Synthetic and Systems Biotechnology 6 (2021) 262–271

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.sciencedirect.com/journal/synthetic-and-systems-biotechnology

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,**], Ya-Wen He [a,*]

[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

ARTICLE INFO

Keywords:
Pseudomonas
Phenazine-1-carboxylic acid
phz cluster
MvaU
Regulatory network

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. β-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 *phz2* cluster. Chromatin immunoprecipitation (ChIP) analysis also indicated that MvaU directly bound to the P5 region of the *phz2* cluster promoter. MvaU repression of PCA biosynthesis was independent of QscR and OxyR in PA1201 and neither PCA or H_2O_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

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, China.
** Corresponding author.
E-mail address: yawenhe@sjtu.edu.cn (Y.-W. He).
[1] Equal contributors.

https://doi.org/10.1016/j.synbio.2021.09.006
Received 15 July 2021; Received in revised form 8 September 2021; Accepted 10 September 2021

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 *Rhizoctonia solani* and *Xanthomonas 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, phzA1B1C1-D1E1F1G1 (phz1) and phzA2B2C2D2E2F2G2 (phz2), 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]. Therefore, 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 *rhlI/rhlR*- and *pqsR*-dependent QS systems are essential for *phz1* and *phz2* 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 *phz1* expression and positively regulates *phz2* expression via multiple mechanisms [27]. OxyR is a LysR-family transcriptional regulator and is responsible for H_2O_2 tolerance in PA1201. Overexpression of *oxyR* increases PCA biosynthesis via regulation by the small RNA phrS [28]. QslA 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 *phz1* cluster transcription, but transcriptionally repressed the expression of the *phz2* gene cluster, consequently resulting in reduced biosynthesis of *phz2*-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 *phz2* 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], KNO_3 [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 Generay plasmid minipreparation purification system. DNA was recovered from gels using an AxyPrep DNA gel extraction kit (Axygen). 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 pK18mobsacB 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 pBBR1MCS-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 (OD_{600}) 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 mm^2 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 *phz1* cluster, the *phz2* 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 P*phz1-lacZ* fusions or P*phz2-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 pFLP2 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 pFLP2 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 *Hin*d III-*Bam*H 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 Na_3PO_4, 10 mM imidazole; pH 7.4) and lysed by sonication.

Recombinant protein was purified by Ni^{2+}-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 KH_2PO_4, 10 mM K_2HPO_4, and 10% (v/v) glycerol (pH 7.4) using an AmiconR 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 $MgCl_2$, 5% glycerol, 40 nM NaCl, and 0.5 mg salmon sperm DNA in a final volume of 20 μL. To evaluate competitive binding, unlabeled 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 Δ*mvaU*(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 OD_{600} 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). P_{mvaU}-dependent β-galactosidase activity was determined at various time points after inoculating the constructed reporter strains PA: P_{mvaU}-*lacZ* and Δ*mvaU*:P_{mvaU}-*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). P_{mvaU}-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 unlabeled 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 Δ*mvaU* in PPM medium (Fig. 2A). However, PCA level in the Δ*mvaU* 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 (Kan^R) in strain Δ*mvaU* significantly reduced PCA biosynthesis to a level much lower than that of MSH in both PPM and SCM media (Fig. 2B–C).

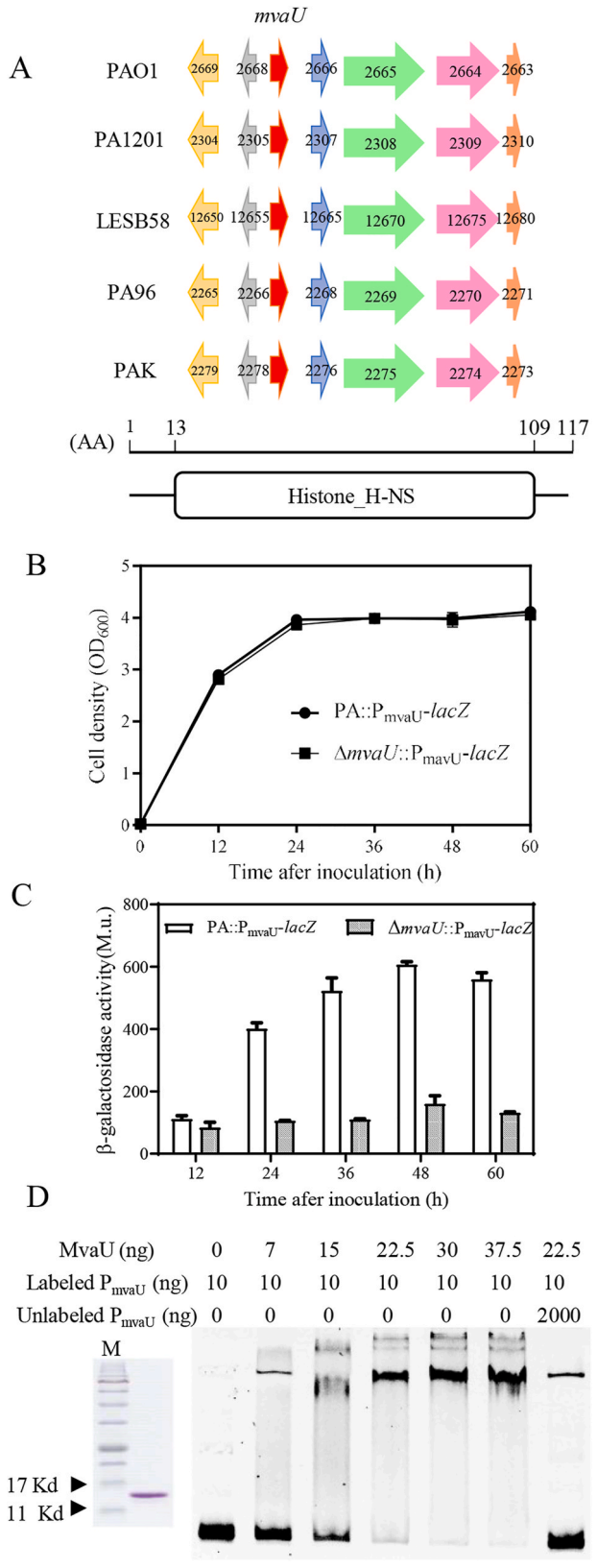

Fig. 1. *mvaU* is a self-regulated gene. (A) Schematic representation of *mvaU* locus in diverse *Pseudomonas aeruginosa* strains showing the conservation of this H-NS family proteins gene. Arrows of the same color represent different gene orthologs with their corresponding numbered genome annotation, and mvaU is represented by a red arrow. All sequences were retrieved from the National Center for Biotechnology Institute (NCBI) Microbial Genomes Resources database. (B) Cellular growth of the reporter strains PA:P_{mvaU}-*lacZ* and ΔmvaU:P_{mvaU}-*lacZ* in PPM medium. (C) β-galactosidase activities of strains PA:P_{mvaU}-*lacZ* and ΔmvaU:P_{mvaU}-*lacZ* during different growth phases. (D) EMSA assays showing MvaU binding to the *mvaU* promoter region P_{mvaU}. Data shown for (B) and (C) comprise means of three replicates and error bars indicate standard deviations.

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 synthesis. In our previous study, two strains were constructed including Δ*phz1* (with *phz1* deleted) and Δ*phz2* (with *phz2* deleted) [19]. PCA biosynthesis is dependent on the activity of enzymes encoded by the *phz2* operon (*phz2*-dependent PCA biosynthesis) in strain Δ*phz1*, while PCA biosynthesis is only dependent on enzymes encoded by the *phz1* operon in Δ*phz2* (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, *mavU* was further deleted in strains Δ*phz1* or Δ*phz2* and the resultant strains were respectively termed Δ*phz1*Δ*mvaU* and Δ*phz2*Δ*mvaU*. No effect on cellular growth was observed among the constructed strains in PPM medium (data not shown).

HPLC analyses demonstrated that PCA levels in Δ*phz1*Δ*mvaU* cultures were significantly higher than in Δ*phz1* 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 Δ*phz1* strain (Fig. 3A). The overexpression of *mvaU* in the Δ*phz1*Δ*mvaU* strain almost abolished PCA biosynthesis (Fig. 3A). In contrast, no significant differences were observed for PCA levels in strains Δ*phz2*Δ*mvaU* and Δ*phz2*. Similarly, overexpression of *mvaU* in Δ*phz2*Δ*mvaU* 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 (Δ*phz2*:P_{phz1}-*lacZ* or Δ*phz1*:P_{phz2}-*lacZ*) to monitor *phz1* and *phz2* activities. The β-galactosidase activity of the resultant strains (Δ*phz2*Δ*mvaU*:P_{phz1}-*lacZ* and Δ*phz1*Δ*mvaU*:P_{phz2}-*lacZ*) were compared against those of their parental reporter strains. The β-galactosidase activity of strain Δ*phz1*Δ*mvaU*:P_{phz2}-*lacZ* was significantly higher than for strain Δ*phz1*:P_{phz2}-*lacZ* at all growth points in cultures within PPM (Fig. 3B). In contrast, deletion in the reporter strain Δ*phz2*:P_{phz1}-*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*.

(caption on next column)

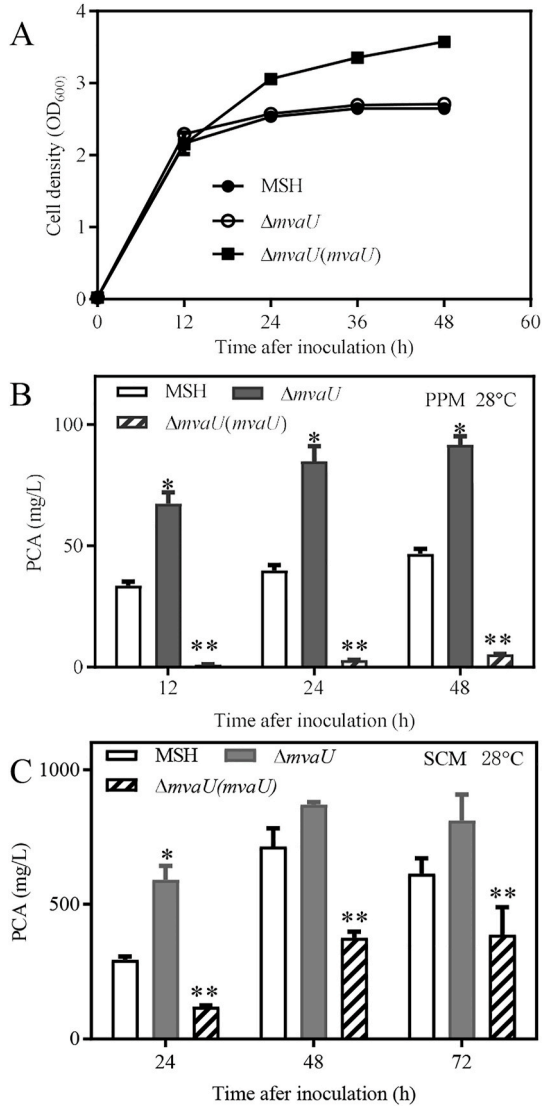

Fig. 2. MvaU negatively regulates PCA biosynthesis in *P. aeruginosa* PA1201. (A) Cellular growth in 50 mL of PPM medium within a 250 mL flask. (B) PCA levels of PA1201-derived strain cultures in PPM medium. (C) PCA levels of PA1201-derived strain cultures in SCM medium. Three replicates were performed 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 ($p < 0.01$).

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 Δ*mvaU* using established protocols [19]. In the resultant strains, *lacZ* expression was driven by the promoter regions P1 (nt −280 to nt +200), P2 (nt −71 to nt +200), or P3 (nt −30 to nt +200) (Fig. 4A). β-galactosidase activity assays indicated that the P3 region did not exhibit promoter activity (Fig. 4B), while the P2 region exhibited weak promoter activity for *lacZ* expression, and the P1 region exhibited full promoter activity (Fig. 4B). P1- or P2-dependent β-galactosidase activities on Δ*mvaU* background were significantly higher than on wild-type MSH background (Fig. 4B). These findings suggest that MvaU-dependent *phz2* repression might be mediated via P2 region.

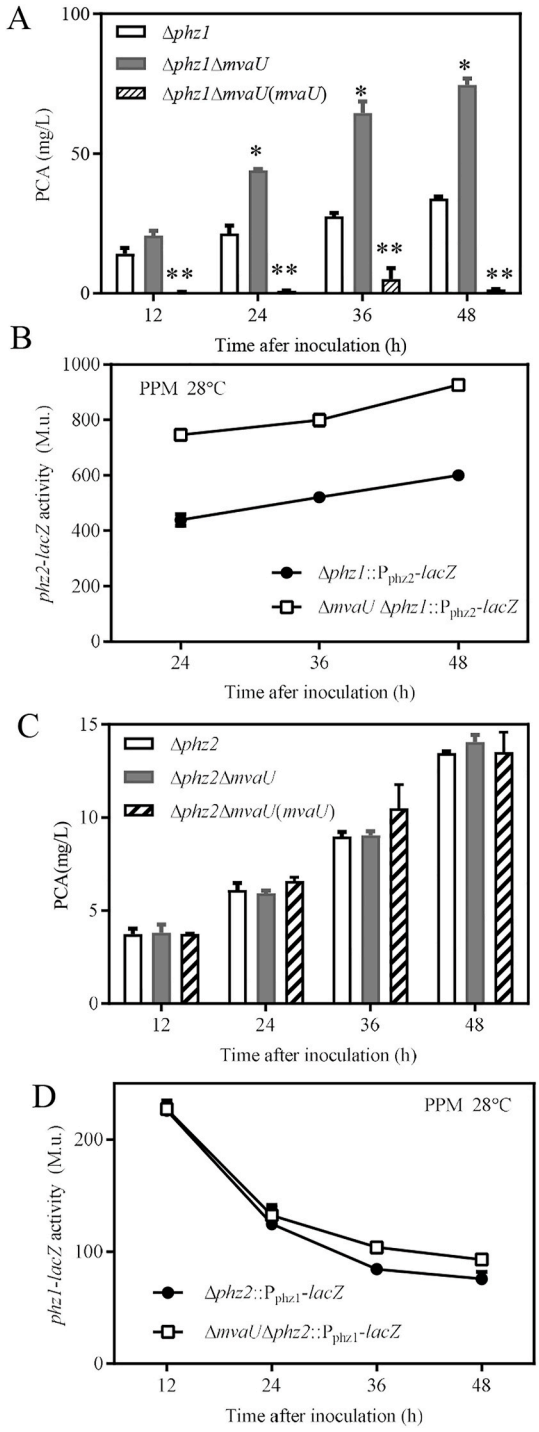

Fig. 3. *mvaU* is a self-regulated gene. (A) *phz2*-dependent PCA production during growth in PPM medium. (B) P_{phz2}-dependent β-galactosidase activities of strains MSH, Δ*mvaU*, and Δ*mvaU*(*mvaU*). (C) *phz1*-dependent PCA production during growth in PPM medium. (D) P_{phz1}-dependent β-galactosidase activities of strains MSH and Δ*mvaU*. Each bar represents the average for three independent experiments and error bars indicate standard deviations. Statistical significance with respect to the Δ*phz1* strain 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 P_{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 P_{phz2} were respectively synthesized as probes (Fig. 5A). EMSA analysis subsequently showed that

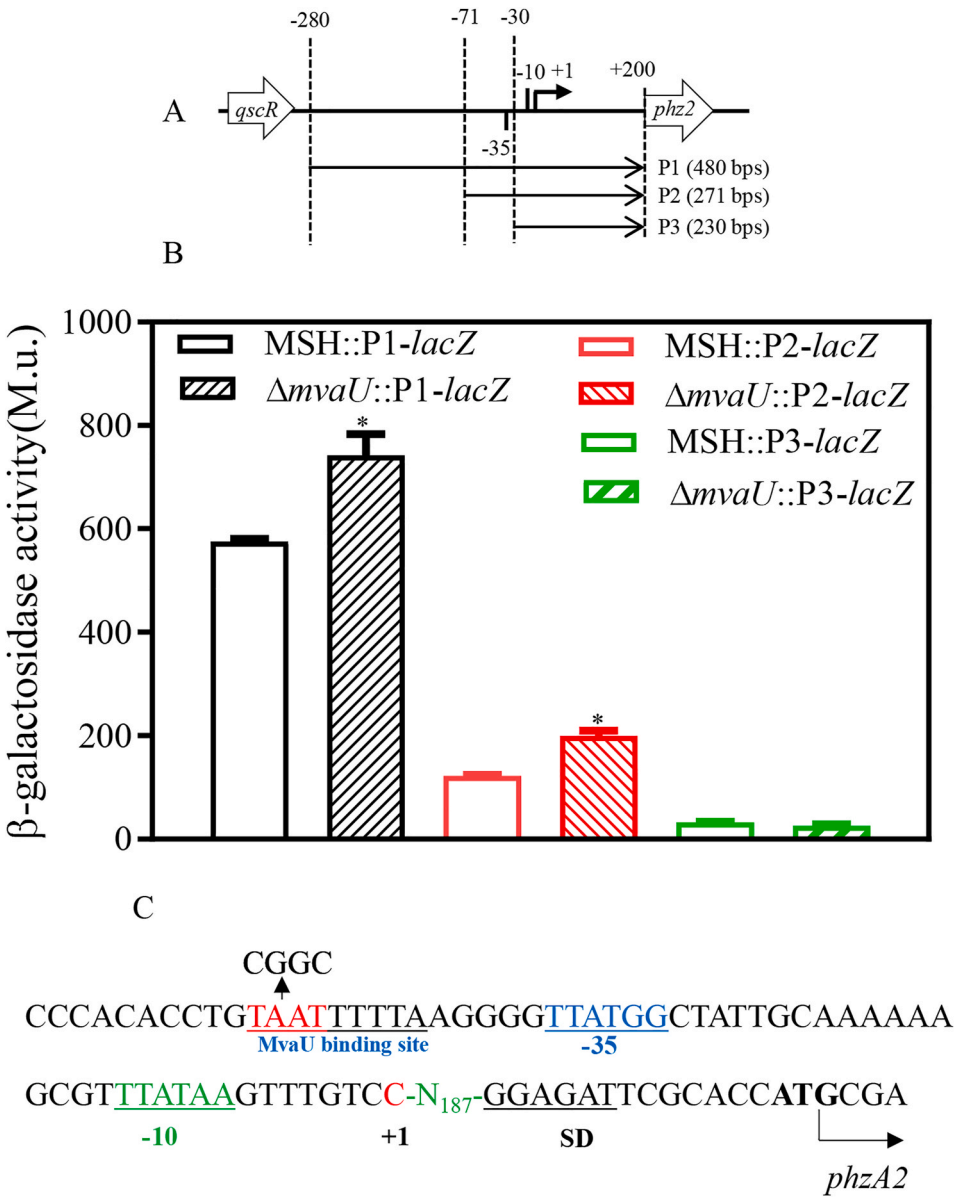

Fig. 4. *phz2* promoter activity in the strains MSH and Δ*mvaU*. (A) Schematic representation of the promoter regions upstream of the start codon of the *phz2* gene cluster. (B) β-galactosidase activities of the constructed reporter strains. (C) Bioinformatics analysis of P_{phzA2}. SD: Shine-Delgarno motif. Each bar represents the average for three independent experiments and error bars indicate standard deviations. Statistical significance with respect to the MSH:P1-*lacZ* strain is indicated by an asterisk ($p < 0.05$).

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 Δ*mvaU*. The resultant strains were termed ΔP5 and ΔP5Δ*mvaU,* 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_{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 Δ*mvaU*. The resultant strains were termed MSH:P1CG-*lacZ* and Δ*mvaU*:P1CG-*lacZ* (Fig. 6C). P1-dependent β-galactosidase activities on Δ*mvaU* 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 Δ*mvaU* (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 Δ*mvaU* overexpressing 6 × His tag [referred to as Δ*mvaU*(His)] and Δ*mvaU* overexpressing His-*mvaU* [referred to as Δ*mvaU*(His-*mvaU*)]. Anti-His tag magnetic beads that contain monoclonal antibodies against His were used to select DNA

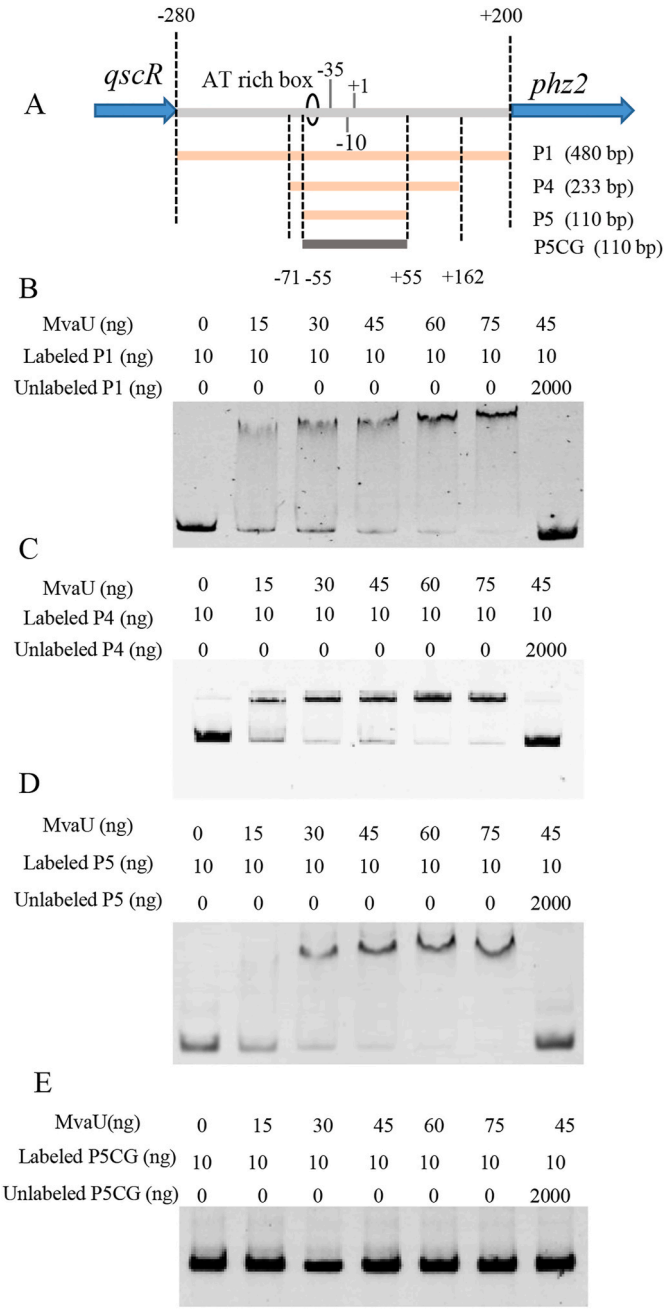

Fig. 5. EMSA analysis of direct binding of MvaU to *phz2* promoter regions. (A) Schematic diagram of the positions of selected probes for EMSA analysis identified in this study. (B) EMSA analysis of MvaU binding to the P1 probe within the *phz2* promoter. (C) EMSA analysis of MvaU binding to the P4 probe within the *phz2* promoter. (D) EMSA analysis of MvaU binding to the P5 probe within the *phz2* promoter. (E) EMSA analysis of no MvaU binding to the P5CG (P5 with point mutations from TAAT to CGGC) probe within the *phz2* promoter. MvaU concentrations (ng) are indicated above the lanes. Unlabeled probe (2000 ng) was added as a specific competitor in the last lane for each EMSA assay.

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 Δ*mvaU*(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

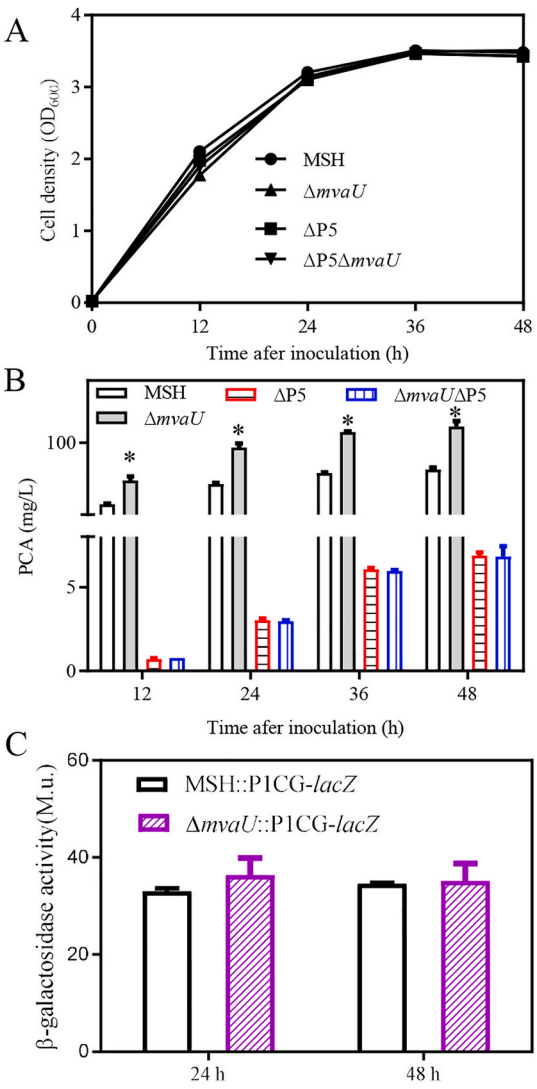

Fig. 6. Verification of the MvaU binding site on the *phz2* promoter. (A) Cellular growth in 50 mL PPM medium within a 250 mL flask. (B) PCA production of MSH-derived strains in PPM medium. (C) β-galactosidase activities of strains MSH:P1CG-*lacZ* and Δ*mvaU*:P1CG-*lacZ*. Each bar represents the average for three independent experiments and error bars indicate standard deviations. Statistical significance is indicated by an asterisk ($p < 0.05$).

demonstrated that overexpression of His-*mvaU* in strain Δ*mvaU* 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 (Δ*qscR* and Δ*qscR*Δ*mvaU*) were compared against that of strain MSH. Deletion of *mvaU* in both MSH and Δ*qscR* significantly increased PCA biosynthesis (Fig. 8A). Further, overexpression of *mvaU* in the double deletion strain Δ*qscR*Δ*mvaU* significantly reduced PCA biosynthesis (Fig. 8A).

OxyR is a LysR-type transcriptional regulator that is involved in H_2O_2

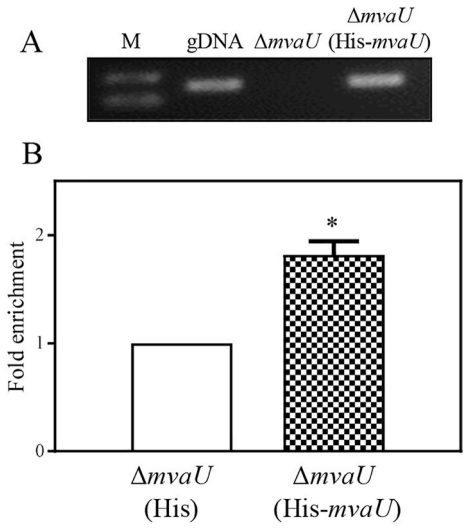

Fig. 7. ChIP assays showing MvaU binding to the *phz2* promoter region. (A) PCR products (110 bp) generated using immunoprecipitated DNA as templates and primers specific to the P5 region. (B) qRT-PCR analysis of the relative levels of the P5 region in the immunoprecipitated DNA derived from the strains Δ*mvaU* and Δ*mvaU*(His-*mvaU*). Each bar represents the average for three independent experiments and error bars indicate standard deviations. Statistical significance with respect to the strain Δ*mvaU* is indicated by an asterisk ($p < 0.05$).

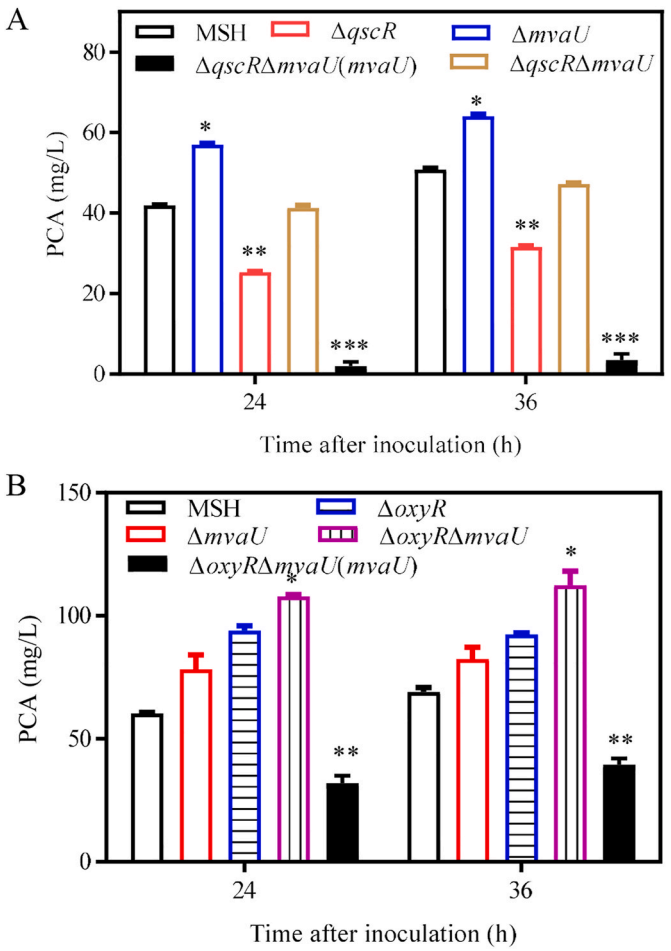

Fig. 8. MvaU negatively regulates PCA synthesis independent of the repressor QscR (A) and OxyR (B). Each bar represents the average for three independent experiments and error bars indicate standard deviations. Statistical significance is indicated by one ($p < 0.05$) or two asterisks ($p < 0.01$).

sensing and the regulation of antioxidant defense systems in many bacterial species [33]. In our previous study, the deletion or overexpression 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 (Δ*oxyR* and Δ*oxyR*Δ*mvaU*) were compared against that of strain MSH. Deletion of *mvaU* in both strains MSH and Δ*oxyR* significantly increased PCA biosynthesis (Fig. 8B). Lastly, overexpression of *mvaU* in the double deletion strain Δ*oxyR*Δ*mvaU* significantly reduced PCA biosynthesis (Fig. 8B).

3.7. PCA and H_2O_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_2O_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_2O_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_2O_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 Δ*phz1*Δ*phz2* (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 *phz2* 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_2O_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 Δ*phz2* 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

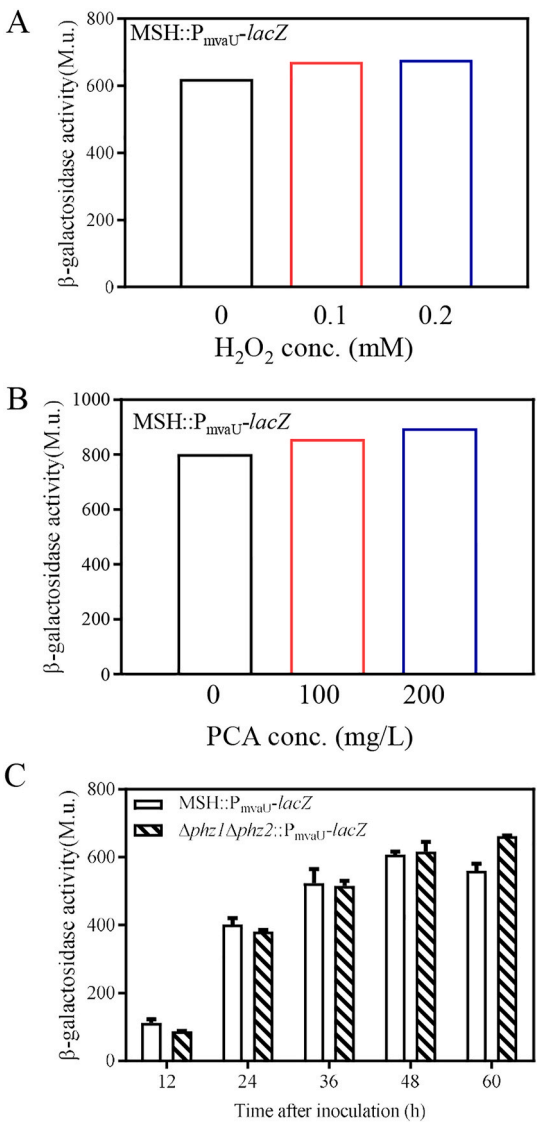

Fig. 9. PCA and H_2O_2 are not the signals that induce *mvaU* expression in PA1201. (A) Effects of P_{mvaU}-dependent β-galactosidase activity in the presence of H_2O_2. (B) P_{mvaU}-dependent β-galactosidase activity of strain MSH:P_{mvaU}-*lacZ* in the presence of PCA. (C) P_{mvaU}-dependent β-galactosidase activity of strains MSH and Δ*phz1*Δ*phz2*. Each bar represents the average for three independent experiments and error bars indicate standard deviations.

temperature of 37 °C [39].

Although this study identified the MvaU binding site as an AT-rich region within the *phz2* promoter, subsequent foot-printing 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 H_2O_2 sensing and the regulation of antioxidant 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 H_2O_2 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.

Acknowledgements

This work was financially supported by grants from National Key R&D Program of China (2018YFA0901901 to Y.-W.He), National Natural Science Foundation of China (No. 31972231 to Y.-W.He). We thank LetPub (www.letpub.com) for linguistic assistance and pre-submission expert review.

Appendix A. Supplementary data

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

References

[1] Winardhi RS, Yan J, Kenney LJ. H-NS regulates gene expression and compacts the nucleoid: insights from single-molecule experiments. Biophys J 2015;109(7):1321–9. https://doi.org/10.1016/j.bpj.2015.08.016.

[2] Gawade P, Gunjal G, Sharma A, Ghosh A. Reconstruction of transcriptional regulatory networks of fis and H-NS in *Escherichia coli* from genome-wide data analysis. Genomics 2020;112(2):1264–72. https://doi.org/10.1016/j.ygeno.2019.07.013.

[3] McMackin EAW, Marsden AE, Yahr TL. H-NS family members MvaT and MvaU regulate the *Pseudomonas aeruginosa* type III secretion system. J Bacteriol 2019;201(14). https://doi.org/10.1128/JB.00054-19. e00054-19.

[4] Landick R, Wade JT, Grainger DC. H-NS and RNA polymerase: a love–hate relationship? Curr Opin Microbiol 2015;24:53–9. https://doi.org/10.1016/j.mib.2015.01.009.

[5] Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL. Repression of phase-variable cup gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. Proc Natl Acad Sci Unit States Am 2005;102(31):11082–7. https://doi.org/10.1073/pnas.0502663102.

[6] Ali SS, Xia B, Liu J, Navarre WW. Silencing of foreign DNA in bacteria. Curr Opin Microbiol 2012;15(2):175–81. https://doi.org/10.1016/j.mib.2011.12.014.

[7] Pfeifer E, Hünnefeld M, Popa O, Frunzke J. Impact of xenogeneic silencing on phage-host interactions. J Mol Biol 2019;431(23):4670–83. https://doi.org/10.1016/j.jmb.2019.02.011.

[8] Tendeng C, Bertin PN. H-NS in Gram-negative bacteria: a family of multifaceted proteins. Trends Microbiol 2003;11(11):511–8. https://doi.org/10.1016/j.tim.2003.09.005.
[9] Tendeng C, Soutourina OA, Danchin A, Bertin PN. MvaT proteins in *Pseudomonas* spp.: a novel class of H-NS-like proteins. Microbiology 2003;149(11):3047–50. https://doi.org/10.1099/mic.0.C0125-0.
[10] Lippa AM, Gebhardt MJ, Dove SL. H-NS-like proteins in *Pseudomonas aeruginosa* coordinately silence intragenic transcription. Mol Microbiol 2020. https://doi.org/10.1111/mmi.14656.
[11] Castang S, Mcmanus HR, Turner KH, Dove SL. H-NS family members function coordinately in an opportunistic pathogen. Proc. Natl. Acad. Sci. U.S.A 2008;105(48):18947–52. https://doi.org/10.1073/pnas.0808215105.
[12] Vallet I, Diggle SP, Stacey RE, Camara M, Ventre I, Lory S, et al. Biofilm formation in *Pseudomonas aeruginosa*: fimbrial cup gene clusters are controlled by the transcriptional regulator MvaT. J Bacteriol 2004;186(9):2880–90. https://doi.org/10.1128/JB.186.9.2880-2890.2004.
[13] Westfall LW, Carty NL, Nancy L, Phillip K, Colmer-Hamood JA, Hamood AN. mvaT mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon mexEF-oprN. FEMS Microbiol Lett 2006;255(2):247–54. https://doi.org/10.1111/j.1574-6968.2005.00075.x.
[14] Zhou L, Jiang H, Jin K, Sun S, Zhang W, Zhang X, et al. Isolation, identification and characterization of rice rhizobacterium *Pseudomonas aeruginosa* PA1201 producing high level of biopesticide" Shenqinmycin" and phenazine-1-carboxamide. Wei sheng wu xue bao= Acta microbiologica Sinica 2015;55(4):401–11. https://doi.org/10.13343/j.cnki.wsxb.20140460.
[15] Zhou L, Jiang HX, Sun S, Yang DD, Jin KM, Zhang W, et al. Biotechnological potential of a rhizosphere *Pseudomonas aeruginosa* strain producing phenazine-1-carboxylic acid and phenazine-1-carboxamide. World J Microbiol Biotechnol 2016;32(3):50. https://doi.org/10.1007/s11274-015-1987-y.
[16] Bilal M, Guo S, Iqbal HMN, Hu HB, Wang W, Zhang XH. Engineering *Pseudomonas* for phenazine biosynthesis, regulation, and biotechnological applications: a review. World J Microbiol Biotechnol 2017;33(10):1–11. https://doi.org/10.1007/s11274-017-2356-9.
[17] Biessy A, Filion M. Phenazines in plant-beneficial *Pseudomonas* spp.: biosynthesis, regulation, function and genomics. Environ Microbiol 2018;20(11):3905–17. https://doi.org/10.1111/1462-2920.14395.
[18] Guttenberger N, Blankenfeldt W, Breinbauer R. Recent developments in the isolation, biological function, biosynthesis, and synthesis of phenazine natural products. Bioorg Med Chem 2017;25(22):6149–66. https://doi.org/10.1016/j.bmc.2017.01.002.
[19] Sun S, Zhou L, Jin KM, Jiang HX, He YW. Quorum sensing systems differentially regulate the production of phenazine-1-carboxylic acid in the rhizobacterium *Pseudomonas aeruginosa* PA1201. Sci Rep 2016;6:30352. https://doi.org/10.1038/srep30352.
[20] Recinos DA, Sekedat MD, Hernandez A, Cohen TS, Sakhtah H, Prince AS, et al. Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity. Proc Natl Acad Sci Unit States Am 2012;109(47):19420–5. https://doi.org/10.1073/pnas.1213901109.
[21] Mentel M, Ahuja EG, Mavrodi DV, Breinbauer R, Thomashow LS, Blankenfeldt W. Of two make one: the biosynthesis of phenazines. Chembiochem 2009;10(14):2295–304. https://doi.org/10.1002/cbic.200900323.
[22] Cui Q, Lv H, Qi Z, Bei J, Xiao B, Liu L, et al. Cross-regulation between the phz1 and phz2 operons maintain a balanced level of phenazine biosynthesis in *Pseudomonas aeruginosa* PAO1. PloS One 2016;11(1):e0144447. https://doi.org/10.1371/journal.pone.0144447.
[23] Askitosari TD, Boto ST, Blank LM, Rosenbaum MA. Boosting heterologous phenazine production in *Pseudomonas putida* KT2440 through the exploration of the natural sequence space. Front Microbiol 2019;10:1990. https://doi.org/10.3389/fmicb.2019.01990.
[24] Li Y, Du X, Lu ZJ, Wu D, Zhao Y, Ren B, et al. Regulatory feedback loop of two phz gene clusters through 5′-untranslated regions in *Pseudomonas* sp. M18. PloS One 2011;6(4):e19413. https://doi.org/10.1371/journal.pone.0019413.
[25] Jin KM, Zhou L, Jiang H, Sun S, Fang YL, Liu JH, et al. Engineering the central biosynthetic and secondary metabolic pathways of *Pseudomonas aeruginosa* strain PA1201 to improve phenazine-1-carboxylic acid production. Metab Eng 2015;32:30–8. https://doi.org/10.1016/j.ymben.2015.09.003.
[26] Ding F, Oinuma KI, Smalley NE, Schaefer AL, Hamwy O, Greenberg EP, et al. The *Pseudomonas aeruginosa* orphan quorum sensing signal receptor QscR regulates global quorum sensing gene expression by activating a single linked operon. mBio 2018;9(4). https://doi.org/10.1128/mBio.01274-18. e01274-18.
[27] Sun S, Chen B, Jin ZJ, Zhou L, Fang YL, Thawai C, et al. Characterization of the multiple molecular mechanisms underlying RsaL control of phenazine-1-carboxylic acid biosynthesis in the rhizosphere bacterium Pseudomonas aeruginosa PA1201. Mol Microbiol 2017;104(6):931–47. https://doi.org/10.1111/mmi.13671.
[28] Sun S, Tan LTH, Fang YL, Jin ZJ, Zhou L, Goh BH, et al. Overexpression of oxyR increases phenazine-1-carboxylic acid biosynthesis via small RNA phrS in the rhizobacterium strain Pseudomonas PA1201. Mol Plant Microbe Interact 2020;33(3):488–98. https://doi.org/10.1094/MPMI-09-19-0264-R.
[29] Fang YL, Chen B, Zhou L, Jin ZJ, Sun S, He YW. The anti-activator QslA negatively regulates phenazine-1-carboxylic acid biosynthesis by interacting with the quorum sensing regulator MvfR in the rhizobacterium *Pseudomonas aeruginosa* strain PA1201. Front Microbiol 2018;9:1584. https://doi.org/10.3389/fmicb.2018.01584.
[30] Li C, Wally H, Miller SJ, Lu CD. The multifaceted proteins MvaT and MvaU, members of the H-NS family, control arginine metabolism, pyocyanin synthesis, and prophage activation in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2009;191(20):6211–8. https://doi.org/10.1128/JB.00888-09.
[31] He YW, Xu M, Lin K, Ng YJ, Wen CM, Wang LH, et al. Genome scale analysis of diffusible signal factor regulon inXanthomonas campestrispv.campestris: identification of novel cell-cell communication-dependent genes and functions. Mol Microbiol 2005;59(2):610–22. https://doi.org/10.1111/j.1365-2958.2005.04961.x.
[32] Orlando V. Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 2000;25(3):99–104. https://doi.org/10.1016/S0968-0004(99)01535-2.
[33] Imlay JA. Transcription factors that defend bacteria against reactive oxygen species. Annu Rev Microbiol 2015;69:93–108. https://doi.org/10.1146/annurev-micro-091014-104322.
[34] Williams P, Camara M. Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol 2009;12(2):182–91. https://doi.org/10.1016/j.mib.2009.01.005.
[35] Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol. Mol. Biol. Rev. 2012;76(1):46–65. https://doi.org/10.1128/MMBR.05007-11.
[36] Balasubramanian D, Schneper L, Kumari H, Mathee K. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. Nucleic Acids Res 2013;41(1):1–20. https://doi.org/10.1093/nar/gks1039.
[37] Higgins S, Heeb S, Rampioni G, Fletcher MP, Williams P, Cámara M. Differential regulation of the phenazine biosynthetic operons by quorum sensing in *Pseudomonas aeruginosa* PAO1-N. Front. Cell. infect. Microbiol. 2018;8:252. https://doi.org/10.3389/fcimb.2018.00252.
[38] García-Reyes S, Soberón-Chávez G, Cocotl-Yanez M. The third quorum-sensing system of *Pseudomonas aeruginosa: Pseudomonas* quinolone signal and the enigmatic PqsE protein. J Med Microbiol 2020;69(1):25–34. https://doi.org/10.1099/jmm.0.001116.
[39] Wu DQ, Li Y, Xu Y. Comparative analysis of temperature-dependent transcriptome of *Pseudomonas aeruginosa* strains from rhizosphere and human habitats. Appl Microbiol Biotechnol 2012;96(4):1007–19. https://doi.org/10.1007/s00253-012-4466-5.
[40] Jin ZJ, Zhou L, Sun S, Cui Y, Song K, Zhang XH, et al. Identification of a strong quorum sensing-and thermo-regulated promoter for the biosynthesis of a new metabolite pesticide phenazine-1-carboxamide in *Pseudomonas* strain PA1201. ACS Synth Biol 2020;9(7):1802–12. https://doi.org/10.1021/acssynbio.0c00161.