



# Differential control of the Pcol/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system

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## KEYWORDS

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GacS/GacA;  
RpoS

## Summary

A quorum-sensing (QS) locus Pcol/PcoR had been identified previously in the biological control bacterium *Pseudomonas fluorescens* 2P24. In this paper, we investigated the upstream regulators that influence the transcription of the *N*-acyl homoserine lactone (AHL) biosynthesis gene *pcol* using a chromosomal *pcol::lacZ* fusion reporter strain. Stationary sigma factor RpoS was identified as a negative regulator of QS system using a random mini-Tn5 mutant procedure. Furthermore, deletion mutagenesis and complementation experiments demonstrated that the two-component system GacS/GacA positively regulated the QS system by upregulating *pcol* transcription. However, compared with the *gacA* or *gacS* mutant, introduction of a second mutation of *rpoS* in the *gacA*<sup>-</sup> or *gacS*<sup>-</sup> background did not lead to further change in the transcriptional expression of the *pcol* gene or the synthesis of AHL. Our results suggest that in strain 2P24, RpoS could only play its negative regulatory role on the *pcol* gene under a functional GacS/GacA system background.

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## Introduction

Many plant-associated Gram-negative microbes produce and monitor the local accumulation of signal molecules, including *N*-acyl-homoserine lactones (AHL), to regulate a broad range of

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biological behaviors in association with their population density (quorum sensing, QS) (Miller and Bassler, 2001). The regulation mechanisms and functions of QS have been intensively studied in recent years. Many reports revealed that the QS system is under the control of specific regulons (Venturi, 2005). In pseudomonades, such regulatory elements include the stationary phase sigma factor RpoS (Whiteley et al., 2000; Aguilar et al., 2003; Bertani and Venturi, 2004), two-component regulatory system GacS/GacA (Reimann et al., 1997; Chatterjee et al., 2003; Bertani and Venturi, 2004), small RNA binding regulator RsmA (Pessi et al., 2001), LuxR-family member VqsR (Juhas et al., 2004) and TetR-family member RsaL (Rampioni et al., 2007).

The two-component system GacS/GacA consisting of the sensor kinase GacS and the cytoplasmic response regulator GacA is present in a wide variety of Gram-negative bacteria. Upon interaction with a yet-unknown signal, the sensor GacS undergoes autophosphorylation, leading to the activation of the regulator GacA via a phosphorelay mechanism; the activated GacA in turn controls the expression of an array of phenotypes, including pathogenicity, plant growth-promoting ability, biofilm formation, production of secondary metabolites and secreted enzymes and proteins (Heeb and Haas, 2001). GacA is known to positively regulate the accumulation of the QS AHL signals by modulating the transcription of the *luxI* family genes in *P. aeruginosa* (Reimann et al., 1997), *P. syringae* (Bertani and Venturi, 2004) and *P. putida* (Chatterjee et al., 2003). Bacterial sigma factors ( $\sigma^{70}$ ,  $\sigma^{32}$ ,  $\sigma^{54}$ ,  $\sigma^{28}$ ,  $\sigma^E$ ) are a type of transcriptional regulator that facilitates the activity of RNA polymerase at a set of promoters defined by specific  $-35$  and  $-10$  sequences (Regine, 2002). Among these, RpoS (also called  $\sigma^S$  or  $\sigma^{38}$ ) is a stationary phase sigma factor that is induced when bacterial growth transits from exponential phase to stationary phase; it also acts as a central regulator of stress tolerance (Regine, 2002; Stockwell and Loper, 2005). The relationship between QS and RpoS is intriguing but confusing, largely because of the distinct regulatory features reported among different bacteria. In *P. aeruginosa*, the QS system RhlR/RhlI appeared to be required for the expression of *rpoS* (Latifi et al., 1996). However, Whiteley et al. (2000) found no quorum-regulated *rpoS::lacZ* transcription; instead, RpoS repressed the transcription of *rhlI*. In a recent transcriptome analysis, Schuster et al. (2004) found that whereas RpoS did not detectably affect expression of *lasI*, it positively regulated the transcription of *lasR* and *rhlR*, but negatively regulated the expression of *rhlI*. Additionally, in *Burkholderia cepacia* strain ATCC 25416, an *rpoS*-

null mutant displayed no difference in the biosynthesis of AHL signal molecules (Aguilar et al., 2003). In *P. putida* WCS358, QS and RpoS were reported to constitute a hierarchical regulatory circuit (Bertani and Venturi, 2004).

We previously identified the QS locus Pcol/PcoR in the plant disease-suppressive bacterium *Pseudomonas fluorescens* 2P24, which is involved in the regulation of root colonization and biological control ability (Wei and Zhang, 2006). In this report, we investigated the roles of *rpoS* gene and the GacS/GacA two-component system in the regulation of QS in *P. fluorescens* 2P24.

## Materials and methods

### Bacterial strains and growth conditions

The strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* DH5 $\alpha$  was grown in Luria–Bertani (LB) medium at 37 °C. *P. fluorescens* 2P24 and its derivatives were grown in LB or ABM minimal medium (Chilton et al., 1974) or King's B medium (King et al., 1954) at 30 °C. When required, growth media were supplemented with ampicillin (50  $\mu\text{g ml}^{-1}$ ), kanamycin (50  $\mu\text{g ml}^{-1}$ ), tetracycline (20  $\mu\text{g ml}^{-1}$ ), chloramphenicol (20  $\mu\text{g ml}^{-1}$ ) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) (40  $\mu\text{g ml}^{-1}$ ).

### DNA manipulations and sequencing

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

### Construction of a *pcol::lacZ* transcriptional fusion in chromosome

Using plasmid pRG970b (Eede et al., 1992) as the template, a 3.1-kb fragment of promoterless *lacZ* gene was PCR-amplified with the primers lacZ-1 and lacZ-2 (Table 1). The PCR product was digested with *Bam*HI and cloned into p47S $\Delta$ I (Wei and Zhang, 2006), resulting in the suicide plasmid p47S $\Delta$ I-lacZ, which was introduced into strain *P. fluorescens* 2P24 by triparental mating to generate a double-crossovered mutant PM101 by the gene replacement method (Link et al., 1997).

**Table 1.** Strains, plasmids and oligonucleotides in this study

Strain, plasmid or oligonucleotide	Description	Reference or source
<b>Strains</b>		
<i>P. fluorescens</i>		
2P24	Wild type, Ap <sup>r</sup>	Wei and Zhang (2005)
PM101	<i>pcol::lacZ</i> reporter fusion of 2P24, Ap <sup>r</sup>	This study
PM201	<i>gacA</i> in-frame deletion; Ap <sup>r</sup>	This study
PM202	<i>gacS</i> in-frame deletion; Ap <sup>r</sup>	Wei and Zhang (2005)
PM203	<i>gacA</i> in-frame deletion of PM101; Ap <sup>r</sup>	This study
PM204	<i>gacS</i> in-frame deletion of PM101; Ap <sup>r</sup>	This study
PM300	<i>rpoS::Tn5</i> of PM101, Ap <sup>r</sup> Km <sup>r</sup>	This study
PM301	<i>rpoS::Tn5</i> of PM101, Ap <sup>r</sup> Km <sup>r</sup>	This study
PM302	<i>rpoS</i> in-frame deletion of PM101; Ap <sup>r</sup>	This study
PM303	<i>rpoS</i> in-frame deletion; Ap <sup>r</sup>	This study
PM304	<i>rpoS</i> , <i>gacA</i> in-frame deletion of PM101; Ap <sup>r</sup>	This study
PM305	<i>rpoS</i> , <i>gacA</i> in-frame deletion; Ap <sup>r</sup>	This study
PM306	<i>rpoS</i> , <i>gacS</i> in-frame deletion of PM101; Ap <sup>r</sup>	This study
PM307	<i>rpoS</i> , <i>gacS</i> in-frame deletion; Ap <sup>r</sup>	This study
<i>A. tumefaciens</i> NTL4 (pZLR4)	<i>traG::LacZ</i> , AHL indicator, Gm <sup>r</sup>	Cha et al. (1998)
<b>Plasmids</b>		
pHSG299	ColE1 origin, cloning vector, Km <sup>r</sup>	TaKaRa
pRG970b	Source of promoterless <i>lacZ</i> gene; Sm <sup>r</sup>	Eede et al. (1992)
pRK415	Cloning vector, Tc <sup>r</sup>	Keen et al. (1988)
pRK600	ColE1 replicon with RK2 transfer region, helper plasmid; Cm <sup>r</sup>	Finan et al. (1986)
pUTkm	Delivery plasmid for Tn5; R6K replicon; Ap <sup>r</sup> ; Km <sup>r</sup>	Herrero et al. (1990)
p299-Δ <i>rpoS</i>	Suicide plasmid containing deleted <i>rpoS</i> gene, Km <sup>r</sup>	This study
p415-A	pRK415 containing intact <i>gacA</i> gene; Tc <sup>r</sup>	Yan et al. (2004)
p415- <i>rpoS</i>	pRK415 containing a 2-kb PCR fragment with <i>rpoS</i> gene, Tc <sup>r</sup>	This study
p415-S	pRK415 containing intact <i>gacS</i> gene; Tc <sup>r</sup>	Wei and Zhang (2005)
p47SΔA	Suicide plasmid pSR47s containing deleted <i>gacA</i>	This study
p47SΔI	pSR47s containing 3.2-kb <i>NotI</i> <i>pcol</i> gene in-frame deletion fragment; Km <sup>r</sup>	Wei and Zhang (2006)
p47SΔI- <i>lacZ</i>	p47SΔI containing a 3.1-kb <i>Bam</i> HI fragment from pRG970b with <i>lacZ</i> gene inserted into <i>Bam</i> HI site; Km <sup>r</sup>	This study
p47SΔS	Suicide plasmid pSR47s containing deleted <i>gacS</i>	Wei and Zhang (2005)
<b>Oligonucleotides<sup>a</sup></b> (5' → 3')		
02R	ATAGGTACCGCAGACACGAAAAAGCC, <i>Kpn</i> I site	
03F	CACAAGCTTTCAGCGCCTGGTACTGGCC, <i>Hind</i> III site	
03R	TCCGATCCATAGCGATGCCGGTCTC, <i>Bam</i> HI site	
04F	GTGGATCCGCCAGACCATCG, <i>Bam</i> HI site	
04R	ACGAATTCATAGTCGTCTATCCCGGC, <i>Eco</i> RI site	
<i>lacZ</i> -1	CCGGATCCCTAGAAGAAGCTTG, <i>Bam</i> HI site	
<i>lacZ</i> -2	ATGGATCCTGCAGACATGGCCTG, <i>Bam</i> HI site	
P1	GCCGATCCTGAACATGGTCTGTCTCC, <i>Bam</i> HI site	
P2	TCCAATTCAGCCAGCATTCTGTG, <i>Eco</i> RI site	
P3	TTGAATTCGATGGTCCAGGC, <i>Eco</i> RI site	
P4	TGGTCGACAGGAACATCACC, <i>Sal</i> I site	

Ap<sup>r</sup>, Cm<sup>r</sup>, Gm<sup>r</sup>, Km<sup>r</sup>, Tc<sup>r</sup>, indicate resistance to ampicillin, chloromycetin, gentamicin, kanamycin and tetracycline, respectively.

<sup>a</sup>Specified restriction sites are underlined.

## Tn5 mutagenesis and cloning of the *rpoS* gene

To identify the potential upstream regulators for *pcol* gene transcription, we mutated the *pcol::lacZ* strain PM101 randomly by a mini-Tn5 transposition according to a previously described method (Herrero et al., 1990). The mutated cells were incubated on the ABM plate containing ampicillin, kanamycin and  $\beta$ -galactosidase substrate X-gal at 30 °C for 30 h. Colonies with increased  $\beta$ -galactosidase activity (indicated by more intensive blue color) were purified and characterized. Mutated genes in clones with verified phenotypes were identified by cloning the genomic DNA fragments harboring the transposon and subsequent sequencing of the flanking DNA. Full length of *rpoS* gene was amplified by primers 03F and 02R (Table 1) designed according to DNA sequences obtained in the analysis of the Tn-5-induced mutants. The *rpoS* gene was inserted into the shuttle vector pRK415 as a *HindIII* and *KpnI* fragment to create the complement plasmid p415-*rpoS*.

## Construction of *gacA*, *gacS* and *rpoS* in-frame deletion mutant strains

The suicide plasmid for the introduction of an in-frame deletion in *rpoS* was constructed as follows. Two fragments flanking *rpoS* gene were amplified by PCR. One was created by primers 03F and 03R (Table 1) which introduced the *HindIII* and *BamHI* sites, respectively, and the other was amplified by primers 04F and 04R (Table 1) which introduced the *BamHI* and *EcoRI* sites, respectively. After being digested by relevant restriction enzymes, these two fragments were ligated into plasmid pHSG299 to create a suicide vector p299 $\Delta$ *rpoS*, which contained a 1.8 kb *HindIII*–*EcoRI* *rpoS* gene fragment with 380 bp deletions in the open reading frame (ORF).

A similar strategy was used to create a *gacA* deletion suicide plasmid, p47S $\Delta$ A, with primers P1 and P2 (Table 1) which introduced the *BamHI* and *EcoRI* sites, with primers P3 and P4 (Table 1) which introduced the *EcoRI* and *Sall* sites, respectively. In this plasmid, an internal fragment of 247 bp was deleted in the ORF of *gacA* gene. Construct p47S $\Delta$ S for in-frame deletion of *gacS* was reported previously (Wei and Zhang, 2005).

Using these suicide plasmids, we constructed *rpoS*, *gacA* and *gacS* deletion mutants of *P. fluorescens* 2P24 with a gene replacement method (Link et al., 1997). All mutations were verified by multiple PCR analysis.

## Isolation and detection of AHL

*P. fluorescens* 2P24 and its derivatives were grown in LB liquid medium at 30 °C for 36 h. About 0.8 ml of cultures were extracted with the same volume of ethyl acetate, the extracts were then dried and resuspended in 0.1 ml of methanol. For quantitative analysis of the AHL, 3  $\mu$ l of the sample was incubated with the 0.2 ml AHL biosensor *A. tumefaciens* NTL4 (pZLR4) (Cha et al., 1998) ( $OD_{600} = 0.8$ ); the reaction mixture was incubated at 28 °C for 3 h and the  $\beta$ -galactosidase activity of biosensor cells was assayed (Miller, 1972).

## $\beta$ -galactosidase assay

For  $\beta$ -galactosidase measurement, *Pseudomonas* and *Agrobacterium* strains that contained  $\beta$ -galactosidase reporter genes were grown in LB and ABM liquid medium respectively at 28 °C with shaking at 130 rpm in 50-ml flasks. Cultures were sampled at different time points and assayed for  $\beta$ -galactosidase specific activity according to Miller method (Miller, 1972).

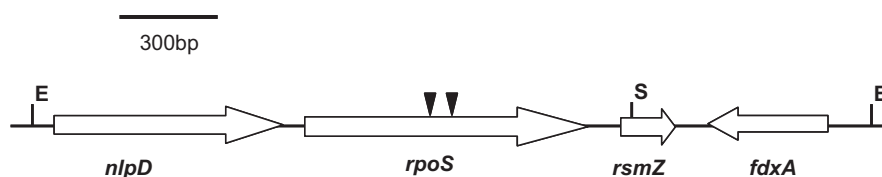
## Nucleotide sequence accession number

The GenBank accession number for the DNA sequence containing the *rpoS* gene of *P. fluorescens* 2P24 is EF587266.

## Results

### Cloning of *rpoS* gene using a mini-Tn5 mutant procedure

To monitor the expression changes of QS in *P. fluorescens* 2P24, we constructed the reporter strain PM101 that contained a chromosomal fusion of a promoterless *lacZ* gene into the ORF of the homoserine lactone biosynthase gene *pcol*. Therefore, the transcription of the *pcol* gene could be quantified by measurement of the  $\beta$ -galactosidase activity. After introducing the mini-Tn5 transposon, ca. 10,000 mutants of strain PM101 were screened for LacZ activity. Two mutants, PM300 and PM301, formed colonies with more intensive blue color on minimal medium supplemented with X-gal were identified. The Tn5 insertion sites were subsequently cloned as *Sall* fragments from the chromosomal DNA of these two mutant strains. Sequence analysis revealed that in both cases, the transposon inserted in the same ORF but at different positions (Figure 1). The disrupted ORF was of 1008 bp in



**Figure 1.** Schematic diagram of *P. fluorescens* 2P24 *rpoS* gene and its flanking regions. The solid triangles represent the position of Tn5 insertion in mutant strains PM300 and PM301, respectively. Restriction enzyme abbreviations: E, *EcoRI*; S, *Sall*.

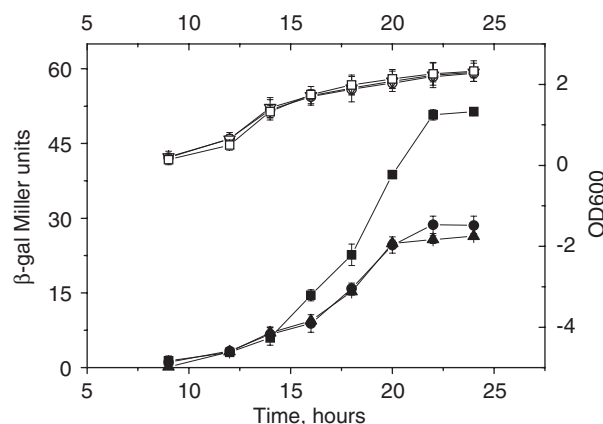
length and was predicted to encode a 38.1 kDa protein. The deduced protein showed a high-level similarity to members of sigma factor RpoS family, with 96.7% identity to RpoS of *P. fluorescens* Pf-5 (Sarniguet et al., 1995; Stockwell and Loper, 2005); 94.0% identity to RpoS of *P. fluorescens* CHA0 (Heeb et al., 2002); and 58.6% identity to KatF of *E. coli* (McCann et al., 1991).

In *P. fluorescens* CHA0, the *rpoS* gene was flanked by the *nlpD* and *rsmZ* genes (Heeb et al., 2002), encoding a novel lipoprotein (NlpD) and a small RNA regulator (RsmZ), respectively. Similar gene arrangement is present in the *rpoS* region of *P. fluorescens* 2P24 (Figure 1).

### *rpoS* negatively regulated the *pcol* gene transcription and the accumulation of AHL

To avoid the potential polar effect that may affect further analysis of the gene regulation, an in-frame deletion of *rpoS* gene in strain *P. fluorescens* PM101 (*pcol::lacZ*) was constructed. When grown in LB broth, the in-frame deletion mutant PM302 (*rpoS*<sup>-</sup>, *pcol::lacZ*) showed significantly higher  $\beta$ -galactosidase activity from the early-logarithmic phase than that of strain PM101; the most significant difference was observed when the bacteria grew into late-logarithmic and stationary phases (Figure 2). The *pcol::lacZ* activity of the *rpoS* gene mutant PM302 can be complemented to the wild-type level by the plasmid-born wild-type *rpoS* gene (Figure 2). These results suggested that RpoS was involved in the negative regulation of *pcol* transcription.

To further analyze the effect of *rpoS* deletion, we quantitatively compared the accumulation of QS signals AHL in *P. fluorescens* 2P24 and its *rpoS* mutant. An *rpoS* in-frame deletion mutant strain PM303 was constructed with wild-type strain 2P24 to analyze the effect of *rpoS* on the AHL accumulation. The AHL produced by 2P24 and its derivatives were extracted and detected after 36 h shaking in LB broth as described in the Materials and methods. The results (Figure 5) indicated that mutant PM303



**Figure 2.** Regulation of *rpoS* on the transcription of *pcol::lacZ* fusion.  $\beta$ -galactosidase activity (solid) and the growth curve (open) of each strain were measured at various time points after inoculation into 30-ml LB medium. All experiments were performed in triplicate, and the mean values  $\pm$ SD are indicated. (●, ○) The parent strain PM101/pRK415; (■, □) the *rpoS* mutant PM302/pRK415; (▲, △) the complemented PM302/p415-*rpoS*.  $\beta$ -gal,  $\beta$ -galactosidase; OD<sub>600</sub>, optical density at 600 nm.

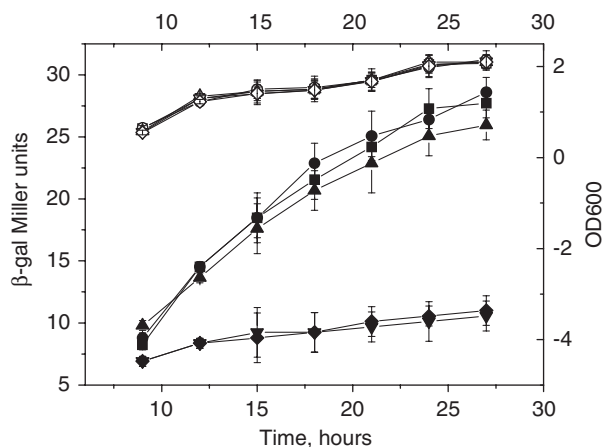
(*rpoS*<sup>-</sup>) produced a significantly larger amount of AHL signals than the wild-type 2P24. In complementation experiments, the plasmid p415-*rpoS*, but not the vector, restored the production of the QS signals to the wild-type level (Figure 5). This result was consistent with that of the reporter-mediated assay, indicating that *rpoS* negatively regulated the transcription of the *pcol* gene in strain 2P24.

### GacS/GacA positively regulated the *pcol* gene transcription and the accumulation of AHL

The two-component regulatory system GacS/GacA has been identified as an important regulator in many pseudomonades (Heeb and Haas, 2001). In 2P24, GacS/GacA was determined to play an important role in biocontrol of wheat take-all disease (Yan et al., 2004; Wei and Zhang, 2005).

To detect whether the GacS/GacA influences the expression of *pcol* in 2P24, *gacA* and *gacS* deletion mutants were constructed in the reporter strain PM101 (*pcol::lacZ*), resulting in PM203 (*gacA*<sup>-</sup>, *pcol::lacZ*) and PM204 (*gacS*<sup>-</sup>, *pcol::lacZ*), respectively. When grown in LB liquid medium, the *pcol::lacZ* activity increased with the increase of cell density in strain PM101, but did not increase in the mutants PM203 and PM204, in which the GacS/GacA two-component regulatory systems were impaired (Figure 3). When the plasmids harboring the wild-type *gacA* and *gacS* gene (p415-A and p415-S) were introduced into PM203 and PM204, respectively, the *pcol::lacZ* activities of these complemented mutants were parallel to that of strain PM101 (Figure 3).

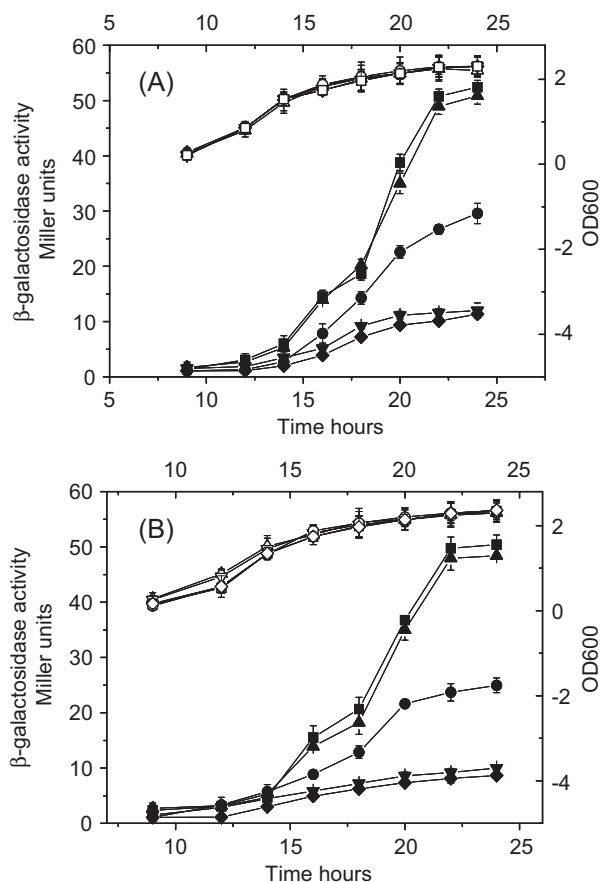
The *gacA* and *gacS* deletion mutant strains, PM201 (*gacA*<sup>-</sup>) and PM202 (*gacS*<sup>-</sup>), were also constructed in wild-type 2P24 to determine their influence on AHL molecules accumulation. The AHL production of PM201 and PM202 decreased markedly compared to the wild-type strain 2P24 (Figure 5), and the mutant phenotypes of PM201 and PM202 were completely complemented by the plasmid-born *gacA* and *gacS* genes, respectively (Figure 5). Taken together, these results genetically indicated that the GacS/GacA system positively regulates the transcription of the *pcol* gene and the accumulation of AHL.



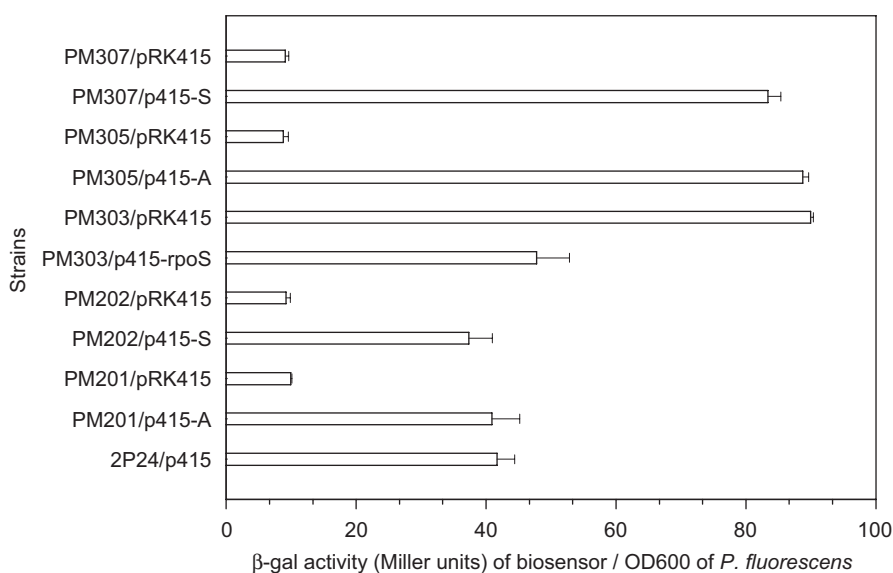
**Figure 3.** Regulation of *gacS* and *gacA* on the transcription of *pcol::lacZ* fusion.  $\beta$ -galactosidase activity (solid) and the growth curve (open) of each strain were measured at various time points after inoculation into 30-ml LB medium. All experiments were performed in triplicate, and the mean values  $\pm$ SD are indicated. (●, ○) The parent strain PM101/pRK415; (◆, ◇) the *gacA* mutant PM203/pRK415; (■, □) the complemented PM203/p415-A; (▼, ▽) the *gacS* mutant PM204/pRK415; (▲, △) the complemented PM204/p415-S.  $\beta$ -gal,  $\beta$ -galactosidase; OD<sub>600</sub>, optical density at 600 nm.

### The effect of *GacS/GacA* and *rpoS* double mutant on the *pcol* expression and the AHL production

Since the above results demonstrated the opposite regulatory roles of sigma factor RpoS and the two-component system GacS/GacA on QS in *P. fluorescens* 2P24, we were interested in determining the effect of a double deletion mutant of these two regulatory factors on the expression of



**Figure 4.** The transcriptional activity of *pcol::lacZ* fusion in parent strain PM101 and mutant derivatives.  $\beta$ -galactosidase activity (solid) and the growth curve (open) of each strain were measured at various time points after inoculation into 30-ml LB medium. All experiments were performed in triplicate, and the mean values  $\pm$ SD are indicated. (A) Regulation of *gacA* and *rpoS* on the transcription of *pcol::lacZ* fusion gene; (●, ○) the parent strain PM101/pRK415; (◆, ◇) the *gacA* mutant PM203/pRK415; (■, □) the *rpoS* mutant PM302/pRK415; (▼, ▽) the *rpoS-gacA* double mutant PM304/pRK415; (▲, △) the complemented PM304/p415-A. (B) Regulation of *gacS* and *rpoS* on the transcription of *pcol::lacZ* fusion gene. (●, ○) The parent strain PM101/pRK415; (◆, ◇) the *gacS* mutant PM204/pRK415; (■, □) the *rpoS* mutant PM302/pRK415; (▼, ▽) the *rpoS-gacS* double mutant PM306/pRK415; (▲, △) the complemented PM306/p415-S.  $\beta$ -gal,  $\beta$ -galactosidase; OD<sub>600</sub>, optical density at 600 nm.



**Figure 5.** Regulation of *gacS*, *gacA* and *rpoS* on the production of AHL. The  $\beta$ -galactosidase activity of *traG::LacZ* fusion in biosensor strain *A. tumefaciens* NTL4 (pZLR4) was measured after incubation with AHL signals that were extracted from the wild-type 2P24 and its derivative strains. The wild-type 2P24/pRK415; the *rpoS* mutant PM303/pRK415; the *gacA* plus *rpoS* mutant PM305/pRK415; the complemented PM305/p415-A; the *gacS* plus *rpoS* mutant PM307/pRK415; the complemented PM307/p415-S; the *gacA* mutant PM201/pRK415; the complemented PM201/p415-A; the *gacS* mutant PM202/pRK415; the complemented PM202/p415-S. All experiments were performed in triplicate, and the mean values  $\pm$ SD are indicated.

*pcol* and the accumulation of AHL signals. Double mutant strains PM304 (*gacA*<sup>-</sup>, *rpoS*<sup>-</sup>, *pcol::lacZ*) and PM306 (*gacS*<sup>-</sup>, *rpoS*<sup>-</sup>, *pcol::lacZ*) were constructed using reporter strain PM101 (*pcol::lacZ*) by the method described above. PM304 and PM306 showed a similar phenotype on the expression of *pcol::lacZ* with the single mutants on GacS/GacA system, PM203 (*gacA*<sup>-</sup>, *pcol::lacZ*) and PM204 (*gacS*<sup>-</sup>, *pcol::lacZ*), respectively (Figure 4), i.e., the double mutant strains PM304 and PM306 reduced the expression level of *pcol::lacZ* significantly compared to the single deletion mutant of *rpoS* PM302 (*rpoS*<sup>-</sup>, *pcol::lacZ*) and the reporter strain PM101 (*pcol::lacZ*). Plasmids harboring the wild-type *gacA* (p415-A) and *gacS* (p415-S), but not the *rpoS* gene (data not shown) and the vector, complemented the lost phenotype of strains PM304 and PM306, respectively. Interestingly, The *pcol::lacZ* activities of their complemented mutants were restored to the level similar to that of mutant strain PM302 (*rpoS*<sup>-</sup>, *pcol::lacZ*), but not to that of reporter strain PM101 (*pcol::lacZ*) (Figure 4), indicating that the sigma factor RpoS could only play its negative regulatory role on the *pcol* gene under a functional GacS/GacA system background.

Analysis of AHL signal accumulation of the double deletion mutants of GacS/GacA system and sigma factor RpoS in wild-type strain 2P24 received the results consistent with that of the transcriptional regulation assay. Double deletion mutant strains

PM305 (*gacA*<sup>-</sup>, *rpoS*<sup>-</sup>) and PM307 (*gacS*<sup>-</sup>, *rpoS*<sup>-</sup>) were generated by two-step homologous recombination using wild-type 2P24 as a receptor. Both mutants produced much lower amounts of AHL than that of a single mutant of *rpoS* (PM303) or the wild type, but similar to the single mutant of *gacA* (PM201) or *gacS* (PM202) (Figure 5). Complementation of PM305 and PM307 with plasmids p415-A and p415-S, respectively, resulted in a large amount of AHL signal accumulation, which is significantly higher than that of wild-type 2P24 and comparable to that of mutant PM303 (*rpoS*<sup>-</sup>). These results indicated in the absence of the Gac system, expression of *pcol* no longer was repressed by RpoS.

## Discussion

In this study, we provided a genetic evidence to demonstrate that in *P. fluorescens* strain 2P24, the GacS/GacA two-component system plays a positive role in the transcriptional expression of *pcol*. Furthermore, using a random mini-Tn5 mutagenesis procedure, we identified the stationary sigma factor RpoS as a negative regulator of the transcription of *pcol*. The observation that *pcol* was expressed at a basal level in a mutant lacking both RpoS and the Gac system suggested that the control of *pcol* by RpoS was via the function of the

two-component system. RpoS may function via a third factor to repress the Gac system. However, given the large number of mutant candidates we have screened through and the high randomness of Tn-5 insertion in pseudomonads, we preferred a model in which RpoS directly functioned to repress the activity of the Gac system, which in turn acted to turn on the QS reporter. Since very little is known about the genetic elements such as promoters and *trans*-acting proteins necessary for the expression of the Gac system, currently it is difficult to determine the mechanisms of this RpoS-mediated control of Gac system. Sigma factors were generally recognized as the key factors in gene transcription initiation (Regine, 2002), it is very likely that the RpoS negatively controls the Gac system at transcriptional level.

It has been reported that RpoS participates in the regulation of QS systems in various bacterial strains but the effect of the regulation differed among these systems. In *P. putida* strain WCS358, RpoS positively regulates the Ppnl/PpuR QS system (Bertani and Venturi, 2004), but in *Burkholderia cepacia* genomovar I type strain, ATCC 25416, this sigma factor does not detectably affect the accumulation of AHL (Aguilar et al., 2003). In *P. aeruginosa* strain PAO1, RpoS and QS reciprocally regulate the expression to each other (Schuster et al., 2004). Our observation that RpoS negatively affected expression of *pcol* in strain 2P24 added one more layer to the complexity of the relationship between QS system and RpoS. Further study will be directed in understanding the mechanisms underlying how the Pcol/PcoR system is affected by RpoS in strain 2p24.

It has been reported that the GacS/GacA two-component system positively controlled the expression of *rpoS* in *P. fluorescens* Pf-5 (Whistler et al., 1998) and in *Azotobacter vinelandii* (Castaneda et al., 2001). Similarly, we observed a positive regulation of GacA on the transcription of *rpoS* gene in *P. fluorescens* 2P24 (data not shown). Together with the above results, we proposed the existence of a negative feedback relationship between RpoS and the Gac system, induction of RpoS by the two-component system may provide a mechanism to lower the level of the Gac when environmental cues favorable for hyperactivity of this system no longer are available.

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## References

- Aguilar C, Bertani I, Venturi V. Quorum-sensing system and stationary-phase sigma factor (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416. *Appl Environ Microbiol* 2003; 69:1739–47.
- Bertani I, Venturi V. Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Appl Environ Microbiol* 2004; 70:5493–502.
- Castaneda M, Sanchez J, Moreno S, Nunez C, Espin G. The global regulators GacA and  $\sigma^S$  form part of a cascade that controls alginate production in *Azotobacter vinelandii*. *J Bacteriol* 2001;183:6787–93.
- Cha C, Gao P, Chen YC, et al. Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Mol Plant-Microbe Interact* 1998;11:1119–29.
- Chatterjee A, Cui Y, Yang H, Collmer A, Alfano JR, Chatterjee AK. GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant-Microbe Interact* 2003;16:1106–17.
- Chilton MD, Currier TC, Farr SK, et al. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci USA* 1974;71:3672–6.
- Eede VD, Deblaere GR, Goethals KM, et al. Broad host range and promoter selection vectors for bacteria that interact with plants. *Mol Plant-Microbe Interact* 1992; 5:228–34.
- Finan TM, Kunkel B, de Vos GF, et al. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* 1986;167:66–72.
- Heeb S, Haas D. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol Plant-Microbe Interact* 2001;14:1351–63.
- Heeb S, Blumer C, Haas D. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* 2002;184:1046–56.
- Herrero M, Lorenzo VD, Timmis KN. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* 1990;172:6557–67.

- Juhas M, Wiehlmann L, Huber B, et al. Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* 2004;150:831–41.
- Keen NT, Tamaki S, Kobayashi D, et al. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 1988;70:191–7.
- King EO, Ward MK, Raney DE. Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* 1954;44:301–7.
- Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* 1996;21:1137–46.
- Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 1997;179:6228–37.
- McCann MP, Kidwell JP, Matin A. The putative  $\sigma$  factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *J Bacteriol* 1991;173:4188–94.
- Miller JH. *Experiments in molecular genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1972.
- Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu Rev Microbiol* 2001;55:165–99.
- Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Camara M, et al. The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* 2001;183:6676–83.
- Rampioni G, Polticelli F, Bertani I, Righetti K, Venturi V, Zennaro E, et al. The *Pseudomonas* quorum-sensing regulator rsal belongs to the tetrahelical superclass of H-T-H proteins. *J Bacteriol* 2007;189:1922–30.
- Regine HA. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* 2002;66:373–93.
- Reimann C, Beyeler M, Latifi A, Winteler H, Foglino M, Lazdunski A, et al. The global activator GacA of *Pseudomonas aeruginosa* PAO1 positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* 1997;24:309–19.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
- Sarniguet A, Kraus J, Henkels MD, Muehlchen AM, Loper JE. The sigma factor  $\sigma^S$  affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proc Natl Acad Sci USA* 1995;92:12255–9.
- Schuster M, Hawkins AC, Harwood CS, Greenberg EP. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 2004;51:973–85.
- Stockwell VO, Loper JE. The sigma factor RpoS is required for stress tolerance and environmental fitness. *Microbiology* 2005;151:3001–9.
- Venturi V. Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* 2005;30:274–91.
- Wei HL, Zhang LQ. Cloning and functional characterization of the *gacS* gene of the biocontrol strain *Pseudomonas fluorescens* 2P24. *Acta Microbiol Sin* 2005;45:368–72.
- Wei HL, Zhang LQ. Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24. *Antonie Van Leeuwenhoek* 2006;89:267–80.
- Whistler CA, Corbell NA, Sarniguet A, Ream W, Loper JE. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor  $\sigma^S$  and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* 1998;180:6635–41.
- Whiteley M, Parsek MR, Greenberg EP. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J Bacteriol* 2000;182:4356–60.
- Yan XX, Zhang LQ, Yang ZW, Tang WH. The role of regulatory gene *gacA* in the suppression of soil-borne diseases by *Pseudomonas fluorescens* 2P24. *Acta Phyto Pathol Sin* 2004;34:272–9.