H-NS family protein MvaU downregulates phenazine-1-carboxylic acid (PCA) biosynthesis via binding to an AT-rich region within the promoter of the phz2 gene cluster in the rhizobacterium *Pseudomonas* strain PA1201

Yun-Ling Fang\(^{a,1}\), Ying Cui\(^{a,1}\), Lian Zhou\(^{a}\), Chitti Thawai\(^{b}\), Tatheer Alam Naqvi\(^{c}\), Hong-Yan Zhang\(^{d,2}\), Ya-Wen He\(^{a,1}\)

\(^{a}\) State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic and Developmental Sciences, SJTU-Shanghai Nong Le Joint R&D Center on Biopesticides and Biofertilizers, Shanghai Jiao Tong University (SJTU), Shanghai, 200240, China

\(^{b}\) Department of Biology, Faculty of Science, King Mongkut’s Institute of Technology Ladkrabang, Bangkok, Thailand

\(^{c}\) Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, Abbottabad, Pakistan

\(^{d}\) Shanghai Nong Le Biological Products Company Limited, Shanghai, 201419, China

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ABSTRACT

Histone-like nucleoid-structuring (H-NS) proteins are key regulators in gene expression silencing and in nucleoid compaction. The H-NS family member proteins MvaU in *Pseudomonas aeruginosa* are thought to bind the same AT-rich regions of chromosomes and function to coordinate the control of a common set of genes. Here, we explored the molecular mechanism by which MvaU controls PCA biosynthesis in *P. aeruginosa* PA1201. We present evidence suggesting that MvaU is self-regulated. Deletion of *mvaU* significantly increased PCA production, and PCA production sharply decreased when *mvaU* was over-expressed. MvaU transcriptionally repressed *phz2* cluster expression and consequently reduced PCA biosynthesis. \(\beta\)-galactosidase assays confirmed that base pairing near the –35 box is required when MvaU regulates PCA production in PA1201. Electrophoretic mobility shift assays (EMSA) and additional point mutation analysis demonstrated that MvaU directly bound to an AT-rich motif within the promoter of the *pha* cluster. Chromatin immunoprecipitation (ChIP) analysis also indicated that MvaU directly bound to the P5 region of the *pha* cluster promoter. MvaU repression of PCA biosynthesis was independent of QscR and OxyR in PA1201 and neither PCA or \(\text{H}_2\text{O}_2\) were the environmental signals that induced *mvaU* expression. These findings detail a new MvaU-dependent regulatory pathway of PCA biosynthesis in PA1201 and provide a foundation to increase PCA fermentation titer by genetic engineering.

1. Introduction

Histone-like nucleoid structuring proteins (H-NS) are global regulators of gene expression in many different Gram-negative bacteria [1–4]. The N-terminal regions of H-NS form coiled structures that function as oligomerization domains, while C-terminal regions are responsible for DNA binding [5]. Several genome-wide association studies have shown that H-NS display a marked preference for binding AT-rich regions of target genes [6,7].

MvaT and MvaU have been identified in *Pseudomonas* as paralogs of H-NS that share structural and functional similarities to H-NS, despite only sharing 20% sequence similarity with H-NS. MvaT and MvaU are thought to be structurally similar to H-NS, wherein a DNA binding domain is located at the C-terminus of MvaT, and MvaU is comparatively more conserved relative to H-NS, while the multimerization domain located at the N-terminus is more divergent [7–10]. Chromatin immunoprecipitation analysis coupled with DNA microarray analysis have shown that MvaU influences the expression of many genes in *P. aeruginosa*, including genes previously identified that may be important for virulence [11–13]. In addition, MvaU has been shown to
coordinate with MvaT and occupy the same regions of chromosomes, while co-regulating the expression of 350 genes [11]. It was consequently proposed that MvaT and MvaU are equally important for cell function. The deletion of either MvaT or MvaU leads to increased production of the other one, indicating cross-regulation of the two proteins and that they can functionally compensate for each other, while the synthetic lethality that ensues is due to mis-regulated expression of certain target genes [5].

PA1201 is a *P. aeruginosa* strain that was originally isolated from rice rhizospheres and displays strong inhibitory activity towards the rice pathogens *Rhiocizonia solani* and *Xanthomomas oryzae* pv. *oryzae* [14,15]. PA1201 also produces a high level of the phenazine derivatives, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), which are redox-active, nitrogen-containing, heterocyclic, colored, and aromatic secondary metabolites that display broad-spectrum antibiotic activity toward many fungal, bacterial, and oomycete plant pathogens [16–18]. Two gene clusters, phaA1B1C1-D1E1F1G1 (pha1) and phaA2B2C2D2E2F2G2 (pha2), are involved in PCA and PCN biosynthesis [19–24]. PA1201 is less toxic to both human cell lines and *Drosophila melanogaster* in contrast to the clinically isolated *P. aeruginosa* strains PAO1 and PA14, and is also amenable to genetic modification [25]. It has become a model strain to study the molecular regulation of phenazine biosynthesis. Research over the past decade has shown that production of phenazines is controlled by complex regulatory networks that comprise multiple components including quorum-sensing (QS) systems, small noncoding RNAs, and several specific and global transcriptional regulators in PA1201. The *rhl/rhlR* and *pqsR*—dependent QS systems are essential for *pha1* and *pha2* expression and PCA biosynthesis [19]. QscR is an orphan QS signal receptor and regulates phenazine biosynthesis through indirectly affecting the expression of QS-regulated genes [26]. Rsal is a potent repressor of PCA biosynthesis and negatively regulates *pha1* expression and positively regulates *pha2* expression via multiple mechanisms [27]. OxyR is a LyS-family transcriptional regulator and is responsible for *H₂O₂* tolerance in PA1201. Overexpression of oxyR increases PCA biosynthesis via regulation by the small RNA *phrR* [28]. OxyA is an anti-activator, which negatively regulates PCA biosynthesis by interacting with the QS regulator MvfR in PA1201 [29].

MvaU has been demonstrated to negatively regulate the biosynthesis of pyocyanin (PYO), a derivative of PCA, in the clinically isolated PAO1 [30]. However, the detailed regulatory mechanism underlying this association remains unknown. Herein, we provide evidence that the expression of *mvaU* is self-regulated and that MvaU negatively regulates PCA production. MvaU had no effect on *pha1* cluster transcription, but transcriptionally repressed the expression of the *pha2* gene cluster, consequently resulting in reduced biosynthesis of *pha2*-dependent PCA. β-galactosidase assays, EMSA analysis, and chromatin co-immunoprecipitation analyses revealed that MvaU binds to a region close to the −35 elements of the *pha2* cluster promoter. Finally, MvaU-dependent negative regulation of PCA biosynthesis was shown to be independent of OxyR and QscR. The findings of this study provide evidence for a new regulatory pathway of PCA biosynthesis in PA1201 and represent a foundation for increasing PCA fermentation titer by genetic engineering.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. *E. coli* was routinely grown in LB medium at 37 °C. PA1201 and its derivatives were grown at 28 °C in 50 mL pigment-producing medium (PPM; comprising tryptone [22 g/L], glucose [20 g/L], KNO₃ [5 g/L], pH 7.5) in a shaker at 200 rpm. When required, 20 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used for blue/white colony screening. *P. aeruginosa* and its derivative strains were cultured with antibiotics at the indicated final concentrations (μg/mL): gentamicin (Gm), 45; kanamycin (Km), 50; spectinomycin (Sp), 100; tetracycline (Tc), 120; and chloramphenicol (Cm), 200. Antibiotics amended for *E. coli* cultures included 50 μg/mL Km, 10 μg/mL Gm, 10 μg/mL Tc, and 40 μg/mL Cm.

2.2. DNA manipulations, gene deletion and functional complementation analysis

All molecular biological techniques followed standard protocols unless otherwise described. Taq polymerase (TaKaRa), Q5® High-Fidelity DNA Polymerase (NEB), KOD Plus DNA polymerase (Toyobo), RNA reverse transcriptase, restriction endonucleases, DNA ligase, DNA molecular mass markers (TaKaRa), and other associated products were used as recommended by their respective manufacturers. Genomic DNA was extracted using an EZ-10 spin column genomic DNA isolation kit (Bio Basic Inc, China). Plasmid DNA was purified using a Genery plasmid minipreparation purification system. DNA was recovered from gels using an AsyPrep DNA gel extraction kit (Asyxygen). Lastly, DNA was synthesized and sequenced by the Sangon Biotechnology Corporation.

The in-frame deletion mutants were generated using previously described methods [31]. Briefly, the upstream and downstream regions of the target gene to be deleted were fused using overlap extension PCR. The fusion product was then subcloned into the vector pKI8mosbacB carrying the sucrose-sensitive *sacB* gene. The resultant recombinant plasmid was introduced into PA1201 through mating and the plasmid was subsequently integrated within the target gene via homologous recombination. The resultant strain was then plated on LB agar plate with 100 μg/mL Sp and 5% (w/v) sucrose for a second single-crossover homologous recombination event, resulting in allelic exchange. The resultant mutant was verified by PCR and subsequent DNA sequencing. For complementation analysis, the target gene was PCR amplified and cloned into the multiple cloning site of the expression plasmid pBRR1MC-2. The resultant constructs were transferred into PA1201 by triparental mating. The primers used are listed in Table S3.

2.3. Extraction and quantification of PCA

Cells from fresh overnight cultures of *P. aeruginosa* PA1201 and its derivative strains were inoculated into 50 mL of PPM in a 250 mL Erlenmeyer flask at a final concentration equivalent to an optical density at 600 nm (OD₆₀₀) of 0.025, followed by incubation at 28 °C with shaking at 200 rpm. PCA was extracted with chloroform (pH 4.0) and quantified with high performance liquid chromatography (HPLC), as previously described [25,29]. HPLC with a UV–Vis detector was used for qualitative and quantitative analysis of PCA in the reverse phase on an Agilent Technologies 1260 Infinity instrument. A 2 μL sample was injected and separation was achieved on a reverse phase C 18 column (XDB-C18,5 μm 4.6 × 12.5 mm² Agilent Eclipse) using 5 mM ammonium acetate–acetonitrile (40:60, v/v) at a flow rate of 0.7 mL/min. The column temperature was maintained at 30 °C in the column oven.

2.4. Construction of lacZ reporter gene fusions

Transcriptional fusions of the *pha1* cluster, the *pha2* cluster upstream region, and lacZ were engineered in plasmid mini-CTX-lacZ and then introduced into the *P. aeruginosa* PA1201 chromosome at the phage D113 attB site using standard protocols. Plasmid mini-CTX-lacZ and its derivatives were mobilized into *P. aeruginosa* PA1201 by biparental conjugation after six or 18 h of incubation. Transconjugants carrying chromosomal inserts of mini-CTX-lacZ and its derivatives were recovered on LB containing tetracycline (50 μg/mL) and spectinomycin (50 μg/mL). The plasmid backbone was then cured from the chromosome of each transconjugant using the pFLP2-encoded Flp recombinase, leaving only the Phb1-lacZ fusions or Phb2-lacZ fusions in the chromosome. The pFLP2 plasmid was then introduced into *P. aeruginosa* via electroporation and pFLP2-containing carbenicillin-resistant colonies were
subsequently streaked onto LB containing 10% (w/v) sucrose to select for pFPL2 plasmid loss following excision of the mini-CTX backbone from the chromosome. Sucrose-resistant colonies were patched on LB plates containing tetracycline (50 μg/mL) or carbenicillin (100 μg/mL) to confirm the loss of the mini-CTX backbone (i.e., via tetracycline sensitivity) and the pFPL2 plasmid (via carbenicillin sensitivity).

2.5. Site-directed mutagenesis of the AT-rich motif of phz2 promoter regions

lacZ reporter plasmids with the AT-rich motif of the phz2 promoter region altered by site-directed mutagenesis were constructed to assess the importance of the AT-rich motif for MvaU regulation of phz2 expression. The phz2 promoter mutant fragment containing the 4-nucleotide (nt) substitution in the AT-rich motif was PCR amplified with primers specific for the mutant.

2.6. β-galactosidase (β-gal) activity assays

P. aeruginosa PA1201 and its derivative strains that carried different fusion plasmids were cultured at 28 °C with shaking at 200 rpm in a 250 mL Erlenmeyer flask containing 50 mL of PPM broth. Samples were collected after various time intervals and β-gal activity was monitored following previously described methods [19].

2.7. MvaU protein expression, purification, and SDS-PAGE analysis

The MvaU coding region was amplified by PCR with primers listed in Table S3 and then cloned into the pET28a expression vector after digestion with Hind III-BamHI I (Merck KGAa, Darmstadt, Germany). Cloning was conducted in frame with the sequence coding for a 6-histidine tag at the N-terminus of the gene. The resultant plasmids were introduced into E. coli BL21 (DE3) cells for heterologous expression. Strain BL21(DE3) cells transformed with pET28a-mvaU were grown overnight at 16 °C with shaking at 180 rpm within 2 L of LB medium, followed by induction with 0.1 mM of isopropyl-β-thiogalactopyranoside. Cell pellets were resuspended in 50 mL of equilibration buffer (20 mM NaCl, 300 mM Na₂HPO₄, 10 mM imidazole; pH 7.4) and lysed by sonication.

Recombinant protein was purified by Ni²⁺-affinity chromatography from the soluble cellular fraction using His-Pur Ni-NTA resin (Thermo Scientific, Pierce Biotechnology, USA) according to the manufacturer’s instructions. MvaU was then eluted with buffer containing 20 mM sodium phosphate, 300 mM sodium chloride, and 250 mM imidazole (pH 7.4). The purified protein was then dialyzed against buffer containing 10 mM KH₂PO₄, 10 mM K₂HPO₄, and 10% (v/v) glycerol (pH 7.4) using an Amicon Ultra-0.5 centrifugal filter device (AmiconR, Merck KGAa, Germany). Protein purity was determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.8. Electrophoretic mobility shift assays (EMSA)

The interaction of MvaU with its putative binding site on the phz2 promoter was analyzed by EMSA, as previously described [19], but with minor modifications. Briefly, the corresponding region of the P. aeruginosa PA1201 genomic DNA was amplified using a Cy5-labelled sense primer and an antisense primer (Table S3). The amplified PCR product was purified for EMSA following standard protocols. Various concentrations of purified MvaU proteins were incubated for 20 min with 10 ng of Cy5-labelled PCR product in buffer containing 4 mM Tris/HCl (pH 8), 4 mM MgCl₂, 5% glycerol, 40 mM NaCl, and 0.5 mg salmon sperm DNA in a final volume of 20 μL. To evaluate competitive binding, unlabelled oligonucleotides (Table S3) were used in excess as indicated. Samples were separated on 4% polyacrylamide gels and DNA-protein complexes were visualized using a molecular imager (Typhoon Trio Plus, GE Health Sciences).

2.9. Chromatin immunoprecipitation (ChIP) assay

P. aeruginosa strain ΔmvaU(pLAFR3-His-mvaU) carrying a plasmid that expresses His-MvaU was used for ChIP experiments. The strain was grown in PPM medium with shaking at 28 °C until reaching an OD₆₀₀ of ~2, at which point formaldehyde was added to a final concentration of 1%. Cultures were then maintained with shaking at 28 °C for 15 min. To quench the cross-linking reaction, glycine was added to a final concentration of 250 mM, followed by a 15 min incubation at room temperature. Cell pellets were then washed twice with 1x PBS and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.5 mM DTT, 10% glycerol, and 5 mM protein inhibitor). Chromosomal DNA was then sheared by sonication to an average size of 2–5 Kbp. After removal of cell debris by centrifugation, 50 μL of each sample was removed to serve as an input control. Immunoprecipitation (IP) reactions were initiated by adding anti-His magnetic beads (Medical & Biological Laboratories, Inc. Cat #D291-11) for His-tagged proteins, to each of the remaining samples. After incubation at 4 °C overnight, beads were pelleted and washed three times with 0.1 M sodium phosphate buffer (pH 8.0). Beads were then resuspended in elution buffer (50 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8), and 1% SDS) and immunoprecipitated complexes were removed from the beads by incubation at 65 °C for 15 min. The recovered supernatants were incubated at 65 °C overnight to reverse the cross-links. A control mock immunoprecipitation (without MvaU protein) was also prepared. PCRs were conducted using 1 μL of reverse solution as template within 25 μL reactions and with 30 cycles. An aliquot of the amplicons (5 μL) was loaded onto a 2% agarose gel for evaluation of PCR success. After electrophoresis, PCR products were visualized with GoldView staining. The primers used for amplification are described in Table S3.

2.10. Statistical analyses

Each experiment was independently repeated at least three times, with triplicate parallel samples within each experiment. Unless stated otherwise, each value represents the mean and standard deviation (SD) of three replicate measurements. Student’s t tests were used to evaluate the significance of difference between various treatments using the GraphPad Prism 5 program.

3. Results

3.1. mvaU is self-regulated in PA1201

PA1201 harbors the gene PA2306 that encodes a H-NS family protein homologous to the MvaU protein of PAO1 (Fig. 1A). Comparative genomic analyses revealed that MvaU is present in the sequenced Pseudomonas aeruginosa strains PAO1, LESB58, PA96, PA1201, and PAK (Fig. 1A). P₄mvaU-dependent β-galactosidase activity was determined at various time points after inoculating the constructed reporter strains PA: P₄mvaU-lacZ and ΔmvaUP₄mvaU-lacZ into PPM medium. Specifically, measurements were taken every 12 h until 60 h of cultivation, as shown in Fig. 1B-C. mvaU was significantly induced after 24 h of cultivation in PPM medium, indicating a cell density-dependent expression pattern (Fig. 1B-C). P₄mvaU-dependent β-galactosidase activity in mvaU deletion reporter strain was relatively stable, but significantly lower than that in the wild-type reporter strain (Fig. 1B-C).

Purified MvaU protein expressed in a heterologous system was used for EMSA assays (Fig. 1D). Cy5-labelled probes were synthesized that encompassed the 304 bp region upstream of the mvaU transcriptional start site. EMSA analysis showed that MvaU (7 ng–37.5 ng) could form stable complexes with the Cy5-labelled probe (termed Pro-mvaU, 10 ng). Addition of unlabelled Pro-mvaU (2000 ng) to the reaction mixture containing 22.5 ng of MvaU and 10 ng of labelled Pro-mvaU hampered the formation of stable MvaU/Pro-mvaU complexes (Fig. 1D). Taken together, these findings indicate that mvaU is a self-regulated gene of PA1201.
3.2. MvaU negatively regulates PCA biosynthesis in PA1201

The regulatory role of mvaU in PCA biosynthesis was assessed by using a previously constructed MSH strain (ΔMSH) that produces PCA as the sole phenazine and carries three deletions: phzM, phzS, and phzH [25]. No significant difference in cell growth was observed between MSH and ΔmvaU in PPM medium (Fig. 2A). However, PCA level in the ΔmvaU culture was about 50% higher than in the MSH culture (Fig. 2B). Similar trends in PCA biosynthesis were observed in the mvaU deletion strain that was grown in optimized SCM medium for high PCA yields (Fig. 2C). Overexpression of mvaU via the broad host vector pBBR-1-MCS (KanR) in strain ΔmvaU significantly reduced PCA biosynthesis to a level much lower than that of MSH in both PPM and SCM media (Fig. 2B–C).

3.3. MvaU negatively regulates phz2-dependent PCA biosynthesis and phz2 expression

The PA1201 genome contains two nearly identical PCA biosynthetic operons, phz1 and phz2, that contribute to PCA biosynthesis in our previous study, two strains were constructed including Δphz1 (with phz1 deleted) and Δphz2 (with phz2 deleted) [19]. PCA biosynthesis is dependent on the activity of enzymes encoded by the phz2 operon (phz2-dependent PCA biosynthesis) in strain Δphz1, while PCA biosynthesis is only dependent on enzymes encoded by the phz1 operon in Δphz2 (i.e., phz1-dependent PCA biosynthesis). To determine if MvaU exerts its repression on PCA biosynthesis at the level of phz1, phz2, or both loci, mvaU was further deleted in strains Δphz1 or Δphz2 and the resultant strains were respectively termed Δphz1ΔmvaU and Δphz2ΔmvaU. No effect on cellular growth was observed among the constructed strains in PPM medium (data not shown).

HPLC analyses demonstrated that PCA levels in Δphz1ΔmvaU cultures were significantly higher than in Δphz1 cultures after incubation between 24 and 48 h (Fig. 3A). After 36 h, PCA levels were 64.3 mg/L, representing a 2.3-fold higher level than for the Δphz1 strain (Fig. 3A). The overexpression of mvaU in the Δphz1ΔmvaU strain almost abolished PCA biosynthesis (Fig. 3A). In contrast, no significant differences were observed for PCA levels in strains Δphz2ΔmvaU and Δphz2. Similarly, overexpression of mvaU in Δphz2ΔmvaU did not have a significant effect on PCA biosynthesis (Fig. 3C). These results clearly indicate that MvaU negatively regulates phz2-dependent PCA biosynthesis.

Given the confirmed regulatory effect of MvaU on each phz cluster, mvaU was deleted in previously described reporter strains (Δphz2::Pphz2-lacZ or Δphz1::Pphz1-lacZ) to monitor phz1 and phz2 activities. The β-galactosidase activity of the resultant strains (Δphz2ΔmvaU::Pphz1-lacZ and Δphz1ΔmvaU::Pphz2-lacZ) were compared against those of their parental reporter strains. The β-galactosidase activity of strain Δphz1ΔmvaU::Pphz2-lacZ was significantly higher than for strain Δphz1::Pphz2-lacZ at all growth points in cultures within PPM (Fig. 3B). In contrast, deletion in the reporter strain Δphz2::Pphz1-lacZ had no significant effect on β-galactosidase activity (Fig. 3D). These results together suggest that MvaU negatively regulates phz2 transcriptional activity independently of phz1.
3.4. MvaU negatively regulates phz2 expression by directly binding to an AT-rich 5'-UTR region of phz2

To further understand how MvaU negatively regulates phz2 expression, phz2 promoter regions of different length were amplified using primers shown in Table S3. The PCR amplicons were then cloned into the mini-CTX-lacZ plasmid and integrated into the chromosomes of strains MSH and ΔmvaU using established protocols [19]. Three replicates were performed for each strain and error bars indicate standard deviations. Statistical significance with respect to the MSH strains is indicated by one (*p < 0.05) or two asterisks (**p < 0.01).

To investigate whether MvaU directly binds to the putative target region within phz2, EMSA assays were conducted. Cy5-labelled DNA fragments corresponding to the three regions, P1, P4 (nt 71 to nt +162), and P5 (nt 55 to nt +55) within Pphz2 were respectively synthesized as probes (Fig. 5A). EMSA analysis subsequently showed that MvaU-dependent phz2 repression might be mediated via P2 region.
MvaU can bind to any of the three probes (Fig. 5B–D). These findings confirmed the promoter-lacZ fusion reporter assay results shown in Fig. 4B, but also provided evidence for a putative MvaU binding site for the 110 bp probe P5.

To further verify the role of the identified region in MvaU-dependent repression of PCA biosynthesis, the P5 region was deleted in strains MSH and ΔmvaU. The resultant strains were termed ΔP5 and ΔP5ΔmvaU, respectively. No significant differences in cell growth were observed among the strains (Fig. 6A). Deletion of the P5 region in the MSH strain significantly reduced PCA biosynthesis in PPM medium. Further deletion of mvaU in strain ΔP5 had little effect on PCA biosynthesis (Fig. 6B).

Previous results demonstrated that the MvaU binding site is an AT-rich motif. In the identified P5 region of P\textsubscript{phz2}, an AT-rich region (TAATTTTTAA) was present (Fig. 4C). To verify whether this AT-rich region is the MvaU-binding site, point mutations were introduced. Specifically, the “TAAT” residues of the AT-rich region in the reporter plasmid mini-CTX-P1:lacZ were mutated to “CGGC”. The resultant plasmid was then integrated into the chromosomes of strains MSH and ΔmvaU. The resultant strains were termed MSH:P1CG-lacZ and ΔmvaU:P1CG-lacZ (Fig. 6C). P1-dependent β-galactosidase activities on ΔmvaU background were significantly higher than on wild-type MSH background (Fig. 4B). However, the point-mutated promoter P1CG-dependent β-galactosidase activity in strain MSH was not significantly different from that of strain ΔmvaU (Fig. 6C). Furthermore, the “TAAT” in P5 probe was mutated into “CGGC” (P5CG in this study) and labelled with Cy5 (Fig. 4C). EMSA assay revealed no binding between MvaU and P5CG (Fig. 5E). These findings suggest that the TA-rich region is required for MvaU-dependent phz2 repression.

3.5. Chromatin immunoprecipitation (ChIP) analysis demonstrates that MvaU directly binds to the promoter region of phz2 cluster

ChIP analysis is a powerful and versatile technique used for probing protein-DNA interactions within the natural chromatin context of cells [32]. ChIP assays were consequently used to investigate whether MvaU binds to the P5 region of the phz2 promoter in vivo (Fig. S1). Two strains were constructed for this assay including ΔmvaU(His) and ΔmvaU overexpressing His-mvaU [referred to as ΔmvaU(His-mvaU)]. Anti-His tag magnetic beads that contain monoclonal antibodies against His were used to select DNA
fragments that were cross-linked with His-MvaU. ChIP-polymerase chain reactions (PCRs) were then used to identify recovered DNA fragments (Fig. S1). A 110-bp PCR product corresponding to the P5 region of the phz2 promoter was detected in the anti-His immunoprecipitate, while these products were not detected in the control mock immunoprecipitate from strain ΔmvaU(His) (Fig. 7A). Subsequent sequencing of the 110-bp PCR product verified its identity of the P5 region. Further, quantitative real-time PCR analysis of anti-His immunoprecipitates demonstrated that overexpression of His-mvaU in strain ΔmvaU significantly increased the enrichment level of immunoprecipitated DNA of the P5 region of the phz2 promoter (Fig. 7B). These results suggest that MvaU directly binds the P5 region of the phz2 promoter.

3.6. MvaU repression of PCA biosynthesis is independent of QscR and OxyR

qscR is located just upstream of the phz2 cluster and encodes an orphan QS signal receptor that regulates global QS Genes [26]. Deletion of qscR significantly increased pyocyanin (PYO) production in PAO1 [26]. To investigate whether QscR is involved in MvaU-dependent repression of PCA biosynthesis, it was deleted in strains MSH and ΔmvaU. The PCA biosynthesis of the resultant strains (ΔqscR and ΔqscRΔmvaU) were compared against that of strain MSH. Deletion of mvaU in both MSH and ΔqscR significantly increased PCA biosynthesis (Fig. 8A). Further, overexpression of mvaU in the double deletion strain ΔqscRΔmvaU significantly reduced PCA biosynthesis (Fig. 8A).

OxyR is a LysR-type transcriptional regulator that is involved in H$_2$O$_2$...
sensing and the regulation of antioxidant defense systems in many bacterial species [33]. In our previous study, the deletion or over-expression of oxyR in PA1201 led to significant increases in PCA biosynthesis [28]. To investigate the role of OxyR in MvaU-dependent repression of PCA biosynthesis, it was deleted in strains MSH and ΔmvaU. The PCA biosynthesis of the resultant strains (ΔoxyR and ΔoxyRΔmvaU) were compared against that of strain MSH. Deletion of mvaU in both strains MSH and ΔoxyR significantly increased PCA biosynthesis (Fig. 8B). Lastly, overexpression of mvaU in the double deletion strain ΔoxyRΔmvaU significantly reduced PCA biosynthesis (Fig. 8B).

3.7. PCA and H$_2$O$_2$ are not the environmental signals that induce mvaU expression

Previous studies reported that MvaU responds to different environmental stresses and regulates gene expression in several bacterial species [3,11]. In this study, the effects of exogenous addition of H$_2$O$_2$ and PCA on mvaU expression was investigated with PA$_P$$_{mvaU}$-lacZ cultures. The addition of 100–200 mg/L PCA or 0.1–0.2 mM H$_2$O$_2$ to PA$_P$$_{mvaU}$-lacZ cultures had no significant effect on cellular growth. Further, exogenous addition of 100–200 mg/L PCA or 0.1–0.2 mM H$_2$O$_2$ to the PA$_P$$_{mvaU}$-lacZ cultures resulted in no significant effects on mvaU expression, as based on β-galactosidase activity assays (Fig. 9A-B). The latter result was further supported by the lack of significant difference in $P_{mvaU}$-dependent β-galactosidase activity between the MSH and PCA-deficient strain Δphz1Δphz2 (Fig. 9C).

4. Discussion

In this study, the roles of the histone-like nucleoid structuring protein MvaU on PCA biosynthesis and associated underlying molecular mechanisms were investigated in the rhizosphere strain Pseudomonas aeruginosa PA1201. These results indicate that MvaU is a self-regulated regulator and negatively regulates PCA biosynthesis. The negative regulation of MvaU on PCA is dependent on the $P_{mvaU}$ gene cluster. Further, lacZ-dependent reporter assays, EMSA analyses, point mutation-based analysis, and ChIP assays suggested that MvaU binds to an AT-rich site within the promoter of the phz2 cluster. Finally, this study demonstrated that MvaU repression of PCA biosynthesis is independent of the two key regulators QscR and OxyR, and that two key metabolite signals PCA and H$_2$O$_2$ are not the environmental signals that trigger mvaU expression. Taken together, this study provides new insights into the regulatory signaling pathway involving MvaU within PCA biosynthesis in the rhizosphere bacterium PA1201.

Phenazines are one of the most important metabolites produced by P. aeruginosa and play diverse roles in cellular growth, development, and virulence. The regulation of the phenazine PYO in P. aeruginosa has been thoroughly studied in the clinically isolated PAO1 strain [19,20,28,29,34–38] and both MvaT and MvaU have been demonstrated to negatively regulate PYO production in PAO1 [30]. Both mvaT and mvaU are present in PA1201. Deletion of mvaT had little effect on PCA biosynthesis (unpublished data) and deletion of mvaU significantly increased PCA biosynthesis in PA1201. MvaU had little effect on phz1 cluster expression and bound to an AT-rich region within the phz2 cluster to negatively regulate phz2 expression (Fig. 3). Our previous results showed that PCA level in the strain Δphz2 is extremely low and that the phz2 contributes much more PCA than the phz1 in the wild-type PA1201 [19]. Thus, MvaU binding to phz2 is enough to shut down PCA biosynthesis in PA1201. Whether this regulatory mechanism in PA1201 also occurs in strain PAO1 requires further investigation since both strains have different origins and different optimal growth temperature. PA1201 is a melon rhizosphere strain with the optimal growth temperature of 28 °C while PAO1 is a clinically isolated strain with the growth optimal
temperature of 37 °C [39]. Although this study identified the MvaU binding site as an AT-rich region within the phz2 promoter, subsequent footprinting analysis failed to identify the specific MvaU binding sequence. Similarly, very few MvaU binding sites have been identified in other Pseudomonas strains [10,30]. These results are probably due to the unique characteristics of MvaU. MvaU is a small transcriptional regulator without the typical helix-turn-helix (HTH) DNA binding domain. MvaU typically acts as a gene silencer and regulates the expression of more than 300 genes [10,11]. Its binding to DNA requires specific co-factors or unique in vivo conditions that warrant further investigation.

H-NS Family Proteins are present in diverse bacterial strains. How MvaU senses the cytoplasmic or environmental clues to regulate gene expression remains to be elucidated. The LysR-type transcriptional regulator OxyR is involved in H2O2 sensing and the regulation of antioxidative defense systems in many bacterial species [33]. In PA1201, deletion or overexpression of oxyR was shown to increase PCA biosynthesis [28]. QscR is a third AHL-responsive LuxR homologue and regulates global quorum sensing gene expression and PYO biosynthesis in P. aeruginosa [26]. Deletion of mvaU had no significant effect on the production of AHL and PQS signals. Moreover, MvaU repression of PCA biosynthesis is independent of qscR and oxyR (Fig. 8). These findings ruled out the possibility that quorum sensing signals, cell density and reactive oxygen species are the environmental signals sensed by MvaU. This is further supported by the finding that exogenous addition of PCA or H2O2 had little effects on mvaU expression (Fig. 9).

Due to its broad-spectrum antibiotic activity and the ability to trigger plant immunity against pathogen attacks, PCA has been used as a major active component in the development of the new metabolite pesticide “Shenqinmycin” in China. As of 2016, 1% Shenqinmycin suspensions have been shown to prevent nine fungal plant diseases [40]. To improve PCA yields, the engineered strain PAIV was generated to achieve PCA yields of 9.8 g/L [25]. In this study, deletion of mvaU in strain MSH increased PCA yields by about 50% (Fig. 2). Nevertheless, it is unclear if deletion of mvaU can be used to further improve PCA yields in industrial PCA-producing strains, which warrants further investigation.

Declaration of competing interest

The authors have no interests to declare.

CRediT authorship contribution statement

Yun-Ling Fang: Methodology, Validation, Investigation, Writing – original draft. Ying Cui: Data curation, Formal analysis, Writing – review & editing. Lian Zhou: Supervision, Data curation, Conceptualization. Chitti Thawai: Validation, Writing – review & editing. Tatheer Alam Naqvi: Formal analysis, Writing – original draft, Supervision. Hong-Yan Zhang: Supervision, Writing – review & editing, Funding acquisition, Project administration. Ya-Wen He: Supervision, Writing – review & editing, Funding acquisition, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sysbio.2021.09.006.

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