LasR Might Act as an Intermediate in Overproduction of Phenazines in the Absence of RpoS in *Pseudomonas aeruginosa*

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As an opportunistic bacterial pathogen, *Pseudomonas aeruginosa* PAO1 contains two phenazine-producing gene operons, *phzA1B1C1D1E1F1G1* (*phz1*) and *phzA2B2C2D2E2F2G2* (*phz2*), each of which is independently capable of encoding all enzymes for biosynthesizing phenazines, including phenazine-1-carboxylic acid and its derivatives. Other previous study reported that the RpoS-deficient mutant SS24 overproduced pyocyanin, a derivative of phenazine-1-carboxylic acid. However, it is not known how RpoS mediates the expression of two *phz* operons and regulates pyocyanin biosynthesis in detail. In this study, with deletion of the *rpoS* gene in the $\text{PA}\Delta\text{phz1}$ mutant and the $\text{PA}\Delta\text{phz2}$ mutant respectively, we demonstrated that RpoS exerted opposite regulatory roles on the expression of the *phz1* and *phz2* operons. We also confirmed that the *phz1* operon played a critical role and especially biosynthesized much more phenazines than the *phz2* operon when the *rpoS* gene was knocked out in *P. aeruginosa*. By constructing the translational reporter fusion vector *lasR'*-*lacZ* and the chromosomal fusion mutant $\text{PA}\Delta\text{lasR}::\text{lacZ}$, we verified that RpoS deficiency caused increased expression of *lasR*, a transcription regulator gene in a first quorum sensing system (*las*) that activates overexpression of the *phz1* operon, suggesting that in the absence of RpoS, LasR might act as an intermediate in overproduction of phenazine biosynthesis mediated by the *phz1* operon in *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, RpoS, LasR, *phz1*, *phz2*, phenazine

Introduction

Due to its high adaptability, *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, can be found in different types of environments, such as water and soil [1]. In fact, it can dwell in a variety of life forms, including mammals, insects, nematodes, and plants, and cause infectious diseases in its hosts [2–6]. For human beings, infections with this pathogen most commonly found in cystic fibrosis (CF) patients often cause both acute and chronic diseases that lead to progressive lung tissue destruction [7–9]. Pathophysiological effects of *P. aeruginosa* are often associated with a number of virulence factors secreted by

this bacterium [10, 11]. Among these virulence factors secreted by *P. aeruginosa* is pyocyanin (PCN), a phenazine redox pigment with low molecular weight. In all isolated strains of *P. aeruginosa*, it has been confirmed that 90% to 95% of them produced PCN [12, 13]. Meanwhile, the existence of PCN with high concentration in the sputum of CF patients also indicated that PCN could play a critical role in pulmonary tissue damage observed with chronic infection of the lung [14, 15]. This finding was in accord with several recent studies showing that PCN contributed in different methods to the pathophysiological functions observed in airways infected with *P. aeruginosa* [16–19]. Besides PCN, in fact, *P. aeruginosa* could biosynthesize

several phenazine derivatives, all of them being converted from a precursor, phenazine-1-carboxylic acid (PCA) [20, 21].

Interestingly, *P. aeruginosa* is found to contain two copies of the phenazine-producing operons that are designated *phz1* (*phzA1B1C1D1E1F1G1*) and *phz2* (*phzA2B2C2D2E2F2G2*) in its chromosome, each of them being independent and sufficient for encoding all enzymes involved in PCA biosynthesis. Genome sequences showed that both phenazine-producing operons, *phz1* and *phz2*, displayed >98% identity at the nucleic acid level. However, they are very far from each other in the chromosome (up to 2.6 Mb) and their promoter sequences are completely different, suggesting that the *phz1* and *phz2* may be governed through different regulation pathways [13]. Actually, in three quorum sensing systems (LasI/LasR, RhlI/RhlR, and PQS), both the PQS and RhlI/RhlR systems positively regulate *phz1* expression [22, 23], while the orphan LuxR-type quorum sensing regulator QscR negatively regulates *phz1* and *phz2* expression [8, 24]. The regulation of *phz2* was not known much.

RpoS, an alternative sigma factor S, has been widely studied in many genera of bacteria and serves as a global regulator that triggers expression of a lot of genes, many of which are expressed in general stress response or especially during the stationary phase in *P. aeruginosa* and *E. coli* [25–27]. Furthermore, based on the genome-wide transcription analysis, it had been confirmed that up to 10% of total genes were governed by RpoS in *E. coli*, directly or indirectly [25]. In one previous published work, it was shown that PCN production was enhanced remarkably when the *rpoS* gene of *P. aeruginosa* was inactivated with an insertion of gentamicin resistance gene cassette (*aacC1*) in the mutant SS24 [28]. Until now, however, we do not know whether or how RpoS regulates expression of each of the *phz1* and *phz2* in the wild-type PAO1 strain. To answer these questions, in this study, we first constructed the *rpoS*-deletion mutants using the wild-type strain PAO1, the *phz1*-defect mutant PAΔ*phz1* and the *phz2*-negative mutant PAΔ*phz2* as receptor strains, respectively. By quantitatively measuring PCA and PCN production, we confirmed that RpoS down-regulates the expression of *phz1* and up-regulates the expression of *phz2*, and the *phz1* contributes much more to overproduction of phenazines than the *phz2* in the *rpoS*-inactivated mutant. By creating reporter fusion *lasR*'-'*lacZ* on the plasmid and chromosome, we found that LasR might act as an intermediate for much more phenazine production mediated by the *phz1* operon in the *rpoS*-deleted mutants.

Materials and Methods

Strains, Plasmids, Primers, and Culture Condition

All bacterial strains, plasmids and primers used in this work are detailed in Tables 1 and 2, respectively. *E. coli* strains were regularly grown at 37°C in Luria-Bertani (LB) broth at 37°C with shaking at 180 rpm [29]. *P. aeruginosa* strains were routinely cultivated in LB broth with the same conditions above, or on LB agar plates, or in GA medium supplemented with glycerol and alanine for phenazine production and β-galactosidase activity assay [29, 30]. When required, LB or GA medium was supplemented with the following antibiotics: for *E. coli*, 100 μg/ml ampicillin (Amp), 25 μg/ml tetracycline (Tet), 20 μg/ml gentamicin (Gen), 50 μg/ml kanamycin (Kan); for *P. aeruginosa*, 125 μg/ml Tet, 40 μg/ml Gen, 100 μg/ml Kan, 100 μg/ml spectinomycin (Spe). To screen mutants with double crossover after conjugal mating, LB agar was supplemented with 10% (w/v) sucrose.

DNA Manipulations

Regular DNA manipulations, including DNA extraction, endonuclease digestion, ligation, agarose gel electrophoresis and DNA isolation and purification from agarose gel, were carried out with standard molecular methods [29]. Plasmid isolation was performed using a Plasmid Extraction Mini Kit (Solarbio, China). Genomic DNA was extracted from *P. aeruginosa* with the methods as described by Chen and Kuo [31] or using the Bacterial Genomic DNA Extraction Kit (Solarbio). Cells of *P. aeruginosa* were transformed with plasmid DNA using electroporation [32].

Polymerase chain reactions (PCRs) were typically performed with 2.5 U of Phusion High-Fidelity DNA Polymerase as previously described [33]. DNA sequencing was completed with the DNA sequencing facilities at Qingdao Agriculture University.

Construction of *P. aeruginosa* Deletion and Fusion Mutants

To generate the *rpoS*-deleted mutant, allelic exchange was obtained by creation of the pEXSG plasmid at first. Briefly, the upstream and downstream DNA fragments of *rpoS* were amplified from the genomic DNA of the wild-type strain PAO1 with primer pairs *rpoS*-1F/*rpoS*-1R_{XbaI} and *rpoS*-2F_{XbaI}/*rpoS*-2R, respectively. Two purified amplicons were mixed, then cleaved with restriction endonuclease *XbaI*, and finally self-ligated. The purified ligation was used as the template for a new round of PCR that was performed with a pair of primers *rpoS*-3F_{SacI}/*rpoS*-3R_{PstI}. After cleaving with restriction endonuclease *SacI* and *PstI* simultaneously, the new PCR amplicons were ligated and cloned into the suicide plasmid pEX18Tc [34], generating pEXS. The gentamicin resistance gene cassette (*aacC1*) isolated from the plasmid pUCGm by *XbaI*-digestion was then ligated into the *XbaI* site on pEXS to create pEXSG [35].

After confirmation by sequencing, homologous recombination and allelic exchange occurred in the wild-type strain PAO1 following conjugal mating with *E. coli* SM10 λ*pir* bearing pEXSG as a donor strain and sucrose counter selection was carried out as

Table 1. Strains and plasmids.

Strain/plasmid	Characteristics	Source/reference
Strains		
<i>E. coli</i>		
DH5 α	F Φ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17 recA1endA1deoRgyrA96 thi-1relA1supE44</i>	Lab collection
SM10	F <i>leuB6 recA tonA21 lacY1thi-1 thr-1 supE44</i> (Mu κ ⁺) λ Kan ^R	Lab collection
<i>P. aeruginosa</i>		
PAO1	Phenazines producer, Wild type, Amp ^R Spe ^R	Lab collection
PA Δ <i>phz1</i>	<i>phz1</i> locus deleted and inserted with <i>aacC1</i> , Spe ^R Gen ^R	[33]
PA Δ <i>phz2</i>	<i>phz2</i> locus deleted and inserted with <i>aph</i> , Spe ^R Kan ^R	[33]
PA Δ <i>phz1</i> Δ <i>phz2</i>	<i>phz1</i> deleted and inserted with <i>aacC1</i> , <i>phz2</i> deleted and inserted with <i>aph</i> , simultaneously, Spe ^R Gen ^R Kan ^R	[33]
PA Δ <i>rpoS</i>	<i>rpoS</i> knocked out and replaced with <i>aacC1</i> , Spe ^R Gen ^R	This study
PA Δ <i>phz1</i> Δ <i>rpoS</i>	<i>rpoS</i> deleted in the mutant Δ <i>phz1</i> , Spe ^R Gen ^R	This study
PA Δ <i>phz2</i> Δ <i>rpoS</i>	<i>rpoS</i> knocked out and replaced with <i>aacC1</i> in the mutant Δ <i>phz2</i> , Spe ^R Gen ^R Kan ^R	This study
PA Δ <i>lasR::lacZ</i>	<i>lasR</i> deleted and fused in-frame with the truncated <i>lacZ</i> , Amp ^R Spe ^R	This study
PA Δ <i>lasR::lacZ</i> Δ <i>rpoS</i>	<i>rpoS</i> deleted in the fusion mutant PA Δ <i>lasR::lacZ</i> , Spe ^R Gen ^R	This study
Plasmids		
pBluescript II SK	Cloning vector with high copies, ColE, Amp ^R	Stratagene
pUCm-T	T-vector for cloning, ColE, Amp ^R	Sangon
pEX18Tc	Suicide vector with MCS from pUC18, <i>sacB</i> ⁺ <i>oriT</i> ⁺ , Tet ^R	[34]
pEXS	pEX18Tc containing a 2.0-kb <i>rpoS</i> -flanking DNA fragment, Tet ^R	This study
pEXSG	A gentamicin resistance cassette inserted in pEXS, Tet ^R Gen ^R	This study
pEXR	a 2.0-kb <i>lasR</i> -flanking DNA fragment cloned in pEX18Tc, Tet ^R	This study
pEXRZ	The upstream of <i>lasR</i> fused in frame with the truncated <i>lacZ</i> in pEXR, Tet ^R	This study
pMS	A 1.7-kb <i>rpoS</i> gene cloned in pME6010, Tet ^R	This study
pME15Z1	A 0.9-kb <i>phz1</i> upstream DNA fragment and its first 8 <i>phzA1</i> codons cloned in pME6015, Tet ^R	[33]
pME15Z2	A 0.9-kb <i>phz2</i> upstream DNA fragment and its first 8 <i>phzA2</i> codons cloned in pME6015, Tet ^R	[33]
pSI	A 0.9-kb <i>lasI</i> upstream DNA fragment and its first 8 <i>lasI</i> codons cloned in pME6015, Tet ^R	This study
pSR	A 0.9-kb <i>lasR</i> upstream DNA fragment and its first 8 <i>lasR</i> codons cloned in pME6015, Tet ^R	This study
pLI	A 0.9-kb <i>rhlI</i> upstream DNA fragment and its first 10 <i>rhlI</i> codons cloned in pME6015, Tet ^R	This study
pLR	A 0.9-kb <i>rhlR</i> upstream DNA fragment and its first 10 <i>rhlR</i> codons cloned in pME6015, Tet ^R	This study
pME22Z1	A 902-bp upstream DNA fragment of <i>phz1</i> (from -902 to +1) and transcriptional fusion <i>phz1-lacZ</i> cloned in pME6522, Tet ^R	[33]
pME22Z2	A 517-bp upstream DNA fragment of <i>phz2</i> (from -517 to +1) and transcriptional fusion <i>phz2-lacZ</i> cloned in pME6522, Tet ^R	[33]
pME6010	Low-copy shuttle vector between <i>E. coli</i> and <i>Pseudomonas</i> sp., Tet ^R	[38]
pME6015	pVS1-p15A shuttle vector for translational <i>lacZ</i> fusion, Tet ^R	[38]
pNM480	' <i>lacZ</i> fusion vector, Amp ^R	[37]
pUCGm	Gentamicin resistance gene cassette flanked with multiple restriction endonuclease sites, Amp ^R Gen ^R	[35]

previously described [33, 36]. To delete the *rpoS* gene in the *phz1*-deficient mutant (PA Δ *phz1*), the conjugal mating was performed with the mutant PA Δ *phz1* as a receptor strain and *E. coli* SM10 bearing pEXS as a donor strain, generating the mutant PA Δ *phz1* Δ *rpoS*. Similarly, the mutant PA Δ *phz2* Δ *rpoS* was obtained

with conjugation using the mutant PA Δ *phz2* as a receptor strain and *E. coli* SM10 harboring pEXSG as a donor strain. All of the *rpoS*-deletion mutants (PA Δ *rpoS*, PA Δ *phz1* Δ *rpoS*, and PA Δ *phz2* Δ *rpoS*) were verified by PCR (Data not shown).

To construct the fusion mutant PA Δ *lasR::lacZ*, the disruption

Table 2. Primers used in this work.

Name of primer	Sequence (5'-3') and artificial restriction endonuclease site (bold and in italics)
rpoS-1F	GTGCACCTGAACGCGACCGAGC
rpoS-1R _{XbaI}	CTATTATCTAGAAGAATGGCCTGTCTGAGTGACG (XbaI)
rpoS-2F _{XbaI}	GAATTATCTAGAGTGCCATGTCGTTATCCCTTGC (XbaI)
rpoS-2R	CT AACGGCCCGATCGCCTGAG
rpoS-3F _{SacI}	GAATTAGAGCTCAAGCGCCTGGAAACCACCAGC (SacI)
rpoS-3R _{PstI}	CTAATACTGCAGATGGATAAGGGGGAAGGATTGAG (PstI)
lasR-1F	TTCATCCTCTTCATGCTGCCGACC
lasR-1R _{Scal}	GAACTTAGTACTCTCAAGAAAACCGTCAACCAAGGC (Scal)
lasR-2F _{Scal}	GTTCAAAGTACTTTTCGGACGTTTCTTCGAGCCTAG (Scal)
lasR-2R	CCTGATAGCGAGTCCGGAGCGGGTC
lasR-3F _{SacI}	CAAGTTGAGCTCGTGATCGCCGCGATCCTGTTC (SacI)
lasR-3R _{HindIII}	CAAGTTAAGCTTTACAGCGGATTCGGCATCGAC (HindIII)
rpoS-CF	CGATCCCCAGTGCTCCCGCAACCTG
rpoS-CR	GACCAAGCCTACCCCGTTCCACCC
lasI-TF	CAATTAGAATTCTGGGAACCGTCCATCTACCAG (EcoRI)
lasI-TR	GTAATTGGATCCGACCAATTTGTACGATCATCTTC (BamHI)
lasR-TF	CAATTAGAATTCTCTCTGGGAGCTGGAGCATGG (EcoRI)
lasR-TR	CTAATTCTGCAGCTCAAGAAAACCGTCAACCAAG (PstI)
rhII-TF	CAATTAGAATTCCCTTCACCCGGCCGAAGACC (EcoRI)
rhII-TR	GTAATTCTGCAGCGATTCAGAGCAATTCGATC (PstI)
rhIR-TF	CAATTAGAATTGCGCCGAGGGCGTGGTCTG (EcoRI)
rhIR-TR	GTAATAGGATCCACAGCAAAAAGCCTCCGTC (BamHI)

plasmid was also created. With the same methods above, pEXR was constructed after the upstream DNA region (including the first 10 codons of *lasR*) and the downstream DNA region of *lasR* were amplified with primers (lasR-1F/lasR-1R_{Scal}, lasR-2F_{Scal}/lasR-2R, and lasR-3F_{SacI}/lasR-3R_{HindIII}). Then, a 3.2-kb truncated *lacZ* fragment obtained with *SmaI*-*DraI* digestion of pNM480 was in-frame fused and inserted in the *Scal* site in the pEXR to generate pEXRZ [37]. Conjugal mating was performed using the wild-type strain PAO1 as a receptor strain. Allelic exchange occurred and the fusion mutant PAΔ*lasR*::*lacZ* was finally selected on LB plates supplemented with sucrose. Similarly, with this fusion mutant PAΔ*lasR*::*lacZ* as a receptor strain, conjugal mating was performed using *E. coli* SM10 bearing pEXSG as a donor strain, generating an *rpoS*-deleted fusion mutant PAΔ*rpoS*Δ*lasR*::*lacZ*. These fusion mutants, PAΔ*lasR*::*lacZ* and PAΔ*rpoS*Δ*lasR*::*lacZ*, were verified with PCR amplification method (Data not shown).

Complementation Assay of the *rpoS* Gene

To complement the *rpoS* gene, an amplicon with 1,840 bp in length covering the whole open reading frame of the *rpoS* and its promoter region was amplified using a pair of primers (rpoS-WF/rpoS-WR). After cleaving with restriction endonuclease *KpnI* and

HindIII simultaneously, the PCR amplicon with 1,761-bp in length was ligated and cloned into the low-copy vector pME6010, creating pMS [38]. After sequencing, the plasmid DNA was transformed into competent PAO1 cells or its derivatives by electroporation. The positive colonies developed on LB agar plates supplemented with tetracycline were confirmed by plasmid isolation and restriction enzyme digestion analysis.

Creation of the Translational Fusion Vectors: *lasI*'-'*lacZ*, *lasR*'-'*lacZ*, *rhII*'-'*lacZ* and *rhIR*'-'*lacZ*

To assess the expression levels of the two QS system components in the absence of RpoS, the translational fusion vectors, including *lasI*'-'*lacZ*, *lasR*'-'*lacZ*, *rhII*'-'*lacZ* and *rhIR*'-'*lacZ*, were constructed with plasmid pME6015, respectively. Briefly, about 900-bp DNA fragments covering the first several codons of *lasI*, *lasR*, *rhII* and *rhIR* and their upstream regions were amplified with primer pairs lasI-TF/lasI-TR, lasR-TF/lasR-TR, rhII-TF/rhII-TR, and rhIR-TF/rhIR-TR, respectively. The relevant PCR products were double-cleaved, and ligated and fused in-frame with the truncated reporter gene *lacZ* in pME6015, generating pSI, pSR, pLI, and pLR, respectively [38]. All of the fusion vectors were confirmed by sequencing reports before they were transformed into host cells.

PCA and PCN Assays

To quantify PCA, the strains were inoculated and cultivated in 150 ml of GA broth in 500-ml shake flasks at 37°C for 72 h. Then, 900 µl of cultures were harvested every 12 h. After acidifying to pH 4.0, a 3-fold sample volume of chloroform was added to each of the samples. After being vortexed, chloroform extracts were then clarified with centrifugation at 12,000 rpm for 5 min. PCA in samples was diluted appropriately, and determined quantitatively at 252 nm with a spectrophotometer [33]. The concentration of PCA was obtained according to the equation $[C_A (\mu\text{g/ml}) = 2.9667 \times OD_{252} - 0.0979]$, $R^2 = 0.9998$ that was created with a purified sample of PCA.

PCN was extracted with chloroform from cultures grown in 150 ml of GA or LB broth with shaking at 37°C. Samples were harvested and PCN was determined quantitatively every 12 h. Briefly, 2.5 ml of cultures were mixed with 1.5 ml of chloroform. After vortexing for 5 min, supernatants were discarded and 1 ml of 0.2 mol/l HCl was added to the sample. PCN extracted in the top layer was determined quantitatively at 520 nm with a spectrophotometer [39]. Concentrations of PCN in cultures were obtained $[C_N (\mu\text{g/ml}) = 17.072 \times OD_{520}]$ [40]. An authentic PCN was purchased from Sigma-Aldrich (USA).

β-Galactosidase Activity Assay

All bacterial strains were grown in 150 mL LB or GA broth with shaking at 180 rpm at 37°C. Samples of the transformants or other relevant derivatives were harvested every 12 h. β-Galactosidase activity was quantified with the regular method as described by Dr. Miller [29, 41].

Statistical Analysis

Relevant data were analyzed with one-way analysis of variance using the statistical software package SPSS (USA). Duncan's multiple range test was applied for means separation of

phenazine production and β-galactosidase activity in LB or GA broth. Values of $p < 0.05$ were considered statistically significant, and values of $p < 0.01$ were extremely significant.

Results and Discussion

RpoS Acts as a Repressor for Phenazines Biosynthesis in *P. aeruginosa*

To confirm how phenazine biosynthesis is regulated by RpoS in *P. aeruginosa*, we generated the *rpoS*-deletion mutant $\text{PA}\Delta\text{rpoS}$ with the wild-type strain PAO1 as acceptor cell in conjugation. Both the mutant $\text{PA}\Delta\text{rpoS}$ and the wild-type strain PAO1 were separately grown in GA broth. Their PCA and PCN production were quantified at 12 hour intervals. As shown in Fig. 1A, in comparison with the wild-type strain PAO1, the mutant $\text{PA}\Delta\text{rpoS}$ biosynthesized much more PCA. At the same time, it was found that the amount of PCA was reduced dramatically as the *rpoS* was re-delivered into the mutant $\text{PA}\Delta\text{rpoS}$ through pMS, but the amount of PCA production did not alter as the mutant $\text{PA}\Delta\text{rpoS}$ was transformed with the empty shuttle vector pME6010, suggesting that the knockout of *rpoS* contributed to more production of PCA.

As shown in Fig. 1B, although PCN is converted through other metabolic pathways from its precursor, PCA, its production in the mutant $\text{PA}\Delta\text{rpoS}$ cultivated with GA medium was about 2-fold higher than that produced in the wild-type strain PAO1. We also found that the amounts of PCN in the mutant $\text{PA}\Delta\text{rpoS}$ bearing pMS decreased and were almost equal to those in the parental strain PAO1. Meanwhile, PCN production in the transformant $\text{PA}\Delta\text{rpoS}/\text{pME6010}$ was almost the same as that in the parental strain

Fig. 1. Phenazines produced in the wild-type strain PAO1 and its derivatives in GA broth.

Phenazine-1-carboxylic acid (A) and pyocyanin (B) were quantified while the wild-type strain PAO1 and its derivatives were separately cultivated in GA broth for 72 h. All experiments were performed at least three times. Error bars represented standard deviations of three determinations. Different superscript letters indicated extremely significant difference ($p < 0.01$) according to Duncan's multiple range test.

PA Δ rpoS. The results indicated that the inactivation of *rpoS* contributed to more production of PCN. Taken together, it was suggested that RpoS could act as a repressor for phenazine biosynthesis in *P. aeruginosa*.

The *phz1* Operon Contributes Much More to Increased Production of Phenazine in the Absence of RpoS than the *phz2* Operon

Although RpoS deficiency caused a remarkable increase of phenazines biosynthesized in the *rpoS*-negative mutant PA Δ rpoS, this conclusion is not enough for us to understand whether or how RpoS governs each of *phz1* and *phz2*. To further study if RpoS exerts regulatory roles on the expression of the *phz1* operon, we knocked out the *rpoS* in the mutant PA Δ phz2, creating the mutant PA Δ phz2 Δ rpoS. Two strains were separately cultivated into GA broth for measuring phenazine production, applying the double mutant PA Δ phz1phz2 as a negative control. As shown in Fig. 2A, compared with the parental mutant PA Δ phz2, the amount of PCA biosynthesized in the mutant PA Δ phz2 Δ rpoS was dramatically enhanced. As shown in Fig. 2B, the amount of PCN produced in the mutant PA Δ phz2 Δ rpoS was dramatically increased nearly 4-fold, compared to that produced in the mutant PA Δ phz2. When re-introduced with the *rpoS* of *P. aeruginosa*, the transformant PA Δ phz2 Δ rpoS/pMS biosynthesized the same amount of phenazines as the mutant PA Δ phz2. In fact, only *phz1* operon is present in the mutants PA Δ phz2 and PA Δ phz2 Δ rpoS, suggesting that their phenazine production (PCA and PCN) are just produced with the presence of the *phz1*. So, these results showed that the knockout of the *rpoS* gene makes the *phz1* biosynthesize

much more phenazine and RpoS downregulates the phenazine biosynthesis mediated by the *phz1*.

To further examine whether RpoS has regulatory effects on the function of the *phz2* operon, we also knocked out the *rpoS* gene in the mutant PA Δ phz1, generating the mutant PA Δ phz1 Δ rpoS. Both of the mutants PA Δ phz1 and PA Δ phz1 Δ rpoS were respectively cultivated in GA broth for phenazine assay, while the double mutant PA Δ phz1phz2 cultures were used as negative controls. Compared to the parental mutant PA Δ phz1, as shown in Fig. 3A, the amount of PCA produced in the mutant PA Δ phz1 Δ rpoS was remarkably reduced 2-fold. Meanwhile, PCN produced in the mutant PA Δ phz1 Δ rpoS was remarkably decreased 3-fold compared to that in the mutant PA Δ phz1 (Fig. 3B). For only the *phz2* is present in the mutants PA Δ phz1 and PA Δ phz1 Δ rpoS, and phenazines are only produced by the *phz2*. Therefore, these results showed that the knockout of the *rpoS* gene makes the *phz2* biosynthesize less phenazine and RpoS upregulates the phenazine biosynthesis mediated by the *phz2*. Taken together, because phenazine production with the *phz1* operon was so much higher than the decreased phenazine production with the *phz2* operon, the net amount of phenazine eventually increased a lot in the absence of RpoS in *P. aeruginosa*.

RpoS Does Down-Regulate the *phz1* Expression, but Up-Regulates *phz2* Expression at the Posttranscriptional Level, Not at the Transcriptional Level

To check whether RpoS exerts regulatory effects on the expression of *phz1* and *phz2*, we transformed the translational fusion vector pME15Z1 (*phzA1*'-'*lacZ*), pME15Z2 (*phzA2*'-

Fig. 2. Phenazines produced in the *phz2*-deleted derivatives in GA broth.

Phenazine-1-carboxylic acid (A) and pyocyanin (B) were biosynthesized by the *phz2*-deleted mutant PA Δ phz2, PA Δ phz2 Δ rpoS, and their derivatives in 150 ml of GA broth at 37°C for 72 h. All experiments were carried out at least three times. Error bars represented standard deviations of three determinations. Different superscript letters indicated extremely significant difference ($p < 0.01$) based on Duncan's multiple range test.

Fig. 3. Phenazines produced in the *phz1*-knockout derivatives in GA broth.

Phenazine-1-carboxylic acid (A) and pyocyanin (B) were biosynthesized by the *phz1*-deleted mutant PAΔ*phz1*, PAΔ*phz1*Δ*rpoS*, and their derivatives in 150 ml of GA broth at 37°C for 72 h. All experiments were carried out at least three times. Error bars represented standard deviations of three determinations. Different superscript letters indicate extremely significant difference ($p < 0.01$) based on Duncan's multiple range test.

'*lacZ*) and the transcriptional fusion vector pME22Z1 (*phzA1-lacZ*), pME22Z2 (*phzA2-lacZ*) into the mutant PAΔ*rpoS* and the wild-type strain PAO1, respectively, using the pME6015 and pME6522 as negative controls. After being inoculated into GA medium, these transformants were grown in a shaker at 37°C and their β-galactosidase activities were quantified with a standard method once every 12 hours. As showed in Fig. 4, compared to the wild-type strain PAO1, β-galactosidase activities of the translational fusion pME15Z1 (*phzA1'-lacZ*) in the mutant PAΔ*rpoS* were increased 2-fold. β-Galactosidase activities of the transcriptional fusion

pME22Z1 (*phzA1-lacZ*), however, were equivalent in strains PAΔ*rpoS* and PAO1. These results suggested that the *rpoS*-knockout up-regulated the expression of the *phz1* at the post-transcriptional level, not at the transcriptional level, indicating that RpoS has negative regulatory effect on the expression of the *phz1* at the post-transcriptional level. At the same time, compared to the wild-type strain PAO1, β-galactosidase activities of the translational fusion pME15Z2 (*phzA2'-lacZ*) in the mutant strain PAΔ*rpoS* were reduced 2-fold. β-Galactosidase activities of the transcriptional fusion pME22Z2 (*phzA2-lacZ*), however, were almost the same in both strains. These results

Fig. 4. β-Galactosidase activity assay with the translational fusion pME15Z1 (A), pME15Z2 (B), and the transcriptional fusion pME22Z1 (C), pME22Z2 (D) in the wild-type strain PAO1 and its mutant PAΔ*rpoS*, respectively.

Relevant strains were separately cultivated in 150 ml of GA broth for 72 h at 37°C. All experiments were carried out at least three times. Error bars represents standard deviations of three determinations.

indicated that the knockout of the *rpoS* down-regulated the expression the *phz2* at the post-transcriptional level, not at the transcriptional level, suggesting that RpoS plays a positive regulatory role on the *phz2* expression at the post-transcriptional level.

LasR Might Act as an Intermediate in Overproduction of Phenazine Biosynthesis in the *rpoS*-Deleted Mutant

To investigate how RpoS regulates the expression of the *phz1* and *phz2* operons, we tried to confirm whether the LasI-LasR and RhII-rhIR quorum sensing systems are regulated by RpoS. The fusion vectors pSI (*lasI*'-'*lacZ*), pSR (*lasR*'-'*lacZ*), pLI (*rhII*'-'*lacZ*), and pLR (*rhIR*'-'*lacZ*) were introduced into the mutants *PAΔphz2* and *PAΔphz2ΔrpoS*, respectively. All transformants were cultivated in GA medium and their β-galactosidase activities were quantified with standard method. As shown in Fig. 5, β-galactosidase

Fig. 5. β-Galactosidase activity assay with the translational fusion pLR (A), pLI (B), pSR (C), and pSI (D) in the mutant *PAΔphz2* and *PAΔphz2ΔrpoS*, respectively.

Relevant strains were separately cultivated in 150 ml of GA broth at 37°C for 72 h. All experiments were carried out at least three times. Error bars represent standard deviations of three determinations.

activities only produced by the translational fusion pSR (*lasR*'-'*lacZ*) in the mutant *PAΔphz2ΔrpoS* were 2-fold higher than those in the mutant *PAΔphz2*, while β-galactosidase activities produced by each of the other fusion vectors (pSI, pLI, and pLR) were almost the same in both of the mutants *PAΔphz2* and *PAΔphz2ΔrpoS*, respectively, indicating that RpoS deficiency would up-regulate the expression of *lasR*.

To confirm upregulation of the *lasR* expression in the absence of RpoS, we also constructed the fusion mutants *PAΔlasR::lacZ* and *PAΔlasR::lacZΔrpoS*. We quantified their β-galactosidase activities while they were respectively grown in GA medium at 37°C for 72 h. As shown in Fig. 6, β-galactosidase activities produced by the fusion mutant *PAΔlasR::lacZΔrpoS* were almost 2-fold higher than those produced in the fusion mutant *PAΔlasR::lacZ*, indicating that RpoS deficiency caused overexpression of *lasR*. As we all know, the LasI/LasR system activates RhII/RhIR and PQS systems that in turn positively regulate the expression of the *phz1* operon. Therefore, we suggested that LasR might act as an intermediate for overproduction of the *phz1*-mediated phenazine biosynthesis in the absence of RpoS.

P. aeruginosa is one of the most common pathogens that may cause infectious disease in hospitals and communities. During its infection, it usually biosynthesizes and secretes several virulence factors into the host. It had been reported

Fig. 6. β-Galactosidase activity assay with the fusion mutant *PAΔlasR::lacZ* and *PAΔlasR::lacZΔrpoS*.

Relevant strains were separately cultivated in 150 ml of GA broth at 37°C for 72 h. All experiments were carried out at least three times. Error bars represent standard deviations of three determinations. **, indicates extremely significant difference ($p < 0.01$), and * indicates no significant difference ($p > 0.05$) according to two-tailed paired Student's *t* test.

that PCN was an extremely important virulence factor and played a critical role during infection. Although it was also reported that the absence of RpoS led to more PCN production in the *rpoS*-inactivated mutant, it has not been known whether or how each of two *phz* operons is regulated by RpoS in detail. To get a better insight into this regulatory pathway, in this study, we first generated a set of mutants and then regulation of *phz1* and *phz2* mediated by RpoS was investigated by quantifying and analyzing their phenazine production. It was finally concluded that RpoS exerts opposite regulatory effects on the expression of each of the two *phz* operons and PCN biosynthesis. In the absence of RpoS, the *phz2* produces less phenazine, but the *phz1* produces much more, indicating that phenazine production was balanced very well due to there being two copies of *phz* operons in the PAO1 strain.

Meanwhile, with creation of the transcriptional and translational fusion vectors with the promoterless *lacZ*, it was also demonstrated that the regulation mediated by RpoS on the expression of *phz1* and *phz2* happened at the post-transcriptional level, not at the transcriptional level. Based on these results, it is reasonably concluded that RpoS up- or down-regulates *phz1* and *phz2* expression indirectly, not directly. There probably is an intermediate that locates between RpoS and two phenazine-producing operons. The production of this intermediate should be regulated by RpoS and directly governs the *phz* expression. Although the regulation of the *rpoS* has been identified to be involved with the *las*, the *rhl* system, and other quorum sensing systems [42, 43], this intermediate has not been found and identified. To screen such intermediate regulators, we examined whether the first two quorum sensing systems were at downstream of the RpoS-mediated regulatory pathway. It was demonstrated that the *lasR* was overexpressed in the absence of RpoS. In fact, it was confirmed previously that the expression of *phz1* was directly upregulated by *rhlI/rhlR* and PQS system [44]. LasI/LasR system activates synthesis of PQS, which in turn activates *rhlI* expression [45, 46]. According to these data, we reasonably suggested that LasR might act as an intermediate for phenazine overproduction mediated by the *phz1* in the RpoS-negative mutant. However, we still did not learn more about the regulation of *phz2*. In addition, it was just reported that inactivation of LasR and RhlR did not block the expression of some virulence factors, suggesting the regulation of virulence factor production could be complicated [47]. Further study on the specific regulation of phenazine biosynthesis mediated by RpoS will be needed later.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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