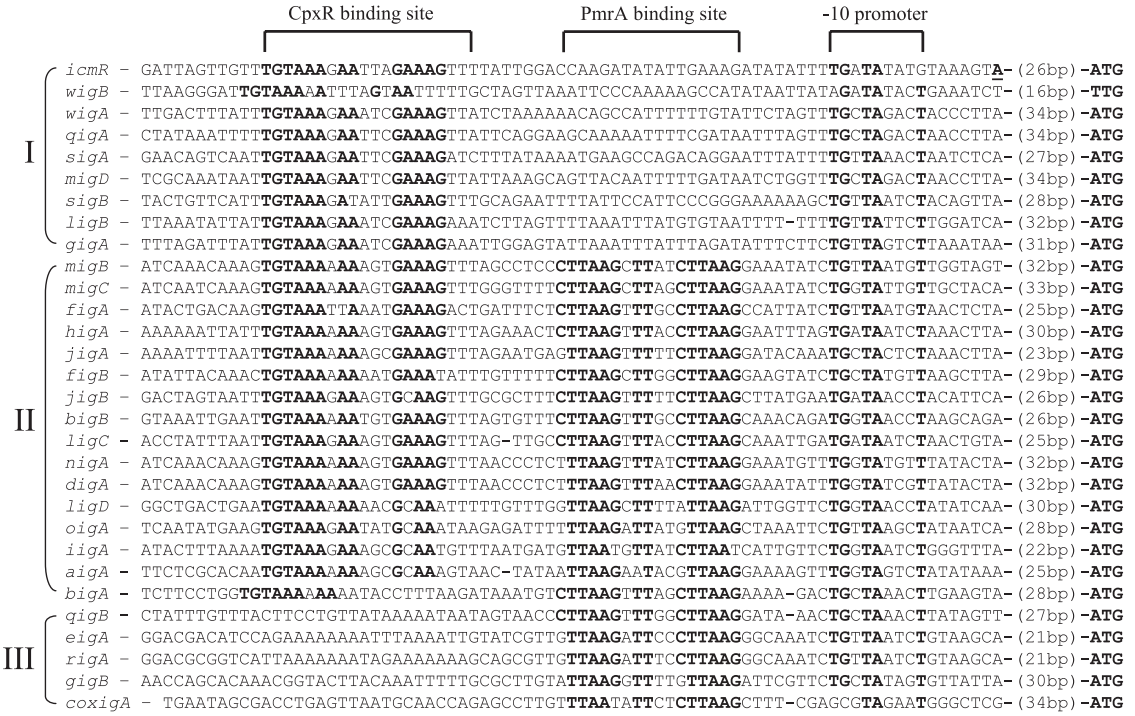

FIG. 1. Thirty *fir* genes contain the CpxR and/or the PmrA regulatory element. The regulatory sequences of the *fir* genes from 29 *Legionella* species and *C. burnetii* were aligned. The name of the *fir* gene is indicated to the left of each sequence. The regulatory elements are in bold, and the regulator that recognizes each motif is indicated above. The −10 promoter is indicated according to the transcription start site that was previously found for the *icmR* gene, which is in bold and underlined (16). The regulatory regions are divided into three groups (indicated on the left) according to the presence or absence of the regulatory elements, and the distances from the first ATG are indicated. The names of the *Legionella* species from which the *fir* genes were aligned (and their accession numbers), from the top, are *L. pneumophila* (*icmR*, Y12705), *L. waltersii* (*wigB*, AY860648), *L. worsleiensis* (*wigA*, AY860646), *L. quateirensis* (*qigA*, AY860645), *L. shakespearei* (*sigA*, AY860647), *L. moravica* (*migD*, AY860644), *L. spiritensis* (*sigB*, AY860657), *L. longbeachae* (*ligB*, AY512558), *L. gratiana* (*gigA*, AY860642), *L. micdadei* (*migB*, AY512559), *L. maceachernii* (*migC*, AY860654), *L. feeleii* (*figA*, AY753535), *L. hackeliae* (*higA*, AY753534), *L. jamestowniensis* (*jigA*, AY860649), *L. fairfieldensis* (*figB*, AY860653), *L. jordanis* (*jigB*, AY860651), *L. brunensis* (*bigB*, AY860650), *L. lansingensis* (*ligC*, AY860652), *L. nautarum* (*nigA*, AY860655), *L. drozanskii* (*digA*, AY860662), *L. londiniensis* (*ligD*, AY860660), *L. oakridgensis* (*oigA*, AY860643), *L. israelensis* (*iigA*, AY860663), *L. adelaidensis* (*aigA*, AY860661), *L. birminghamensis* (*bigA*, AY860641), *L. quinlivanii* (*qigB*, AY860656), *L. erythra* (*eigA*, AY860658), *L. rubrilucens* (*rigA*, AY860659), and *L. geestiana* (*gigB*, AY860664). *coxigA* is the *fir* gene of *C. burnetii*.

procedure starting with the *L. pneumophila pmrA* mutant HK-PQ1 (57) as previously described (48).

β-Galactosidase assay. A β-galactosidase assay was used to measure the levels of expression of the *lacZ* translational fusions. β-Galactosidase assays for *E. coli* strain MC1061 and *L. pneumophila* strains were performed as previously described (17). To carry out this experiment with *L. micdadei*, bacteria were grown on charcoal-yeast extract plates for 36 h (exponential phase) or 72 h (stationary phase) and scraped from the plates directly into AC buffer, pH 6.5 [4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 3.4 mM Na-citrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4], and this suspension was used for the β-galactosidase assay as previously described (17).

RESULTS

The *fir* genes contain similar regulatory elements. It has been previously found that the expression of the *L. pneumophila icmR* gene is regulated by the response regulator CpxR (16), and we were interested in examining whether the rest of the *fir* genes contain the CpxR consensus binding site in the regulatory region. We aligned the regulatory regions of all the *fir* genes available (Fig. 1). The CpxR binding site was found to be present in most sequences and to be highly conserved. However, to our surprise, the alignment revealed that many of the *fir* genes contained an additional putative regulatory element located between the −10 promoter and the CpxR binding site (Fig. 1). The *L. pneumophila* CpxR binding site was shown before to be similar to the *E. coli* recognition site (Fig. 2A), and a literature search revealed that the new putative regulatory element is highly similar to a sequence known as the consensus binding site of the PmrA response regulator (Fig. 2B), which is known to be part of the PmrAB two-component system (29). The PmrAB two-component system was identified first in *S. enterica* serovar Typhimurium as required for resistance of the bacteria to antimicrobial peptides such as polymyxin B (41), and since then, it has been shown in other pathogenic bacteria, such as *P. aeruginosa* (32), *E. carotovora* (23), and *E. coli* (19) to be involved in the regulation of genes that are related to virulence. The alignment of the regulatory regions shown in Fig. 1 enabled us to divide the *Legionella fir* genes into three groups, i.e., (i) genes that contain only the CpxR binding site, (ii) genes that contain both sites, and (iii) genes that contain only the suspected PmrA binding site. Most *fir* genes were found to belong to the second group (Fig. 1). When a phylogenetic tree was generated from the IcmQ protein sequences (which resulted in the same phylogenetic tree as was published before for the *mip* gene) (39) for a few repre-

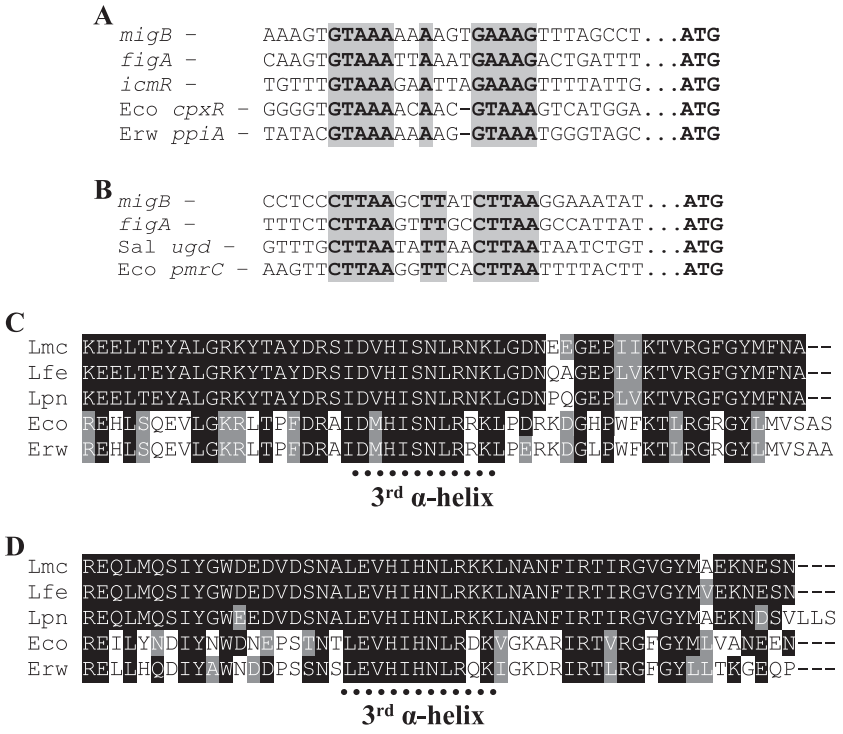

FIG. 2. The CpxR and PmrA proteins and their binding sites are highly conserved. The regulatory regions of the *migB* and *figA* genes were aligned with other regulatory sequences which are known to be regulated by CpxR (A) or PmrA (B) in other bacteria. The names of the bacteria and genes are indicated to the left of each sequence. Abbreviations: Eco, *E. coli*; Erw, *E. carotovora*; Sal, *S. enterica* serovar Typhimurium. The CpxR and PmrA consensus sequences are in bold and surrounded by gray. Sequence alignment of the C-terminal ends of the CpxR (C) and PmrA (D) proteins from different bacteria. Abbreviations: Lmc, *L. micdadei*; Lfe, *L. feeleii*; Lpn, *L. pneumophila*; Eco, *E. coli*; Erw, *E. carotovora*. The location of the third α-helix sequence of these proteins is indicated at the bottom of each alignment. The third α-helix was predicted by the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred). Accession numbers of the *migB*, *figA*, and *icmR* regulatory regions are as listed in the legend to Fig. 1. The rest of the accession numbers are as follows: *E. coli cpxR* and *pmrC* genes, NC000913; *E. carotovora ppiA* gene, NC004547; *S. enterica ugd* gene, NC003198. Accession numbers of the CpxR proteins: *L. micdadei*, EF094475; *L. feeleii*, EF094473; *L. pneumophila*, AAQ18123; *E. coli*, NP418348; *E. carotovora*, YP052398. Accession numbers of the PmrA proteins: *L. micdadei*, EF094474; *L. feeleii*, EF094472; *L. pneumophila*, AAU27375; *E. coli*, AAV92780; *E. carotovora*, YP052131.

sentatives from each group (Fig. 3), it was clear that the evolutionary events that led to the generation of the three "*fir* regulatory groups" perfectly correlate with it. The phylogenetic tree shown in Fig. 3 indicated that the suspected PmrA binding site entered prior to the entry of the CpxR binding site (it is present also in the *Coxiella burnetii fir* homologue *coxigA*), and the disappearance of the PmrA binding site is probably an event that occurred later during the course of evolution.

The CpxR and PmrA proteins from *L. micdadei* and *L. feeleii* are highly conserved. For further analysis, we chose to continue with *L. micdadei* and *L. feeleii*. *L. micdadei* is known as the second most common Legionnaires' disease agent in the world (2), and it was found to be less virulent than *L. pneumophila* in guinea pig and tissue culture models of infection (14, 53). It has been reported that *L. micdadei* does not inhibit phagosome-lysosome fusion and does not multiply within a ribosome-studded phagosome (26, 40, 53). *L. feeleii*, on the other hand, was never a subject of any kind of genetic research; however, it was shown to cause very few cases of Legionnaires' disease (55). The *L. micdadei migB* and *L. feeleii figA* genes were chosen to be investigated since they belong to the second group of *fir* genes containing both regulatory elements. The existence of a CpxR binding site and a PmrA putative binding

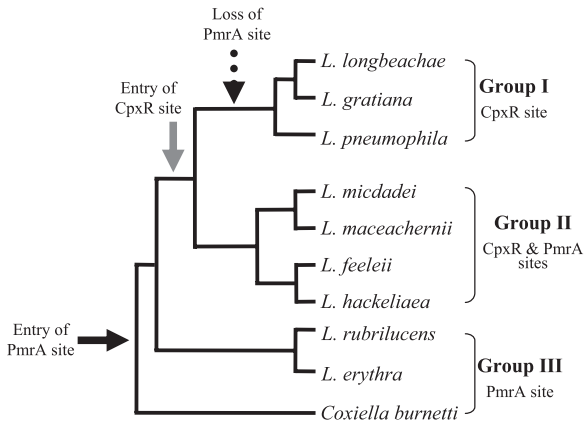

FIG. 3. The presence of the CpxR and PmrA binding sites is correlated with the evolutionary tree of the different *Legionella* species. A rectangular cladogram generated by the sequences of IcmQ proteins from nine *Legionella* species (accession numbers are the same as those listed for the *fir* genes in the legend to Fig. 1) and *C. burnetii* as an outgroup by the ClustalW program with the SRS server (http://srs.ebi.ac.uk/srsbin/cgi-bin/). The nine *Legionella* species chosen for this analysis are representatives of the three regulatory groups indicated on the right.

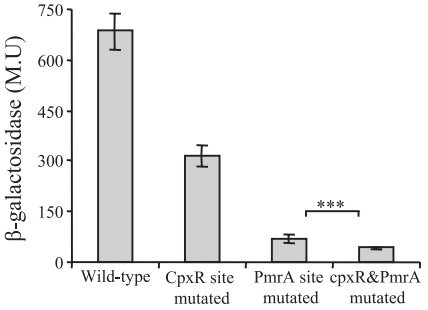

FIG. 4. The *L. micdadei* CpxR and PmrA binding sites are required for expression of *migB*. Plasmids containing the *migB*::*lacZ* (wild-type or mutated CpxR and/or PmrA binding sites) were introduced into *L. micdadei*, and their expression was measured at exponential phase by the β-galactosidase assay as described in Materials and Methods. The results (in Miller units [M.U.]) are the averages ± standard deviations of at least three independent experiments. Statistical analysis was performed with the standard *t* test. ***, $P < 0.0001$.

site in the same regulatory region represents a new type of regulation which has not been described before that occurs in the largest group of *fir* genes. CpxR and PmrA are both members of the OmpR family of response regulators which contain a winged helix-turn-helix DNA binding motif (1, 30, 31), and their third α-helix was shown to be involved in DNA binding and is highly conserved (5). To determine whether both CpxR and PmrA are directly involved in the regulation of the *fir* genes, we used low-stringency Southern hybridization with the *L. pneumophila cpxR* and *pmrA* genes as probes (in *L. pneumophila*, Lpg1292 was identified as the *pmrA* gene by a BLAST search) to clone the *L. micdadei* and *L. feeleii cpxR* and *pmrA* genes. As expected, the third α-helix was found to be highly conserved among the CpxR and PmrA proteins from *L. micdadei*, *L. feeleii*, and *L. pneumophila*, as well as *E. coli* and *E. carotovora* (Fig. 2C and D, respectively), strongly indicating that the expected target regulatory elements of these proteins in *L. micdadei* and *L. feeleii* will be similar to those of the other bacteria. When we compared the full-length CpxR and PmrA proteins, we found 41 to 50% identity between the *Legionella* proteins and those of *E. coli* and *E. carotovora*, while the identity of the third α-helix of the two proteins among the different bacteria was found to be 82% (Fig. 2C and D). This information, together with the fact that the *cpxR* and *pmrA* homologous genes from both *Legionella* species were found to be located upstream from *cpxA* and *pmrB* homologues, respectively (data not shown), strongly indicates that the genes identified are indeed the *Legionella* homologues of the CpxR and PmrA response regulators. It is interesting that the conservation of the third α-helix of the different PmrA proteins was found to be higher than among the different CpxR proteins, which were found to be highly conserved among the *Legionella* species but slightly different in comparison to *E. coli* and *E. carotovora* CpxR. This observation fits the differences found in the CpxR binding element in the *Legionella* species in comparison to the *E. coli* and *E. carotovora* genes (Fig. 2A) and might explain the inability of the *E. coli* CpxR response regulator to activate the expression of the *L. pneumophila icmR* gene (14).

The CpxR and PmrA binding sites are significant for the expression of the *L. micdadei migB* gene. To examine whether

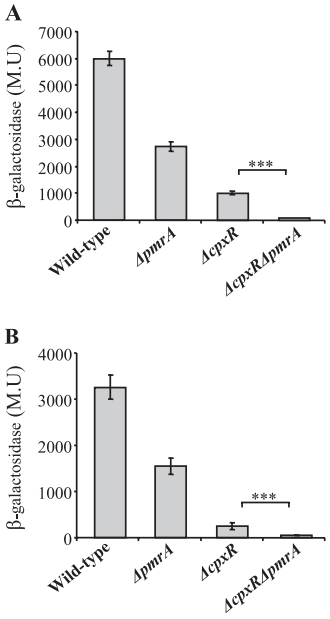

FIG. 5. The *L. pneumophila* CpxR and PmrA proteins regulate the expression of the *migB* and *figA* genes. Plasmids containing the *migB*::*lacZ* (A) or the *figA*::*lacZ* (B) fusion were introduced into four *L. pneumophila* strains, i.e., JR32 (wild type), *pmrA* mutant strain HK-PQ1 (Δ*pmrA*), *cpxR* mutant strain OG2002 (Δ*cpxR*), and *cpxR pmrA* double-mutant strain EA-CRPA (Δ*cpxR* Δ*pmrA*). Expression was measured at stationary phase by β-galactosidase assay. The results (in Miller units [M.U.]) are the averages ± standard deviations of at least three independent experiments. Statistical analyses were performed with the standard *t* test. ***, $P < 0.0001$.

CpxR and PmrA are involved in the regulation of the *migB* gene, we constructed a *migB*::*lacZ* fusion and three additional plasmids based on it, containing substitutions in the CpxR binding site or the putative PmrA binding site or in both of these sites together. The four resulting plasmids were introduced into *L. micdadei*, and the level of expression of the *migB* gene was determined by β-galactosidase assay as described in Materials and Methods. The results obtained showed that the mutations in the CpxR binding site decreased the expression of the *migB* gene to approximately half of the wild-type levels (Fig. 4). The mutation in the putative PmrA binding site was found to influence the expression of *migB* even more severely, while the combined mutation lowered the expression to nearly zero levels (Fig. 4). These results point out the relevance of these two regulatory elements for the regulation of the *migB* gene.

***L. pneumophila* CpxR and PmrA regulate the expression of the *migB* and *figA* genes.** To find out whether the *L. micdadei migB* gene is indeed regulated by the CpxR and PmrA proteins, we introduced the plasmid containing the *migB*::*lacZ* fusion into *L. pneumophila* containing insertions in the *cpxR* and/or the *pmrA* genes. The level of expression of the *migB* gene was drastically lowered in each of the single-mutant strains, whereas in the double-mutant strain, the expression was more severely lowered (Fig. 5A). Similar results were obtained for the *figA*::*lacZ* fusion in the same four *L. pneumophila* strains (Fig. 5B), indicating that the expression of the

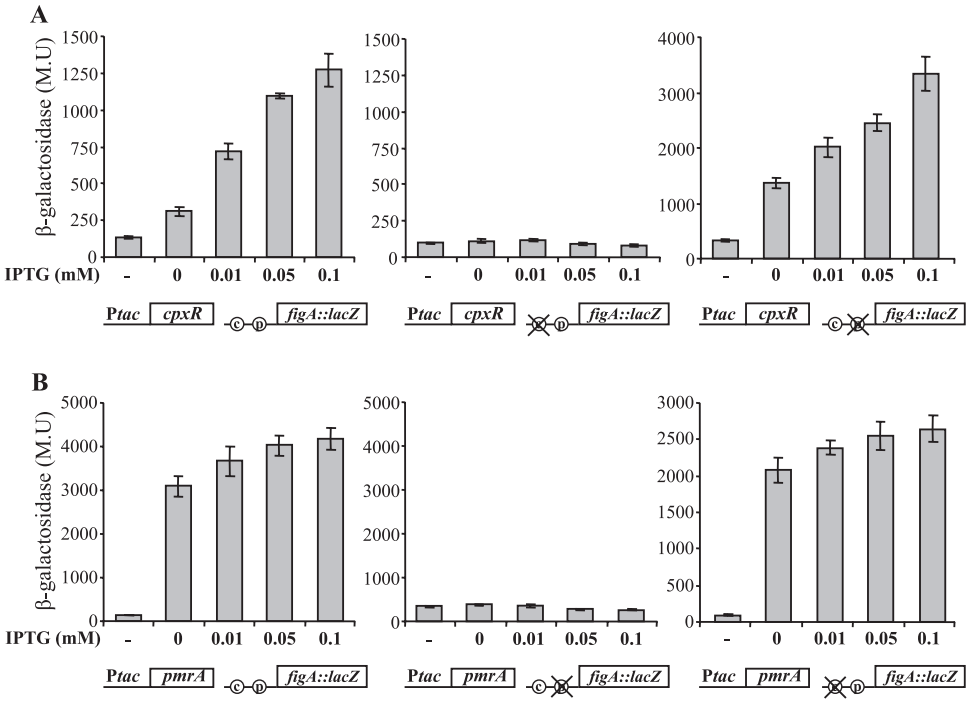

FIG. 6. The *L. feeleii* CpxR and PmrA proteins are direct positive regulators of the *figA* gene. Plasmids containing the *figA*::*lacZ* fusion (wild-type or mutated CpxR or PmrA binding sites, as indicated in the schemes below the bars as follows: c, CpxR binding site; p, PmrA binding site; X, mutated site) and the *L. feeleii cpxR* (A) or *pmrA* (B) gene under the control of the P_{tac} promoter (as indicated in the schemes below the bars) were examined in *E. coli* MC1061. Levels of expression of the different plasmids were measured at different IPTG concentrations (indicated below the bars) by the β-galactosidase assay at exponential phase. The scales of the two graphs on the right are different because of the differences in the initial levels of expression of the mutated fusions examined. As negative controls (indicated by a minus sign), the plasmids containing the *figA*::*lacZ* fusion (with or without the mutations) without the *cpxR* (A) or the *pmrA* (B) gene were used. The results (in Miller units [M.U.]) are the averages ± standard deviations of at least three independent experiments.

migB and *figA* genes is regulated by the CpxR and PmrA response regulators in an additive manner.

The *L. feeleii* CpxR and PmrA proteins are direct regulators of the *figA* gene. To find out whether the CpxR and PmrA response regulators directly influence the expression of the *fir* genes examined and if they function independently, we used the *L. feeleii figA*::*lacZ* fusion and constructed two additional plasmids containing substitutions in the CpxR or the PmrA binding sites in a way similar to what was described for *migB*. We then cloned into these three plasmids the *L. feeleii cpxR* or *pmrA* gene under the control of the P_{tac} promoter (induced by IPTG). The resulting plasmids were introduced into *E. coli* MC1061, and the expression of the *figA* gene with or without the mutations at the two regulatory elements was determined with different concentrations of IPTG in such a way that in each experiment a single plasmid containing one regulatory sequence and one regulator under the control of the P_{tac} promoter was examined. The results in Fig. 6 show that *L. feeleii* CpxR positively regulates the *figA* gene only when its binding site is intact, and the activation increased as the concentration of IPTG added increased, indicating direct regulation by the CpxR response regulator. Moreover, the *figA*::*lacZ* fusion that contained the substitution in the CpxR site was not influenced at all by the addition of IPTG (Fig. 6A). Similar results were obtained with *L. feeleii* PmrA, but in this case the presence of the *pmrA* gene on the same plasmid with the *figA*::*lacZ* fusion without the addition of IPTG drastically increased the expression of *figA* (Fig. 6B). This result probably occurred because of the leakiness of the P_{tac} promoter but strongly indicates that low levels of the PmrA regulator were sufficient for the activation of the *figA* gene (a result that fits the strong effect obtained with the mutation of the PmrA site in the *migB* gene [Fig. 4]). As expected, the *figA*::*lacZ* fusion containing the substitution in the PmrA binding site was not affected by the addition of IPTG (Fig. 6B). Reciprocal experiments showed that the expression of the *figA*::*lacZ* fusion containing a mutation in the PmrA binding site was activated by the CpxR protein (Fig. 6A), and similarly, the *figA*::*lacZ* fusion containing a mutated CpxR binding site was activated by the PmrA protein (Fig. 6B). These results show that each of the regulators activates the expression of the *figA* gene independently, even if the site of the second regulator is missing. The results presented strongly relate the two response regulators with their binding sites and show that they both positively regulate the expression of the same gene directly and independently.

The CpxR and PmrA proteins bind directly to the *migB* and *figA* regulatory regions. After showing that the CpxR and PmrA response regulators positively regulate the *migB* and *figA* genes, we wanted to prove the direct binding between the proteins and the regulatory regions. To do that, we purified the *L. micdadei* and *L. feeleii* CpxR and PmrA proteins, tagged all four of them with an N-terminal six-histidine tag, and performed gel mobility shift assays with the purified proteins in increasing amounts and the *migB* or *figA* regulatory region

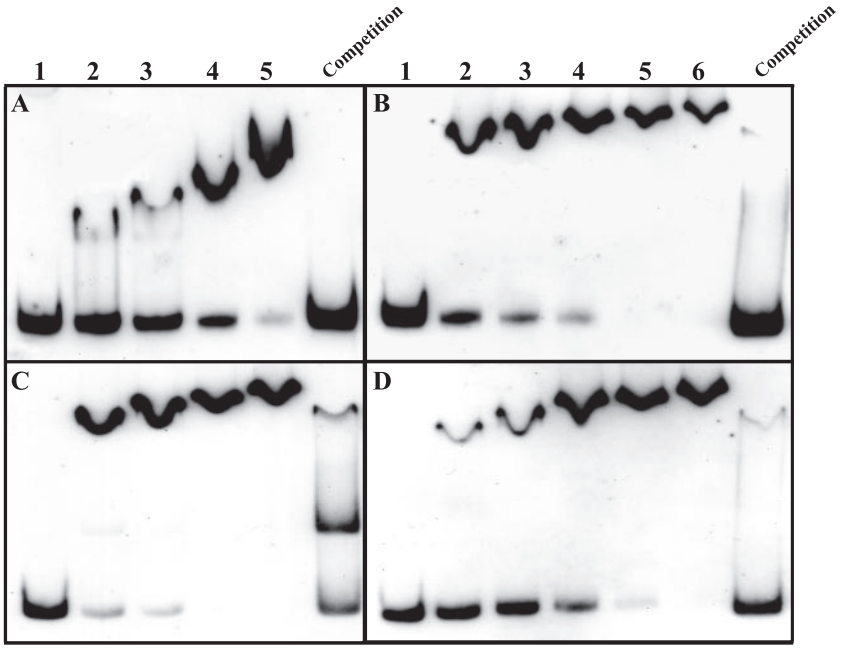

FIG. 7. The *L. micdadei* and *L. feeleii* CpxR and PmrA proteins bind the regulatory regions of the *migB* and *figA* genes. Mobility shift assays were performed with the *L. micdadei* pure His6-CpxR (A) and His6-PmrA (B), the *L. feeleii* pure His6-CpxR (C) and His6-PmrA (D), and the DIG-labeled *migB* (A and B) and *figA* (C and D) regulatory regions. The first lane in each gel did not contain any protein. The rest of the numbered lanes contained increasing amounts of the relevant proteins in twofold increments, starting from 0.125 μg (A), 0.25 μg (B), 0.5 μg (C), and 0.25 μg (D). Competition was performed by incubating the protein amount added to the second lane (the smallest amount) with 200 ng of the unlabeled probe as a specific competitor for 15 min prior to the addition of the DIG-labeled probe. The lane numbers and competition in each lane are indicated.

labeled with DIG-11-ddUTP. The results of these experiments showed direct binding of the *L. micdadei* CpxR (Fig. 7A) and PmrA (Fig. 7B) proteins to the *migB* regulatory region and of the *L. feeleii* CpxR (Fig. 7C) and PmrA (Fig. 7D) proteins to the *figA* regulatory region. To examine whether this binding was specific, we added unlabeled probe to the binding reaction mixture and found that the unlabeled probes competed with the labeled probes for association with the relevant protein (Fig. 7). To further investigate the association of these regulators with their target sequences, we examined the PmrA (Fig. 8A and B) and CpxR (Fig. 8C and D) proteins for binding to the mutated *migB* and *figA* regulatory regions. Binding of the PmrA protein to the *migB* (Fig. 8A) and *figA* (Fig. 8B) regulatory regions that contained a mutation in the PmrA binding site was significantly reduced. In addition, the CpxR regulator did not bind at all to the *migB* (Fig. 8C) and *figA* (Fig. 8D) regulatory regions which contained a mutation in the CpxR binding site. Moreover, as shown in Fig. 8E, the CpxR and PmrA regulators were able to bind simultaneously to the same regulatory region, as indicated by a shift that is stronger than the one observed for each of the proteins by itself. The results obtained by the different gel mobility shift assays prove that binding of these two response regulators to their target sequences is direct, specific, and independent.

CpxR and PmrA exist in the genomes of species from the three groups. As shown in Fig. 1, the *L. pneumophila fir* gene—*icmR*—does not contain the PmrA binding site although a PmrA-encoding gene is present in the *L. pneumophila* genome. Therefore, we were interested in examining whether there is a connection between the presence of the CpxR and/or PmrA binding site in the *fir* regulatory region and the presence of its corresponding regulator in *Legionella* species that belong to each of the three *fir* regulatory groups described above. To find out whether these species contain the coding sequence of the *cpxR* and *pmrA* genes, low-stringency Southern hybridizations were performed. The genomic DNAs from two representatives of each of the three groups (group I, *L. pneumophila* and *L. longbeachae*, whose *fir* genes contain only the CpxR binding site; group II, *L. micdadei* and *L. feeleii*, whose *fir* genes contain both regulatory elements; and group III, *L. rubrilucens* and *L. erythra*, whose *fir* genes contain only the PmrA binding site) were hybridized with the *cpxR* and *pmrA* genes from *L. pneumophila* under low-stringency conditions. The hybridizations results showed that all six of the species examined contained both genes (see Fig. S1 in the supplemental material), indicating that these two response regulators (CpxR and PmrA) might regulate the expression of other genes and that the appearance or loss of the regulatory element from the regulatory region of the different *fir* genes is not due to the existence or disappearance of the corresponding regulatory protein.

DISCUSSION

L. pneumophila is known to infect and replicate inside human macrophages and amoebae (22) using the Icm/Dot type IV secretion system, which is encoded by 25 genes (44, 45, 51, 52). The IcmR and IcmQ proteins are two components of the Icm/Dot system that were shown to be located in the bacterial

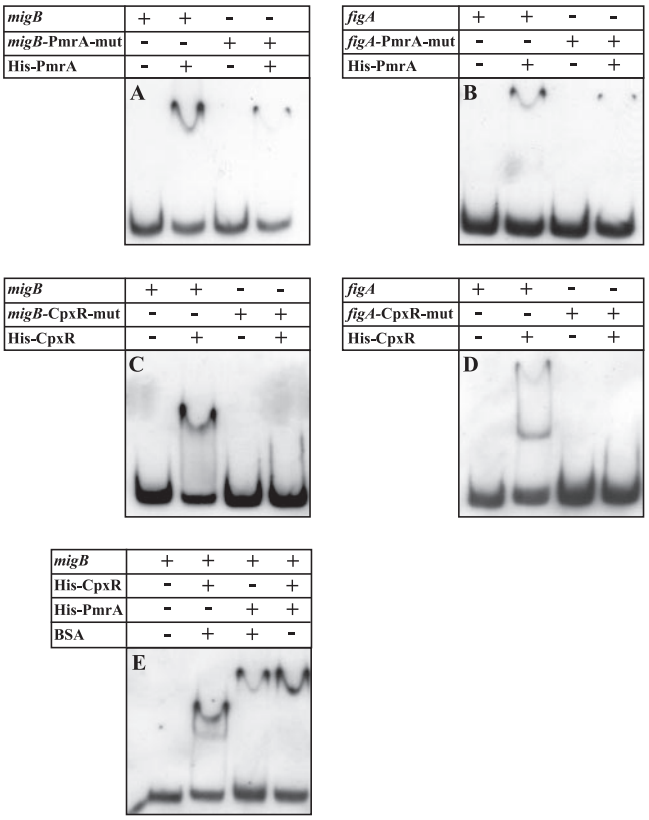

FIG. 8. Binding of the CpxR and PmrA proteins to their target genes is specific and independent. Binding of the PmrA protein to the *migB* and *figA* probes was decreased when the PmrA site was mutated (A and B, respectively), and the CpxR protein did not bind to these probes when the CpxR site was mutated (C and D, respectively). The probe or protein added to each reaction mixture is indicated above each lane. (E) Independence of binding of the CpxR and PmrA proteins was examined by incubating the *L. micdadei* CpxR and/or PmrA proteins with equal amounts of the *migB* probe. Bovine serum albumin was added to the reaction mixtures containing the same amounts of individual proteins. The addition of each protein (60 ng) is indicated above each lane.

cytoplasm (5, 43). IcmQ was shown to consist of pore-forming activity inside lipid membranes by self-interaction, which was found to be regulated by the association of IcmQ with IcmR—its chaperone (11). The genome of *C. burnetii*, the causative agent of Q fever, was also found to contain a complete Icm/Dot system, except for the *icmR* gene (56, 58), which was shown to be replaced with a different gene—*coxigA*—that encodes a protein that was shown to interact with its corresponding IcmQ protein (13). It was previously shown that, similarly to the situation in *C. burnetii*, several other *Legionella* species that were found to grow within human macrophages and different types of protozoa and to cause Legionnaires' disease (15) contain in their genomes completely different genes upstream from a highly conserved *icmQ* gene. These genes were found to function similarly together with their corresponding *icmQ* genes (12, 13) and therefore were named *fir* genes, for functional homologues of *icmR* (13).

In this study, we examined the regulation of the hypervariable *fir* genes in order to learn more about the functional similarities between them. Alignment of the regulatory regions of 30 *fir* genes clearly showed that most of them contain a CpxR binding site, and to our surprise, the alignment revealed an additional element which was identified as the consensus binding sequence of the PmrA response regulator (Fig. 1). The existence of these two binding sites divided the *fir* genes into three regulatory groups, which contain either one of these binding sites or both of them (there is not even one *fir* gene that does not contain at least one of these sites). It is interesting that each of the three groups contains at least one *Legionella* species that was previously isolated from patients, for example, *L. pneumophila* (group I), *L. micdadei* (group II), and *L. erythra* (group III) (15). This information indicates the lack of correlation between the existence of these regulatory elements and the ability of the relevant species to cause pneumonia in humans. In the present study, we chose to further analyze the regulatory regions of *L. micdadei migB* and *L. feeleii figA*, both containing both binding sites. We showed that the CpxR and PmrA proteins directly bind to the regulatory sequences of the *migB* and *figA* genes and positively regulate their expression.

Regulation of one gene by two different two-component systems could be explained by the necessity of a certain gene to be expressed in response to different signals which activate different two-component systems, and few such cases have been described before. For example, the *S. enterica ugd* gene is triggered by the PmrAB system, which is activated by a high concentration of extracytoplasmic Fe^{3+} and also by the RcsCB system that responds to cell envelope stress, thus enabling one gene to be expressed under different stress conditions (33). The expression of the *ugd* gene was also shown to be elevated in response to low levels of Mg^{2+}, which activate the PhoPQ two-component system, which activates the expression of the PmrD protein that consequently activates the PmrAB system in a posttranscriptional manner and results in up-regulation of the *ugd* gene (25). The latter is an example of a case in which two response regulators, PhoP and PmrA, which are both members of the winged helix-turn-helix protein family (1) control the expression of a single gene. The regulation of the *csgD* gene in *E. coli* by the OmpR and CpxR response regulators upon two distinct signals is another example of the activation of one gene by two members of the winged helix-turn-helix family under different conditions (24).

The CpxR and PmrA response regulators that were shown here to bind the same regulatory regions and activate the same *fir* genes are both members of the winged helix-turn-helix protein family (1), but they have never been shown to directly regulate the expression of the same gene. We show here evidence regarding the evolution of regulatory sequences among a large number of *Legionella* species regardless of the existence of the corresponding regulators in the bacteria, an evolution which might have occurred in order to allow optimal adaptation of a certain species to its environment. Group I was shown to include *Legionella* species that contain only the PmrA regulatory element, and since this regulatory element was also found in the regulatory region of the *C. burnetii fir* gene (which is not part of the genus *Legionella*), it is most likely that this is an ancestral regulatory element. At some point during evolution, a second regulatory element was acquired, the CpxR regulatory element, and group II was formed, probably in order to enable the relevant *fir* genes to be expressed as a

response to an additional environmental signal sensed by the cognate sensor kinase CpxA. The disappearance of the ancestral PmrA regulatory element formed the third regulatory group and might have happened since the corresponding species existed in a niche where the expression of the *fir* genes was no longer required as a response to the signal sensed by the PmrB sensor kinase. However, the fact that the CpxR and PmrA regulators are able to activate the *fir* genes independently from each other might lead to the hypothesis that each of the three regulatory groups exists in an environment that requires different expression patterns of the relevant *fir* gene.

ACKNOWLEDGMENTS

This work was supported by a grant from the Israel Science Foundation (393/03).

We thank Efrat Altman for the construction of the *L. pneumophila cpxR pmrA* double mutant.

REFERENCES

1. **Aravind, L., V. Anantharaman, S. Balaji, M. M. Babu, and L. M. Iyer.** 2005. The many faces of the helix-turn-helix domain: transcription regulation and beyond. FEMS Microbiol. Rev. **29:**231–262.
2. **Benin, A. L., R. F. Benson, and R. E. Besser.** 2002. Trends in Legionnaires' disease, 1980–1998: declining mortality and new patterns of diagnosis. Clin. Infect. Dis. **35:**1039–1046.
3. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
4. **Chen, J., K. S. de Felipe, M. Clarke, H. Lu, O. R. Anderson, G. Segal, and H. A. Shuman.** 2004. *Legionella* effectors that promote nonlytic release from protozoa. Science **303:**1358–1361.
5. **Chen, Y., W. R. Abdel-Fattah, and F. M. Hulett.** 2004. Residues required for *Bacillus subtilis* PhoP DNA binding or RNA polymerase interaction: alanine scanning of PhoP effector domain transactivation loop and alpha helix 3. J. Bacteriol. **186:**1493–1502.
6. **Coers, J., J. C. Kagan, M. Matthews, H. Nagai, D. M. Zuckman, and C. R. Roy.** 2000. Identification of Icm protein complexes that play distinct roles in the biogenesis of an organelle permissive for *Legionella pneumophila* intracellular growth. Mol. Microbiol. **38:**719–736.
7. **Conover, G. M., I. Derre, J. P. Vogel, and R. R. Isberg.** 2003. The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol. Microbiol. **48:**305–321.
8. **De Wulf, P., A. M. McGuire, X. Liu, and E. C. Lin.** 2002. Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in *Escherichia coli*. J. Biol. Chem. **277:**26652–26661.
9. **Dong, J., S. Iuchi, H. S. Kwan, Z. Lu, and E. C. Lin.** 1993. The deduced amino-acid sequence of the cloned *cpxR* gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of *Escherichia coli*. Gene **136:**227–230.
10. **Duménil, G., and R. R. Isberg.** 2001. The *Legionella pneumophila* IcmR protein exhibits chaperone activity for IcmQ by preventing its participation in high-molecular-weight complexes. Mol. Microbiol. **40:**1113–1127.
11. **Duménil, G., T. P. Montminy, M. Tang, and R. R. Isberg.** 2004. IcmR-regulated membrane insertion and efflux by the *Legionella pneumophila* IcmQ protein. J. Biol. Chem. **279:**4686–4695.
12. **Feldman, M., and G. Segal.** 2004. A specific genomic location within the *icm*/*dot* pathogenesis region of different *Legionella* species encodes functionally similar but nonhomologous virulence proteins. Infect. Immun. **72:**4503–4511.
13. **Feldman, M., T. Zusman, S. Hagag, and G. Segal.** 2005. Coevolution between nonhomologous but functionally similar proteins and their conserved partners in the *Legionella* pathogenesis system. Proc. Natl. Acad. Sci. USA **102:**12206–12211.
14. **Fields, B. S., J. M. Barbaree, E. B. Shotts, Jr., J. C. Feeley, W. E. Morrill, G. N. Sanden, and M. J. Dykstra.** 1986. Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. Infect. Immun. **53:**553–559.
15. **Fields, B. S., R. F. Benson, and R. E. Besser.** 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15:**506–526.
16. **Gal-Mor, O., and G. Segal.** 2003. Identification of CpxR as a positive regulator of *icm* and *dot* virulence genes of *Legionella pneumophila*. J. Bacteriol. **185:**4908–4919.
17. **Gal-Mor, O., T. Zusman, and G. Segal.** 2002. Analysis of DNA regulatory elements required for expression of the *Legionella pneumophila icm* and *dot* virulence genes. J. Bacteriol. **184:**3823–3833.
18. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835–1842.
19. **Hagiwara, D., T. Yamashino, and T. Mizuno.** 2004. A genome-wide view of the *Escherichia coli* BasS-BasR two-component system implicated in iron-responses. Biosci. Biotechnol. Biochem. **68:**1758–1767.
20. **Haydel, S. E., W. H. Benjamin, Jr., N. E. Dunlap, and J. E. Clark-Curtiss.** 2002. Expression, autoregulation, and DNA binding properties of the *Mycobacterium tuberculosis* TrcR response regulator. J. Bacteriol. **184:**2192–2203.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
22. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
23. **Hyytiäinen, H., S. Sjoblom, T. Palomaki, A. Tuikkala, and E. Tapio Palva.** 2003. The PmrA-PmrB two-component system responding to acidic pH and iron controls virulence in the plant pathogen *Erwinia carotovora* ssp. *carotovora*. Mol. Microbiol. **50:**795–807.
24. **Jubelin, G., A. Vianney, C. Beloin, J. M. Ghigo, J. C. Lazzaroni, P. Lejeune, and C. Dorel.** 2005. CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. J. Bacteriol. **187:**2038–2049.
25. **Kox, L. F., M. M. Wosten, and E. A. Groisman.** 2000. A small protein that mediates the activation of a two-component system by another two-component system. EMBO J. **19:**1861–1872.
26. **Levi, M. H., A. W. Pasculle, and J. N. Dowling.** 1987. Role of the alveolar macrophage in host defense and immunity to *Legionella micdadei* pneumonia in the guinea pig. Microb. Pathog. **2:**269–282.
27. **Luo, Z. Q., and R. R. Isberg.** 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc. Natl. Acad. Sci. USA **101:**841–846.
28. **Machner, M. P., and R. R. Isberg.** 2006. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. Dev. Cell **11:**47–56.
29. **Marchal, K., S. De Keersmaecker, P. Monsieurs, N. van Boxel, K. Lemmens, G. Thijs, J. Vanderleyden, and B. De Moor.** 2004. In silico identification and experimental validation of PmrAB targets in *Salmonella typhimurium* by regulatory motif detection. Genome Biol. **5:**R9.
30. **Martínez-Hackert, E., and A. M. Stock.** 1997. The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. Structure **5:**109–124.
31. **Martínez-Hackert, E., and A. M. Stock.** 1997. Structural relationships in the OmpR family of winged-helix transcription factors. J. Mol. Biol. **269:**301–312.
32. **McPhee, J. B., S. Lewenza, and R. E. Hancock.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**205–217.
33. **Mouslim, C., and E. A. Groisman.** 2003. Control of the *Salmonella ugd* gene by three two-component regulatory systems. Mol. Microbiol. **47:**335–344.
34. **Murata, T., A. Delprato, A. Ingmundson, D. K. Toomre, D. G. Lambright, and C. R. Roy.** 2006. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. Nat. Cell Biol. **8:**971–977.
35. **Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn, and C. R. Roy.** 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science **295:**679–682.
36. **Ninio, S., D. M. Zuckman-Cholon, E. D. Cambronne, and C. R. Roy.** 2005. The *Legionella* IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation. Mol. Microbiol. **55:**912–926.
37. **Raivio, T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Annu. Rev. Microbiol. **55:**591–624.
38. **Raivio, T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J. Bacteriol. **179:**7724–7733.
39. **Ratcliff, R. M., J. A. Lanser, P. A. Manning, and M. W. Heuzenroesder.** 1998. Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. J. Clin. Microbiol. **36:**1560–1567.
40. **Rechnitzer, C., and J. Blom.** 1989. Engulfment of the Philadelphia strain of *Legionella pneumophila* within pseudopod coils in human phagocytes. Comparison with other *Legionella* strains and species. APMIS **97:**105–114.
41. **Roland, K. L., L. E. Martin, C. R. Esther, and J. K. Spitznagel.** 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. J. Bacteriol. **175:**4154–4164.
42. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
43. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.
44. **Segal, G., M. Feldman, and T. Zusman.** 2005. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. FEMS Microbiol. Rev. **29:**65–81.
45. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial

conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
46. **Segal, G., and E. Z. Ron.** 1993. Heat shock transcription of the *groESL* operon of *Agrobacterium tumefaciens* may involve a hairpin-loop structure. J. Bacteriol. **175:**3083–3088.
47. **Segal, G., and H. A. Shuman.** 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila*. Infect. Immun. **65:**5057–5066.
48. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilize the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
49. **Shohdy, N., J. A. Efe, S. D. Emr, and H. A. Shuman.** 2005. Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. Proc. Natl. Acad. Sci. USA **102:**4866–4871.
50. **Tamayo, R., A. M. Prouty, and J. S. Gunn.** 2005. Identification and functional analysis of *Salmonella enterica* serovar Typhimurium PmrA-regulated genes. FEMS Immunol. Med. Microbiol. **43:**249–258.
51. **Vincent, C. D., J. R. Friedman, K. C. Jeong, E. C. Buford, J. L. Miller, and J. P. Vogel.** 2006. Identification of the core transmembrane complex of the *Legionella* Dot/Icm type IV secretion system. Mol. Microbiol. **62:**1278–1291.
52. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
53. **Weinbaum, D. L., R. R. Benner, J. N. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz.** 1984. Interaction of *Legionella micdadei* with human monocytes. Infect. Immun. **46:**68–73.
54. **Wiater, L. A., A. B. Sadosky, and H. A. Shuman.** 1994. Mutagenesis of *Legionella pneumophila* using Tn*903*dll*lacZ*: identification of a growth-phase-regulated pigmentation gene. Mol. Microbiol. **11:**641–653.
55. **Yu, V. L., J. F. Plouffe, M. C. Pastoris, J. E. Stout, M. Schousboe, A. Widmer, J. Summersgill, T. File, C. M. Heath, D. L. Paterson, and A. Chereshsky.** 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J. Infect. Dis. **186:**127–128.
56. **Zamboni, D. S., S. McGrath, M. Rabinovitch, and C. R. Roy.** 2003. *Coxiella burnetii* express type IV secretion system proteins that function similarly to components of the *Legionella pneumophila* Dot/Icm system. Mol. Microbiol. **49:**965–976.
57. **Zusman, T., G. Aloni, E. Halperin, H. Kotzer, E. Degtyar, M. Feldman, and G. Segal.** 2007. The response regulator PmrA is a major regulator of the *icm*/*dot* type-IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. Mol. Microbiol. **63:**1508–1523.
58. **Zusman, T., G. Yerushalmi, and G. Segal.** 2003. Functional similarities between the *icm*/*dot* pathogenesis systems of *Coxiella burnetii* and *Legionella pneumophila*. Infect. Immun. **71:**3714–3723.