

The Response Regulator CpxR Directly Regulates Expression of Several *Legionella pneumophila* *icm/dot* Components as Well as New Translocated Substrates^{∇†}

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Legionella pneumophila has been shown to utilize the *icm/dot* type IV secretion system for pathogenesis. This system was shown to be composed of *icm/dot* complex components and accessory proteins, as well as a large number of translocated substrates. Bioinformatic analysis of the regulatory regions of all the genes revealed that several *icm/dot* genes, as well as two genes encoding *icm/dot* translocated substrates, contain the conserved CpxR regulatory element, a regulator that has been shown previously to control the expression of the *icmR* gene. An experimental analysis, which included a comparison of gene expression in a *L. pneumophila* wild-type strain and gene expression in a *cpxR* deletion mutant, construction of mutants with mutations in the CpxR conserved regulatory elements, controlled expression studies, and mobility shift assays, demonstrated the direct relationship between the CpxR regulator and the expression of the genes. Furthermore, genomic analysis identified nine additional genes that contain a putative CpxR regulatory element; five of these genes (two *legA* genes and three *ceg* genes) were suggested previously to be putative *icm/dot* translocated substrates. The three *ceg* genes identified, which were shown previously to contain a putative PmrA regulatory element, were found here to be regulated by both CpxR and PmrA. The other six genes (two *legA* genes and four new genes products) were found to be regulated by CpxR. Moreover, using the CyaA translocation assay, these nine gene products were found to be translocated into host cells in an Icm/Dot-dependent manner. Our results establish that the CpxR regulator is a fundamental regulator of the *icm/dot* type IV secretion system in *L. pneumophila*.

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular pathogen that multiplies in nature inside amoebae and in human macrophages during disease (24, 47). The *icm/dot* type IV secretion system is the major virulence system known in *L. pneumophila*; this system consists of 26 Icm/Dot proteins that probably constitute the secretion complex itself, as well as accessory proteins, such as chaperones (for a review, see reference 49). In the last 5 years, a growing number of protein substrates (RalF, LidA, Lep, Sid, Vip, Wip, Ylf, Leg, Vpd, Drr, and Ceg) that are translocated into host cells via the *icm/dot* secretion system were identified (for a review, see reference 42); in most cases the function of these proteins is not known, but two of them (SidF and SdhA) were shown to be required for inhibition of host cell death (5, 27) and four others (RalF, LidA, DrrA/SidM, and SidJ) were shown to be required for manipulation of host cell vesicular trafficking (28, 31, 37, 38), as well as other functions (11). Even though many *icm/dot* genes and many more *icm/dot* translocated substrates (IDTS), as well as potential IDTS, have been identified, there is only a limited amount of information about direct regulators that control the levels of expression of these genes.

A group of stationary-phase-related regulatory factors, in-

cluding the stationary-phase sigma factor RpoS (2, 4, 20, 62), the two-component system LetAS (3, 19, 21, 30, 51), the ppGpp synthetase RelA (62), the translational repressor CsrA (16, 17, 36), the posttranscriptional regulator Hfq (32), and the response regulator LqsR (56), have been shown to be involved in *L. pneumophila* gene expression, activation of virulence traits, and physiological changes that occur in the stationary phase, but no direct target genes among the *icm/dot* genes themselves or among the IDTS-encoding genes (or any other genes) have been found for this regulatory network.

The only two regulators that were shown previously to directly regulate the expression of *icm/dot* genes or IDTS-encoding genes are PmrA and CpxR (18, 61). The PmrA response regulator, which is part of the PmrAB two-component system, was shown to directly regulate the expression of a large number of IDTS-encoding genes (61). The CpxR response regulator, which is part of the CpxRA two-component system, was shown to directly regulate the expression of *icmR* (18), as well as many functional homologues of *icmR* (encoding FIR proteins) in other *Legionella* species (15). In addition, after the discovery that CpxR is a direct regulator of *icmR* (18), this protein was identified in two additional screens related to pathogenesis. The CpxR regulator was found in a screen aimed at identification of suppressors of the IcmO/DotL lethality phenotype (57), as well as in a screen that was conducted to identify *L. pneumophila* mutants with defects in Rab1 recruitment (37). However, no additional genes directly regulated by CpxR were identified in these two screens.

The aims of the work described in this paper were to identify additional *icm/dot*-related genes (encoding complex compo-

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nents and accessory proteins, as well as IDTS) which are directly regulated by the CpxR response regulator and to characterize their roles in the *icm/dot* system. Our data clearly indicate that CpxR regulates the expression of different components of the *icm/dot* system, and its regulon includes at least 11 IDTS.

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 (48); OG2002, a *cpxR* mutant (18); HK-PQ1, a *pmrA* mutant (61); EA-CRPA, a *cpxR pmrA* double mutant (15); and GS3011, an *icmT* mutant (63). The *Escherichia coli* strains used were MC1022 containing the M15 deletion of the *lacZ* gene (9), MC1061 containing a deletion in the *lacZ* gene (10), BL21(DE3) (53), and *cpxR* deletion mutant PAD215, a kind gift from Thomas Silhavy. The bacterial media, plates, and antibiotic concentrations used have been described previously (50). The plasmids and primers used in this study are shown in Tables S1 and S2 in the supplemental material, respectively.

Construction of *lacZ* translational fusions. To generate *lacZ* translational fusions, the regulatory regions of the genes of interest were amplified by PCR with the primers shown in 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 plasmids shown in Table S1 in the supplemental material. The levels of expression from these plasmids were determined by the β -galactosidase assay as previously described (34, 61).

Generation of substitutions in the CpxR and PmrA binding sites. To generate substitutions in the CpxR or PmrA binding sites in the regulatory regions of the genes examined, site-directed mutagenesis was performed with the consensus sequences by using the PCR overlap extension approach (22) and a method similar to the method described previously (61). In all the mutations generated the upstream part of the CpxR binding site was changed from GTAAA to TGAAA, and the upstream part of the PmrA binding site was changed from CTTAA to CTATA. The primers used for mutagenesis are shown in Table S2 in the supplemental material, and the plasmids resulting from site-directed mutagenesis are shown in Table S1 in the supplemental material.

Construction of isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *cpxR*. The *L. pneumophila cpxR* gene was amplified by PCR with primers CpxR-EcoRI and CpxR-pET-R (see Table S2 in the supplemental material). The PCR products were then digested with EcoRI and BamHI and cloned into pUC-18 to generate pEA-puc-CpxR; this plasmid was sequenced and then digested with the same enzymes and cloned into pMMB207 downstream from the P_{lac} promoter to generate pEA-pTac-CpxR. The resulting plasmid was then digested with XbaI and EheI, and the resulting fragment, containing P_{lac} -*cpxR* together with the *lacI* gene, was cloned into plasmids containing the regulatory region of the *sidM* and *lvG4* genes, as well as plasmids containing the mutations in the CpxR binding site of these two genes, resulting in plasmids shown in Table S1 in the supplemental material.

Construction of *cyaA* fusions. Plasmid pMMB-cyaA-C (61) was used to clone all of the *cyaA* fusions constructed. All genes examined were amplified by PCR using a pair of primers (see Table S2 in the supplemental material) containing suitable restriction sites at the 5' end. The PCR products were subsequently digested with the relevant enzymes and cloned into the pMMB-cyaA-C vector to generate plasmids shown in Table S1 in the supplemental material; all the inserts were sequenced to verify that no mutations were incorporated during the PCR. To construct *cyaA* fusions which were expressed under regulation of the natural promoter of the gene, the regulatory regions of the relevant genes were amplified by PCR using primers shown in Table S2 in the supplemental material, digested, and cloned into plasmids containing the corresponding *cyaA* fusion instead of the P_{lac} promoter to generate plasmids shown in Table S1 in the supplemental material.

Protein purification and gel mobility shift assays. *L. pneumophila* His₆-CpxR was purified from *E. coli* BL21(DE3) containing plasmids pOG-ECP2 (18) and pRep4 using 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 purified protein was then stored at -20°C . Gel mobility shift assays were performed as previously described (61), with a few modifications. The regulatory regions of the *sidM*, *lvG4*, and *cegC2* genes (~ 200 bp) were amplified by PCR

with primers shown in 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 136 pg of the *sidM*-labeled probe, 154 pg of the *lvG4*-labeled probe, or 123 pg of the *cegC2*-labeled probe in buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 $\mu\text{g/ml}$ poly(dI-dC), 5% glycerol, and 10 ng/ml herring sperm DNA. For samples containing unlabeled probe, 50 ng (for *sidM* and *lvG4*) or 150 ng (for *cegC2*) of the probe was allowed to bind the CpxR protein for 15 min before 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 \times Tris-acetate-EDTA gels in 0.5 \times Tris-acetate-EDTA running buffer. Following electrophoresis, the gels were transferred to nylon membranes and fixed by UV cross-linking. The DIG-labeled DNA fragments were detected by following the manufacturer's instructions.

Quantitative RT-PCR. RNA was prepared as described elsewhere (18). Total RNA was digested with an RNase-free DNase reagent (Promega). The reverse transcription (RT)-PCR mixture contained the following components: 10 μg of total RNA, 20 pmol of reverse primer (see Table S2 in the supplemental material), 40 U of RNase inhibitor (RNasin; Promega), and 10 U of avian myeloblastosis virus reverse transcriptase (Promega). The reaction mixture was incubated for 5 min at 45°C , and cDNA was synthesized at 37°C for 1 h. The following cycle conditions were used for PCR: 30 s at 95°C , 30 s at 50°C , and 30 s at 72°C for extension. The number of cycles varied depending on the levels of expression of the various mRNAs to ensure that the comparison was performed in the linear range of amplification. 16S rRNA was used as the internal control to confirm that equal amounts of total RNA were used in the reactions.

CyaA translocation assay. Differentiated HL-60-derived human macrophages plated in 24-well tissue culture dishes at a concentration of 2.5×10^6 cells/well were used for the assay. Bacteria were grown on ABCYE [*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal yeast extract] plates containing chloramphenicol for 48 h. The bacteria were scraped off the plates and suspended in AYE (ACES-buffered yeast extract) medium, the optical density at 600 nm (OD_{600}) was adjusted to 0.1 in AYE containing chloramphenicol, and the resulting cultures were grown on a roller drum for 17 to 18 h until an OD_{600} of about 3 (stationary phase) was reached. The bacteria were then diluted in fresh AYE medium to obtain an OD_{600} of 0.2 and grown for 2 h. IPTG was added to final concentration of 1 mM, and the cultures were grown for an additional 2 h. Cells were infected with bacteria harboring the appropriate plasmids at a multiplicity of infection of 4, and the plates were centrifuged at $180 \times g$ for 5 min, followed by incubation at 37°C under CO₂ (5%) for 2 h. Cells were then washed twice with ice-cold phosphate-buffered saline buffer (1.4 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄) and lysed with 200 μl of lysis buffer (50 mM HCl, 0.1% Triton X-100) at 4°C for 30 min. Lysed samples were boiled for 5 min and neutralized with NaOH. The levels of cyclic AMP (cAMP) were determined using the cAMP Biotrak enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. The CyaA fusion proteins were detected by Western blotting, using monoclonal anti-CyaA 3D1 antibody (Santa Cruz Biotechnology, Inc.) diluted 1:500 and goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Jackson Immuno-research Laboratories, Inc.) diluted 1:10,000.

RESULTS

Deletion in CpxR reduces the level of expression of several *Icm/Dot* components. Previously, we demonstrated that the *L. pneumophila* CpxR regulator directly controls the expression of the *icmR* gene (18). In addition, the levels of expression of the *icmV* and *icmW* genes were also found to be reduced in the *cpxR* mutant strain (18, 57). Sequence analysis of the regulatory regions of all the *icm/dot* components, which include the *icm/dot* genes themselves, genes encoding accessory proteins (such as *LvgA*), and IDTS-encoding genes, indicated that a predicted CpxR binding site is present in the upstream regulatory region of several of these genes (Fig. 1A), including (i) the *icmV* and *icmW* genes, both of which are part of an operon along with one additional gene (*icmV-dotA* and *icmW-icmX*) and which were found to contain the CpxR regulatory element on the complementary strand (the CpxR regulatory element

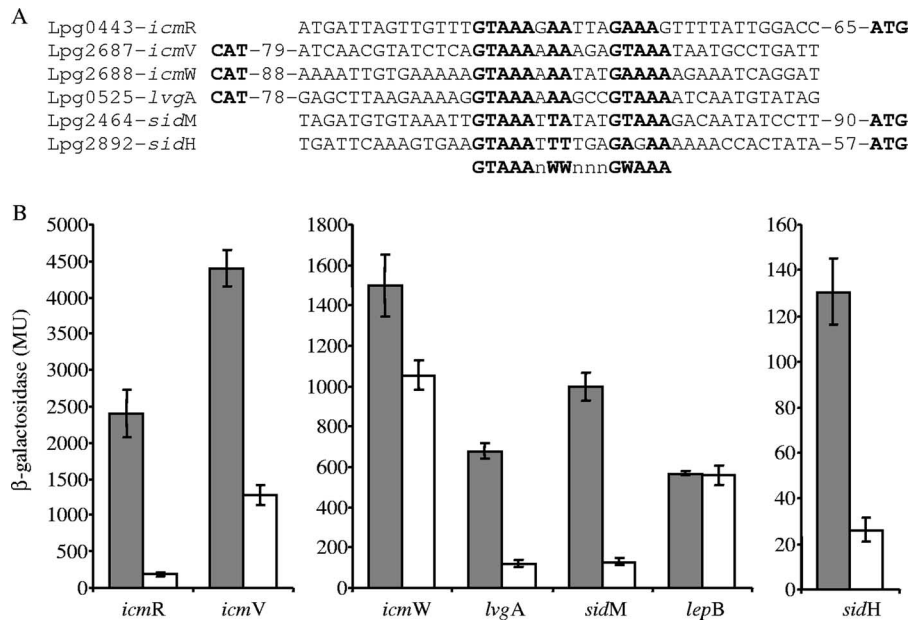


FIG. 1. Analysis of the expression of known pathogenesis-related genes containing the CpxR consensus sequence. (A) Sequence alignment of the regulatory regions of *icm/dot* genes (*icmR*, *icmV*, and *icmW*), the *icm/dot* accessory gene *lvgA*, and two IDTS-encoding genes (*sidM* and *sidH*). The putative consensus sequence is indicated by boldface type, and the distances to the first ATG codon are also indicated. The complementary strand is shown for genes in which the consensus regulatory element was identified on the reverse strand (*icmV*, *icmW*, and *lvgA*). (B) Expression of *lacZ* fusions in wild-type *L. pneumophila* and the *cpxR* deletion mutant. The expression of *lacZ* fusions of three *icm/dot* genes (*icmR*, *icmV*, and *icmW*), the *lvgA* gene, two IDTS-encoding genes (*sidM* and *sidH*) that contain the CpxR consensus regulatory element, and *lepB* that does not contain the consensus regulatory element in wild-type strain JR32 (shaded bars) and the *cpxR* deletion mutant OG2002 (open bars) was examined. β -Galactosidase activity was determined as described in Materials and Methods. The data (in Miller units [MU]) are the averages \pm standard deviations (error bars) of at least three different experiments.

that was characterized previously in the *icmR* regulatory region was located on the direct strand [Fig. 1A]; (ii) the *lvgA* gene, whose product was shown previously to interact with the IcmS protein (58) and which was also found to contain the CpxR regulatory element on the complementary strand; and (iii) the *sidH* and *sidM/drrA* genes, whose products were shown previously to be translocated into host cells in an Icm/Dot-dependent manner (31, 37, 43) and which were found to contain the CpxR regulatory element on the direct strand.

LacZ fusions were constructed for all the genes described above and examined to determine their levels of expression in the *L. pneumophila* wild-type strain and the *cpxR* deletion mutant, and they were all found to be expressed at lower levels in the *cpxR* mutant (Fig. 1B). As a negative control, an IDTS-encoding gene (*lepB*) that was shown previously to be regulated by the PmrA regulator (61), which belongs to the same family of regulators as CpxR (1), was not affected by deletion of the CpxR regulator (Fig. 1B). To further support the *lacZ* fusion results, the levels of expression of the *icmV* and *sidM* genes were also examined at the mRNA level using quantitative RT-PCR and were found to be reduced in the *cpxR* deletion mutant compared to the wild-type strain (Fig. 2). These results demonstrate that the CpxR regulator participates in the regulation of the pathogenesis-related genes examined and that it can also regulate the expression of genes when its regulatory element is located on the complementary strand (as it is in the case of *icmV*, *icmW*, and *lvgA*) (Fig. 1A).

Consensus sequence identified is the target sequence of the CpxR response regulator. To examine the relationship be-

tween the consensus sequence identified (Fig. 1A) and the CpxR regulator, the consensus sequence of the five genes identified (*icmV*, *icmW*, *lvgA*, *sidM*, and *sidH*) was mutagenized by changing the upstream part of the consensus sequence from GTAAA to TGAAA, and the results of this analysis are shown in Fig. 3. The results obtained clearly demonstrate that the mutations in the regulatory element had the same effect as deletion of the CpxR regulator. Moreover, when the fusions containing the mutated consensus sequence were introduced into the *cpxR* deletion mutant, no additional reduction in the level of expression was observed (Fig. 3), supporting the conclusion that the conserved regulatory element identified is recognized by the CpxR response regulator itself.

CpxR activates the expression of the genes containing the conserved regulatory element. In order to define the role of CpxR in the regulation of the genes that contain the conserved regulatory element, the effect of CpxR in a surrogate host, *E. coli*, was examined. In this analysis the *L. pneumophila cpxR* gene was cloned under control of the P_{tac} promoter (induced by IPTG) and introduced into *E. coli* together with the *sidM* fusion (containing the CpxR binding site in the direct orientation) or the *lvgA lacZ* fusion (containing the CpxR binding site on the complementary strand). The results obtained in this analysis clearly demonstrated that the concentration of IPTG used (which controls the level of expression of the CpxR regulator) determined the levels of expression of the *sidM* and *lvgA lacZ* fusions in *E. coli* (Fig. 4), proving that CpxR has a role in their regulation. Moreover, the *sidM* and *lvgA lacZ* fusions that contained substitutions in the CpxR consensus

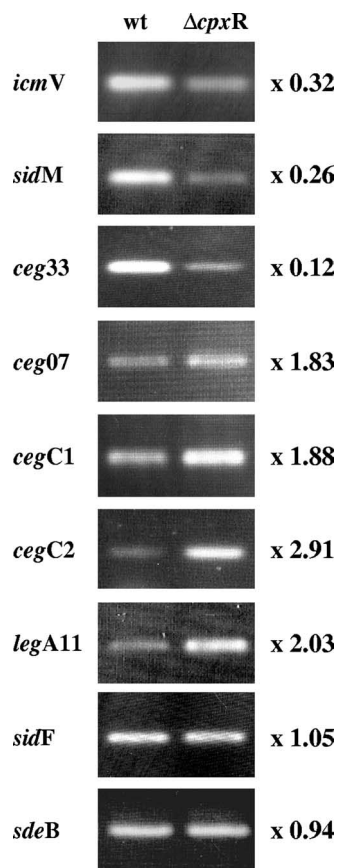


FIG. 2. Analysis of mRNA levels for genes containing the CpxR consensus sequence in the wild type and a *cpxR* deletion mutant. Total RNA derived from the wild type (wt) and the *cpxR* deletion mutant ($\Delta cpxR$) was used in RT-PCR to assess the mRNA levels for seven genes containing the CpxR consensus sequence (*icmV*, *sidM*, *ceg33*, *ceg07*, *cegC1*, *cegC2*, and *legA11*) and two genes that do not contain the CpxR consensus sequence (*sidF* and *sdeB*). The PCR was carried out for 19 cycles for *icmV*, *legA11*, and *sidM*, for 21 cycles for *ceg07*, *ceg33*, *cegC1*, *sidF*, and *sdeB*, and for 23 cycles for *cegC2*. Quantification (values are indicated on the right) was performed using the TINA image analysis software, and the values were obtained by dividing the mRNA level in the *cpxR* mutant by the mRNA level in the wild-type strain.

sequence were not influenced by the addition of IPTG (Fig. 4). The results described here show that the CpxR response regulator is strongly related to the consensus sequence identified and show that it positively regulates the expression of the genes examined.

***L. pneumophila* His₆-CpxR protein directly binds to the regulatory regions of the genes examined.** To further support the direct relationship between the CpxR regulator and its binding site, the *L. pneumophila* CpxR protein was His tagged, overexpressed, purified, and used for gel mobility shift assays with ~200-bp fragments that cover the *sidM* and *legA* regulatory regions. The *L. pneumophila* His₆-CpxR was found to bind to the regulatory regions of both genes, as shown by a shift in the migration of the DNA probe (Fig. 5). The degree of the band shift and the amount of the shifted probe were correlated with increased amounts of the His₆-CpxR protein (Fig. 5A and B). In addition, competition with an unlabeled probe reduced the

band shift (Fig. 5A and B, compare the second and sixth lanes), and no effect was observed when the same amount of herring sperm DNA was added (Fig. 5A and B, compare the second and seventh lanes).

The mobility shift assay, together with the examination of gene expression in the *cpxR* null mutant, the controlled expression studies, and the analysis of mutations in the consensus sequence, strongly demonstrated the direct relationship between the CpxR regulator and its binding site, as well as its key role in the regulation of different components of the Icm/Dot secretion system in *L. pneumophila*.

Identification of new *L. pneumophila* CpxR-regulated genes. The relative conservation of the CpxR regulatory element identified and the involvement of the CpxR-regulated genes in pathogenesis led us to search for additional genes that contain this conserved regulatory element in the *L. pneumophila* genome. This analysis was performed using whole-genome DNA pattern analysis (from the Regulatory Sequence Analysis Tools), which allowed one base mismatch from the consensus described in Fig. 1 only in the AAA part of the consensus sequence. The analysis which we performed resulted in identification of nine *L. pneumophila* genes that contain a putative CpxR consensus sequence (Table 1 and Fig. 6A and 7A). These genes include (i) two *legA* (*Legionella* eukaryotic gene) genes (lpg0038 [*legA10*] and lpg0436 [*legA11*]) that have been suggested previously to be putative IDTS-encoding genes due to the presence of an ankyrin domain (6, 12); (ii) three *ceg* (coregulated with effector-encoding genes) genes (lpg0227 [*ceg7*], lpg0898 [*ceg18*], and lpg2591 [*ceg33*]) that have been suggested previously to be putative IDTS-encoding genes due to the presence of a PmrA-encoding consensus sequence in their regulatory region (Fig. 7B) (61); and (iii) four *cegC* (coregulated with effector-encoding genes by CpxR) genes (lpg0012 [*cegC1*], lpg0126 [*cegC2*], lpg1144 [*cegC3*], and lpg2200 [*cegC4*]) that are unique genes with unknown functions that have not been described previously. A previous analysis of large groups of predicted *L. pneumophila* IDTS indicated that even though the genes encoding many of these proteins exhibit no homology to any entry in the GenBank database, several of them were found to have paralogues in the *L. pneumophila* genome (29), and it was also shown that the genes coding for some of these IDTS are occasionally located next to one another (28, 29, 61). Analysis of the genes identified (Table 1) revealed that three of these genes are located next to previously identified IDTS-encoding genes and three other genes have one or more paralogues in the *L. pneumophila* genome (Table 1).

Four *cegC* genes and two *legA* genes are regulated by CpxR. To examine if the four *cegC* genes and two *legA* genes identified are regulated by CpxR, they were all fused to the *lacZ* reporter. The six resulting fusions were examined to determine their levels of expression in the *L. pneumophila* wild-type strain compared to their levels of expression in a *cpxR* deletion mutant. Similar to the results obtained for the CpxR-regulated genes described above, the levels of expression of all the genes examined were found to be affected in the *cpxR* mutant compared to the wild-type strain (Fig. 6B). However, the levels of expression of some of the genes examined were found to be higher in the *cpxR* mutant strain than in the wild-type strain, indicating that CpxR acts as a repressor for these genes, a

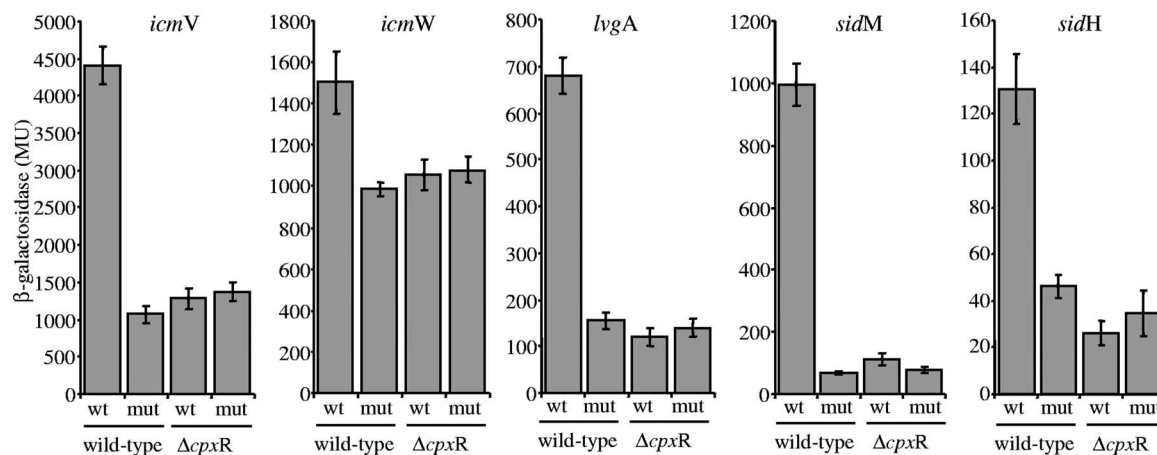


FIG. 3. Analysis of the relationship between the *cpxR* consensus and the CpxR regulator. Expression of *icmV*, *icmW*, *lvgA*, *sidM*, and *sidH lacZ* fusions in *L. pneumophila* was determined. For each of the fusions, data are shown for the wild-type regulatory region (wt) and the mutated regulatory region (mut) in the JR32 wild-type strain (wild-type) and *cpxR* mutant strain OG2002 ($\Delta cpxR$). β -Galactosidase activity was determined as described in Materials and Methods. The data are the averages \pm standard deviations (error bars) of at least three different experiments. MU, Miller units.

result similar to the results described previously for the CpxR regulator in other systems (44). In addition, the CpxR consensus sequence of the four *cegC* genes was mutated, and when the mutated fusions were examined in the *L. pneumophila* wild-type strain, effects (increases in the levels of expression of *cegC1* and *cegC2* and decreases in the levels of expression of *cegC3* and *cegC4*) similar to the effects obtained when the corresponding wild-type fusions were examined in the *cpxR* deletion mutant were obtained (Fig. 6B). To confirm the CpxR repression observed, the *cegC1*, *cegC2*, and *legA11* genes were also examined at the mRNA level using quantitative RT-PCR, and the levels of their mRNAs were found to be higher in the *cpxR* deletion mutant (Fig. 2), as expected based on the *lacZ* fusions results. Furthermore, since the two genes (*sidM* and *lvgA*) that were examined in the gel mobility shift assay were activated by CpxR, we examined the regulatory region of the *cegC2* gene (repressed by CpxR) using this assay as well. *L.*

pneumophila His₆-CpxR was found to bind to the regulatory regions of the *cegC2* gene, as shown by a shift in the migration of the DNA probe (Fig. 5C). Increases in the size of the band shift and in the amount of the shifted probe were correlated with increased amounts of the His₆-CpxR protein (Fig. 5C). It is interesting that no band shift was observed when the assay was performed with amounts of the CpxR protein that were sufficient to obtain a clear band shift with *sidM* and *lvgA* (between 6 and 50 ng) and that larger amounts of CpxR (250 ng) were required to obtain a clear band shift. These results might indicate that the CpxR protein has higher affinity for the sites on which it acts as an activator than for the sites on which it acts as a repressor, or they might be related to the degree of identity of each site to the CpxR consensus sequence.

Three *ceg* genes are regulated by both PmrA and CpxR. In a previous study we found that PmrA regulates the expression of many *L. pneumophila* IDTS-encoding genes; in addition, the

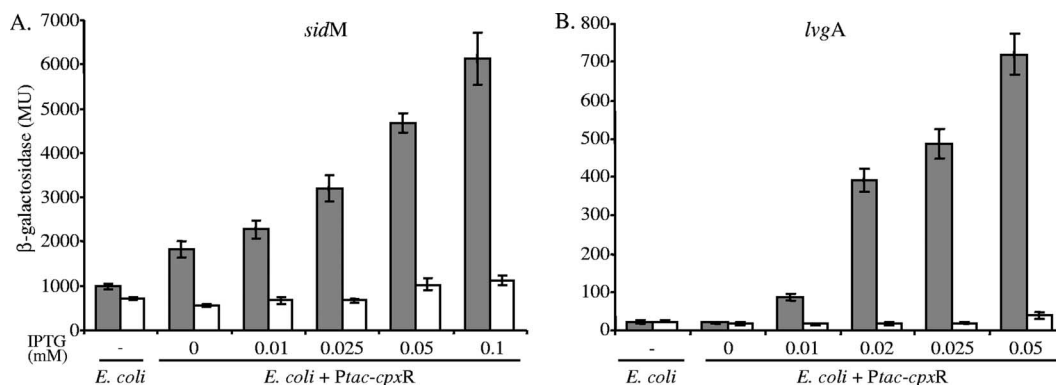


FIG. 4. CpxR directly regulates the genes containing the consensus sequence. The expression of wild-type fusions (grey bars) of *sidM::lacZ* (A) and *lvgA::lacZ* (B), as well as the same fusions containing a mutation in the CpxR regulatory element (open bars), was examined in *E. coli* having a deletion in the *cpxR* gene (PAD215). The bacteria examined contained a plasmid with the *L. pneumophila cpxR* gene cloned under control of the *P_{tac}* promoter (activated by IPTG), and they were grown in media containing different concentrations of IPTG (indicated below the bars). Bacteria containing the same *lacZ* fusions without the *cpxR* gene were used as controls (left columns in both panels). β -Galactosidase activity was determined as described in Materials and Methods. The data are the averages \pm standard deviations (error bars) of at least three different experiments. MU, Miller units.

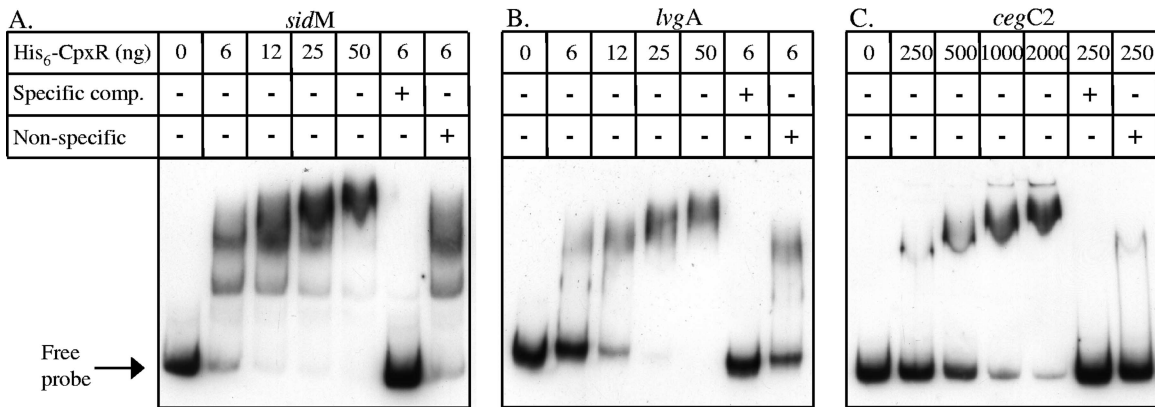


FIG. 5. *L. pneumophila* His₆-CpxR protein binds to the *sidM*, *lvgA*, and *cegC2* regulatory regions. Mobility shift assays were performed with pure His₆-CpxR protein and DIG-labeled *sidM* (136 pg) (A), *lvgA* (154 pg) (B), and *cegC2* (123 pg) (C) regulatory regions in the presence (plus sign) or absence (minus sign) of unlabeled probe (Specific comp.) (50 ng in panels A and B and 150 ng in panel C) or the same amount of herring sperm DNA (Non-specific). The amount of His₆-CpxR is indicated above each lane.

PmrA consensus sequence was found in the regulatory region of 35 uncharacterized genes (61). As described above, three of these genes (*ceg7*, *ceg18*, and *ceg33*) were also found to contain a putative CpxR binding site (Fig. 7A). The organizations of the CpxR and PmrA putative regulatory elements in relation to one another were found to be different in these three genes. In *ceg7* the two sites overlap, and the CpxR site was found to be located on the complementary strand; in *ceg18* the two sites were found to be located about 35 bp apart, and the CpxR site was located on the complementary strand; and in *ceg33* the two sites were found to be located about 10 bp apart, and they both were located on the direct strand (Fig. 7B) (this type of organization was observed previously for the *fir* genes in different *Legionella* species [15]). To investigate the effect of the CpxR and PmrA regulators on the levels of expression of these genes, three *lacZ* fusions were constructed and examined in the *L. pneumophila* wild-type strain, the *cpxR* deletion mutant (OG2002), the *pmrA* deletion mutant (HK-PQ1), and the *cpxR pmrA* double mutant (EF-CRPA), and the results are shown in Fig. 7C. The results obtained clearly show that both the CpxR and PmrA regulators affect the expression of the three genes examined. PmrA always serves as an activator, and CpxR serves as a repressor for two of the genes (*ceg7* and *ceg18*) and as an activator for *ceg33*. The effect of the *cpxR* deletion mu-

tation on the expression of the *ceg7* and *ceg33* genes was also examined at the mRNA level, and the results were similar to the results obtained for the *lacZ* fusions (Fig. 2). The data indicated very clearly that the results for the *cpxR pmrA* double mutant were always the same as the results for the *pmrA* single mutant, indicating that the relief of repression that was observed in the *cpxR* deletion mutant with *ceg7* and *ceg18* requires activation of the PmrA regulator. The effects of both regulators on the level of expression of the *ceg18::lacZ* fusion were the most pronounced effects and opposite each other (a 6.5-fold increase in the *cpxR* mutant and 6-fold decrease in the *pmrA* mutant). Therefore, this gene was analyzed further by constructing mutants with mutations in both the *pmrA* and *cpxR* putative regulatory elements, and the resulting fusions were analyzed in the four strains indicated above; the results are shown in Fig. 7D. The results clearly demonstrated that the expression of the *ceg18* gene is completely dependent on the presence of the PmrA regulatory element and the PmrA regulator, since no expression was obtained when either of these two factors was missing. Mutations in the *cpxR* binding site, in the CpxR regulator, or in both resulted in an increase in the level of expression of the gene, but this effect was observed only if the *pmrA* regulatory element and the PmrA regulator were intact, indicating that CpxR represses the PmrA activation of this gene.

Nine new genes identified code for new *L. pneumophila* IDTS. To determine whether the proteins encoded by the nine new CpxR-regulated genes identified (two *legA* genes, three *ceg* genes, and four *cegC* genes) are translocated into host cells by the *L. pneumophila* *icm/dot* system, the *Bordetella pertussis* CyaA reporter gene fusion that has been used previously to study the translocation of many bacterial effectors into eukaryotic cells (52) was utilized. The *legA10*, *legA11*, *ceg7*, *ceg18*, *ceg33*, *cegC1*, *cegC2*, *cegC3*, and *cegC4* genes were fused to the CyaA reporter at their N-terminal ends (leaving their C-terminal ends exposed) and transformed into wild-type strain JR32 and an *icmT* mutant in which the *icm/dot* system is inactive. HL-60-derived human macrophages were infected with *L. pneumophila* expressing the CyaA hybrid proteins or unfused CyaA, and translocation was examined (Fig. 8A). In-

TABLE 1. Properties of proteins encoded by new genes containing the CpxR consensus sequence

Gene ^a	lpg no.	Nearby gene	Paralog(s)	Motif or domain
<i>legA10</i>	lpg0038	<i>ceg1</i> (lpg0035)		Ankyrin repeat
<i>legA11</i>	lpg0436	<i>ceg14</i> (lpg0437)		Ankyrin repeat
<i>ceg7</i>	lpg0227		lpg2248	
<i>ceg18</i>	lpg0898			
<i>ceg33</i>	lpg2591	<i>legS1</i> (lpg2588)		
<i>cegC1</i>	lpg0012		lpg0502, lpg1455	
<i>cegC2</i>	lpg0126		lpg2433 (<i>ceg30</i>), lpg1120, lpg1484 (<i>ceg22</i>)	
<i>cegC3</i>	lpg1144			Coiled coil
<i>cegC4</i>	lpg2200			

^a All the genes are also present in the genomes of *L. pneumophila* strains Lens, Paris, and Corby.

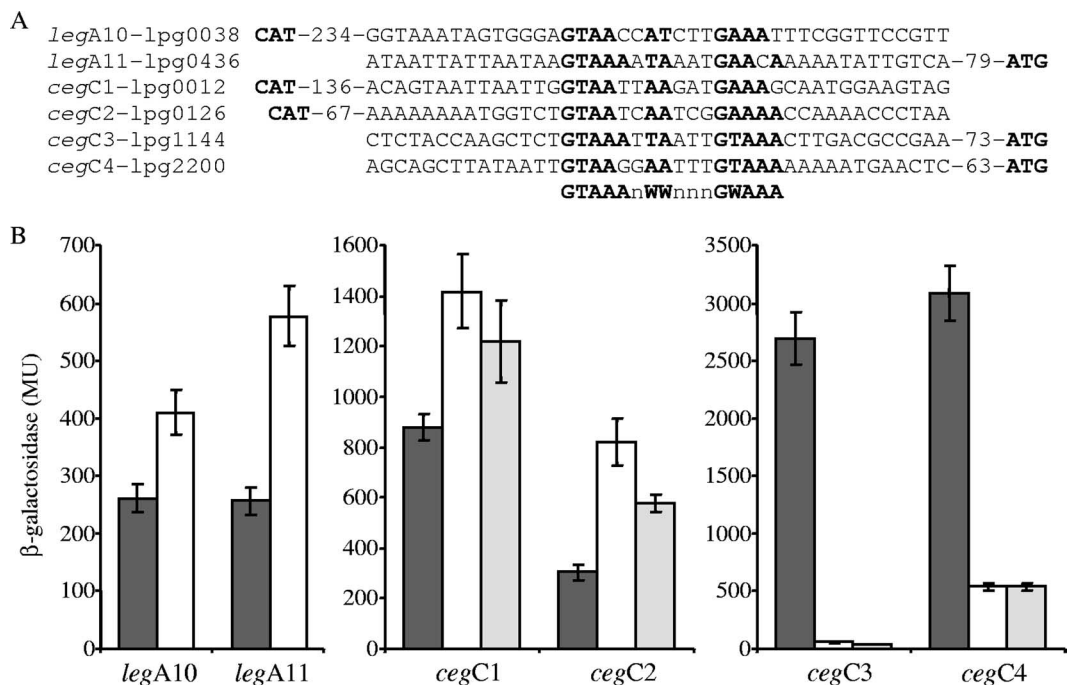


FIG. 6. *legA* and *cegC* genes are regulated by the CpxR regulator. (A) Sequence alignment of the regulatory regions of the *legA* and *cegC* genes (*legA10*, *legA11*, *cegC1*, *cegC2*, *cegC3*, and *cegC4*). The putative consensus sequence is indicated by boldface type, and the distances to the first ATG codon are also indicated. The complementary strand is shown for genes (*legA10*, *cegC1*, and *cegC2*) in which the consensus regulatory element was identified on the reverse strand. (B) Expression of the four *cegC* genes and the two *legA* genes in *L. pneumophila* wild-type strain (dark grey bars) and *cpxR* mutant strain OG2002 (open bars). For the *cegC* genes *lacZ* fusions containing a mutation in the CpxR consensus sequence were also examined in the *L. pneumophila* wild-type strain (light grey bars). β -Galactosidase activity was determined as described in Materials and Methods. The data are the averages \pm standard deviations (error bars) of at least three different experiments. MU, Miller units.

fection with bacteria containing all the genes examined resulted in very large differences in the cAMP levels produced when the wild-type and *icmT* mutant strains were used (although the levels of the CyaA fusion proteins were similar, as deduced by Western blot analysis using a CyaA antibody [data not shown]), indicating that a functional Icm/Dot secretion system was required for translocation (Fig. 8A). A construct expressing only CyaA or several unrelated proteins fused to the CyaA reporter resulted in very low levels of cAMP (Fig. 8A and data not shown), indicating that increased cAMP levels required the presence of the proteins examined. We concluded that the nine proteins examined (five proteins that were suggested previously to be putative IDTS, as well as the four newly identified proteins) that contain the CpxR consensus sequence in their regulatory regions are new IDTS.

Deletion in *cpxR* affects IDTS translocation into host cells.

To examine the effect of the *cpxR* deletion on IDTS translocation, the translocation of two *cyaA* fusions (*sidM* and *cegC4*) was examined. These two *cyaA* fusions were examined when they were expressed from the P_{tac} promoter (induced by IPTG), as well as when each of them was expressed from its natural promoter (which should reflect the overall effect of the *cpxR* deletion on the translocation of the genes). When the genes were under P_{tac} control, high levels of translocation were observed in the wild-type strain for both fusions, and a clear reduction in translocation was seen in the *cpxR* deletion mutant (Fig. 8B). The reduction in translocation was probably due to the effect of the *cpxR* regulator on the expression of *icm/dot*

components (see above). When the same *cyaA* fusions were expressed from their natural promoters (P_{sidM} -*cyaA*-*sidM* and P_{cegC4} -*cyaA*-*cegC4*), moderate levels of translocation were observed in the wild-type strain and a dramatic reduction in translocation was observed in the *cpxR* deletion mutant (Fig. 8B). The reduction in translocation most likely resulted from the combined effects of the *cpxR* deletion mutation on the level of expression of *icm/dot* components and the level of expression of the IDTS themselves. These results established that the CpxR regulator is a fundamental regulator of the *icm/dot* type IV secretion system in *L. pneumophila*.

DISCUSSION

The *L. pneumophila* Icm/Dot type IV secretion system is composed of about 23 complex components, three accessory proteins, and a large number of IDTS (for reviews, see references 42 and 49), and many additional proteins have been suggested to be putative IDTS (6, 12, 29, 61). This very large number of components must be coordinated at the level of gene expression in order to result in a successful infection and intracellular multiplication.

In a previous study, we identified the PmrA response regulator as a direct regulator of 16 IDTS, and 32 potential IDTS were found to contain a putative PmrA site. In addition, the PmrA regulator was found to be required for intracellular growth in the protozoan host *Acanthamoeba castellanii* and to be partially required for intracellular growth in HL-60-derived

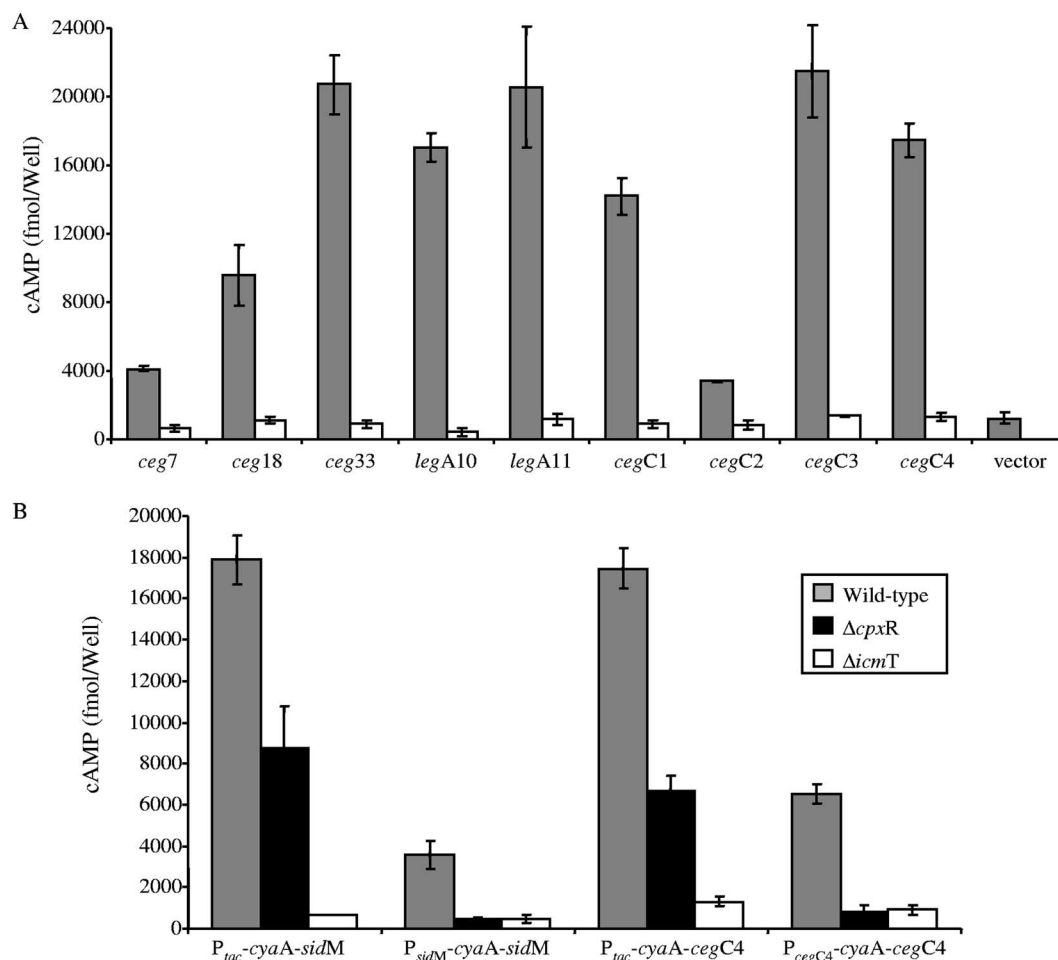


FIG. 8. Icm/Dot-dependent translocation of CpxR-regulated genes. (A) Wild-type strain JR32 (grey bars) and *icmT* mutant GS3011 (open bars) harboring the CyaA fusion proteins (indicated below the bars) were used to infect HL-60-derived human macrophages, and the cAMP levels of the infected cells were determined as described in Materials and Methods. (B) Effect of the *cpxR* deletion mutant on the translocation of *sidM* and *cegC4 cyaA* fusions in wild-type strain JR32 (grey bars), *cpxR* mutant OG2002 (black bars), and *icmT* mutant GS3011 (open bars). The fusions were cloned under control of P_{tac} or under control of their natural promoter. The data are the means for the amount of cAMP per well obtained in three independent experiments, and the error bars indicate standard deviations.

an intracellular growth phenotype in a different host similar to the phenotype observed for the *pmrA* mutant in *A. castellanii*. Even though no intracellular growth defect was observed for the *cpxR* deletion mutant, the CpxR regulator was found to affect several pathogenesis-related phenotypes: (i) the CpxR regulator was found in a screen aimed at identification of suppressors of the IcmO/DotL lethality phenotype (57); (ii) the CpxR regulator was identified in a screen that was conducted to identify *L. pneumophila* mutants with defects in Rab1 recruitment (37); and (iii) the *cpxR* deletion mutation was found to strongly affect IDTS translocation (this study). All these results indicate that CpxR is a central regulator of *L. pneumophila* pathogenesis.

Although the PmrA and CpxR regulators belong to the same group of DNA binding proteins containing a winged helix-turn-helix motif (1) and are both involved in the regulation of *L. pneumophila* pathogenesis-related genes, their effects on their target genes and the locations of their regulatory elements were found to be different. The PmrA binding sites were found to be located at a precise distance from the -10 pro-

motor, and they were always found to be located on the direct strand (61). In contrast, the regulatory elements of the CpxR regulator were found to be located at different distances from the predicted -10 promoter element and on both the direct and complementary strands (Fig. 9). A situation similar to the situation described here has been observed for the CpxR regulators in other bacteria (14). Besides the differences in the locations of the two regulators and in the orientations of their regulatory elements, the PmrA regulator was found to function as an activator of its target genes in all the genes examined thus far, whereas the CpxR regulator was found to function as a repressor or as an activator of its target genes (Fig. 9). The CpxR regulator was also shown to function as an activator as well as a repressor in other bacteria (44).

The CpxRA and PmrAB two-component systems have both been found to be involved in the pathogenesis of bacteria other than *L. pneumophila*. The CpxR response regulator has been shown to be involved in the pathogenesis of *Salmonella enterica*, *Shigella sonnei*, enteropathogenic *E. coli*, and *Yersinia* sp., and in most cases its involvement was related to contact

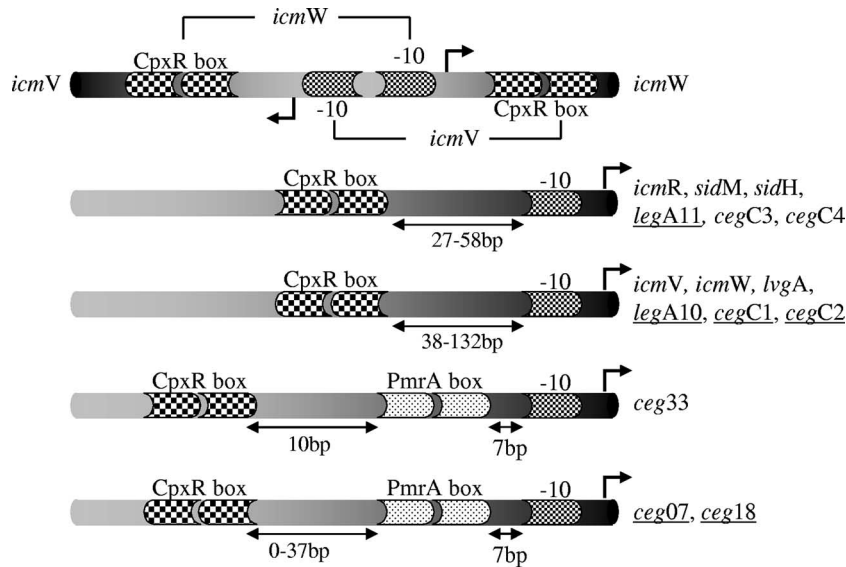


FIG. 9. Schematic diagrams of CpxR-regulated promoters. The CpxR boxes, PmrA boxes, -10 promoter elements, and transcription start sites (arrow) are indicated along with their orientations with respect to the first ATG codon of the gene (black region). For *icmV* and *icmW* (upper bar) the relevant regulatory elements for each of the two genes are indicated. The genes that were found to have a certain type of regulation are indicated on the right; the genes that were found to be repressed by CpxR are underlined.

with eukaryotic cells (7, 8, 25, 40, 41). The PmrA response regulator has been shown to be involved in the virulence of *S. enterica*, *Pseudomonas aeruginosa*, and *Francisella* sp., and its involvement was mainly related to its relationship with the PhoP response regulator and resistance to antimicrobial peptides (13, 33, 35, 55). The CpxR and PmrA regulators are assumed to function together with their cognate sensor histidine kinases (CpxA and PmrB, respectively) (Fig. 10), which are located immediately downstream from them. Since these two response regulators are key regulators of the *L. pneumophila icm/dot* pathogenesis system, it is important to determine

the environmental signals that control PmrB and CpxA phosphorylation, as these stimuli might provide clues about how the intracellular environment influences the expression of the different IDTS. Currently, the environmental signals that control the phosphorylation of *L. pneumophila* CpxA and PmrB are not known, but analysis of the activation of these systems in other bacteria produced interesting and relevant findings. The CpxRA system in *E. coli* was shown to be activated in response to periplasmic stress and accumulation of unfolded proteins in the periplasm, and this activation was shown to involve the CpxP protein (26, 46); however, no CpxP homolog was found

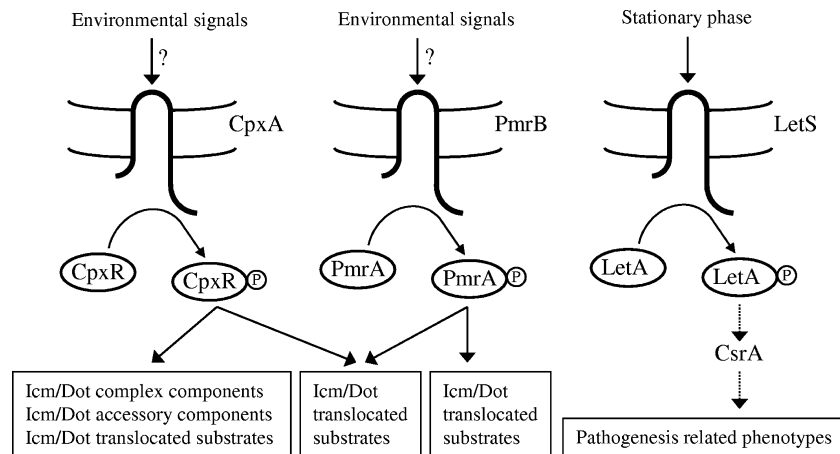


FIG. 10. Model of regulation of the *L. pneumophila* Icm/Dot pathogenesis system. The three two-component systems (CpxRA, PmrAB, and LetAS) which were found to be involved in the regulation of pathogenesis-related genes and/or phenotypes are shown. The environmental signals sensed by CpxA and PmrB are currently not known, and the phosphorylation of these components is expected to activate by transfer of the phosphate group to the cognate response regulators CpxR and PmrA, respectively, which then activate or repress the transcription of their target genes. The target genes of CpxR and PmrA overlap in some cases, but only CpxR was found to control the expression of *icm/dot* genes; both regulators control the expression of IDTS-encoding genes. No target genes are currently known for the LetA-CsrA regulatory cascade. The dotted line indicates connections that might be indirect.

to be present in *L. pneumophila*. In *S. enterica*, the CpxA protein was shown to be involved in sensing low pH (39). The *S. enterica* PmrAB system was shown to be activated by low pH, as well as in response to high levels of Fe(III) (45, 60). Since *L. pneumophila* resides in a phagosome that does not fuse with lysosomes early during infection (23, 59) but it was shown to acquire lysosomal markers late during infection (54), it is tempting to believe that one or both of these sensor histidine kinases respond to changes in pH. However, since we found three *ceg* genes which were shown here to be regulated by both CpxR and PmrA and since for two of these genes CpxR was found to act as a repressor and PmrA was found to act as an activator, it is unlikely that both CpxA and PmrB sense pH and that they control the levels of expression of the same target genes in opposite ways. The environmental stimuli that activate the PmrAB and CpxRA two-component systems in *L. pneumophila* and the time during infection when these two-component systems are activated remain to be elucidated.

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