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Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24

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Abstract

Pseudomonas fluorescens 2P24 is a biocontrol agent isolated from a wheat take-all decline soil in China. This strain produces several antifungal compounds, such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide and siderophore(s). Our recent work revealed that strain 2P24 employs a quorum-sensing system to regulate its biocontrol activity. In this study, we identified a quorum-sensing system consisting of PcoR and PcoI of the LuxR–LuxI family from strain 2P24. Deletion of *pcoI* from 2P24 abolishes the production of the quorum-sensing signals, but does not detectably affect the production of antifungal metabolites. However, the mutant is significantly defective in biofilm formation, colonization on wheat rhizosphere and biocontrol ability against wheat take-all, whilst complementation of *pcoI* restores the biocontrol activity to the wild-type level. Our data indicate that quorum sensing is involved in regulation of biocontrol activity in *P. fluorescens* 2P24.

Introduction

Numerous bacteria utilize specific signals called autoinducers to establish intercellular communication. Expression of many physiological traits and behaviors relies on the size of a bacterial community, which is gauged by extracellular accumulation of specific self-produced signals. This phenomenon, named quorum sensing (QS), is a widespread regulatory mechanism that plays a central role in the physiology and development of bacteria (Gray and Garey 2001; Miller and Bassler 2001). The first QS system was discovered in the bioluminescent marine bacterium *Vibrio fischeri*, in which LuxI is responsible for biosynthesis of the intercellular signal *N*-(3-oxohexanoyl)-homoserine lactone, and LuxR is a transcriptional regulator (Hanzelka and Greenberg 1995). As the concentration of the QS signal reaches a critical level, it interacts with the LuxR protein and the LuxR-autoinducer complex binds to the *luxICDABE* promoter region and activates transcription of the target genes (Hanzelka and Greenberg 1995).

QS has been studied for more than 25 years and such systems have been identified in over 100 species of bacteria (Miller and Bassler 2001; Bodman et al. 2003). In Gram-negative bacteria, *N*-acylhomoserine lactones (AHLs) differing in the length of their *N*-linked side chains and the nature of the substitute at the C3 position, are the most common QS signals (Cámara et al. 1998). By detection of QS signals using biosensors based on a variety of well-studied systems, a large number of bacteria were found to produce AHLs (or AHL-like activities) (Brelles-Mariño and Bedmar 2001). Among these, many QS

signals producing microorganisms are plant-associated bacteria, and a few of them are plant growth-promoting and biocontrol bacteria such as *Pseudomonas aureofaciens*, *Pseudomonas putida* and *Pseudomonas fluorescens* (Pierson et al. 1998). For instance, in *P. aureofaciens* 30-84, a well-characterized biocontrol agent which protects wheat from take-all disease caused by the ascomycete fungus *Gaeumannomyces graminis* var. *tritici*, the PhzI–PhzR QS system is employed to regulate the *phzFABCD* operon responsible for synthesizing a phenazine antibiotic (Wood et al. 1997). Recently, a second QS system, CsaI–CsaR, was identified in *P. aureofaciens* strain 30-84, which appears to regulate cell surface properties and plays a role in rhizosphere competitiveness but not in production of phenazine (Zhang and Pierson 2001). In *P. putida* IsoF, a bioremediation strain that also can promote plant growth, PpuI–PpuR activates *ppuA* and positively influences biofilm development (Steidle et al. 2002). Another biocontrol agent *P. fluorescens* F113 also produces AHL signals, but the signal synthase, HdtS, is not a member of the LuxI family and the phenotype regulated by this QS system is unknown (Laue et al. 2000).

Pseudomonas fluorescens 2P24 was first isolated from wheat roots taken from a field affected by take-all disease in Shandong province, China. This strain produces several secondary metabolites such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide and siderophore(s) (Wei et al. 2004a). Among them, the antibiotic 2,4-DAPG was genetically proven to be a key determinant in the suppression of plant diseases (Wei et al. 2004b). In addition to wheat take-all disease, strain 2P24 is able to protect plants against tomato bacterial wilt caused by *Ralstonia solanacearum* and cotton rhizoctoniosis caused by *Rhizoctonia solani* (Wei et al. 2004a, b). In this study, we report the identification of a quorum-sensing system, PcoI–PcoR, in *P. fluorescens* 2P24 and the characterization of several biocontrol-related phenotypes regulated by this system.

Materials and methods

Strains, plasmids and growth conditions

The strains and the plasmids are listed in Table 1. *Pseudomonas* spp. and *Agrobacterium tumefaciens* were grown in Luria-Bertani (LB) medium or King's B medium (King et al. 1954) at 28 °C, whereas *Escherichia coli* DH5α and S17-1 (λ-pir) were grown at 37 °C in LB medium. For antagonistic determination, *R. solani* and *G. graminis* were grown on potato dextrose agar (PDA) plate at 25 °C. ABM minimal medium (Chilton et al. 1974) was used for detection of AHLs. For plasmid propagation and selection of transformants, media were supplemented with antibiotics at appropriate concentration as follows: streptomycin sulfate (200 μg/ml), ampicillin (50 μg/ml), kanamycin (50 μg/ml), gentamicin (30 μg/ml) and tetracycline (20 μg/ml).

DNA manipulations and sequencing

Chromosomal DNA of bacteria was isolated by the CTAB method (Del Sal et al. 1988). Plasmid DNA extractions and other molecular assays were performed according to standard procedures (Sambrook et al. 1989). Nucleotide sequencing was performed by Bioasia Biotechnology Co. Ltd. Nucleotide and deduced amino acid sequences were analyzed with the on-line BLAST search engine in GenBank.

Cloning of pcoI *gene*

The gene coding for AHL synthase in *P. fluorescens* 2P24 was identified by screening cosmid clones that are able to activate the *traG*::*lacZ* reporter in the biosensor strain *A. tumefaciens* NTL4 (pZLR4) (Cha et al. 1998). Briefly, a genomic library of *P. fluorescens* 2P24 was constructed in the broad-host-range cosmid vector pLAFR5 (Keen et al. 1988) using the restriction enzyme *Sau*3A. Recombinant clones were spotted on LB plate containing 5% (vol/vol) biosensor strain and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). After incubation at 37 °C for 16–20 h, a positive reaction was determined by the presence of a blue zone diffusing out of the testing clones.

Construction of pcoI *in-frame deletion mutant strain*

To create a *pcoI* gene deletion allele, two fragments flanking *pcoI* gene were amplified by

Table 1. Strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
P. fluorescens		
2P24	Ap^r wild type	Wei et al. (2004a)
2P24ΔI	Ap^r *pcoI* deletion mutant	This study
2P24-I	Ap^r 2P24ΔI containing p415-I	This study
G. graminis var. *tritici*	Plant pathogen causing wheat take-all	Lab stock
R. solani	Plant pathogen causing cotton damping-off	Lab stock
E. coli		
DH5α	F^- *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ (*argF-lacZYA*)*I169* Z *80lacZ* Δ*M15*	Hanahan (1983)
S17-1 (λ-pir)	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn*7* *λ-pir*	de Lorenzo and Timmis (1994)
A. tumefaciens NTL4(pZLR4)	NT1 derivative carrying a *traG::lacZ* reporter fusion	Cha et al. (1998)
Plasmids		
pLAFR5	Tc^r; *oriT* cosmid	Keen et al. (1988)
p83-51	Tc^r; a cosmid containing *pcoI* and *pcoR* genes	This study
pHSG299	Km^r; ColE1 origin	TaKaRa
p299-83-51	Km^r; pHSG299 containing ca. 10 kb *Bam*HI fragment from p83-51 with *pcoI* and *pcoR* genes	This study
pBluescriptII SK +	Ap^r; ColE1 origin	Stratagene
pBS-83-51	Ap^r; pBluescript containing 4.3 kb *Eco*RI fragment from p299-83-51 with *pcoI* gene	This study
pRK415	Tc^r; IncP1 replicon, polylinker of pUC19, Mob^+	Keen et al. (1988)
p415-I	Tc^r; pRK415 containing 0.8 kb PCR fragment from pBS-83-51 with *pcoI* gene	This study
pBSΔIA	Ap^r; pBluescript containing 1.58 kb *Pst*I -*Bam*HI fragment	This study
pBSΔIB	Ap^r; pBluescript containing 1.63 kb *Eco*RI-*Bam*HI fragment	This study
pBSNot6	Ap^r; a *Not*I site inserted into pBluescript following the *Kpn*I site	Zhou et al. (2005)
pBSNΔI	Ap^r; pBSNot6 containing 3.2 kb *Pst*I -*Eco*RI fragment in which ca. 270 bp of *pcoI* gene was deleted	This study
pSR47S	Km^r; *oriT sacB*	Andrews et al. (1998)
p47SΔI	Km^r; pSR47S containing 3.2 kb *Not*I fragment from pBSNΔI	This study
pRK600	Cm^r; ColE1 replicon with RK2 transfer region, helper plasmid	Finan et al. (1986)

Ap^r, Km^r, Cm^r, and Tc^r indicate resistance to ampicillin, kanamycin, chloromycetin and tetracycline, respectively.

PCR. One was created by primers I473 (5′-TCGTTCGCTGCAGGAAACC-3′) which included the *Pst*1 site (underlined) and I2056 (5′-ATGGATCCCGGAGTTTGTGCATGCCC-3′) which introduced the *Bam*H1 site (underlined), and the other was created by primers I2328 (5′-AAGGATCCGGGTTCGGCTTCTCTGACAC-3′) which introduced the *Bam*H1 site (underlined) and I3966 (5′-ATGAATTCAATGGCCCACTC-AACGCA-3′) which introduced the *Eco*R1 site (underlined). The standard PCR reaction involved 5 min at 94 °C, the following 35 cycles of 40 s at 94 °C, 40 s at 60 °C and 1 min at 72 °C, and finally 10 min at 72 °C. After being digested with relevant restriction enzymes, the two fragments were inserted into pBSNot6 (Zhou et al. 2005) to create pBSNΔIA and pBSNΔIB. The *Pst*1-*Bam*H1 fragment from pBSNΔIA was inserted into pBSNΔIB resulting in pBSNΔI, from which a 3.2-kb *Not*1 fragment, including a *pcoI* gene with 270 bp deletion, was lifted and ligated into pSR47S (Andrews et al. 1998). The last suicide plasmid p47SΔI was used in a two-step strategy to introduce the shortened *pcoI* locus into the chromosome of *P. fluorescens* 2P24 according to previous method (Wei and Zhang 2005). Primers I1867 (5′-ATGAATTCATCCCAGTCAGATTC CGGG-3′) and I2676 (5′-AAGAATTCGCAAA GATGGGTAACGAAGCA-3′) which introduced the same *Eco*R1 site (underlined) were used to confirm a double crossover event.

To complement the *pcoI* mutant, a 0.8-kb fragment, containing the putative upstream promoter and coding region of *pcoI* gene, was amplified by primers I1867 and I2676. The amplified PCR fragment was inserted into the shuttle vector pRK415 (Keen et al. 1988) to create p415-I, and the resulting plasmid was introduced into strain 2P24ΔI by triparental mating using helper strain DH5α containing plasmid pRK600 (Finan et al. 1986).

Isolation and detection of AHLs

Thin-layer chromatography (TLC) in combination with an AHL biosensor was used to detect the AHL species as described previously (Shaw et al. 1997). AHLs were extracted with equal volume of ethyl acetate from 40 ml of filter-sterilized supernatants from stationary-phase cultures of *P. fluorescens* 2P24 and its derivatives grown overnight in LB medium at 28 °C. *E. coli* DH5α containing p415-I was also analyzed for AHLs production similarly except that the bacteria were grown at 37 °C. The extracts were brought to dryness by evaporation and the residuals were dissolved in 0.2 ml of methanol. Three microliters of samples were then applied to C_{18} reversed-phase TLC plates (catalog no. 1.15389; Merck, Darmstadt, Germany) and dried with a stream of cold air. Samples were separated using methanol (60%, vol/vol) in water as the mobile phase. Detection of AHLs was done by overlaying the TLC plate with a 3 mm thin film of 0.8% (wt/vol) LB agar (100 ml) seeded with 5 ml of an exponentially grown AHL biosensor *A. tumefaciens* NTL4 (pZLR4) and X-gal (40 μg/ml). The overlayed TLC plates were incubated at 28 °C for 24 h. AHL activities were determined by the appearance of blue spots on the plate.

AHL standards *N*-hexanoyl-homoserine lactone (C_6-HSL) and *N*-octanoyl-homoserine lactone (C_8-HSL) were purchased from Sigma; *N*-(3-oxo-hexanoyl)-homoserine lactone (oxo-C_6-HSL) and *N*-(3-oxo-octanoyl)-homoserine lactone (oxo-C_8-HSL) were the gift of Dr. Z. Q. Luo, Purdue University.

Assays for secondary metabolites and fungal growth inhibition

Qualitative assays for the production of hydrogen cyanide and protease were performed as described previously (Pierson and Thomashow 1992). Siderophore(s) activity was measured by CAS assays (Schwyn and Neilands 1987). For extraction of 2,4-DAPG, strain 2P24 and its derivatives were grown in King's medium B at 28 °C for 48 h. At 12, 24, 36 and 48 h, absorbance of each culture at 600 nm was determined. Simultaneously, 3 ml of each culture were extracted with ethyl acetate and the organic phase was evaporated. Extracts were resuspended into 0.2 ml methanol for HPLC assay (Duffy and Défago 1997).

The antibiosis of *P. fluorescens* against *R. solani* on PDA was performed as follows. A fresh mycelial disk (diameter, 5 mm) of *R. solani* was inoculated onto the centers of fresh PDA plates (diameter, 90 mm) and 5 μl of saturated *P. fluorescens* culture was dotted around the

inocula at a distance of 30 mm. The plates were incubated at 25 °C for 2–3 days and antibiosis ability were determined by measuring the distance of inhibitory zone.

The qualitative assays for the production of hydrogen cyanide, protease and siderophore(s) were performed three times. The quantitative assays for 2,4-DAPG production and fungal growth inhibition activity were performed in triplicate at least twice independently.

Biofilm formation assays

To determine biofilm formation on untreated polyvinylchloride (PVC) plastic, an assay similar to that described previously was used with a few modifications (Stepanovic et al. 2000). Briefly, testing strains were grown to saturation in LB medium and then diluted (1:1000) with fresh LB broth. 0.5 ml diluted culture was transferred to an Eppendorf tube. Bacteria were incubated without agitation for 4, 8, 12 and 16 h at 28 °C and biofilm was quantified at each time points. Biofilm was stained with 0.1% (w/v) crystal violet (CV) for 15 min at room temperature and then rinsed thoroughly and vigorously with water to remove unattached cells and residual dye. Ethanol (95%) was used to solubilize the dye that had stained the biofilm cells. The absorbance of the solubilized dye (A_{570}) was determined with a spectrophotometer. All experiments were performed in triplicate at least twice independently.

Assay for colonization

All the bacterial strains used in colonization assay were selected and labeled with streptomycin sulfate resistance (Weller 1983). Wheat seeds were surface sterilized and treated with bacterial cultures (10^8 cfu/ml) as described previously (Pierson et al. 1998). To determine bacterial colonization, sterile soil and natural soil from a northern suburb of Beijing were used to germinate wheat seeds, respectively. Assays for seedlings were performed as described previously (Zhang and Pierson 2001). After 7, 14 and 21 days, ten plants were harvested randomly from each treatment and bacterial population in rhizosphere (1 g) and the root tip (1 cm) were determined as described by Hoben and Somasegaran (1982). The experiment was carried out twice.

Wheat take-all suppression assay

For wheat take-all suppression assay, *G. graminis* var. *tritici* was cultured on PDA at 25 °C and the inoculum was prepared as colonized wheat bran, dried and stored at room temperature. 2P24 and its derivatives were cultured in King'S B medium for 48 h. Wheat seeds coated with bacterial cultures (10^8 cfu/ml) were planted into autoclaved soil mixed with 0.8% (wt/wt) *G. graminis* var. *tritici* inoculum. Three weeks after germination, roots were washed to be free of soil, and take-all severity was rated on a scale of 0–4 where 0 = no visible symptoms, healthy plant; 1 = 5% of roots black; 2 = 5–25% of roots black; 3 = 25–50% of roots black; 4 = 50–100% of roots black as far as dead plant or nearly so (Ownley et al. 1992). The assay was designed as three repeats every treatment and 20 plants for each repeat. The experiment was carried out twice.

Nucleotide sequence accession number

The nucleotide sequences of the *P. fluorescens* 2P24 *pcoI* and *pcoR* genes have been deposited in the GenBank database under accession no. AF115381.

Results

Identification of QS components PcoI *and* PcoR

A genomic bank of *P. fluorescens* 2P24 consisting of 4472 clones was screened on LB medium containing the biosensor *A. tumefaciens* NTL4 (pZLR4) and X-Gal. Nine positive clones were identified based on their capacity to produce compounds of autoinducer activity in *E. coli* strain DH5α. Subcloning of one selected cosmid p83-51 narrowed the positive fragment down to a 4.5-kb *Eco*R1 fragment (Figure 1a). Sequence analysis of this fragment revealed three distinct open reading frames (ORF) (Figure 1a). Blast analysis suggested that a 576-bp ORF encoding a polypeptide of 191 amino acids with a molecular mass of 21.2 kDa is

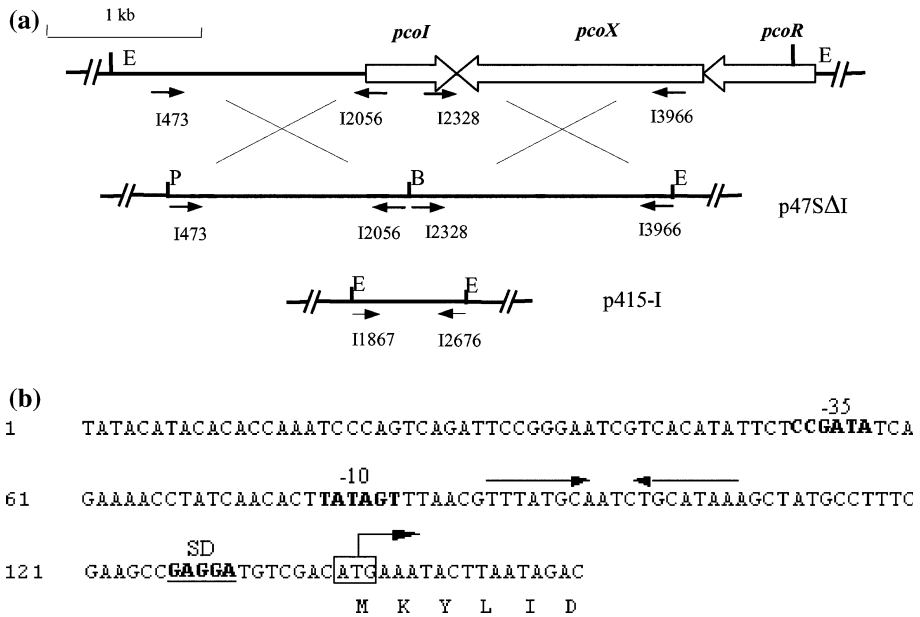

Figure 1. (a) Genetic analysis of *pcoI* gene. Hollow arrows represent the location and orientation of the genes in *P. fluorescens* 2P24 chromosome. Suicide plasmid p47SΔI containing deleted *pcoI* gene was constructed by ligating the *Pst*I-*Bam*HI and *Bam*HI-*Eco*RI fragments amplified by primer pairs I473/I2056 and primer pairs I2328/I3966, respectively. Plasimd p415-I contained intact *pcoI* gene via PCR with primers I1867 and I2676. The solid triangles represent the location and orientation of primers used in this study. Restriction enzyme abbreviations: E, *Eco*RI; P, *Pst*I; B, *Bam*HI. (b) Nucleotide sequence analysis of the 5′ upstream region of *pcoI*. The putative −10 and −35 promoter sequences are in bold. Two convergent arrows above the 19-bp inverted repeats indicate a possible PcoR-binding site. The putative Shine–Dalgarno (SD) sequences is denoted with a bold line. The predicted start codon is framed and the arrow shows the orientation of transcription.

responsible for biosynthesis of the autoinducer. The deduced PcoI protein showed high level similarity to members of LuxI family, with 89% identity to MupI of *P. fluorescens* NCIMB 10586, in which a QS system regulates the biosynthesis of a polyketide antibiotic mupirocin (El-Sayed et al. 2001). The deduced PcoI protein also shows 56% identity to LasI of *P. aeruginosa* PA01 (Passador et al. 1993; Pearson et al. 1994) and 53% identity to PpuI of *P. putida* IsoF (Steidle et al. 2002). The deduced PcoI contains all the 10 amino acids conserved in members of LuxI family (Parsek et al. 1997). The *pcoI* gene was preceded by a putative promoter at −35 (CCGATA) and −10 (TATAGT) and a potential ribosome binding site GAGGA. A 19-bp perfect inverted repeat element was found at 30 bp upstream of the putative start codon of PcoI (Figure 1b), which does not show any similarity to known *lux* box sequences. Whether this inverted repeat sequence is the binding site of the putative regulator PcoR (see below) is currently unknown.

Another ORF, *pcoR*, divergently oriented with respect to *pcoI*, was located 1573 bp downstream of *pcoI* and was predicted to encode a polypeptide of 234 amino acids. The deduced PcoR protein showed high similarity to members of LuxR family, with 82% identity to MupR (El-Sayed et al. 2001), 40% identity to PpuR (Steidle et al. 2002) and 36% identity to LasR (Gambello and Iglewski 1991). PcoR contains all of the seven highly conserved amino acids among LuxR homologs. However, no obvious promoter region was found in the region upstream of *pcoR*.

A further ORF, temporarily named *pcoX*, lies in the intergenic region between *pcoR* and *pcoI* and divergently orientated with respect to *pcoI*. This ORF encodes a 521-amino-acid protein with 83% identity to MupX of *P. fluorescens* NCIMB 10586, a gene encoding a putative amidase or hydrolase (Nardini and Dijkstra 1999; Carl et al. 2004; Cooper et al. 2005).

PcoI *directs the biosynthesis of several AHLs*

To characterize the AHLs synthesized by *P. fluorescens* 2P24, we used the biosenser strain *A. tumefaciens* NTL4 (pZLR4), which allows the detection of multiple AHLs with different *N*-linked side chain lengths and chemical modifications at the C3 position (Cha et al. 1998). TLC overlays were performed with crude AHL extracts from wild type strain 2P24 and its derivatives (Figure 2). Three major AHLs and three weak signals were readily detected from culture supernatants of the wild type strain 2P24, in which two rapidly moving spots co-migrated with the synthetic 3-oxo-C6-HSL and 3-oxo-C8-HSL, respectively. None of these AHL species was detected in supernatants from the growth of the *pcoI* deletion mutant 2P24ΔI. The *pcoI* gene complemented strain 2P24-I produced a similar AHL signal profile as the wild type 2P24 (Figure 2). These results indicated genetically that *pcoI* is a functional AHL biosynthase gene responsible for the synthesis of at least six different AHL signals. AHL signals were also detected from *E. coli* DH5α harboring the *pcoI* gene, indicating that *pcoI* from *Pseudomonas* is functional under the *E. coli* genomic background (Figure 2). A similar observation was reported in the cloning of the *luxI* homolog *ppuI* from *P. putida* strain IsoF (Steidle et al. 2002). The above data demonstrate the existence of a LuxI–LuxR family QS system in strain 2P24 and that the *pcoI* gene directs the biosynthesis of all the AHL signals detected in our assay system.

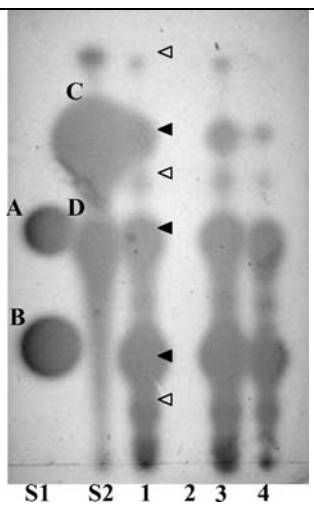

Figure 2. TLC analysis of AHLs produced by the wild-type 2P24 (lane 1), the *pcoI* mutant 2P24ΔI harboring pRK415 (lane 2), 2P24ΔI harboring plasmid p415-I (*pcoI*+) (lane 3), and *E. coli* DH5α harboring p415-I (*pcoI*+) (lane 4). The spots represent the AHLs detected by biosensor *A. tumefaciens* NTL4 (pZLR4). Three major signals produced by 2P24 are marked with closed triangles, three minor ones are marked with open triangles. S1, 3-unsubstituted-acyl-HSL standards; S2, 3-oxo-acyl-HSL standards. A, C_6-HSL; B, C_8-HSL; C, oxo-C_6-HSL; D, oxo-C_8-HSL.

Effect of pcoI *gene on secondary metabolites production*

Our analyses indicate that production of hydrogen cyanide, protease and siderophore(s) was indistinguishable among the wild type 2P24, *pcoI* mutant 2P24ΔI and the complemented mutant 2P24-I (Table 2). Since the 2,4-DAPG was regarded as the key weapon of strain 2P24 to suppress plant soilborne diseases (Wei et al. 2004b), this antibiotic was extracted from culture broth of strain 2P24, 2P24ΔI and 2P24-I, and quantified by HPLC. No significant difference in the cell density (OD at 600 nm) of each strain was observed during the culturing (data not shown). Production of 2,4-DAPG also showed no significant difference among the tested strains after growth for 48 h (Table 2). Similarly, the antifungal activity of the pseudomonads against *R. solani* grown on plates was not detectably affected by deletion of the *pcoI* gene (Table 2).

The pcoI *gene is involved in biofilm formation and plant colonization*

QS has been documented to play a key role in bacterial biofilm development in a number of genera. Our analyses of the strain 2P24 in a PVC plastic-biofilm test system points to a similar conclusion. At all time points tested, the biofilm produced by the *pcoI* mutant on PVC plastic was markedly less than that of the wild type or the complemented mutant (Figure 3). Extended incubation did not detectably result in increase in biofilm formation of the mutant. Although occasionally the biofilm produced by the complemented strain 2P24-I was more than that of the wild type 2P24, no significant difference was consistently observed between these two strains.

Table 2. Phenotypic characterization of *P. fluorescens* 2P24 and it derivatives.

Strain	Secondary-metabolites production				Antibiosis ability to plant pathogens[c]	
	Siderophore(s)[a]	HCN[a]	Proteinase[a]	2,4-DAPG(μM)[b,c]	*G. gramini* (mm)	*R. solani* (mm)
2P24	+	+	+	823.5 ± 224.9	7.1 ± 0.5	7.4 ± 0.6
2P24ΔI	+	+	+	863.5 ± 478.1	6.9 ± 0.5	7.7 ± 0.8
2P24-I	+	+	+	925.2 ± 125.5	6.9 ± 0.7	7.6 ± 0.5

[a] + represents producing secondary-metabolite.
[b] Pseudomonads strains were allowed to enter into stationary phase and there was no significant difference on the cell density (OD at 600 nm) compared to each other.
[c] Assays were performed three times in triplicate and the values represent the mean of triplicate ± SEM.

We analyzed the *pcoI* mutant for its ability to colonize the wheat rhizosphere and on the wheat tip. In autoclaved soil, the population levels in the wheat rhizosphere colonized by the 2P24ΔI (pRK415) were ten-fold lower than to those of strains 2P24 and 2P24-I (Figure 4c). Similar experiments conducted in natural soil revealed a 15-fold difference between 2P24ΔI (pRK415) and 2P24 and 2P24-I (Figure 4d). These results demonstrate that QS plays an important role in the survival of strain 2P24 on wheat roots. These observations were consistent with the previous report that PhzI–PhzR was required for rhizosphere survival of *P. aureofaciens* 30-84 (Zhang and Pierson 2001). Similar results were obtained on the wheat tip except that the overall recovered populations of the various strains were generally lower on the wheat tip than in the wheat rhizosphere (Figure 4a, b). The similar colonization characteristics and population reduction tendency of the *pcoI* defective mutant found on both root tips and rhizosphere suggests that QS exerts a stronger influence on the attachment than on the movement of *P. fluorescens* 2P24 on wheat roots.

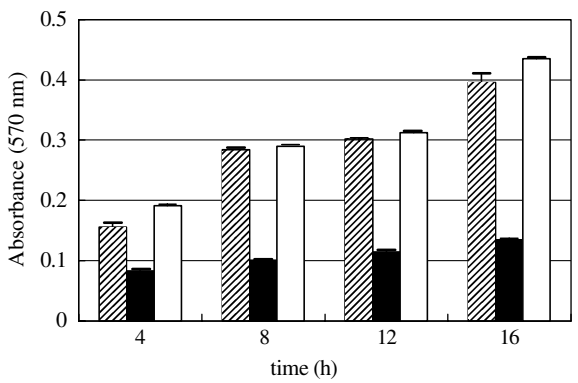

Figure 3. Quantification of bacteria in biofilm formed on PVC plastic by the wild-type 2P24, the *pcoI* mutant, and the complemented mutant. Biofilm was allowed to form in Eppendorf tubes. The tubes were incubated for 4, 8, 12 and 16 h at 28 °C. The unattached bacteria were rinsed, and the biofilms were stained with crystal violet. The residual dye was solubilized and the absorbance of strain 2P24 (▨), 2P24ΔI (pRK415) (■) and 2P24-I (□) were determined a spectrophotometrically at 570 nm. Error bars denotes standard deviation from three experiments.

Effect of pcoI *gene on biocontrol activity*

Since PcoI affects physiological fitness and competitiveness, we examined whether the QS system mutant was defective in the biocontrol ability of *P. fluorescens* 2P24 in the suppression of wheat take-all disease. Twenty-one days after planting, wheat seedlings were harvested and the roots were washed and scored for the severity of take-all by calculating disease index. The disease index of *pcoI* mutant 2P24ΔI (pRK415) treatment decreased to 46.3, which was significantly lower than that of untreated disease control (86.3). However, the disease index of the mutant was still significantly higher than that of the wild type 2P24 (26.3) and of the complemented mutant 2P24-I (27.5) (Figure 5). Thus, comparing to the wild-type strain, we observed a 35% decrease in the biocontrol efficiency of the *pcoI* mutant and that such a decrease can be fully complemented by introducing the *pcoI* gene into mutant. These results clearly show that the PcoI–PcoR QS system regulates functions contributing to the biocontrol activities of *P. fluorescens* strain 2P24.

Discussion and conclusion

In this paper we reported the identification, cloning and characterization of a LuxR–LuxI type QS

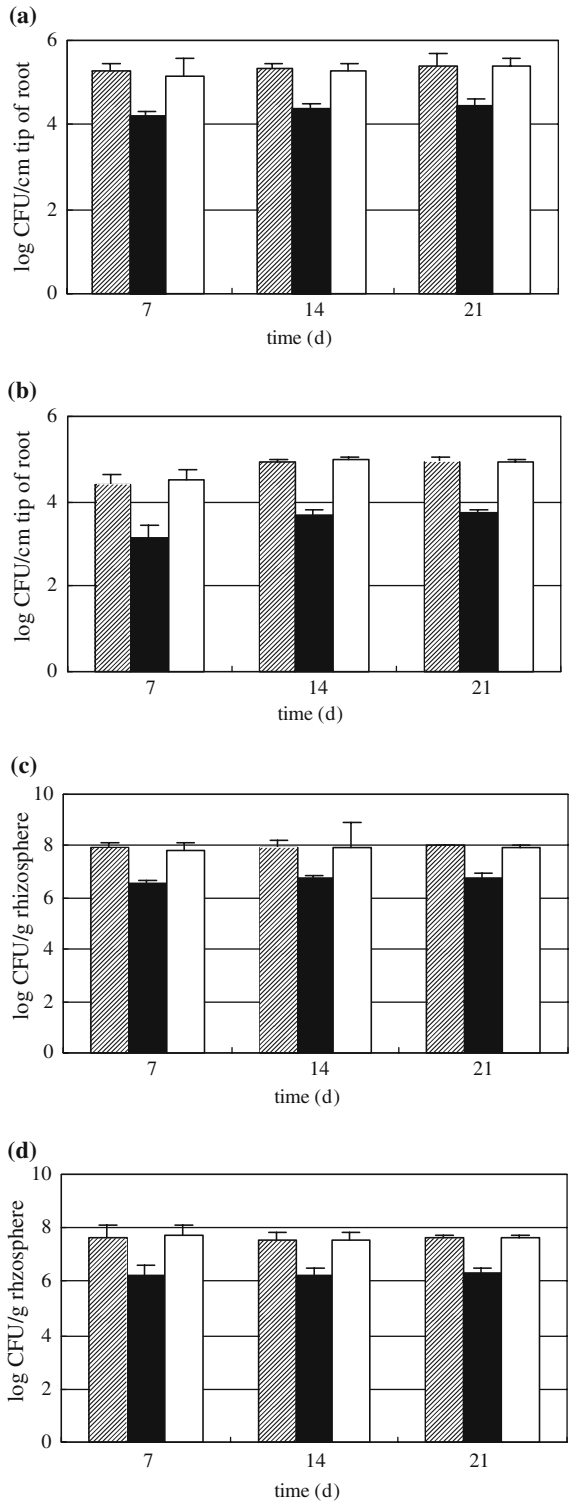

Figure 4. Colonization of wheat rhizosphere and on the tip of root by 2P24 and its derivatives in sterile and natural soils. The population of strain 2P24 (▨), 2P24ΔI (pRK415) (■) and 2P24-I (□) were determined at 7, 14 and 21 days, respectively. (a) tip of root in sterile soil; (b) tip of root in nature soil; (c) rhizosphere in sterile soil; (d) rhizosphere in nature soil. Error bars denoting standard deviation from three experiments are shown.

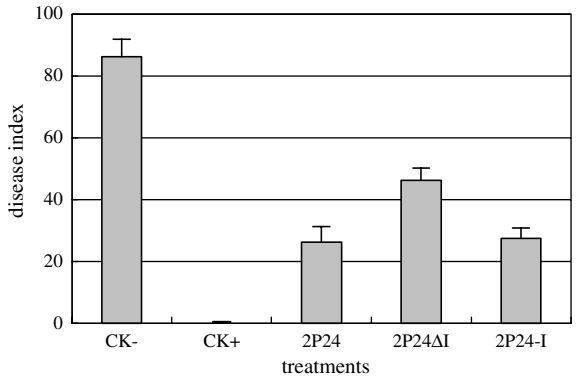

Figure 5. Suppression of 2P24 and its derivatives on wheat take-all disease in greenhouse. CK+ denotes healthy control plants without treatment with both pathogen and Pseudomonads. CK− denotes the diseased control plants only treated with pathogen. The mean values were calculated for three repeats. Error bars denoting standard deviation from three experiments are shown.

system from *P. fluorescens* strain 2P24. The putative signal receptor PcoR and the autoinducer synthase PcoI show a high similarity to QS elements of related bacterial species, with the highest similarity to the MupI–MupR protein pair of *P. fluorescens* NCIMB 10586. PcoR–PcoI clearly is involved in regulation of very different target genes or gene sets. In *P. fluorescens* NCIMB 10586, *mupI–mupX–mupR* genes locate distal to a large gene set for the biosynthesis of the polyketide antibiotic mupirocin. Reporter gene fusions and deletion analyses of *mupR* and *mupI* indicated that this QS system activates the *mup* operon and is involved in the expression of mupirocin biosynthesis pathway (El-Sayed et al. 2001). In *P. fluorescens* 2P24, however, no mupirocin has been identified so far but this strain produces 2,4-DAPG, a polyketide compound that is toxic to bacteria, fungi, nematodes, anthelminthic worms and plants (Maurhofer et al. 2004; Wei et al. 2004b). Genes for the biosynthesis of 2,4-DAPG in strain 2P24, *phlACBDE*, have been cloned (GenBank accession no. DQ083928). Studies of a *phlD* mutation suggested that 2,4-DAPG is the only antibiotic which showed antibiosis activity on plate to phytopathogenic fungi such as *G. graminis* var. *tritici*, *R. solani* and the phytopathogenic bacterium *R. solanancearum* (Wei et al. 2004b). Our analyses of the *pcoI* mutant revealed that the QS system does not detectably affect the production of 2,4-DAPG in strain 2P24 (Table 2). Taken together, these observations indicate that the two highly homologous QS systems, *pcoI–pcoR* and *mupI–mupR*, regulate some very different biological processes in pseudomonad strains NCIMB 10586 and 2P24.

pcoX, a 1530-bp ORF located between *pcoI* and *pcoR* in *P. fluorescens* 2P24, is similar to *mupX* of *P. fluorescens* NCIMB 10586, which is located between the *mupI* and *mupR* genes (El-Sayed et al. 2001). At the amino acid level, PcoX can be categorized into a class of hydrolases belonging to the family of α/β hydrolase fold proteins (Nardini and Dijkstra 1999). In *P. putida* KF715 and *P. putida* 86, one class of putative amidases, showing 27% identity to PcoX, are considered to be involved in the degradation of biphenyl and quinoline, which could be used as sole source of carbon for strain KF715 and 86 (Hayase et al. 1990; Carl et al. 2004). In *P. fluorescens* NCIMB 10586, a recent study showed that deletion mutant of *mupX* slightly affected the production of mupirocin. MupX appears to be dispensable for antibiotic biosynthesis but somehow is involved in the modulation of either the expression of the genes or the efficiency of one or more of these enzymes for the biosynthetic pathway (El-Sayed et al. 2003; Cooper et al. 2005). The biological function of PcoX and the relationship between QS and PcoX in strain *P. fluorescens* 2P24 are under active investigation.

Cis-acting components of standard QS circuits are also present in the PcoI–PcoR system. These elements are of great importance in bridging QS signals and the expression of target genes. Similar to the MupI–MupR system, a 19-bp inverted repeat sequence is present upstream of *pcoI*. Although this DNA element does not show significant similarity to the *lux* box of the LuxR–LuxI system, in *P. fluorescens* NCIMB 10586 it was regarded as a possible binding site for MupR (El-Sayed et al. 2001). However, *lux* box-like elements, mostly situated in the promoter regions of *luxI* homologs, have been shown as the binding site of signal receptors in *P. putida* IsoF (Steidle et al. 2002), *Burkholderia cepacia* (Lewenza et al. 1999) and *A. tumefaciens* (Hwang et al. 1994). In some *luxI* family members, genes coding for the AHL biosynthases are co-transcribed with the QS-regulated genes, such as the bioluminescence gene cluster in *Vibrio fisheri* (Engebrecht et al. 1983)

and the phenazine antibiotic operon in *P. aureofaciens* 30-84 (Pierson et al. 1995). Remarkably, the 19-bp palindromic sequence upstream of the *pcoI* gene in strain 2P24 locates downstream of the −10 region of the putative promoter, whereas in most characterized QS systems, such protein binding sites locate upstream of the promoter. Localization of the regulatory DNA sequence downstream of the −10 element of the promoter suggests that the binding proteins function as transcriptional repressors (Collado-Vides et al. 1991). Indeed some quorum-sensing transcriptional activators have been converted into repressors by artificially positioning the protein binding sites downstream of the −10 element of a promoter (Luo and Farrand 1999; Egland and Greenberg 2000).

At least three distinct QS signals produced by *pcoI* gene were readily detected by the *A. tumefaciens* reporter strain NTL4 (pZLR4), and other three weak signals were also recognizable on the TLC assay (Figure 2). It is known that *P. fluorescens* F113 produces C6-HSL, C10-HSL and 3-OH-$C_{14:1}$-HSL (Laue et al. 2000), and three signals, 3-OH-C6-HSL, 3-OH-C8-HSL and 3-OH-C10-HSL were detected from *P. fluorescens* 2-79 (Cha et al. 1998). *mupI*, the closest homolog of *pcoI*, directs the production of three detectable AHL signals in *P. fluorescens* NCIMB 10586 (El-Sayed et al. 2003), whereas the PcoI of strain 2P24 produces at least six different signals. These compounds may derive from incomplete degradation of the bona fide product of the synthase or by sloppy chemistry performed by the signal synthases and they may not be all biologically active. For instance, TraI from the conjugation constitutive Ti plasmid pTiC58ΔaccR directs the synthesis of more than five distinct compounds on the TLC assay, but only the 3-oxo-C8 HSL significantly stimulates expression of *tra* gene directly from the Ti plasmid (Zhu et al. 1998). Since the molecular structure of the AHLs from each strain remains unknown, it is difficult to determine which of these molecules is the cognate ligand for the predicted receptor PcoR. Clearly, determining the chemical structure of the products of the PcoI and other similar signal synthase is crucial for further understanding of the functions of these QS systems.

QS systems in Gram-negative *Proteobacteria* are involved in regulation of very diverse functions, ranging from bioluminescence, antibiotic synthesis, biofilm formation, to production of exoenzyme and siderophores (Hanzelka and Greenberg 1995; Wood et al. 1997; Lewenza et al. 1999; Steidle et al. 2002). Our results show that the QS system in *P. fluorescens* 2P24 controls biocontrol activity of this agent, but not by directly regulating the production of several metabolites, including 2,4-DAPG, HCN, siderophores and proteinases that are important for its biocontrol capacity. Nevertheless, these observations are in agreement with previous findings that synthesis of 2,4-DAPG in *P. fluorescens* CHA0 and F113 might not be under the control of QS, although the production of this compound occurs in a cell population-dependent manner (Delany et al. 2000; Schnider-Keel et al. 2000). So far no QS system has been described in *P. fluorescens* CHA0 or *P. fluorescens* Q2-87, in which 2,4-DAPG is known as a key factor against plant diseases (Brodhagen et al. 2004; Maurhofer et al. 2004). In *P. fluorescens* F113, biosynthesis of 2,4-DAPG is repressed by the binding of a pathway-specific repressor PhlF on an inverted repeated sequence, *phO* downstream of the *phlA* transcriptional start site. The antibiotic 2,4-DAPG functions as an autoinducer-like molecule that interacts with PhlF to destabilize the PhlF-*phO* complex (Abbas et al. 2002). This process is bacterial population-dependent, but not under the control of classical QS regulation. Recent studies have identified additional QS systems in related bacterial species, such as CsaI–CasR in *P. aureofaciens*, BviI–BviR in *B. cepacia* and RhlI–RhlR in *P. aeruginosa* (Brint and Ohman 1995; Lewenza et al. 1999; Zhang and Pierson 2001). It will be of great interest to investigate whether biosynthesis of 2,4-DAPG is regulated by QS system(s) distinct from the classical LuxI–LuxR family.

Our greenhouse experiments indicate that the PcoI–PcoR QS system in *P. fluorescens* 2P24 is involved in its biocontrol capacity. This is reminiscent of the function of the PhzI–PhzR system in *P. aureofaciens* 30-84, which also is important for its biocontrol activity. However, the PhzI–PhzR contributes to disease suppression at least in part, due to its positive regulation of the *phzFABCD* operon responsible for synthesizing phenazine antibiotic, a key factor for antibiosis (Wood et al. 1997). One possible role of the PcoI–PcoR QS system in biocontrol of 2P24 is its influence on the rhizosphere competence of strain 2P24 because in

both sterilized and natural soils, the rhizosphere population and root tip population of wild type strain 2P24 were significantly higher than those of the *pcoI*-minus mutant. Given the fact that microbial communities on the root surface can be viewed as a form of biofilm and biofilm formation of the *pcoI* mutant is impaired (Figure 3), it is conceivable that the PcoI–PcoR system affects the development of root attachment or colonization structures of strain 2P24.

In summary, our data show that the PcoI–PcoR system in *P. fluorescens* 2P24 plays an important role in its ability to colonize on wheat roots (Figure 4) and to suppress take-all of wheat (Figure 5). Clearly, other factors, such as production of the antibiotic 2,4-DAPG are also important in this process. Further analysis of the target genes regulated by PcoR–PcoI as well as the relationship between this QS system and other regulatory systems involved in biocontrol capacity, such as the GacS-GacA two-component system, should provide insight into the understanding of signal cascades leading to the disease suppression phenotype.

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