RESEARCH ARTICLE

Cross-Regulation between the \textit{phz1} and \textit{phz2} Operons Maintain a Balanced Level of Phenazine Biosynthesis in \textit{Pseudomonas aeruginosa} PAO1

Qinna Cui\textsuperscript{1,2}, Huinan Lv\textsuperscript{1}, Zhuangzhuang Qi\textsuperscript{1}, Bei Jiang\textsuperscript{2}, Bo Xiao\textsuperscript{1}, Linde Liu\textsuperscript{1}, Yihe Ge\textsuperscript{1,*}, Xiaomei Hu\textsuperscript{2,*}

1 Department of Applied and Environmental Microbiology, School of Biological Sciences, Ludong University, Yantai, China, 2 Department of Microbiology, College of Basic Medical Sciences, Third Military Medical University, Chongqing, China

* These authors contributed equally to this work.
¤ Current address: State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

Abstract

Gene duplication often provides selective advantages for the survival of microorganisms in adapting to varying environmental conditions. \textit{P. aeruginosa} PAO1 possesses two seven-gene operons [\textit{phz1} (\textit{phzA1B1C1D1E1F1G1}) and \textit{phz2} (\textit{phzA2B2C2D2E2F2G2})] that are involved in the biosynthesis of phenazine-1-carboxylic acid and its derivatives. Although the two operons are highly homologous and their functions are well known, it is unclear how the two \textit{phz} operons coordinate their expressions to maintain the phenazine biosynthesis. By constructing single and double deletion mutants of the two \textit{phz} operons, we found that the \textit{phz1}-deletion mutant produced the same or less amount of phenazine-1-carboxylic acid and pyocyanin in GA medium than the \textit{phz2}-knockout mutant while the \textit{phz1}-\textit{phz2} double knockout mutant did not produce any phenazines. By generating \textit{phzA1} and \textit{phzA2} translational and transcriptional fusions with a truncated \textit{lacZ} reporter, we found that the expression of the \textit{phz1} operon increased significantly at the post-transcriptional level and did not alter at the transcriptional level in the absence of the \textit{phz2} operon. Surprisingly, the expression the \textit{phz2} operon increased significantly at the post-transcriptional level and only moderately at the transcriptional level in the absence of the \textit{phz1} operon. Our findings suggested that a complex cross-regulation existed between the \textit{phz1} and \textit{phz2} operons. By mediating the upregulation of one \textit{phz} operon expression while the other was deleted, this crosstalk would maintain the homeostatic balance of phenazine biosynthesis in \textit{P. aeruginosa} PAO1.

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Introduction

Phenazines are an array of secondary metabolites that are biosynthesized and secreted by fluorescent pseudomonad. Many studies have reported that phenazines play a major role in microbial competitiveness [1,2], suppression of soil-borne plant fungal pathogens [3–6], and affect their pathogenicity in human or animal hosts [7,8].

Of all the phenazine-producing microorganisms, the major opportunistic pathogen *Pseudomonas aeruginosa* is the most widely studied phenazine-producing bacterium. *P. aeruginosa* has been identified as a common pathogen in animals, insects, nematodes, and plants [8–11]. In the human host, *P. aeruginosa* causes severe and chronic infections in immunocompromised, burned, and injured patients [12]. Additionally, *P. aeruginosa* is the most commonly found pathogen associated with cystic fibrosis (CF) in patients’ lung and is responsible for progressive lung tissue destruction leading to respiratory failure [13,14].

*P. aeruginosa* produces a common precursor phenazine-1-carboxylic acid (PCA) that is biosynthesized into its main derivatives pyocyanin (PYO), 1-hydrophenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) [1, 15–17]. It was reported that at least 90% of *P. aeruginosa* isolates could produce PYO [17,18]. Moreover, PYO was detected at high concentrations in the sputum of cystic fibrosis patients, suggesting that phenazine compounds could act as virulence factors and play a crucial role in host-pathogen interactions [19,20]. This hypothesis is supported by several studies on the pathophysiological effects of PYO and other phenazine derivatives found in the airways of individuals infected with *P. aeruginosa*. For example, it was proposed that PCA and PYO were responsible for increasing oxidant production, neutrophil chemokine IL-8 and leukotriene B4 release, and the expression of intercellular adhesion molecule-1 (ICAM-1) by human airway epithelial cells [21–23]. PYO could also inhibit the cytokine-dependent expression of RANTES, and monocyte chemoattractant protein-1 (MCP-1) [23–25]. Moreover, PYO was recently shown to cause airway goblet cell hyperplasia and metaplasia and mucus hypersecretion in airway epithelial cells [26].

Two copies of the seven-gene operon *phz1* (*phzA1B1C1D1E1F1G1*) and *phz2* (*phzA2B2C2D2E2F2G2*) are known to be responsible for the biosynthesis of PCA in *P. aeruginosa* and *Streptomyces cinnamomensis* [17,27,28]. In these strains, the *phz1* and *phz2* operons share 99% identity and possess similar flanking genes respectively. Gene duplication is often found in many microorganisms and is thought to provide several selective advantages when the bacteria encounter various environments [29]. For example, the maintenance of duplicate genes may be favored when spatial or temporal differences in expression enable tissue-specific variation or survival under varying environmental conditions [30,31]. In *P. aeruginosa* PA14, the two *phz* operons showed environment-dependent expression and played differential roles in its pathogenicity [32].

In the PAO1 strain, the *phz1* is located at positions 4,713,795 to 4,720,062 bp in the genome, while the *phz2* is located approximately 2.6 Mb from *phz1* at positions 2,070,685 to 2,076,985 bp. Although the two *phz* operon exhibit 98.3% identity at the DNA level, their promoter regions are quite different, indicating that *phz1* and *phz2* may be modulated via different regulation mechanisms [17]. Both the PQS and rhl systems positively regulate *phz1* expression [29,30], while the orphan LuxR-type quorum sensing regulator QscR negatively regulates *phz1* and *phz2* expression [12,31]. Although both *phz1* and *phz2* contribute to the production of phenazines, *phz1* expression has been proposed to account for the majority of phenazines biosynthesis based on regulation analysis [33,34]. However, it is now known if the *phz1* and *phz2* operons cross-regulate each other during phenazine biosynthesis. In this study, we first generated mutants lacking the *phz1* and/or *phz2* operons and evaluated phenazine biosynthesis in the PAO1 strain. Because PCA and PYO of phenazines produced by the *phz1* or *phz2* operons
differed from those reported in the PA14 strain during growth in liquid batch cultures [32], we employed promoterless lacZ fusions constructed on a plasmid and the chromosome to examine the expression of the phz1 and phz2 operons at the transcriptional and post-transcriptional level. Our results indicated that a cross talk could exist between the phz1 and phz2 operons in the PAO1 strain. This cross-regulation between the two phz operons may function to balance phenazine biosynthesis homeostatically.

**Materials and Methods**

**Bacterial strains, plasmids, primers and culture conditions**

All bacterial strains and the primary plasmids and primers used in this study are shown in Tables 1 and 2, respectively. Cultures of *Escherichia coli* were routinely grown in Luria-Bertani (LB) medium at 37°C [35]. *P. aeruginosa* PAO1 and its derivatives were routinely grown at 37°C in LB broth with shaking at 180 rpm, or on LB agar sometimes amended with sucrose (10%) for screening double-cross mutants, or in glycerol-alanine supplemented (GA) medium for the PCA and PYO assays [36]. The antibiotics applied to the medium included spectinomycin (Sp, 100 μg/ml), tetracycline (Tc, 125 μg/ml), kanamycin (Km, 300 μg/ml) or gentamycin (Gm, 40 μg/ml) in the experiments with the PAO1 strain and its derivatives and ampicillin (Ap, 50 μg/ml), Tc (25 μg/ml), Km (50 μg/ml) or Gm (20 μg/ml) in the experiments with *E. coli*.

**DNA manipulation and cloning procedure**

Small-scale plasmids were prepared from *P. aeruginosa* derivatives or *E. coli* using the alkaline lysis method or Plasmid DNA Extraction Kit (Sangon, Shanghai, China). Chromosomal DNA was isolated from *P. aeruginosa* with the method as described by Chen and Kuo [37] or by using the Genomic DNA Extraction Kit (Sangon, Shanghai, China). Standard DNA recombinant techniques were applied for digestion, agarose gel electrophoresis, dephosphorylation, isolation of DNA fragments from agarose gels, and ligation. *E. coli* or *Pseudomonas sp.* cells were transformed with plasmid DNA by CaCl₂ treatment or electroporation, respectively [38].

Polymerase chain reactions (PCRs) were typically performed with 2.5 U of thermostable DNA polymerase in a reaction mixture containing 100 ng of target DNA. A 250 μM concentration of each of the four dNTPs, 10 pmol of two primers, 5 mM MgCl₂, and 1× buffer in a final volume of 25 μl were used for the amplification reaction. A total of 30 or 33 cycles (2 min at 94°C, 30 sec at 50 to 55°C, and 1 min 72°C) was followed by a final elongation step for 7 min at 72°C. PCR products were cloned into pGEM-T or pBluescript II SK for verification by sequencing.

**Deletion mutation of the phz1 and/or phz2 operons**

To delete the phz1 operon, a disruption plasmid was first created. A 1114-bp fragment covering a partial sequence of the phzM gene and a partial upstream region of the phzA1 gene was amplified with primers phz1-1F and phz1-1R. A second fragment of 1170-bp which was located at the downstream region of the phzG1 and contained a partial sequence of the phzS was amplified using primers phz1-2F and phz1-2R. The two PCR products were pooled, purified using the PCR purification kit (Sangon, Shanghai, China), digested with *Xho*I, repurified, and ligated. The resultant ligation served as the template, and nested PCR was performed with primers phz1-3F and phz1-3R. After double digestion with *Sac*I and *Hind*III, the PCR product was cloned into the suicide plasmid pEX18Tc [39], resulting in pEXZ1. A gentamycin
resistance cassette \((\text{aacC}1)\) was obtained via the \(X\text{ba}1\)-digestion of the cloning vector \(\text{pUCGm}\) [40], and cloned into the unique \(X\text{ba}1\) site in \(\text{pEXZ1}\) to generate \(\text{pEXZ1G}\).

To knock out the \(\text{phz2}\) operon, the same nested PCRs were performed to construct the suicide plasmid \(\text{pEXZ2}\). Briefly, a first fragment with a length of 1987 bp containing whole \(\text{qscR}\) sequence and partial upstream region of \(\text{phzA2}\) and a second fragment of 1087 bp covering the

| Strain/plasmid | Relevant characteristics | Source/reference |
|---------------|--------------------------|------------------|
| **Strains**   |                          |                  |
| \(\text{E. coli}\) | \(\Phi 80 \text{lacZ}\Delta\text{M15} \Delta (\text{lacZYA-argF}) \text{U169 hsdR17 recA1endA1 thi-1}\) | Lab collection |
| \(\text{SM10}\) | \(\text{F}^- \text{thi-1 thr-1 leuB6 recA1 tonA1 lacY1 supF44} (\text{M15})\) \(\lambda\) | Lab collection |
| \(\text{P. aeruginosa}\) | \(\text{PAO1}\) Phenazine-1-carboxylic acid and its derivatives producer, Wild type, Ap\(^r\)Sp\(^r\) | Lab collection |
| \(\Delta\text{phz1}\) | \(\text{phz1} \text{locus deleted} \text{and inserted with aacC1, Sp}\(^r\)\) | This study |
| \(\Delta\text{phz2}\) | \(\text{phz2} \text{locus deleted} \text{and inserted with aph, Sp}\(^r\)\) | This study |
| \(\Delta\text{phz1phz2}\) | \(\text{phz1} \text{deleted} \text{and inserted with aacC1, phz2 deleted} \text{and inserted with aph, simultaneously, Gm}\(^r\)\) | This study |
| \(\Delta\text{phz1phz2}\) | The partial \(\text{phz2B2}\) deleted and chromosomally fused with the truncated \(\text{lacZ}\) in frame, \(\text{Sp}\(^r\)\) | This study |
| \(\Delta\text{phz1phz2}\) | \(\text{phz1} \text{deleted} \text{and inserted with aacC1 in the mutant \(\Delta\text{phz1}\), Sp}\(^r\)\) | This study |
| \(\Delta\text{phz1phz2}\) | \(\text{the partial phz1B1} \text{deleted} \text{and chromosomally fused with the truncated \(\text{lacZ}\) in frame, Sp}\(^r\)\) | This study |
| **Plasmids**  |                          |                  |
| \(\text{pBluescript II SK}\) | Clone vector, CoIE, Ap\(^r\) | Stratagene |
| \(\text{pGEM-T}\) | T-vector, CoIE, Ap\(^r\) | Promega |
| \(\text{pEX18Tc}\) | Gene replacement vector with MCS from \(\text{pUC18}\), ori\(\text{T}\) sacB\(^+\), Tc\(^r\) | [39] |
| \(\text{pEX18Tc}\) | \(\text{phz1} \text{flanking PCR fragment, Tc}\(^r\)\) | This study |
| \(\text{pEXZ1}\) | A 2.0-kb \(\text{phz1}\)-flanking PCR fragment inserted with \(\text{aacC1}\), Tc\(^r\)\) | This study |
| \(\text{pEXZ1}\) | A 2.4-kb \(\text{phz1B1}\) deleted PCR fragment cloned in \(\text{pEX18Tc}\), Tc\(^r\) | This study |
| \(\text{pEXZ1}\) | A 2.4-kb \(\text{phz1B1}\)-deleted PCR fragment fused in frame with the truncated \(\text{lacZ}\) in \(\text{pEX18Tc}\), Tc\(^r\) | This study |
| \(\text{pEXZ2}\) | A 3.0-kb \(\text{phz2}\)-flanking PCR fragment, Tc\(^r\) | This study |
| \(\text{pEXZ2}\) | A 3.0-kb \(\text{phz1}\)-flanking PCR fragment inserted with \(\text{aph}\) in \(\text{pEX18Tc}\), Tc\(^r\)\) | This study |
| \(\text{pEXZ2}\) | A 2.5-kb \(\text{phz2B2}\)-deleted PCR fragment cloned in \(\text{pEX18Tc}\), Tc\(^r\) | This study |
| \(\text{pEXZ2}\) | A 2.5-kb \(\text{phz2B2}\)-deleted PCR fragment fused in frame with the truncated \(\text{lacZ}\) in \(\text{pEX18Tc}\), Tc\(^r\) | This study |
| \(\text{pME10Z1}\) | \(\text{pME6010}\) containing a 6.9-kb \(\text{phz1}\) cluster, Tc\(^r\) | This study |
| \(\text{pME10Z2}\) | \(\text{pME6010}\) containing a 6.8-kb \(\text{phz2}\) cluster, Tc\(^r\) | This study |
| \(\text{pME15Z1}\) | A 0.9-kb \(\text{phz1}\) upstream fragment and a translational \(\text{phz1}\)-\(\text{lacZ}\) fusion with first 8 \(\text{phz1}\) codons in \(\text{pME6015}\), Tc\(^r\) | This study |
| \(\text{pME15Z2}\) | A 0.9-kb \(\text{phz2}\) upstream fragment and a translational \(\text{phz2}\)-\(\text{lacZ}\) fusion with first 8 \(\text{phz2}\) codons in \(\text{pME6015}\), Tc\(^r\) | This study |
| \(\text{pME22Z1}\) | \(\text{pME6522}\) carrying a 902-bp upstream region of \(\text{phz1}\) (from -902 to +1) and transcriptional fusion \(\text{phz1}\)-\(\text{lacZ}\), Tc\(^r\) | This study |
| \(\text{pME22Z2}\) | \(\text{pME6522}\) carrying a 517-bp upstream region of \(\text{phz2}\) (from -517 to +1) and transcriptional fusion \(\text{phz2}\)-\(\text{lacZ}\), Tc\(^r\) | This study |
| \(\text{pME6010}\) | \(\text{Low capy vector in} \text{Pseudomonas sp.}, \text{Tc}\(^r\)\) | [43] |
| \(\text{pME6015}\) | \(\text{pVS1-p15A}\) shuttle vector for translational \(\text{lacZ}\) fusion, Tc\(^r\) | [43] |
| \(\text{pME6522}\) | \(\text{pVS1-p15A}\) shuttle vector for transcriptional \(\text{lacZ}\) fusion and promoter probing, Tc\(^r\) | [44] |
| \(\text{pNM481}\) | \(\text{lacZ}\) fusion vector, Ap\(^r\) | [45] |
| \(\text{pNM482}\) | \(\text{lacZ}\) fusion vector, Ap\(^r\) | [45] |
| \(\text{pUC18-19Km}\) | CoIE, aph, kanamycin resistance cassette flanked with multiple restriction sites, Ap\(^r\)\(\text{Km}\(^r\)\) | [42] |
| \(\text{pUCGm}\) | CoIE, aacC, gentamycin resistance cassette flanked with multiple restriction sites, Ap\(^r\)\(\text{Gm}\(^r\)\) | [40] |
partial downstream region of \textit{phzG2} were amplified with two pairs of primers (phz2-1F/phz2-1R and phz2-2F/phz2-2R, respectively). After purification, digestion with \textit{Kpn}I, and re-purification, the two PCR products were mixed and ligated. Using the ligation product as a template, an approximately 3.0-kb nested PCR product was amplified with primers phz2-3F/phz2-3R and then cloned into pEX18Tc to obtain pEXZ2. A \textit{Kpn}I-digested kanamycin resistance cassette (\textit{aph}) from pUC18-19Km was cloned into the unique \textit{Kpn}I site in pEXZ2 to generate pEXZ2K [41,42].

After confirmation, the suicide plasmids pEXZ1G and pEXZ2K were mobilized from \textit{E. coli} SM10 (donor strain) to \textit{P. aeruginosa} PAO1 (receptor strain) by biparental mating. The \textit{phz1}-deficient mutant (designated as \textit{Δphz1}) was selected on plates containing 10% sucrose and

**Table 2. PCR primers used in this study.**

| Primers  | Sequences (5’-3’, artificial restriction enzyme site underlined and in italics) |
|----------|--------------------------------------------------------------------------------|
| phz1-1F  | GGA CGG CAC CTC TTG CAG CAT G                                                   |
| phz1-1R  | AAA TTT \textit{TCT AGA} TTT TCA GCG TCA TTC CGT G (XbaI)                       |
| phz1-2F  | CAA TTA \textit{TCT AGA} GCC CAT CTA ACC GCA CGC GGT C (XbaI)                   |
| phz1-2R  | CCA GCT CGA TGC CGT CCA GGA TTG C                                               |
| phz1-3F  | AAA TTT \textit{GAG CTC} CCC TGC CAA CAG GCT GG (Sacl)                         |
| phz1-3R  | GTA TAT \textit{AAG CTT} GCG AAG CCG CGC TGG CG (HindIII)                      |
| phz2-1F  | CAT CCA \textit{TTT GTT} CCA GGT GAT GCC                                       |
| phz2-1R  | TTA ATT \textit{GCT ACC} TAA TGC CGA ATT GCC ATG ACC G (Acc65I)                |
| phz2-2F  | CAA TAT \textit{GCT ACC} TGC AAC CGT GAC ACG ACC G (Acc65I)                    |
| phz2-2R  | GCC CGC CCG AGA AGC TTC AAC G                                                  |
| phz2-3F  | AAT TAA GAG \textit{CTC GAC ACC TGG AGG ATG TTG AGG AAG} (Sacl)               |
| phz2-3R  | GTF \textit{TCT AAG CTT} CGA GCA CGC CGC CCA ACG (HindIII)                     |
| phz2-12F | GTF \textit{CAT AGT ACT} CGA TGT CGA GGG GTG TTT CCC TG (ScaI)                 |
| phz2-12R | CAT GGG TCG AAC CGA GAT AGA C                                                   |
| phz2-13F | TAA ATT \textit{AAG CTT} GCT CGT CCT CGC GCA GCA TCG (HindIII)                |
| phz2-13R | CTC TCC CGA CGA CGA TGG AGC GTG C                                               |
| phz2-1F  | CTA TAT \textit{CCC GGG} TTT CGA AGA CGG CTT GGA G (SmaI)                     |
| phz2-2F  | CAA TAT \textit{CCC GGG} TTT CGA AGA CGG CTT GGA G (SmaI)                     |
| phz2-2R  | CCA CTT GGG CAG CCA GTC GTC GTC C                                              |
| phz2-3F  | CAT ATA \textit{GCT ACC GCC GTG AGG CCC ATC GGA GAC C} (Acc65I)                |
| phz2-3R  | GTF \textit{CTA TCT AGA CCG CCG TGC TCG TGC GTC ATG C} (XbaI)                 |
| phz1-WF  | GAT TAC \textit{AAG CTT} AGC AAT CCC GCA TAC CCT GTC (HindIII)                |
| phz1-WR  | ATA ATT \textit{GCT ACC GCC GTG ATG AAA CGT CGG AG} (KpnI)                     |
| phz2-WF  | GAA TAA GAG \textit{CTC GTG TGG TTC GGC ACG CTA GTG} (ScaI)                   |
| phz2-WR  | GTF \textit{ATT GCT CAG GTC CGC GCA GGA GCG ATG} (ScaI)                       |
| phz1-LF  | CTA TTA \textit{GAA TTC} GTC GAT CCC GCT CTC GATC (EcoRI)                     |
| phz1-LR  | GTF \textit{ATA GCT CAG TTC CCT GTC GGG GTG AC} (PstI)                        |
| phz2-LF  | GTF \textit{ATA GAA TTC CAC GGC ATC GGT CAC} (EcoRI)                         |
| phz2-LR  | CTT AAT \textit{GGA TTC CAA CCG TTG GTA CTC} (BamHI)                          |
| phz1-CF  | CAA \textit{TAA GAA TTC GCC GAC ACC GCC ACC GAC} (EcoRI)                     |
| phz1-PR  | GTF \textit{GTA TTA C7G CAG ATT GCA TAA AAC ACA GAA CGG TC} (PstI)            |
| phz2-CF  | GAA TAT \textit{GAA TTC GGC GAC CTG CTG GCG CC} (EcoRI)                      |
| phz2-PR  | GTF \textit{ATA GCT CAG ACA AAC TTA TAA ACG CCT TTT TG} (PstI)                |

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gentamycin due to its gentamycin resistance and tetracycline sensitivity. The \( \text{phz2} \) knockout mutant (called \( \Delta \text{phz2} \)) was obtained with the same selection methods described above based on its kanamycin resistance and tetracycline sensitivity. Then, the double-deletion mutant \( \Delta \text{phz1}\text{phz2} \) was constructed by mating the mutant \( \Delta \text{phz1} \) with the \( \text{pEXZ2K} \)-harboring cells of \( \text{E. coli} \) SM10, or by mating the mutant \( \Delta \text{phz2} \) with the \( \text{pEXZ1G} \)-bearing cells of \( \text{E. coli} \) SM10. All of the mutant constructs involved in this study are shown in Fig 1A. The insertion of the \( \text{aacC1} \) and \( \text{aph} \) resistance cassette was verified by PCR in all mutants and relevant data were available on figshare (http://dx.doi.org/10.6084/m9.figshare.1612163).

Cloning and complementation expression of the \( \text{phz1} \) or \( \text{phz2} \) operons
To clone the \( \text{phz1} \) operon, a 6.9-kb fragment containing the whole \( \text{phz1} \) DNA region was amplified with a primer pair (\( \text{phz1-WF} \) and \( \text{phz1-WR} \)). After double digestion with \( \text{HindIII} \) and \( \text{KpnI} \), the PCR product was cloned into the low-copy shuttle vector \( \text{pME6010} \) to obtain \( \text{pME10Z1} \) [43]. Using the same methods, \( \text{pME10Z2} \) covering the whole \( \text{phz2} \) DNA region was constructed using primers \( \text{phz2-WF} \) and \( \text{phz2-WR} \). After sequencing, the plasmids were transformed into competent PAO1 cells or its derivatives by electroporation. The positive colonies formed on LB plates supplemented with tetracycline were confirmed by plasmid isolation and restriction enzyme digestion analysis.

Creation of the translational fusion constructs: \( \text{phz1} \) '-' \( \text{lacZ} \) and \( \text{phz2} \) '-' \( \text{lacZ} \) and the transcriptional fusion constructs: \( \text{phz1-lacZ} \) and \( \text{phz2-lacZ} \)
To quantify the expression levels of the two phenazine-producing operons, the translational fusion constructs \( \text{phz1} \) '-' \( \text{lacZ} \) and \( \text{phz2} \) '-' \( \text{lacZ} \) were created in plasmid (Fig 1B). Briefly, a 0.9-kb DNA fragment covering the first ten codons of \( \text{phzA1} \) and its upstream region was amplified with a primer pair, \( \text{phz1-LF} \) and \( \text{phz1-LR} \). The relevant PCR product was purified, double-cleaved with \( \text{EcoRI-PstI} \), re-purified, and then fused in-frame with the truncated \( \text{lacZ} \) in plasmid \( \text{pME6015} \) to create \( \text{pME15Z1} \) [43]. Similarly, \( \text{pME15Z2} \) (a translational fusion construct \( \text{phz2} \) '-' \( \text{lacZ} \)) was constructed in \( \text{pME6015} \) with a 0.9-kb fragment containing the first eight codons of \( \text{phzA2} \) and its upstream region amplified with a primer pair \( \text{phz2-LF/phz2-LR} \). To assess the two \( \text{phz} \) operons at the transcription level, the transcriptional fusion constructs \( \text{pME22Z1 (phz1-lacZ)} \) and \( \text{pME22Z2 (phz2-lacZ)} \) were created in plasmid (Fig 1C). Briefly, a 0.9-kb DNA fragment covering the partial downstream region of the \( \text{phzM} \) and the \( \text{phz1} \) promoter region (to transcription start site +1) were amplified with a pair of primers, \( \text{phz1-CF/} \) \( \text{phz1-} \text{CR} \), then double digested with \( \text{EcoRI-PstI} \), and cloned into \( \text{pME6522} \) to generate \( \text{pME22Z1} \) [28, 44]. Similarly, a 0.5-kb fragment of the \( \text{phz2} \) promoter region with the partial \( \text{qscR} \) gene was amplified using a primer pair \( \text{phz2-CF/phz2-CR} \), and then cloned into the \( \text{EcoRI-PstI} \) site in \( \text{pME6522} \) to create \( \text{pME22Z2} \). All of the fusions were verified by sequencing analysis prior to transformation.

Creation of the translational \( \text{phz1} \) or \( \text{phz2} \) fusion mutants with the truncated \( \text{lacZ} \) in frame
To precisely reflect the expression of the \( \text{phz1} \) and \( \text{phz2} \) gene clusters in PAO1 and its derivatives, mutants in which the \( \text{phz1} \) or \( \text{phz2} \) were deleted and insertionally fused in frame with a truncated \( \text{lacZ} \) in their chromosome were further created. To obtain the \( \text{phz1} \) fusion mutant, two fragments were amplified with two pairs of primers (\( \text{phz1-1F/phz1z-1R} \) and \( \text{phz1z-2F/phz1-2R} \)) to obtain a 1432-bp fragment covering eight codons of \( \text{phzA1} \) and its upstream
Fig 1. Structures of two phz operons in P. aeruginosa PAO1 and its derivatives and two types of plasmid fusions with the truncated lacZ. (A) phz1 (light grey arrows) and phz2 (heavy grey arrows) indicate two phenazine operons of phzA1B1C1D1E1F1G1 and phzA2B2C2D2E2F2G2, respectively. aacC1 (horizontally striped arrow) and aph (vertically striped arrow) indicate the gentamycin and kanamycin resistance cassettes inserted into chromosome, respectively. lacZ (black arrow) indicates the truncated β-galactosidase gene inserted and fused in frame with the first several codons of phzA1 or phzA2 and their upstream region in the chromosome. The translational plasmid fusion (B) and the transcriptional plasmid fusion (C) were generated in plasmids pME6015 and pME6522, respectively. MCS stands for the multi-cloning site.

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Quantitative assay for PCA and PYO
For PCA, the cell cultures were grown in 500-ml shaking flasks with 150 ml GA or LB broth at 37°C for 72h. 900 μl of samples were collected every 12 hours and then acidified to pH 4.0 with HCl before adding 2.7 ml of chloroform. Chloroform extracts were clarified by centrifugation at 10,000 rpm for 5 min. Phenazine samples were diluted with chloroform appropriately, and PCA was quantified spectrophotometrically at 252 nm [47]. The equation of linear regression [concentration (μg/ml) = 2.9667×OD252-0.0979, R² = 0.9998] was generated with a purified sample of PCA provided by Dr. Xu (Shanghai Jiaotong University, Shanghai, China) as a gift.

PYO was extracted with chloroform from cultures grown in 500-ml flasks containing 150 ml of GA or LB medium with shaking at 37°C. Samples were collected and PYO was quantified once every 12 hours. Briefly, a 5-ml volume of culture was mixed with 3 ml of chloroform. After vortexing for 5 min, the sample supernatant was removed and 2 ml of 0.2 M HCl was added to the tube. PYO was extracted in the aqueous pink layer and spectrophotometrically determined at 520 nm [48,49]. Concentrations converted to micrograms of PYO produced per milliliter of culture were measured by multiplying the optical density at 520 nm (OD520) by 17.072 [50]. A standard sample of PYO was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Supplementation of the cultures with exogenous PCA or PYO
To determine whether phenazine feedback affected the expression of the two phz operons, the cultures of mutants with the truncated lacZ fusions in the chromosome were supplemented...
with different concentrations of exogenous PCA or PYO during the exponential phases. The PCA sample was generously provided as a gift by Dr. Xu's research group. PYO was prepared and collected by our laboratory as described by Frank & Demoss [51]. Briefly, one volume of cell-free culture supernatant was added to two volumes of chloroform and shaken for at least 5 min. PYO was extracted from the chloroform into a 0.2 N HCl solution (deep red). When the color changed from red to blue with the addition of NaOH buffer (pH = 10), the blue PYO was again extracted into chloroform. This procedure was repeated 5 times, finally generating PYO powder following the evaporation of the chloroform. High concentration PCA and PYO were dissolved in ethanol; the same volume of ethanol was supplied to the cultures as the negative control. During the following cultivation, the samples were collected at fixed intervals and β-galactosidase-specific activities were analyzed.

**β-Galactosidase assay**

All bacterial strains were grown with shaking at 200 rpm in 500-ml conical flasks containing 150 ml LB or GA medium at 37°C. Samples of strain PAO1 and its derivatives were collected after a specified periods of growth. β-Galactosidase-specific activities were determined according to the method of Miller using SDS- and chloroform-treated cells in appropriate amounts [35,52].

**Statistical analysis**

All data were analyzed with one-way analysis of variance using the statistical software package SPSS (Chicago, IL, USA).

**Results**

Both phz1 and phz2 operons contribute to phenazine production in culture condition

To quantitatively evaluate the specific contribution of the two phz loci to phenazine compound production, the two single-deletion mutants (Δphz1 and Δphz2) were cultivated in GA or LB medium. The wild-type strain PAO1 was used as the positive control and the double-deletion mutant (Δphz1phz2) as the negative control. Bacterial growth was determined at optical density 600 nm (OD600) at 12 hour intervals.

Although the bacterial growth of the pseudomonad strains differed from one another in different media, there were no significant differences in the growth curves in GA or LB medium between the wild-type strain PAO1 and its derivatives (Fig 2). Thus, the deletion of the two phz loci exerted no effects on bacterial growth. As shown in Fig 3, PCA production was decreased in the single-deletion mutant Δphz1 and Δphz2 compared to the wild-type strain PAO1. However, the amount of PYO produced respectively by the mutant Δphz1, Δphz2 and the parental strain PAO1 were same and negligible in LB medium following spectrophotometric analysis, suggesting that LB medium was not suitable for PYO biosynthesis. As shown in Fig 4, PCA and PYO produced in the single-deletion mutant Δphz1 and Δphz2 in the GA medium were lower than those obtained in the wild-type strain PAO1. No matter which medium (LB or GA medium) was used to culture them, the single-deletion mutant Δphz1 and Δphz2 produced less amounts of PCA and PYO than the wild-type strain PAO1. Moreover, the Δphz2 mutant did not produce much more PCA and PYO compared with the Δphz1 mutant, suggesting that the two phz operons contributed equally to phenazine production.

To further confirm the contribution of phz1 and phz2 operons to PCA and PYO production, complementation experiments were performed by expression of the phz1 and phz2 on a shuttle
We found that PCA and PYO produced in the Δphz1 and Δphz2 mutants harboring the pME6010 plasmid were equal to those produced in the Δphz1 and Δphz2 mutants without the pME6010 plasmid. As shown in Figs 3 and 4, When pME10Z1 harboring the whole phz1 operon or pME10Z2 bearing the whole phz2 operon were introduced into the Δphz1 or Δphz2 mutants, respectively, PCA and PYO production were restored to the level produced by the wild-type strain PAO1.

Total expression levels of phz2 and phz1 operon are cross-upregulated in the absence of phz1 and phz2 operon respectively

To explore whether phz2 exerts any regulatory effects on the expression of the phz1 operon, the translational fusion construct pME15Z1 (phzA1'-lacZ) was transferred into the single-deletion mutant Δphz1, Δphz2 or the double-deletion mutant Δphz1phz2. We found that the β-galactosidase activity of the phzA1'-lacZ fusion construct in the Δphz2 or Δphz1phz2 mutants was enhanced by 50% compared to the Δphz1 mutant (Fig 5A). These results suggested that deletion of the phz2 operon led to increased expression of the phz1 operon.

To determine whether the phz1 exerts any influences on the expression of the phz2 locus, the translational fusion construct pME15Z2 (phzA2'-lacZ) was delivered into the single-deletion mutants Δphz1, Δphz2 or the double-deletion mutant Δphz1phz2. We found that the β-galactosidase activity of the phzA2'-lacZ fusion construct in the double-deletion mutant Δphz1phz2 or the single-deletion mutant Δphz1 was enhanced 3-fold compared to the single-deletion mutant Δphz2 (Fig 5B). These results indicated that deletion of the phz1 operon led to enhancement of phz2 operon expression.

To truly and precisely reflect the expression of the two phz operons under natural conditions and to eliminate the negative effects due to copies of the translation fusion plasmid in the deletion mutants, a set of fusion mutants in which phzA1 or phzA2 was fused in frame on the chromosome with a truncated lacZ reporter were constructed using the wild-type strain PAO1,
the single deletion mutant Δphz1 or Δphz2 as receptor strains. The nearly identical growth curves of the translational fusion mutants ΔphzA1Z, ΔphzA1Zphz2, ΔphzA2Z, and Δphz1phzA2Z grown in LB or GA broth indicated that their growth rates were not affected by
the mutation or fusion in the two phz loci regions (data available on figshare). As shown in Fig 6A, the expression of the translational fusion construct phzA1-lacZ on the chromosome in the ΔphzA1Zphz2 mutant was enhanced 2- to 4-fold compared to the ΔphzA1Z mutant. This result was consistent with the result of the translational fusion expressed from the plasmid discussed above, suggesting that the expression of the phz1 operon was up-regulated in the absence of the phz2 operon. As shown in Fig 6B, the expression of the translational fusion construct phz2-lacZ on the chromosome in the Δphz1phzA2Z mutant was elevated 6 folds compared to the Δphz2 mutant. This result was similar to the result obtained in the translational fusion on the plasmid, indicating that the expression of the phz2 operon was up-regulated in the absence of the phz1 operon.

Fig 4. PCA and PYO produced by P. aeruginosa PAO1 and its derivatives in GA medium. PCA (A) and PYO (B) were biosynthesized by the wild-type strain PAO1 (solid circle) and its derivatives, the single-deletion mutant Δphz1 (solid square) and Δphz2 (solid triangle), the double-deletion mutant Δphz1phz2 (solid diamond), the Δphz1 mutant harboring pME10Z1 (open square) and the Δphz2 mutant containing pME10Z2 (open triangle) in GA medium. All experiments were performed in triplicate, and each value was presented as the average ± standard deviation. doi:10.1371/journal.pone.0144447.g004
The transcription of the *phz2* operon increases in the absence of the *phz1*, the transcription of the *phz1* does not in the absence of the *phz2*

To determine whether the cross-regulation between the two *phz* operons occurred at the transcriptional or post-transcriptional level, two transcriptional fusion constructs [pME22Z1 (*phz1*-lacZ) and pME22Z2 (*phz2*-lacZ)] were created in pME6522. The β-galactosidase activities of the two transcriptional fusion constructs were measured in the wild-type strain PAO1 and its mutation derivatives. The β-galactosidase activity of pME22Z1 in the double-deletion Δ*phz1phz2* mutant was nearly identical to that in the single-deletion Δ*phz1* mutant (Fig 7A), suggesting that the transcription of the *phz1* operon was not affected by the presence or absence of the *phz2* operon. However, the β-galactosidase activity of pME22Z2 was higher (20 to 30%) in the double-deletion mutant Δ*phz2* than that in the single-deletion mutant Δ*phz2* (Fig 7B), suggesting that the transcription of the *phz2* operon was moderately enhanced by the absence of the *phz1* operon.

Roles of PCA and PYO in the regulation of *phz* expression

Because PCA and PYO are the main exo-products of the enzymes encoded by the *phz* operons, we tested whether these products have a regulatory effect on the *phz* expression. When a higher concentration of exogenous PYO (>0.32 μg/ml) was added, the β-galactosidase activity in the Δ*phzA1Zphz2* mutant’s culture was reduced compared to that supplemented with ethanol as negative control. These results suggested that PYO accumulation in the culture suppressed the expression of the *phz1* operon. If the concentration of PYO added was low (<0.16 μg/ml), no
effect on phz1 operon expression was observed (Fig 8A). Similar results were obtained in the Δphz1phzA2Z mutant’s culture with the addition of PYO (Fig 8B). The β-galactosidase activity in the Δphz1phzA2Z mutant’s culture was not affected by the addition of exogenous PCA, suggesting that higher concentrations of PCA did not exert negative regulatory effects on the expression of the phz2 operon. However, expression of β-galactosidase in the Δphz1A1Zphz2 mutant’s culture was repressed by the addition of exogenous PCA, indicating that the expression of phz1 was inhibited when high level of PCA accumulated in the culture.

Discussion

In this study, we constructed a series of phz deletion mutants and evaluated the specific contribution of two phz operons to phenazine biosynthesis in PAO1. In LB or GA medium, the mutant Δphz2 produced slightly more phenazines than the mutant Δphz1. However, in P. aeruginosa PA14, the phz1-deficient mutant produced more PCA than the phz2-deleted mutant, suggesting that regulation should be different for the expression of the two phz operons in two strains despite the fact that the sequences of phz operons and their promoter regions in both strains were extremely identical (>99%) [32]. Our results obtained in two types of media
supported the conclusion that the \textit{phz2} operon is active and functional in the wild-type strain PAO1. The function of the \textit{phz2} should not be ignored because it produces phenazines in the LB or GA medium. This conclusion was also supported by the previous work by Mavrodi et al. [16,17]. In their report, PCA was detected in extracts from the transformants when each copy of the two \textit{phz} operons was cloned into an \textit{E. coli—P. aeruginosa} shuttle vector and then

\begin{figure}
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\includegraphics[width=\textwidth]{fig7}
\caption{The transcription level assay of one \textit{phz} operon in the absence or in the presence of the other one \textit{phz} operon. (A) \textbeta-Galactosidase activities were produced by pME22Z1 in the double-deletion mutant \textit{Δphz1phz2} (black column), the single-deletion mutant \textit{Δphz1} (grey column) and mutant \textit{Δphz2} (light grey column). pME6522 in the mutant \textit{Δphz1} (white column) served as the negative control. (B) \textbeta-Galactosidase activities were produced by pME22Z2 in the mutant \textit{Δphz1phz2} (grey column), \textit{Δphz1} (light grey column) and \textit{Δphz2} (black column). pME6522 in the \textit{Δphz2} mutant (white column) served as the negative control. All experiments were performed in triplicate, and each value was presented as the average ± standard deviation. *indicates \textit{P} > 0.05, **indicates \textit{P} < 0.01, two-tailed paired Student t test.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8}
\caption{Effects of exogenous phenazines on expression of the \textit{phz} operon. (A) \textbeta-Galactosidase activities expressed in the mutant \textit{ΔphzA1Zphz2} in the presence of exogenous PYO or PCA. (B) \textbeta-Galactosidase activities expressed in the mutant \textit{Δphz1phzA2Z} in the presence of exogenous PYO or PCA. a-e, PYO was added to samples with final concentration of 0.08, 0.16, 0.32, 0.64 and 1.28 \textmu g/ml; f-j, PCA was added with 0.025, 0.25, 0.5, 1.0, and 2.0 \textmu g/ml. All values were measured after 24 hour of addition. Data reported were the means of triplicate experiments ± standard deviations. * indicates \textit{P} > 0.05, two-tailed paired Student t test.}
\end{figure}
introduced into the non-phenazine-producing strain *P. fluorescens* M4-80R or *E. coli* JM109. However, their study just verified that the *phz2* was similar to the *phz1* and had the same ability to produce phenazines. In other previous reports, the *phz1* operon was shown to play a major role in producing phenazines because it produced the majority of PYO in the wild-type strain PAO1 in LB medium. Therefore, it was suggested that the *phz2* could not substitute for the *phz1* in the biosynthesis of PYO [33,34]. However, it was possible that the *phzC1* mutant did not produce blue pigment in LB medium in their report because the amount of PYO produced by the *phz2* operon was too low to make the LB medium plate blue, not because the *phz2* produced no PYO. As a matter of fact, the *phz2* operon produced the same amount of PYO in GA medium as the *phz1* during the first 36 hours of cultivation (Fig 4B). Therefore, the blue pigment (PYO) could be biosynthesized by both the *phz1* and *phz2* operons in the parental strain PAO1. This conclusion was also confirmed by the results from our translational fusion *phz1*-lacZ and *phz2*-lacZ constructed in the pME6015 plasmid in the PAO1 strain.

However, the mechanism by which the two *phz* operons function under natural conditions in the parental strain PAO1 is not clear. To answer this question, we examined the expression of two operons in PAO1 and its derivatives using the lacZ reporter gene. We found that the expression of one *phz* operon dramatically increased when the other operon was deleted, suggesting that one *phz* operon could compensate for the absence of the other operon by up-regulating its expression level. We postulated that there would be a homeostatic regulatory mechanism which mediates the expression of the two *phz* operons. To test this hypothesis, we further constructed the translational fusion mutants on the chromosome with the truncated lacZ in frame. The assessment of ß-galactosidase activities in two pair of mutants (ΔphzA1Z/ΔphzA1Zphz2, ΔphzA2Z/Δphz1phzA2Z) confirmed that a homeostatic balance did exist between the two *phz* operons. Thus, when one *phz* operon (*phz1* or *phz2*) does not function, the other operon would be up-regulated to compensate for the decrease in phenazine production. This similar finding had been reported before in other pseudomonad species. For example, 2,4-diacetylphloroglucinal (DAPG) and pyoluteorin (PLT) display an inverse relationship in *P. fluorescens* CHA0 in which each metabolite activates its own biosynthesis while repressing the synthesis of the other metabolite [53,54]. Moreover, phloroglucinol (a precursor of DAPG) is responsible for the inhibition of pyoluteorin production in *P. fluorescens* Pf-5 [55]. In *Pseudomonas* sp. M18, one *phz*-deletion mutant M18Z1 produced less PCA, but more pyoluteorin (PLT) [56]. In bio-control strains, homeostatic balance exists during the biosynthesis of secondary metabolites and will compensate for the loss of one antibiotic by overproducing another, thereby maintaining total antibiotic production and bio-control ability [57]. Similarly, the maintenance of the two *phz* operons in *P. aeruginosa* PAO1 by the homeostatic balance would keep phenazine production stable, which would be beneficial to its pathogenicity in the host.

In an attempt to gain additional insight into the mechanism for this homeostatic regulation, we created the transcriptional fusion pME22Z1 and pME22Z2 and transformed them into the derivative mutants. ß-Galactosidase activities shown that no significant changes occurred at the *phz1* transcription level in the presence or absence of the *phz2*. Combined with the translational fusions’ data, we speculated that the cross-regulation mediating the *phz1* expression occurred at the post-transcriptional level and less likely at the transcriptional level. Interestingly, the transcription of the *phz2* increased moderately in the absence of the *phz1*. Meanwhile, the translational fusion analysis shown that the expression level of the *phz2* increased significantly (more than 3 times) in the absence of the *phz1*. Therefore, we suggested that the cross-regulation between the two *phz* operons might mediate the *phz2* expression at both the transcriptional and post-transcriptional levels.
Based on sequence analysis, the two phz loci differed markedly in their upstream regions although they possessed 98.3% identity in their open reading frame regions [31]. These differences may serve as a platform for cross-regulating two phz operons and contribute to phenazine biosynthesis. In *P. aeruginosa* M18, the 5' long region in the phz1 and phz2 mRNA was demonstrated to post-transcriptionally mediate the expression of two phenazine producing loci [28, 58]. Meanwhile, it was confirmed that RsmA could negatively regulate the phz1 expression and positively mediate the phz2 expression at post-transcriptional level [58]. Due to the high identity between strains M18 and PAO1 in their phz operons, they may share similar structures or mechanisms involved in the differential mediation of the two phenazine biosynthesis operons. In PCA and PYO feedback assay, exogenous PYO inhibition in PAO1 strain in our study was consistent with the previous work did by Dietrich et al. [27]. However, while exogenous PCA did not exert an effect on the phz2 expression, it exhibited a negative effect on the phz1 expression. These results may also provide some clues into the homeostatic interplay between the two phz operons. Although we described an initial characterization of the relationship between the two phz operons and identified a homeostatic balance between them, we could not explore the precise expression levels of the two phz operons in the wild-type strain under natural conditions. This issue should be addressed in future studies.

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**Author Contributions**

Conceived and designed the experiments: YG XH. Performed the experiments: QC HL ZQ BJ. Analyzed the data: BX. Contributed reagents/materials/analysis tools: LL. Wrote the paper: YG XH.

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