Regulation of the Pcol/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by the PhoP/PhoQ two-component system

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A quorum-sensing locus, *pcol/pcor*, which is involved in the regulation of root colonization and plant disease-suppressive ability, was previously identified in *Pseudomonas fluorescens* 2P24. In this study, we performed random mutagenesis using mini-Tn5 in order to screen the upstream transcriptional regulators of *pcol*, a biosynthesis gene responsible for the synthesis of *N*-acylhomoserine lactone signal molecules. Two mutants, PM400 and PM410, with elevated *pcol* gene promoter activity, were identified from ~10 000 insertion clones. The amino acid sequences of the interrupted genes in these two mutants were highly similar to PhoQ, a sensor protein of the two-component regulatory system PhoP/PhoQ, which responds to environmental Mg^{2+} starvation and regulates virulence in *Salmonella typhimurium* and antimicrobial peptide resistance in *Pseudomonas aeruginosa*. The promoter activity of *pcol* was also induced under low- Mg^{2+} conditions in the 2P24 strain of *P. fluorescens*. Deletion mutagenesis and complementation experiments demonstrated that the transcription of *pcol* was negatively regulated by the sensor PhoQ but positively regulated by the response regulator PhoP. Genetic evidence also indicated that transcription of the outer-membrane protein gene *oprH* was induced by Mg^{2+} starvation through regulation of the wild-type PhoP/PhoQ system. Additionally, PhoQ was involved in biofilm formation by 2P24 under low- Mg^{2+} conditions through a PhoP-independent pathway.

INTRODUCTION

Quorum sensing (QS) is a widespread mechanism of cell-to-cell communication used by many bacteria to monitor their population densities and to adapt their behaviours by regulating gene expression in response to fluctuations in cell population density (for reviews see Miller & Bassler, 2001; Keller & Surette, 2006). QS plays an important role in a diverse array of physiological activities, including symbiosis, virulence, competence, conjugation, antibiotic production, swarming, sporulation and biofilm formation (González & Keshavan, 2006; de Kievit & Iglewski, 2000; Sakuragi & Kolter, 2007; Waters *et al.*, 2008). The regulation of QS has been intensively studied in recent years (Venturi, 2006). In pseudomonads, identified regulatory elements of QS include the stationary-phase sigma factor RpoS (Bertani & Venturi, 2004), the two-component regulatory system GacS/GacA (Reimann *et al.*, 1997), the small RNA-binding regulator RsmA (Pessi *et al.*, 2001), the LuxR family member VqsR

(Juhas *et al.*, 2004) and the tetrahelical H-T-H superclass member RsaL (Rampioni *et al.*, 2007). The discovery of new regulators of QS will help to further elucidate the signal transduction mechanism in bacteria that survive under various environmental conditions.

The two-component PhoP/PhoQ system was first recognized in *Salmonella typhimurium* (Kier *et al.*, 1979) as a regulatory system that responds to extracellular Mg^{2+} starvation (Soncini *et al.*, 1996; Vécovi *et al.*, 1996). PhoQ is a bifunctional sensor protein that detects environmental changes in Mg^{2+} concentration and, by modulating its phosphatase/kinase activity, defines the phosphorylation state of the regulator PhoP (Castelli *et al.*, 2000). Multiple studies have indicated that the PhoP/PhoQ system is a master regulator of virulence in *S. typhimurium* (for a review see Groisman, 2001). Recent studies on PhoP/PhoQ in *Pseudomonas aeruginosa* PAO1 have revealed that this system plays an important role in the expression of the outer-membrane protein OprH (Macfarlane *et al.*, 1999), the modification of lipid A (Ernst *et al.*, 1999) and the resistance to cationic antimicrobial peptides and aminoglycosides (Macfarlane *et al.*, 2000). It has also been demonstrated to regulate several virulence determinants, including swarming, lipase production and virulence in a neutropenic mouse model (Brinkman *et al.*, 2001).

Abbreviations: AHL, *N*-acylhomoserine lactone; PVC, polyvinyl chloride; QS, quorum sensing.

The GenBank/EMBL/DDBJ accession number for the DNA sequence containing the *oprH*, *phoP* and *phoQ* genes of *P. fluorescens* 2P24 is EU569830.

Supplementary material is available with the online version of this paper.

In the present study, a random mini-Tn5 mutagenesis procedure was used to identify the upstream regulator(s) of QS in *Pseudomonas fluorescens* 2P24, a plant-disease-suppressive bacterium isolated from the wheat rhizosphere (Wei & Zhang, 2006). The Tn5-interrupted gene exhibited high amino acid similarities to the *phoQ* gene of several bacteria and the results indicated that the PhoP/PhoQ system may be an important upstream regulator of QS in *P. fluorescens* 2P24.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. *P. fluorescens* 2P24 and its derivatives were grown in Luria–Bertani (LB) or ABM minimal medium (Chilton *et al.*, 1974) at 28 °C. To study the effect of Mg²⁺ concentration, LB medium containing 30 mM NaCl (low-Mg²⁺ condition) or 15 mM MgCl₂ (high-Mg²⁺ condition) (see Supplementary Fig. S1, available with the online version of this paper), and ABM minimal medium containing 0.1 mM MgSO₄ (low-Mg²⁺ condition) or 15 mM MgSO₄ (high-Mg²⁺ condition) were used. When required, the growth media were supplemented with ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), tetracycline (20 µg ml⁻¹), or X-Gal (40 µg ml⁻¹).

DNA manipulations. Plasmid DNA extractions and other molecular assays were performed according to standard procedures (Sambrook *et al.*, 1989). Nucleotide sequencing was performed by Sun Biotechnology. Nucleotide and deduced amino acid sequences were analysed using the National Center for Biotechnology Information BLAST server (<http://www.ncbi.nlm.nih.gov>).

Tn5 mutagenesis and cloning of *oprH*, *phoP* and *phoQ*. In order to identify the potential upstream regulators of *pcrI* gene transcription, we randomly mutated a 2P24 reporter strain, PM101 (*pcrI::lacZ*), using a mini-Tn5 mutagenesis procedure according to a previously described method (Herrero *et al.*, 1990). The mutated cells were incubated on ABM plates containing ampicillin, kanamycin and the β-galactosidase substrate X-Gal at 28 °C for 30 h. Colonies with increased β-galactosidase activity (indicated by a more intense blue colour) were selected and purified. The mini-Tn5-disrupted genes in these mutants were identified by cloning and sequencing the genomic DNA fragments flanking the transposon. Using primers phoQT3/phoQT7 (see Supplementary Table S1, available with the online version of this paper, for details of primers), which were designed according to the flanking sequence, three cosmids (p18-25, p22-31 and p28-18) were screened out by PCR from the genomic DNA library of 2P24 (Wei & Zhang, 2005). A 5.7 kb *HindIII* fragment from p18-25 was subcloned into pBluescript II SK(+), giving rise to the plasmid pBS-6Q. Sequencing of this fragment identified the entire gene sequences of *oprH*, *phoP* and *phoQ* (Fig. 1a).

Table 1. Bacterial strains and plasmids used in this study.

Ap^r, Gm^r, Km^r, Sm^r and Tc^r indicate resistance to ampicillin, gentamicin, kanamycin, streptomycin and tetracycline, respectively

Strain or plasmid	Description	Reference or source
<i>P. fluorescens</i>		
2P24	Wild-type, Ap ^r	Wei & Zhang (2005)
PM101	<i>pcrI::lacZ</i> reporter fusion of 2P24, Ap ^r	Yan <i>et al.</i> (2008)
PM400, PM410	<i>phoQ::Tn5</i> in PM101, Ap ^r Km ^r	This study
PM401	<i>phoQ</i> in-frame deletion in PM101, Ap ^r	This study
PM402	<i>phoQ</i> in-frame deletion in 2P24, Ap ^r	This study
PM403	<i>phoP</i> in-frame deletion in PM101, Ap ^r	This study
PM404	<i>phoP</i> in-frame deletion in 2P24, Ap ^r	This study
<i>A. tumefaciens</i>		
NTL4(pZLR4)	<i>traG::lacZ</i> , AHL indicator, Gm ^r	Cha <i>et al.</i> (1998)
Plasmids		
pBluescript II SK(+)	Cloning vector, Ap ^r	Stratagene
pHSG299	ColE1 origin, cloning vector, Km ^r	TaKaRa
pRK415	<i>Escherichia</i> – <i>Pseudomonas</i> shuttle vector, Tc ^r	Keen <i>et al.</i> (1988)
p18-25	Cosmid clone containing entire <i>phoQ</i> gene, Tc ^r	This study
pBS-6Q	5.7 kb <i>HindIII</i> fragment from p18-25 containing entire <i>oprH</i> , <i>phoP</i> and <i>phoQ</i> cloned into pBluescript II SK(+)	This study
pUTKm	Delivery plasmid for Tn5; R6K replicon, Ap ^r Km ^r	Herrero <i>et al.</i> (1990)
pRG970	Cloning vector containing promoterless <i>lacZ</i> Y for construction of transcriptional fusions, Sm ^r	Van den Eede <i>et al.</i> (1992)
pRG970Km	A derivative plasmid of pRG970b, Km ^r	This study
p970- <i>pcrI</i>	pRG970Km containing <i>pcrI::lacZ</i> transcriptional fusion, Km ^r	This study
p970-H	pRG970Km containing <i>oprH::lacZ</i> transcriptional fusion, Km ^r	This study
p299Δ <i>phoP</i>	Suicide plasmid containing deleted <i>phoP</i> gene on pHSG299, Km ^r	This study
p299Δ <i>phoQ</i>	Suicide plasmid containing deleted <i>phoQ</i> gene on pHSG299, Km ^r	This study
p415- <i>phoP</i>	<i>phoP</i> , as a 2.2 kb <i>NcoI</i> – <i>PstI</i> fragment from pBS-6Q, was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study
p415- <i>phoQ</i>	<i>phoQ</i> , as a 1.9 kb <i>SalI</i> – <i>EcoRV</i> fragment from pBS-6Q, was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study

Fig. 1. (a) Schematic diagram of *P. fluorescens* 2P24 *oprH*, *phoP* and *phoQ* genes. The single-headed arrows represent the location and orientation of the genes in the *P. fluorescens* 2P24 chromosome. The main restriction enzyme sites are shown. Tn5 in PM400 and PM410 was inserted in the *phoQ* gene at position 1025 bp and 1163 bp, respectively. The construction of strains PM400–PM404 and of plasmids p415-*phoP* and p415-*phoQ* is described in Methods. 574F and 1016R (Table S1) indicate the primers used in the PCR to construct the reporter plasmid p970-H. (b) Upstream nucleotide sequence and part of the translated *oprH* gene in *P. fluorescens* 2P24. The start of *oprH* is labelled. The hexanucleotide direct repeats (PhoP box) upstream of *oprH* are indicated by underlines.

The promoter region, including the proposed PhoP box and the upstream fragment of *oprH* (Fig. 1), was amplified by PCR using primers H-574F and H-1016R (Table S1), and a 442 bp *Bam*HI fragment was cloned ahead of a promoterless *lacZ* in p970Km, a derivative plasmid of pRG970 (Van den Eede *et al.*, 1992). The resulting plasmid, p970-H, was used to examine the activity of the *oprH* promoter.

Construction of *phoP* and *phoQ* in-frame deletion mutants.

phoP and *phoQ* deletion mutants were constructed using a two-step homologous recombination strategy. The detailed protocol and PCR primers (Table S1) are given in the online supplementary material. The in-frame deletion structures of *phoP* and *phoQ* were ligated into plasmid pHSG299 to generate p299Δ*phoP* and p299Δ*phoQ*, respectively. Allelic exchange using p299Δ*phoP* or p299Δ*phoQ* with the wild-type 2P24 resulted in mutants PM404 and PM402, respectively; using p299Δ*phoP* or p299Δ*phoQ* with the reporter strain PM101 (*pcol::lacZ*) resulted in mutants PM403 and PM401, respectively (Fig. 1a).

In order to construct a complementary plasmid, the *phoP* gene was inserted as a 2.2 kb *Nco*I–*Pst*I fragment into the shuttle vector pRK415 to create p415-*phoP*. Similarly, the *phoQ* gene was inserted as

a 1.9 kb *Sal*I–*Eco*RV fragment into pRK415 to create p415-*phoQ* (Fig. 1a).

Isolation and detection of *N*-acylhomoserine lactone (AHL).

P. fluorescens 2P24 and its derivatives were grown in LB liquid medium at 28 °C for 36 h. Aliquots (0.8 ml) of the cultures were extracted with the same volume of ethyl acetate; the extracts were then dried and resuspended in 0.1 ml methanol. For quantitative analysis of AHL, 3 μl aliquots of the samples were incubated with 0.2 ml of the AHL biosensor *Agrobacterium tumefaciens* NTL4(pZLR4) (Cha *et al.*, 1998) (OD₆₀₀ 0.8). The reaction mixture was incubated at 28 °C for 3 h and the β-galactosidase activity of the biosensor cells was assayed (see below).

β-Galactosidase assay and statistical analysis.

For β-galactosidase measurement, *Pseudomonas* and *Agrobacterium* strains that contained β-galactosidase reporter genes were grown in LB and ABM liquid medium, respectively, at 28 °C with shaking at 130 r.p.m. in 50 ml flasks. Cultures were sampled at different time points and assayed for β-galactosidase specific activity according to the method of Miller (1972). The data collected from various time points were analysed and compared by performing a two-sample independent *t* test (*P* < 0.01) using Origin 7.0 software (Originlab Corporation).

Biofilm formation assay. In order to determine biofilm formation on polyvinyl chloride (PVC) plastic, an assay similar to that described previously was used with a few modifications (Wei & Zhang, 2006). Briefly, test strains were grown to saturation in LB medium and then diluted (1 : 100) into fresh LB broth. A 0.5 ml volume of diluted culture was transferred to an Eppendorf tube. Bacteria were incubated without agitation for 20 h at 28 °C and the resulting biofilm was quantified. The biofilm was stained with 0.1 % (w/v) crystal violet for 20 min at room temperature and then unattached cells and residual dye were removed. Ethanol (95 %) was used to dissolve the dye that had stained the biofilm cells and the A_{570} of the dissolved dye was determined.

RESULTS

Identification of *oprH-phoP-phoQ* homologues in *P. fluorescens* 2P24

Random mutagenesis using the transposon mini-Tn5 was performed in the reporter strain *P. fluorescens* PM101 (*pcoI::lacZ*) to identify the potential upstream regulators of *pcoI* transcription. A total of 10 000 inserted clones were screened on ABM plates containing kanamycin and X-Gal. Two mutants, PM400 and PM410, which formed deeper blue colonies compared with their parental strain PM101, were identified and purified for further studies. The sequences of the Tn5 flanking DNA in both PM400 and PM410 were found to be highly similar to that of *phoQ*, the sensor gene of the two-component regulatory system PhoP/PhoQ found in several bacteria. This finding raised the possibility that the PhoP/PhoQ system is involved in the regulation of QS in *P. fluorescens* 2P24.

A 5.7 kb *Hind*III fragment containing the entire *phoQ* gene was cloned from the genomic DNA of the wild-type 2P24 (see Methods). Sequencing and BLAST analysis of this fragment revealed three ORFs (Fig. 1a), which exhibited high similarity to the *oprH-phoP-phoQ* locus from *P. aeruginosa* PAO1 (Macfarlane *et al.*, 1999), and the *phoP-phoQ* locus from *S. typhimurium* (Miller *et al.*, 1989) and *Escherichia coli* K-12 (Kasahara *et al.*, 1992). Identities with the *P. aeruginosa* proteins were 59 % for OprH, 85 % for PhoP and 68 % for PhoQ. Identities with the *S. typhimurium* proteins were 53 % for PhoP and 31 % for PhoQ. Identities with the *E. coli* proteins were 53 % for PhoP and 30 % for PhoQ (Fig. S2). The ORFs of PhoP and PhoQ overlapped by 4 nucleotides, indicating that they could form a single transcriptional unit.

Four hexanucleotide (A/G)TTCA(G/A) direct repeats, each separated by 5 bp, were situated 61 bp upstream of *oprH* (Fig. 1b). Similar direct repeats proposed to be PhoP-binding motifs have been found at a similar distance upstream of *oprH-phoP-phoQ* in *P. aeruginosa* PAO1 (Macfarlane *et al.*, 1999).

Regulation of *oprH* and *pcoI* transcriptional expression by Mg^{2+}

In *P. fluorescens* 2P24, the deeper blue colonies of the *phoQ*-defective mutants PM400 and PM410 grown on

ABM plates containing X-Gal indicated a negative regulation of *pcoI* expression by PhoQ and suggested the potential involvement of Mg^{2+} in the regulation of the QS system. To investigate this possibility, the effect of Mg^{2+} on the expression of *pcoI* in LB medium was measured using a *pcoI::lacZ* transcriptional fusion in the plasmid p970-*pcoI*. The results revealed that *pcoI* transcription under low- Mg^{2+} conditions was significantly enhanced compared with that under high- Mg^{2+} conditions (Fig. 2a). Furthermore, elevated transcription of *pcoI* was observed when the Mg^{2+} concentration was changed from the high level to the low level during growth (Fig. S3). To avoid the potential influence of the anion, $MgSO_4$ instead of $MgCl_2$ was used to assay the *pcoI::lacZ* transcriptional level in LB and ABM medium (Fig. 2b, c). The results were consistent with those in LB medium containing $MgCl_2$, suggesting that it was the Mg^{2+} , and not the anion, that regulated the *pcoI* expression. Taken together, these results indicated that the expression of *pcoI* is induced by the depletion of Mg^{2+} in *P. fluorescens* 2P24. Similarly, the transcription of the outer-membrane protein gene *oprH*, a verified PhoP/PhoQ-regulated gene in *P. aeruginosa* PAO1 (Macfarlane *et al.*, 1999), was highly elevated under low- Mg^{2+} conditions, but was fully repressed under high- Mg^{2+} conditions (Fig. 2d). However, the Mg^{2+} signal exhibited a significantly stronger effect on *oprH* transcription, with an activation/repression ratio of 45-fold; this was considerably higher than that on *pcoI* transcription, for which the ratio was only 2-fold. These results revealed that in strain 2P24 the transcriptions of *pcoI* and *oprH* were induced by depletion of Mg^{2+} to a certain level.

Differential regulation of *oprH* transcription by PhoP and PhoQ

In order to avoid polar effects on downstream genes, in-frame deletion mutants, PM402 (*phoQ*) and PM404 (*phoP*) (Fig. 1a), were constructed in wild-type *P. fluorescens* 2P24 to study the role of PhoP/PhoQ on target genes. Under both low- and high- Mg^{2+} conditions, the transcription of *oprH* was fully repressed in the *phoP* deletion strain PM404 (Fig. 3), indicating that PhoP is a positive regulator of *oprH* transcription. In the *phoQ*-defective strain PM402, *oprH* maintained a similar transcription level under both growth conditions (Fig. 3), suggesting that Mg^{2+} loses its regulatory effect on *oprH* expression in the absence of PhoQ. It was interesting that, compared with the wild-type 2P24, expression of *oprH* in PM402 was upregulated significantly in high- Mg^{2+} medium but downregulated significantly in low- Mg^{2+} medium (Fig. 3). These results suggested that in strain 2P24 the transcriptional regulation of *oprH* by PhoQ is highly dependent on the environmental concentration of Mg^{2+} . In addition, the transcription of *oprH* was generally maintained at a significantly higher level in PM402 (*phoQ*) than in PM404 (*phoP*), suggesting a difference in the regulation of *oprH* expression by the response regulator PhoP and the sensor PhoQ.

Fig. 2. Mg^{2+} regulates *pcol* and *oprH* expression in *P. fluorescens* 2P24. β -Galactosidase activity of the plasmid transcriptional fusion *lacZ* in the wild-type 2P24 was measured at various time points after inoculation into LB medium (a, c, d) and ABM medium (b) containing high or low Mg^{2+} . All experiments were performed in triplicate; means $\pm$ SD are plotted. Statistical analyses were performed with the two-sample independent *t* test. **, $P < 0.01$; ***, $P < 0.001$.

Fig. 3. Mg^{2+} regulates *oprH* expression in a PhoP/PhoQ-dependent manner. β -Galactosidase activity of the plasmid *p970-H* (*oprH::lacZ*) in *P. fluorescens* strains was measured at various time points after inoculation into LB medium. All experiments were performed in triplicate; means $\pm$ SD are plotted. (a) High- Mg^{2+} condition: LB medium containing 15 mM $MgCl_2$. (b) Low- Mg^{2+} condition: LB medium containing 30 mM NaCl.

Regulation of QS by the two-component PhoP/PhoQ system

In order to determine the potential regulatory effect of the PhoP/PhoQ system on *pcoI* expression, the β -galactosidase activity of a genomic *pcoI::lacZ* transcriptional fusion was measured in the mutants PM401 (*phoQ*, *pcoI::lacZ*) and PM403 (*phoP*, *pcoI::lacZ*), and also in their parental strain PM101 (*pcoI::lacZ*). The expression of *pcoI::lacZ* was elevated significantly in the *phoQ* deletion strain PM401 under both low- and high- Mg^{2+} conditions. Introduction of a *phoQ*⁺ plasmid, p415-*phoQ*, into strain PM401 restored the *pcoI::lacZ* activity to the wild-type level (Fig. 4), suggesting that PhoQ acts as a negative regulator on *pcoI* expression. In contrast, when the bacteria were grown in high- Mg^{2+} medium, there was no significant difference in the *pcoI::lacZ* activity between the *phoP* deletion strain PM403 and its wild-type PM101. The *pcoI::lacZ* activity, however, was significantly suppressed in PM403 under low- Mg^{2+} conditions (Fig. 4b). Introduction of a *phoP*⁺ plasmid, p415-*phoP*, into strain PM403 resulted in a slight overexpression of *pcoI::lacZ* in high- Mg^{2+} medium, which was considerably greater than that in low- Mg^{2+} medium (Fig. 4). These observations indicated that PhoP positively regulates *pcoI* expression and that this regulation is more pronounced under low- Mg^{2+} conditions.

The accumulation of QS signals (AHL) in *P. fluorescens* 2P24 and its derivatives was measured and compared quantitatively using the biosensor strain *A. tumefaciens* NTL4(pZLR4). The result (Fig. 5) was consistent with the *pcoI::lacZ* transcriptional assay described above and confirmed the involvement of the PhoP/PhoQ system in the regulation of the QS system in strain 2P24.

Effect of PhoP/PhoQ on biofilm formation

Since the PhoP/PhoQ system is involved in the regulation of *pcoI* transcription in strain 2P24, we reasoned that the characteristics controlled by the QS system might be also influenced by PhoP/PhoQ. Biofilm formation on PVC plastic is known to be positively controlled by QS in strain 2P24 (Wei & Zhang, 2006). Therefore, an experiment was performed to examine whether biofilm formation was affected by PhoP/PhoQ. When cultured in high- Mg^{2+} medium (Fig. 6), neither the *phoP* nor the *phoQ* mutant caused a significant change in the biofilm formation in Eppendorf tubes. In contrast, when cultured in low- Mg^{2+} medium, mutant PM402 (*phoQ*) exhibited a significantly enhanced biofilm formation, and this mutation could be restored by the plasmid p415-*phoQ* (*phoQ*⁺). Mutation of *phoP*, however, had no significant influence on biofilm formation. These observations indicated the negative role of PhoQ in biofilm formation under the low- Mg^{2+} condition; however, this regulation did not appear to be mediated via the regulator PhoP.

Effect of PhoP/PhoQ on bacterial colony formation

When grown on LB plates, the *phoQ* mutant PM402 formed significantly smaller colonies compared with the wild-type under both low- and high- Mg^{2+} conditions. In contrast, the *phoP* mutant PM404 formed larger colonies on both media (Fig. 7). Introduction of the complementary plasmids p415-*phoQ* and p415-*phoP*, respectively, restored the colony size to the wild-type level. Soncini *et al.* (1996) reported that the mutation of *phoP* resulted in a larger cell size in *S. typhimurium*. However, no such morphological change was observed in the *phoP* or *phoQ* mutant of 2P24 (data not shown). The number of living cells from

Fig. 4. Regulation of the transcriptional expression of *pcoI* by PhoP/PhoQ. β -Galactosidase activity of the genomic fusion *pcoI::lacZ* in PM101 and its derivatives was measured at various time points after inoculation into LB medium. All experiments were performed in triplicate; means $\pm$ SD are plotted. (a) High- Mg^{2+} condition: LB medium containing 15 mM MgCl_2 . (b) Low- Mg^{2+} condition: LB medium containing 30 mM NaCl .

Fig. 5. Regulation of AHL production by PhoP/PhoQ. β -Galactosidase activity of the *traG::lacZ* fusion in the biosensor strain *A. tumefaciens* NTL4(pZLR4) was measured after incubation with AHL that had been extracted from the wild-type 2P24 and its derivatives. The cell density (OD_{600}) of each strain was measured. All experiments were performed in triplicate; means $\pm$ SD are plotted.

Fig. 6. Regulation of biofilm formation by PhoP/PhoQ. A biofilm was allowed to form in Eppendorf tubes. The tubes were incubated for 20 h at 28 °C. The unattached bacteria were rinsed off and the biofilm was stained with crystal violet. The residual dye was dissolved and the A_{570} of the solution was determined. All experiments were performed in triplicate; means $\pm$ SD are plotted.

individual colonies was checked by plating and it was found that the number of such cells in colonies of the *phoQ* mutant (2.5×10^6 cells per colony) was considerably less than that in the wild-type 2P24 (1.9×10^8 cells per colony), indicating that the different colony sizes might be a result of differences in cell population. This result is consistent with the slower growth rate of the *phoQ* mutant in LB broth under both low- and high-Mg²⁺ conditions (Fig. S4). Additionally, the changes in colony size of the PhoP/PhoQ mutants were unlikely to have been caused by Mg²⁺ starvation in the growth media, since similar regulation of colony size by PhoP/PhoQ was observed when the mutant cells were cultured in high-Mg²⁺ medium, although the bacterial colonies of 2P24 strains were generally smaller in low-Mg²⁺ medium.

DISCUSSION

The PhoP/PhoQ locus identified in *P. fluorescens* strain 2P24 has the highest similarity to that in *P. aeruginosa* PAO1, in which the expression of the outer-membrane protein OprH is regulated by environmental Mg²⁺ signals via the PhoP/PhoQ system (Macfarlane *et al.*, 1999). Similarly, the transcriptional expression of *oprH* was also induced by Mg²⁺ starvation in wild-type 2P24 (Fig. 1b), indicating that Mg²⁺ concentration is an environmental cue for *oprH* expression in strain 2P24. But the expression of *oprH* was not changed in the *phoP* or *phoQ* mutant, irrespective of the Mg²⁺ level in the medium (Fig. 3). These observations suggest that the presence of both PhoP and PhoQ is necessary for the Mg²⁺ regulation of *oprH* expression. In strain 2P24, PhoP functions as a positive regulator of *oprH* expression under both low- and high-Mg²⁺ conditions. This observation is consistent with the results of a Western blot analysis of OprH expression in *P. aeruginosa* PAO1 (Macfarlane *et al.*, 1999). In strain 2P24, however, the sensor PhoQ plays opposite roles in *oprH* transcription in response to changes in Mg²⁺ level in the growth medium (Fig. 3). Compared with the wild-type strain, *oprH* expression in the *phoQ* mutant was down-regulated in low-Mg²⁺ medium but up-regulated in high-Mg²⁺ medium. This result differed from the expression of OprH observed in *P. aeruginosa* PAO1, which was consistently activated in the absence of PhoQ in both low- and high-Mg²⁺ media (Macfarlane *et al.*, 1999). Given the fact that the active form of response regulators is usually the phosphorylated form, our results support the hypothesis that in strain 2P24, PhoQ acts as a kinase to activate PhoP under low-Mg²⁺ conditions but acts as a phosphatase to repress PhoP under high-Mg²⁺ conditions. This is consistent with an *in vitro* assay indicating that phosphatase activity is the target for Mg²⁺ regulation of the sensor PhoQ in *S. typhimurium* (Castelli *et al.*, 2000). However, this hypothesis does not adequately address the observation that *oprH* transcription is maintained at a higher level in the *phoQ* mutant than in the *phoP* mutant under both low- and high-Mg²⁺ conditions, which

Fig. 7. Regulation of colony formation by PhoP/PhoQ. The wild-type strain 2P24 and its derivatives were cultured on LB plates for 3 days at 28 °C. High, high-Mg²⁺ condition: LB medium containing 15 mM MgCl₂. Low, low-Mg²⁺ condition: LB medium containing 30 mM NaCl. Bar, 1 mm.

indicates that PhoP alone is capable of activating the transcription of *oprH*. A possible explanation for this observation is that one or more alternative kinases present in strain 2P24 mediate the PhoP phosphorylation in the PhoQ-negative background. It is well known that two-component signal-transduction systems are often linked. In *E. coli* strain MG1655, EvgS/EvgA regulates PhoP/PhoQ through a small membrane protein B1500 (Eguchi *et al.*, 2007). Moreover, Matsubara *et al.* (2000) described a cross-phosphorylation between ArcA/ArcB and EnvZ/OmpR in *E. coli* K-12. Further experiments are needed to determine the phosphorylation mechanism of the response regulator PhoP in strain 2P24.

Our genetic evidence suggests that the PhoP/PhoQ two-component system acts as a new regulator of QS in strain *P. fluorescens* 2P24. Two-component regulatory systems represent one of the most common mechanisms of transmembrane signal transduction in bacteria. The QS signalling in many Gram-negative bacteria is often subservient to the two-component system, to connect with environmental signals. In *P. aeruginosa* strain SCV20265, phosphate limitation activates the *rhl* QS system through the PhoB/PhoR two-component system (Jensen *et al.*, 2006); the global two-component system GacS/GacA, which responds to an unknown signal, regulates the *rhl* and *las* QS system in *P. aeruginosa* (Reimann *et al.*, 1997) and the *pco* QS system in *P. fluorescens* 2P24 (Yan *et al.*, 2008). The regulation of *pcoI* expression by PhoP/PhoQ described in this study linked Mg²⁺ signal with the *pco* QS system in strain 2P24, and reflected the complex mechanism of QS, which not only responded to the cell density but also connected with various environmental stimuli.

The deletion and complementation experiments in this study demonstrated that PhoP positively regulates both *pcoI* transcription (Fig. 4) and AHL production (Fig. 5).

Moreover, the regulatory effect was more prominent under low-Mg²⁺ conditions. Similar to the regulation of *oprH* transcription, the *phoQ* mutant exhibited a consistently higher level of *pcoI* transcription than that in the *phoP* mutant under both low- and high-Mg²⁺ conditions, which also supports the existence of a second phosphorylation agent for PhoP. However, unlike the opposite regulatory effect of PhoQ on *oprH* in response to different Mg²⁺ conditions, PhoQ merely acts as a negative regulator of *pcoI* transcription irrespective of the Mg²⁺ level in the medium (Fig. 4). These findings demonstrate a clearly different regulation of downstream phenotypes by the PhoP/PhoQ system in 2P24, and reflect the complexity of the signal transduction pathway mediated by the PhoP/PhoQ system in this strain.

The response regulator PhoP has been demonstrated to directly activate many genes by binding with direct repeats upstream of the target genes in *E. coli* strains K-12 (Kato *et al.*, 1999) and MC4100 (Minagawa *et al.*, 2003) and in *P. aeruginosa* PAO1 (McPhee *et al.*, 2006), and also appears to directly activate the expression of *oprH* in strain 2P24 by binding with similar direct hexanucleotide repeats upstream of *oprH* (Fig. 1b). PhoP was also found to positively regulate *pcoI* transcription under low-Mg²⁺ conditions, but compared with the 77-fold reduction in *oprH* expression caused by the *phoP* mutation (Fig. 3b), only a 3-fold reduction of *pcoI* expression was observed (Fig. 4b). These observations indicate different regulatory mechanisms of PhoP on the expression of *oprH* and *pcoI*. Combined with the observation that there is no similar binding motif located around the putative promoter of *pcoI* (Wei & Zhang, 2006), we propose that PhoP regulates *pcoI* expression through an indirect pathway.

The QS systems LasI/LasR and RhlI/RhlR play important roles in biofilm formation in *P. aeruginosa* PA14 by

regulating the expression of *pel* genes (Sakuragi & Kolter, 2007). Similarly, the *pcrI* gene was previously demonstrated to positively influence biofilm formation in strain 2P24 (Wei & Zhang, 2006). The biofilm assay performed in this study demonstrated that biofilm formation in 2P24 is negatively regulated by PhoQ in low-Mg²⁺ medium, but that this regulation is independent of PhoP since no significant influence was observed following *phoP* deletion or Mg²⁺ depletion (Fig. 6). This observation is contrary to the regulatory model of QS by PhoP/PhoQ described in this paper, indicating that the influence of PhoQ on biofilm formation was probably not via the QS system and that, in 2P24, PhoQ probably acts through a response regulator(s) other than PhoP. In contrast to the situation in 2P24, a dynamic biofilm assay in *P. aeruginosa* PA14 demonstrated that PhoQ positively regulates the biofilm formation (Ramsey & Whiteley, 2004). Additionally, a *phoP*-insertion mutant in *S. typhimurium* resulted in the earlier maturation of biofilms on gallstones and enhanced biofilm formation on glass (Prouty & Gunn, 2003). The differential regulation of biofilms by PhoP/PhoQ may imply that different strains have evolved fine-tuned regulatory features of PhoP/PhoQ to adapt to and enhance colonization in particular niches and environments.

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