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MOLECULAR PATHOGENESIS

The *Legionella pneumophila* Incomplete Phosphotransferase System Is Required for Optimal Intracellular Growth and Maximal Expression of PmrA-Regulated Effectors

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ABSTRACT The nitrogen phosphotransferase system (PTS^{Ntr}) is a regulatory cascade present in many bacteria, where it controls different functions. This system is usually composed of three basic components: enzyme I^{Ntr} (EI^{Ntr}), NPr, and $EIIA^{Ntr}$ (encoded by the *ptsP*, *ptsO*, and *ptsN* genes, respectively). In *Legionella pneumophila*, as well as in many other *Legionella* species, the $EIIA^{Ntr}$ component is missing. However, we found that deletion mutations in both *ptsP* and *ptsO* are partially attenuated for intracellular growth. Furthermore, these two PTS^{Ntr} components were found to be required for maximal expression of effector-encoding genes regulated by the transcriptional activator PmrA. Genetic analyses which include the construction of single and double deletion mutants and overexpression of wild-type and mutated forms of EI^{Ntr}, NPr, and PmrA indicated that the PTS^{Ntr} components affect the expression of PmrA-regulated genes via PmrA and independently from PmrB and that EI^{Ntr} and NPr are part of the same cascade and require their conserved histidine residues in order to function. Furthermore, expression of the *Legionella micdadei* EII^{Ntr} component in *L. pneumophila* resulted in a reduction in the levels of expression of PmrA-regulated genes which was completely dependent on the *L. pneumophila* PTS components and the *L. micdadei* EII^{Ntr} conserved histidine residue. Moreover, reconstruction of the *L. pneumophila* PTS *in vitro* indicated that EI^{Ntr} is phosphorylated by phosphoenolpyruvate (PEP) and transfers its phosphate to NPr. Our results demonstrate that the *L. pneumophila* incomplete PTS^{Ntr} is functional and involved in the expression of effector-encoding genes regulated by PmrA.

KEYWORDS *Legionella*, effector gene expression, phosphotransferase system (PTS), PmrA two-component system

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Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular pathogen which utilizes the Icm/Dot type IV secretion system for pathogenesis (1, 2). The Icm/Dot secretion system was shown to translocate a cohort of approximately 300 effector proteins into host cells during infection (3). The levels of expression of many of the genes encoding these effectors were found to be regulated by three two-component systems (TCSs): (i) the PmrAB TCS, which consists of the PmrA response regulator (RR) and the PmrB sensor histidine kinase (SHK), was shown to directly activate the expression of 43 effector-encoding genes (4, 5); (ii) the CpxRA TCS, which consists of the CpxR RR and the CpxA SHK, was shown to directly activate or repress the expression of 27 effector-encoding genes and four Icm/Dot components (6–8); (iii) the LetAS TCS, which consists of the LetA RR and the LetS SHK (9, 10), was shown to regulate the transcription of two small regulatory RNAs, RsmY and RsmZ, which act in

a redundant fashion to jointly antagonize CsrA, an RNA-binding protein that negatively regulates the expression of 26 effector-encoding genes at the translational level (11–15). In addition, the *L. pneumophila* Lqs system and its signaling molecule LAI-1 positively regulate the transcription of these small regulatory RNAs and negatively regulate the RNA-binding protein CsrA (16). Such TCSs are usually activated by phosphorylation of a conserved histidine residue located in their SHKs, from which the phosphate group is then transferred to a conserved aspartic acid residue located in the receiver domain of the cognate RR, which in turn directly activates or represses gene expression (17).

In many bacteria, global regulatory systems such as the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) are involved in various regulatory functions. The sugar-related PTS consists of two cytoplasmic proteins, enzyme I (EI), which is phosphorylated by PEP, and histidine phosphocarrier protein (HPr), which is phosphorylated by the EI component, both of which lack sugar specificity, and sugar-specific enzyme II (EII) components (18). Many Gram-negative bacteria also contain the nitrogen PTS (PTS^{Ntr}). The PTS^{Ntr} constitutes another phosphorylation cascade which proceeds sequentially from PEP to EI^{Ntr} encoded by *ptsP*, to NPr encoded by *ptsO*, and to $EIIA^{Ntr}$ encoded by *ptsN*, proteins which are homologous to the sugar PTS components EI, HPr, and EIIA, respectively (19, 20). In different bacterial species the PTS^{Ntr} regulates diverse processes implicated in metabolism of nitrogen and carbon (21), it plays a role in potassium homeostasis (22) and biofilm formation (23), and is essential for virulence in some bacteria such as *Salmonella enterica* (24) and *Brucella melitensis* (25), as well as in *L. pneumophila* (26).

In this report, we demonstrate by *in vitro* and *in vivo* experiments that the incomplete PTS^{Ntr} (lacking an $EIIA^{Ntr}$) found in *L. pneumophila* functions as a phosphorelay. We also demonstrate a link between the PTS^{Ntr} and the RR PmrA which controls the expression of numerous effector-encoding genes. We show that *ptsP* and *ptsO* deletion mutants are defective for intracellular growth and that the levels of expression of PmrA-regulated genes are reduced in these mutants, thus for the first time connecting the PTS^{Ntr} to effector gene expression in *L. pneumophila*.

RESULTS

***L. pneumophila* contains an incomplete PTS^{Ntr} lacking an $EIIA^{Ntr}$ component.** A genomic search performed at the NCBI database revealed that *L. pneumophila* contains only the first two components of the PTS^{Ntr}, the *ptsP* gene encoding EI^{Ntr} and the *ptsO* gene encoding NPr (Fig. 1A to C). The overall operon organization of the PTS^{Ntr} genes is known to be conserved in bacteria (20). In most cases the *rpoN* gene encoding the nitrogen sigma factor (RpoN) is located first in an operon which also includes *ptsN* (encoding $EIIA^{Ntr}$) and *ptsO* (encoding NPr) (Fig. 1A). This operon was also shown to contain additional genes, some of which are conserved in most bacterial species (such as the *yhbH* gene, encoding a ribosome binding protein), and in several bacteria additional genes belonging to the PTS^{Ntr} are also part of this operon (such as *ptsK* encoding HPr kinase/phosphorylase and *ptsI* encoding an EI-like protein). Examination of this operon in many bacteria indicated that the *ptsN* gene, when present, is always located between the *rpoN* and the *ptsO* genes (Fig. 1A). However, this was found not to be the case in *L. pneumophila* (as well as in *Acinetobacter baumannii*), which does not contain a *ptsN* gene as part of the *rpoN* operon. In addition, a BLAST search indicated that there is no $EIIA^{Ntr}$ homologue in *L. pneumophila* (an E score of 0.1 was used as a cutoff to determine similarity). Importantly, the absence of the *ptsN* gene encoding $EIIA^{Ntr}$ is not unique to *L. pneumophila*. This was also found to be the case in all the *Legionella* species belonging to the *L. pneumophila* clade (27), which includes 21 species, while it is present in most of the other *Legionella* species and in all of the members of the *Legionella micdadei* clade (Fig. 2). Even though the $EIIA^{Ntr}$ is absent in the *L. pneumophila* clade, the EI^{Ntr} and NPr proteins are conserved in the entire *Legionella* genus (see Fig. S1 in the supplemental material and data not shown), suggesting that they are functional.

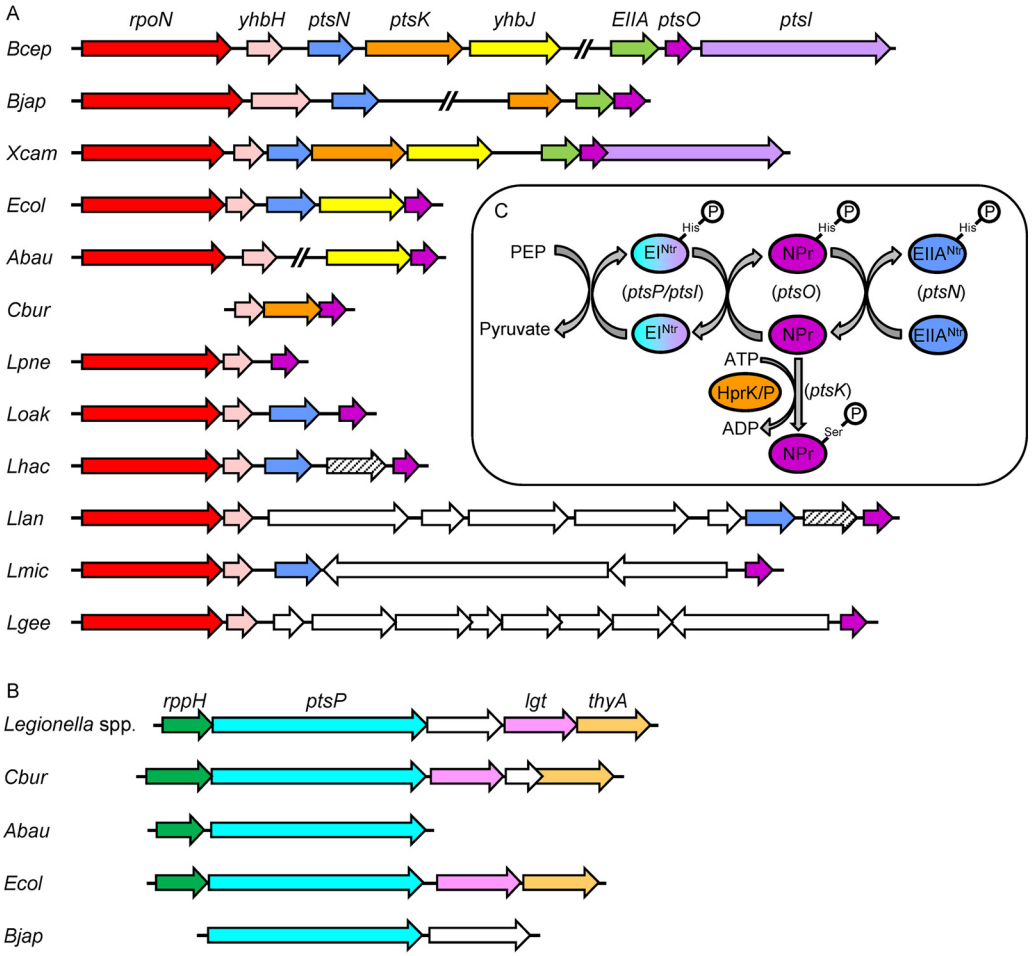

FIG 1 The PTS^{Ntr} components in different bacteria. The operon organization of genes encoding the PTS^{Ntr} components NPr (A) and EI^{Ntr} (B) in various bacteria is shown. Homologous genes are shown with the same color; open reading frames that have no homologues in the other regions presented are represented by white arrows. *rpoN* encodes the nitrogen sigma factor, *yhbH* encodes a ribosome binding protein, *ptsN* encodes EIIA^{Ntr}, *ptsK* encodes NPr kinase/phosphorylase, *ptsO* encodes NPr, *ptsI* encodes an EI-like protein, *rppH* encodes RNA pyrophosphohydrolase, *ptsP* encodes EI^{Ntr}, *lgt* encodes diacylglyceryl transferase, and *thyA* encodes thymidylate synthetase. Bcep, *Burkholderia cepacia*; Bjap, *Bradyrhizobium japonicum*; Xcam, *Xanthomonas campestris*; Ecol, *Escherichia coli*; Abau, *Acinetobacter baumannii*; Cbur, *Coxiella burnetii*; Lpne, *L. pneumophila*; Loak, *Legionella oakridgensis*; Lhac, *Legionella hackeliae*; Llan, *Legionella lansingensis*; Lmic, *L. micdadei*; and Lgee, *Legionella geestiana*. The *ptsO* operon organization found in *L. pneumophila* is the same in 21 other *Legionella* species (Fig. 2). (C) Schematic representation of the PTS^{Ntr} phosphorylation cascade. Phosphoryl groups are sequentially transferred on histidine residues from PEP to EI^{Ntr}, NPr, and subsequently to EIIA^{Ntr}. Phosphorylation of NPr by HPrK/P on serine is also indicated.

The absence of the *ptsN* gene from *L. pneumophila*, together with the conservation of the NPr protein in all members of the *Legionella* genus, might indicate that the PTS^{Ntr} of *L. pneumophila* participates in other functions which probably involve the transfer of phosphate from EI^{Ntr} to NPr and then to a component which is different from EIIA^{Ntr}.

The *L. pneumophila ptsP* and *ptsO* genes are required for optimal intracellular growth. Since only two components of the PTS^{Ntr} are present in *L. pneumophila*, we constructed deletion mutations in each of the genes encoding these components and examined them for intracellular growth in the amoeba host *Acanthamoeba castellanii* (28). Since *ptsP* is located in the middle of an operon (29), we generated a nonpolar in-frame deletion mutation in this gene and a kanamycin deletion substitution mutant in *ptsO*. Examination of these mutants revealed that both the *ptsP* and *ptsO* deletion mutants are partially defective for intracellular growth in *A. castellanii* (Fig. 3A and B). The intracellular growth phenotype of these mutants was complemented by introducing plasmids containing the *ptsP* and *ptsO* genes, respectively, cloned under the control of the P*tac* promoter (induced by isopropyl-β-D-thiogalactopyranoside [IPTG]) (Fig. 3A

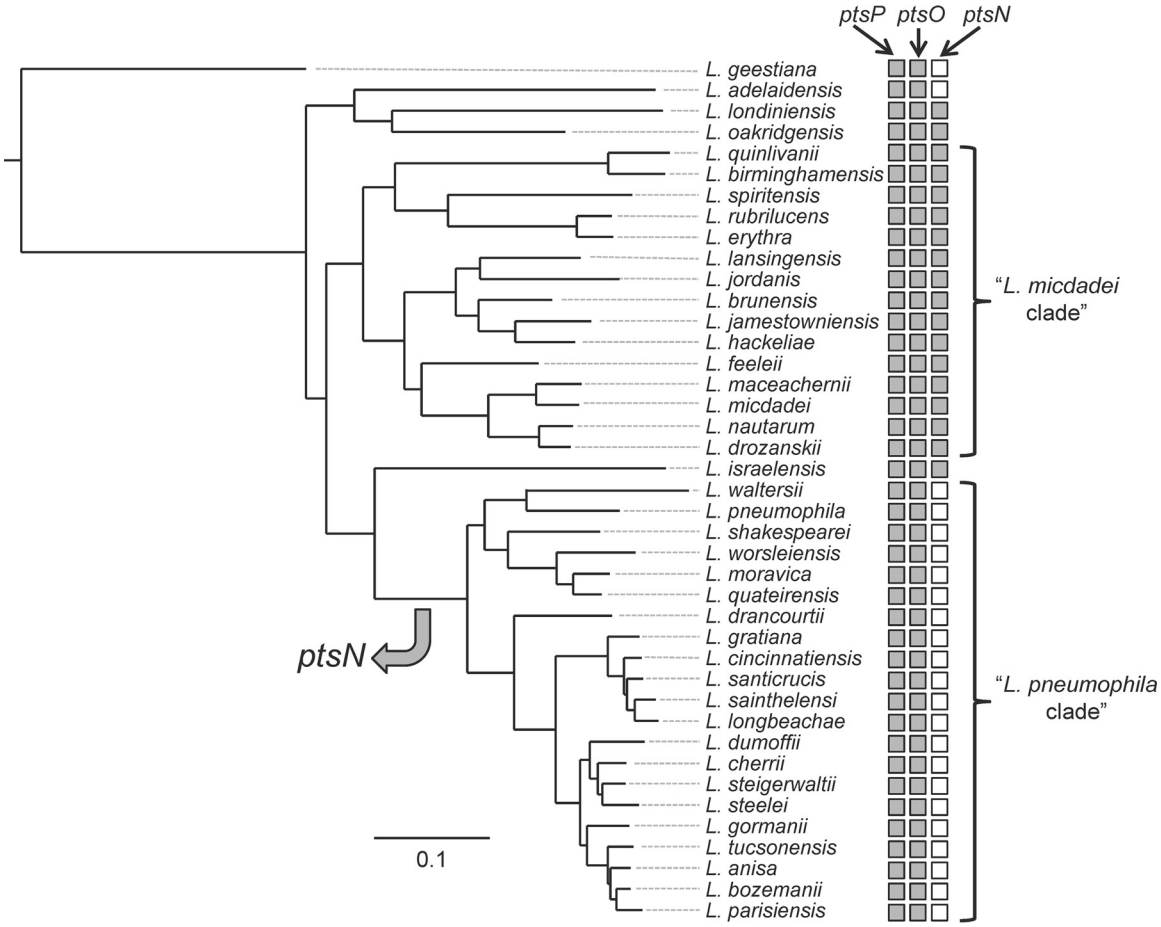

FIG 2 Presence and absence of PTS^{Ntr} components in the *Legionella* genus. A maximum-likelihood tree of 41 sequenced *Legionella* species was reconstructed on the basis of concatenated amino acid alignment of 78 orthologous open reading frames (27). For each species, the presence (gray) or absence (white) of the *ptsP*, *ptsO*, and *ptsN* genes of the PTS^{Ntr} is indicated.

and B). These two deletion mutants were also examined for intracellular growth in HL-60-derived human macrophages (Fig. 3C and D) (28). In these cells, both the *ptsP* and *ptsO* deletion mutants showed a mild intracellular growth phenotype which was also complemented using the plasmids described above (Fig. 3C and D). Examination of a double deletion mutant of both *ptsP* and *ptsO* (*ptsP-ptsO*) did not result in an additive effect on intracellular growth in comparison to the single deletion mutants (data not shown). The growth rates of *ptsP* and *ptsO* single deletion mutants, as well as the growth rate of the double *ptsP-ptsO* deletion mutant, are similar to the growth rate of the wild-type strain when the mutants are examined *in vitro* in regular growth medium (Fig. S2).

The results described above agree with previous results showing that the *L. pneumophila ptsP* gene is required for full virulence in guinea pigs (26) and clearly indicate that the PTS^{Ntr} is functional in *L. pneumophila* even though it lacks the EIIA^{Ntr} component.

Identification of genes whose levels of expression are reduced in the *ptsP* deletion mutant. Thus far, three regulatory systems (CpxRA, PmrAB, and LetAS) (see introduction) which are activated by phosphorylation were shown to be involved in the expression of *L. pneumophila* virulence-related genes (30). Therefore, we decided to examine the possibility that one of these systems is regulated by the PTS^{Ntr} and leads to the intracellular growth phenotype observed. To this end, genes known to be regulated by these two-component regulatory systems were examined: (i) an effector-encoding gene (*cegC3*) and an Icm/Dot-encoding gene (*icmR*) activated by the CpxRA

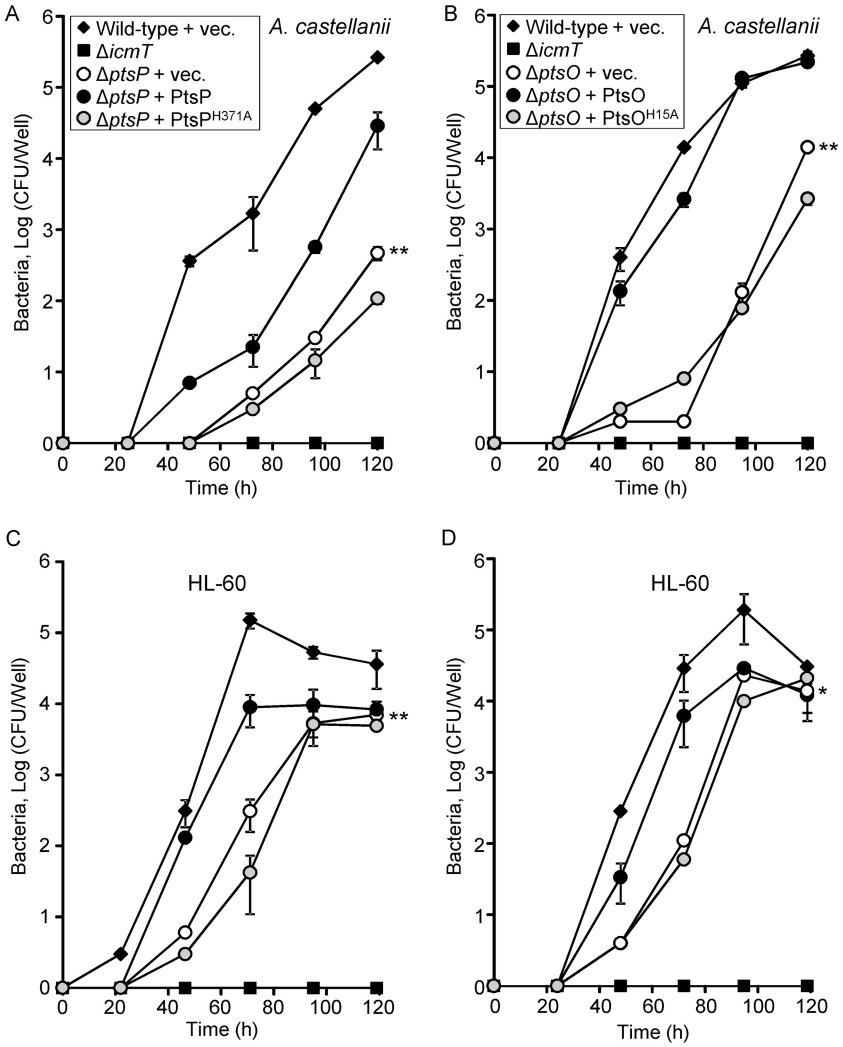

FIG 3 The PTS^{Ntr} is required for *L. pneumophila* optimal intracellular multiplication. The ability of *ptsP* (A and C) and *ptsO* (B and D) deletion mutants to grow intracellularly was examined in *A. castellanii* (A and B) and HL-60-derived human macrophages (C and D). Symbols: diamonds, *L. pneumophila* wild-type strain JR32; squares, the *icmT* mutant strain GS3011; open circles, *ptsP* and *ptsO* deletion mutants SY-ΔptsP and SY-ptsO-Km, respectively, containing the vector (pMMB207); black circles, *ptsP* and *ptsO* deletion mutants SY-ΔptsP and SY-ptsO-Km, respectively, containing the complementing plasmids expressing the wild-type EI^{Ntr} and NPr (pSY-207-Ptac-ptsP in panels A and C and pSY-207-Ptac-ptsO in panels B and D); and gray circles, *ptsP* and *ptsO* deletion mutants SY-ΔptsP and SY-ptsO-Km, respectively, containing plasmids expressing the mutated EI$^{Ntr\text{-}H371A}$ and NPr^{H15A} (pSY-207-Ptac-ptsP-H371A in panels A and C and pSY-207-Ptac-ptsO-H15A in panels B and D). The experiments were performed as described in Materials and Methods. The experiments were done three times, and similar results were obtained; error bars indicate standard deviations. The intracellular growth rates were found to be significantly different (*, $P < 0.01$; **, $P < 0.001$, two-way repeated analysis of variance) in comparisons between results for the *pstP* and *ptsO* deletion mutants and those of the wild-type strain.

TCS (7), (ii) two effector-encoding genes (*ceg20* and *ceg23*) activated by the PmrAB TCS (4), (iii) two effector-encoding genes (*ralF* and *ylfB*) regulated by the LetAS-RsmYZ-CsrA regulatory cascade (12, 13), and (iv) two genes encoding small RNAs (sRNAs) (*rsmY* and *rsmZ*) which are directly activated by the LetAS TCS (13, 14). The examination of these eight genes revealed that only the expression levels of the PmrA-regulated genes were reduced in the *ptsP* deletion mutant (Fig. 4), indicating that the PTS^{Ntr} specifically affects the levels of expression of PmrA-regulated genes.

The two PTS^{Ntr} components affect the levels of expression of PmrA-regulated genes. To further substantiate the result described above, the levels of expression of *lacZ* fusions of nine PmrA-regulated genes (*ceg3*, *ceg4*, *ceg11*, *ceg19*, *ceg20*, *ceg21*,

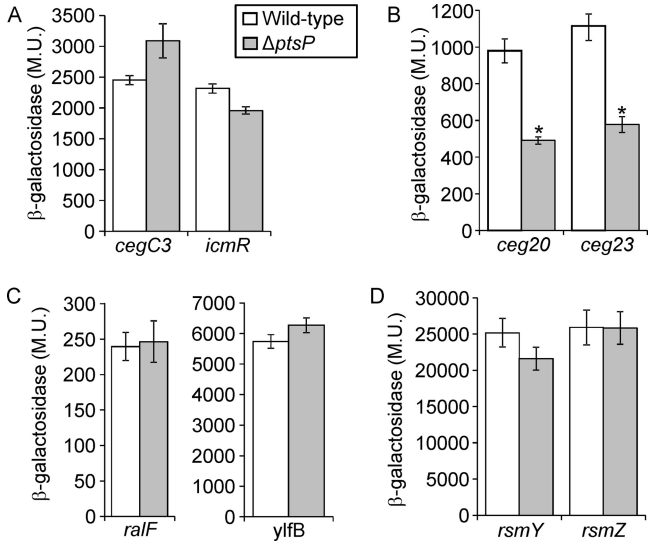

FIG 4 The absence of PtsP affects the expression levels of PmrA-regulated genes. The expression of effector translational *lacZ* fusions regulated by CpxR (A), PmrA (B), and CsrA (C) as well as sRNA transcriptional *lacZ* fusions regulated by LetA (D) (the genes examined are indicated below the bars) were examined in the wild-type strain (JR32) and in the *ptsP* deletion mutant (SY-ΔptsP) at the stationary phase. β-Galactosidase activity was measured as described in Materials and Methods. The levels of expression of the *lacZ* fusions were found to be significantly different (*, $P < 10^{-5}$, Student's *t* test) in comparisons between results in the wild-type strain and those in the *pmrA* deletion mutant. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments.

ceg23, *lepB*, and *legA14*) (4, 5) were examined in the wild-type strain and in the *pmrA*, *ptsP*, and *ptsO* deletion mutants. The expression levels of all these genes were found to be completely dependent on PmrA, and their levels of expression were very low in the absence of PmrA (Fig. 5). Importantly, all the examined genes had reduced levels of expression in the *ptsP* and *ptsO* deletion mutants (Fig. 5). The reduction in the levels of expression of the PmrA-regulated effector-encoding genes in these two mutants was about 2-fold in comparison to their expression levels in the wild-type strain (for complementation analysis of the levels of expression using *ptsP* and *ptsO*, see below). When the levels of expression of the PmrA regulated genes were further examined in a *ptsP-ptsO* double deletion mutant, no additional reduction in expression levels were observed in comparison to the levels in the *ptsP* and *ptsO* single deletion mutants (Fig. 5). These results indicate that the two PTS^{Ntr} components present in *L. pneumophila* affect the levels of expression of PmrA-regulated genes similarly and are most likely part of a single regulatory cascade.

The PTS^{Ntr} components do not affect the expression of the *pmrA* gene. The similar effect of the *ptsP*, *ptsO*, and the double *ptsP-ptsO* deletion mutants on the levels of expression of PmrA-regulated genes (Fig. 5) might result from the PTS^{Ntr} affecting (i) the level of expression of the *pmrA* gene itself or (ii) the function of the PmrAB TCS. To distinguish between these possibilities, we examined the level of expression of the *pmrA* gene in the *ptsP* and *ptsO* single deletion mutants and in the *ptsP-ptsO* double deletion mutant and found that the *pmrA* gene is similarly expressed in these three mutants and in the wild-type strain (Fig. 6A). The lack of effect on the level of expression of the *pmrA* gene in the deletion mutant examined indicates that the PTS^{Ntr} most likely affects the functionality of the PmrA regulator since it had no effect on its level of expression.

The PTS^{Ntr} components affect the levels of expression of the PmrA-regulated effector-encoding genes via PmrA. To examine if the PTS^{Ntr} functions via PmrA, a double deletion mutant of *ptsP* and *pmrA* was constructed. Because the deletion of the *pmrA* gene leads to very low levels of expression of most of its target genes (Fig. 5), we

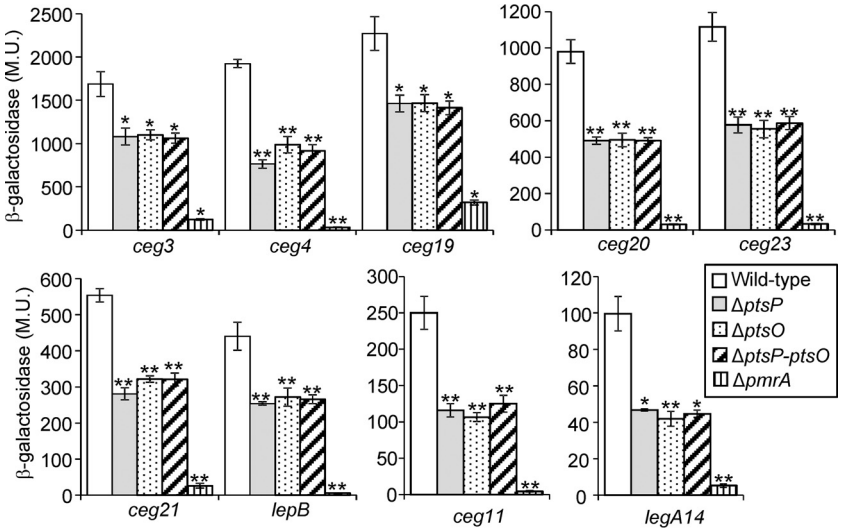

FIG 5 The levels of expression of PmrA-regulated effector-encoding genes are reduced in mutants of the PTS^{Ntr}. The expression of effector translational *lacZ* fusions (the effectors examined are indicated below the bars) was examined in the wild-type strain (JR32), the *ptsP* deletion mutant (SY-ΔptsP), the *ptsO* deletion mutant (SY-ptsO-Km), the *ptsP-ptsO* double deletion mutant (SY-ΔptsP-ptsO-Km), and the *pmrA* deletion mutant (HK-PQ1) at the stationary phase. The levels of expression of the *lacZ* fusions were found to be significantly different (*, $P < 0.001$; **, $P < 10^{-5}$, Student's *t* test) in comparisons between results in the wild-type strain and those in the different deletion mutants. β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments. The effector-encoding genes were divided according to their levels of expression.

chose the *ceg3* and *ceg4 lacZ* fusions for this analysis since their levels of expression, in the absence of the PmrA activator, would still make it possible to observe an additional reduction in their levels of expression in the *ptsP-pmrA* double deletion mutant. Examination of the *ceg3* and *ceg4 lacZ* fusions in the wild-type strain, in the *ptsP* and *pmrA* single deletion mutants, and in the *ptsP-pmrA* double deletion mutant resulted in no additive reduction in the levels of expression of these two genes in the double deletion mutant (Fig. 6B), suggesting that the PTS^{Ntr} affects the level of expression of PmrA-regulated genes only in the presence of PmrA.

To further substantiate the requirement of PmrA in order to observe the effect of the PTS^{Ntr} on the levels of expression of PmrA-regulated effector-encoding genes, we constructed a system in which the EI^{Ntr} and NPr were expressed under the P*tac* promoter (activated by IPTG), and their ability to affect the levels of expression of PmrA-regulated genes was examined. Overexpression of EI^{Ntr} and NPr from a P*tac* promoter resulted in an increase in the levels of expression of *ceg3* and *lepB lacZ* fusions as the concentrations of IPTG increased (Fig. 6C), indicating that when the components of the PTS^{Ntr} are overexpressed, they can increase the levels of expression of the PmrA-regulated genes above the level found in the wild-type strain. However, in the absence of PmrA, overexpression of EI^{Ntr} and NPr did not result in an increase in the levels of expression of *ceg3* and *lepB* (Fig. 6C).

Collectively, these results clearly show that the PTS^{Ntr} requires the presence of PmrA in order to exert its effect on the levels of expression of PmrA-regulated effector-encoding genes.

The PTS^{Ntr} components affect the levels of expression of PmrA-regulated genes in the absence of PmrB. The requirement of the PmrA activator in order for the PTS^{Ntr} components to affect the expression levels of the PmrA-regulated effector-encoding genes suggests that this effect might be mediated also via the PmrA cognate SHK PmrB. To this end, a *pmrB* single deletion mutant and a *pmrB-ptsP* double deletion mutant were constructed. If the PTS^{Ntr} components function via PmrB, the expected result would be that in the absence of PmrB the *ptsP* deletion would not affect the

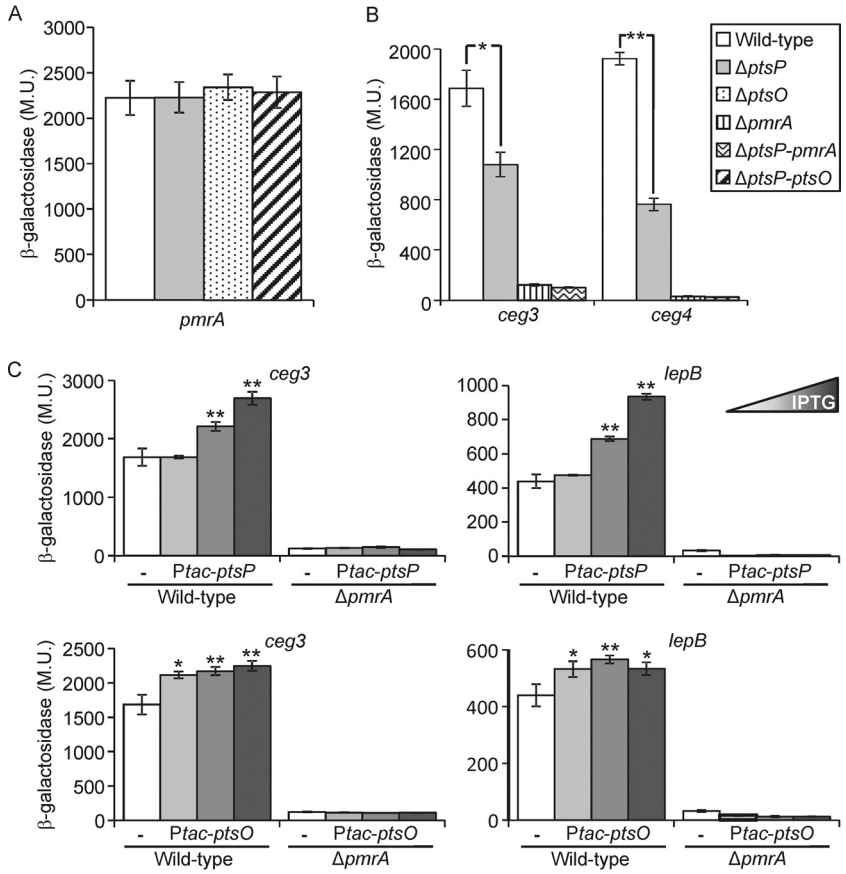

FIG 6 The PTS^{Ntr} affects the levels of expression of PmrA-regulated genes via PmrA. (A) The expression of the PmrA translational *lacZ* fusion was examined in the wild-type strain (JR32), in the *ptsP* deletion mutant (SY-ΔptsP), in the *ptsO* deletion mutant (SY-ptsO-Km), and in the *ptsP-ptsO* double deletion mutant (SY-ΔptsP-ptsO-Km) at the stationary phase. (B) The expression of effector translational *lacZ* fusions (the effectors examined are indicated below the bars) was examined in the wild-type strain (JR32), in the *ptsP* deletion mutant (SY-ΔptsP), in the *pmrA* deletion mutant (HK-PQ1), and in the *ptsP-pmrA* double-deletion mutant (SY-ΔptsP-pmrA-Km) at the stationary phase. The levels of expression of the marked *lacZ* fusions were found to be significantly different (*, $P < 0.01$; **, $P < 0.001$, Student's *t* test). For panels A and B, strains are identified according to the legend on the figure. (C) The levels of expression of two PmrA-regulated effector-encoding genes (*ceg3* and *lepB*) were examined in the *L. pneumophila* wild-type strain (JR32) and the *pmrA* deletion mutant (HK-PQ1). The bacteria contained a plasmid with the *ptsP* gene (two upper graphs) or the *ptsO* gene (two lower graphs) cloned under the control of the IPTG-inducible P*tac* promoter. The plasmids containing the corresponding *lacZ* fusion of the examined gene without the *ptsP* or *ptsO* gene were used as controls (white bars). The IPTG concentrations used to express the wild-type and mutated *ptsP* were 0.005, 0.01, and 0.05 mM, and they were 0, 0.005, and 0.01 mM for *ptsO*. The levels of expression of the *lacZ* fusions were found to be significantly different (*, $P < 0.01$; **, $P < 0.001$, Student's *t* test), in comparisons between results with the fusions with the *ptsP* or *ptsO* gene expressed from the P*tac* promoter and those with the corresponding *lacZ* fusions without the *ptsP* and *ptsO* genes. β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments.

expression levels of the PmrA-regulated genes. In contrast, if the PTS^{Ntr} components function independently from PmrB, the expected result would be that in the absence of PmrB, the *ptsP* deletion would continue to affect the levels of expression of the PmrA-regulated genes in a way similar to that of the wild-type strain.

To examine this, we determined the levels of expression of two PmrA-regulated genes (*ceg3* and *ceg4*) in the *pmrB* deletion mutant and in the *ptsP-pmrB* double deletion mutant. Examination of the levels of expression of PmrA-regulated genes in a deletion mutant of *pmrB* revealed that these genes are expressed at higher levels in the absence of PmrB than in the wild-type strain (Fig. 7 and S3). Previous studies have found that removal of the PmrB SHK results in a higher level of expression due to the

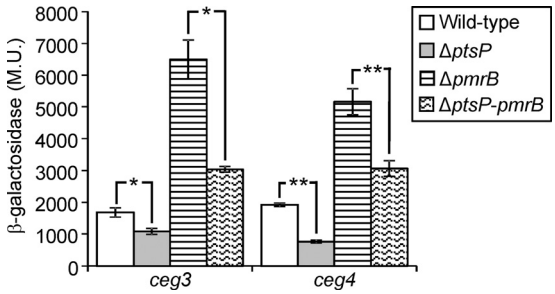

FIG 7 The PTS^{Ntr} affects the levels of expression of PmrA-regulated genes and is independent of PmrB. The expression of effector translational *lacZ* fusions (the effectors examined are indicated below the bars) was examined in the wild-type strain (JR32), in the *ptsP* deletion mutant (SY-ΔptsP), in the *pmrB* deletion mutant (EA-pmrB), and in the *ptsP-pmrB* double deletion mutant (SY-ΔptsP-pmrB-Km). The levels of expression of the marked *lacZ* fusions were found to be significantly different (*, $P < 0.001$; **, $P < 10^{-5}$, Student's *t* test). β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments.

absence of the phosphatase activity usually elicited by PmrB (31). Importantly, examination of the levels of expression of the *ceg3* and *ceg4 lacZ* fusions in the *ptsP-pmrB* double deletion mutant showed that their expression levels were reduced in comparison to their levels in the *pmrB* deletion mutant (Fig. 7), indicating that the effect of PtsP is independent of the presence of PmrB in the bacterial cell. In addition, similar reductions were observed between the *pmrB* deletion mutant and the *pmrB-ptsP* double deletion mutant and between the wild-type strain and the *ptsP* deletion mutant (Fig. 7).

The similar reductions in the levels of expression of PmrA-regulated genes mediated by the deletion of *ptsP* in the presence or absence of PmrB indicate that the PTS^{Ntr} affects the levels of expression of these genes independently from PmrB.

Both EI^{Ntr} and NPr require their conserved phosphorylation sites in order to function. Components of the PTS^{Ntr} are highly conserved throughout bacterial species, and the histidine residues used for phosphorylation are universally conserved in all of them (19). We therefore constructed mutant versions of the *L. pneumophila* EI^{Ntr} and NPr in which the highly conserved histidine residues of both proteins were replaced with alanines (EI$^{Ntr\text{-}H371A}$ and NPr^{H15A}, respectively). These mutants were examined for their ability to activate the expression of the PmrA-regulated genes. Overexpression of the wild-type EI^{Ntr} (encoded by *ptsP*) and NPr (encoded by *ptsO*) from a P*tac* promoter in the *ptsP* and *ptsO* deletion mutants, respectively, resulted in an increase in the levels of expression of *ceg3* and *lepB lacZ* fusions as the concentrations of IPTG increased (Fig. 8). However, when the mutated EI$^{Ntr\text{-}H371A}$ or NPr^{H15A} was expressed in the same mutants, under the same conditions, no increase in the expression levels of these fusions was observed. Moreover, when the mutated EI$^{Ntr\text{-}H371A}$ and NPr^{H15A} were used for complementation of the *ptsP* and *ptsO* deletion mutants for intracellular growth in amoeba, no complementation was observed (Fig. 3).

Collectively, the observation that the conserved histidine residues of the *L. pneumophila* EI^{Ntr} and NPr are required for their function suggests that their effect on PmrA-regulated genes is mediated by phosphorylation.

Both EI^{Ntr} and NPr require the presence of each other in order to function. To determine if EI^{Ntr} and NPr require one another in order to affect the levels of expression of PmrA regulated effector-encoding genes, we examined whether the wild-type EI^{Ntr} protein can increase the expression levels of PmrA-regulated genes in the absence of NPr and vice versa. Expression of the wild-type EI^{Ntr} (encoded by *ptsP*) and NPr (encoded by *ptsO*) proteins from a P*tac* promoter in the *ptsO* and *ptsP* deletion mutants, respectively, resulted in no increase in the expression levels of *ceg3* and *lepB lacZ* fusions as opposed to the increase in the levels of expression that was obtained when each of them was expressed in its corresponding deletion mutant (Fig. 8, compare the

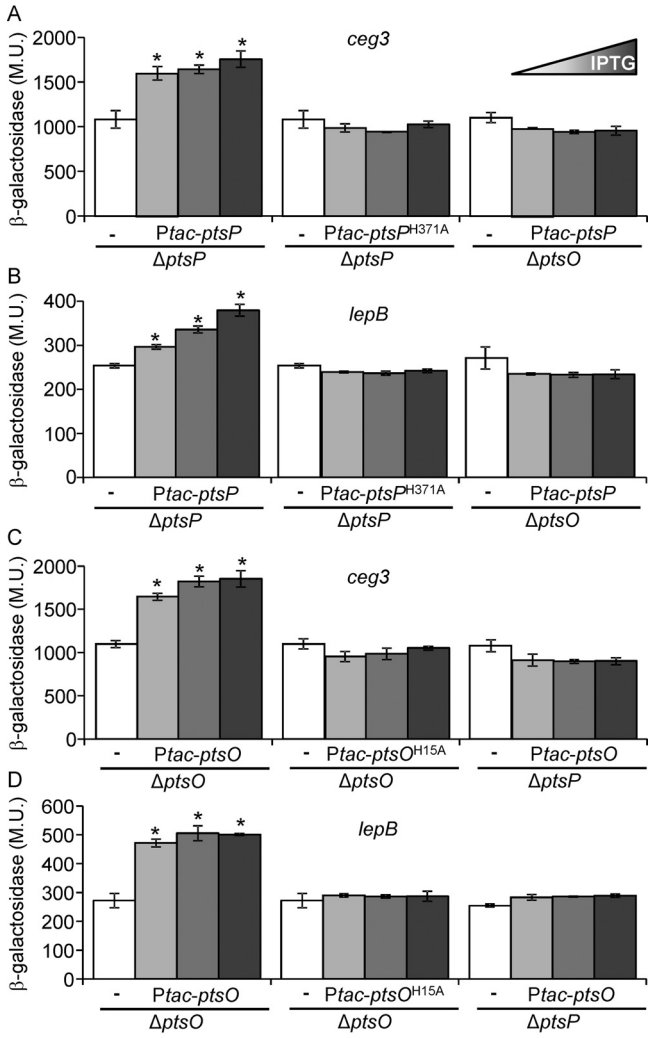

FIG 8 The conserved histidine residues of EI^{Ntr} and NPr are required for their function. The levels of expression of two PmrA-regulated effector-encoding genes (*ceg3* and *lepB*) were examined in the *L. pneumophila ptsP* deletion mutant (SY-ΔptsP) and the *ptsO* deletion mutant (SY-ΔptsO-Km). The bacteria contained a plasmid carrying the wild-type *ptsP* gene, the wild-type *ptsO* gene, the mutated *ptsP*^{H371A} gene, or the mutated *ptsO*^{H15A} gene cloned under the control of the IPTG inducible P*tac* promoter. The plasmids containing the corresponding *lacZ* fusion of the examined gene without the *ptsP* or *ptsO* gene were used as controls (white bars). The IPTG concentrations used to express the wild-type and mutated *ptsP* genes were 0.005, 0.01, and 0.05 mM, and they were 0, 0.005, and 0.01 mM for *ptsO*. The levels of expression of the *lacZ* fusions were found to be significantly different (*, $P < 0.001$, Student's *t* test) in comparisons between results for the *lacZ* fusions containing the *ptsP* or *ptsO* gene expressed from the P*tac* promoter and those for the fusions without the *ptsP* and *ptsO* genes. β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments.

left and right bar groups). These results indicate that EI^{Ntr} and NPr can affect the expression levels of PmrA-regulated genes only when both components are present in the bacterial cell.

The expression of the *L. micdadei ptsN* in *L. pneumophila* abolishes the effect of the PTS^{Ntr} on PmrA-regulated genes. As described above (Fig. 2), about 20 *Legionella* species belonging to the *L. micdadei* clade (27) contain a *ptsN* gene encoding the EIIA^{Ntr} component, which is missing in the *L. pneumophila* clade. To further explore the function of the *L. pneumophila* incomplete PTS^{Ntr}, we examined the effect of expressing the *L. micdadei ptsN* gene on the expression levels of PmrA-regulated genes in *L. pneumophila*. To this end, we cloned and expressed the *L. micdadei* EIIA^{Ntr} (encoded by *ptsN*) under the control of the P*tac* promoter (activated by IPTG) in *L. pneumophila* and

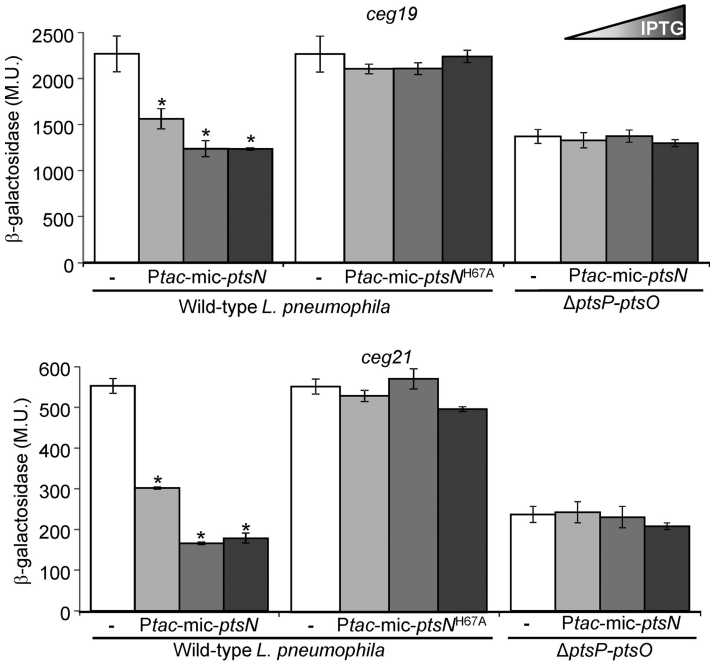

FIG 9 Overexpression of the *L. micdadei ptsN* abolishes the effect of PTS^{Ntr} on PmrA-regulated genes in *L. pneumophila*. The levels of expression of two PmrA-regulated effector-encoding genes (*ceg19* and *ceg21*) were examined in the *L. pneumophila* wild-type strain (JR32) or the *ptsP-ptsO* double deletion mutant (SY-ΔptsP-ptsO-Km). The bacteria contained a plasmid with the *L. micdadei ptsN* gene (P*tac*-mic-*ptsN*) or the mutated *L. micdadei ptsN*^{H67A} gene (P*tac*-mic-*ptsN*^{H67A}) cloned under the control of the IPTG-inducible P*tac* promoter. The plasmids containing the corresponding *lacZ* fusions of the examined genes without the *ptsN* gene were used as controls (white bars). The IPTG concentrations used to express the wild-type and mutated *L. micdadei ptsN* genes were 0, 0.1, and 1 mM. The levels of expression of the *lacZ* fusions were found to be significantly different (*, $P < 10^{-5}$, Student's *t* test) in comparisons between results for the *lacZ* fusions containing the wild-type *L. micdadei ptsN* gene expressed from the P*tac* promoter and those for the fusions without the *ptsN* gene. β-Galactosidase activity was measured as described in Materials and Methods. Data (expressed in Miller units [M.U.]) are the averages ± standard deviations (error bars) of the results of at least three different experiments.

examined its effect on the levels of expression of two PmrA-regulated effector-encoding genes, *ceg19* and *ceg21*. The expression of the *L. micdadei ptsN* gene in *L. pneumophila* resulted in a reduction in the levels of expression of both the *ceg19* and *ceg21 lacZ* fusions, and the reduction strengthened as the concentration of the added IPTG increased (Fig. 9). At the highest IPTG concentration (1 mM), the expression levels of both genes were reduced to about half of their levels of expression in the absence of the *L. micdadei ptsN*. The extent of this reduction in the expression is similar to the reduction obtained when these two genes were examined in the *ptsP* and *ptsO* single deletion mutants or the *ptsP-ptsO* double deletion mutant (compare Fig. 5 and 9). This result suggests that the overexpression of *L. micdadei ptsN* completely abolished the effect of the *L. pneumophila* PTS^{Ntr} on the PmrA-regulated effector-encoding genes. To further support the conclusion that the effect of the *L. micdadei ptsN* resulted from its natural function encoding a protein that is phosphorylated by NPr, we constructed a mutated form of the *L. micdadei ptsN* in which the conserved histidine residue, which is phosphorylated by NPr, was mutated to alanine (EIIA$^{Ntr\text{-}H67A}$). Overexpression of this mutated form of *ptsN* had no effect on the levels of expression of the PmrA-regulated effector-encoding genes *ceg19* and *ceg21* (Fig. 9). Moreover, when the *L. micdadei ptsN* was expressed in the *ptsP-ptsO* double deletion mutant, no reduction in the levels of expression of the *ceg19* and *ceg21 lacZ* fusions was observed, indicating that the *L. pneumophila* PTS^{Ntr} components are required in order for the *L. micdadei ptsN* to affect the expression levels of PmrA-regulated genes in *L. pneumophila*.

Collectively, the effect of the wild-type *L. micdadei ptsN* and the lack of effect of the mutated *L. micdadei ptsN* on the levels of expression of the PmrA-regulated genes in *L.*

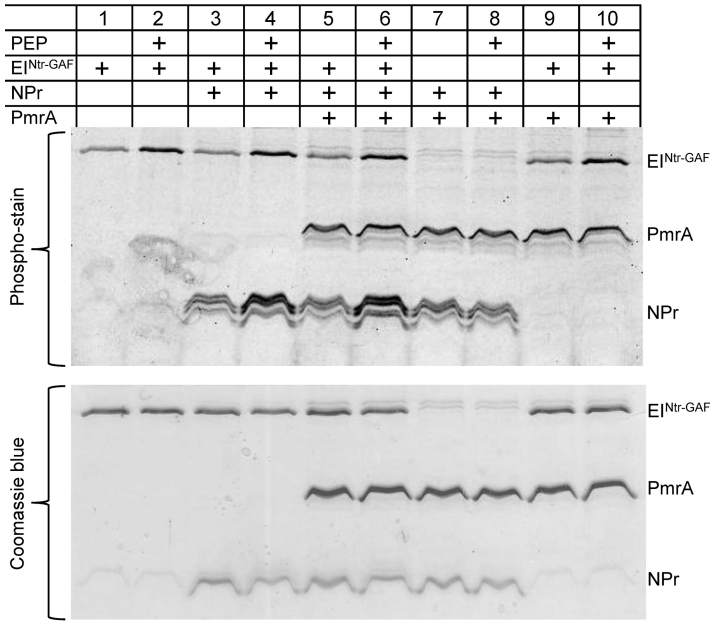

FIG 10 EI^{Ntr} and NPr are phosphorylated by PEP. Results of an *in vitro* phosphorylation assay of EI^{Ntr}, NPr, and PmrA are shown. Wild-type His$_6$-tagged EI^{Ntr} without its GAF domain (His$_6$-EI$^{Ntr\Delta GAF}$), NPr-His$_6$, and His$_6$-PmrA were overexpressed and purified. Different combinations of the purified proteins were incubated with or without PEP and separated on 15% SDS-polyacrylamide gels, as described in Materials and Methods. Phosphorylated proteins were specifically detected by the Pro-Q Diamond phosphoprotein gel stain technique, while total protein was visualized by Coomassie blue.

pneumophila indicate that the *L. micdadei* EIIA^{Ntr} functions as a phosphate sink for the PTS^{Ntr} in *L. pneumophila*. This result further supports the possibility that the *L. pneumophila* NPr transfers its phosphate directly or indirectly to PmrA.

EI^{Ntr} and NPr are phosphorylated *in vitro* by PEP. To determine if the PTS^{Ntr} phosphorylation cascade can be recapitulated *in vitro*, the EI^{Ntr}, NPr, and PmrA proteins were His tagged, overexpressed, purified, and used for an *in vitro* phosphorylation assay using PEP as a phosphate donor (Fig. 10). The results obtained show that the phosphorylation level of EI^{Ntr} was increased 3- to 4-fold following incubation with PEP (Fig. 10, compare lanes 1 and 2, lanes 3 and 4, lanes 5 and 6, and lanes 9 and 10), and the phosphorylation level of NPr was increased similarly following incubation with PEP and EI^{Ntr} (Fig. 10, compare lanes 3 and 4 and lanes 5 and 6). However, no increase in the phosphorylation level of NPr was observed in the absence of EI^{Ntr} with or without PEP (compare lanes 7 and 8). When EI^{Ntr} and NPr were incubated together with PmrA, a very slight increase (1.2-fold) in the PmrA levels of phosphorylation was observed in the presence of PEP (Fig. 10, compare lanes 5 and 6); this slight increase in phosphorylation was not observed when the same reactions were performed without NPr (compare lanes 9 and 10). The slight increase in the phosphorylation of PmrA might occur since there is another component present *in vivo* which functions between NPr and PmrA, or the slight increase may arise because an unknown component is required in order to stabilize a direct phosphotransfer between NPr and PmrA or because the conditions *in vitro* do not allow the phosphotransfer from NPr to PmrA to take place in an efficient manner. Attempts to change the concentrations of the proteins as well as the PEP concentration did not result in phosphorylation of PmrA. In addition, PmrA was phosphorylated *in vitro* by acetyl-phosphate, but no reverse phosphorylation from PmrA to NPr or EI^{Ntr} was observed (data not shown).

The results obtained demonstrate that the *L. pneumophila* PTS^{Ntr} is functional. EI^{Ntr} is phosphorylated by PEP and can transfer its phosphate group to NPr, similarly to what was previously shown in other bacterial PTSs. It is currently not possible to draw a definite conclusion about whether NPr can transfer its phosphate directly to PmrA or to

another component which functions between NPr and PmrA in order to mediate the effect on the PmrA-regulated genes.

DISCUSSION

The term "phosphotransferase system" is used for a group of proteins that transfer phosphate derived from PEP from one member of the system to the next in a given order. Two general types of PTSs are known (19): the sugar PTS, which is responsible for phosphorylation and transport of sugars into the cell, and the nitrogen PTS (PTS^{Ntr}), which does not transport sugars but exerts regulatory functions. In both PTSs, the active phosphate moiety is derived from PEP and then transferred through two general phosphotransferase proteins: enzyme I (EI or EI^{Ntr} in PTS^{Ntr}) and histidine protein (HPr or NPr in PTS^{Ntr}). In the sugar PTS, HPr subsequently phosphorylates the sugar-specific components enzyme IIA (EIIA) and enzyme IIB (EIIB) allowing uptake of the sugar (18). The sugar PTSs do not exclusively catalyze carbohydrate uptake but also regulate the activities of a huge number of genes and proteins in response to available carbon sources (18, 32). In the PTS^{Ntr}, NPr phosphorylates EIIA^{Ntr}; however, the EIIA^{Ntr} is not active in transport. The PTS^{Ntr} regulates diverse processes implicated in the metabolism of nitrogen and carbon (21), it plays a role in potassium homeostasis (22) and biofilm formation (23), and is essential for virulence in several bacteria such as *S. enterica* (24), *B. melitensis* (25), and *L. pneumophila* (26). In many bacteria the PTS^{Ntr} constitutes a phosphorylation cascade that works in parallel with the sugar PTS, while in other bacteria only one of the two systems exists. In addition to the three basic PTS components (EI, HPr, and EIIA), some gammaproteobacteria also contain an HPr kinase/phosphorylase (HPrK/P). HPrK/P controls the phosphorylation state of HPr at a serine residue, whereas EI phosphorylates HPr at a histidine. When phosphorylated on a serine residue, HPr mediates different functions involved in gene regulation (33).

All the components of the sugar PTS are absent in *L. pneumophila*; no gene encoding HPrK/P was found in this bacterium, and only the first two components of the PTS^{Ntr} are present in it (Fig. 2). Even though *L. pneumophila* possesses an incomplete PTS^{Ntr}, the remaining phosphorylation cascade (which includes only EI^{Ntr} and NPr) is functional. We could demonstrate that EI^{Ntr} becomes phosphorylated by PEP, that NPr becomes phosphorylated by EI^{Ntr} (Fig. 11), and that the conserved histidine residues of both components are essential for their function (Fig. 3 and 8). The observation that inactivation of both EI^{Ntr} and NPr, as well as mutations in their conserved histidine residues, similarly affected *L. pneumophila* intracellular growth suggests that NPr might phosphorylate or interact with a protein or an unknown regulatory factor involved in intracellular growth. Along these lines, our results demonstrate that all the PmrA-regulated genes examined are downregulated in the absence of EI^{Ntr} and NPr (Fig. 5). The effect of the PTS^{Ntr} components on PmrA-regulated genes was completely dependent on the presence of PmrA (Fig. 6) and independent of the presence of PmrB (Fig. 7). In addition, the ability of EI^{Ntr} and NPr to activate the expression of the PmrA-regulated effector-encoding genes was dependent on the presence of their conserved histidine residues and on the presence of both components (Fig. 8). Moreover, adding the *L. micdadei* EIIA^{Ntr} reduced the levels of expression of PmrA-regulated effector-encoding genes to levels similar to those of the *ptsP* and *ptsO* deletion mutants (Fig. 9). Together, our results indicate that the *L. pneumophila* incomplete PTS^{Ntr} functions in a manner similar to that of bacteria containing a complete PTS^{Ntr}, but in *L. pneumophila* it was rewired to affect the activity of the PmrA response regulator which regulates the expression of the largest regulon of effector-encoding genes in *L. pneumophila* (more than 40 effectors).

Even though we could demonstrate *in vitro* phosphorylation of EI^{Ntr} by PEP and phosphorylation of NPr by EI^{Ntr}, we did not observe significant phosphorylation of PmrA by NPr. However, all our results suggest that the phosphorylated NPr can activate the expression of PmrA-regulated genes via PmrA. There are several possible explanations of how this activation might occur: (i) PmrA is directly phosphorylated by the phosphorylated NPr (which we were unable to observe *in vitro*); (ii) an unknown

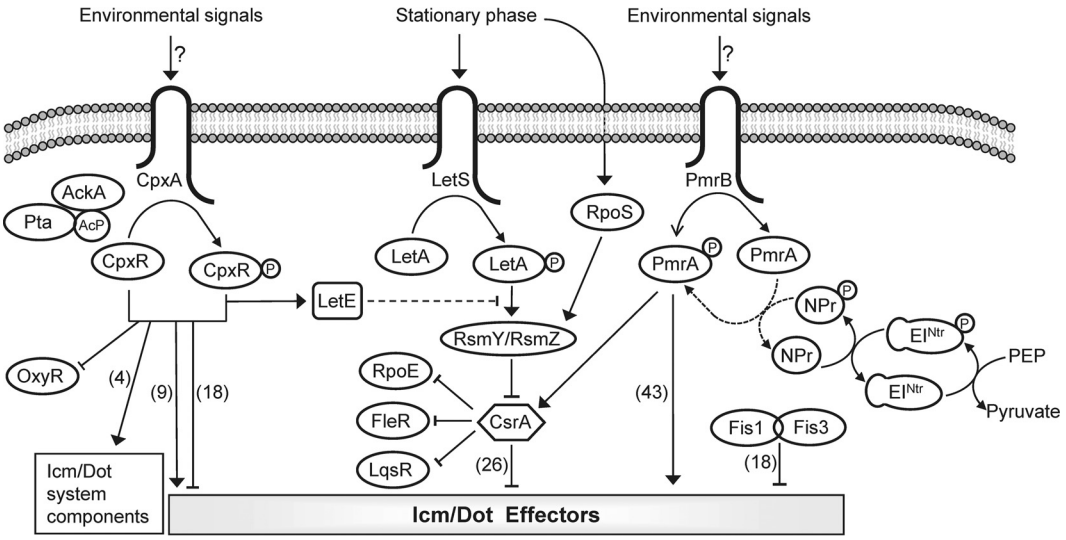

FIG 11 Model of the regulatory systems that control the expression of the *L. pneumophila* pathogenesis-related genes. The three TCSs (CpxRA, PmrAB, and LetAS), the components of the LetAS-RsmYZ-CsrA regulatory cascade, and the two Fis regulators (Fis1 and Fis3) are schematically illustrated. The environmental signals sensed by CpxA and PmrB are currently not known, and the phosphorylation of these components is expected to be activated by transfer of the phosphate group to their cognate response regulators CpxR and PmrA, respectively, which then directly activate or repress the transcription of their target effector-encoding genes. Acetyl-phosphate (Ac-P) produced by AckA and Pta was previously shown to transfer a phosphate group to CpxR. The PTS^{Ntr} was shown in this study to be functional in *L. pneumophila*; EI^{Ntr} is phosphorylated by PEP and then transfers the phosphate group to NPr. In addition, the PTS^{Ntr} affects the levels of expression of PmrA-regulated effector-encoding genes via PmrA in a direct or indirect manner. The numbers of effector-encoding genes which were shown to be regulated by each of the regulatory systems are indicated in parentheses. Solid arrows and T-shaped symbols indicate activation and repression, respectively; the dotted line indicates a possible indirect effect.

regulatory factor is phosphorylated by the phosphorylated NPr, which in turn transfers its phosphate to PmrA; (iii) an unknown regulatory factor stabilizes the direct phosphotransfer between the phosphorylated NPr and PmrA; or (iv) a metabolite or second messenger affects the interaction/phosphotransfer of phosphorylated NPr with PmrA.

Direct involvement of the PTS and PTS^{Ntr} in regulation of gene expression was described before. For example, the SsrB RR (which is part of the SsrAB TCS) directly promotes the transcription of multiple genes within *Salmonella* pathogenicity island 2 (SPI-2). It was found that EIIA^{Ntr} controls SPI-2 genes by acting on the SsrB protein at the posttranscriptional level. EIIA^{Ntr} was found to directly interact with SsrB, preventing the SsrB protein from binding to its target promoters (24). However, since there is no EIIA^{Ntr} in *L. pneumophila*, this is not the mechanism by which the *L. pneumophila* PTS^{Ntr} functions. In firmicutes, PTS regulation domains (PRDs) are found fused to transcription regulators. Transcription activators such as MltR, LicR, and LevR contain different numbers of PRDs, and they were found to become phosphorylated by EI and HPr. However, no transcription regulators containing a PRD were found in *L. pneumophila*, and they are very rarely found in gammaproteobacteria (18).

In addition to the two modes of involvement of PTS and PTS^{Ntr} in transcription regulation described above, direct involvement of HPr/NPr in regulation of gene expression was also described previously. In *Bacillus subtilis* it was found that the AraC type transcriptional activator YesS, which regulates the expression of pectin/rhamnogalacturonan utilization genes, interacts with HPr. YesS was found to interact with HPr and His-phosphorylated HPr but not with Ser-phosphorylated HPr (34). In *Neisseria meningitidis* HPr and Ser-phosphorylated HPr were found to interact with the LysR-type transcriptional repressor CrgA involved in capsule production in response to host cell contact (35). Our attempts to find interaction between EI^{Ntr} or NPr and PmrA as well as between their mutated forms did not reveal any interaction (data not shown).

The PmrAB TCS controls the level of expression of the largest regulon of effector-encoding genes (more than 40 effectors) in *L. pneumophila* (4, 5) (Fig. 11). The results described in the manuscript suggest that the levels of expression of these PmrA-

regulated effector-encoding genes are affected by at least three factors: (i) the level of expression of the *pmrAB* operon itself, which was found to be relatively high (Fig. 6A) even though no regulators which control the expression of this operon were described; (ii) the function of PmrB SHK as a kinase or phosphatase of PmrA that can activate or inactivate PmrA, respectively; and (iii) the activity of the PTS components EI^{Ntr} and NPr that can directly or indirectly affect the activity of PmrA. The PmrA protein must be phosphorylated in order to activate gene expression (see Fig. S4 in the supplemental material), and PmrB seems to function as a phosphatase of PmrA under the growth conditions examined (Fig. 7); but it is highly likely that PmrB also functions as a kinase of PmrA under specific conditions. In *S. enterica* the PmrB SHK was also shown to function as a phosphatase of PmrA (31), but under conditions of mildly acidic pH (36) or the presence of Fe^{3+} (37); *in vivo* during infection of macrophages (38), PmrB was shown to function as a kinase of PmrA. The conditions under which the *L. pneumophila* PmrB SHK phosphorylates and thus activates PmrA are currently not known. The finding that the *L. pneumophila* PTS^{Ntr} also contributes to the activity of PmrA suggests that cytoplasmic signals activate the expression of the PmrA-regulated effector-encoding genes as well (Fig. 11). Moreover, the deletion of the *L. pneumophila* PTS^{Ntr} resulted in a reduction of only 2-fold in the expression of PmrA-regulated genes in comparison to a very strong reduction in their levels of expression in the *pmrA* deletion mutant (Fig. 5), indicating that additional components probably phosphorylate PmrA. The most notifiable difference between the EI of the sugar PTS and the EI^{Ntr} is the presence of a GAF (cyclic GMP phosphodiesterase, *Anabaena* adenylate cyclase, and *Escherichia coli* FhlA) domain at the N terminus of EI^{Ntr} (39). This domain was also found to be present in the N terminus of the *L. pneumophila* EI^{Ntr}. N-terminal GAF domains are often responsible for detection of small-molecule signals, such as cyclic GMP (cGMP), formate, glutamine, and α-ketoglutarate (tricarboxylic acid [TCA] cycle intermediate) (40–42), and they frequently exert regulatory effects on adjacent catalytic domains. The putative ligands of many of the GAF domains remain unidentified, and such is the case with the *L. pneumophila* EI^{Ntr} GAF domain. Nonetheless, the cytoplasmic/metabolic signal recognized by the *L. pneumophila* EI^{Ntr} GAF domain together with the environmental signal sensed by the *L. pneumophila* PmrB SHK is expected to result in activation of the expression levels of the effector-encoding genes regulated by PmrA.

To conclude, the way by which the *L. pneumophila* PTS^{Ntr} activates the levels of expression of PmrA-regulated genes via PmrA is currently not known. However, our results demonstrate that the *L. pneumophila* incomplete PTS^{Ntr} is functional, that it is required for optimal intracellular growth, and that it mediates its effect on intracellular growth by controlling the expression levels of more than 40 effector-encoding genes regulated by PmrA.

MATERIALS AND METHODS

Bacterial strains and media. The *L. pneumophila* parental strain used in this work was JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which behaves as a wild-type strain in terms of intracellular growth (43). In addition, mutant strains derived from JR32 which contain a kanamycin (Km) cassette instead of the *icmT* gene (GS3011) (44), the *pmrA* gene (HK-PQ1) (4), the *pmrB* gene (EA-pmrB) (this study), and the *ptsO* gene (SY-ptsO-Km) (this study) and a clean deletion mutation in the *ptsP* gene (SY-ΔptsP) (this study) were used. In addition, three double deletion mutants were constructed, all of them containing the clean deletion in the *ptsP* gene together with a deletion in the *pmrA* gene (SY-ΔptsP-pmrA-Km), the *pmrB* gene (SY-ΔptsP-pmrB-Km), or the *ptsO* gene (SY-ΔptsP-ptsO-Km). The *L. micdadei* strain used in this work was ATCC 33218 (45). The *E. coli* strains used in this work were MC1022, MC1061 (46), BL21(DE3) (47), and SY327 λ*pir* (48). Bacterial media, plates, and antibiotics were as previously described (49).

Plasmid construction. To construct *lacZ* translational fusions, the 300-bp regulatory regions of four effector-encoding genes (*ceg3*, *ceg4*, *ceg11*, and *legA14*) and 900 bp of one regulator (*pmrA*) (see Data Set S1 in the supplemental material) were amplified by PCR using the primers listed in Data Set S2. The PCR products were then digested with BamHI and EcoRI, cloned into pGS-lac-02, and sequenced. These five new *lacZ* fusions, as well as 11 *lacZ* fusions that were previously constructed and used in this study, are listed in Data Set S1.

To construct IPTG-inducible *ptsP* and *ptsO* genes, the *L. pneumophila ptsP* and *ptsO* genes were amplified by PCR using the primers listed in Data Set S2. The PCR products were then digested with NdeI and BamHI for *ptsP* and EcoRI and BamHI for *ptsO* and cloned into pMMB207 downstream of the P*tac*

promoter to generate pSY-207-Ptac-ptsP and pSY-207-Ptac-ptsO, respectively. In addition, the *ptsP* and *ptsO* genes were mutated by PCR to encode substitutions of alanines for the conserved histidines at position 371 (*ptsP*^{H371A}) for EI^{Ntr} (EI$^{Ntr\text{-}H371A}$) and at position 15 (*ptsO*^{H15A}) for NPr (NPr^{H15A}) using the primers listed in Data Set S2 and cloned into pMMB207 downstream of the P*tac* promoter to generate pSY-207-Ptac-ptsP-H371A and pSY-207-Ptac-ptsO-H15A, respectively (Data Set S1). The resulting four plasmids were then digested with PstI and EheI, and the resulting fragment, containing P*tac-ptsP*, P*tac-ptsO*, P*tac-ptsP*^{H371A}, or P*tac-ptsO*^{H15A} together with the *lacI* gene, was cloned into plasmids containing the *lacZ* fusions of the *ceg3* and *lepB* genes digested with PstI and XmnI, resulting in the plasmids listed in Data Set S1.

To construct IPTG-inducible wild-type and mutated *L. micdadei ptsN* genes, the *ptsN* gene was amplified by PCR, using the primers listed in Data Set S2, and cloned into pMMB207 downstream of the P*tac* promoter to generate pZT-207-mic-ptsN (Data Set S1). In addition, the *ptsN* gene was mutated by PCR to encode a substitution of alanine for the conserved histidine residue at position 67 (*ptsN*^{H67A}), using the primers listed in Data Set S2, and cloned into pMMB207 downstream of the P*tac* promoter to generate pZT-207-mic-ptsNmut (Data Set S1). These two plasmids were then digested with PstI and EheI, and the resulting fragment, containing either P*tac-ptsN* together with the *lacI* gene or P*tac-ptsN*^{H67A} together with the *lacI* gene, was cloned into plasmids containing the *lacZ* fusions of the *ceg19* and *ceg21* genes digested with PstI and XmnI, resulting in the plasmids listed in Data Set S1.

To construct a deletion substitution in the *L. pneumophila ptsO* and *pmrB* genes, a 1-kb DNA fragment located on each side of the planned deletions was amplified by PCR using the primers listed in Data Set S2. The primers were designed to contain an SalI site at the place of the deletion. The four fragments that were amplified were cloned into pUC-18 digested with suitable enzymes, and the inserts were sequenced to generate the plasmids listed in Data Set S1. The resulting plasmids were digested with suitable enzymes, and the inserts were used for a four-way ligation containing the Km resistance cassette (Pharmacia) digested with SalI and the pUC-18 vector digested with EcoRI and BamHI. The correct plasmids were identified by restriction digests. The generated plasmids (Data Set S1) were digested with PvuII, which cuts on both sides of the pUC-18 polylinker, and the resulting fragments were cloned into the pLAW344 allelic exchange vector digested with EcoRV to generate the plasmids pEA-pmrB-Km-GR and pSY-ptsO-Km-GR that were used for allelic exchange, as previously described (49).

To construct a nonpolar in-frame deletion mutation in the *L. pneumophila ptsP* gene, a 1-kb DNA fragment located on each side of the planned deletion was amplified by PCR using the primers listed in Data Set S2. The primers were designed to contain an SalI site at the place of the deletion. The two fragments that were amplified were cloned into pUC-18 digested with suitable enzymes, and the inserts were sequenced to generate the plasmids listed in Data Set S1. The resulting plasmids were digested with suitable enzymes, and the inserts were used for a four-way ligation containing the Km resistance cassette (Pharmacia) digested with SalI and the pUC-18 vector digested with EcoRI and BamHI. The generated plasmid (Data Set S1) was digested with PvuII, and the resulting fragment was cloned into the pSY100 allelic exchange vector digested with EcoRV to generate the plasmid pSY100-ptsP-Km, which was then digested with SalI and self-ligated to generate pSY100-ptsP-GR, which was used for allelic exchange, as previously described (44). To construct double deletion *ptsP-ptsO*, *ptsP-pmrA*, and *ptsP-pmrB* mutants, the *ptsP* clean deletion mutant was used instead of the wild-type strain.

To overexpress the NPr, EI^{Ntr}, and the EI^{Ntr} protein without its GAF domain (EI^{NtrΔGAF}) for *in vitro* phosphorylation assays, a fragment containing the *L. pneumophila ptsO* or *ptsP* gene or the *ptsP* gene encoding a deletion of the GAF domain (*ptsP*^{ΔGAF}) was amplified by PCR using the primers listed in Data Set S2. The resulting fragments were digested with NdeI and BamHI and cloned into the pET-21a vector for *ptsO* and into pET-15b for *ptsP* and *ptsP*^{ΔGAF} digested with the same enzymes to generate the plasmids listed in Data Set S1. The resulting plasmids express the full-length NPr fused to a His_6 tag on the C terminus, the full-length EI^{Ntr} fused to a His_6 tag on the N terminus, and the EI^{NtrΔGAF} protein fused to a His_6 tag on the N terminus. The plasmid pZT-His-lpn-pmrA (Data Set S1) was used to overexpress the His-tagged PmrA protein.

Growth analysis. Intracellular growth assays of *L. pneumophila* mutants were performed in *Acanthamoeba castellanii* (ATCC 30234) and HL-60-derived human macrophages (ATCC CCL-240), as previously described (28). *In vitro* growth assays were performed in AYE growth medium [*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract]. Bacteria grown on plates were scraped and resuspended to an optical density at 600 nm (OD_{600}) of 0.10 for time zero measurement, and OD_{600} measurements were conducted in a Synergy HT2011 plate reader in triplicate.

***β*-Galactosidase assay.** The levels of expression of the individual *lacZ* fusions were examined in the *L. pneumophila* wild-type and mutant strains at stationary phase, as previously described (4).

***In vitro* phosphorylation assay.** *L. pneumophila* His_6-PmrA, His_6-EI^{Ntr}, His_6-EI^{NtrΔGAF}, and NPr-His_6 were purified from *E. coli* BL21(DE3) containing the plasmid pZT-His-lpn-pmrA (4), pZT-His-ptsP, pZT-His-ptsP-GAF, or pZT-His-ptsO, respectively. The four proteins were purified by nickel-affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Protein purification was performed as previously described (4, 8). *In vitro* phosphorylation was carried out by incubating 10 mM PEP (Sigma) with different combinations of purified 4 μM His_6-PmrA, 1 μM His_6-EI^{NtrΔGAF}, and 10 μM NPr-His_6 at 37°C for 20 min in a buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM KCl, and 0.5 mM dithiothreitol (DTT). The results presented were obtained with His_6-EI^{NtrΔGAF}, and similar results were obtained also with the full-length His_6-EI^{Ntr} as well as after treatment of the PmrA protein with a phosphatase (data not shown). Samples were separated on 15% SDS-polyacrylamide gels, and phosphorylated proteins were detected by a Pro-Q Diamond phosphoprotein gel stain (Invitrogen), according

to the manufacturer's instructions. Bands were quantified by densitometry of the autoradiograms using ImageQuant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00121-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, XLS file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLS file, 0.1 MB.

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REFERENCES

1. Segal G, Feldman M, Zusman T. 2005. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. FEMS Microbiol Rev 29:65–81. https://doi.org/10.1016/j.femsre.2004.07.001.
2. Isberg RR, O'Connor TJ, Heidtman M. 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. Nat Rev Microbiol 7:13–24. https://doi.org/10.1038/nrmicro1967.
3. Finsel I, Hilbi H. 2015. Formation of a pathogen vacuole according to *Legionella pneumophila*: how to kill one bird with many stones. Cell Microbiol 17:935–950. https://doi.org/10.1111/cmi.12450.
4. Zusman T, Aloni G, Halperin E, Kotzer H, Degtyar E, Feldman M, Segal G. 2007. The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. Mol Microbiol 63:1508–1523. https://doi.org/10.1111/j.1365-2958.2007.05604.x.
5. Al-Khodor S, Kalachikov S, Morozova I, Price CT, Abu Kwaik Y. 2009. The PmrA/PmrB two-component system of *Legionella pneumophila* is a global regulator required for intracellular replication within macrophages and protozoa. Infect Immun 77:374–786. https://doi.org/10.1128/IAI.01081-08.
6. Altman E, Segal G. 2008. The response regulator CpxR directly regulates the expression of several *Legionella pneumophila icm/dot* components as well as new translocated substrates. J Bacteriol 190:1985–1996. https://doi.org/10.1128/JB.01493-07.
7. Feldheim YS, Zusman T, Speiser Y, Segal G. 2016. The *Legionella pneumophila* CpxRA two-component regulatory system: new insights into CpxR's function as a dual regulator and its connection to the effectors regulatory network. Mol Microbiol 99:1059–1079. https://doi.org/10.1111/mmi.13290.
8. Gal-Mor O, Segal G. 2003. Identification of CpxR as a positive regulator of *icm* and *dot* virulence genes of *Legionella pneumophila*. J Bacteriol 185:4908–4919. https://doi.org/10.1128/JB.185.16.4908-4919.2003.
9. Gal-Mor O, Segal G. 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. https://doi.org/10.1016/S0882-4010(03)00027-5.
10. Hammer BK, Tateda ES, Swanson MS. 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol Microbiol 44:107–118. https://doi.org/10.1046/j.1365-2958.2002.02884.x.
11. Hovel-Miner G, Pampou S, Faucher SP, Clarke M, Morozova I, Morozov P, Russo JJ, Shuman HA, Kalachikov S. 2009. SigmaS controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. J Bacteriol 191:2461–2473. https://doi.org/10.1128/JB.01578-08.
12. Nevo O, Zusman T, Rasis M, Lifshitz Z, Segal G. 2014. Identification of *Legionella pneumophila* effectors regulated by the LetAS-RsmYZ-CsrA regulatory cascade, many of which modulate vesicular trafficking. J Bacteriol 196:681–692. https://doi.org/10.1128/JB.01175-13.
13. Rasis M, Segal G. 2009. The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. Mol Microbiol 72:995–1010. https://doi.org/10.1111/j.1365-2958.2009.06705.x.
14. Sahr T, Bruggemann H, Jules M, Lomma M, Albert-Weissenberger C, Cazalet C, Buchrieser C. 2009. Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. Mol Microbiol 72:741–762. https://doi.org/10.1111/j.1365-2958.2009.06677.x.
15. Shi C, Forsbach-Birk V, Marre R, McNealy TL. 2006. The *Legionella pneumophila* global regulatory protein LetA affects DotA and Mip. Int J Med Microbiol 296:15–24. https://doi.org/10.1016/j.ijmm.2005.09.003.
16. Schell U, Simon S, Sahr T, Hager D, Albers MF, Kessler A, Fahrnbauer F, Trauner D, Hedberg C, Buchrieser C, Hilbi H. 2016. The alpha-hydroxyketone LAI-1 regulates motility, Lqs-dependent phosphorylation signalling and gene expression of *Legionella pneumophila*. Mol Microbiol 99:778–793. https://doi.org/10.1111/mmi.13265.
17. Gao R, Stock AM. 2010. Molecular strategies for phosphorylation-mediated regulation of response regulator activity. Curr Opin Microbiol 13:160–167. https://doi.org/10.1016/j.mib.2009.12.009.
18. Deutscher J, Ake FM, Derkaoui M, Zebre AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P. 2014. The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol Mol Biol Rev 78:231–256. https://doi.org/10.1128/MMBR.00001-14.
19. Peterkofsky A, Wang G, Seok YJ. 2006. Parallel PTS systems. Arch Biochem Biophys 453:101–107. https://doi.org/10.1016/j.abb.2006.01.004.
20. Pfluger-Grau K, Gorke B. 2010. Regulatory roles of the bacterial nitrogen-related phosphotransferase system. Trends Microbiol 18:205–214. https://doi.org/10.1016/j.tim.2010.02.003.
21. Powell BS, Court DL, Inada T, Nakamura Y, Michotey V, Cui X, Reizer A, Saier MH, Jr, Reizer J. 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIA^{Ntr} affects growth on organic nitrogen and the conditional lethality of an *era*^{ts} mutant. J Biol Chem 270:4822–4839.
22. Lee CR, Cho SH, Yoon MJ, Peterkofsky A, Seok YJ. 2007. *Escherichia coli* enzyme IIA^{Ntr} regulates the K^+ transporter TrkA. Proc Natl Acad Sci U S A 104:4124–4129. https://doi.org/10.1073/pnas.0609897104.
23. Cabeen MT, Leiman SA, Losick R. 2016. Colony-morphology screening uncovers a role for the *Pseudomonas aeruginosa* nitrogen-related phosphotransferase system in biofilm formation. Mol Microbiol 99:557–570. https://doi.org/10.1111/mmi.13250.
24. Choi J, Shin D, Yoon H, Kim J, Lee CR, Kim M, Seok YJ, Ryu S. 2010. *Salmonella* pathogenicity island 2 expression negatively controlled by EIIA^{Ntr}-SsrB interaction is required for *Salmonella* virulence. Proc Natl Acad Sci U S A 107:20506–20511. https://doi.org/10.1073/pnas.1000759107.
25. Dozot M, Poncet S, Nicolas C, Copin R, Bouraoui H, Maze A, Deutscher J, De Bolle X, Letesson JJ. 2010. Functional characterization of the incomplete phosphotransferase system (PTS) of the intracellular pathogen *Brucella melitensis*. PLoS One 5:e12679. https://doi.org/10.1371/journal.pone.0012679.
26. Higa F, Edelstein PH. 2001. Potential virulence role of the *Legionella pneumophila ptsP* ortholog. Infect Immun 69:4782–4789. https://doi.org/10.1128/IAI.69.8.4782-4789.2001.
27. Burstein D, Amaro F, Zusman T, Lifshitz Z, Cohen O, Gilbert JA, Pupko T,

Shuman HA, Segal G. 2016. Genomic analysis of 38 *Legionella* species identifies large and diverse effector repertoires. Nat Genet 48:167–175. https://doi.org/10.1038/ng.3481.
28. Segal G, Shuman HA. 1999. *Legionella pneumophila* utilize the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect Immun 67:2117–2124.
29. Hindre T, Bruggemann H, Buchrieser C, Hechard Y. 2008. Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology 154:30–41. https://doi.org/10.1099/mic.0.2007/008698-0.
30. Segal G. 2013. The *Legionella pneumophila* two-component regulatory systems that participate in the regulation of Icm/Dot effectors. Curr Top Microbiol Immunol 376:35–52. https://doi.org/10.1007/82_2013_346.
31. Chen HD, Groisman EA. 2013. The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. Annu Rev Microbiol 67:83–112. https://doi.org/10.1146/annurev-micro-092412-155751.
32. Deutscher J, Francke C, Postma PW. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol Mol Biol Rev 70:939–1031. https://doi.org/10.1128/MMBR.00024-06.
33. Nessler S. 2005. The bacterial HPr kinase/phosphorylase: a new type of Ser/Thr kinase as antimicrobial target. Biochim Biophys Acta 1754:126–131. https://doi.org/10.1016/j.bbapap.2005.07.042.
34. Poncet S, Soret M, Mervelet P, Deutscher J, Noirot P. 2009. Transcriptional activator YesS is stimulated by histidine-phosphorylated HPr of the *Bacillus subtilis* phosphotransferase system. J Biol Chem 284:28188–28197. https://doi.org/10.1074/jbc.M109.046334.
35. Derkaoui M, Antunes A, Poncet S, Nait Abdallah J, Joyet P, Maze A, Henry C, Taha MK, Deutscher J, Deghmane AE. 2016. The phosphocarrier protein HPr of *Neisseria meningitidis* interacts with the transcription regulator CrgA and its deletion affects capsule production, cell adhesion, and virulence. Mol Microbiol 100:788–807. https://doi.org/10.1111/mmi.13349.
36. Perez JC, Groisman EA. 2007. Acid pH activation of the PmrA/PmrB two-component regulatory system of *Salmonella enterica*. Mol Microbiol 63:283–293. https://doi.org/10.1111/j.1365-2958.2006.05512.x.
37. Wosten MM, Kox LF, Chamnongpol S, Soncini FC, Groisman EA. 2000. A signal transduction system that responds to extracellular iron. Cell 103:113–125. https://doi.org/10.1016/S0092-8674(00)00092-1.
38. Merighi M, Ellermeier CD, Slauch JM, Gunn JS. 2005. Resolvase-in vivo expression technology analysis of the *Salmonella enterica* serovar Typhimurium PhoP and PmrA regulons in BALB/c mice. J Bacteriol 187:7407–7416. https://doi.org/10.1128/JB.187.21.7407-7416.2005.
39. Anantharaman V, Koonin EV, Aravind L. 2001. Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. J Mol Biol 307:1271–1292. https://doi.org/10.1006/jmbi.2001.4508.
40. Lee CR, Park YH, Kim M, Kim YR, Park S, Peterkofsky A, Seok YJ. 2013. Reciprocal regulation of the autophosphorylation of enzyme I^{Ntr} by glutamine and α-ketoglutarate in *Escherichia coli*. Mol Microbiol 88:473–485. https://doi.org/10.1111/mmi.12196.
41. Martinez-Argudo I, Little R, Dixon R. 2004. Role of the amino-terminal GAF domain of the NifA activator in controlling the response to the antiactivator protein NifL. Mol Microbiol 52:1731–1744. https://doi.org/10.1111/j.1365-2958.2004.04089.x.
42. Ho YS, Burden LM, Hurley JH. 2000. Structure of the GAF domain, a ubiquitous signaling motif and a new class of cyclic GMP receptor. EMBO J 19:5288–5299. https://doi.org/10.1093/emboj/19.20.5288.
43. Sadosky AB, Wiater LA, Shuman HA. 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect Immun 61:5361–5373.
44. Zusman T, Yerushalmi G, Segal G. 2003. Functional similarities between the *icm/dot* pathogenesis systems of *Coxiella burnetii* and *Legionella pneumophila*. Infect Immun 71:3714–3723. https://doi.org/10.1128/IAI.71.7.3714-3723.2003.
45. Vickers RM, Brown A, Garrity GM. 1981. Dye-containing buffered charcoal-yeast extract medium for differentiation of members of the family *Legionellaceae*. J Clin Microbiol 13:380–382.
46. Casadaban MJ, Cohen SN. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol 138:179–207. https://doi.org/10.1016/0022-2836(80)90283-1.
47. Studier FW, Moffatt BA. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189:113–130. https://doi.org/10.1016/0022-2836(86)90385-2.
48. Donnenberg MS, Kaper JB. 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect Immun 59:4310–4317.
49. Segal G, Shuman HA. 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila*. Infect Immun 65:5057–5066.