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Research Article

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

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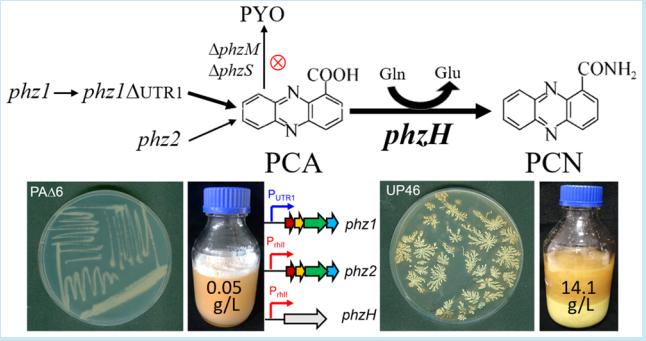
ABSTRACT: Phenazine-1-carboxamide (PCN) produced by multifarious *Pseudomonas* strains represents a promising candidate as a new metabolite pesticide due to its broad-spectrum antifungal activity and capacity to induce systemic resistance in plants. The rice rhizosphere *Pseudomonas* strain PA1201 contains two reiterated gene clusters, *phz1* and *phz2*, for phenazine-1-carboxylic acid (PCA) biosynthesis; PCA is further converted into PCN by this strain using a functional *phzH*-encoding glutamine amino-transferase. However, PCN levels in PA1201 constitute approximately one-fifth of PCA levels and the optimal temperature for PCN synthesis is 28 °C. In this study, the *phzH* open reading frame (ORF) and promoter region were investigated and reannotated. *phzH* promoter P_{phzH} was found to be a weak promoter, and PhzH levels were not sufficient to convert all of the native PCA into PCN. Following RNA Seq and promoter-*lacZ* fusion analyses, a strong quorum sensing (QS)- and thermo-regulated promoter P_{rhlI} was identified and characterized. The activity of P_{phzH} is approximately 1% of P_{rhlI} in PA1201. After three rounds of promoter editing and swapping by P_{rhlI}, a new PCN-overproducing strain UP46 was generated. The optimal fermentation temperature for PCN biosynthesis in UP46 was increased from 28 to 37 °C and the PCN fermentation titer increased 179.5-fold, reaching 14.1 g/L, the highest ever reported.



KEYWORDS: *Pseudomonas, biopesticide, phenazine-1-carboxamide, phzH, promoter swapping*

Phytopathogens have long been recognized as major threats to plant species. These plant pathogens result in significant crop yield losses while also affecting biodiversity. To mitigate these effects, synthetic pesticides have played critical roles in ensuring agricultural productivity since the 1950s. However, the overuse and mishandling of pesticides in agriculture have adversely and chronically impacted soils due to the accumulation of pollutants. Because of growing concerns for human health and environmental pollution, biological pesticides have become promising alternatives to mitigate the detrimental effects of phytopathogenic fungi on agricultural plants. Microbial metabolite pesticides, which represent a key group in the biological pesticide family, are naturally occurring products used for pest-control in agriculture. Since 1970, several Streptomyces-derived metabolite pesticides, including blasticidin-S, kasugamycin, polyoxins, validamycin, avermectins, and spinosad, have been developed to control the growth of bacterial and fungal pathogens as well as pests in crops.[1−5] Phenazines, which comprise a group of nitrogen-containing redox active compounds, have long been known for their broad-spectrum antifungal properties.[6] Natural phenazines are secondary metabolites produced by fluorescent pseudomonads, streptomycetes, and archaeal Methanosarcina species.[7−9] Phenazines have been shown to play important roles in the biological control of plant diseases in a range of plant growth-promoting rhizobacteria (PGPR)[10−13]

M18 and PA1201, two well-studied *Pseudomonas* strains, were respectively identified in melon and rice rhizospheres.[14,15] The genomes of these strains are organized in a similar manner and they harbor similar genetic sequences. Furthermore, phenazine-1-carboxylic acid (PCA) represents the dominant phenazine produced in both M18 and PA1201. Two duplicated gene clusters, $phzA_1B_1C_1D_1E_1F_1G_1$(*phz1*) and $phzA_2B_2C_2D_2E_2F_2G_2$ (*phz2*), are responsible for PCA biosynthesis in both *Pseudomonas* strains.[16] Over the last 10 years, PCA biosynthesis in M18 or PA1201 has been shown to be regulated by an untranslated region (UTR) in the *phz1* promoter region,[17] by three quorum sensing (QS) systems

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(LasI/LasR, RhlI/RhlR, and PQS/PQSR),[16] and by the transcription factors RsaL, QslA, and OxyR.[18−20] On the basis of these findings, at least five rounds of genetic engineering have been conducted in both PA1201 and M18, and the PCA fermentation titer in the engineered strain PA-IV was reported to reach 9.8 g/L.[21] PCA was commercially rebranded as Shenqinmycin in 2005. Until 2016, 1% Shenqinmycin suspension was successfully registered in China as a new metabolite biopesticide to prevent nine plant fungal diseases in rice, wheat, pepper, watermelon, and cucumber (Registration Certificate No. PD20110315).

Some phenazine-producing bacteria also contain one or more accessory genes, such as *phzO*, *phzH*, *phzM*, and *phzS*, that encode terminal-modifying enzymes for the conversion of PCA into 2-hydroxyphenazine (2OH-PCA), phenazine-1-carboxamide (PCN), and pyocyanin (PYO).[22] PCN exhibits antifungal activity against a number of important plant pathogens, including *Fusarium oxysporum*, *Rhizoctonia solani*, *Pythium ultimum*, and *Verticillium alboatrum*.[23−26] The results of an *in vitro* assay revealed that the antifungal activity of PCN was 5−10 times greater than that of PCA at a pH of 5.7 or higher.[10,23] Recently, PCN has been reported to inhibit the growth and virulence of the plant pathogenic fungus *Fusarium graminearum* by directly affecting the activity of a histone acetyltransferase FgGcn5.[25] PCN was shown to inhibit the activities of cell-wall chitin synthetase and complex I of the mitochondrial electron transport chain in *Rhizoctonia solani*, a causal agent of rice sheath blight.[26] Moreover, PCN produced by *Pseudomonas* sp. CMR12a was found not only to be important in direct antagonism against soilborne pathogens, but also to play a role in enhancing plant innate immunity.[27,28] These findings suggest that PCN might represent a promising candidate as a new metabolite pesticide.

The conversion of PCA into PCN is catalyzed by a glutamine aminotransferase encoded by *phzH* in *Pseudomonas* strains.[10,25] M18 harbors a nonfunctional version of gene *phzH* containing a point mutation (C to T) at the 904th nucleotide. Because of the presence of this mutation, M18 cannot produce PCN.[29] PA1201 contains a functional copy of *phzH*; however, PCN levels are approximately one-fifth of PCA levels in PPM culture.[15] In this study, a strong quorum sensing (QS)- and thermo-regulated promoter P_{rhlI} was identified and used to replace the promoters of *phzH* and *phz2* in the PCA-overproducing strain. The optimal fermentation temperature for PCN biosynthesis of the resultant strain UP46 was increased from 28 to 37 °C. Furthermore, the PCN fermentation titer exhibited a 179.5-fold increase (Supporting Information, Figure S1), reaching 14.1 g/L, the highest yield ever reported for PCN produced using biosynthetic strategies.

MATERIAL AND METHODS

Bacterial Strains, Media, and Growth Conditions. The bacterial strains used in this study are described in Table S1. *E. coli* strains were grown aerobically at 37 °C in lysogeny broth (LB) medium. When required, the media was supplemented with 20 μg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or 100 μg/mL ampicillin to facilitate blue/white colony screening. The PA1201 strain has been described previously by Zhou *et al.*[16] Unless otherwise stated, PA1201-derived strains were grown in 50 mL of PPM medium (Pigment-Producing Medium; 22 g/L Tryptone, 20 g/L glucose, 5 g/L KNO3, pH 7.5)[30] in an Erlenmeyer flask (250 mL) at 250 rpm in a rotary shaker (ZQWY-200N, SHzhichu, China). When required, 50 μg/mL spectinomycin (Sp) or 50 μg/mL kanamycin (Kan) was added into the media. All chemicals were purchased from Sangon Biotech (Shanghai) Co., Ltd.

Two-Step Recombination Method for in-Frame Gene Deletion in PA1201. The method used for marker-less gene deletion was previously described elsewhere.[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 suicide vector pK18mobsacB carrying the sucrose-sensitive *sacB* gene.[32] 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 50 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. The primers used for PCR and subsequent selection are listed in Table S2.

Quantitative Analysis of PCA and PCN Production in PPM Cultures. PCA or PCN extraction and quantitative analysis were performed as previously described by Zhou *et al.*[15] Briefly, 180 μL of the appropriate culture were collected and mixed with 20 μL of 6 M HCl. This was followed by an extraction step with 540 μL of chloroform. The organic phase was subsequently collected and dried at 4 °C. The resultant residues were dissolved in 50 μL of methanol. Five microliter-aliquots of PCA or PCN crude extracts were subsequently collected for HPLC analysis (Agilent Technologies 1260 Infinity) under the following conditions: C18 reversed-phase column (5 μm, 4.6 mm × 12.5 mm) eluted with acetonitrile/5 mM ammonium acetate (60:40, v/v). PCA or PCN production was quantified using the corresponding peak area (A) in the HPLC elute according to an established formula as previously described by Zhou *et al.*[15]

Total RNA Extraction and Quantitative Real Time Reverse Transcription (RT)-PCR Analysis. Total RNAs were extracted using RNeasy mini-prep kit (Qiagen, USA). The PrimeScript RT reagent kit was applied for quantitative RT-PCR analysis. Quantification of *phzH* cDNA level and melting curve analysis were performed using Mastercycler ep Realplex 4S (Eppendorf) and SYBR Premix Ex Taq (Takara) according to the manufacture's protocol.

RhlI Protein Expression, Purification, and Western Blot Analysis. The coding region of *rhlI* was amplified with the primers listed in Table S2 and subcloned into the plasmid pET-28A (GenScript). The recombinant construct was then transformed into *E. coli* strain BL21. For expression of RhlI protein, the cells were grown at 37 °C in LB medium supplemented with 100 μg/mL kanamycin until an OD_{600} value of 0.6 was reached. Protein expression was subsequently induced at 18 °C with 0.1 mM IPTG for 16 h. The bacterial cells were lysed by sonication on ice and the debris were removed by centrifugation at 14 000 rpm for 30 min (Thermo Scientific, Sorvall Legend X1R). The soluble proteins were loaded onto a column containing Ni-NTA agarose resin as described in the protocol supplied. The eluted RhlI protein was concentrated using an ultra-0.5 centrifugal filter and subsequently stored at −80 °C in a suitable storage buffer (25 mM Tris, 200 mM NaCl and 10% glycerol, pH 8.0).

The polyclonal antisera against the RhlI protein were generated by ABclonal Biotechnology Company (Wuhan,

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China). Western blot analysis was performed as described by Green and Sambrook.[33] The hybridization signal was detected using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE, USA). The monoclonal antibody against the α-subunit of RNA polymerase (NeoClone) was used as a control for sample loading. ImageJ (https://imagej.nih.gov/ij) was used for image processing to determine the signal intensity of the Western blot bands.

Generation of *lacZ*-Dependent Reporter Strains for Promoter Assay. The method for the generation of the promoter-*lacZ* fusion reporter strain in PA1201 was previously described by Sun *et al.*[16] Briefly, the promoter regions approximately 500 bp upstream of the start codon of the target genes (*lasB*, *oprI*, *pqsA*, *rhlI*, *rhlR*, *phz1*, *phz2*, and *phzH*) were individually amplified by PCR using the primers listed in Table S2. The resultant PCR products were then cloned into the vector mini-CTX-*lacZ*.[34] The resultant recombinant plasmids were integrated into the *attP* site in the chromosomes of PA1201-derived strains following the protocol described by Becher and Schweizer.[33] The resultant reporter strains were verified by PCR using the primers in Table S2. The β-galactosidase activity of the reporter strain was determined.

Promoter Swapping Analysis in PA1201. The procedure for promoter swapping in PA1201 was previously described by Jin *et al.*[21] Briefly, the DNA fragment corresponding to P_{rhlI} was amplified by PCR with primers P_{rhlI}::*phzH/phz2*-F2 and P_{rhlI}::*phzH/phz2*-R2, and the ∼500-bp DNA fragments corresponding to the upstream (L) or downstream (R) regions of the of target promoter P_{phzH} or P_{phz2} were separately amplified by PCR with primes P_{rhlI}::*phzH/phz2*-F1 and P_{rhlI}::*phzH/phz2*-R1, P_{rhlI}::*phzH/phz2*-F3, and P_{rhlI}::*phzH/phz2*-R3 which were listed in Table S2. The DNA fusion fragments containing P_{rhlI} flanked by fragments L and R were obtained via two rounds of fusion PCR amplification (Figure S2). These fragments were subsequently subcloned into the vector pK18*mobsacB*. The recombinant constructs were integrated into PA1201 by homologous recombination. Transformants were selected on PPM medium supplemented with 50 μg/mL Sp and 50 μg/mL Kan (Figure S2). Resultant colonies were subsequently suspended in 1 × PBS buffer and plated on a PPM agar plate containing 5% (w/v) sucrose and 50 μg/mL Sp to select for a second crossover event. The strains with swapped promoters were further verified by PCR and DNA sequencing.

Batch Fermentation of the Strain UP46. PPM medium was used for seed culture preparation. The seed cultures were prepared by inoculating UP46 colonies into 50 mL of PPN medium (22 g/L Tryptone, 20 g/L glucose, and 15 g/L KNO_3, pH 7.5) in a 250 mL flask and incubating at 28 °C and 200 rpm for 12 h. The seed culture was then transferred to a 5 L fermenter (BLBIO-5GJG-4-H, China) containing 3.5 L of PPN medium. Fermentation was carried out at 37 °C with an air flow rate of 3 L/min. The dissolved oxygen was kept at 70% by adjusting the agitation speed from 300 to 600 rpm. Antifoam (Sangon Biotech, China) was added automatically as needed.

Statistical Analysis. All the experiments were performed in triplicate at different stages in the associated growth curves. All figures were prepared using Graphpad Prism7 software. Data are presented as mean ± standard deviation (SD). Statistically significant differences between two groups were analyzed by Student's *t* test.

RESULTS

PCA Is Partially Converted into PCN in the PCA-Overproducing Strains. In the previous study by Jin *et al.*,[21] deletion of the six sets of cytotoxicity-associated genes in PA1201 (resulting in strain PAΔ6) did not significantly affect phenazine production. In this study, *phzM* and *phzS* were further deleted in strain PAΔ6, and the resultant strain was named PA26. Upon growth in PPM medium at 28 °C for 24 h, PA26 produces only PCA and PCN, and 62.3% of the total phenazines are PCA (Figure 1).

Deletion of the 5′-untranslated region (UTR) of the *phz1* gene cluster in PA1201 was previously shown to significantly

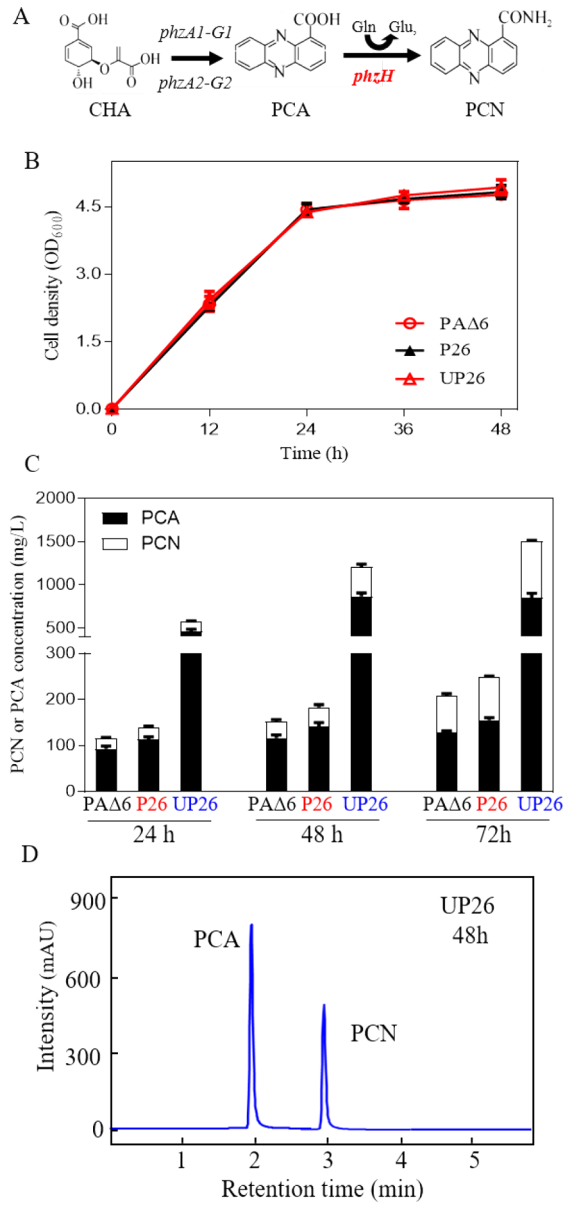

Figure 1. Only partial PCA is converted into PCN in PCA-overproducing strains. (A) PhzH is responsible for PCA conversion into PCN. (B) Growth time curves of the three strains PAΔ6, PA26, and UP26 in PPM medium at 28 °C. (C−D) HPLC analysis of PCA and PCN production in the strains PAΔ6, PA26, and UP26 in PPM medium. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to the strain PAΔ6 are indicated by one ($p \leq 0.05$), or two ($p \leq 0.01$) asterisks.

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increase PCA biosynthesis.[21] In this study, to improve PCN biosynthesis, the 5′-UTR of the *phz1* cluster was deleted in strain PA26 and the resultant strain was named UP26. We found that PCA is still the predominant phenazine in strain UP26. PCN levels in the UP26 cultures were 110 mg/L at 24 h postinoculation (hpi), 342.5 mg/L at 48 hpi, and 652 mg/L at 72 hpi. In contrast, PCA levels were 463.5 mg/L at 24 hpi, 865 mg/L at 48 hpi, and 849.5 mg/L at 72 hpi (Figure 1). These results indicate that PCA cannot be fully converted into PCN in the PCA-overproducing strains PA26 and UP26. This phenomenon is most likely caused by low *phzH* expression in these strains.

Characterization of the *phzH* Open Reading Frame and Promoter Region. To evaluate *phzH* expression levels in PA1201, we attempted to identify the exact *phzH* open reading frame (ORF) and its promoter region. Bioinformatics analysis revealed four putative start codons, TGT, ATG_{+1}, ATG, and ATG_{-1}, for *phzH* (Figure 2A), and ATG was originally predicted as the start codon by Wu *et al.*[35] To verify the predicted start codon, the four putative *phzH* ORFs were respectively amplified by PCR and subcloned into the *Hin*dIII and *Bam*HI recognition sequences of the multiple cloning sites of vector pBBR-1-MCS; gene expression is driven by the promoter P_{lac} in this vector. The resultant plasmids were then transformed into *phzH* deletion strain Δ*phzH* to generate the strains Δ*phzH*(ATG_{-1}), Δ*phzH*(ATG), Δ*phzH*(ATG_{+1}), and Δ*phzH*(TGT) (Figure 2B). The results showed that overexpression of the *phzH* ORFs starting from ATG_{-1} or ATG had little effect on PCN conversion, whereas overexpression of the *phzH* ORFs starting from ATG_{+1} or TGT could fully convert PCA into PCN (Figure 2C). These findings ruled out the possibility that ATG or ATG_{-1} is the start codon of *phzH* ORF. On the basis of the presence of the Shine-Dalgarno (SD) sequence, ATG_{+1} was reassigned as the start codon of the *phzH* ORF (Figure 2A).

We subsequently identified the *phzH* transcript start site by 5′-RACE using mRNAs prepared from cells at 24 hpi in PPM medium. The start site (T^{+1}) of the *phzH* transcript was identified at nucleotide −133 relative to the start codon ATG_{+1}, and the putative −35 element and −10 element (TTAACT N21-TATCAT) were assigned on the basis of conformity to the *E. coli* σ-70 promoter consensus sequence (Figure 2A).

To further verify the predicted −10 and −35 elements, the *phzH* ORF together with the three putative promoter regions of different lengths (P_{108}, P_{180}, and P_{504}) were amplified and respectively cloned into the *Bam*HI and *Hin*dIII recognition sequences of the multiple cloning site of pBBR-1-MCS (Figure 3). In resultant plasmids, *phzH* transcription was not driven by the vector-born promoter P_{lac}, but by each of the three putative promoters, P_{108}, P_{180}, or P_{504} (Figure 3). The resultant

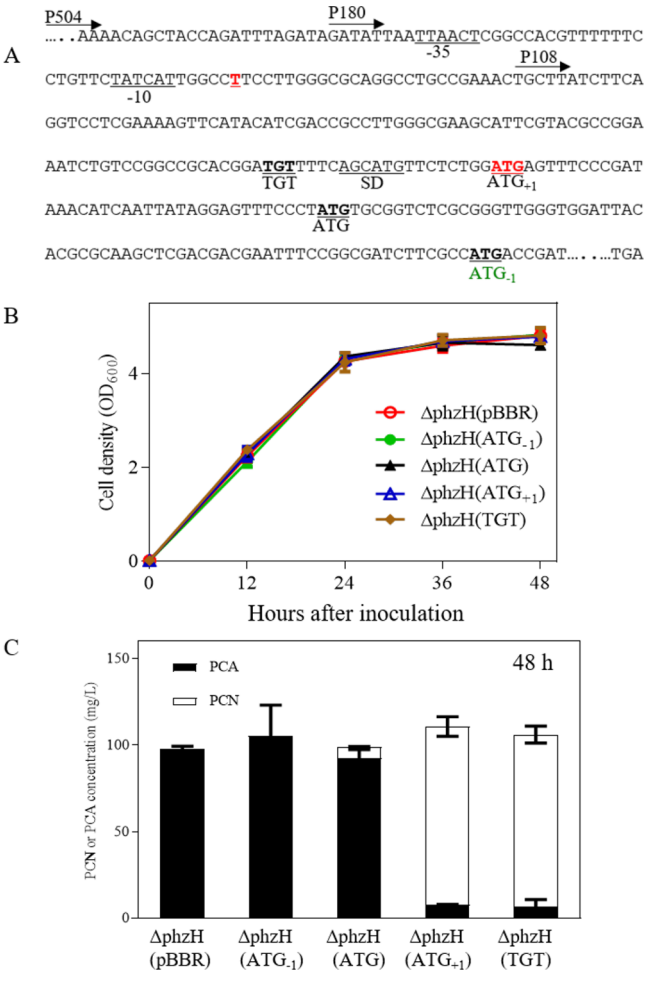

Figure 2. Determining *phzH* open reading frame (ORF). (A) DNA sequence of *phzH* promoter region with predicted elements. (B) Growth curves of Δ*phzH*-derived strains in PPM medium. (C) PCA and PCN production of the Δ*phzH*-derived strains. Shown are averages for three technical repeats with standard deviation.

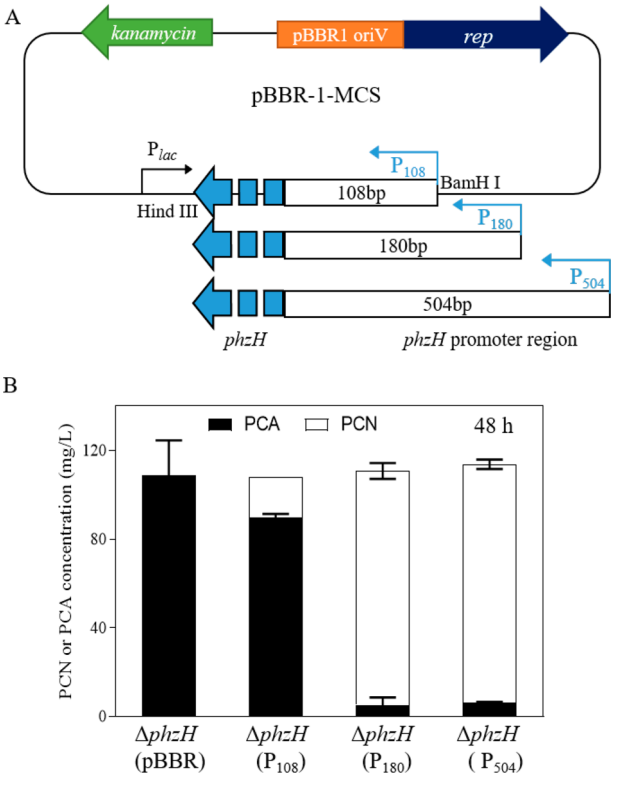

Figure 3. Determining *phzH* promoter region. (A) A schematic diagram showing the three putative promoter regions driving *phzH* expression in pBBR-1-MCS. (B) PCA and PCN production of the Δ*phzH* strains driven by three promoter regions P_{108}, P_{180}, and P_{504}. Shown are averages for three technical repeats with standard deviation.

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recombinant plasmids were further transformed into Δ*phzH* strain to generate the strains Δ*phzH*(P_{108}), Δ*phzH*(P_{180}), and Δ*phzH*(P_{504}), respectively. In strains Δ*phzH*(P_{180}) or Δ*phzH*(P_{504}), PCA could be fully converted into PCN. Conversely, no PCA was converted into PCN in the control strain Δ*phzH*(pBBR) (Figure 3). These results showed that only promoter region P_{180} or P_{504} could drive *phzH* expression, which is consistent with the fact that P_{180} and P_{504} contain the predicted −10 and −35 elements.

The Activity of P_{phzH} Is Approximately 1% of that of P_{UTR1} in PA1201. To evaluate P_{phzH} activity, a DNA fragment spanning the 504-bp region upstream of the start codon ATG_{+1} of *phzH* was amplified and subcloned into the vector mini-CTX-*lacZ* to generate a recombinant plasmid mini-CTX-P_{phzH}-*lacZ*. This recombinant plasmid was subsequently introduced and integrated into the *attP* site in the chromosome of UP26 strain to generate the reporter strain UP26::P_{phzH}-*lacZ* (Figure S3). In a previous study by Sun *et al.*[16] three recombinant plasmids P_{phz1}-*lacZ*, P_{phz2}-*lacZ*, and P_{UTR1}-*lacZ* were constructed to monitor the promoter activity of gene clusters *phz1*, *phz2*, or *phz1* without a 232-bp untranslated region (UTR1) in PA1201. In this study, the three recombinant plasmids were integrated into strain UP26 to generate three reporter strains UP26::P_{phz1}-*lacZ*, UP26::P_{phz2}-*lacZ*, and UP26::P_{UTR1}-*lacZ* to monitor the activity of P_{phz1}, P_{phz2}, or P_{UTR1} in strain UP26.

The above four reporter strains, together with the negative control strain UP26::*lacZ*, were individually grown in PPM medium for 48 h and *β*-galactosidase activities were determined. As shown in Figure 4, P_{phzH}-dependent *β*-galactosidase activities were 155.5 m.u. at 24 hpi and 162 m.u. at 48 hpi; these results are comparable with the P_{phz1}-dependent *β*-galactosidase activities (177 m.u. at 24 hpi and 194.5 m.u. at 48 hpi) (Figure 4). P_{phz2}-dependent *β*-galactosidase activities (37.5 m.u. at 24 hpi and 49 m.u. at 48 hpi) were significantly lower than those of P_{phzH}-dependent ones (Figure 4). In contrast, P_{UTR1}-dependent *β*-galactosidase activity was 14 976 m.u. at 24 hpi and 19 988 m.u. at 48 hpi (Figure 4). Thus, P_{phzH}-dependent *β*-galactosidase activities were only 0.8%−1.0% of P_{UTR1}-dependent ones. This reduction in promoter activity is most likely the reason why PCA is not fully converted into PCN in the PCA-overproducing strains PA26 and UP26 (Figure 1).

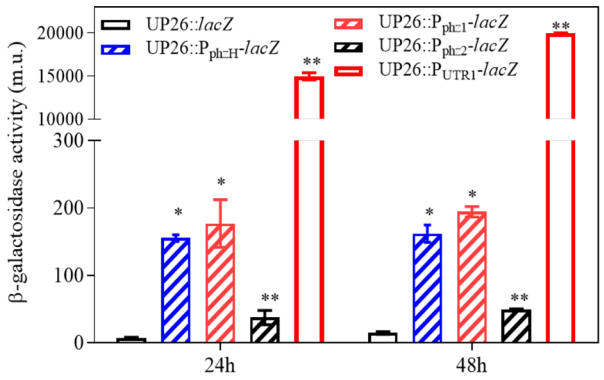

Figure 4. Relative promoter activities of *phzH*, *phz1*, and *phz2* as indicated by *β*-galactosidase assay. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to strain UP26::*lacZ* are indicated by one or two asterisks ($p \leq 0.05$).

Identification of Strong Promoters in PA1201 by RNA_Seq Analysis. RNA-Seq analysis was previously utilized for the identification of PCA or OxyR-regulated genes in three PA1201-derived strains, PAΔ6, Δ*phz1*Δ*phz2* and Δ*oxyR*.[20,21] In this study, we reanalyzed all the published transcriptome data and identified a total of 19 genes with ≥5000 RPKM (reads per kilobase per million mapped reads) in the four data sets (Figure S4). We speculated that each of these genes would have a strong promoter to drive transcription. To verify this hypothesis, the promoter activity of each of the 19 genes in strain UP26 were respectively analyzed following the construction of promoter-*lacZ* fusion reporter strains (Figure S3). The promoter-dependent *β*-galactosidase activities of *pqsA*, *lasB*, *rhlR*, *oprI*, and *rhlI* are shown in Figure 5. The activity of P_{rhlI} was shown to be the highest among the five promoters tested (Figure 5B). P_{rhlI}-dependent *β*-galactosidase activity was 38253 m.u. at 24 hpi and 79465.5 m.u. at 48 hpi, which is respectively 246-fold and 490-fold greater than that observed for P_{phzH} in PPM medium at the same time points (Figure 5C). In addition, P_{rhlI}-dependent *β*-galactosidase activities at 24 hpi and 48 hpi were approximately 2.1−4.0-fold greater than those observed for P_{UTR1} at the same time points (Figure 5C).

rhlI encodes an acyl homoserine lactone (AHL) synthase responsible for *N*-butanoyl-homoserine lactone (abbreviated as C4-HSL) biosynthesis in PA1201.[16] As shown in Figure 5D, the transcription start site of P_{rhlI} was experimentally identified as C^{1+} by Kievit *et al.*[36] In addition, P_{rhlI} also contains the −35 and −10 elements (TTGCCT-N14-TTTAGT), and a putative UTR region upstream of the SD sequence (Figure 5D). This promoter region was used for swapping analyses in the following experiments.

Swapping P_{phzH} and P_{phz2} with P_{rhlI} Significantly Increased PCN Biosynthesis. In this study, markerless promoter swapping was performed using an established method previously described by Jin *et al.*[21] (Figure S3). The P_{phzH} was first replaced by P_{rhlI} in strain UP26 to generate the strain UP36. In PPM medium at 28 °C, no statistically significant difference in growth was observed between the strains UP26 and UP36. However, UP36 produced only PCN (Figure 6), indicating that all PCA was converted into PCN. As expected, PCN levels in strain UP36 were comparable with the total levels of PCA and PCN in strain UP26 at 24 hpi or 48 hpi (Figure 6). To determine whether the increased PCN levels in the strain UP36 result from increased *phzH* transcription, we measured *phzH* mRNA levels by quantitative real time RT-PCR (qRT-PCR). As shown in Figure 6D, the *phzH* mRNA level at 24 hpi in the strain UP36 is 116.6-fold higher than that in the strain UP26. In strain UP36, P_{UTR1}-driven gene cluster *phz1* and P_{phz2}-driven gene cluster *phz2* cocontribute to PCN biosynthesis. In this study we observed that the activity of P_{phz2} was far lower than that of P_{rhlI} (Figures 4 and 5). This result prompted us to replace P_{phz2} with P_{rhlI} in UP36 to improve PCN biosynthesis. The resultant strain UP46 produced similar levels of PCN to UP36 at 24 hpi in PPM medium (Figure 6). However, PCN levels in strain UP46 were 1720.5 mg/L at 48 hpi and 2561 mg/L at 72 hpi, which is about 36.4% and 68.0% higher than those observed for UP36 (1261.4 mg/L at 48 hpi and 1524 mg/L at 72 hpi) at the same time points, respectively (Figure 6C).

P_{rhlI} Is a Thermo-regulated Promoter in PA1201. The optimal fermentation temperature for PCA biosynthesis in the rhizosphere bacteria M18 and PA1201 is 28 °C.[21,35] However,

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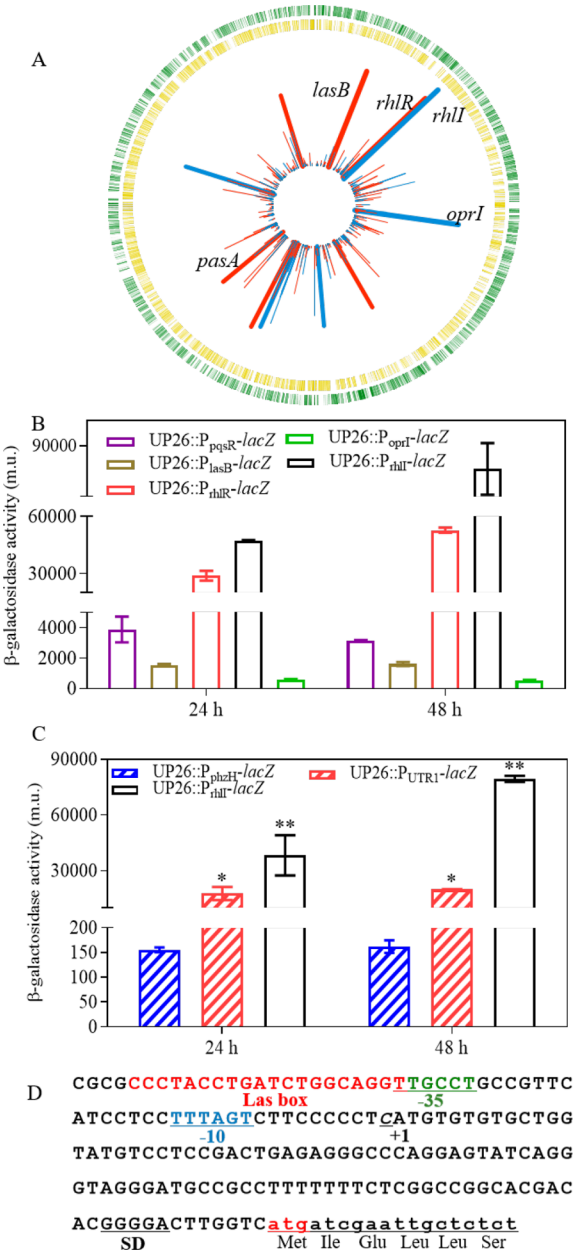

Figure 5. Identification of a high strength promoter P_{rhlI} in PA1201. (A) The relative expression levels (as indicated by RPKM) of all PA1201 genes by RNA_Seq analysis. (B) The relative activities of P_{pqsR}, P_{lasB}, P_{oprI}, P_{rhlR}, and P_{rhlI} as indicated by LacZ assay. (C) The relative activities of P_{rhlI}, P_{phzH}, and P_{UTR1} as indicated by LacZ assay. (D) DNA sequence of P_{rhlI} showing −35 element, −10 element, transcription initiation site, Las-box, SD region, and start codon. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to strain UP26::P_{phzH}-*lacZ* are indicated by one or two asterisks.

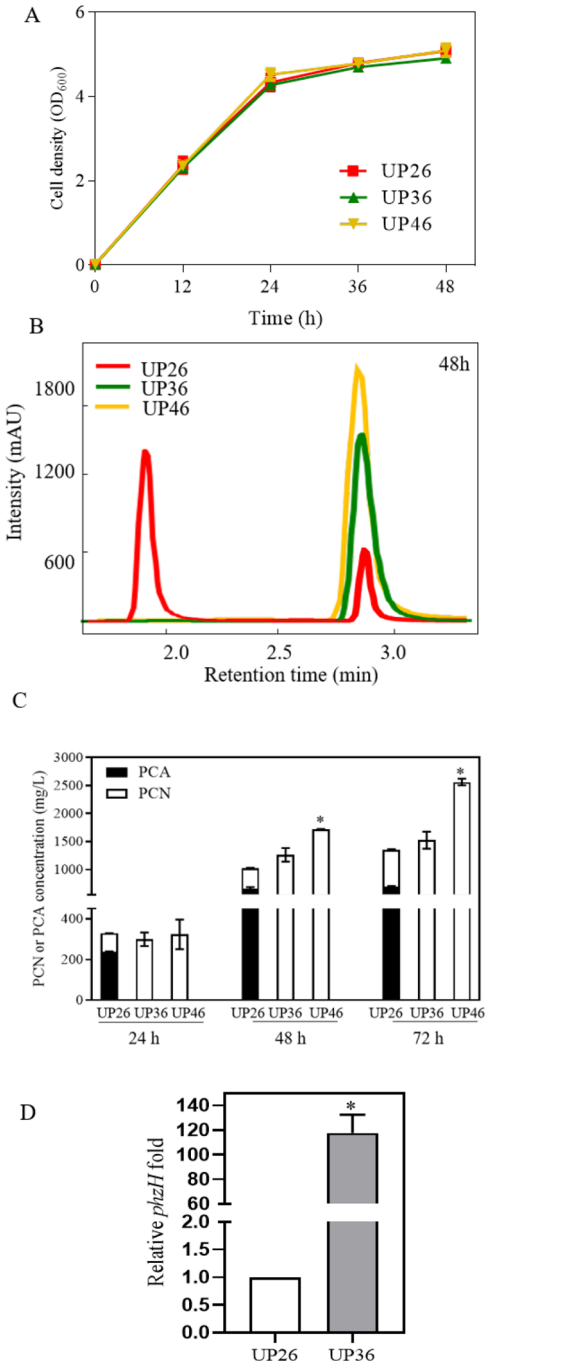

Figure 6. Replacement of P_{phzH} and P_{phz2} by P_{rhlI} in UP26 significantly increased PCN production. (A) Growth time curves of three strains, UP26, UP36, and UP46 in PPM cultures. (B) HPLC analysis of PCA and PCN production of three strains fermented at 48 hpi. (C) Quantitative analysis of PCA and PCN production of the three strains at 24 hpi, 48 hpi, and 72 hpi. (D) Relative *phzH* fold in strains UP26 and UP36. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to the strain UP26 are indicated by one or two asterisks ($p \leq 0.05$).

many clinically isolated *P. aeruginosa* strains grow well at 37 °C or higher.[37] In this study, the P_{rhlI}-dependent reporter strain UP46::P_{rhlI}-*lacZ* was generated and grown at 28 °C, 32 °C, 37 °C, and 42 °C in PPM medium. No statistically significant difference in bacterial growth was observed in the reporter strain at 28, 32, and 37 °C (Figure 7A). The P_{rhlI}-dependent *β*-galactosidase activity of the strain UP46::P_{rhlI}-*lacZ* at 37 °C was the highest in the 24-hpi cultures (Figure 7B). At different growth points, the *β*-galactosidase activities of the strain UP46::P_{rhlI}-*lacZ* at 37 °C were 55.3%−90.2% higher than the activities observed at 28 °C, respectively (Figure 7A). To further confirm these results, RhlI protein was expressed in *E. coli* BL21 (Figure S5). Polyclonal antibody against purified RhlI protein was generated (Figure S5). Western blot analysis showed that RhlI protein levels at 37 °C were 2.2-fold higher than those at 28 °C at 48 hpi (Figure 7C).

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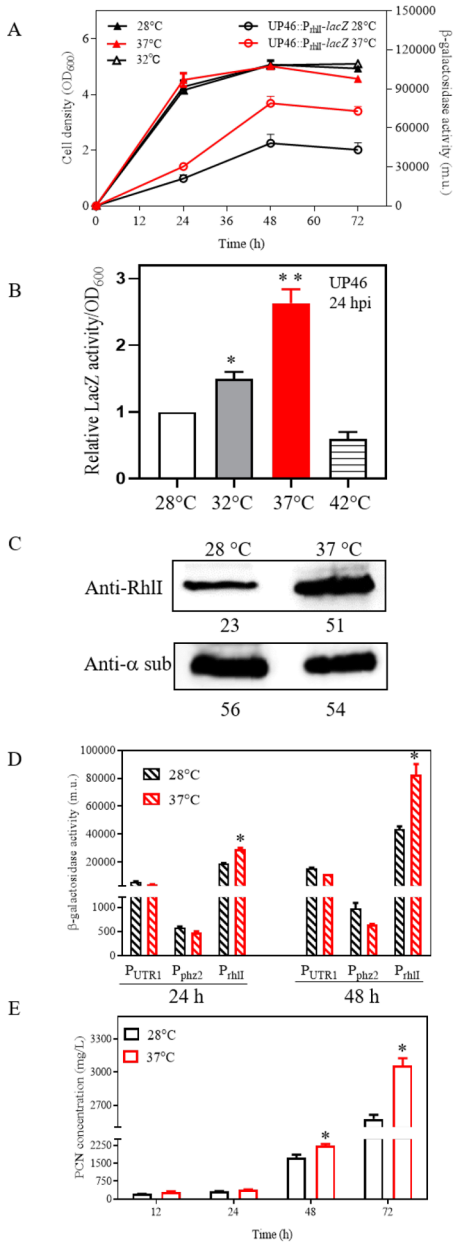

Figure 7. P_{rhlI} is a thermo-regulated promoter. (A) Growth curves of UP46 strain in PPM medium and the relative activity of P_{rhlI} at 28 °C, 32 °C, and 37 °C. (B) Relative LacZ activity per OD_{600} of UP46 at 28 °C, 32 °C, 37 °C, 42 °C at 24 hpi. (C) RhlI protein levels in UP46 strain at 28 and 37 °C. (D) The P_{UTR1}-, P_{phz2}-, and P_{rhlI}-dependent β-galactosidase activity at 28 and 37 °C in PPM medium. (E) PCN production of the strain UP46 in PPM medium at 28 and 37 °C conditions. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to 28 °C are indicated by one asterisk ($p \leq 0.05$).

Using a similar strategy, we also compared the activities of P_{phz2} and P_{UTR} at 28 and 37 °C in a PPM culture using the generated reporter strains UP46::P_{phzH}-*lacZ* and UP46::P_{UTR1}-*lacZ* (Figure 7D). A statistically significant difference was not observed in P_{phz2}- or P_{UTR1}-dependent β-galactosidase activity between 28 and 37 °C (Figure 7D), suggesting that P_{phz2} and P_{UTR} are not thermo-regulated promoters.

PCN Biosynthesis in Strain UP46 Is QS- and Thermo-regulated. We analyzed PCN levels of strain UP46 during growth in PPM medium at 28 and 37 °C. No significant difference was observed in PCN levels at early growth stages (at 12 hpi and 24 hpi) between 28 and 37 °C (Figure. 7E). However, PCN levels at 37 °C during later growth stages (48 hpi and 72 hpi) were 2236 mg/L and 3060.5 mg/L, respectively; these levels were 28.4% and 18.8% higher than those at 28 °C (1741 mg/L at 48 hpi and 2577 mg/L at 72 hpi) at the same time points, respectively (Figure 7E).

Modification of Medium Composition Significantly Increased PCN Production. PPM used in this study was originally designed to promote PYO production in clinically isolated *Pseudomonas aeruginosa* strains.[30] PPM is composed of 22 g/L Tryptone, 20 g/L glucose, and 5 g/L KNO_3. Since Tryptone and glucose are the major carbon sources for bacterial growth, the optimal concentration of tryptone or glucose for PCN production in strain UP46 was first investigated in this study (Figure S6). The results showed that the addition of 22 g/L tryptone or 20 g/L glucose resulted in the highest PCN level in PPM medium; a reduction in the tryptone or glucose concentration significantly decreased PCN production (Figure S6). Because glutamine is essential for the conversion of PCA into PCN,[23] we subsequently investigated the effects of different nitrogen sources on PCN biosynthesis following the addition of KNO_3, glutamine, or $(NH_4)_2SO_4$ at a final concentration of 5 g/L (Figure 8). The results showed that the addition of these nitrogen sources had no significant effect on UP46 growth (Figure 8A). The addition of 5 g/L $(NH_4)_2SO_4$ to PPM medium had an adverse effect on PCN biosynthesis, whereas the addition of 5 g/L KNO_3 or 5 g/L glutamine significantly increased PCN biosynthesis at 48 hpi and 72 hpi (Figure 8B).

Since KNO_3 is industrially available at a lower cost than glutamine, the dosage effect of KNO_3 on PCN biosynthesis was further investigated in UP46 at 37 °C. The results revealed that the growth profile exhibited by UP46 at 37 °C in PPM medium supplemented with 5−10 g/L KNO_3 is different from that at 28 °C: the OD_{600} values of the cell cultures dramatically decreased at 60−72 hpi (Figure 8C). However, the addition of 7.5 to 10 g/L KNO_3 to PPM medium significantly increased PCN biosynthesis at 48 hpi and 72 hpi (Figure 8D). The PCN fermentation titer of strain UP46 in PPM medium supplemented with 10 g/L KNO_3 reached 7306 mg/L at 72 hpi, which is 92.7-fold greater than that of strain PAΔ6 (Figure S1). This medium is referred to as PPN in subsequent analyses.

Furthermore, the optimal pH for PCN production in PPN medium was found to be 7.5 (Figure S7). The bacterial cells were observed to form cell clumps during later growth stages when strain UP46 was grown in 50 mL of PPN medium in a 250 mL flask (Figure S8). Upon growth on PPN agar plates, strain UP46 was observed to grow well secreting large amounts of PCN outside the cell. Indeed, yellow PCN particles were observed to crystallize around the colonies (Figure 9A). After being subcultured for 96 at 24 h intervals in PPN medium, strain UP46 was reinoculated in PPN medium and cultured for 72 h. The PCN level in the culture is comparable to that found in the fresh 72 h culture.

Batch Fermentation in 5 L Fermenter for PCN Production. To generate higher PCN yields, a batch fermentation with strain UP46 was performed in a 5 L fermenter containing 3.5 L of PPN liquid medium. Bacterial growth reached the stationary stage at 48 hpi. PCN production increased over time, and the maximum PCN production (14.1 g/L) was observed at 72 hpi (Figure 9C). No clear cell clumps,

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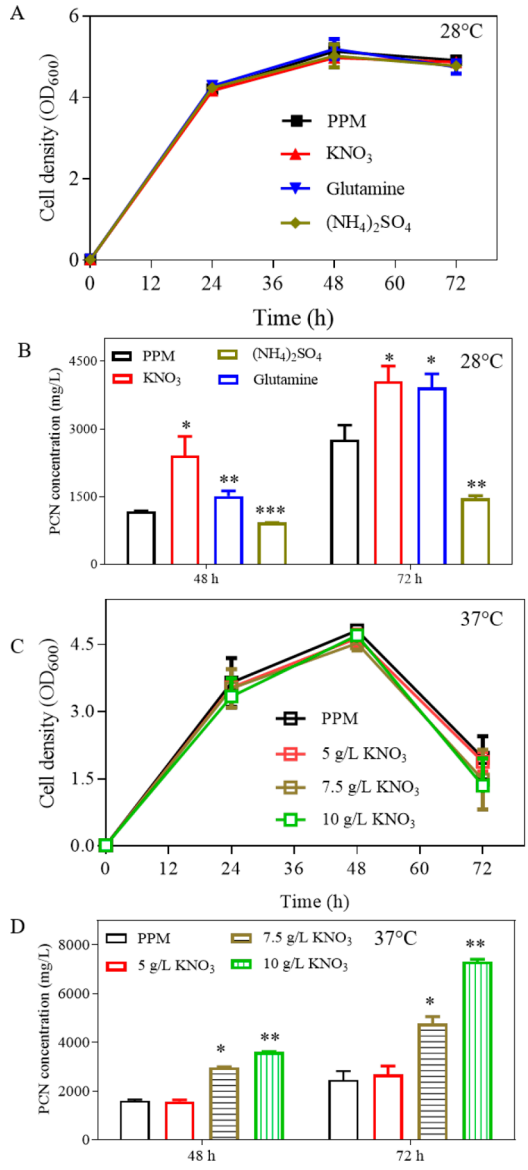

Figure 8. Optimization of nitrogen source significantly increased PCN production in the strain UP46. (A) Growth curves of strain UP46 in PPM medium supplemented with 5 g/L nitrogen sources at 28 °C. (B) PCN production of strain UP46 in PPM medium supplemented with 5 g/L nitrogen sources 48 hpi and 72 hpi. (C) Growth curves of the strain UP46 in PPM medium supplemented with 5−10 g/L KNO_3 at 37 °C. (D) PCN production of the strain UP46 in PPM medium supplemented with 5−10 g/L KNO_3 at 37 °C. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to PPM medium are indicated by one ($p \leq 0.05$) or more asterisks ($p \leq 0.01$).

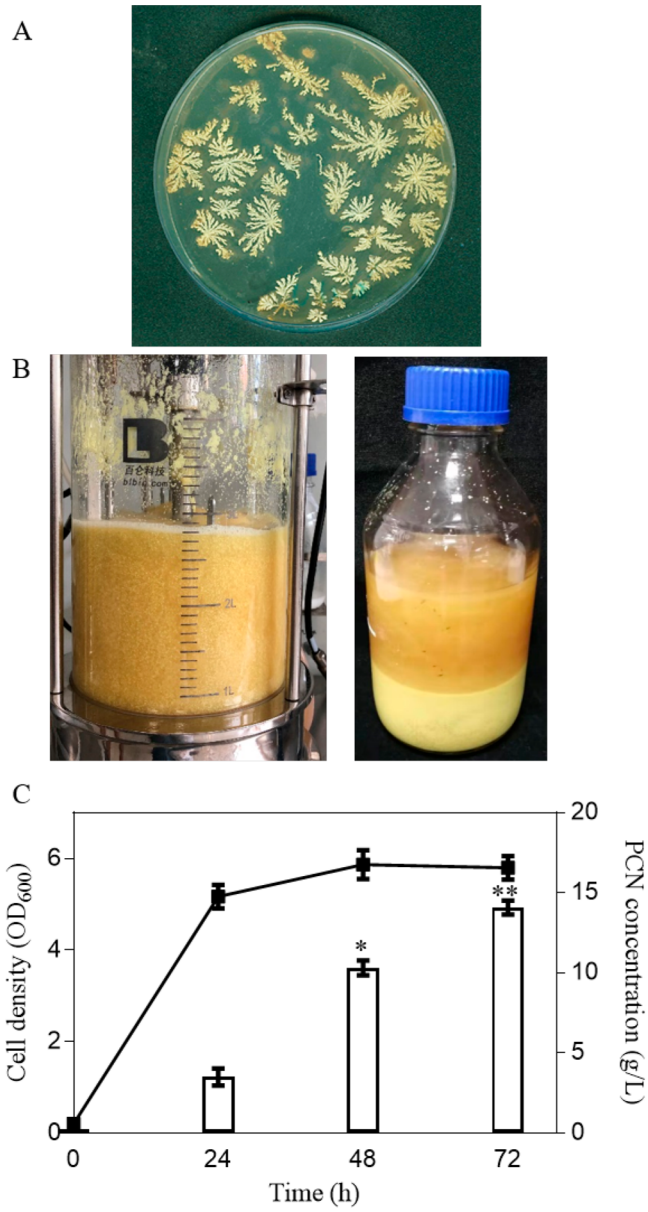

Figure 9. Batch fermentation of the strain UP46 in 5 L fermenter containing 3.5 L of PPN liquid media at 37 °C. (A) Colonies with secreted PCN crystals. (B) Cultures at 5 L fermenter and 500 mL flask at 72 hpi. (C) Cell growth and PCN production during growth at different time points. Shown are averages for three technical repeats with standard deviation. Statistically significant differences with respect to 24 h after inoculation are indicated by one or more asterisks ($p \leq 0.05$).

as shown in Figure S8, were observed during fermentation in the 5 L fermenter; however, PCN precipitate was observed as a deposit at the bottom of the bottle after fermentation (Figure. 9B).

DISCUSSION

As a bacterial secondary metabolite, PCN has long been recognized for its broad-spectrum antibiotic activity in the biological control of a range of fungal phytopathogens.[7,8,25] Moreover, PCN has also been reported as a lead molecule in the development of antitumor drugs, antimicrobial coatings on silicone urethral catheters, and diamide derivatives for selective herbicides.[8,38,39] Chemical synthesis of PCN is technically feasible; however, the yield is low, and toxic byproducts such as aniline, azobenzoate, lead oxide, or o-phenylenediamine are produced.[40,41] Therefore, the biocatalytic synthesis of PCN using engineered microorganisms provides an attractive alternative.

Over the past two decades, a range of PCN-producing *Pseudomonas* strains have been identified.[15,23,24,28] Genetic and metabolic strategies have been used to improve phenazine biosynthesis. For example, Chin-A-Woeng *et al.*[23] expressed *phzH* under the control of the *tac* promoter in the PCA-producing biocontrol strains *P. fluorescens* 2−79 and *P.*

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aureofaciens 30−84 to facilitate the production of PCN instead of PCA. Yao *et al.*[42] generated a PCN-overproducing strain by knocking out three regulatory genes, *lon*, *pars*, and *psrA*, in *P. chlororaphis* HT66. The resultant strain produced 4.10 g/L of PCN in a defined medium. In all these cases, two factors limited PCN industrial production and its application in plant protection. First, the optimal fermentation temperature for PCN biosynthesis is 28 °C. In industry, a fermentation temperature of 28 °C does not favor the large-scale catalytic production of PCN because the temperature inside a fully agitated fermenter is usually above 28 °C and additional cooling systems inevitably increase production costs. Second, PCN fermentation titer remains relatively low in these strains. To address the aforementioned disadvantages, the rice rhizosphere strain PA1201 was used as a starter strain since it has been successfully engineered as an industrial producer of a new metabolite pesticide Shenqinmycin.[21] A strong QS- and thermo-regulated promoter P_{rhlI} was identified as part of the latter genomic promoter analysis. After three rounds of promoter swapping, the optimal fermentation temperature for PCN biosynthesis in the resultant strain UP46 was increased from 28 to 37 °C, and PCN fermentation titer increased 179.5-fold. Thus, this strategy significantly improves PCN production efficiency and reduces the associated production costs, facilitating the feasibility of its application in plant protection.

phzH was initially observed to facilitate the conversion of PCA into PCN in *P. chlororaphis* PCL1391.[23] However, PCN production in the *phzH* deletion mutant PCL1120 could be largely restored following the introduction of the *phzH* gene under the control of P_{tac} or P_{lac}. The authors of the latter study proposed that the incomplete complementation in PCL1120 harboring the *phzH* gene *in trans* may be caused by weak expression of the promoter in this strain.[23] In this current study, following the utilization of the P_{phzH}-*lacZ* fusion reporter strain, we confirmed that P_{phzH} is a weak promoter in PPM medium. The activity of P_{phzH} is only comparable to that of P_{phz1}, and is far lower than that of P_{UTR1} or P_{rhlI} (Figure 4; Figure 5). This is the most likely the reason why PCA could not be completely converted in the PCA-overproducing strains (Figure 1). Further promoter sequence analyses performed during the present study suggest that two DNA motifs within P_{phzH} might contribute to its low activity in PPM culture. One of the latter motifs is the 21-bp space between the −10 and −35 elements of P_{phzH} (Figure 2A). This space is greater than the optimal space of 17 ± 1 bp previously observed for gene expression.[43] Similar long spaces between the −10 and −35 elements have been reported to inhibit promoter activity in *Xanthomonas* and *E. coli* genes.[43,44] It has been proposed that additional transcriptional factors are required to bind to the long spacer sequences. The latter binding reaction either twists or bends the DNA to reorientate the −10 and −35 elements so that they can be bound by the RNAP complex.[44,45] Another motif is the UTR region downstream of the transcription start site T^+ within the P_{phzH} (Figure 2A). UTRs have been shown to assist in the regulation of gene expression by folding into specific secondary structures such as RNA thermometers or riboswitches in bacteria.[46] The role of the long spacer region between the −10 and −35 elements and the UTR region in P_{phzH} deserves further investigation. Nevertheless, although P_{phzH} activity was low in PPM medium, we cannot rule out the possibility that higher *phzH* expression could be induced by certain unknown environmental signals in the rhizosphere of host plants either via a transcription factor or via the UTR region at the post-transcriptional level. Further investigation of the *in vivo* expression of *phzH* in the plant rhizosphere may help to elucidate the true role of *phzH* in the adaptation and evolution of PA1201 to natural conditions.

In *P. aeruginosa*, the *rhlI* gene encodes the enzyme that produces the QS signal C4-HSL, which interacts with RhlR. The RhlR/C4-HSL complex in turn binds to the promoters of the *rhlI* and *phz* operon to promote C4-HSL and PCA biosynthesis in a cell density-dependent manner.[47] In the PCN-overproducing strain UP46, which contains three P_{rhlI} regions, P_{rhlI} activity remains cell density-dependent during growth at both 28 and 37 °C (Figure 7A). PCN production also occurs in a cell density-dependent manner: lower production is observed during the early growth stages and maximal production of PCN occurred at 72 hpi (Figure 7D). This cell density-dependent PCN production profile lends itself to industrial PCN production by promoting expedited bacterial growth during early stages and elevated PCN production during later stages thereby inhibiting high concentrations of PCN building up inside the cells and killing the producing bacteria.

The thermo-regulation of gene expression is common in *P. aeruginosa*. For example, maximal PCA biosynthesis in the sweet melon rhizosphere bacterial strain M18 is only observed at 28 °C. This is because that *phzM* expression is induced at 37 °C, resulting in more PCA being converted into PYO[48] and the PCA biosynthetic gene clusters *phz1* and *phz2* being down-regulated via unknown mechanisms at 37 °C.[35] In addition, extensive RNA thermometers present in the promoters of target genes are also responsible for the thermos-regulation. For example, in *P. aeruginosa* PAO1 or PA14, the production of QS-dependent virulence factors is thermoregulated by diverse RNA thermometers in the promoter regions.[37] In the present study, we showed that the QS-regulated promoter P_{rhlI} is also thermo-regulated (Figure 7). Our preliminary results suggest that the anti-SD structure and a UTR region present within P_{rhlI} are likely responsible for the thermos-regulation of P_{rhlI}. The detailed roles of the two structures in the thermos-regulation of P_{rhlI} are currently under investigation.

Recently, the 5′ UTR within P_{rhlI} was found to encode a sRNA named rhlS, which regulates RhlR-dependent quorum sensing in *P. aeruginosa* strain PAO1.[49] P_{rhlI} in PA1201 contains the same rhlS DNA sequence, and overexpression of the small RNA rhlS in strain UP46 had no effect on PCN biosynthesis. These findings suggest that rhlS is not involved in the regulation of PCN biosynthesis in strain UP46.

CONCLUSIONS

The *Pseudomonas* strain PA1201 produces a low level of PCN through a functional *phzH*-encoding glutamine aminotransferase. A strong quorum sensing- and thermo-regulated promoter P_{rhlI} was identified. After multiple rounds of gene deletion, promoter editing and swapping by P_{rhlI}, a new PCN-overproducing strain UP46 was generated. The optimal fermentation temperature for PCN biosynthesis was increased from 28 to 37 °C and the PCN fermentation titer in UP46 reached 14.1 g/L, the highest ever reported. To further reduce PCN industrial production cost, the PCN yield of strain UP46 might be further enhanced through additional genetic and metabolic engineering as well as medium and process optimization.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00161.

Lists of primers, strains, and plasmids used in this study; additional figures (PDF)

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Author Contributions

H.Y.W., Z.L., and Z.X conceived the experimental design, interpreted the results, and wrote the manuscript. J.Z.J., S.S., and C.Y. performed the experiments. S.K. developed the assay method for promoter swapping.

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Notes

The authors declare no competing financial interest.

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