JOURNAL OF BACTERIOLOGY, Aug. 2003, p. 4908–4919 Vol. 185, No. 16
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.16.4908–4919.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Identification of CpxR as a Positive Regulator of *icm* and *dot* Virulence Genes of *Legionella pneumophila*

Ohad Gal-Mor and Gil Segal*

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

Received 21 November 2002/Accepted 23 May 2003

To date, 24 *Legionella pneumophila* genes (*icm* and *dot* genes) have been shown to be required for intercellular growth and host cell killing. A previous report indicated that the regulation of these genes is complicated and probably involves several regulatory proteins. In this study, a genetic screen performed in *Escherichia coli* identified the CpxR response regulator as an activator of the *L. pneumophila icmR* gene. Construction of an *L. pneumophila cpxR* insertion mutant showed that the expression of *icmR* is regulated by CpxR. In addition, a conserved CpxR binding site (GTAAA) was identified in the *icmR* regulatory region and *L. pneumophila* His-tagged CpxR protein was shown to bind to the *icmR* regulatory region using a mobility shift assay. Besides its dramatic effect on the *icmR* level of expression, the CpxR regulator was also found to affect the expression of the *icmV-dotA* and *icmW-icmX* operons, but to a lesser extent. The role of CpxA, the cognate sensor kinase of CpxR, was also examined and its effect on the *icmR* level of expression was found to be less pronounced than the effect of CpxR. The RpoE sigma factor, which was shown to coregulate genes together with CpxR, was examined as well, but it did not influence *icm* and *dot* gene expression. In addition, when the *cpxR* mutant strain, in which the expression of the *icmR* gene was dramatically reduced, and the *cpxA* and *rpoE* mutant strains were examined for their ability to grow inside *Acanthamoeba castellanii* and HL-60-derived human macrophages, no intracellular growth defect was observed. This study presents the first evidence for a direct regulator (CpxR) of an *icm-dot* virulence gene (*icmR*). The CpxR regulator together with other regulatory factors probably concerts with the expression of *icm* and *dot* genes to result in successful infection.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a broad-host-range facultative intracellular pathogen. *L. pneumophila* is able to infect, multiply within, and kill human macrophages as well as the free-living amoebae that serve as their environmental reservoir (19, 37). Two regions of *icm* and *dot* genes required for human macrophage killing and intercellular multiplication have been discovered in *L. pneumophila* (reviewed in references 41 and 46). Most of these genes were also shown to be required for intracellular growth in the protozoan host *Acanthamoeba castellanii* (42). Complementation and primer extension analysis indicated that these genes are probably organized in nine transcriptional units (*icmTS*, *icmR*, *icmQ*, *icmPO*, *icmMLKEGCD*, *icmJB*, *icmF-tphA*, *icmWX*, and *icmV-dotA*) (2, 4, 34, 39, 40, 45). The *icm-dot* system encodes a type IV secretion system that translocates effector molecules into the host cell and, in this way, modulates the properties of the phagosome containing bacteria (7, 30, 41, 46). The specific function of most of the Icm proteins is not known, but the IcmS and IcmW proteins as well as the IcmQ and IcmR proteins were shown to interact with one another (6). In addition, the IcmR protein was shown to exhibit chaperone activity for IcmQ, which was shown to form homopolymers (13).

Recently, 12 regulatory sites were identified in the upstream region of eight *icm* and *dot* genes. Seven of these sites were shown to constitute the −10 promoter elements of these genes, whereas the other five are expected to serve as binding sites for regulatory factors (16). To date, several regulatory factors have been shown to be involved in the regulation of *icm* and *dot* genes in *L. pneumophila*, including the stationary-phase sigma factor RpoS, the ppGpp synthetase RelA (50), and the response regulator LetA (15, 26) but none of them was shown to directly regulate any of the *icm* and *dot* genes.

CpxR and CpxA are known to comprise a two-component system, which constitutes a typical signal transduction pathway (reviewed in references 1, 17, and 43). In this system, *cpxR* encodes the cytoplasmic response regulator (12), while *cpxA* encodes an inner membrane sensor histidine kinase (36). In *E. coli* the Cpx system is activated by envelope stress signals, such as accumulation of misfolded proteins in the periplasm. When the signal is received, CpxA is being activated by autophosphorylation on a conserved histidine residue and then acts as a kinase to phosphorylate a conserved aspartate residue of the CpxR regulator. Phosphorylation of CpxR by CpxA-p enhances its binding upstream from target genes, leading to transcriptional activation (reviewed in reference 35). Recent studies of several pathogenic bacteria determined the role of the Cpx system in the regulation of virulence genes. In uropathogenic *Escherichia coli*, Cpx was found to be involved in P-pilus assembly (21, 22). A *Salmonella enterica* serovar Typhi *cpxA* mutant does not invade intestinal epithelial cells (24). In *Shigella sonnei*, the *virF* gene (a master regulator of genes required for host cell invasion) was found to be under the control of the Cpx pathway (31, 32).

The goal of the study presented here was to identify the regulator of the *icmR* gene that was shown to have different

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

TABLE 1. Plasmids used in this study

Plasmid	Feature(s)	Reference or Source
pBR322	*rep* (pMB1) *rop* Ap^r Tc^r	3
pET-15b	*oriR* (ColE1) Ap^r, pT7, N-terminal His_6 tag	Novagen
pGS-lac-02	pAB-1 with a promoterless *lacZ* gene	16
pGS-reg-RP1	pOG-R125 containing a mutation in the promoter of *icmR*	This study
pGS-reg-RP2	pOG-R125 containing a mutation in the promoter of *icmR*	This study
pLAW344	*sacB* MCS[a] *oriT* (RK2) Cm^r *oriR* (ColE1) Ap^r	47
pMMB207	RSF1010 derivative, IncQ Cm^r *oriT* MCS	29
pOG-BCC2	*E. coli cpxR* in pOG-BRR3	This study
pOG-BRM1	pOG-R125 containing a mutation in the BRM1 site of *icmR*	This study
pOG-BRM2	pOG-R125 containing a mutation in the BRM2 site of *icmR*	This study
pOG-BRR1	*icmR*::*lacZ* fusion from pOG-R-125 in pBR322	This study
pOG-BRR2	pOG-BRR1 containing a single *Eco*RI site	This study
pOG-BRR3	P*tac* promoter in pOG-BRR2	This study
pOG-cpxA-Km1	pOG-U-cpxRA with the Km^r cassette in *cpxA*	This study
pOG-cpxA-Km2	Insert of pOG-cpxA-Km1 in pLAW344	This study
pOG-cpx-C2	*E. coli cpxR* in pUC18	This study
pOG-cpxR-1	*L. pneumophila cpxR* in pUC18	This study
pOG-cpxR-2	pOG-cpxR-1 with the Km^r cassette in *cpxR*	This study
pOG-cpxR-3	Insert of pOG-cpxR-2 in pLAW344	This study
pOG-ECP2	*L. pneumophila cpxR* in pET-15b	This study
pOG-R-125	*icmR*::*lacZ* fusion in pGS-lac-01	50
pOG-R-cpx3	*L. pneumophila cpxR* in pOG-R125	This study
pOG-RCPR3	*L. pneumophila cpxR* in pOG-BRR3	This study
pOG-R-cpxRA	*L. pneumophila cpxRA* in pOG-R125	This study
pOG-rpoE-1	*L. pneumophila rpoE* in pUC18	This study
pOG-rpoE-2	pOG-rpoE-1 with the Km^r cassette in *rpoE*	This study
pOG-rpoE-3	Insert of pOG-rpoE-2 in pLAW344	This study
pOG-RR16	*L. pneumophila* genomic insert in pOG-BRR3	This study
pOG-U-cpxRA	*L. pneumophila cpxRA* in pUC18	This study
pOG-U-ECP	*L. pneumophila cpxR* in pUC18	This study
pSS-R27	Regulatory region of *icmR* in pMC1403	50
pUC18	*oriR* (ColE1) MCS Ap^r	49

[a] MCS, multiple cloning site.

levels of expression in *L. pneumophila* and *E. coli*. By applying a genetic complementation screen, the *L. pneumophila* CpxR homolog was found to directly regulate the expression of the *icmR* gene. In addition, besides its dramatic effect on *icmR* gene expression, the CpxR regulator was found to positively regulate the expression of two *icm-dot* operons (*icmV-dotA* and *icmW-icmX*), but to a lesser extent. These results indicate that the CpxR regulator plays a major role in the regulation of the *icm* and *dot* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. *L. pneumophila* strains used in this work were JR32, a streptomycin resistance, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which is a wild-type strain in terms of intracellular growth (38), and a mutant that cannot grow intracellularly, 25D (19). *L. pneumophila* strains constructed in this study are all JR32 derivatives, including a *cpxR* insertion mutant, OG2002, and *cpxA* and *rpoE* deletion substitution mutants OG2004 and OG2003, respectively. The *E. coli* strains used were MC1061 (5) and BL21(DE3) (44). Plasmids and primers used in this work are described in Tables 1 and 2, respectively. Bacterial media, plates, and antibiotic concentrations were used as described before (40).

Genetic screen to identify an *icmR*::*lacZ* activator. To construct the vector for the genetic screen, the plasmid pBR322 was digested with *Hin*dIII and *Eco*RI and the *icmR*::*lacZ* fusion from pOG-R125 digested with the same enzymes was cloned into it, resulting in the plasmid pOG-BRR1. This plasmid was then partially digested with *Eco*RI, filled in and religated. A plasmid containing a single *Eco*RI site was isolated and designated pOG-BRR2. Subsequently, pOG-BRR2 was digested with *Hin*dIII and *Nru*I, and a *Hin*dIII-*Eco*47III fragment containing the P*tac* promoter from pMMB207 was cloned into it to generate pOG-BRR3. In this plasmid the unique *Eco*RI site is located immediately downstream from the P*tac* promoter. To construct an *L. pneumophila* genomic library in pOG-BRR3, chromosomal DNA from the *L. pneumophila* JR32 wild-type strain was partly digested with *Tsp*509I. The digested DNA was separated on 1% agarose gel and the 2.5- to 3.5-kb fragments were purified from the gel. These fragments were cloned into the *Eco*RI site (*Tsp*509I has compatible cohesive ends with *Eco*RI) of pOG-BRR3 and electroporated into *E. coli* MC1061. The resulting transformants were screened on MacConkey plates containing ampicillin and IPTG (isopropyl-β-D-thiogalactopyranoside). The background clones gave pale pink colonies, while the suspected clones, which had higher levels of β-galactosidase, gave dark-red colonies.

Construction of plasmids for allelic exchange. All the primers used for the allelic exchange procedure were designed according to the *L. pneumophila* genome sequence information (http://genome3.cpmc.columbia.edu/~legion/index.html). In order to construct an *L. pneumophila cpxR* insertion mutant, the primers cpxR-F-2 and cpxA-R-2 (Table 2) were used to amplify a 1,784-bp DNA fragment containing the complete *cpxR* gene and part of the *cpxA* gene. This fragment was cloned into pUC18 digested with *Hin*cII to generate pOG-cpxR-1. To knock out the *cpxR* gene, the kanamycin resistance cassette (Pharmacia) was cloned into the *Eco*RV site in *cpxR* to generate pOG-cpxR-2. The plasmid pOG-cpxR-2 was digested with *Eco*RI and *Xmn*I and filled in, and the insert was cloned into the *Eco*RV site of the allelic exchange vector pLAW344, resulting in pOG-cpxR-3. This plasmid was used for allelic exchange, as was previously described (42), to result in the *L. pneumophila cpxR* insertion mutant, OG2002. Several isolates of OG2002 were analyzed by PCR to confirm that the right change occurred on the chromosome (data not shown).

To construct an *L. pneumophila cpxA* deletion substitution mutant, the primers cpxR-F-2 and cpxA-R-long (Table 2) were used to amplify a 3,294-bp DNA fragment containing the entire *cpxR* and *cpxA* genes. This fragment was cloned into pUC18 digested with *Sma*I to generate pOG-U-cpxRA. To knock out the *cpxA* gene, the kanamycin resistance cassette was cloned instead of an internal 85-bp *Sma*I-*Eco*47III fragment to generate pOG-cpxA-Km1. This plasmid was digested with *Eco*RV and the insert was cloned into the *Eco*RV site of the vector pLAW344 to generate pOG-cpxA-Km2. This plasmid was used for allelic ex-

TABLE 2. Primers used in this study

Primer name	Sequence (5′-3′)
cpxA-R-2	GGATGTTTCAATTTCCATGCG
cpxA-R long	GTGGTTTACAAG TACGAGAGG
cpx-box-1F	GTTGTTTTGAAAGAATTAGAAAGTTTTATTGG
cpx-box-1R	CTAATTCTTTCAAAACAACTAATCATACATTAAC
cpx-box-2F	GATATATTGAAAGTAAGAGATTTAGCTCAGG
cpx-box-2R	CTCTTACTTTCAATATATCAAAATATATCTTTCAA
CpxR-Coli-F	ACATGCTGCTCAATCATCAGCCCC
CpxR-Coli R2	GAATTCATGAATAAAATCCTGTTAGTTG
cpxR-F-2	TAAAACAC ATGAAGGACACTGC
cpxR-pET-F	GGCATATGAGCAGCTCTATTCTCATTATTG
cpxR-pET-R	CCGGATCCTACAGACTACGCATTAAACATGTACCC
icmR-Down	ATGGGAACCAAGAATTAGGG
icmR-PRO-1F	TATATTTTGATAGATGTAAAGTAAGAGATTTAGCTC
icmR-PRO-1R	ACTTTACATCTATCAAAATATATCTTTCAATATATC
icmR-PRO-2F	TATATTTTGATCTATGTAAAGTAAGAGATTTAGCTC
icmR-PRO-2R	ACTTTACATAGATCAAAATATATCTTTCAATATATC
icmR-reg-up	CCCTGGATGAGTTAATGTATG
icmR-Up	GAATTCAGGAGTGGTAATAATGGGT
pMC-lac	TAAGTTGGGTAACGCCAGGG
rpoE-F	TTACTTCAATTAAATCTTGGGGC
rpoE-R	CCATTGAAGTGTTCTATCACCG

change, which resulted in an *L. pneumophila cpxA* deletion substitution mutant, OG2004. Several isolates of OG2004 were analyzed by PCR and Southern hybridization to confirm that the right change occurred on the chromosome (data not shown).

An *L. pneumophila rpoE* deletion substitution mutant was constructed by amplification of the *rpoE* gene using the primers rpoE-F and rpoE-R (Table 2). The resulting 2,704-bp PCR fragment was cloned into pUC18 digested with *Sma*I to generate pOG-rpoE-1. The kanamycin resistance cassette was cloned instead of an internal 474-bp *Nsi*I fragment (after being filled in) to result in pOG-rpoE-2. This plasmid was then digested with *Eco*RI and filled in, and the insert was cloned into the *Eco*RV site of the allelic exchange vector pLAW344, resulting in the plasmid pOG-rpoE-3. This plasmid was used to construct the *L. pneumophila rpoE* deletion substitution mutant OG2003. Several isolates of OG2003 were analyzed by PCR to confirm that the correct change occurred on the chromosome (data not shown).

Construction of plasmids for CpxR and CpxA complementation. To complement the expression of the *icmR*::*lacZ* fusion in *E. coli*, the *L. pneumophila cpxR* gene was isolated by digesting the plasmid pOG-cpxR-1 with *Eco*RI and *Pvu*II. The insert (1,135 bp) containing the *L. pneumophila cpxR* gene was filled in and cloned into the vector pOG-BRR3 (after digestion with *Eco*RI and fill in) to generate the plasmid pOG-RCPR3. To complement the expression of the *icmR*::*lacZ* fusion in the *L. pneumophila cpxR* mutant (OG2002), the later insert containing the *cpxR* gene was cloned into pOG-R125 digested with *Xmn*I to generate pOG-R-cpx3. In order to complement the expression of the *icmR*::*lacZ* fusion in the *L. pneumophila cpxA* mutant (OG2004), the plasmid pOG-U-cpxRA was digested with *Pst*I and *Hin*dIII and the insert (2,865 bp) was cloned into the plasmid pOG-R-cpx3 digested with the same enzymes to generate pOG-R-cpxRA.

Cloning of the *E. coli cpxR* gene. In order to determine whether the *E. coli* CpxR protein can activate the *L. pneumophila icmR*::*lacZ* fusion, the intact *E. coli cpxR* gene was amplified by PCR from the *E. coli* MC1061 chromosome, using the primers cpxR-coli-F and cpxR-coli R2 (Table 2). The resulting 858-bp PCR product was cloned into pUC18 digested with *Hin*cII to generate pOG-cpx-C2, which was verified by sequencing. This plasmid was subsequently digested with *Eco*RI and *Hin*dIII and the resulting fragment was cloned into pOG-BRR3 digested with the same enzymes to generate pOG-BCC2, containing the complete *E. coli cpxR* gene under the regulation of the P*tac* promoter.

Site-directed mutagenesis. Site-directed mutagenesis was performed by using the overlap extension PCR method (18), as described before (16). The PCR template for all the mutations constructed in the *icmR* regulatory region was pSS-R27. The resulting PCR products were digested with *Bam*HI and cloned into the vector pGS-lac-02. All of the mutations were confirmed by sequencing of the whole regulatory region. The changes made were always A to C, T to G, C to A, and G to T. The primers used to mutate the BRM1 (for binding of regulator mutation 1) site (pOG-BRM1) were cpx-box-1F and cpx-box-1R; the BRM2 (for binding of regulator mutation 2) site (pOG-BRM2), cpx-box-2F and cpx-box-2R; the second nucleotide of the promoter (pGS-reg-RP1), icmR-PRO-1F and icmR-PRO-1R; and the third nucleotide of the promoter (pGS-reg-RP2), icmR-PRO-2F and icmR-PRO-2R (Table 2).

Northern hybridization analysis. RNA preparation was performed as described before (16). RNA samples (10 μg) were separated on 1.5% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell) by capillary transfer in 10× SSC (0.15 M sodium chloride, 15 mM sodium citrate). To prepare a probe for the *icmR* gene, a 452-bp PCR fragment was amplified using the primers icmR-Up and icmR-Down (Table 2). The *icmR* probe was purified from agarose gel and radiolabeled with [α-^{32}P]dCTP by the random prime labeling kit (Roche). Hybridization was performed at 65°C for 16 h in a solution containing 5× SSPE (0.18 M sodium chloride, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA), 2.5× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin [BSA]), 0.25% sodium dodecyl sulfate (SDS), and 150 mg of denatured herring sperm DNA per ml. After the hybridization, the filter was washed three times (briefly) in 2× SSPE–0.1% SDS at room temperature, once in 1× SSPE–0.1% SDS at 65°C for 20 min, and then in 0.1× SSPE–0.1% SDS at 65°C for 20 min. Then, the membrane was air dried and exposed to X-ray film (Fuji).

Construction of plasmids for *L. pneumophila* His$_6$-CpxR protein purification. A 700-bp fragment containing the *L. pneumophila cpxR* gene was amplified by PCR using the primers cpxR-pET-F and cpxR-pET-R containing an *Nde*I and a *Bam*HI site, respectively (Table 2). The 700-bp insert was cloned into pUC18 digested with *Hin*cII to generate pOG-U-ECP. This plasmid was sequenced and digested with *Nde*I and *Bam*HI and the resulting insert was cloned into the pET-15b vector (Novagen) digested with the same enzymes to generate pOG-ECP2. The resulting plasmid expresses a full-length *L. pneumophila* CpxR protein fused to a His$_6$ tag on its N terminus, with a predicted molecular mass of ~28 kDa, under the T7 promoter.

Protein purification. The *L. pneumophila* His$_6$-CpxR protein was purified from *E. coli* BL-21(DE3) transformed with pOG-ECP-2. The protein was purified by nickel-affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Protein purification was performed at room temperature under native conditions, according to adjusted protocols described in standard protocol 7 and 9 in the QIAexpressionist manual (available from Qiagen). Briefly, strain BL21(DE3) containing pOG-ECP-2 was grown overnight in NZCYM medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 g of Casamino Acids, 20.28 ml of 0.4 M $MgSO_4 \cdot 7H_2O$ in 1 liter of water) containing ampicillin (50 μg/ml) at 37°C with shaking. The culture was then diluted 1/100, and after 2 h, IPTG was added to 1 mM to induce expression of the fusion protein. The bacteria were grown for an additional 4 h at 37°C under vigorous aeration to an optical density at 600 nm (OD_{600}) of approximately 2.0 and harvested by centrifugation at 7,600 × *g* for 10 min. The pellet was resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and lysozyme [1 mg/ml]). Cells were disrupted by sonication and then centrifuged at 10,000 × *g* for 20 min. The soluble fraction was collected and 1 ml of 50% Ni-NTA resin was added to it, and

the mixture was gently shaken on ice for 60 min and then loaded on a column. The column was washed twice with 4 ml of wash buffer (50 mM NaH_2PO_4, 600 mM NaCl, 20 mM imidazole, 0.1% Triton). The protein was eluted in four fractions with 0.2, 0.8, 0.5 and 0.5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole). Fractions 2 to 4 were then collected and dialyzed (at 4°C) initially against 0.1 M sodium phosphate (pH 8.0), 1 mM EDTA, 150 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride and then with the same buffer containing 25% glycerol. Following dialysis the protein was concentrated to ~0.37 mg/ml in a centrifugal filter device (Centricon YM-10; Amicon) and stored at −20°C. Protein concentration was estimated by using the Bio-Rad protein assay reagent (Bio-Rad), using BSA as a standard.

Mobility shift assay. A 200-bp DNA fragment containing the *icmR* regulatory region (consisting of nucleotides −98 to + 102 from the *icmR* transcription start site), was amplified by PCR from the plasmid pOG-R125 using the primers pMC-lac and icmR-reg-up (Table 2). This fragment was labeled with digoxigenin (DIG)-dUTP by the PCR DIG probe synthesis kit (Roche) according to the manufacturer's instructions (but with modified PCR DIG mix that was diluted 1:2.5 with unlabeled deoxynucleoside triphosphates). In each assay 10 fmol of the DIG-labeled probe was used. The His_6-CpxR protein (at the concentration indicated) was incubated in the presence or absence of 50 mM acetyl phosphate (Ac-P) at 37°C for 30 min in a 15-μl reaction volume containing 10 mM Tris (pH 7.4), 50 mM KCl, 1 mM EDTA, 5% glycerol, BSA (50 μg/ml), 1 mM dithiothreitol, 20 mM potassium glutamate, and 10 mM $MgSO_4$. The DIG-labeled probe (10 fmol) was added, and the reaction mixture was incubated for another 30 min at 37°C. Reactions were stopped by the addition of 3 μl of loading dye, and products were electroporated on 6% nondenaturing polyacrylamide gels (29:1) in 0.5× TAE running buffer (0.04 M Tris-acetate, 1 mM EDTA). Subsequently, the gels were electroblotted onto a positively charged nylon membrane (Schleicher & Schuell) and fixed by UV cross-linking. Detection of the DIG-labeled DNA probe by anti-DIG Fab fragment-alkaline phosphatase conjugate (Roche) and substrate CSPD was performed as directed by the manual from Roche.

β-Galactosidase assays. β-Galactosidase assays were performed as described elsewhere (28). *L. pneumophila* strains were grown on ACES (*N*-[2-acetamido]-2-aminoethane-sulfonic acid) buffered charcoal yeast extract (ABCYE) plates containing chloramphenicol for 48 h. The bacteria were scraped off the plate and suspended in ACES yeast extract (AYE) medium, and the bacterial OD_{600} was calibrated to 0.1 in AYE. The resulting cultures were grown on a roller drum for 17 to 18 h until reaching an OD_{600} of about 3.2 (stationary phase), and the assays were performed with 50 μl of culture. *E. coli* strains were grown in LB broth containing ampicillin for 16 h. The cultures were then diluted 1:100 into fresh LB broth with or without IPTG (1 mM) and grown on a roller drum for 2 h until reaching an OD_{600} of about 0.6 (exponential phase); 100 μl from this culture was taken to the assays. The substrate for β-galactosidase hydrolysis was *o*-nitrophenyl-β-D-galactopyranoside (Sigma).

Intracellular growth in *A. castellanii*. Intracellular growth assays were performed in a similar way to what was previously described (42). *A. castellanii* (ATCC 30234) cells (1.5×10^5) in proteose yeast glucose broth were added to wells of a 24-well microtiter plate and the amoebae were incubated for 1 h at 37°C to let the amoebae adhere. Then the proteose yeast glucose was aspirated, and the wells were washed once with 0.5 ml of warm (37°C) *Acanthamoeba* buffer (Ac-buffer), and 0.5 ml of warm Ac-buffer was added to the wells. Then, *L. pneumophila*, in Ac-buffer, was added to the wells at a multiplicity of infection of approximately 0.1. The plate was incubated for 30 min at 37°C, the Ac-buffer was aspirated, the wells were washed three times with 0.5 ml of warm Ac-buffer, and 0.6 ml of warm Ac-buffer was added to the wells. The supernatant of each well was sampled at intervals of about 24 h and CFU were determined by plating multiple dilutions on ABCYE plates.

Intracellular growth in HL-60-derived human macrophages. Intracellular growth assays were performed in a similar way to what was previously described (42). Wells of a 24-well microtiter plate containing 6×10^6 differentiated HL-60-derived macrophages were used for infection. *L. pneumophila* was added to the wells at a multiplicity of infection of approximately 0.1 and the infected HL-60-derived macrophages were incubated for 1 h at 37°C under CO_2 (5%). Then, the wells were washed three times, and 0.6 ml of RPMI 1640 containing 2 mM glutamine and 10% normal human serum was added to the wells. The supernatant of each well was sampled at intervals of about 24 h and CFU were determined by plating multiple dilutions on ABCYE plates.

Nucleotide sequence accession numbers. Sequence data of the *L. pneumophila cpxRA* and *rpoE* genes have been assigned GenBank accession numbers AY295086 and AY295087, respectively.

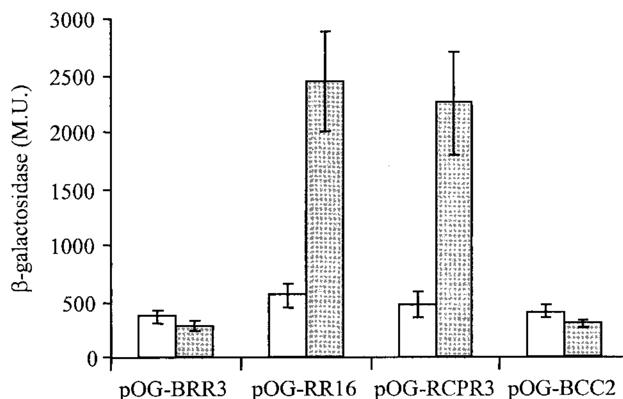

FIG. 1. Level of expression of the *icmR*::*lacZ* fusion in *E. coli. E. coli* containing different plasmids was grown to exponential phase, and the level of expression of the *icmR*::*lacZ* fusion was determined without (white) and with (gray) IPTG induction. The plasmids examined were the vector (pOG-BRR3), the clone isolated from the genetic screen (pOG-RR16), the vector harboring the *L. pneumophila cpxR* gene by itself (pOG-RCPR3), and the vector harboring the *E. coli cpxR* gene (pOG-BCC2). The β-galactosidase specific activity is presented as an average of the results of at least three different experiments. The error bars represent standard deviations.

RESULTS

The levels of expression of nine translational fusions of *L. pneumophila icm* and *dot* genes (*icmT*::*lacZ*, *icmR*::*lacZ*, *icmQ*::*lacZ*, *icmP*::*lacZ*, *icmM*::*lacZ*, *icmJ*::*lacZ*, *icmF*::*lacZ*, *icmW*::*lacZ*, and *icmV*::*lacZ*) have been previously compared between *E. coli* and *L. pneumophila* (16). It was found that the level of expression of the *icmR*::*lacZ* fusion in *E. coli* in comparison to *L. pneumophila* was fourfold lower at exponential phase (295 ± 16 and 1,157 ± 159 Miller units [MU], respectively) and sevenfold lower at stationary phase (369 ± 19 and 2,435 ± 339 MU, respectively) (16). (Unless otherwise noted, results are presented as means ± standard deviations.) These results led us to assume that *L. pneumophila* possesses a regulatory factor(s) required for optimal expression of the *icmR* gene that is missing or functions differently in *E. coli.* These differences in the level of expression of the *icmR*::*lacZ* fusion served as the basis for a genetic screen aimed at the identification of the *L. pneumophila icmR* regulator.

Genetic screen for the identification of an *icmR* regulator. To identify the activator that participates in the regulation of *icmR*, a genetic screen of the *L. pneumophila* library in *E. coli* was performed. The *L. pneumophila* library was constructed using a plasmid (pOG-BRR3) that contains the *icmR*::*lacZ* fusion and at a different position a unique *Eco*RI site located immediately downstream from a P*tac* inducible promoter (see Materials and Methods). All together, about 13,700 *E. coli* transformants were screened on MacConkey plates containing IPTG for higher expression of the *icmR*::*lacZ* fusion, and 24 suspected (dark-red) clones were identified. One clone (pOG-RR16) was found to have a particularly high level of β-galactosidase activity (2,452 ± 446 MU) in comparison to the vector (pOG-BRR3) β-galactosidase background level (283 ± 37 MU). This high level of expression of the *icmR*::*lacZ* fusion was found to be dependent on IPTG induction (Fig. 1). Most of the other 23 clones isolated showed β-galactosidase levels of

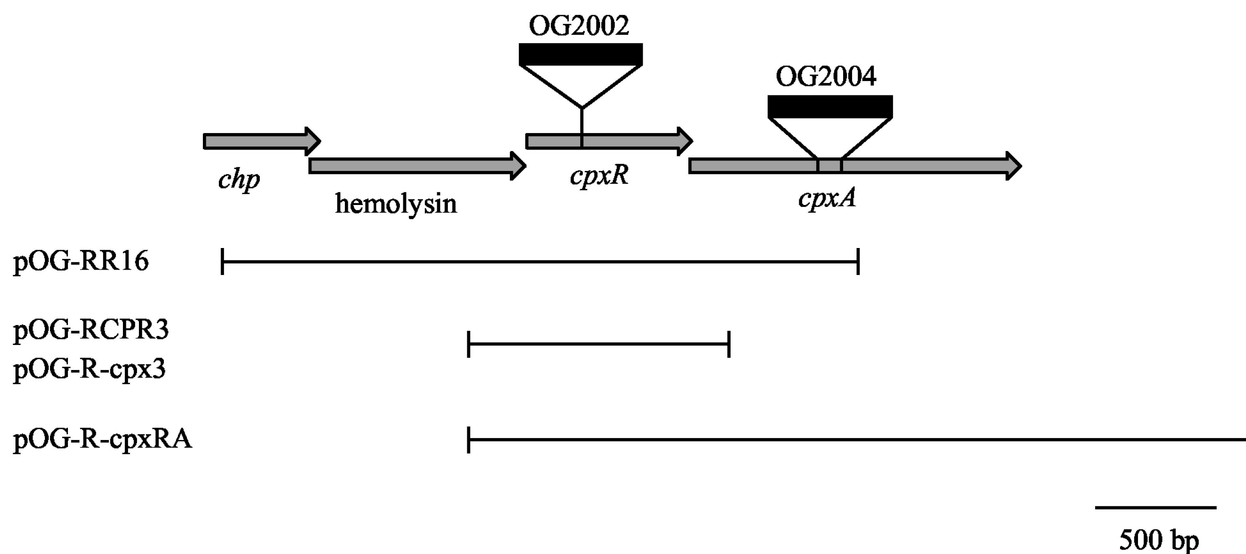

FIG. 2. Linkage map of the *L. pneumophila cpxRA* locus. The arrows indicate ORFs (conserved hypothetical protein, *chp*; hemolysin; *cpxR* and *cpxA*). The position of the kanamycin resistance cassette in the *cpxR* insertion mutant (OG2002) and *cpxA* deletion substitution mutant (OG2004) is indicated. The thin lines indicate the regions covered by the plasmids listed on the left.

expression of about 300 to 350 MU. The positive clone that had a high level of expression (pOG-RR16) was partly sequenced and examined by restriction analysis. pOG-RR16 was found to contain two joined inserts from different regions of the *L. pneumophila* chromosome, 2.6 and 6.4 kb in size. The sequence data from pOG-RR16 was compared to the *L. pneumophila* genome database, which showed that the 2.6-kb fragment (located immediately downstream from the P*tac* promoter) contains the following genes: (i) an open reading frame (ORF) homologous to a conserved hypothetical protein (*chp*), (ii) an ORF homologous to hemolysin, (iii) an ORF homologous to *cpxR*, and (iv) a portion (217 of 455 amino acids) from an ORF homologous to *cpxA* (Fig. 2). The *L. pneumophila* CpxR ortholog was found to be 49% identical and 68% similar to the *E. coli* CpxR, whereas the *L. pneumophila* CpxA ortholog was found to be 27% identical and 46% similar to the *E. coli* CpxA. The *L. pneumophila cpxR* and *cpxA* genes were found to overlap each other by 8 bp, an organization that was also found in other bacteria (10).

***L. pneumophila* CpxR enhances the expression of the *icmR*::*lacZ* fusion in *E. coli*.** The most reasonable candidate for being responsible for the elevated expression of the *icmR*::*lacZ* fusion in the clone described above (pOG-RR16) was the response regulator CpxR (see introduction). In order to test this assumption, the *L. pneumophila cpxR* gene by itself was cloned into the library vector (pOG-BRR3) and the resulting plasmid (pOG-RCPR3, Fig. 2) was examined for the level of expression of the *icmR*::*lacZ* fusion, in the presence and absence of IPTG. As can be seen in Fig. 1, in the presence of IPTG, the level of expression of the *icmR*::*lacZ* fusion from pOG-RCPR3 (2,255 ± 459 MU) was similar to the level of expression obtained with the original clone isolated by the genetic screen (pOG-RR16, 2,452 ± 446 MU) and was eightfold higher than the level of expression of the library vector (pOG-BRR3, 283 ± 37 MU). These data indicate that supplying the *L. pneumophila cpxR* gene on a plasmid significantly elevates the level of expression of the *icmR*::*lacZ* fusion in *E. coli.*

CpxR controls the expression of three *L. pneumophila icm* genes. To examine, whether CpxR also plays a role in the regulation of *icmR* in *L. pneumophila*, an insertion mutant was constructed in the *cpxR* gene. The resulting *L. pneumophila* strain (OG2002, Fig. 2) grows well on bacteriological media with a growth rate similar to that of the wild-type strain (data not shown). To determine the involvement of CpxR in *icm* and *dot* gene expression, the levels of expression of the nine *icm*::*lacZ* fusions described above were compared between the *cpxR* mutant strain (OG2002) and the wild-type strain (JR32). As can be seen in Fig. 3, the level of expression of three *icm*::*lacZ* fusions was found to be significantly lower in OG2002. The level of expression of the *icmV*::*lacZ* and *icmW*::*lacZ* fusions decreased more than threefold (from 4,384 ± 634 to 1,278 ± 213 MU for *icmV*::*lacZ* and from 4324 ± 425 to 1418 ± 130 MU for *icmW*::*lacZ* in JR32 and OG2002, respectively) in the *cpxR* mutant strain (OG2002). However, the most profound change was the reduction in the level of expression of the *icmR*::*lacZ* fusion, which decreased 13-fold from 2,410 ± 329 MU in the wild-type strain (JR32) to 187 ± 26 MU in the *cpxR* mutant strain (OG2002). These results indicate that the response regulator CpxR is involved in the regulation of *icmV*, *icmW*, and especially *icmR* in *L. pneumophila*.

CpxR activates the expression of *icmR* in *L. pneumophila*. In order to give further support to the assumption that the reduction in the level of expression of *icmR* in OG2002 was due to the insertion in the *cpxR* gene, a complementation experiment was performed. The *L. pneumophila cpxR* gene was cloned into a plasmid containing the *icmR*::*lacZ* fusion (pOG-R125) and the resulting plasmid (pOG-R-cpx3) (Fig. 2) was introduced into the *cpxR* insertion mutant (OG2002). When the plasmid containing the *cpxR* gene (pOG-R-cpx3) was introduced into the *cpxR* insertion mutant strain (OG2002), the

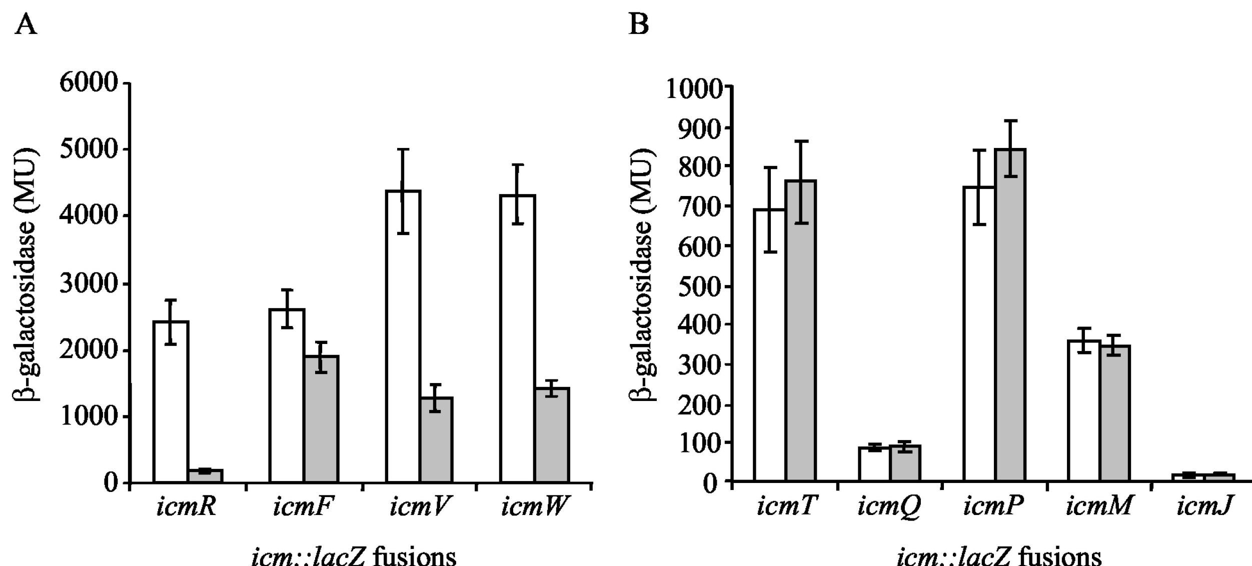

FIG. 3. CpxR affects the level of expression of three *icm*::*lacZ* fusions. The level of expression of nine *icm*::*lacZ* fusions (*icmT*, *icmR*, *icmQ*, *icmP*, *icmM*, *icmJ*, *icmF*, *icmV*, and *icmW*) was examined in the *L. pneumophila* wild-type strain JR32 (white) and the *cpxR* insertion mutant OG2002 (gray). The β-galactosidase activity was measured as described in Materials and Methods. Four *icm*::*lacZ* fusions that had high β-galactosidase activities (A) and five that had low activities (B) are shown. The data are presented as averages of results of at least three different experiments. The error bars represent standard deviations. The level of expression of the vector was 7.3 ± 2.4 MU.

level of expression of *icmR*::*lacZ* was restored (1,901 ± 202 MU) to a level similar to that in the wild-type strain (2,410 ± 329 MU) (Fig. 4).

The differences in the levels of expression of the *icmR* gene

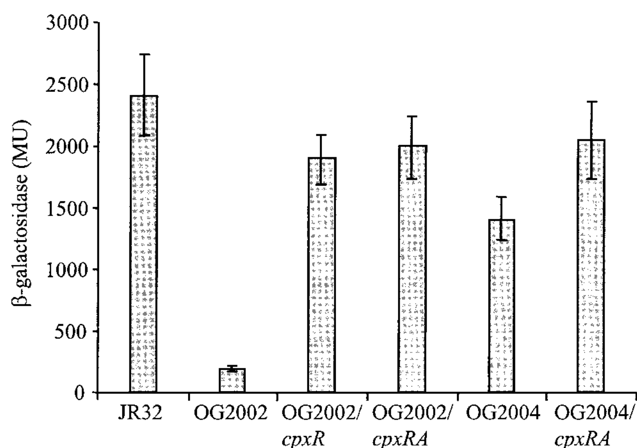

FIG. 4. The expression of the *icmR*::*lacZ* fusion in different *L. pneumophila* strains. Strains of *L. pneumophila* containing different plasmids were grow to stationary phase and analyzed for the level of expression of the *icmR*::*lacZ* fusion. All the strains examined contain an *icmR*::*lacZ* fusion located on a plasmid. The following were used: wild-type strain (JR32), the *cpxR* insertion mutant (OG2002), the *cpxR* insertion mutant containing the *L. pneumophila cpxR* gene (OG2002/*cpxR*), the *cpxR* insertion mutant containing the *L. pneumophila cpxRA* genes (OG2002/*cpxRA*), the *cpxA* deletion substitution mutant (OG2004), and the *cpxA* deletion substitution mutant containing the *L. pneumophila cpxRA* genes (OG2004/*cpxRA*). The β-galactosidase activity was measured as described in Materials and Methods. The data are presented as an average of at least three different experiments. The error bars represent standard deviations. The level of expression of the vector was 7.3 ± 2.4 MU.

between wild-type *L. pneumophila* (JR32), the *cpxR* mutant strain (OG2002) and the *cpxR* mutant strain (OG2002) containing a complementing plasmid (pOG-R-cpx3) were also determined at the mRNA level. Total RNA was purified from these strains and analyzed by Northern hybridization using the *icmR* gene as a probe (Fig. 5). Quantification of the hybridization signals indicated that the level of *icmR* mRNA in the *L. pneumophila* wild-type strain (JR32) was about 10-fold higher than its level in the *cpxR* mutant strain (OG2002) and introduction of the *cpxR* gene resulted in partial complementation. However, the *cpxR* mutant strain (OG2002) was found to hybridize normally with other probes such as the *flaA* gene (data not shown). Therefore, the Northern hybridization results are in agreement with the results obtained with the *icmR*::*lacZ* fusion (compare Fig. 4 and 5), showing that *icmR* gene expression is controlled by the CpxR regulator.

Identification of the CpxR binding site in the *icmR* regulatory region. The binding site of the response regulator CpxR in the upstream regulatory region of its target genes has been identified before in *E. coli* (21, 33) and in *S. sonnei* (31). According to these reports, CpxR has a conserved recognition site that contains the sequence GTAAA (11). This sequence was found twice in the *icmR* regulatory region (Fig. 6A). The first GTAAA site (which will be referred to as BRM1) is located 87 bp upstream from the first IcmR methionine and 60 bp upstream from the *icmR* transcription start site (16). The second GTAAA site (which will be referred to as BRM2) is located 29 bp upstream from the first IcmR methionine and only 2 bp upstream from the transcription start site. To find which of these two potential sites (if any) serves as a binding site for CpxR, each of them was changed separately using site-directed mutagenesis (changing the first two nucleotides from GT to TG). The plasmids containing these mutations (pOG-BRM1 and pOG-BRM2) were introduced into *L. pneu-*

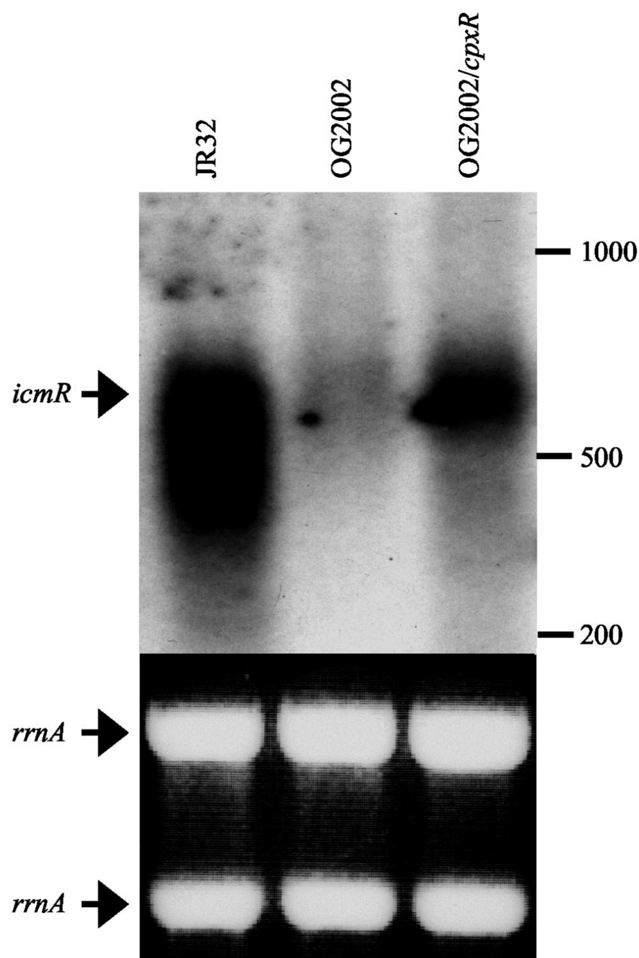

FIG. 5. CpxR affects *icmR* mRNA level. RNA was purified from stationary-phase cultures of the *L. pneumophila* wild-type strain (JR32), the *cpxR* insertion mutant (OG2002), and the *cpxR* insertion mutant containing the complementing plasmid pOG-R-cpx3 (OG2002/*cpxR*). The upper part of the figure shows the *icmR* mRNA levels that were analyzed by Northern hybridization, using the *icmR* gene as a probe. The arrow indicates the *icmR* mRNA signal (about 620 bp); the size maker is indicated to the right. The lower part of the figure shows equal loadings of samples (10 μg) demonstrated by ethidium bromide staining of *rrnA* (23S and 16S) (indicated by arrows) in the agarose gel prior to transfer.

mophila JR32 and the levels of expression of the *icmR*::*lacZ* fusion were determined (Fig. 6B). The expression of the *icmR*::*lacZ* fusion containing the mutation at the BRM1 site (pOG-BRM1) was found to be 11-fold lower than that of the *icmR*::*lacZ* fusion that contains a wild-type regulatory region (209 ± 37 and 2,411 ± 329 MU, respectively), a result expected from a mutation in an activator binding site. In contrast, the mutation constructed at the BRM2 site resulted in a higher level of expression of the *icmR*::*lacZ* fusion than that of the fusion that contains the wild-type regulatory region (3,291 ± 382 and 2,411 ± 329 MU, respectively) (Fig. 6B). This result might be explained by the fact that the mutation at the BRM2 site altered the −10 promoter element of *icmR* from TATATG to TATATT (Fig. 6A). The latter sequence was shown to constitute the −10 promoter element of the *icmV* gene, and our previous studies showed that the *icmV*::*lacZ* fusion has a higher level of expression than the *icmR*::*lacZ* fusion (16). Hence, changing the −10 promoter element of *icmR* to the sequence TATATT might be the cause for the higher level of expression of *icmR*::*lacZ* fusion containing the mutation at the BRM2 site.

To give further support to the hypothesis that the BRM1 site functions as the binding site for CpxR (and not a binding site of another unrelated *icmR* activator), the plasmid containing the mutation at the BRM1 site (pOG-BRM1) was examined in the *L. pneumophila cpxR* mutant strain (OG2002). As can be seen in Fig. 6B, no additive effect was found when the plasmid containing the mutation at the BRM1 site (pOG-BRM1) was examined in the *cpxR* mutant strain (OG2002), indicating that the sequence GTAAA at the BRM1 site constitutes the binding site for the CpxR activator. It was expected that if the BRM1 site was a recognition site for another regulator, and not CpxR, the effect of introducing the *icmR*::*lacZ* fusion containing the mutation at the BRM1 site into the *cpxR* mutant strain would have resulted in a lower level of expression of this fusion than that of the wild-type strain containing the same fusion (additive effect). In agreement with this observation, the level of expression from pOG-BRM1 (209 ± 37 MU) was very similar to the level of expression of the wild-type *icmR*::*lacZ* fusion in the *cpxR* mutant strain (188 ± 26 MU). These results show that the same level of expression could be achieved when either the binding site of CpxR was mutated or when the regulator itself was absent.

In order to learn more about the importance of the BRM1 site in *icmR* gene expression, the effect of the mutation in this site was compared to the effects of two mutations (RP1 and RP2) constructed in the −10 promoter element of *icmR* (Fig. 6A). Similar mutations that were previously constructed in the same two positions in the −10 promoter element of several *icm* genes resulted in dramatic reductions in the levels of expression of the genes located downstream from them (16). As can be seen in Fig. 6B, the level of expression from pOG-BRM1 (209 ± 37 MU) was found to be lower than the level of expression of the *icmR*::*lacZ* fusion carrying the mutations at the −10 promoter element (727 ± 42 MU for RP1 and 293 ± 31 MU for RP2). These results indicate that the CpxR binding site plays a critical role in the expression of *icmR*, and its contribution to the *icmR* gene expression is at least as significant as that of the −10 promoter element.

The *E. coli* CpxR protein does not activate the *L. pneumophila icmR* gene. The finding that the *L. pneumophila* CpxR regulator recognizes a regulatory element similar to that of *E. coli* CpxR is not surprising; however, this finding makes it hard to understand the initial ability to identify the *L. pneumophila* CpxR regulator in the screen performed, as the *E. coli* strain used in the screen contained an intact *cpxR* gene. In order to clarify this issue, we cloned the *E. coli cpxR* gene into the vector that was used for the screen (pOG-BRR3) in the same way in which the *L. pneumophila cpxR* regulator was cloned. The resulting plasmid (pOG-BCC2) was examined for the level of expression of the *icmR*::*lacZ* fusion with or without the addition of IPTG. The expression of the *icmR*::*lacZ* fusion was found to be equal to that obtained with the vector (pOG-BRR3) (Fig. 1), indicating that the *E. coli* CpxR regulator cannot activate the *L. pneumophila icmR* gene. The analysis

A

TTGTTTGTAAAGAATTAGAA...30bp...TATTTTGATATATGTAAAGTAAGAG...20bp...TAATG

B

FIG. 6. Identification of the CpxR binding site in the *icmR* regulatory region. (A) Sequence of *icmR* regulatory region. The first ATG codon is shown in boldface type. The position of the transcription start site is indicated by an asterisk. The two GTAAA sites (BRM1 and BRM2) are indicated by their designations. The two nucleotides that were changed (GT to TG) within the BRM1 and BRM2 sites are underlined. The *icmR* −10 promoter element (TATATG) is indicated and the pair of nucleotides mutated in this site (A to C and T to G) are underlined and indicated by their designations (RP1 and RP2). (B) Effect of different mutations in the regulatory region of *icmR* on the expression of the *icmR*::*lacZ* fusion. The following were used: the wild-type strain containing the *icmR*::*lacZ* fusion (JR32), the *cpxR* insertion mutant containing the *icmR*::*lacZ* fusion (OG2002), the wild-type strain containing the *icmR*::*lacZ* fusion with a mutation at the BRM1 site (JR32/BRM1), the wild-type strain containing the *icmR*::*lacZ* fusion with a mutation at the BRM2 site (JR32/BRM2), the *cpxR* insertion mutant containing the *icmR*::*lacZ* fusion with a mutation at the BRM1 site (OG2002/BRM1), the wild-type strain containing the *icmR*::*lacZ* fusion with a mutation at the second nucleotide of the −10 promoter element (JR32/RP1), and the wild-type strain containing the *icmR*::*lacZ* fusion with a mutation at the third nucleotide of the −10 promoter element (JR32/RP2). The β-galactosidase activity was measured as described in Materials and Methods. The data are presented as averages of results of at least three different experiments. The error bars represent standard deviations. The expression level of the vector was 7.3 ± 2.4 MU.

was performed during exponential as well as stationary phase, and the same result was obtained (Fig. 1 and data not shown).

The *L. pneumophila* His$_6$-CpxR protein binds to the *icmR* regulatory region. To determine whether *L. pneumophila* CpxR binds to the *icmR* regulatory region, *L. pneumophila* CpxR was His tagged, overexpressed, and purified as described in Materials and Methods. We utilized a 200-bp fragment that covers the *icmR* regulatory region as a probe in a mobility shift assay with the His$_6$-CpxR protein. The *L. pneumophila* His$_6$-CpxR was found to bind to the regulatory region of *icmR*, as evidenced by a shift in the migration of the DNA probe after electrophoresis (Fig. 7, lanes 2 to 5), and no effect was observed when BSA (50 pmol) was added (Fig. 7, lane 1). The degree of the band shift as well as the amount of the shifted probe was correlated with increasing amounts of the His$_6$-CpxR protein (Fig. 7, compare lanes 2 to 5). In addition, the amount of the shifted probe was found to be affected by the elimination of Ac-P from the reaction. This result indicates that phosphorylation of the His$_6$-CpxR protein is required for maximum binding (Fig. 7, compare lanes 5 and 6). Finally, a competition with unlabeled probe reduced the band shift (Fig. 7, compare lanes 5 and 7), and no effect was observed when the same amount of Herring sperm DNA was added (data not shown). These data indicate that the *L. pneumophila* His$_6$-CpxR protein binds to the *icmR* regulatory region.

CpxA has moderate effect on the expression of *icmR*. Phosphorylation of CpxR, which usually occurs by its cognate sensor kinase CpxA, allows the upregulation of its target genes. Therefore, it was interesting to examine whether the expression of the *icmR*::*lacZ* fusion is dependent on the presence of a functional CpxA. To illuminate this issue, a deletion substitution was constructed in the *cpxA* gene. The resulting strain

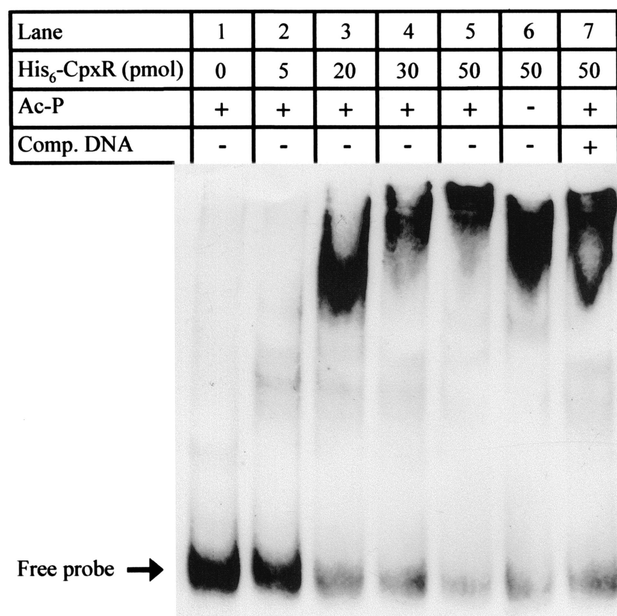

FIG. 7. *L. pneumophila* His_6-CpxR binds to the *icmR* regulatory region. Mobility shift assays were performed with pure His_6-CpxR and the DIG-labeled *icmR* regulatory region in the presence (+) and absence (−) of 100 fmol of the unlabeled *icmR* regulatory region (Comp. DNA) or acetyl phosphate (Ac-P). Each lane contains 10 fmol of the DIG-labeled probe and the amount of His_6-CpxR (pmol) is indicated above each lane. Lane 1 contains 50 pmol of BSA.

(OG2004) (Fig. 2), grows well on bacteriological media with a growth rate similar to that of the wild-type strain (data not shown).

The level of expression of the *icmR*::*lacZ* fusion in the *L. pneumophila cpxA* mutant strain (OG2004) was found to be mildly lower than that of the wild-type strain (1,416 ± 186 and 2,410 ± 329 MU, respectively) (Fig. 4). The lower level of expression of the *icmR*::*lacZ* fusion in OG2004 was complemented by introducing the *cpxRA* genes on a plasmid (pOG-R-cpxRA, 2,045 ± 307 MU) (Fig. 4). When this plasmid was introduced into the *cpxR* mutant strain (OG2002), the level of expression of the *icmR*::*lacZ* fusion (1,991 ± 250 MU) was similar to the level obtained by complementation with the *cpxR* gene by itself (pOG-R-cpx3, 1,901 ± 202 MU) and similar to the level of expression of the *icmR*::*lacZ* fusion in the wild-type strain (2411 ± 329 MU) (Fig. 4). These results indicate that in the conditions used, CpxA has only a moderate effect on the expression of *icmR* and that CpxR may be phosphorylated by factors other than CpxA (see Discussion).

RpoE is not involved in the regulation of the *icm* and *dot* genes. RpoE is an alternative sigma factor that regulates extracytoplasmic functions (reviewed in reference 48). It was shown before that the Cpx pathway and RpoE share a functionally similar mechanism of signal transduction (9, 35) and that both are involved in the regulation of protein turnover and protein-folding activities in the bacterial envelope. In addition, in some cases the RpoE sigma factor and the Cpx pathways overlap in their target genes, as was demonstrated in *E. coli* for the regulation of the periplasmic protease DegP (9) and probably the heat shock sigma factor RpoH (33). Considering this, the role of the RpoE sigma factor in *icm* and *dot* gene expression was examined. Using a BLAST search against the *L. pneumophila* genome database, the sequence coding for the *L. pneumophila* RpoE was identified (the *L. pneumophila* RpoE was found to be 37% identical and 55% similar to the *E. coli* RpoE), and a deletion substitution was constructed in it (OG2003). The nine *icm*::*lacZ* fusions described above were introduced into this strain (OG2003) and their level of expression was determined. As can be seen in Fig. 8, no significant change was found in the levels of expression of all nine *icm*::*lacZ* fusions in OG2003 in comparison to those of the wild-type strain. These results indicate that the *icm* and *dot* genes (including the three genes that were shown to be regulated by CpxR) probably do not contain RpoE-dependent promoters. Analysis of the upstream regulatory region of these genes also revealed that promoter consensus sequences recognized by the *E. coli* RpoE sigma factor (48) are not present there.

CpxR, CpxA, and RpoE are dispensable for intracellular growth of *L. pneumophila*. In the environment, *L. pneumophila* replicates intracellularly within protozoan hosts, such as *A. castellanii* (14), while in humans, during infection, these bacteria grow inside alveolar macrophages (20, 37). Given that CpxR was found to affect the level of expression of three *icm* genes, required for intracellular multiplication, the role of CpxR (as well as of the other two regulators described, CpxA and RpoE) in intracellular growth within *A. castellanii* and HL-60-derived human macrophages was examined. As can be seen in Fig. 9, the *L. pneumophila cpxR*, *cpxA*, and *rpoE* mutant strains (OG2002, OG2004, and OG2003, respectively) replicated to the same extent as the wild-type strain in the protozoan host *A. castellanii* (Fig. 9A) and in HL-60-derived human macrophages (Fig. 9B). Therefore, we concluded that the three regulators examined (CpxR, CpxA, and RpoE) are dispensable for intracellular growth of *L. pneumophila.*

DISCUSSION

We previously reported the construction of nine *icm* translational fusions (*icmT*::*lacZ*, *icmR*::*lacZ*, *icmQ*::*lacZ*, *icmP*::*lacZ*, *icmM*::*lacZ*, *icmJ*::*lacZ*, *icmF*::*lacZ*, *icmW*::*lacZ*, and *icmV*::*lacZ*) and the comparison of their levels of expression in *E. coli* and *L. pneumophila* (16). Most of these fusions (six out of nine) were found to retain similar levels of expression in both bacteria, suggesting that the same basic regulation of these *icm* genes is conserved between *E. coli* and *L. pneumophila.* However, the *icmR*::*lacZ* fusion, was found to have a high level of expression in *L. pneumophila* and a low level of expression in *E. coli* (16). These results led to the assumption that optimal expression of *icmR* requires an *L. pneumophila* regulatory factor(s) that is absent or functions differently in *E. coli.* In order to identify this potential regulator, a genetic screen was applied. In this screen, the *L. pneumophila* response regulator CpxR was identified as an activator of the *icmR* gene. Construction of an *L. pneumophila cpxR* insertion mutant demonstrated the critical role that CpxR plays in controlling *icmR* gene expression. In addition, the binding site (GTAAA) of the CpxR regulator was identified in the *icmR* regulatory region located 60 bp upstream from its transcription start site. The consensus identified is similar to the one found in other bac-

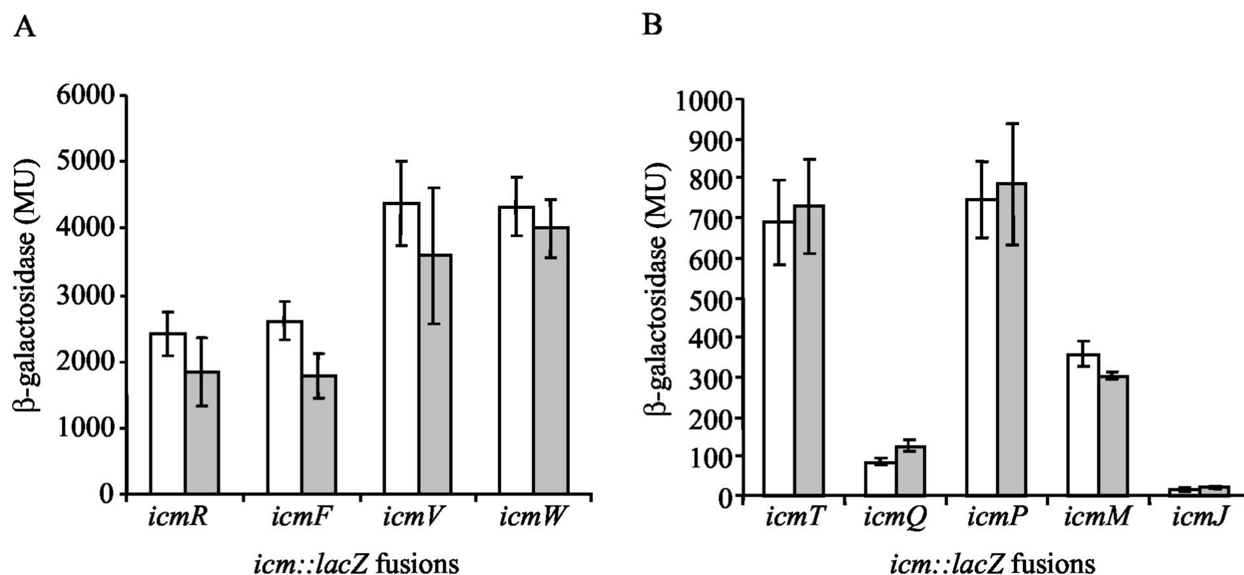

FIG. 8. RpoE does not affect the levels of expression of the *icm*::*lacZ* fusions. The levels of expression of nine *icm*::*lacZ* fusions (*icmT*, *icmR*, *icmQ*, *icmP*, *icmM*, *icmJ*, *icmF*, *icmV*, and *icmW*) were examined in *L. pneumophila* wild-type strain JR32 (white) and the *rpoE* deletion substitution mutant OG2003 (gray). β-Galactosidase activity was measured as described in Materials and Methods. Four *icm*::*lacZ* fusions that had high β-galactosidase activities (A) and five that had low activities (B) are shown. The data are presented in Miller units (MU) and are the averages of the results of at least three different experiments. The error bars represent standard deviations. The expression level of the vector was 7.3 ± 2.4 MU.

teria (11, 21, 33). Mutagenesis of the CpxR binding site was found to reduce the level of expression of the *icmR*::*lacZ* fusion to a lower level than that of mutations constructed in the −10 promoter element of *icmR*, suggesting that the contribution of the CpxR binding site to the expression of *icmR* is highly significant. Moreover, the *L. pneumophila* CpxR regulator was shown to bind the *icmR* regulatory region in vitro by using a mobility shift assay. Furthermore, the presented results indicate that CpxR is also involved in the regulation of *icmV* and *icmW*. Considering that the decrease in their level of expression was mild in comparison to the decrease observed with the *icmR*::*lacZ* fusion and the similar levels of expression that they had in *L. pneumophila* and *E. coli* (16), we speculate that the regulation of *icmV* and *icmW* by CpxR might be indirect.

The finding that CpxR regulates the expression of the *icmR* gene led us to examine the role of its cognate sensor kinase CpxA in the regulation of *icmR.* The expression of *icmR*::*lacZ* fusion in an *L. pneumophila cpxA* mutant strain was found to be moderately low in comparison to that of the wild-type strain. The differences in the effects of the *cpxR* and *cpxA* mutant strains on the level. of expression of the *icmR*::*lacZ* fusion might be explained by the possible substitution of CpxA by the low-molecular-weight compound acetyl-phosphate (Ac-P). This compound might phosphorylate the CpxR response regulator in the absence of CpxA as was shown previously for the *E. coli* CpxR and other response regulators (23, 25, 27) as well as in our gel-shift results. In *E. coli*, the *pta* and *ackA* genes code for a phosphate acyltransferase and an acetate kinase, respectively, and they were shown to be responsible for Ac-P synthesis (27). A BLAST search against the *L. pneumophila* incomplete genome database identified one ORF homologous to the *E. coli pta* and *ackA* genes (23% identity and 41% similarity to *pta*; 33% identity and 51% similarity to *ackA*). This homolog could be responsible for the synthesis of Ac-P in *L. pneumophila*, which might facilitate the phosphorylation of the CpxR regulator in the absence of CpxA.

Interestingly, despite the sequence similarity between the *E. coli* and the *L. pneumophila* CpxRs and their similar recognition sequences, the *E. coli* CpxR does not activate the expression of the *icmR*::*lacZ* fusion when expressed from a plasmid in *E. coli*. This result might indicate that the *E. coli* CpxR regulator does not bind to the *icmR* regulatory region or that it binds but cannot activate the *icmR* promoter. Currently we cannot distinguish these two possibilities. These data may indicate that these two proteins are not interchangeable. However, this is probably not the only difference between the *cpx* systems in these two bacteria. In *E. coli*, CpxP serves as an inhibitor molecule for CpxA (8); however, the *cpxP* gene is thus far absent in the *L. pneumophila* genome database (85% completed).

Although CpxR was shown to directly control the expression of the *icmR* gene and to be involved in the expression of two other *icm* genes (*icmV* and *icmW*), the *L. pneumophila* CpxR regulator was found to be dispensable for intracellular growth in *A. castellanii* and HL-60-derived human macrophages. This information indicates that even a 10-fold reduction in the expression of the *icmR* gene was still sufficient for establishing a successful infection in the eukaryotic hosts examined. It is possible that in other hosts used by *L. pneumophila* the reduction in the amount of IcmR in the bacterial cell would result in an intracellular growth phenotype. Previously, it was shown that an *icmR* insertion mutant retains some ability to kill HL-60-derived human macrophages (40) and another mutation in this gene was shown to permit limited intracellular replication in murine bone marrow macrophages (6). These results might

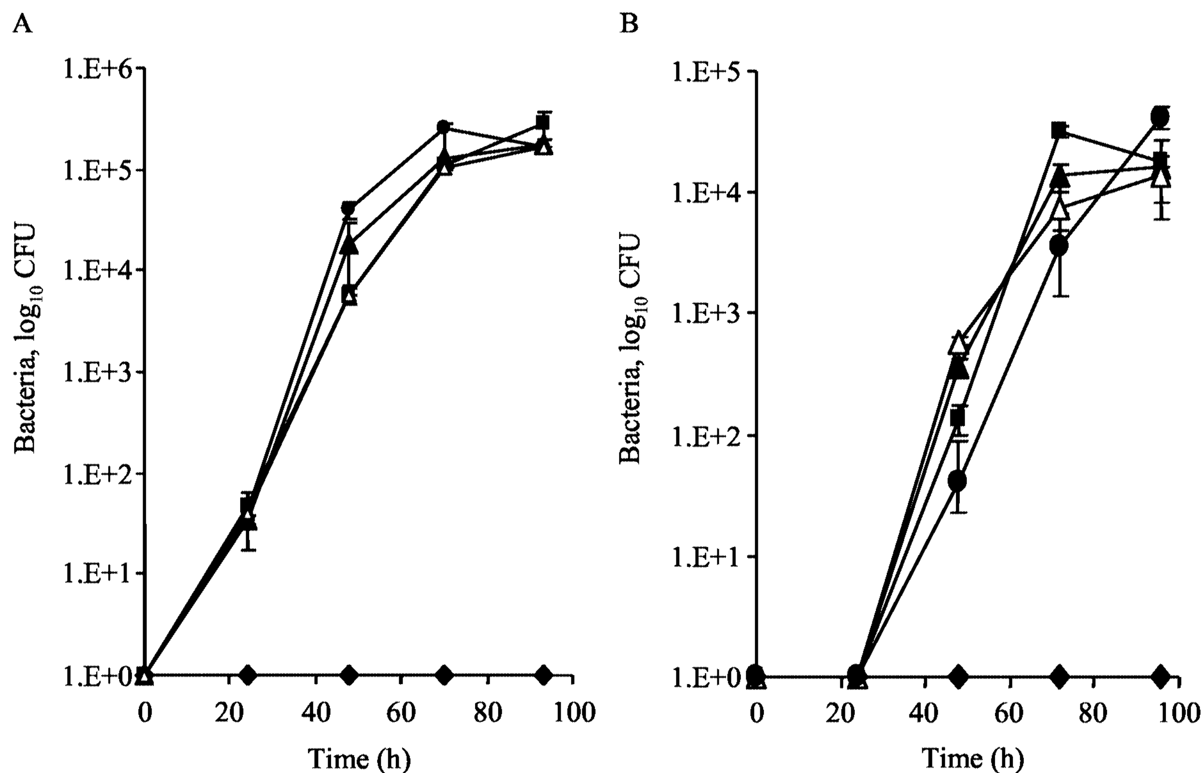

FIG. 9. CpxR, CpxA and RpoE are dispensable for *L. pneumophila* intracellular multiplication. The ability of *cpxR*, *cpxA*, and *rpoE* mutant strains to grow intracellularly was examined in *A. castellanii* (A) and HL-60-derived human macrophages (B). Symbols: squares, *L. pneumophila* wild-type strain (JR32); diamonds, the mutant strain 25D; closed triangles, *cpxR* mutant strain (OG2002); open triangles, *cpxA* mutant strain (OG2004); circles, *rpoE* mutant strain (OG2003). The experiments were performed as described in Materials and Methods. The experiments were done three times, and the same results were obtained; error bars indicate standard errors.

also explain the fact that the reduction in the level of expression of the *icmR* gene in the *cpxR* mutant strain does not result in an intracellular growth defect, as even a complete null mutation in the *icmR* gene still allows some ability to replicate within host cells. In addition, the IcmR gene product was suggested to act as the chaperone of IcmQ (13). This information is consistent with the known role of CpxR, in other bacteria, where it was shown to regulate genes involved in protein-folding functions (9, 21, 35). Since IcmR is probably not a structural component of the Icm/Dot complex, it might be that a reduction in its level can result in a no intracellular growth phenotype.

Thus far, several regulatory factors such as RpoS, RelA, and LetA have been shown to be involved in the regulation of the *icm* and *dot* genes in *L. pneumophila*, but not in a direct manner. The data presented here, showing that the regulation of the *L. pneumophila icmR* virulence gene is mediated by the CpxR response regulator, is the first evidence for a direct regulator of an *icm* or *dot* gene. The CpxR regulator together with other regulatory factors probably concerts with the expression of *icm* and *dot* genes to result in successful infection.

ACKNOWLEDGMENTS

This research was supported by the Charles H. Revson Foundation of the Israel Science Foundation (grant 45/00). G. Segal was supported by the Alon fellowship awarded by the Israeli Ministry of Education.

REFERENCES

1. **Albright, L. M., E. Huala, and F. M. Ausubel.** 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. **23:**311–336.
2. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. Mol. Microbiol. **14:**809–822.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Brand, B. C., A. B. Sadosky, and H. A. Shuman.** 1994. The *Legionella pneumophila icm* locus: a set of genes required for intracellular multiplication in human macrophages. Mol. Microbiol. **14:**797–808.
5. **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.
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. **Danese, P. N., and T. J. Silhavy.** 1998. CpxP, a stress-combative member of the Cpx regulon. J. Bacteriol. **180:**831–839.
9. **Danese, P. N., and T. J. Silhavy.** 1997. The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli.* Genes Dev. **11:**1183–1193.
10. **De Wulf, P., B. J. Akerley, and E. C. Lin.** 2000. Presence of the Cpx system in bacteria. Microbiology **146:**247–248.
11. **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.
12. **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.

13. **Dumenil, 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.
14. **Fields, B. S.** 1996. The molecular ecology of *legionellae.* Trends Microbiol. **4:**286–290.
15. **Gal-Mor, O., and G. Segal.** 2003. The *Legionella pneumophila* GacA homolog (LetA) is involved in the regulation of icm virulence genes and is required for intracellular multiplication in *Acanthamoeba castellanii.* Microb. Pathog. **34:**187–194.
16. **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.
17. **Gross, R., B. Arico, and R. Rappuoli.** 1989. Families of bacterial signal-transducing proteins. Mol. Microbiol. **3:**1661–1667.
18. **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.
19. **Horwitz, M. A.** 1987. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. J. Exp. Med. **166:**1310–1328.
20. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Investig. **66:**441–450.
21. **Hung, D. L., T. L. Raivio, C. H. Jones, T. J. Silhavy, and S. J. Hultgren.** 2001. Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili. EMBO J. **20:**1508–1518.
22. **Jones, C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren.** 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J. **16:**6394–6406.
23. **Kenney, L. J., M. D. Bauer, and T. J. Silhavy.** 1995. Phosphorylation-dependent conformational changes in OmpR, an osmoregulatory DNA-binding protein of *Escherichia coli.* Proc. Natl. Acad. Sci. USA **92:**8866–8870.
24. **Leclerc, G. J., C. Tartera, and E. S. Metcalf.** 1998. Environmental regulation of *Salmonella typhi* invasion-defective mutants. Infect. Immun. **66:**682–691.
25. **Lukat, G. S., W. R. McCleary, A. M. Stock, and J. B. Stock.** 1992. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc. Natl. Acad. Sci. USA **89:**718–722.
26. **Lynch, D., N. Fieser, K. Gloggler, V. Forsbach-Birk, and R. Marre.** 2003. The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii.* FEMS Microbiol. Lett. **219:**241–248.
27. **McCleary, W. R., J. B. Stock, and A. J. Ninfa.** 1993. Is acetyl phosphate a global signal in *Escherichia coli*? J. Bacteriol. **175:**2793–2798.
28. **Miller, J. H.** 1972. Experiments in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39–47.
30. **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.
31. **Nakayama, S., and H. Watanabe.** 1998. Identification of *cpxR* as a positive regulator essential for expression of the *Shigella sonnei virF* gene. J. Bacteriol. **180:**3522–3528.
32. **Nakayama, S., and H. Watanabe.** 1995. Involvement of *cpxA*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. J. Bacteriol. **177:**5062–5069.
33. **Pogliano, J., A. S. Lynch, D. Belin, E. C. Lin, and J. Beckwith.** 1997. Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. Genes Dev. **11:**1169–1182.
34. **Purcell, M., and H. A. Shuman.** 1998. The *Legionella pneumophila icmGC-DJBF* genes are required for killing of human macrophages. Infect. Immun. **66:**2245–2255.
35. **Raivio, T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Annu. Rev. Microbiol. **55:**591–624.
36. **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.
37. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
38. **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.
39. **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.
40. **Segal, G., and H. A. Shuman.** 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila.* Infect. Immun. **65:**5057–5066.
41. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the *Legionella pneumophila* phagosome determined? Trends Microbiol. **6:**253–255.
42. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
43. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
44. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
45. **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.
46. **Vogel, J. P., and R. R. Isberg.** 1999. Cell biology of *Legionella pneumophila.* Curr. Opin. Microbiol. **2:**30–34.
47. **Wiater, L. A., A. B. Sadosky, and H. A. Shuman.** 1994. Mutagenesis of *Legionella pneumophila* using Tn*903*dII*lacZ*: identification of a growth-phase-regulated pigmentation gene. Mol. Microbiol. **11:**641–653.
48. **Wosten, M. M.** 1998. Eubacterial sigma-factors. FEMS Microbiol. Rev. **22:**127–150.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
50. **Zusman, T., O. Gal-Mor, and G. Segal.** 2002. Characterization of a *Legionella pneumophila relA* insertion mutant and roles of RelA and RpoS in virulence gene expression. J. Bacteriol. **184:**67–75.