Multiple-Level Regulation of 2,4-Diacetylphloroglucinol Production by the Sigma Regulator PsrA in Pseudomonas fluorescens 2P24

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Abstract

Background: Pseudomonas fluorescens 2P24 is a rhizospheric bacterium that aggressively colonizes the plant roots. It produces the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), which contributes to the protection of various crop plants against soil borne diseases caused by bacterial and fungal pathogens. The biosynthesis of 2,4-DAPG is regulated at the transcriptional level in the expression of the phlACBD operon as well as at the posttranscriptional level by the Gac/Rsm signal transduction pathway. However, the detailed mechanism of such regulation is not clear.

Methodology/Principal Findings: In this study, we identified a binding site for the sigma regulator PsrA in the promoter region of the phlA gene. Electrophoretic mobility shift experiments revealed direct and specific binding of PsrA to the phlA promoter region. Consistent with the fact that its binding site locates within the promoter region of phlA, PsrA negatively regulates phlA expression, and its inactivation led to significant increase in 2,4-DAPG production. Interestingly, PsrA also activates the expression of the sigma factor RpoS, which negatively regulates 2,4-DAPG production by inducing the expression of the RNA-binding protein RsmA.

Conclusions/Significance: These results suggest that PsrA is an important regulator that modulates 2,4-DAPG biosynthesis at both transcriptional and posttranscriptional levels.

Citation: Wu X, Liu J, Zhang W, Zhang L (2012) Multiple-Level Regulation of 2,4-Diacetylphloroglucinol Production by the Sigma Regulator PsrA in Pseudomonas fluorescens 2P24. PLoS ONE 7(11): e50149. doi:10.1371/journal.pone.0050149

Editor: Ramy K. Aziz, Cairo University, Egypt

Received: April 19, 2012; Accepted: October 22, 2012; Published: November 29, 2012

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Funding: This work was supported by the National High Technology Research and Development Program (“863” Program) of China (2011AA10A205) and the National Natural Science Foundation of China (31071725, 30871666). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Rhizosphere-inhabiting fluorescent Pseudomonas spp. is a group of ubiquitous root colonizing bacteria with remarkable propensity of interacting with plant roots and protecting the roots against pathogenic microorganisms [1–5]. The ability of pseudomonads to suppress soil borne pathogens mainly depends on their ability to secrete secondary antibiotic metabolites, such as pyrrolnitrin, phenazines, pyoluteorin, hydrogen cyanide, and 2,4-diacetylphloroglucinol [2,4-DAPG] [1,4,6,7]. Among these antimicrobial compounds, 2,4-DAPG is a phenolic derivative with antifungal, antimicrobial, and antiviral properties that has been extensively studied [1,8–11]. Besides its anti-microbial activity, 2,4-DAPG induces systemic resistance in plants and promotes exudation of amino acids from roots [12,13].

The products of the four-gene operon phlACBD are responsible for the biosynthesis of 2,4-DAPG. Among these proteins, PhlD shows structural similarity with type III polyketide synthase, which is critical for the biosynthesis of monoacetylphloroglucinol (MAPG). PhlA, PhlC and PhlB are required for the transacetylation of MAPG to produce DAPG [6,14,15]. Biosynthesis of 2,4-DAPG is regulated by multiple factors. First, expression of the phlACBD operon is controlled by the divergent phlF gene, which codes for a transcriptional repressor [16]. Repression by PhlF in the promoter region of phlA [17]. Second, maximal production of 2,4-DAPG occurs in the late exponential phase or stationary phase and is regulated by a number of additional factors, including the GacS/GacA two-component system [4,7,18], the small RNA-binding proteins RsmA and RsmE [19,20], the sigma factors RpoD, RpoN and RpoS [21–23], and the resistance-nodulation-division efflux pump EmhABC [24].

Differing from many bacterial pathogens of plants or animals in which the highly conserved GacS/GacA two-component system regulates important virulence traits [25,26], this system controls disease suppression ability in plant-beneficial pseudomonads [18,25,27]. GacS is a sensor histidine kinase, which by responding to yet unknown signals, undergoes autophosphorylation. The signals are then relayed to the response regulator GacA by phosphotransfer, leading to the activation of a number of diverse genes, including non-coding small RNAs. For examples, expression of csrB and csrC in Escherichia coli, and rsmY and rsmX in P. fluorescens are regulated in this manner [25,28–31]. These small...
RNAs have a high binding affinity for small RNA-binding proteins of the CsrA/RsmA family, which negatively controls the expression of extracellular enzymes [20,25,28,32,33]. In E. coli, RpoS influences the expression of many genes during the transition from exponential to stationary phase to produce proteins usually associated with resistance to starvation or osmotic stress [34]. In Pseudomonas spp., mutations in rpoS lead to pleiotropic phenotypes, such as reduction in bacterial survival under environmental stress or alterations in the production of antibiotics pyoluteorin and 2,4-DAPG [21,35,36]. However, the role of rpoS in the production of secondary metabolites is complex and displays great variations among bacterial species and the products involved. For example, in P. fluorescens strain Pf-5, mutations in rpoS lead to a decrease of pyrrolnitrin production, which is accompanied by an increase in the production of pyoluteorin and 2,4-DAPG [21].

The level of RpoS in bacterial cells increases considerably when the culture enters the stationary phase [35]; the GacS/GacA two-component system is necessary for the timely expression and accumulation of RpoS during the transition from exponential growth to the stationary phase, indicating that RpoS is a component of the regulatory circuits involved in GacS/GacA [37,38].

In this study, we describe the identification and characterization of PsrA, a new regulator involved in 2,4-DAPG synthesis in P. fluorescens [37,38]. We show that PsrA negatively controls the phlA gene at the transcriptional level via direct binding to an operator in the phlA promoter region as well as at the posttranscriptional level by influencing the expression of RpoS and RsmA.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. E. coli was grown in LB medium at 37°C, P. fluorescens was cultured in LB medium or ABM minimal medium [39] at 30°C. When necessary, antibiotics were added at the following concentrations: ampicillin at 50 μg ml⁻¹, chloramphenicol at 20 μg ml⁻¹, gentamicin at 30 μg ml⁻¹, kanamycin at 50 μg ml⁻¹, and tetracycline at 20 μg ml⁻¹.

DNA manipulations

Plasmid and chromosomal DNA isolation, restriction enzyme digestion, ligation, and gel electrophoresis were performed by standard methods [40]. Plasmids were introduced into E. coli via chemical transformation, and into P. fluorescens strains via biparental mating or electroporation [41]. Nucleotide sequences were determined by Sunbiotechnology Co. Ltd (Beijing, China) and were analyzed using BLAST [42].

Construction of bacterial mutants

To generate the psrA gene mutant PM113, two fragments flanking the psrA gene were amplified by PCR using the primers psrA50/prsrA1770 and psrA2360/prsrA4250, respectively (Table S1). After digestion with relevant restriction enzymes, the two PCR fragments were cloned into pHSG299 (TaKaRa), producing p299DpsrA (Fig. 1). Allelic exchange using p299DpsrA with the wild-type 2P24 resulted in mutant PM113. The mutant was verified by diagnostic PCR. To construct the plasmid for complementation, the coding region of the psrA gene was PCR-amplified using primers psrA1660/prsrA2410 and cloned into pRK415 [43] to yield pH15-psrA (Fig. 1). Plasmid pH15-psrA was constructed by PCR amplification of psrA using the PsrA-EcoRI and PsrA-XbaI primer pair and 2P24 genomic DNA as template.

The EcoRI-XbaI restriction fragment was cloned into pJN105 [44], resulting in the psrA expression vector pHN-psrA.

To construct a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope-RsmA fusion, a PCR-generated fragment with the sequence 5'-TATACAGATATGAATTGATATGAGAAA-3' inserted in-frame to the 3' end of the rsmA gene was cloned into pK18mobGII [45]. The resulting plasmid pK18RsmAVSV was verified by sequencing and mobilized into strains 2P24 and the psrA mutant PM303 to generate strains PM114 and PM304, respectively. The plasmid pK18PhaAVSV which contains a -VSV-G fusion was constructed similarly, and was mobilized into strains 2P24, PM113, and PM601 to generate strains PM305, PM306, and PM307, respectively. All mutations were verified by DNA sequencing.

Construction of reporter fusions and β-galactosidase assays

To construct psrA-lacZ, rpoS-lacZ, rsmA-lacZ, rsmE-lacZ, rsmY-lacZ and rsmX-lacZ transcriptional fusions, the DNA fragments containing the upstream region of each of these genes (500-bp for psrA, 470-bp for rpoS, 530-bp for rsmA, 300-bp for rsmE, 280-bp for rsmY, and 270-bp for rsmX) were PCR amplified using the appropriate primer sets (Table S1). Each of these cis-acting regions was inserted as a BamHI or a BglII fragment into pRG970Gm [46] to place them upstream of a promoterless lacZ gene. Plasmid pRG970Gm-rsmZMp was constructed by PCR amplification using primer pair rsmZMp and RsmZ-P3361 and inserted into pRG970Gm as a BamHI fragment. In this construct, an upstream activating sequence (UAS) in the rsmZ promoter was deleted. These plasmids were introduced into appropriate P. fluorescens strains by electroporation. β-galactosidase activity of the testing strains was determined by using the Miller method [47].
Site-directed mutagenesis of the phiA and the rpoS promoters

To introduce specific mutations into the phiA promoter, a 740-bp BamHI fragment containing the phiA promoter region was excised from p970Gm-phiAp and inserted into pHSG399 to create p399phiAp. Oligonucleotides containing the designed mutations (primer pairs phiAMGF-phiApM and phiADTF-phiApM) were used to generate p399phiAp derivatives by inverse PCR (QuickChange site-directed mutagenesis kit; Stratagene). The mutated nucleotides were confirmed by DNA sequencing. The obtained p399phiAp derivatives were digested with BamHI and the fragments containing the mutated phiA promoter were then inserted into pRG970Gm to generate p970Gm-phiApD3T and p970Gm-phiApM3G, respectively.

A 800-bp fragment containing the promoter of rpoS amplified by PCR with primer pairs rpoS1 and rpoSp2, was inserted into pHSG399 to obtain the plasmid p399rpoSp. Oligonucleotides containing the designed mutation (primer pair RpoSMF and RpoSMR) were used to generate p399rpoSp derivative by inverse PCR (see above). The mutated nucleotides were confirmed by DNA sequencing.

Expression and purification of the PsrA protein

The predicted ORF of psrA was amplified by PCR with primers psrA-NdeI and psrA-XhoI (Table S1). After NdeI-XhoI digestion, the PCR product was cloned into pET-22b(+) to give pET-PsrA, which was introduced into E. coli BL21 (DE3) (Novagen) for the production of His6-PsrA. To induce protein expression, IPTG was added to E. coli cultures grown to an optical density of 0.6 at a final concentration of 0.8 mM. His6-PsrA was purified by using a nickel affinity column (Amersham Biosciences) according to the manufacturer’s instructions. Recombinant proteins were eluted by 200 mM imidazole, and the purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining. Protein concentration was determined by Nanodrop ND-1000 (Thermo Scientific) absorbance at 280 nm and by the Bicinchoninic acid (BCA) assay.

Electrophoretic mobility shift assay

The upstream DNA fragments of the psrA, phiA, or rpoS genes containing the putative PsrA-binding sequences were amplified by PCR using the appropriate primer sets, respectively (Table S1). The DNA fragments were purified using a QIAquick gel extraction kit (Qiagen). Protein-DNA interaction assays were performed in 20 μl of 1× binding buffer [20 mM HEPES, pH 7.6; 1 mM EDTA; 10 mM (NH₄)₂SO₄; 1 mM DTT; 150 mM KCl, 5% [wt/vol] glycerol]. The reaction mixtures were incubated at room temperature for 20 min. Each binding reaction was loaded onto a 8% native polyacrylamide gel and ran for 2 h at 90 Volts. Gels were stained with SYBR Green as recommended by the manufacturer of the EMSA kit E33075 (Invitrogen).

Production of PsrA antibodies

Polyclonal antibodies against PsrA of P. fluorescens 2P24 were produced in a mouse by subcutaneous immunization with 200 μg of the purified recombinant His6-PsrA, and this initial immunization was followed by additional immunizations at 3 and 4 weeks, respectively. Six days after the last injection, the blood of the immunized mouse was collected, and its serum was used for Western blot analysis.

Western blot analysis

Cell lysates of the P. fluorescens 2P24 or its derivatives were prepared by sonication in TNT buffer [10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween-20] (Sambrook and Russell, 2001), the soluble fractions separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking with 5% milk in PBST (PBS containing 0.02% Tween-20), membranes were incubated with the appropriate primary antibody: anti-PsrA (1:1,000), anti-VSV-G antibody (1:2,000; Sigma) and anti-3-phosphoglycerate kinase (PGK) (1:2,000; Invitrogen). Blots were washed with PBST, probed with an anti-mouse horseradish peroxidase conjugated secondary antibodies (1:5,000; Sigma). The resulting blots were incubated for 1 min in ECL reagent and detected using O-MAT X-ray film (Kodak).

Extraction and quantification of the 2,4-DAPG production

P. fluorescens 2P24 and its derivatives were cultivated with shaking in 30 ml LB liquid media at 140 rpm at 30°C. 2,4-DAPG was extracted from the culture supernatant and was quantified by HPLC as described previously [10].

Nucleotide sequence accession number

The sequence of the psrA gene from strain 2P24 has been deposited in the GenBank database under accession no. HQ992504.

Results

The phiA-phlF intergenic region in P. fluorescens 2P24 contains a PsrA binding site

The importance of inverted repeat sequences for regulatory protein binding has been established for numerous regulators [48,49]. Previous studies have revealed the presence of several palindromic sequences in the phiA-phlF intergenic region in 2,4-DAPG-producing P. fluorescens [17]. A sequence alignment of the phiA gene promoter region from strain 2P24 with other well-studied 2,4-DAPG producing Pseudomonas strains revealed a very well conserved palindromic sequence GAAACN5GTTTC (Fig. S1). This element is a potential recognition sequence for PsrA (Pseudomonas sigma regulator), which was previously identified as a positive regulator of rpoS expression in P. putida WCS358 and P. aeruginosa PAO1 [50,51] and was involved in the regulation of type III secretion system and quorum sensing [52,53]. Our ongoing genome sequence of strain 2P24 revealed that this strain codes for P. fluorescens Pf0-1 (accession number ABQ79722; 94% identity), P. aeruginosa PAO1 (accession number AAO56983; 91% identity), P. fluorescens DC3000 (accession number AAO56983; 91% identity), and P. aeruginosa PAO1 (accession number AAG06394, 87% identity). The presence of a putative PsrA-binding sequence in the phiA promoter region suggests potential interactions between PsrA and the phiA promoter sequence. We tested this hypothesis by electrophoretic mobility shift assay (EMSA) using the purified His6-PsrA protein and DNA fragments of the promoter. A mobility shift was observed when His6-PsrA was incubated with
the phlA promoter (Fig. 2A). Importantly, no shift was detected when mutations disrupting the putative binding site were introduced (Fig. 2A). These results indicate that PsrA may regulate the expression of the phlA gene by directly interacting with phlA promoter.

PsrA negatively regulates 2,4-DAPG production at transcriptional level

In LB medium, the psrA mutant PM113 produced higher levels of an uncharacterized red pigment, which is a characteristic phenotype associated with the production of the antibiotic 2,4-DAPG [3,17]. Further quantification by HPLC showed that the 2,4-DAPG levels in culture supernatant of the psrA mutant were about 5-fold higher than that of the wild type strain. Such increase could be repressed by introducing the psrA gene into the mutant (Fig. 2B). An earlier study has shown that inactivation of the phlF gene results in overproduction of 2,4-DAPG in P. fluorescens 2P24 [54]. Interestingly, overexpression of PsrA in the phlF mutant caused dramatic reduction in 2,4-DAPG production (Fig. 2B). To test the influence of PsrA on the phlA transcription, p970Gm-phlAp [46] carrying a phlA-lacZ transcriptional fusion were grown in LB, and β-galactosidase activities were determined (C). Analysis of PhlA levels in strain 2P24 and the psrA mutant by immunoblotting. An antibody directed against 3-phosphoglycerate kinase α (α-PGK) is used as a loading control in this and later blots (D). All experiments were performed in triplicate, and the mean values ±SD are indicated. Growth is indicated by the dotted line. doi:10.1371/journal.pone.0050149.g002

Figure 2. EMSA of PsrA with the phlA (30 ng) promoter fragment that contains PsrA-binding sequence showing formation of a PsrA-DNA complex. Lane 1, DNA probe alone; lanes 2–5, DNA probe incubated with 50, 75, 100, or 150 ng PsrA, respectively; lane 6, the mutagenized DNA probe from p399phlAp derivative (a 3-bp substitution [GGG for TTT] in the phlA promoter) incubated with 150 ng PsrA (A).

Biosynthesis of 2,4-DAPG in strain 2P24 and its psrA and phlF mutants was assayed by HPLC (B). For transcriptional assay, strain 2P24 and its psrA mutant carrying p970Gm-phlAp (wild type phlA-lacZ), p970Gm-phlApM3G (PsrA box mutTTT phlA-lacZ) or p970Gm-phlAD3T (PsrA box ΔTTT phlA-lacZ) were grown in LB, and β-galactosidase activities were determined (C). Analysis of PhlA-V levels in strain 2P24 and the psrA mutant by immunoblotting. An antibody directed against 3-phosphoglycerate kinase α (α-PGK) is used as a loading control in this and later blots (D). All experiments were performed in triplicate, and the mean values ±SD are indicated. Growth is indicated by the dotted line.

doi:10.1371/journal.pone.0050149.g002

PhlF and PsrA expressed differently in P. fluorescens 2P24

PhlF is a specific transcriptional repressor that regulates the expression of the 2,4-DAPG biosynthetic operon by binding to the pho site located about 120-bp downstream of the PsrA recognition site in the phlA gene promoter region (Fig. S1). To compare the expression of psrA and phlF, lacZ fusion of these two genes was introduced into strain 2P24. The psrA gene expressed in a cell density-dependent manner and reached its maximum in the stationary phase, whereas the transcription of the phlF gene reached the highest level earlier in logarithmic phase (Fig. 3). Differential expression of PhlF and PsrA during cell growth implied their distinctive effects on phlA transcription. PhlF is likely a major repressor during the early growth phase. In agreement with this notion, 2,4-DAPG normally begins to accumulate during the transition from logarithmic to stationary phase [17].

PsrA regulates the expression of itself in P. putida, P. aeruginosa, and P. syringae by directly binding to its own promoter [51]. In strain 2P24, two putative PsrA-binding sequences (GAAAACG-TATGGTTTG and CAAAAGTGTGTTTG) are present in the upstream region of the psrA gene (Fig. 1). By EMSA, we found that

the phlA promoter (Fig. 2A). Importantly, no shift was detected when mutations disrupting the putative binding site were introduced (Fig. 2A). These results indicate that PsrA may regulate the expression of the phlA gene by directly interacting with phlA promoter.
PsrA directly binds its promoter region (Fig. S2A). Consistently, the psrA-lacZ transcriptional fusion expressed at a higher level in the psrA defective mutant (Fig. S2B).

PsrA positively regulates the translational regulator RsmA

In some Pseudomonas strains, biosynthesis of 2,4-DAPG is tightly regulated at both transcriptional and post-transcriptional level [25,27]. Multiple factors of the Gac/Rsm signal transduction pathway, including the GacS/GacA system, the small RNAs RsmX, RsmY, and RsmZ, and the translational repressors RsmA and RsmE are important components of regulatory circuit that controls the production of 2,4-DAPG at posttranscriptional level [25,33]. In P. fluorescens 2P24, three small RNAs, RsmX, RsmY, and RsmZ, and two small RNA-binding proteins RsmA and RsmE are present in the draft genome sequence. To determine whether PsrA influences expression of these genes, we measured the transcription of these genes in the psrA mutant. Although no difference for rsmZ, rsm1, rsmX or rsmE was observed (Fig. 4A–D), the expression of rsmA was significantly lower in the mutant (Fig. 4E). Consistent with this observation, the rsmA mutant produced significantly higher amounts of PhlA protein (Fig. 2D) and 2,4-DAPG (Fig. 4F) than the wild type strain 2P24. This increase of 2,4-DAPG can be brought to the wild type level by expressing rsmA in the mutant (Fig. 4F), further validating the negative role of RsmA in 2,4-DAPG production. Thus, PsrA positively regulates the transcription of the rsmA gene, which in turn negatively controls the biosynthesis of 2,4-DAPG.

The effect of PsrA on rsmA gene expression is mediated by the sigma factor RpoS

The absence of a PsrA binding site in the promoter region of rsmA suggests an indirect effect of PsrA on rsmA transcription (data not shown). A previous study has shown that RpoS positively regulates rsmA gene in Pectobacterium carotovorum and its homolog CarA in E. coli [55]. Further, RpoS is a negative regulator for 2,4-DAPG production in P. fluorescens CHA0 and Pf-5 [19,21]. In P. fluorescens 2P24, the production of 2,4-DAPG in the rpoS mutant increased about 3-fold with the wild type strain (Fig. 5A). We therefore examined whether RpoS regulates phlA expression. The phlA-lacZ transcriptional fusion in the rpoS mutant expressed at levels similar to those in the mutant (Fig. S4), suggesting that the negative effects of RpoS on 2,4-DAPG production occurs at post-transcriptional level.

The above observations suggest that in strain 2P24, PsrA regulates rpoS expression, which in turn controls rsmA expression. Careful inspection of the promoter region of rpoS of strain 2P24 revealed the presence of a putative PsrA binding site between –427 and –416 relative to its transcription start site. Incubation of His6–PsrA with a DNA fragment containing this region led to the formation of DNA-protein complex, and no shift was detected when mutations disrupting the putative binding site were introduced (Fig. 5D). In the rpoS mutant, expression of the rpoS-lacZ decreased about 4-fold (Fig. 5B), suggesting a positive role of PsrA in rpoS transcription. In the rpoS mutant, the rsmA-lacZ transcriptional fusion expressed at a significantly lower level than in the wild type (Fig. 5C). The defect was caused by the loss of RpoS because introduction of a plasmid-borne allele of this gene completely restored the expression of rsmA (Fig. 5C). To examine the protein level of RsmA, we created a chromosomal vsv-rsmA in the ΔrpoS strain background and found that similar to the results from the lacZ fusion, VSV-RsmA was produced at a lower level in the mutant (Fig. 5E). The effect of RpoS on rsmA was specific because the expression of rsmE or the production of RsmE was not affected by rpoS deletion (data not shown). Taken together, these results establish that in addition to directly affecting phlA transcription, PsrA influences the production of 2,4-DAPG posttranscriptionally via RpoS and RsmA.

The effect of PsrA on 2,4-DAPG production is independent of the Gacs/Gaca system

In Pseudomonas spp., components of the Gac/Rsm signal transduction pathway includes the GacS/GacA system, the noncoding small RNAs and the translational repressors. Among these, the GacS/GacA system functions as a global regulatory system which positively regulates transcription of the noncoding small RNAs by binding to a conserved UAS in the promoter region [29,31]. In P. chlororaphis, the transcription of psrA was abolished in the gacA mutant, suggesting that the psrA gene in this bacterium was under the regulation of the two-component system [56]. To determine whether psrA was regulated by the GacS/GacA system, we measured the expression of the phlA-lacZ fusion in a GacS/GacA-deficient mutant and no difference was detected between the mutant and the wild type strain. Similar results were obtained when the protein levels of PsrA were probed (Fig. 6). Thus, the GacS/GacA two-component system has no effect on the expression of the psrA gene in this bacterium.

Discussion

The antibiotic 2,4-DAPG is one of the major weapons to inhibit the growth of pathogenic bacteria and fungi by some biocontrol P. fluorescens strains. P. fluorescens mutants unable to produce 2,4-DAPG are defective in its protection against black root rot of tobacco, take-all of wheat, and Pythium damping-off of sugarbeet [8], whereas strains 2P24 and CHA0 overproducing 2,4-DAPG exhibit increased plant disease suppression ability [57,58]. However, overproduction of 2,4-DAPG in some Pseudomonas strains led to a notable phytotoxicity [54]. Clearly, precise regulation of 2,4-DAPG production is necessary for proper responses to the ever changing soil environment [4]. A series of transcriptional and translational regulators that control 2,4-DAPG production have been identified in various strains of P. fluorescens, including CHA0, F113, Pf-5, Q2–87, Q8r1-96, and 2P24. These regulatory factors include the specific repressor PhlF [17], the sigma
factors RpoS, RpoD and RpoN [15,21,23], the H-NS family regulators MvaT and MvaV [59], the oxidoreductase DsbA [60], the RNA binding protein Hfq [46], the resistance-nodulation-division efflux pump EmhABC [24], and the Gac/Rsm signal transduction pathway [25,27]. However, the relationships among these factors and how they co-ordinate to control antibiotics production are not fully understood.

In *P. fluorescens* 2P24, both PhlF and PsrA are transcriptional repressors for expression of genes involved in 2,4-DAPG production by directly interacting with specific binding box localized on the *phlA* promoter region (Fig. S1). This observation raises the question of why two seemingly redundant repressors are needed for production of this antibiotic. We show here that the expression pattern of *phlF* and *psrA* is clearly distinct (Fig. 3), and the 2,4-DAPG production in *phlF*- and *psrA*-negative mutants is very different (Fig. 2B), implying that their roles in the production of 2,4-DAPG may differ greatly. 2,4-DAPG production in the *phlF* mutant is more than 20-fold higher than that in the *psrA* mutant (Fig. 2B), indicating that the pathway-specific regulator PhlF is the dominant regulatory factor for 2,4-DAPG production. Transcriptional analysis showed that the *phlF* expressed at a relatively higher level in the early stages of growth (Fig. 3), which is consistent with its role in preventing 2,4-DAPG production in the early log phase and the fact that the antibiotics usually accumulate in the stationary phase (1, 11). Constitutive expression of the *phlACBD* locus could be a metabolic burden, which can lead to reduction in bacterial growth and its ability to compete with adjacent microorganisms. Therefore, additional repressors are necessary to prevent excessive biosynthesis of 2,4-DAPG after the repression by PhlF is lifted. PsrA might play such a role to restrain the overproduction of 2,4-DAPG, particularly in the stationary phase when the expression of the *psrA* gene was significantly higher (Fig. 3). Consistent with this notion, overexpression of PsrA in *phlF* mutant caused a decrease in 2,4-DAPG production (Fig. 2B). Such a regulatory circuit may allow the bacterium to mount a more efficient and precise regulatory response for balanced production of antibiotics to better adapt to the ever-changing environment. In addition, the presence of the recognition sites for PhlF and PsrA in the *phlA* promoter region of strain 2P24 as well other 2,4-DAPG producing pseudomonads, including *P. fluorescens* F113, Q2–87, CHA0, and Pf-5 (GenBank accession numbers AF497760, U41818, AF207529, and CP000076, respectively) further suggests the importance and conservation of PsrA in the regulation of 2,4-DAPG production (Fig. S1).

Consistent with earlier studies, our results revealed that unlike PhlF which is a specific repressor for the *phlACBD* operon, PsrA is a global regulator that positively regulates the expression of the sigma factor RpoS in bacteria [51,61]. In other *Pseudomonas* spp., PsrA is known to be involved in the regulation of the type III secretion system (TTSS), quorum sensing, fatty acid degradation, and the production of secondary metabolites [52,53,56,62]. Thus, PsrA participates in various regulatory networks involved in various physiological functions. In *P. fluorescens* strain 2P24, in addition to the biosynthesis of 2,4-DAPG, several important

**Figure 4.** Transcription of small non-coding RNA genes *rsmX* (A), *rsmY* (B), and *rsmZ* (C) and their cognate regulator genes *rsmE* (D) and *rsmA* (E) in *P. fluorescens* 2P24, its *psrA* mutant and its *gacA* mutants. (F) HPLC analysis of 2,4-DAPG production by strain 2P24 and its *rsmA* mutant. All experiments were performed in triplicate, and the mean values ±SD are indicated. Growth is indicated by the dotted line. doi:10.1371/journal.pone.0050149.g004
biological control traits, such as the production of N-acyl-homoserine lactone (AHL), biofilm formation and swarming motility were also significantly impaired in the \( \text{psrA} \) mutant (data not shown). \( \text{PsrA} \) was previously described as a transcriptional regulator involved in positive regulation of RpoS and in negative autoregulation, by direct binding to the conserved motif \((\text{G/CAACN} \_\text{4GTTTG/C})\) in the promoter regions [51]. As also confirmed in this study, direct interaction of \( \text{PsrA} \) with the \( \text{phlA} \) gene promoter in strain 2P24 requires the similar binding motif -5\( ^{9} \text{GAAACGGATC} \_\text{GTTTC}^{9} \_\text{9} \)- (Fig. S1). Genome searching in \( \text{P. aeruginosa} \) revealed that at least 26 genes contain the \( \text{PsrA} \) binding motif in their promoter regions [61]. But interestingly, this motif seems not always essential for \( \text{PsrA} \) function. In \( \text{P. aeruginosa} \), \( \text{PsrA} \) is required for the full activation of the TTSS regulatory operon \( \text{exiCEBD} \) by binding to its promoter region despite the absence of a recognizable binding site, but some sequences \((\text{GAAAC} \text{ at the position } -56 \text{ from the transcriptional start site})\) was resemble a partial binding site [53]. Thus further identification of \( \text{PsrA} \)-binding genes in strain 2P24 will help us to better understand the global functions of \( \text{PsrA} \) in response to the environmental changes.

Our results indicate that \( \text{PsrA} \) influences 2,4-DAPG biosynthesis at the post-transcriptional level by activating the expression of the \( \text{rsmA} \) gene through the sigma factor RpoS (Fig. 5). The GacS/GacA two-component system has been known as a major post-transcriptional regulon controlling at least three non-coding small RNAs \( \text{RsmZ}, \text{RsmY}, \text{and RsmX}\), which together with the translational regulator RsmA/CsrA family proteins, coordinate the expression of secondary metabolites, production of extracellular enzymes, and biocontrol properties [33]. RsmA binds to specific mRNA targets, stabilizing some and inducing the degradation of others [63]. Interestingly, the results in this study differ from a recent finding in \( \text{P. fluorescens} \) CHA0, in which a potential \( \text{PsrA} \) recognition site \((\text{CAAGN}_{4} \text{CTTTT})\) overlapping with the putative GacA binding site in the \( \text{rsmZ} \) promoter was identified and the mutation of this sequence abolished the effect of \( \text{PsrA} \) on \( \text{rsmZ} \) expression [64]. In \( \text{P. fluorescens} \) 2P24, the GacS/GacA two-component system also positively regulates three small non-coding RNAs \( \text{RsmX}, \text{RsmY}, \text{and RsmZ}\), and the UAS sequence is necessary for this regulatory role (Fig. 4A–C). However, a \( \text{PsrA} \)-binding site is not present in the promoter region of \( \text{rsmZ} \) and \( \text{PsrA} \) did not detectably affect \( \text{rsmZ} \) transcription (Fig. S3; Fig. 4C). These results imply that, although the regulation at the transcriptional level is conserved, the effects of \( \text{PsrA} \) on 2,4-DAPG biosynthesis at the posttranscriptional level differ distinctively in pseudomonads. Thus, we have proposed a model to illustrate our current understanding of the roles of \( \text{PsrA} \) in biosynthesis of 2,4-DAPG in \( \text{P. fluorescens} \) 2P24 (Fig. 7). Further study is necessary to identify genes potentially regulated by \( \text{PsrA} \) to better understand the function of \( \text{PsrA} \) in other properties of \( \text{P. fluorescens} \), such as antibiotic production, host interaction, and niche adaptation.

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**Figure 5. RpoS regulates the 2,4-DAPG production via RsmA in** \( \text{P. fluorescens} \) **2P24.** Biosynthesis of 2,4-DAPG in strains 2P24 and its \( \text{rpoS} \) mutant was assayed by HPLC (A). The expression of the \( \text{rpoS} \) gene is activated by \( \text{PsrA} \) in strain 2P24 (B). Expression of the \( \text{rsmA} \) gene in the wild type strain 2P24 and the \( \text{rpoS} \) mutant PM303 (C). Binding assay of \( \text{PsrA} \) to the \( \text{rpoS} \) promoter. 30 ng DNA probe was incubated with increasing amounts of \( \text{PsrA} \). Lane 1, DNA probe alone; lanes 2-5, DNA probe incubated with 25, 50, 75, or 100 ng \( \text{PsrA} \), respectively; lane 6, the mutated DNA probe from p399rpoSp derivative (a 3-bp substitution [GGG for TTT] in the \( \text{rpoS} \) promoter) incubated with 100 ng \( \text{PsrA} \) (D). Western blot analysis of RsmA-V in strain 2P24 and the \( \text{rpoS} \) mutant (E).

**doi:**10.1371/journal.pone.0050149.g005
Supporting Information

Figure S1 Alignment of the phlA promoter of P. fluorescens 2P24 with homologous sequences in other pseudomonads. The alignment was made using Clustal W (Thompson JD, Higgins DG, Gibson TJ. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680). Asterisks denote conserved nucleotides. The putative PsrA binding element and the putative Shine-Dalgarno (SD) site of the phlA gene are boxed. RsmA/RsmE proteins are expected to bind the SD region and inhibit the translation of the phlA mRNA (Karine L, Elena S, Magnus L, Katja S, Carol SB, et al. (2007) Mechanism of hcnA mRNA recognition in the Gac/Rsm signal transduction pathway of Pseudomonas fluorescens. Mol Microbiol 66: 341–356). The −10 and −35 promoter element, PhlF binding site: phO, and transcription start site of phlA have been determined (Abbas A, Morrissey JP, Marquez PC, Sheehan MM, Delany I R, et al. (2002) Characterization of interactions between the transcriptional repressor PhlF and its binding site at the phlA promoter in Pseudomonas fluorescens F113. J Bacteriol 184: 3008–3016).

Figure S2 EMSA of PsrA with the psoI promoter fragment that contains PsrA-binding sequence showing formation of a PsrA-DNA complex. 30 ng DNA probe was incubated with increasing amounts of PsrA (A). β-Galactosidase assay showing the expression profile of a plasmidborne psoI-lacZ reporter fusion in strain 2P24 and its psoI mutant (B). All experiments were performed in triplicate, and the mean values ±SD are indicated. Growth is indicated by the dotted line. doi:10.1371/journal.pone.0050149.g007

Figure S3 Alignment of the rsmZ promoter of P. fluorescens 2P24 with homologous sequences in other pseudomonads. The alignment was made using Clustal W as in Fig. S1. Asterisks denote conserved nucleotides. The −10 and −35 promoter element, the putative upstream activating sequence (UAS), the terminator of rpoS, the transcription start site and the terminator of rsmZ, and the putative PsrA binding site in the rsmZ promoter region have been determined (Heeb S, Blumer C, and Haas D. (2002) Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in Pseudomonas fluorescens CHA0. J Bacteriol 184:1046–1056; Humair, B., Wackwitz, B., Haas, D. (2010) GacA-Controlled Activation of Promoters for Small RNA Genes in Pseudomonas fluorescens. Appl Environ Microbiol 76: 1497–1506).

Figure S4 The expression of phlA gene is not regulated by rpoS gene at transcriptional level. β-Galactosidase assay showing the expression profile of a plasmidborne phlA-lacZ reporter fusion in strain 2P24 and its rpoS mutant. This experiment was performed in triplicate, and the mean values ±SD are indicated. Growth is indicated by the dotted line. doi:10.1371/journal.pone.0050149.g007

Table S1 Bacteria strains, plasmids and oligonucleotides used in this study.

Acknowledgments
We thank Drs. Gail P. Ferguson, Zhaoqing Luo, Martin Schuster and Rui Zhou for plasmids and reagents, and Dr. Xiaoxue Yan and Mr. Zhenhua Guo for technical support.
Author Contributions
Conceived and designed the experiments: XGW LQZ. Performed the experiments: XGW JCL WZ. Analyzed the data: XGW LQZ. Wrote the paper: XGW LQZ.

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