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OmpR Regulates the Intracellular Concentration of 2,4-Diacetylphloroglucinol by Affecting the Transcriptional Activity of the Hydrolase *phlG* in *Pseudomonas fluorescens* 2P24

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The broad-spectrum polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) is a major determinant of the ability of the biocontrol agent *Pseudomonas fluorescens* 2P24 to exert its suppressive activities towards a variety of soil-borne plant pathogens. Previously, we demonstrated that dysfunction of the transcriptional regulator OmpR of the two-component regulatory system EnvZ/OmpR resulted in elevated 2,4-DAPG production in *P. fluorescens* 2P24. Herein, we provide evidence that OmpR might regulate the transcriptional activity of the hydrolase *phlG* to influence the intracellular concentrations of 2,4-DAPG. The OmpR deletion mutant PM1102 showed increased transcriptional activity (60%) at the promoter of the *phlACBD* locus and elevated intracellular concentrations (two-fold) of 2,4-DAPG. A subsequent promoter assay for *phlH* in PM1102 and for *phlG* in PM1103 (*phlH*$^-$) showed a negative regulatory relationship for *ompR* on *phlH* and for *phlH* on *phlG*. Additionally, we used EMSA to establish the direct binding of OmpR to the ompR promoter region and the indirect regulatory relationship of OmpR on *phlACBD* and *phlH* transcription. Based on these findings, we propose that OmpR influences the intracellular concentrations of 2,4-DAPG by regulating the transcriptional activity of the hydrolase *phlG* in *P. fluorescens* 2P24.

Key words: *Pseudomonas fluorescens*, 2,4-diacetylphloroglucinol (2,4-DAPG), OmpR, PhlG.

To survive in the fiercely competitive rhizosphere environment, some plant root-associated fluorescent pseudomonads are equipped with diverse secondary metabolites, such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide, phenazine, pyoluteorin, and pyrrolnitrin, which can antagonize hostile micro-organisms. Furthermore, they are endowed with outstanding nutritional versatility and robust adaptability[1,2]. Therefore, some *Pseudomonas fluorescens* strains are exploited as biocontrol agents (BCA) to protect crops from various soil-borne phytopathogens, such as *Gaeumannomyces graminis*, *Pythium ultimum*, *Thielaviopsis basicola*, *Rhizoctonia solani*, and *Ralstonia solanacearum*[3]. The polyketide compound 2,4-DAPG has been shown to be a critical ingredient for fluorescent pseudomonads to carry out their biocontrol functions in the *P. fluorescens* strains Q2-87, F113, CHA0, and 2P24[4-8]. The *phl* gene cluster is composed of eight ORFs, designated *phlH*, *phlG*, *phlF*, *phlA*, *phlC*, *phlB*, *phlD*, and *phlE*[9,10]. Among them, four structural genes (*phlA*, *phlC*, *phlB*, and *phlD*) are responsible for the biosynthesis of 2,4-DAPG, as well as the putative permease gene *phlE* that is jointly transcribed, whereas the transcription repressor gene *phlF* is transcribed in an opposite orientation. The hydrolase gene *phlG* and the TetR-like family

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regulator gene *phlH*, which is adjacent to *phlF*, are divergently transcribed[11-13].

In recent decades, intensive study of the regulatory mechanism of 2,4-DAPG production has revealed a number of environmental and genetic factors that influence the biosynthesis of 2,4-DAPG. The effects of environmental factors on 2,4-DAPG production are complex. In strain F113, the production of 2,4-DAPG can be elevated by sucrose and fructose, and can be inhibited by glucose and sorbose. However, in strains Pf-5 and CHA0, glucose promotes the expression of genes in the *phlACBD* cluster[14-16]. In strain CHA0, some cations, including Zn^{2+}, Cu^{2+}, and Mo^{2+}, can improve the yield of 2,4-DAPG, and this could also be promoted by Fe^{3+} in strain F113. To achieve a maximum yield of 2,4-DAPG, the optimal temperature for 2,4-DAPG accumulation is 12ºC in strains 2P24 and F113[7,14,16]. The biosynthesis of 2,4-DAPG is auto-induced, but can also be strongly repressed by the bacterial extracellular metabolites salicylate and pyoluteorin, as well as by the fungal metabolite fusaric acid[6]. Recent studies have revealed a number of transcriptional and post-transcriptional regulatory factors, including the transcription repressor PhlF[6], the two-component regulatory system (TCS) GacS/GacA[9], snRNA[17], the H-NS family of regulators MvaT and MvaV[18], the hydrolase PhlG[12], the oxidoreductase DsbA[19], the sigma factors RpoD, RpoN, and RpoS[20], the sigma regulator PsrA[21], the resistance-nodulation-division (RND) efflux pump EmhABC[3], and the chaperone Hfq[22].

To screen for novel regulators of 2,4-DAPG production, strain 2P24 was subjected to random Tn5 insertional mutagenesis using the plasmid pUT-Km[23]. The color of mutant sesu-25 colonies appeared to be deep red as a consequence of increased production of an unknown pigment, which is a characteristic phenotype that has been linked to 2,4-DAPG production[4,24]. Sequence analysis revealed that the transcriptional regulator OmpR of the EnvZ/OmpR TCS, one of the most extensively studied TCSs[25], is disrupted in the mutant sesu-25, implying that EnvZ/OmpR TCS might negatively regulate 2,4-DAPG production in the *P. fluorescens* strain 2P24[26].

A typical EnvZ/OmpR TCS is composed of a sensor kinase, EnvZ, and its cognate response regulator, OmpR[27,28]. EnvZ monitors deviations in environmental osmolarity. Upon sensing increased osmolarity, EnvZ auto-phosphorylates and transfers the phosphate group of EnvZ-P to OmpR, forming phosphorylated OmpR (OmpR-P). OmpR-P then binds to the promoter regions of target genes to regulate their expression. EnvZ can also act as a phosphatase that dephosphorylates OmpR-P. Thus, the function of OmpR is "tuned" by modulating the ratio of kinase-to-phosphatase of EnvZ in response to osmolarity changes[29,30]. It has been well-established that EnvZ/OmpR plays a central role in mediating signal transduction in response to osmotic stress in certain strains[25,31]. In some bacteria, not only the secretion of proteases and lipases, swarming, expression of flagellin, and regulation of type III and type VI secretion systems, but also of other TCSs and snRNAs are regulated by the EnvZ/OmpR TCS[32-35].

Herein, we aimed to explore the potential pathways by which the EnvZ/OmpR TCS, a novel regulator involved in 2,4-DAPG biosynthesis, influences 2,4-DAPG production in *P. fluorescens* 2P24. We demonstrate that the transcriptional regulator OmpR negatively influences 2,4-DAPG biosynthesis at the transcriptional level, and exerts positive effects on the hydrolase PhlG and negative effects on the TetR-like family regulator PhlH in *P. fluorescens* 2P24.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study were described in Table 1. *P. fluorescens* strains were grown in Luria-Bertani (LB) broth or King's B[36] at 28°C. *E. coli* DH5α, which was used for cloning experiments, was grown in LB broth at 37°C. When necessary, growth medium was supplemented with X-gal (40 μg ml^{-1}), ampicillin (50 μg ml^{-1}), gentamycin (30 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (20 μg ml^{-1}), or chloramphenicol (20 μg ml^{-1}).

DNA manipulation and sequencing

Plasmid DNA extraction and other molecular assays were performed according to standard procedures[37] or protocols recommended by the respective manufacturer. Electroporation of bacterial cells with plasmid DNA was performed as described previously[8]. Oligonucleotide primers for

PCR amplification are listed in Table 2. DNA sequencing was performed by Genewiz Biotechnology and was analyzed using the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST).

Construction and complementation of *envZ*, *ompR*, and *phlH* deletion mutants

Individual deletion mutants of the *envZ*, *ompR*, and *phlH* genes were constructed using a two-step homologous recombination strategy. The in-frame deletion structures of these genes were amplified (primers listed in Table 2) and ligated into plasmid pHSG299 (TaKaRa) to generate the p299ΔenvZ, p299ΔompR, and p299ΔphlH plasmids (Table 1), respectively. Allelic exchange using these plasmids with the wild-type strain, 2P24, generated the mutants PM1101 (*envZ*$^-$), PM1102 (*ompR*$^-$), and PM1103 (*phlH*$^-$), and all deletion mutants were confirmed by PCR amplification and sequencing (data not shown).

Complementary plasmids for the *envZ*, *ompR* and *phlH* deletion mutants were constructed using the shuttle vector pRK415[38] and were termed p415-envZ, p415-ompR, and p415-phlH (primers listed in Table2 and plasmids listed in Table 1), respectively.

Construction of *ompR::lacZ*, *phlG::lacZ*, and *phlH::lacZ* transcriptional fusion reporters

To construct plasmid-borne reporters, the promoter regions of *ompR* and *phlG* (*phlH* and *phlG* are inversely transcribed and share a common promoter region) were amplified by PCR using the primers ompRproF/ompRproR and phlGproF/phlGproR (Table 2) and then were cloned ahead of a promoter-less *lacZ* allele in pRG970Km[40]. The resulting plasmids, p970K-ompR, p970K-phlG, and p970K-phlH, were used for *ompR*, *phlG*, and *phlH* promoter analysis, respectively.

Extraction and detection of the antibiotic 2,4-DAPG

P. fluorescens 2P24 and its derivatives were cultivated with shaking in 100-ml flasks containing of 30 ml liquid KB medium at 140 rpm and 28°C; 2,4-DAPG production was quantified by HPLC using the method described by Shanahan[14]. Extracellular 2,4-DAPG was extracted from supernatants after centrifugation, and cell-associated 2,4-DAPG was extracted from lysed cells that were prepared using a freeze-thaw procedure.

β-Galactosidase activity assay

For β-galactosidase measurements, *P. fluorescens* strains were grown with shaking in 100-ml flasks containing 30 ml liquid medium at 140 rpm and 28°C. Cultures were sampled at different time points and β-galactosidase-specific activities were quantified using the Miller method[41].

Electrophoretic mobility shift assay (EMSA)

EMSA was performed according to the method described by Zhang with some modifications[35]. The upstream DNA fragments of the *ompR*, *phlA*, and *phlG* (*phlH*) sequences were amplified by PCR using the respective primer sets (Table 2). DNA fragments were purified using a QIAquick gel extraction kit (Qiagen). The reaction mixture (20 ml) contained 20 mM Tris/HCl (pH 7.4), 4 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.5 mg poly(dI:dC), 100 ng BSA, 200 ng DNA probes, and 0-400 ng OmpR protein. For the phosphorylation of OmpR, 100 mM acetyl phosphate (acetyl-P, Sigma) was added to the reaction mixture. OmpR phosphorylation and binding to probes were performed at 37°C for 1 h. As a negative control, a DNA fragment from the *phlC* coding region amplified with the primers phlC297F and phlC297R was included in the binding assays. Samples were then loaded onto a 7.0% native polyacrylamide gel. Electrophoresis was carried out in 0.5× TBE buffer for 4 h on ice. DNA bands were visualized by staining gels with ethidium bromide.

RESULTS

OmpR negatively influences 2,4-DAPG production at the transcriptional level

Previously, we showed that strain Sesu-25, a mini-Tn5 transposon insertion mutant of *ompR* of the *P. fluorescens* strain, 2P24, showed increased (2.2-fold) 2,4-DAPG production, which implied that EnvZ/OmpR TCS might be involved in the regulation of 2,4-DAPG biosynthesis in strain 2P24. To determine the influence of the EnvZ/OmpR TCS on 2,4-DAPG production in strain 2P24, the *envz* in-frame deletion mutant PM1101 and the *ompR* in-frame deletion mutant PM1102 were constructed using a two-step homologous recombination strategy to avoid of any polar effects of transposons. The production of 2,4-DAPG in the PM1101, PM1102, and 2P24 (parental) strains

Table 1. The strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5?	F- recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 (lacZYA-argF) U169λ- (ϕ80dlacz M15)	This lab
P. fluorescens		
2P24	Wild-type, Apr, Cm^r	8 Wei and Zhang, 2005
PM901	A chromosomal phlA::lacZ fusion reporter gene in strain 2P24	3 Tian et al., 2010
PM1101	envZ in-frame deletion in strain 2P24, Apr, Cm^r	This study
PM1102	ompR in-frame deletion in strain 2P24, Apr, Cm^r	This study
PM1103	phlH in-frame deletion in strain 2P24, Apr, Cm^r	This study
Plasmids		
pBluescript II SK(+)	Cloning vector, Ap^r	Stratagene
pBS-Fa10	pBluescript II SK(+) containing 4.5 kb SalI fragment (incomplete phlA; complete phlF, phlG and phlH), ApR	This study
pHSG299	ColE1 origin, cloning vector, Kmr	TaKaRa
p299 envZ	Suicide plasmid containing deleted envZ on pHSG299, Km^r	This study
p299 ompR	Suicide plasmid containing deleted ompR on pHSG299, Km^r	This study
p299 phlH	Suicide plasmid containing deleted phlH on pHSG299, Km^r	This study
pRK415	Escherichia-Pseudomonas shuttle vector, Tc^r	38
p415-envZ	EnvZ was cloned downstream of the lac promoter in pRK415, Tc^r	This study
p415-ompR	ompR was cloned downstream of the lac promoter in pRK415, Tc^r	This study
p415-phlH	phlH was cloned downstream of the lac promoter in pRK415, Tc^r	This study
pRG970	Cloning vector containing promoter-less lacZYA for constructing transcriptional fusions, Sm^r	39
pRG970Km	A derivative plasmid of pRG970, Km^r	40
p970K-ompR	pRG970Km containing a ompR::lacZ transcriptional fusion, Km^r	This study
p970K-phlA	pRG970Km containing a phlA::lacZ transcriptional fusion, Km^r	3 Tian et al., 2010
p970K-phlG	pRG970Km containing a phlG::lacZ transcriptional fusion, Km^r	This study
p970K-phlH	pRG970Km containing a phlH::lacZ transcriptional fusion, Km^r	This study

was evaluated by HPLC. The production of 2,4-DAPG in the PM1101 and PM1102 strains was increased ~1.6-fold compared to that in the wild-type strain, 2P24, and these changes could be restored by complementation with the wild-type allele (Fig. 1a).

To further test whether the transcription of the 2,4-DAPG biosynthetic locus *phlACBD* was altered in the mutants, a plasmid p970K-phlA, harboring a *phlA::lacZ* transcriptional fusion, was introduced into strain 2P24 and its derivatives (PM1101 and PM1102). Transcriptional analysis of the *phlACBD* locus promoter showed that the expression of *phlA::lacZ* were increased ~60% in strains PM1101 and PM1102 (Fig. 1b). These findings indicated that the negative effect of EvnZ/OmpR on the production of 2,4-DAPG occurred at the transcriptional level.

Auto-induction of the *ompR* response regulator

By using promoter analysis software (Virtual Footprint version 3.0), we predicted that the *ompR* promoter region in strain 2P24 harbors a putative OmpR binding site (GAAAAAT), which indicated that the response regulator OmpR could be auto-induced. To test this assumption, the expression of the *ompR* promoter in PM1102 (*ompR*$^-$) and in 2P24 (wild-type) strains was measured in LB medium using an *ompR*::*lacZ* transcriptional fusion reporter (p970K-ompR). The transcription of *ompR* was significantly reduced to half of the level in strain PM1102 compared with the parental strain 2P24 at the both logarithmic- and stationary-phases (Fig. 2a).

To determine whether OmpR actually binds to the *ompR* promoter region, a 294 bp DNA fragment (ompRp) containing the *ompR* promoter region was used in a electrophoretic mobility shift assay (EMSA). As shown in Fig. 2b, when increased amounts of phosphorylated OmpR (OmpR-P) were incubated with ompRp, there an apparent step-wise probe shift, but the 300 bp control DNA (phlAp) amplified from the promoter region of *phlACBD* locus did not show any detectable shift. This finding suggests the direct

Table 2. Oligonucleotides used in this study. Specified restriction sites are underlined

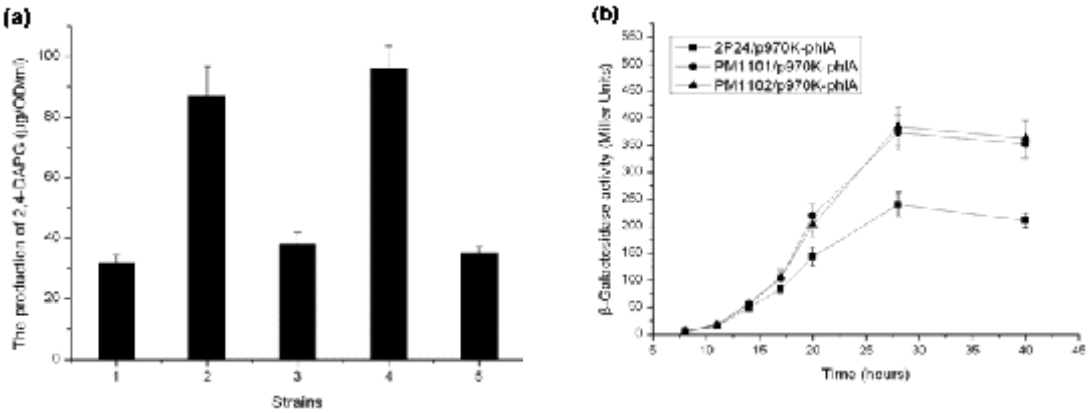
Primer	Sequence (5^1-3^1)	Restriction enzyme
ompRdel-SalF	ATGTCGACGTTGATAGATCTCGCTGTAG	*Sal*I
ompRdel-BamR	ATGGATCCTCAAGGCCCTGGTGATGA	*Bam*HI
ompRdel-BamF	ATGGATCCATCTGCTCGGTGTTC	*Bam*HI
ompRdel-Sac	ATGAGCTCAGGTACAAGTCTTCGAGGC	*Sac*I
envZdel-SalF	ATGTCGACCACCACCAGCAAGAAC	*Sal*I
envZdel-BamR	ATGGATCCGATGAGAAAGTGCACCTGC	*Bam*HI
envZdel-BamF	ATGGATCCTGCATCTGGCGTTGATAG	*Bam*HI
envZdel-SacR	ATGAGCTCCAACAACCAGATCCCGATC	*Sac*I
phlH del-PstF	GTACTGCAGGGGTTGAGTCTCGAG	*Pst*I
phlH del-HinR	CAGAAGCTTTACACTGGTGGG	*Hin*dIII
envZcom-BamF	TGAGGATCCTGCAAGTGTGTAGCGGTT	*Bam*HI
envZcom-EcoR	CAGGAATTCGGTCTCAGCC AGACTGGGAT	EcoRI
ompRcom-PstF	GTACTGCACGTATAACGGCAGCTAGGC	PstI
ompRcom-EcoR	CAGGAATTCGAATAACCGCTACACACTTG	EcoRI
phlHcom-XbaF	AGCTCTAGACGACGATGATGATGGCGG	XbaI
phlHcom-SalR	GTTGTCGACGGACAATGACGGGACC	SalI
ompRproF	TGAGGATCCGGCTGTTGATGCTGTCCAT	*Bam*HI
ompRproR	TGAGGATCCCCCTTGATACGGCTCAGTT	*Bam*HI
phlGproF	TGAGGATCCGCCGGCAAGTTCA	*Bam*HI
phlGproR	TGAGGATCCAATGGATGCCACATG	*Bam*HI
phlpro300F	TGAGGATCCAGAATCTGCAGCCGCGTTG	*Bam*HI
phlpro300R	TGAGGATCCTGTTGATTGGCCAGGAACTGC	*Bam*HI
opmR294F	TGGCATGCGCCGCTGC	
opmR294R	TGCTGCTCATAGGAAGCTCCTTTGATCTC	
phl297F	ATACGACATCGTTCTGTGTGGC	
phl297R	CATGCTTCGCTGCTTTTGAG	

binding of this protein to the *ompR* promoter region, which was consistent with the observations of the previous report[42].

Binding to the promoter regions of target genes is a fundamental strategy whereby OmpR regulates target gene expression[29,30]. As no direct interactions have been detected between OmpR and the promoter region of the *phlACBD* locus (Fig. 2b), it can be speculated that the influence of OmpR on *phlACBD* expression might be indirect.

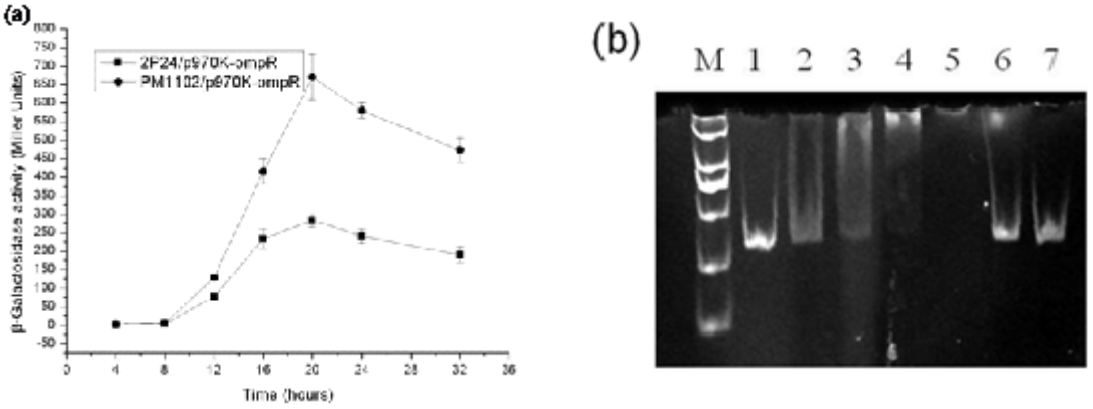
The positive influence of OmpR on the hydrolase gene *phlG*

To investigate the possible pathway whereby OmpR influences the production of 2,4-DAPG, the effects of OmpR on a variety of regulatory factors, such as Gacs/GacA, Hfq, sRNA,



(a) Analysis of 2,4-DAPG accumulation by *P. fluorescens* 2P24 and its derivatives. The numbers 1~5 of x axis represent the strains 2P24/p415, PM1101/p415, PM1101/p415-envZ, PM1102/p415, PM1102/p415-ompR. Extractions were made from at least 3 independent cultures in 30 ml of KB medium, and the 2,4-DAPG production level was determined by HPLC. (b) Expression of the plasmid transcriptional *phlA::lacZ* fusion in *P. fluorescens* 2P24, PM1101, and PM1102. β-galactosidase activity of each strain were measured in a time course after inoculation into 30-ml KB medium. The error bars indicate the standard deviations

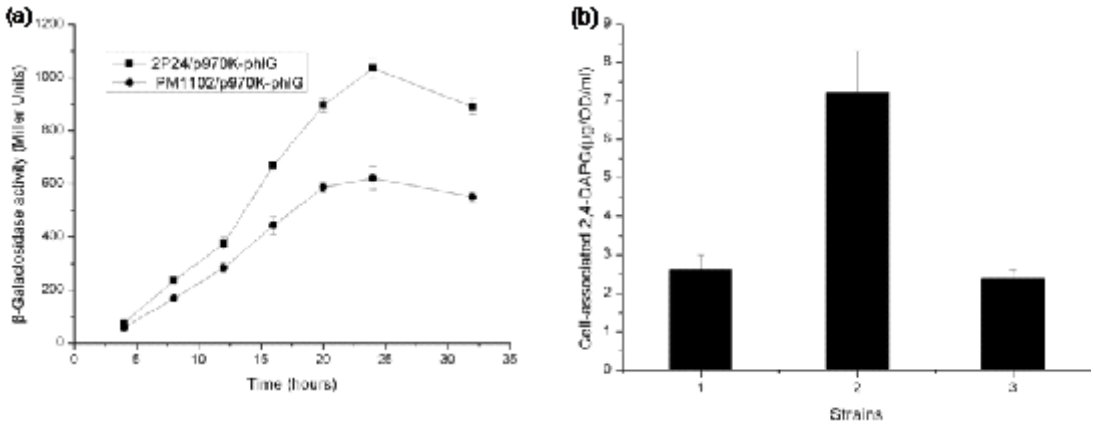
Fig. 1. EnvZ/OmpR negatively regulated the production of 2,4-DAPG at a transcriptional level



(a) Expression of the plasmid transcriptional *ompR::lacZ* fusion in *P. fluorescens* 2P24 and PM1102. β-galactosidase activity of each strain was measured in a time course after inoculation into 30-ml LB medium. The error bars indicate the standard deviations. (b) EMSA was carried out to analyze interactions between phosphorylated OmpR (OmpR-P) and *ompR* promoter (ompRp), *phlA* promoter (phlAp), and *phlG*/*H* promoter (phlGHp). Lane M: molecular weight standard; Lanes 1~4: ompRp with increasing quantities of OmpR-P (0, 100, 200, and 400 ng); Lane 5: 400 ng ompRp; Lane 6: phlAp with 400 ng OmpR-P; Lane 7: phlGHp with 400 ng OmpR-P. DNA fragments and OmpR-P were incubated for 2 h at room temperature and assayed for an electrophoretic mobility shift

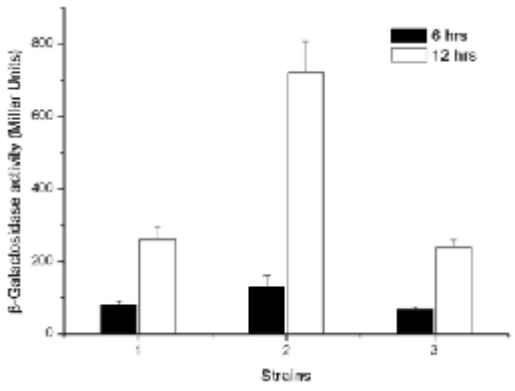
Fig. 2. Autoregulation of *ompR* expression

PhlF, and the resistance-nodulation-division (RND) efflux system EmhR-EmhABC, which are known elements that influence 2,4-DAPG production in strain 2P24[3,22], were tested using a promoter activity assay. When we introduced promoter transcriptional activity reporter plasmids for these regulators, no obvious differences in the transcriptional activity of these reporters were observed between the mutant PM1102 and wild-type 2P24 strains (data not shown). In the BCA *P. fluorescens* strain CHA0, the hydrolase PhlG specifically degrades 2,4-DAPG to MAPG[12]. The *phl* gene clusters are conserved among known 2,4-DAPG-producing fluorescent *Pseudomonas* spp., and PhlG carries out a similar function in strain 2P24[43,44]. Hence, the influence of OmpR on the transcriptional activity of *phlG* was investigated by measuring β-galactosidase activity of the *phlG*



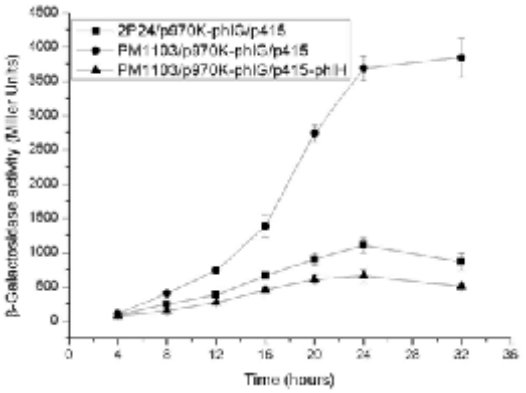
(a) Influence of OmpR on the transcription of *phlG::lacZ* fusion in *P. fluorescens* 2P24 and its *ompR* mutant PM1102. β-galactosidase activity of each strain were measured at various time points after inoculation into 30-ml LB medium. (b) Intracellular accumulation of 2,4-DAPG in *P. fluorescens* 2P24 and its derivatives. Bacterial cells were sampled at 24 hrs after inoculation. Cell-associated 2,4-DAPG was extracted from freeze-thaw cell lysates and quantified by HPLC. The numbers 1~3 of x axis represent the strains 2P24/p415, PM1102/p415, PM1102/p415-ompR, respectively. All experiments were performed in triplicate, and the error bars indicate the standard deviations

Fig. 3. OmpR positively influences the expression of hydrolase gene *phlG*



β-galactosidase activity of each strain were measured at 6hrs and 12hrs after inoculation into 30-ml LB medium. The numbers 1~3 of x axis represent the strains 2P24/p415, PM1102/p415, PM1102/p415-ompR. All experiments were performed in triplicate, and the error bars indicate the standard deviations

Fig. 4. OmpR negtively influences the expression of TetR-like regulator PhlH



β-galactosidase activity of each strain were measured in a time course after inoculation into 30-ml LB medium. The error bars indicate the standard deviations.

Fig. 5. PhlH controls of *phlG* expression in *P.fluorescens* 2P24

promoter fused to a *lacZ* gene. We found that the transcriptional level of phlG was reduced by ~45% in strain PM1102 compared to the parental strain 2P24 (Fig. 3a). Considering the 2,4-DAPG degrading function of PhlG, we inferred that the positive regulation of OmpR on the transcription of the *phlG* might result in elevated levels of intracellular 2,4-DAPG in strain PM1102 (*ompR*$^-$). Therefore, cell-associated 2,4-DAPG production in strain 2P24 and mutant PM1102 was assessed. As expected, the intracellular levels of 2,4-DAPG were approximately two-fold higher in strain PM1102 compared to the wild-type strain 2P24 in the stationary phase (Fig. 3b).

In stain 2P24, *phlG* is adjacent to *phlH*, but they are separated by a putative promoter region (108 bp), and are divergently transcribed[7]. Using Virtual Footprint, no probably binding site could be predicted in this promoter region (phlGHp). The interaction of phosphorylated OmpR (OmpR-P) with phlGHp was assessed by EMSA, no obvious retarded mobility of the probe could be detected (Fig. 2b), which implies that the influence of OmpR on PhlG might be indirect.

Negative control of the TetR-like regulator PhlH by OmpR

The expression of *phlG* was negatively controlled by the TetR-like regulator PhlH in the *P. fluorescens* strain CHA0[12], it has been speculated that in the mutant PM1102 (*ompR*$^-$), the reduced expression of *phlG* might be a consequence of the increased levels of PhlH. The promoter transcriptional activities of *phlH* in strain 2P24 and its derivative PM1102 were determined in LB medium using an *phlH*::*lacZ* transcriptional fusion reporter (p970K-phlG). The transcription levels of *phlH* were significantly elevated (two-fold) both from the exponential phase (6 h) and from stationary phase (12 h) in the *ompR* deletion strain PM1102 compared to the levels in the wild-type 2P24 strain (Fig. 4).

The expression of *phlG* was tested in the *phlH* in-frame deletion mutant PM1103 and in the PhlH-overexpression strain (2P24/415phlH, 2P24 with several copies of *phlH*) to verify the influence of the *phlH* gene on the *phlG* gene in strain 2P24. Additionally, β-galactosidase activity from the plasmid carrying the *lacZ* gene fused to the *phlG* promoter was increased approximately three-fold in strain PM1103 (*phlH*$^-$), and the expression of phlG was reduced by ~40% in the PhlH over-producing strain, compared with that in the wild-type strain 2P24 (Fig. 5). Our findings were confirmed the negative control of *phlG* by *PhlH* in the 2P24 strain. Considering that *phlG* shares a common promoter region with *phlH* and that indirect interactions occur between OmpR and *phlG*, we deduced that the effects of OmpR on phlH could be indirect.

DISCUSSION

P. fluorescens strains are well-known to produce a variety of secondary metabolites, including phenazines, siderophores, pyrrolnitrin, pyoluteorin, and 2,4-DAPG[1,45]. Among them, the polyketide antibiotic 2,4-DAPG has been shown to be critical for biocontrol by some fluorescent pseudomonads to protect crops against a variety of root and seedling phytopathogens, including fungi, bacteria, helminthes, and nematodes; moreover, the inhibition of fungal growth is caused by the impairment of mitochondrial function[43,45]. It has been demonstrated that the elevated expression of 2,4-DAPG can, to a certain degree, enhance plant disease suppression capacity in strains 2P24 and CHA0[7,46]. However, overproduction of 2,4-DAPG could be harmful to bacterium itself and to root development in the host plant as a consequence of its phytotoxic properties[47-49]. It has been proposed that 2,4-DAPG might play a role in the intimate interaction between *P. fluorescens* and certain plants[10]. Carefully regulating the expression of 2,4-DAPG could be critical for fluorescent pseudomonads to colonize the complex rhizhospheric micro-ecosystem and carry out their biocontrol functions[50].

In the past decade, intensive studies have focused on the regulatory mechanism for the biosynthesis of 2,4-DAPG in biocontrol fluorescent pseudomonads. To date, a variety of transcriptional and post-transcriptional regulators have also been reported to participate in the regulation of 2,4-DAPG biosynthesis, in which the production of 2,4-DAPG is carefully calibrated[21,50]. Notably, the transcriptional regulators PhlF and PsrA directly bind to the regulatory sequence of the promoter region of the *phlACBD* locus and repress transcription[21,49,51,52]. The repressive effect of PhlF can be relieved by interactions with 2,4-DAPG, but

not for interactions with PsrA[6,21]. Moreover, the transcription levels of the genes encoded by the *phlACBD* locus are influenced by the sigma factors RpoD/N/S[20], the H-NS family regulators MvaT/V[18], and the oxidoreductase DsbA[19], as assessed by direct or indirect approaches. Furthermore, the RNA-binding proteins RsmA/E can interact with *phlACBD* mRNA, resulting in repression of translation that influences 2,4-DAPG biosynthesis. Small noncoding RNAs (snRNA), RsmX/Y/Z, can capture these small RNA-binding proteins and sequester its repressive effect on the translation of *phlACBD* mRNA[17]. Meanwhile, the activity of snRNAs are regulated by a number of regulatory elements, including GacS/A TCS[53], the integration host factor IHF, the sigma regulator PsrA, the global regulator RetS[54], the chaperone Hfq[22], and the sensor kinase LadS[17]. Moreover, intracellular 2,4-DAPG can be degraded to monoacetylphloroglucinol (MAPG) and acetate by the hydrolase PhlG, and can be effluxed by the pump EmhABC. The expression of *phlG* is positively controlled by GacS/GacA and can be negatively regulated by PhlF and PhlH; however, the regulatory mechanism of EmhABC remains unclear[3,12].

In this present study, we provide evidence that EnvZ/OmpR TCS negatively influences 2,4-DAPG production at the transcriptional level in the *P. fluorescens* strain 2P24 (Fig. 1a, b), which expands the regulatory profile of the EnvZ/OmpR TCS. As a well-studied regulatory element, the EnvZ/OmpR TCS has been shown to regulate many important bacterial physiological activities, including the secretion of proteins and lipases, swarming, flagellin expression, and the type III secretion system[32-34]. Additionally, some novel traits of this TCS have been recently characterized. In *E. coli*, MzrA, a type II membrane protein, specifically interacts with EnvZ, resulting either enhanced EnvZ kinase activity or reduced phosphatase activity, thereby affecting the steady state levels of OmpR-P[55]. In the *Streptomyces venezuelae* strain ISP5230, the OmpR-type atypical response regulator JadR1 can activate the transcription of jadomycin B (JdB) biosynthetic genes, while simultaneously repressing its own expression[56]. In *Salmonella enterica* serovar Typhi GIFU10007, OmpR positively regulates the YehU/YehT TCS by binding to the upstream region of the *yehU* gene under hypotonic growth conditions[57]. Moreover, OmpR can directly bind to the promoter of the Type VI secretion system operon, thereby influencing bacterial survival under acidic conditions and regulating its expression in the enteric pathogen *Yersinia pseudotuberculosis*[35].

Binding to the promoter region of target genes could be the major mechanism by which OmpR directly influences its expression. Consistent with earlier studies[42], our results verified that OmpR directly binds to the promoter region of the *ompR* gene, and auto-induces its own expression (Fig. 2a, b). However, when OmpR-P is incubated with the promoter region of *phlA*, no retarded mobility of the probe was observed (Fig. 2b), implying that the influence of OmpR on *PhlACBD* expression could be indirect. To determine the potential signal transduction pathways by which OmpR influences the biosynthesis of 2,4-DAPG in strain 2P24, the transcriptional activities of several known regulatory elements of fluorescent pseudomonads were studied. Except for the *phlG* gene, no obvious transcriptional differences in the other genes could be detected. In strain PM1102 (ompR$^-$), the expression of *phlG* was reduced by ~45%, and the intracellular levels of 2,4-DAPG were elevated about two-fold compared with that in the wild-type strain 2P24 (Fig. 3a, b). Considering the auto-induction propensity of 2,4-DAPG and the 2,4-DAPG degrading function of PhlG, we hypothesized that the knockout of *ompR* might restrain the expression of *phlG*, resulting in increased concentrations of intracellular 2,4-DAPG. A gel shift assay for the *phlG* promoter fragment indicated that the influence of OmpR on PhlG could be indirect. In *P. fluorescens* CHA0, the activity of *phlG* was tested with a positive control by the GacS and against a negative control by the PhlF and PhlH[12]. As OmpR exerts no obvious influence on the expression of GacA or PhlF in strain 2P24, it is possible that PhlH might be an intermediate in the signal transduction pathway (from EnvZ/OmpRTCS to the *phlACBD* locus). In strain 2P24, the expression of *phlG* was negatively influenced by PhlH (Fig. 5), and *phlH* was subjected to negative regulation by OmpR (Fig. 4). Together, we propose that EnvZ/OmpR TCS might fine tune the biosynthesis of 2,4-DAPG via the phlH and phlG signal transduction pathways.

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