A Pair of Highly Conserved Two-Component Systems Participates in the Regulation of the Hypervariable FIR Proteins in Different Legionella Species
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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 GTAAAnnmmGTAAA (8), whereas in Legionella species, it was shown to be GTAAAnnmmnGAAAG (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...
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 $f_i$ 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, mutation-negative mutant of L. pneumophila Philadelphia-1, which is a wild-type strain in terms of intrinsic virulence (43); OG2002, a pmrA 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.

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 with genomic DNA of L. micdadei and L. feeleii cpxR, pMF-mic-His-pmrA, pMF-feel-His-cpxR, and pMF-feel-His-pmrA plasmids as described previously (47). For the plasmids and primers used in this study, see Tables S1 and S2 in the supplemental material.

Cloning of the cpxR, pmrA, and migB genes. The genomic DNAs 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 pUC18 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 by electroporation, and the membrane was 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.

Cloning 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 pUC18 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 by electroporation, and the membrane was 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.

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 pUC18 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 by electroporation, and the membrane was 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.

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 BamH and EcoRI, and sequenced to generate the pmF-migB-lacZ and pmF-figA-lacZ plasmids, respectively (see Table S1 in the supplemental material).

Consortium construction in the CpxR and PmrA binding sites. To generate substitutes 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 GTAAA to AGCCC, and the downstream parts 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 CpxR site of migB, migB-pmrA-mut-F and migB-pmrA-mut-R for the PmrA site of migB, figA-cpx-mut-F and figA-cpx-mut-R for the regulation region of the CpxR site of figA, and figA-pmrA-mut-F and figA-pmrA-mut-R for the PmrA site of figA (see Table S2 in the supplemental material). The resulting fragments were digested with BamH and EcoRI, cloned into pGS-lac-02, and sequenced. The resulting pmF-MigB-cpx-mut and pmF-MigB-pmrA-mut plasmids contain the substitutes in the regulatory region of the migB gene and the migB-pmrA-mut F and R for the mutagenesis of the CpxR site of migB, and the pmF-MigB-cpx-mut and pmF-MigB-pmrA-mut R for the mutagenesis of the CpxR site of migB, and the figA-cpx-mut-F and figA-cpx-mut-R for the mutagenesis of the CpxR site of figA, and the 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).

Construction of isopropyl-$\beta$-D-thiogalactopyranoside (IPTG)-inducible cpxR and pmrA genes. The L. feeleii cpxR and pmrA genes were amplified by PCR with the primers feel-CpxR-EcoI and feel-CpxR-His-Bam for the cpxR gene under the control of the PcpxR regulon, and feel-CmrA-EcoI and feel-CmrA-His-Bam for the pmrA gene under the control of the PpmrA regulon (see Table S2 in the supplemental material). The PCR products were then digested with EcoRI and BamHI and cloned into the pMB207 downstream from the $P_{cpxR}$ promoter to generate the pmF-feel-cpxR-207 and pmF-feel-pmrA-207 plasmids. The resulting fragments were then digested with XbaI and EcoRI, and the fragment resulting from the double-strand cut containing the lacZ gene, as well as the fragment containing the mutations in the CpxR or PmrA binding site described above, were digested with XbaI and Smal, to generate the pmF-fac, pmF-fap, pmF-cdc, pmF-cdp, pmF-pdc, and pmF-pdp plasmids (see Table S1 in the supplemental material).

Consortium construction of the L. pneumophila cpxR pmrA double mutant. To generate an L. pneumophila cpxR pmrA double mutant, the gentamicin resistance cassette was ligated into EcoRV plasmid pOG-cpxR with EcoRV digested by pOG-cpxR and digested with an insert in the cpxR gene, which was then ligated into Smal and cloned into pLAW344 (54) digested with EcoRV to generate pEA-cpxR-Gm-GR. This plasmid was used for an allelic-exchange
This is an image of a page from a scientific paper. The text appears to be discussing the regulatory elements of the \textit{Legionella} species and their role in virulence. The paper uses a phylogenetic tree to show the relationship between different \textit{Legionella} species and their regulatory elements.

The results section of the paper is particularly highlighted, discussing the consistency of regulatory elements across different species and their implications for virulence.

The text includes several figures and tables, one of which is a phylogenetic tree showing the relationships between different \textit{Legionella} species. The tree is used to illustrate the evolutionary relationships and the conservation of regulatory elements.

The paper also mentions the use of translational fusions and the CpxR binding site as key regulatory elements in \textit{Legionella} species. The gene\textit{icmR} is found to be conserved across different species, indicating its importance in the regulation of virulence genes.

Overall, the paper provides a comprehensive analysis of the regulatory networks in \textit{Legionella} species, highlighting the significance of these networks in the context of virulence and pathogenesis.

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**FIG. 1.** Thirty \textit{fir} genes contain the CpxR and/or the PmrA regulatory element. The regulatory sequences of the \textit{fir} genes from 29 \textit{Legionella} species and \textit{C. burnettii} are shown. The name of the \textit{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 \textit{CpxR} binding site was found to be present in most sequences and to be highly conserved. When a phylogenetic tree was generated from the \textit{IcmQ} protein sequences (which resulted in the same phylogenetic tree as that shown in Fig. 1), the \textit{IcmQ} protein sequences were aligned. The name of the \textit{IcmQ} protein sequence is indicated on the right of each sequence.

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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 coxiG), 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 CpxR and PmrA proteins from these bacteria are highly conserved. For further analysis, we chose to continue with L. micdadei and L. feeleii.

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.

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.
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. felleii 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. felleii, 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. felleii 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 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
pmrA gene under the control of the P\textsubscript{L} sequence and one regulator under the control of the P\textsubscript{L}.

In each experiment a single plasmid containing one regulatory region was examined in E. coli MC1061. Levels of expression of the different plasmids were measured at different IPTG concentrations (indicated below the bars in Miller units [M.U.]) are the averages ± standard deviations of at least three independent experiments.

**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 fig\textit{A} genes examined and if they function independently, we used the L. feeleii fig\textit{A}:lac\textit{Z} 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 \textit{migB}. We then cloned into these three plasmids the \textit{L. feeleii cpxR} or \textit{pmrA} gene under the control of the P\textsubscript{L} promoter (induced by IPTG). The resulting plasmids were introduced into \textit{E. coli} MC1061, and the expression of the fig\textit{A} 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\textsubscript{L} promoter was examined. The results in Fig. 6 show that \textit{L. feeleii} CpxR positively regulates the fig\textit{A} 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 fig\textit{A}:lac\textit{Z} 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 \textit{L. feeleii PmrA}, but in this case the presence of the pmr\textit{A} gene on the same plasmid with the fig\textit{A}:lac\textit{Z} fusion without the addition of IPTG drastically increased the expression of fig\textit{A} (Fig. 6B). This result probably occurred because of the leakiness of the P\textsubscript{L} promoter but strongly indicates that low levels of the PmrA regulator were sufficient for the activation of the fig\textit{A} gene (a result that fits the strong effect obtained with the mutation of the PmrA site in the \textit{migB} gene [Fig. 4]). As expected, the fig\textit{A}:lac\textit{Z} 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 fig\textit{A}:lac\textit{Z} fusion containing a mutation in the PmrA binding site was activated by the CpxR protein (Fig. 6A), and similarly, the fig\textit{A}:lac\textit{Z} 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 fig\textit{A} 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 \textit{migB} and \textit{figA} regulatory regions.** After showing that the CpxR and PmrA response regulators directly regulate the \textit{migB} and \textit{figA} genes, we wanted to prove the direct binding between the proteins and the regulatory regions. To do that, we purified the \textit{L. micdadei} and \textit{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 \textit{migB} or \textit{figA} regulatory region.
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 IcmO proteins are two components of the Icm/Dot system that were shown to be located in the bacterial
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. feelei* 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. burnettii* 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

![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 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. burnettii*, 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. burnettii*, 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
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.

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REFERENCES


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