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The PhoP-PhoQ Two-Component Regulatory System of *Photorhabdus luminescens* Is Essential for Virulence in Insects

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Photorhabdus luminescens **is a symbiont of entomopathogenic nematodes. Analysis of the genome sequence of this organism revealed a homologue of PhoP-PhoQ, a two-component system associated with virulence in intracellular bacterial pathogens. This organism was shown to respond to the availability of environmental magnesium. A mutant with a knockout mutation in the regulatory component of this system (*phoP*) had no obvious growth defect. It was, however, more motile and more sensitive to antimicrobial peptides than its wild-type parent. Remarkably, the mutation eliminated virulence in an insect model. No insect mortality was observed after injection of a large number of the *phoP* bacteria, while very small amounts of parental cells killed insect larvae in less than 48 h. At the molecular level, the PhoPQ system mediated Mg^{2+}-dependent modifications in lipopolysaccharides and controlled a locus (*pbgPE*) required for incorporation of 4-amino-arabinose into lipid A. Mg^{2+}-regulated gene expression of *pbgP1* was absent in the mutant and was restored when *phoPQ* was complemented in *trans*. This finding highlights the essential role played by PhoPQ in the virulence of an entomopathogen.**

Pathogens have to overcome the defenses of their hosts. *Photorhabdus luminescens*, an insect pathogen, faces particularly challenging conditions. This bacterium has a complex life cycle that involves two completely different environments: a symbiotic stage, in which bacteria colonize the nematode gut, and a pathogenic stage, in which susceptible insects are killed by the combined action of the nematode and the bacteria. After entering the insect host, a nematode releases its bacterial symbionts into the insect hemocoel. Once released, the bacteria proliferate rapidly, disregarding both the humoral insect immune response (e.g., antibacterial peptides) and the cell-mediated insect immune response (hemocytes) (14, 17). While multiplying, the bacteria produce exo- and endotoxins, to which the insect succumbs within 48 h of infection. They also produce antibiotics that inhibit the growth of competing microorganisms in the insect cadaver (16, 57) and enhance conditions for nematode reproduction by providing nutrients and other growth factors utilized by the nematodes (25).

How the infecting bacteria overcome or escape from the insect immune system is still largely an open question. The recent sequencing of the strain TT01 genome revealed a plethora of candidate virulence factors (18). Some of these factors have previously been found to be involved in processes that result in insect death. The Mcf toxin (makes caterpillars floppy) was shown to be a dominant virulence factor critical for pathogenesis. The putative apoptosis action of this toxin in insect cells caused the larvae to loose body turgor and die (15). Purified high-molecular-weight toxin complexes had both oral and injectable activities with specific effects on the midgut epithelium of a wide range of insects (7, 70). The protease and lipase fractions did not significantly affect mortality rates (8, 68), while the role of purified lipopolysaccharide (LPS) in virulence was uncertain (19). Recently, *phlA*, a locus encoding a hemolysin belonging to the two-partner secretion family of proteins, was identified in the TT01 genome. Although PhlA appears to be produced in the hemolymph during insect infection, the high virulence of a *phlA* mutant indicates that this hemolysin is not a major virulence determinant (9).

To colonize the nematode gut and multiply in the insect hemocoel (two environments whose physical and chemical properties differ), *P. luminescens*, like other bacterial pathogens (36), has evolved two-component signal transduction systems (66). These systems comprise a membrane-associated sensor kinase and a cytoplasmic transcriptional regulator. In response to an external stimulus, the sensor component autophosphorylates at a conserved histidine residue in an ATP-dependent reaction. In the second step, the phosphoryl group is transferred to the regulator component, promoting its binding to DNA. The two-component PhoP-PhoQ system has been found in many gram-negative bacteria (30), and its primary function seems to be control of physiological adaptation to Mg^{2+} availability (26). Mg^{2+} (or Ca^{2+}) binding to the periplasmic domain of PhoQ promotes the dephosphorylation of phospho-PhoP. Expression of PhoP-activated genes is induced when the Mg^{2+} concentration is low (micromolar) and is repressed when the Mg^{2+} concentration is high (millimolar) (26, 27).

In spite of its presence in both pathogenic and nonpathogenic species, PhoP-PhoQ is an important regulator of virulence genes in a number of intracellular bacterial pathogens, including *Salmonella* sp., *Shigella* sp., *Mycobacterium tubercu-*

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losis, and *Neisseria meningitidis* (38, 45, 46, 52) This system has been studied extensively in *Salmonella enterica* serovar Typhimurium, in which it regulates directly or indirectly more than 40 different genes (29, 44, 65). Many of these genes are species specific and confer unique properties to the microorganism, including survival within macrophages, resistance to host antimicrobial peptides (APs) and acidic pH, invasion of epithelial cells, and antigen presentation (31, 45, 50, 51). These properties are often linked to modifications of many components in the bacterial cell envelope governed by PhoP-PhoQ (22, 32, 43, 51).

In an attempt to identify some of the regulatory systems involved in pathogenicity control in *P. luminescens*, we investigated the role of the PhoP-PhoQ homologue. A knockout mutant with a mutation in the *phoP* regulator was generated. The mutation affected several components of the bacterial envelope and, remarkably, resulted in an avirulence phenotype in an insect model, *Spodoptera littoralis*. Our results suggest that PhoPQ may sense conditions in the insect hemocoel and subsequently promote resistance to the innate immunity of the insect.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Permanent stocks of all strains were maintained at −80°C in Luria-Bertani broth supplemented with glycerol. *P. luminescens* was grown at 30°C. The *P. luminescens* strains used were TT01 (23) and its *phoP* derivative PL2104 (this study). The *Escherichia coli* strains used were TG1 (59) for plasmid maintenance and S17-1 (61) for conjugation. *E. coli* strains were routinely grown in Luria-Bertani medium at 37°C, whereas *P. luminescens* strains were grown at 30°C in Schneider medium (BioWhittaker). To study the effects of Mg^{2+} concentration, ED*-glucose defined medium [15 mM NH_4Cl, 11 μg of ferric citrate per ml, 0.4% glucose, 2 mM K_2SO_4, 80 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM Na_3-citrate, 50 μM $FeCl_3$, 7.5 μM $MnCl_2$, 12.5 μM $ZnCl_2$, 2.5 μM $CuCl_2$, 2.5 μM $CoCl_2$, 2.5 μM Na_2MoO_4] containing 20 μM $MgCl_2$ (low-Mg^{2+} medium) or 10 mM $MgCl_2$ (high-Mg^{2+} medium) was used for both *E. coli* and *P. luminescens*. The final concentrations of the antibiotics used for selection were as follows: 30 mg of gentamicin per liter, 20 mg of kanamycin per liter, and 20 mg of chloramphenicol per liter for *E. coli*; and 15 mg of chloramphenicol per liter for *P. luminescens*. All experiments were performed in accordance with the European regulation requirements concerning the contained use of group I genetically modified organisms (agreement no. 2736 CAII).

DNA manipulations and plasmid construction. Chromosomal DNA preparation, ligation, *E. coli* electroporation, and Southern blotting were carried out by using standard procedures (59). Plasmid DNA was isolated with a GenElute plamid miniprep kit (Sigma). Restriction enzymes were obtained from Roche, and enzymatic reaction products were purified with a MinElute reaction cleanup kit (Qiagen).

Plasmid pDIA604 was constructed by two steps of PCR amplification. Briefly, the chloramphenicol acetyltransferase gene of pACYC184 (Biolabs) was amplified by PCR with oligonucleotides Cat5 (5′-TTGATCGGCACGTAAGAGGT-3′) and Cat3 (5′-AATTTCTGCCATTCATCCGC-3′), which resulted in an 850-bp DNA fragment. Two 1.1-kb DNA fragments containing either the 5′ upstream region of *phoP* or the end of the coding region of *phoP* and the downstream region were also generated by PCR by using either oligonucleotides phoP3 (5′-AAACTGCAGCTCACCGGATGGAACGCCAG-3′) and phoP4 (5′-ACCTCTTACGTGCCGATCAAGGATCCGGTGTCACGAAGCTGTAC C-3′) or oligonucleotides phoP5 (5′-GCGGATGAATGGCAGAAATTGGAT CCCGATGCTGAACTGCGCGAA-3′) and phoP6 (5′-GCTCTAGAGCGCAT ACTGGCACGATGCAG-3′) and genomic DNA from *P. luminescens* TT01. The first 20 bases of primers phoP4 and phoP5 are complementary to primers Cat5 and Cat3, respectively. After purification with a QIAquick PCR purification kit (Qiagen), 100-ng portions of the three previously amplified fragments were mixed and used as a template to generate a new 3.1-kb DNA fragment by a second PCR performed with oligonucleotides phoP3 and phoP6. The resulting amplicon, which corresponded to a *phoP*::Cm fragment, was purified, restricted with *Pst*I and *Xba*I, and ligated to the pJQ200KS vector (53) to obtain pDIA604.

Plasmid pDIA605 was constructed by cloning into the multiple cloning site of the pBluescript SK plasmid (Stratagene), restricted at *Pst*I and *Xba*I sites, the previously amplified 3.1-kb *phoP*::Cm DNA fragment containing the *phoP* promoter region and the beginning of the *phoP* coding sequence.

To construct the pDIA607 plasmid, a DNA fragment containing the whole *phoPQ* locus was generated by PCR with primers PhoP1 (5′-AAACTGCAGA TTGAAAGCCATGACGCCAG-3′) and PhoQ2 (5′-TGCTCTAGACATCCTG AGTGTGAAGTGAA-3′). The 2.5-kb amplified fragment was purified, restricted with *Xba*I and *Pst*I (underlined sites), and cloned into the pBBR1MCS-5 vector, a low-copy-number mobilizable plasmid (40). The exact DNA sequence of pDIA606 was confirmed by sequencing (GENOME express, Montreoil, France).

Construction of a *phoP* mutant and complementation of the mutant. Strain PL2104 was created by allelic exchange with pDIA604 (which contains a *cat* cassette in the *phoP* coding region). pDIA604 was transformed into *E. coli* S17-1 and introduced into *P. luminescens* by mating. Cm^r Gm^s Sac^r exconjugants were selected on proteose peptone agar (1% proteose peptone, 0.5% NaCl, 0.5% yeast extract, 1.5% agar) containing 2% sucrose. The exconjugants had undergone allelic exchange and lost the wild-type copy of *phoP* and the plasmid vehicle. Insertions were confirmed by Southern blot hybridization (data not shown) by using a PCR-amplified digoxigenin-labeled *phoP* gene probe, oligonucleotides phoP3 and phoP4, and a PCR digoxigenin probe synthesis kit (Roche).

Complementation was performed by using mating experiments. pDIA607 was used to transfer the *phoPQ* operon from *E. coli* S17-1 into the recipient *P. luminescens* PL2104. Cm^r Gm^r exconjugants containing the pDIA607 vector were selected.

Swimming capacity. Tryptone motility plates containing 1% Bacto Tryptone (Difco), 0.5% NaCl, and 0.3% Bacto Agar (Difco) were used to test bacterial motility as previously described (5).

In vivo pathogenicity assays. The pathogenicity assays were performed with the common cutworm *S. littoralis* as previously described (28). Briefly, 20 μl of exponentially growing bacteria diluted in phosphate-buffered saline was injected into the hemolymph of 20 fifth-instar larvae of *S. littoralis* reared on an artificial diet. The insect larvae were then individually incubated at 23°C for up to 115 h, and bacterial CFU were determined by plating dilutions on Luria-Bertani agar. Insect death was monitored several times after injection. Three independent experiments were performed.

Antibacterial activity. In vitro susceptibility tests to determine MICs were performed by the broth microdilution method according to National Committee for Clinical Laboratory Standards proposed guidelines (49), with some modifications. Stock solutions of colistin methanesulfonate (Sigma) and polymyxin B (Sigma) were prepared in sterile water to obtain concentrations of 20 and 0.5 mg/ml, respectively. Stock solutions of cecropin A and B were prepared in 0.5% acetic acid to obtain a concentration of 0.4 mg/ml. The antibiotics were then added directly to 96-well microtiter plates in twofold serial dilutions. A total of 10^4 CFU of bacteria that had been grown overnight was dispensed into each microdilution well. The MICs were determined in Mueller-Hinton broth (Biokar) following incubation at 30°C for 48 h. The microtiter plates were read by visual observation.

RNA manipulations. Total RNA was prepared from 10-ml cultures of *P. luminescens* as previously described (16). Primer extension was performed by using standard procedures (59) with some modifications, as previously described (16). Briefly, 10 ng of an end-labeled primer was annealed with total RNA, and a reverse transcriptase reaction was performed with avian myeloblastosis virus reverse transcriptase (Roche) at 42°C for 90 min. As a reference, sequencing reactions were performed with a Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham) with the same primer used to map the 5′ termini of *phoP* mRNA, phoP4. The oligonucleotide used in primer extension experiments was end labeled with phage T4 polynucleotide kinase (BioLabs) and [γ-^{32}P]ATP (3,000 Ci/mmol) by using standard procedures (59).

LPS preparation. *P. luminescens* strains TT01 and PL2104 were grown to the log phase on ED*-glucose synthetic medium supplemented with $MgCl_2$ at either a micromolar or millimolar concentration. LPS was obtained by hot phenol-water extraction. Next, LPS extracts were mixed with 1 volume of loading buffer (Sigma) containing 7% β-mercaptoethanol and boiled for 5 min. LPS profiles were analyzed by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by using a 16.5% acrylamide gel. The gel was fixed overnight in 25% isopropanol–4% acetic acid and then silver stained by using the method of Tsai and Frasch (67).

Sequence comparisons. Amino acid sequence similarity searches were carried out by using the BLASTP software (2, 3).

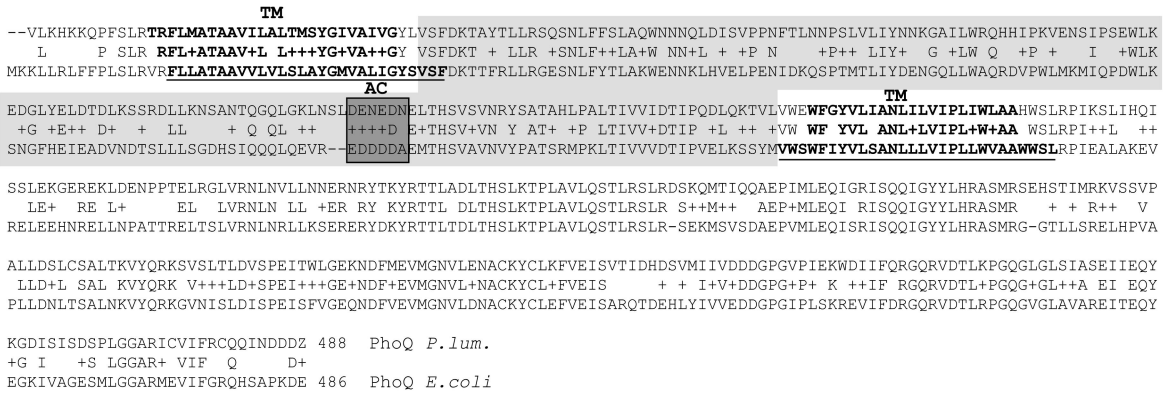

FIG. 1. Sequence alignment of *P. luminescens* and *E. coli* sensor PhoQ showing the ligand binding site, designated the acidic cluster (AC), which is enclosed in a shaded box. Periplasmic domains are delineated by two hydrophobic transmembrane sequences (TM) indicated by boldface type. *P. lum.*, *P. luminescens*.

Nucleotide sequence accession number. *phoPQ* and *pbgP*123*E*1234 sequence data have been deposited in the EMBL databases under accession number BX470251.

RESULTS

Identification of the PhoP-PhoQ two-component system in *P. luminescens*. The genome sequence of *P. luminescens* TT01 was recently completely deciphered (18). Nineteen two-component regulatory systems were found in the genome, including a counterpart of a known PhoP-PhoQ system found in gram-negative bacteria. The organization of the *P. luminescens* *phoPQ* locus is similar to that of the loci in *Salmonella* sp. and *E. coli*; both genes are located downstream of *purB*, encoding adenylsuccinate lyase. The sizes of the predicted *P. luminescens* regulator (222 residues) and sensor (487 residues) are similar to the sizes of the *Salmonella* and *E. coli* regulator (224 and 222 residues, respectively) and sensor (487 residues), and the sequences exhibit 70 and 48% identity at the amino acid level. In addition, the domain organization is similar. PhoP is a typical response regulator, containing an N-terminal receiver domain and a C-terminal helix-turn-helix motif. The putative site of phosphorylation (D58) is conserved. PhoQ contains an N-terminal periplasmic sensor domain, delineated by two hydrophobic transmembrane sequences predicted in silico (13), coupled to a cytoplasmic transmitter domain whose sequence is particularly well conserved. There are differences in the extracellular sensor domain (Fig. 1). One interesting example of the divergence is in the ligand binding site for the divalent cations Mg^{2+} and Ca^{2+}, called the acidic cluster (69). Although the amino acid sequences were strikingly different in this cluster (DENEDNE in *P. luminescens* and EDDDDAE in *E. coli*), conservative conversions maintain several acidic amino acids clustered in the same region, suggesting that this region also serves as a ligand binding site in the *P. luminescens* PhoQ sensor (69).

Characterization and expression of the *phoPQ* operon from *P. luminescens*. *phoQ* starts 19 bases downstream from the coding sequence of *phoP*, suggesting that the two genes form a single transcription unit. Primer extension analysis with total *P. luminescens* RNA (up to 50 μg of RNA) revealed very faint traces of *phoP* mRNA in exponentially growing *P. luminescens* cells (data not shown). To map precisely the transcription start point, we overexpressed the transcriptional regulatory region using plasmid pDIA607. Total RNA was extracted during the exponential growth phase at 30°C, and primer extension analysis was performed with 25 μg of RNA and primer phoP4 (Fig. 2B). A single start point was mapped at an adenosine residue located 99 bp upstream from the translation start codon of *phoP* (Fig. 2A). This start point was preceded by −35 and −10 sequences (TTGCTG-17 bp-TAACAT) with significant similarity to the consensus boxes for sigma 70 promoters in enterobacteria.

To examine the sensing function of PhoPQ in *P. luminescens*, the effect of the Mg^{2+} and Ca^{2+} divalent cations on *phoP* expression was investigated by primer extension. Total RNAs from *P. luminescens* cells transformed with plasmid pDIA607 were prepared following exposure to various Mg^{2+} and Ca^{2+} concentrations (Fig. 2C). Expression of *phoP* was inversely proportional to the Mg^{2+} concentration; the maximal activation was observed in medium containing a micromolar concentration of $MgCl_2$. Addition of a millimolar concentration of Mg^{2+} decreased the *phoP* mRNA abundance about fivefold. Growth in the presence of Ca^{2+} slightly repressed (less than twofold) *phoP* expression compared with expression in low-Mg^{2+} medium. Thus, *phoP* expression in *P. luminescens* is controlled by a unique Mg^{2+}- and Ca^{2+}-inducible promoter. It is interesting that in *Salmonella* and *E. coli*, the *phoPQ* operon is transcribed from two promoters (Fig. 2A), one which is active during growth in the presence of a low concentration of Mg^{2+} and is dependent on PhoPQ and one which is constitutive (26, 39).

Construction and phenotypic characterization of a *phoP* mutant. To obtain clues concerning the functional role of PhoPQ in *P. luminescens*, the regulator PhoP was inactivated by allelic exchange. A mutant strain (PL2104) was constructed with plasmid pDIA604 harboring a chloramphenicol cassette in place of the *phoP* internal coding region (see Materials and Methods).

The mutant was compared to the wild-type strain in an analysis of several phenotypic traits specific to *P. luminescens*. Both strains adsorbed dye from nutrient bromothymol blue agar and produced antibiotics and paracrystalline inclusion bodies at levels indistinguishable from those produced by the

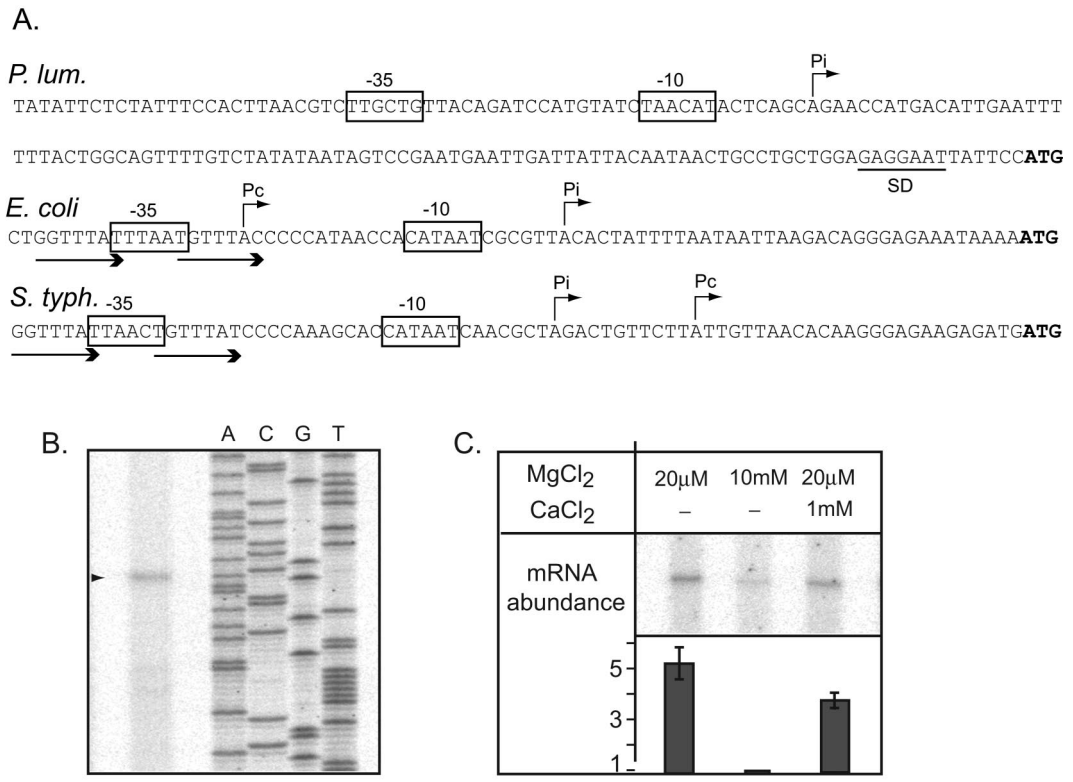

FIG. 2. (A) Nucleotide sequences of the 5′ region of *phoP* from *P. luminescens*, *E. coli*, and *S. enterica* serovar Typhimurium. Mg^{2+}-responsive promoter sequences (−35 and −10 boxes) are enclosed in boxes, and transcriptional start sites are indicated by arrows (Pi, position 1 of Mg^{2+}-inducible promoter; Pc, position 1 of constitutive promoter). The Shine-Dalgarno sequence (SD) is underlined. The first codon of the coding sequence of *phoP* is indicated by boldface type. The newly identified PhoP box, which consists of a direct repeat of the heptanucleotide sequence (T)G(T)TT(AA), is underlined by arrows. *P. lum*., *P. luminescens*; *S. typh*., *S. enterica* serovar Typhimurium. (B) Primer extension analysis of *phoP* transcripts in a *P. luminescens* strain overexpressing *phoPQ* (pDIA607). Total RNA was extracted from an exponential culture grown at 30°C. The arrowhead indicates the position of the extension product obtained. (C) Analysis of *P. luminescens phoP* mRNA abundance following exposure to various magnesium and calcium concentrations during exponential growth at 30°C in ED* synthetic medium. Left lane, ED* medium supplemented with 20 μM $MgCl_2$; middle lane, ED* medium supplemented with 10 mM $MgCl_2$; right lane, ED* medium supplemented with 20 μM $MgCl_2$ and 1 mM $CaCl_2$. Equal amounts of each RNA (25 μg) were used. Experiments were done in triplicate, and the data shown are the data from one representative experiment. For the graph the relative *phoP* mRNA abundance obtained with the lowest level of mRNA was defined as 1.

wild-type strain. The *phoP* mutation did not have any effect on exponential- or stationary-phase cell morphology in Schneider medium (data not shown). We next examined the growth characteristics of strains TT01, PL2104, and PL2104 complemented with copies of *phoPQ* supplied on the low-copy-number mobilizable plasmid pDIA607 in synthetic medium containing either 10 mM or 20 μM $MgCl_2$. Except for a longer lag phase for strain PL2104, no significant differences were detected among the strains in the exponential or stationary growth phase (Fig. 3A). The growth rate appeared to be only slightly lower when the magnesium level was low for PL2104. At very low concentrations of magnesium (less than 0.1 μM), all strains grew slowly and reached the stationary phase at a low cell density (optical density, 0.6 to 0.7). At intermediate magnesium concentrations (10 to 20 μM), all strains had similar specific growth rates and reached the stationary phase at an optical density of 1.2 to 1.5. At both concentrations, loss of pigmentation was observed in 24-h-old cultures in all cases. At a relatively high concentration of magnesium (10 mM), all strains grew to an optical density of 5 to 5.5, and the culture broths were pigmented. These findings differ from what was observed with *Salmonella* or *Neisseria*; *phoP* mutants of these organisms did not grow at reduced (micromolar) magnesium levels (38, 65).

The first trait markedly affected by the mutation was swarming in semisolid agar (Fig. 3B). In 0.3% (wt/vol) agar the *phoP* mutant reproducibly migrated farther from the point of inoculation than the parent migrated. This was due to early onset of the spreading behavior; the mutant started to spread 15 h after inoculation, while the wild-type strain started to spread only after 20 h (Fig. 3B). Wild-type motility was restored when PL2104 was complemented with pDIA607 (data not shown).

Effect of the *phoP* mutation on virulence in insects. To examine the effect of the *phoP* mutation on virulence in insects, we injected similar doses (500 to 1,000 CFU) of parental (TT01), *phoP* (PL2104), and *phoP*-complemented [PL2104 (pDIA607)] cells directly into the hemocoel of *S. littoralis* larvae and monitored insect mortality after injection (Fig. 3C). Remarkably, no mortality was observed with PL2104. Furthermore, septicemia was observed 24 h after injection of wild-type bacteria, while no bacteria were observed in the hemolymph of larvae that received the *phoP* mutant. This is remarkable because living cells of wild-type *P. luminescens* are highly virulent when they are injected into the hemolymph of insects. As

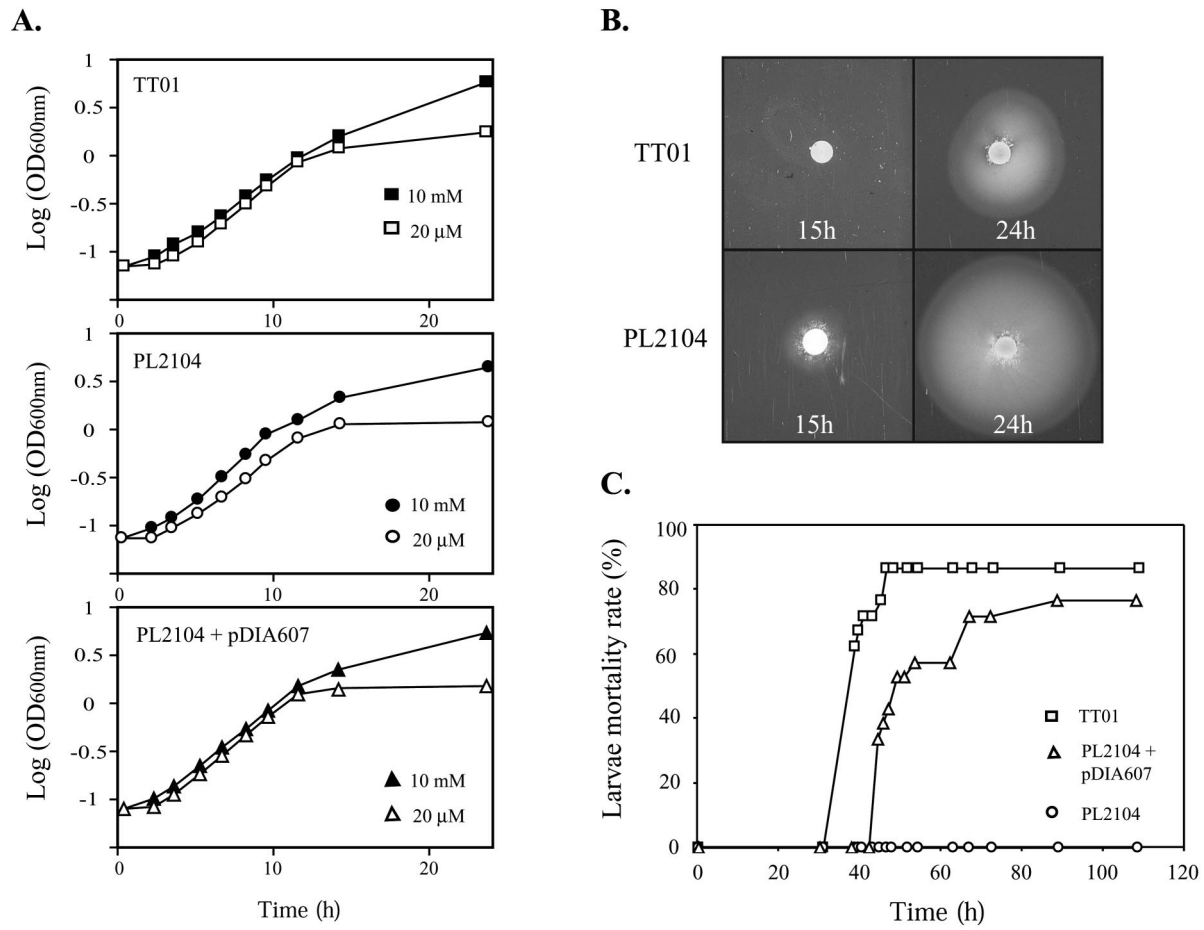

FIG. 3. Phenotypic analysis of the *phoP* mutation in *P. luminescens*. (A) Growth curves for *P. luminescens* wild-type strain TT01, *phoP* knockout mutant PL2104, and complemented *phoP* mutant PL2104(pDIA607) grown in ED* synthetic medium containing 10 mM $MgCl_2$ (solid symbols) or 20 μM $MgCl_2$ (open symbols). OD600nm, optical density at 600 nm. (B) Motility of the *P. luminescens* TT01 and PL2104 strains on semisolid (0.3% [wt/vol] agar) medium plates. Plates were incubated for 15 to 24 h at 30°C. (C) Mortality of *S. littoralis* infected with the *P. luminescens* wild-type strain, *phoP* knockout mutant, and complemented *phoP* mutant. Bacteria obtained at the end of the exponential phase were injected into fourth-instar larvae. The mortality values are based on data obtained after injection into 20 larvae. All experiments were repeated at least three times.

previously reported (9), TT01 killed 90% of the larvae in less than 48 h. Substantiating the role of *phoPQ*, complementation with pDIA607 restored virulence, although with a slight delay. This delay has two possible explanations. Some of the transformed cells may have lost the plasmid during the in vivo infection and therefore may have behaved like *phoP* mutants. Without antibiotic selection pressure, in vitro 70% of the bacterial population lost pDIA607 after 48 h of culture. Alternatively, tight regulation of *phoPQ* expression may be necessary for full virulence.

According to the definition proposed by Bucher (11), insect-pathogenic bacteria are bacteria that produce a lethal septicemia from inocula, usually less than 10,000 cells per insect. Injection of high doses (about 10^5 CFU) revealed that the *phoP* mutant is avirulent (data not shown). Identification of the genes regulated by PhoPQ is therefore crucial for identifying major virulence determinants for entomopathogenicity.

PhoP-PhoQ governed LPS modification in Mg^{2+}-limited medium. The bacterial envelope is the first barrier against environmental aggression. Since LPS is the major surface molecule and pathogenic factor of gram-negative bacteria, a role for LPS in *P. luminescens* virulence has been proposed (20). Several gram-negative bacteria have the ability to modify, in a PhoP-dependent manner, their LPS in response to environmental conditions, especially in Mg^{2+}-depleted media. The PhoPQ regulon plays a key role in the regulation of LPS production in *S. enterica* serovar Typhimurium and in *Pseudomonas aeruginosa* (21, 35) and in the regulation of lipooligosaccharide production in *Yersinia pestis* (37).

Before investigating whether the LPS of *P. luminescens* was affected by a *phoP* deletion, we examined the *P. luminescens* LPS biosynthetic pathway in silico. To do this, BLASTP searches were performed with the translation products of the coding sequences present in the genome to identify putative LPS biosynthetic genes. This analysis revealed that *P. luminescens* possesses four large loci similar to the *lpx*/*dnaE* (lipid A), *waa* (LPS core), *wbl* (O antigen), and *wec* (enterobacterial common antigen) clusters found in other *Enterobacteriaceae* (Fig. 4) (54). Both the organization and the putative functions of genes found in the TT01 clusters homologous to *lpx*/*dnaE*

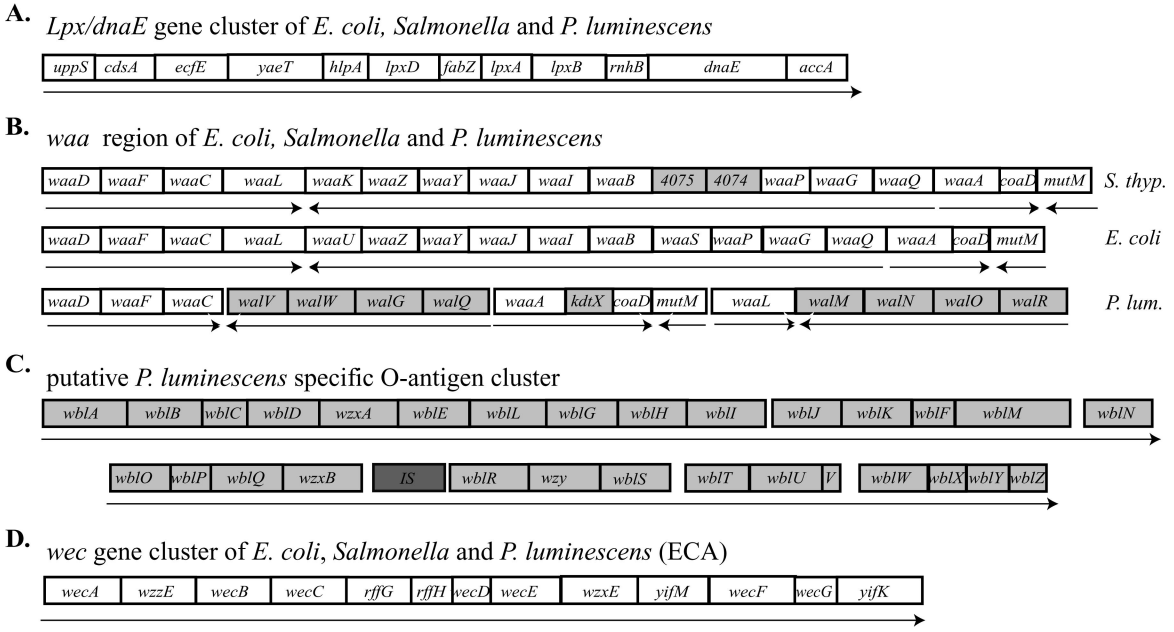

FIG. 4. Organization of genes in *lpx* (A), *waa* (B), *wbl* (C), and *wec* (D) gene clusters in *P. luminescens* TT01 and comparison with the genes of *E. coli* and *S. enterica* serovar Typhimurium. Genes without homologues in the *E. coli* genome are indicated by shading. The arrows indicate the direction of transcription of the genes (approximately drawn to scale). The *lpx*, *waa*, and *wec* regions are involved in initial steps of lipid A synthesis, core assembly, and enterobacterial common antigen (ECA) synthesis, respectively. *S. thyp*., *S. enterica* serovar Typhimurium; *P. lum*., *P. luminescens*.

and *wec* were identical to those of *E. coli* and *S. enterica* serovar Typhimurium. The organization and genes in the *waa* region were more divergent, indicating that the number and nature of the carbohydrate subunits that compose the core oligosaccharide of the LPS are different in *P. luminescens*. The putative strain-specific O-antigen *wbl* locus of *P. luminescens* has few genes homologous to those of the *E. coli wbb* operon. It is composed of 29 genes interrupted by a putative transposase. Of the 29 *wbl* genes, 9 encode proteins that are similar to sugar or UDP-sugar dehydrogenases (WblAB), epimerase (WblH), dehydratase (WblV), kinase (WblW), isomerase (WblX), phosphatase (WblZ), hydrolyase (WblK), and a homologue of the protein encoded by the *trsG* gene (WblM) involved in O-antigen biosynthesis in other organisms. Three other genes encode proteins that are similar to amino or hexapeptide transferases (WblCDQ). The gene cluster is also predicted to code for two sugar 1-phosphate nucleotidyltransferases (WblOY) and six glycosyltransferases used for transfer of sugars to build the O unit (WblGIJFTU). Several genes were also found to be similar to genes which carry out specific assembly or processing steps during conversion of the O unit to the O antigen as part of the complete LPS, such as two putative O antigen translocase genes (*wzxAB*), one potential O-antigen polymerase gene (*wzy*), and one gene coding for a protein weakly similar to polymer ligase (*wblL*), but not to the chain length determinant gene (*wzz*). Finally, the cluster contains four genes with unknown functions, including three genes encoding putative transmembrane proteins (*wblENR*). Therefore, *P. luminescens* likely produces an LPS consisting of three distinct structural regions: lipid A, the core oligosaccharide, and the O-antigen polymer (strain specific or enterobacterial common antigen).

The LPS was extracted from strains TT01, PL2104, and complemented PL2104 grown in low-Mg^{2+} or high-Mg^{2+} medium and was subjected to Tricine-SDS-PAGE analysis (Fig. 5). A comparison of the extracted LPS produced in the presence of a millimolar concentration of $MgCl_2$ (Fig. 5, lanes 1 to 3) did not reveal any difference among the strains, either in the O-antigenic region (upper portion of the gel) or in the core-lipid A region (lower part of the gel). In an Mg^{2+}-limited environment (lanes 4 to 6), the LPS structure in the core-lipid A region was modified, as shown by the greater intensity of the top band than of the two lower bands. This modification was not observed in the mutant LPS (lane 5), suggesting that it was PhoP dependent. Indeed, in the *phoPQ*-complemented mutant, this band was clearly overproduced (lane 6). As the PhoPQ regulon was induced during growth of *P. luminescens* in low-Mg^{2+} medium, the inability to express the PhoPQ regulon must have been responsible for the change observed in the lipid A-core structure.

Identification of a PhoP-dependent Mg^{2+}-responsive locus involved in lipid A modification. In order to identify the possible nature of PhoP- and Mg^{2+}-dependent LPS alteration in *P. luminescens*, a BLASTP comparison of the genomic DNA sequence of *P. luminescens* with the few known *Salmonella* PhoP-activated genes involved in LPS modification was performed. *lpxO* and *pagL* were found to be unique to *Salmonella*, but homologues of *pagP* and the *pbgPE* operon (also designated *pmrHFIJKLM*) were identified in *P. luminescens*. *pagP* encodes an outer membrane protein responsible for incorporation of palmitate into the lipid A moiety of the LPS (55). The seven-gene *pbgPE* operon mediates the synthesis of 4-amino-arabinose and incorporation of this molecule into the 4′-terminal phosphate of lipid A (33, 34) in association with *pmrE* (formerly *pagA* or *ugd*) (33, 47), a gene predicted to encode a

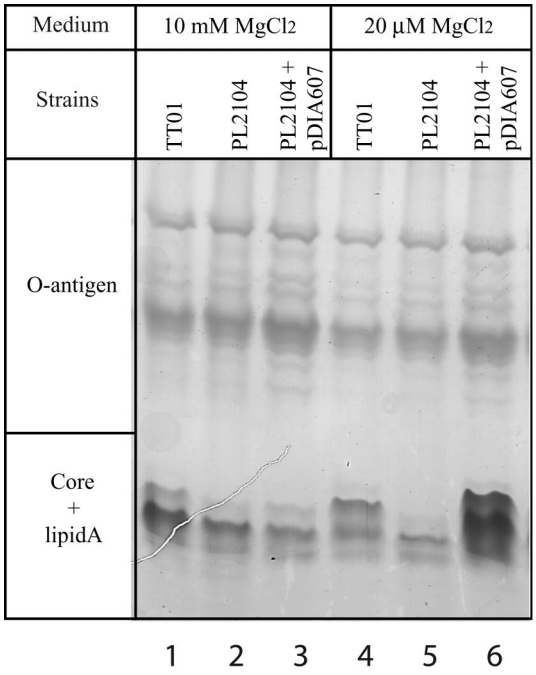

FIG. 5. LPS Tricine-SDS-PAGE profiles of the parental *P. luminescens* strain (TT01), the *phoP* mutant (PL2104), and the complemented *phoP* mutant [PL2104(pDIA607)]. The positions of O-antigen and lipid A-core regions are indicated. Lane 1, TT01 control in high-Mg^{2+} medium; lane 4, TT01 control in low-Mg^{2+} medium; lane 2, PL2104 in high-Mg^{2+} medium; lane 5, PL2104 in low-Mg^{2+} medium; lane 3, PL2104(pDIA607) ($PhoPQ^{+}$) in high-Mg^{2+} medium; lane 6, PL2104(pDIA607) ($PhoPQ^{+}$) in low-Mg^{2+} medium.

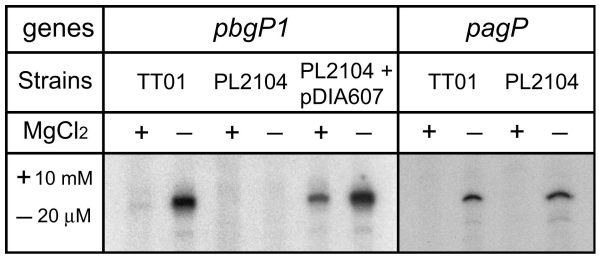

FIG. 6. Primer extension analysis of *pbgP1* and *pagP* mRNA abundance in strain TT01, strain PL2104, and strain PL2104 complemented with pDIA607 at various magnesium concentrations during *P. luminescens* exponential growth at 30°C in ED*-glucose synthetic medium supplemented with 20 μM $MgCl_2$ (−) or 10 mM $MgCl_2$ (+). Equal amounts of each RNA sample (50 μg) were used. Experiments were performed in triplicate.

UDP-glucose dehydrogenase that has several homologues in *P. luminescens*. The *P. luminescens pbgPE* locus is predicted to contain seven genes transcribed unidirectionally, with no more than 33 bp separating any two open reading frames. These genes encode seven proteins whose sequences and sizes are similar to those of the *Salmonella* or *E. coli* homologues. In both *S. enterica* serovar Typhimurium and *E. coli*, the *pbgPE* operon is preceded by the divergently transcribed homologous gene *pmrG* (previously designated *pagH* and *ais*, respectively) and is immediately followed by the divergently transcribed *pmrD* gene; both genes are thought to be PhoP regulated (58, 71). In *P. luminescens*, the *pbgPE* operon is flanked by two genes that are somewhat similar to the vitamin B_{12} transport system genes (*btuCD*) and by a gene similar to the gene encoding the putative lipoprotein NlpC precursor.

The transcription start site of *pbgP1* and *pagP* was determined by primer extension, and the abundance of the corresponding mRNA was determined in strains TT01, PL2104, and PL2104(pDIA607). *P. luminescens* total RNA was extracted from cells grown to the mid-log phase in ED*-glucose medium supplemented with a low concentration (20 μM) or a high concentration (10 mM) of $MgCl_2$. We found that expression of both *pagP* (Fig. 6 and data not shown) and *pbgP1* (Fig. 6) were dependent on the extracellular Mg^{2+} concentration. The expression of these genes was induced by a micromolar concentration of Mg^{2+} but was repressed by a millimolar concentration. *pbgP1* expression was suppressed in the *phoP* mutant strain PL2104 under both conditions. *phoPQ* copies supplied by pDIA607 restored normal Mg^{2+}-regulated expression of *pbgP1* in PL2104, and higher levels of expression were observed in the complemented strain. Curiously, *pagP* expression was not significantly affected by the *phoP* deletion. This indicated that PhoPQ positively controls *pbgPE* expression in an Mg^{2+}-dependent manner, while *pagP* expression is induced in Mg^{2+}-depleted medium in a PhoPQ-independent manner.

Sensitivity to APs. *phoP* mutants of several intracellular pathogens are highly susceptible to a variety of APs (30). The PhoPQ-controlled ability to modify the lipid A moiety of LPS is one of the main determinants that mediate resistance to these molecules. Indeed, addition of aminoarabinose or palmitate to lipid A in a low-Mg^{2+}-concentration environment results in a reduction in the overall LPS negative charge, leading to decreased binding of APs to the bacterial surface (6, 33, 22). This prompted us to examine the sensitivity of both TT01 and PL2104 to these compounds.

P. luminescens antipeptide resistance was determined by the broth microdilution method in Mueller-Hinton medium (which contained 750 μM $MgCl_2$ according to the manufacturer [Biokar]). Different APs were tested (Table 1). The *phoP* mutant was more sensitive to colistin, cecropins A and B, and polymyxin B than the wild type and the *phoP*-complemented mutant were. Cecropins are considered to be the most active antimicrobial components. These small cationic peptides are naturally produced in many insects, including *S. littoralis* larvae (12).

TABLE 1. MICs of four APs for the *P. luminescens* wild type, *phoP* mutant, and complemented mutant grown in Mueller-Hinton broth

Strain	MIC (μg/ml) of:			
	Colistin	Cecropin A	Cecropin B	Polymyxin B
Wild type (TT01)	>10,000	>25[a]	>25[a]	>250
phoP mutant (PL2104)	20	1.56	6.25	1–3
Complemented *phoP* mutant [PL2104 (pDIA607)]	>10,000	12.5	>25[a]	>250

[a] Higher concentrations were not tested due to the susceptibility of *P. luminescens* to the acetic acid used as a solvent.

DISCUSSION

A homologue of the two-component signal transduction system PhoPQ has been identified in *P. luminescens*. A knockout mutant (PL2104) has many of the characteristics of other *phoP* mutants of mammalian pathogens.

(i) PL2104 is more motile than its parent, strain TT01. A similar PhoPQ-dependent effect on motility has been observed in *Salmonella* and *P. aeruginosa*. In *Salmonella*, decreased flagellin expression and cell motility are coregulated by low pH and are dependent on activation of the *phoPQ* pathway, which directly or indirectly negatively regulates transcription of the flagellin gene *fliC* (1). Similarly, in *P. aeruginosa*, a *phoQ* mutant had a decreased ability to swim on soft agar, while a *phoP* null strain was considered a superswimmer (10). Preliminary data indicate that *fliC* is also up-regulated in PL2104 (unpublished data).

(ii) We demonstrated that the LPS, which comprises 40% of the outer membrane layer, is altered in a PhoP-dependent manner when there is a change in the magnesium concentration. Identification in *P. luminescens* of the *pbgPE* locus, an operon involved in synthesis of aminoarabinose and incorporation of this molecule into the lipid A moiety, and characterization of this locus as an Mg^{2+}- and PhoP-dependent operon, substantiated the likely role played by PhoPQ in this process.

(iii) PL2104 showed enhanced sensitivity to several APs (i.e., cecropins A and B, colistin, and polymyxin). This may have been partly correlated with its inability to perform PhoPQ-controlled LPS modification in low-Mg^{2+}-concentration conditions. One mechanism of AP resistance consists of reduced electrostatic interactions between an AP and the negatively charged bacterial surface, which may occur in *P. luminescens* through modifications of lipid A phosphate with aminoarabinose or changes in the overall charge of the peculiar O antigen that it harbors. Another mechanism may involve the presence of long O-specific side chains that sterically hinder the ability of AP to bind to the deeper parts of LPS and prevent disruption of the bacterial outer membrane.

(iv) Finally, deletion of *phoP* resulted in a complete loss of virulence when insects were infected with *P. luminescens*, while complementation restored virulence. PhoPQ-dependent defects, such as envelope alterations, greater susceptibility to insect antibacterial peptides, increased motility promoting the early recognition of flagellar components by an immune response, or the inability to impair immune response activation through LPS signaling, may contribute to the observed avirulence of the mutant.

Surprisingly, although the primary function of PhoPQ in other gram-negative bacteria is to control physiological adaptations in response to an Mg^{2+}-limiting environment (30, 65), the *P. luminescens* mutant is able to grow in medium containing a low level (micromolar) of magnesium without apparent difficulty. This observation might reflect a difference in control of Mg^{2+} uptake in *P. luminescens*. In *S. enterica*, three transporters mediate Mg^{2+} uptake; these are the P-type ATPases MgtA and MgtB, whose expression is transcriptionally induced in the presence of a low Mg^{2+} concentration by PhoPQ, and the constitutive major Mg^{2+} transporter CorA (62). *Salmonella* mutants defective in *mgtA*, *mgtB*, *phoQ*, or *phoP* are defective for growth in the presence of a low Mg^{2+} concentration (65), even though CorA is expressed and functional (63). BLASTP searches revealed little homology between *Salmonella* MgtA and MgtB and various putative P-type ATPases in *P. luminescens*, including a probable copper-transporting ATPase (AtcU), a zinc-transporting ATPase (ZntA), and a potassium-transporting ATPase (KdpB). In contrast, CorA, the most phylogenetically widespread Mg^{2+} transporter, is conserved. Further work is needed to determine the process of Mg^{2+} import in *P. luminescens*, but our analysis suggests that PhoPQ might not play a crucial role in this transport. Consistent with this hypothesis, the PhoPQ-independent *pagP* induction in the presence of a low Mg^{2+} concentration indicates that there must be additional systems that regulate gene expression in response to an Mg^{2+}-limiting environment.

As in *Salmonella*, *phoP* gene expression responds to the extracellular Mg^{2+} concentration, indicating that this cation may be one of the physiological signals that affect the *phoPQ*-dependent response in *P. luminescens*. *phoP* expression also responds to a low Ca^{2+} concentration but not to the same extent (Fig. 2C). The promoter region of *phoP* differs from that of *E. coli* and *Salmonella*. In both species, the autogenously controlled *phoPQ* operon is transcribed from two promoters, a PhoPQ-dependent promoter that is active only during growth in the presence of a low Mg^{2+} concentration and another promoter that is constitutive (26, 39, 64). The relative positions of the constitutive and regulated promoters differ in the two organisms, but an authentic PhoP binding site consisting of a direct repeat of the heptanucleotide sequence (T)G(T)TT (AA) is conserved 25 bp upstream of the Mg^{2+}-inducible transcription start site of *phoPQ* (39, 72). In *P. luminescens*, one Mg^{2+}-inducible transcript was found under the culture conditions used. This transcript has a long 5′ untranslated region, and no obvious PhoP box is apparent upstream of its transcription start point. The very faint level of *phoP* mRNA detected suggested that *P. luminescens* cells may be sensitive to very small changes in the amount of PhoP. It is also possible that factors present in the insect larvae control expression of the operon. Several overlapping promoter-like sequences can be identified upstream of the transcriptional start that could play a role in particular environments. Interestingly, the regulation of the Mg^{2+}-dependent expression of *pagP* and *pbgPE*, coding for enzymes involved in LPS modifications, also slightly differs from the regulation of the homologs in *Salmonella*. In *Salmonella*, both loci are activated by PhoP. The control is direct for *pagP* but indirect for *pbgPE* and occurs via PmrAB, a two-component system that itself is induced by PhoPQ (30). In *P. luminescens*, PmrAB is not conserved, and although expression of both loci is Mg^{2+} dependent, expression of only *pbgPE* seems to be PhoPQ dependent. We do not know at present the regulatory mechanisms operating in this organism for *pagP* and *pbgPE*. Nevertheless, our results strongly support the hypothesis that the PhoPQ signal transduction system is able to respond to the Mg^{2+} content of the host environment and transduce the signal to either induce or repress expression of genes needed to establish an infection in insects. However, whether the Mg^{2+} concentration is directly sensed by PhoPQ or by a PhoPQ-independent mechanism which in turn regulates *phoPQ* is still an open question.

The essential role played in vivo by PhoPQ in *P. luminescens* pathogenicity is somewhat unexpected for the following rea-

sons. This system is known to control virulence, especially in intracellular pathogens, while experiments suggest that *P. luminescens* is extracellular in insects (60). In *Salmonella* and other mammalian pathogens, the external magnesium level sensed by PhoQ is thought to be the signal that indicates to the organism whether it is residing in an intracellular or extracellular compartment in the host (29). Mammalian extracellular fluids contain high Mg^{2+} and Ca^{2+} levels (millimolar). Pathogens encounter low intracellular levels (micromolar) of both cations inside macrophages or epithelial cells. Phytophagous insects, such as *Spodoptera*, have high Mg^{2+} and Ca^{2+} levels in the hemocoel (i.e., 33 mM Mg^{2+} and 3 mM Ca^{2+} in *Spodoptera exigua*) (48). Although these concentrations should theoretically repress *P. luminescens phoPQ* expression, inactivation of *phoP* prevents bacterial proliferation in the hemolymph in vivo and eliminates virulence. This prompted us to make several predictions to account for these apparent discrepancies, noting that compartmentalization is ubiquitous in biological processes. (i) Efficient *Photorhabdus* infection might start with a microenvironment in which the Mg^{2+} concentration is particularly low. Within minutes of its appearance in the hemolymph, the bacterium is recognized by the insect hemocytes and encapsulated in nodules, from which it rapidly reemerges (17). The actual mineral ion concentrations surrounding the bacterium inside the nodules are difficult to ascertain but might be quite different from those in the hemolymph. (ii) It is also possible that *Photorhabdus* has an intracellular phase at some point during the infection. This occurs with *Y. pestis*, a pathogen that is normally present extracellularly. In this organism, PhoP is necessary early in infection during an intracellular phase within phagocytic cells (50). (iii) It is possible that PhoPQ is sensitive to other signals present in vivo in the hemocoel. It has been suggested previously that in *Erwinia chrysanthemi* and *Providencia stuartii* (42, 56) the PhoPQ system may sense chemical signals other than divalent cations. In *E. coli*, the system has been shown to respond to a mildly acidic pH and acetate in addition to Mg^{2+} (4, 41).

In conclusion, the work described in this report showed the central role played by the *phoPQ* regulon in *Photorhabdus* virulence in insects. Further studies are required to understand how and why the PhoP mutant of *P. luminescens* is completely impaired in terms of virulence. Identification of the essential role played in virulence by the PhoPQ signal transduction system is an important step towards understanding how entomopathogenic bacteria such as *P. luminescens* perform the switch from symbiosis to pathogenicity. This finding highlights the fact that the PhoPQ regulatory system promotes pathogen resistance to host innate immunity in vertebrates (22) and plants (24, 42) and also in insects.

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