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Engineering the central biosynthetic and secondary metabolic pathways of *Pseudomonas aeruginosa* strain PA1201 to improve phenazine-1-carboxylic acid production

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ABSTRACT

The secondary metabolite phenazine-1-carboxylic acid (PCA) is an important component of the newly registered biopesticide Shenqinmycin. We used a combined method involving gene, promoter, and protein engineering to modify the central biosynthetic and secondary metabolic pathways in the PCA-producing *Pseudomonas aeruginosa* strain PA1201. The PCA yield of the resulting strain PA-IV was increased 54.6-fold via the following strategies: (1) blocking PCA conversion and enhancing PCA efflux pumping; (2) increasing metabolic flux towards the PCA biosynthetic pathway through the over-production of two DAHP synthases and blocking the synthesis of 21 secondary metabolites; (3) increasing the PCA precursor supply through the engineering of five chorismate-utilizing enzymes; (4) engineering the promoters of two PCA biosynthetic gene clusters. Strain PA-IV produced 9882 mg/L PCA in fed-batch fermenation, which is twice as much as that produced by the current industrial strain. Strain PA-IV was also genetically stable and comparable to *Escherichia coli* in cytotoxicity.

1. Introduction

Phenazines are bacterial, secondary metabolites that have long been recognized for their broad-spectrum antibiotic activity and been widely used in the biological control of a range of fungal phytopathogens (Rane et al., 2007; Pierson and Pierson, 2010; Mavrodi et al., 2013; Thomashow, 2013). In addition, due to potential biotechnological applications in areas such as environmental sensing, microbial fuel cell production, and antitumoractivity, phenazines have also attracted the attention of the scientific community as lead molecules (Pierson and Pierson, 2010). Chemical synthesis of phenazine compounds is technically feasible, however the yield is low (Cheluvappa, 2014), and toxic byproducts such as aniline, azobenzoate, lead oxide, or o-phenylenediamine are produced (Chincholkar et al., 2013). Therefore, the biocatalytic synthesis of phenazines using recombinant microorganisms provides an attractive alternative.

Phenazine-1-carboxylic acid (PCA), one of the major phenazines produced by the fluorescent pseudomonads, was commercially named as Shenqinmycin. 1% Shenqinmycin suspension was registered as a new biopesticide to prevent rice sheath blight, pepper blight and cucumber seedling damping-off in 2011 (Xu, 2013, Fig. S1). PCA was produced by catalytic fermentation with *Pseudomonas aeruginosa* M18 (Hu et al., 2005), a strain which was originally isolated from the melon rhizosphere, and subsequently engineered to improve PCA production. However, due to the low industrial fermentation titer of PCA, the production costs of the biopesticide (1% Shenqinmycin suspension) are much higher than other chemical pesticides, limiting the feasibility of its application. (Fig. 1).

The gene cluster *phzABCDEFG* (*phzA-G* for short) is responsible for phenazine biosynthesis in all phenazine-producing pseudomonads (Blankenfeldt, 2013). A comparison of *phz* clusters from different species reveals that five enzymes, *phzB*, *phzD*, *phzE*, *phzF*, and *phzG*, are conserved among all phenazine-producing bacteria. These enzymes convert chorismate, the end product of the shikimate pathway, into PCA (Mentel et al., 2009; Blankenfeldt, 2013). In most phenazine-producing bacteria, these core biosynthetic genes are flanked by one or more accessory genes, *phzO*, *phzH*, *phzM* and *phzS* (Chin-A-Woeng et al., 2001; Wu et al., 2011). These genes encode different terminal-modifying enzymes that result in the production of the additional phenazine derivatives 2-hydroxyphenazine (2OH–PCA), phenazine-1-carboamide (PCN), and pyocyanin (PYO) (Chin-A-Woeng et al., 2001; Greenhagen et al., 2008).

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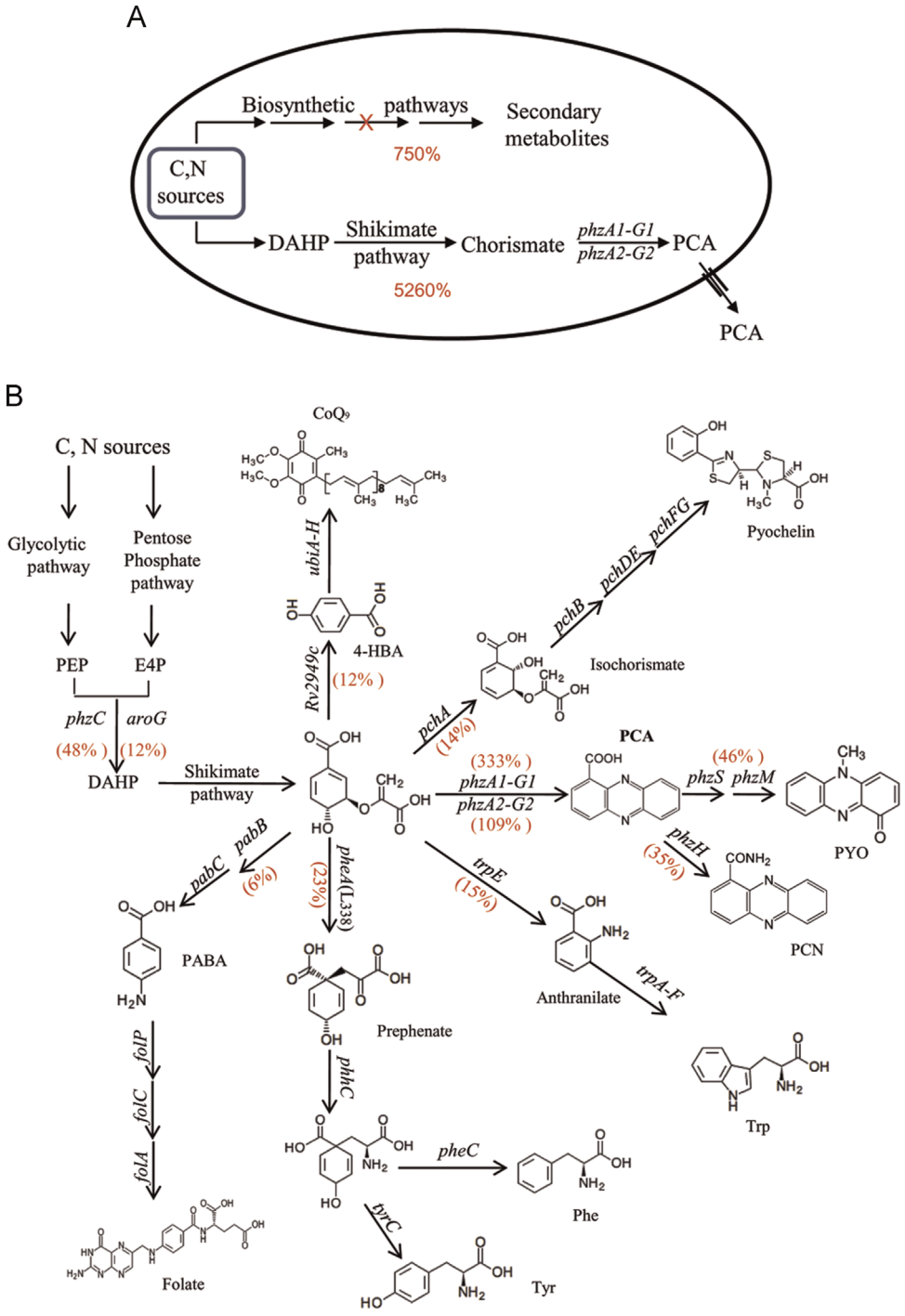

Fig. 1. The general strategies used in this study to improve PCA production in strain PA1201. (A) PCA competes with other secondary metabolites for carbon and nitrogen sources. Deletion of 21 secondary metabolite biosynthetic pathways resulted in a 750% increase in PCA production, and engineering the PCA biosynthetic system resulted in a 5260% increase in PCA production. (B) PCA biosynthetic pathway genes targeted for engineering in this study. Percentages indicate the increase in PCA production after gene engineering. DAHP: 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. PCA: phenazine-1-carboxylic acid.

Several strategies have been used to increase the fermentative production of PCA in *P. aeruginosa* strain M18. First, factorial design and response surface methodology were applied to screen for critical media components and to determine optimal culture conditions in SCM medium (Zhou et al., 2010). Second, several regulatory genes were genetically modified to improve PCA production. The *gacA*-knock-out strain M18G produced 600 mg/L of PCA in King's medium B (KMB) (Ge et al., 2004). The *gacA* and *qscR* double mutant strain M18GQ produced 4,000 mg/L of PCA under optimal culture conditions. Recently, strain M18UMS was constructed by deleting the 5′-UTRs of *phzA1-G1* and the two genes *phzM*, *phzS* in strain M18 (Du et al., 2013). After optimizing growth conditions and medium composition by a central composite design, a maximum PCA yield of 4771 mg/L was observed (Du et al., 2013).

PA1201 is a *Pseudomonas aeruginosa* strain originally isolated from the rice rhizosphere which displays strong inhibitory activity towards the rice pathogens *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* (Zhou et al., 2015). The PCA production of strain PA1201 in PPM medium was about 2-fold that of strain M18 (Zhou

et al., 2015). Similar to the rhizobacterium M18 and several clinically isolated *P. aeruginosa* strains, PA1201 was toxic to both human cell lines and *Drosophila melanogaster* (Zhou et al., 2015). Thus, the goal of this work was to generate a safe, efficient, PCA-producing strain. To achieve this, various genetic engineering methods were applied to modify virulence factors, secondary metabolism and the central PCA biosynthetic pathway in strain PA1201. A genetically stable strain PA-IV was obtained which exhibits a 54.6-fold PCA yield increase over the wild type. Strain PA-IV was comparable to *E. coli* in cytotoxicity and produced 9882 mg/L of PCA with a yield of 3514.6 mg/g DCW in fed-batch fermentation.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains and growth media used in this study are described in Tables S1 and S3, respectively. *E. coli* strains were grown aerobically at 37 °C in LB medium. For PCA production, single colonies were picked from petri plates and used to inoculate 4 mL of PPM (with 100 mg/L spectinomycin, Sp) in 50 mL-flasks. Cultures were then grown at 28 °C with 200 rpm of shaking overnight. Portions of these cultures were then inoculated into 250 mL flasks containing 50 mL ppm or SCM with 100 mg/L Sp to achieve an initial OD_{600}=0.05. After 24–72 h of growth at 28 °C and 250 rpm. Cultures were then collected for the measurement of PCA production and OD_{600}.

2.2. P. aeruginosa cytotoxicity assay and oral infection of Drosophila

The cytotoxicity of PA1201 strains towards the human airway epithelial cell line A549 was measured as described previously (Wang et al., 2013). After a 4 h infection at 37 °C, cell viability was determined by using a Cytotoxicity Detection Kit (LDH), following the manufacturer's instructions (Roche). *Drosophila melanogaster* was maintained in glass vials (90 mm × 25 mm) containing DM medium. Oral infections were performed as described previously (Korgaonkar et al., 2013).

2.3. Two-step recombination method for in-frame marker-less gene deletion in PA1201

The method for marker-less gene deletion was previously described (He et al., 2006) and is summarized in Fig. S2. Briefly, the downstream and upstream regions of the target gene to be deleted are combined by using overlap extension PCR. The fusion products were further cloned into the suicide vector pEX18Gm carrying sucrose-sensitive *sacB* gene. The resulting chimeric plasmid is integrated within the target sequence via homologous recombination and is then excised by a second single-crossover homologous recombination event, resulting in allelic exchange. The resulting mutants were verified by PCR and DNA sequencing.

2.4. Integration of the fusion gene P_{RPO}-phzC into PA1201 chromosome

phzC driven by the P_{RPO} promoter was integrated into PA1201 using the Mini-Tn7 transposon system (Choi and Schweizer, 2006). Briefly, promoter P_{RPO} and *phzC* were separately amplified by PCR using the primers listed in Table S2. The fused P_{RPO}-*phzC* fragment was obtained by fusion PCR and inserted into the mini-Tn7T-Gm vector. The mini-Tn7 construct along with the helper plasmid pTNS2 encoding the Tn7 site-specific transposase genes were delivered into PA1201 by electroporation. Insertions at *att*Tn7 were selected with gentamycin (Gm) and further confirmed by colony PCR using the specific primers listed in Table S2. Removal of the Gm selection marker was achieved by Flp-mediated excision. The unmarked sucrose-resistant colonies were confirmed by PCR and DNA sequencing.

2.5. Promoter replacement in PA1201 chromosome

Promoters P_{RPO}, P_{UTR}, and flanking DNA fragments L and R (~500 bps) of *phzA2-G2*, *mexGHI-opmD* and *aroG* promoters were separately amplified by PCR using the primers in Table S2. DNA fragments containing P_{RPO} or P_{UTR} flanked by fragments L and R were obtained via 2 rounds of fusion PCR amplification. The fused DNA fragments were then cloned into the suicide vector pEX18Gm. The recombinant constructs were subsequently mobilized into strain PA1201 by conjugation. Transconjugants were selected on LB medium supplemented with Sp (100 μg/mL) and Gm (20 μg/mL). Colonies were then resuspended in PBS buffer and plated on LB medium containing 5% (w/v) sucrose and Sp (100 μg/mL) to select for a second cross-over event. The strains with replaced promoters were further verified by PCR and DNA sequencing.

2.6. Replacement of ubiC by Rv2949c in the chromosome

Rv2949c was amplified by PCR using *Mycobacterium tuberculosis* genomic DNA as template and the primers listed in Table S2. The flanking DNA fragments L2 and R2 of target gene *ubiC* of PA1201 were amplified by PCR using the primers listed in Table S2. The DNA fragment containing *Rv2949c* flanked by fragments L2 and R2 was obtained via 2 rounds of fusion PCR and cloned into the suicide vector pEX18Gm. The conjugation, selection and verification procedures were described in Section 2.5.

2.7. Generating a point mutation in W^{323} of PheA in the chromosome

The gene *pheA* was amplified by PCR and cloned into T vector pTA2 (Toyobo, Japan). Mutation was conducted using QuickChange®Site-Directed Mutagenesis Kit following the manufacturer's instructions. After verification by DNA sequencing, the mutated *pheA* was then cloned into the suicide vector pEX18Gm. The conjugation, selection and verification procedures were as described in Section 2.5.

2.8. Western blotting analysis of phzC

Western blotting was performed as described (Green and Sambrook, 2012). The bound His_6-*PhzC* protein was used as an antigen to obtain polyclonal antisera by immunizing rabbits through subcutaneous injections at 2-week intervals. The hybridization signal was detected using SuperSignal®West Pico Chemiluminescent Substrate (PIERCE, USA).

2.9. Measurement of PCA production and yield

In order to extract PCA, 180 μl of fermentation culture was mixed with 20 μl of 6 M HCl and then extracted with 540 μl chloroform as previously described (Ge et al., 2004). A three microliter aliquot of extracted PCA sample was then taken for HPLC analysis (Agilent Technologies 1260 Infinity) under the following conditions: C18 reversed-phase column (5 μm, 4.6 × 12.5 mm^2) eluted with acetonitrile −5 mM ammonium acetate (60:40, v/v). PCA production was quantified using peak area (A) in HPLC elute according to the following formula: PCA (mg/L)=0.0146A−0.341, which was derived from a dose–peak area plot using purified PCA with a correlation coefficiency (R^2) of 0.999. PYO production was quantified as

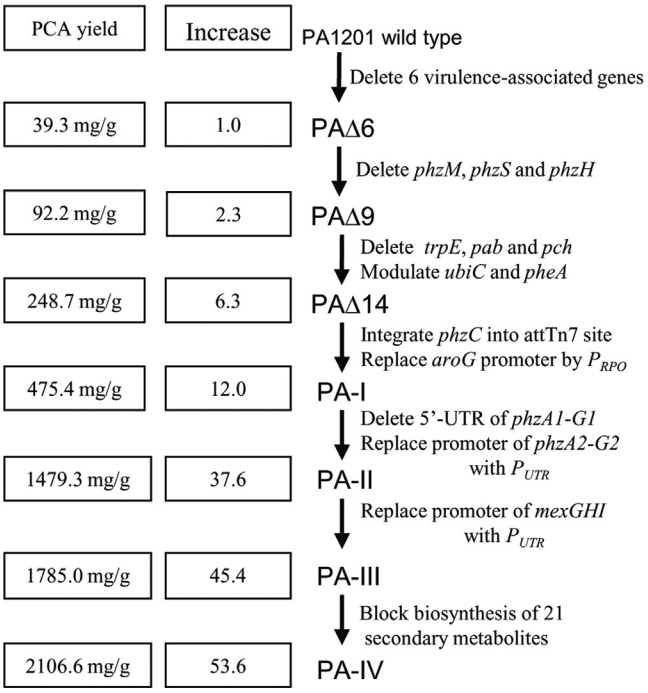

Fig. 2. A summary of the steps in the genetic and metabolic engineering of PA1201 for PCA production.

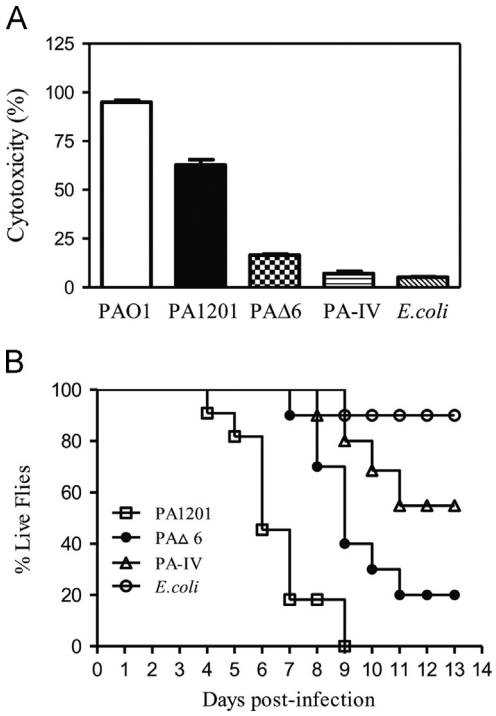

Fig. 3. Cytotoxicity and virulence assay of PA1201-derived strains. (A) The human cell line A549 cytotoxicity assay. (B)Virulence assay based on the *Drosophila-P. aeruginosa* oral infection method. PAΔ6: PA1201 strain carrying deletions of 6 virulence factor genes; *P. aeruginosa* PAO1 and *E. coli* were used as positive and negative controls.

previously described (Essar et al., 1990). Dry cell weight (DCW) in PPM medium was calculated from the optical density at 600 nm (1 OD_{600}=0.4971 g DCW L^{-1}). To determine the OD_{600} of bacterial culture in SCM medium, 100 µl of culture was sampled and diluted in 900 µl of PBS buffer. 1/10 SCM medium was used as blank for OD_{600} determination. DCW in SCM medium was calculated from the optical density at 600 nm (1 OD_{600}=0.4535 g DCW L^{-1}). The results represent the means ± S.D. of three independent experiments.

2.10. Fed-batch fermentation of strain PA-IV

PPM medium was used for seed culture preparation. This was prepared by inoculating 2 colonies into a 250 mL flask containing 50 mL PPM medium, and incubating at 28 °C and 250 rpm for 12 h. The seed culture was then transferred to a 5 L fermentor (Sartrius & BIOSTAT®Aplus) containing 2.5 L PCM medium (Table S3) with an initial OD_{600} of 0.05. Fermentation was carried out at 28 °C with an air flow rate of 3 L/min. The dissolved oxygen was kept at 20–30% by adjusting the agitation speed from 300 rpm to 600 rpm. The pH was maintained at 7.5 by automatic addition of 5 M ammonia hydroxide. A solution containing (per liter) 30.0 g soybean meal, 7.5 g corn steep liquor, 12.0 g glucose and 21.7 mL ethanol was fed to the fermentor during the first 4 days with a feed rate of 10 mL/h. Antifoam (Sigma) was added automatically as needed. (Fig. 2)

3. Results

3.1. Genetic engineering to reduce cytotoxicity and virulence of strain PA1201

The virulence factors of *P. aeruginosa* have been well studied in the model strains PAO1 and PA14 (Veesenmeyer et al., 2009). In this study, the genes encoding global activator of the type III secretion system (ExsA), nonpilus adhesin, exotoxin A, and pilus formation were deleted in strain PA1201 (Fig. S3). The resulting strain PAΔ6 displayed similar growth patterns and PCA yields as strain PA1201 (data not shown), and its cytotoxicity to human cell line A549 was one fifth that of strain PA1201 (Fig. 3A). Using the *Drosophila-P. aeruginosa* oral infection model, we showed that strain PAΔ6 was significantly less virulent than strain PA1201 (Fig. 3B).

3.2. Blocking PCA conversion pathways to improve PCA production

PA1201 contains functional *phzM*, *phzS* and *phzH* genes which are responsible for converting PCA into PYO and PCN in the late log phase of bacterial growth (Zhou et al., 2015). To prevent PCA conversion, *phzS* and *phzM* were deleted from strain PAΔ6, and the resulting strain PAΔ8 failed to produce PYO (Fig. 4A). As expected, strain PAΔ8 produced 57% higher PCA than strain PAΔ6 (Fig. 4B). *phzH* was further deleted from strain PAΔ8 and the resulting strain PAΔ9 produced no PCN (Fig. 4A). Strain PAΔ9 produced 188 mg/L PCA with a yield of 39.3 mg/g DCW in PPM shake culture after 72 h (Fig. 4B), and this was a yield increase of about 2.3-fold over the parent strain PA1201 (Fig. 2).

3.3. Engineering chorismate-utilizing pathways to improve PCA production

PA1201 synthesizes PCA with the enzymes encoded by the *phz* cluster using chorismate as precursor. This strain also contains five additional chorismate-utilizing pathways for synthesis of folate, pyochelin, tryptophan, tyrosine, phenylalanine, and co-enzyme Q (Fig. 1B). These pathways compete with the PCA biosynthetic pathway for the central intermediate precursor, chorismate. During fermentation, bacteria do not need to folate or pyochelin. They can utilize amino acids and CoQ from the growth medium, or use alternative pathways to synthesize them. Therefore, these chorismate-utilizing pathways can be partially or fully blocked. Thus, *pabB/pabC* encoding para-aminobenzoate synthase, *trpE*

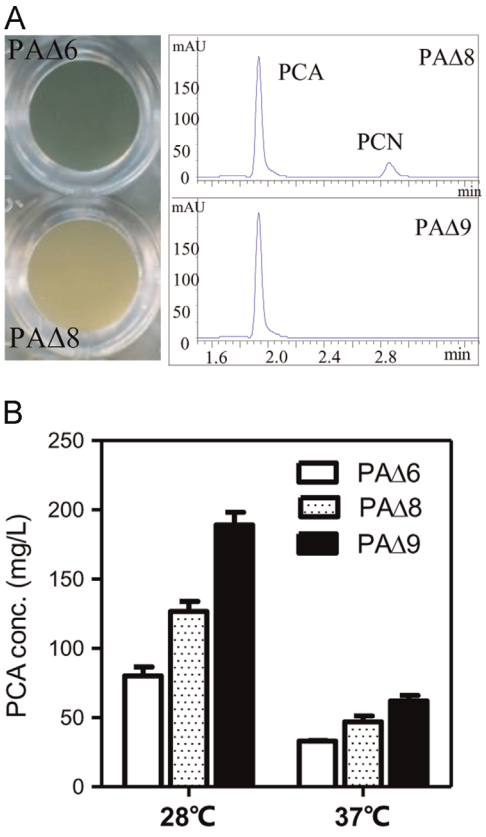

Fig. 4. Deletion of *phzM* and *phzS* (PAΔ8), or *phzM*, *phzS* and *phzH* (PAΔ9) increased PCA production. (A) PYO and PCN production of deletion strains. Left: cell cultures at microplate wells. The blue color indicates pyocyanin (PYO) production. Right: the HPLC chromatograms of PCA and phenazine-1-carboxyamine (PCN). (B) Quantitative analysis of PCA production of deletion strains at 28 °C and 37 °C in PPM medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

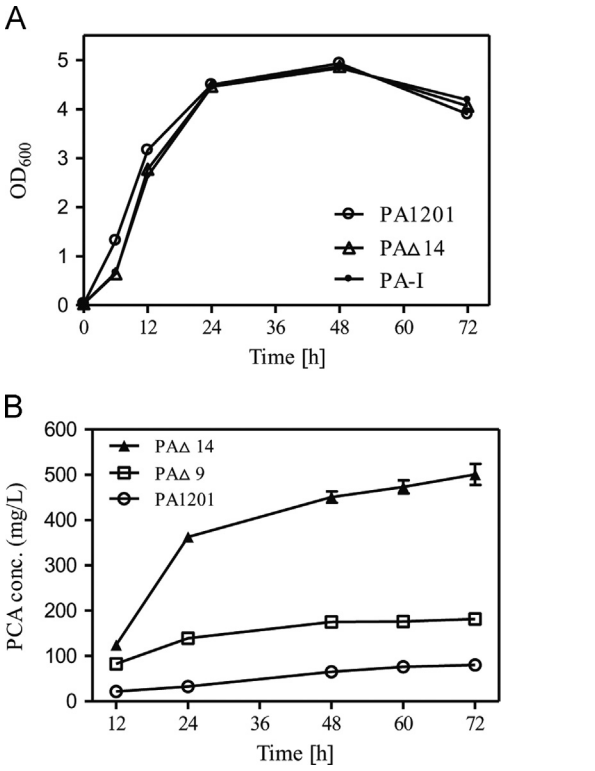

Fig. 5. PCA production of strains with engineered genes for chorismate-utilizing enzymes. (A) Growth time course of parent (PA1201) and engineered strains. (B) PCA production in PPM medium. PAΔ14: PAΔ9 strain carrying *Rv2949c*, *pheA*^{L323} and deletions of *pabB/C*, *trpE* and *pchA*. Three replications were performed for each strain, and error bars indicate the standard deviations.

encoding anthranilate synthase, and *pchA* were all deleted from PA1201. The resulting strains Δ*pabB/C*, Δ*trpE*, or Δ*pchA* produced 6%, 15%, or 14% higher PCA respectively than strain PA1201 in PPM medium (Fig. 1B). Deletion of these 3 genes had little effect on bacterial growth.

pheA encodes a bifunctional chorismate mutase/prephenate dehydratase. Point mutation of the tryptophan residue W^{338} to L^{338} resulted in a partial loss of enzymatic activity in *E. coli* (Zhang et al., 2000). PA1201 PheA contains the same residue (W^{323}) (Fig. S4). Mutation of W^{323} into L^{323} in the strain PA1201 resulted in a 23% increase in PCA production (Fig. 1B). Point mutation of W^{323} had little effect on bacterial growth.

ubiC encodes a chorismate pyruvate lyase which converts chorismate to 4-HBA in *P. aeruginosa*. *Mycobacterium tuberculosis* protein Rv2949c is a new chorismate pyruvate lyase with lower enzymatic activity (Stadthagen et al., 2005). We replaced *ubiC* in the PA1201 chromosome with *Rv2949c*, and although this had little effect on bacterial growth, it boosted PCA production in PPM medium by 12% (Fig. 1B).

All of the above 5 modifications (Δ*pabB/C*, Δ*trpE*, Δ*pchA*, *pheA*-L^{323}, *Rv2949c*) were subsequently combined in the same strain PAΔ9. The resulting strain PAΔ14 grew a little slower than strain PAΔ9 at 6 h and 12 h, and then reached similar OD_{600} as strain PA1201 at 24 h in PPM medium (Fig. 5A). The strain PAΔ14 produced 507 mg/L PCA with a yield of 248.7 mg/g DCW after 72 h in PPM medium (Fig. 5B), and this was an approximately 6.3-fold increase in yield over the parent strain PA1201 (Fig. 2).

3.4. Overexpressing two DAHP synthase genes to improve PCA production

The shikimate pathway links carbohydrate metabolism to the biosynthesis of PCA and other aromatic compounds. The first step of this pathway is the condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) to yield 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) by DAHP synthase. The biological function of DAHP synthase is to regulate the metabolic flux towards the shikimate pathway, and therefore it is one of the key branch points in the shikimate pathway (Herrmann and Weaver, 1999). PA1201 contains two type-I DAHP synthase genes, one type-II DAHP synthase, and two almost identical genes *phzC1* and *phzC2* which encode putative type-II DAHP synthase (Fig. S5). Our preliminary results showed that overexpression of *aroG* or *phzC1* via the plasmid pBBR significantly increased PCA production in PA1201.

We identified a high strength promoter called P_{RPO} by RNA-Seq analysis and subsequent lacZ fusion experiment in PA1201 (Fig. S6A). This promoter was used to replace plasmid-based gene overexpression. First, the coding region of *phzC1* driven under P_{RPO} was integrated into the *att*Tn*7* site in the chromosome of strain PAΔ14 (Fig. S7A). The resulting strain PAΔ14::*phzC* produced 782 mg/L PCA at 72 h (Fig. 6), and this was 54.2% higher than strain PAΔ14. Second, the original promoter of *aroG* was replaced by promoter P_{RPO} (Fig. S7B) and the resulting strain PAΔ14:*aroG* produced 650 mg/L PCA in PPM medium at 72 h (Fig. 6), 28.2% more than PAΔ14. Both modifications were combined in strain PAΔ14 and the resulting strain PA-I produced 969 mg/L PCA with a yield of 475.4 mg/g DCW (Fig. 6), which was a 12-fold increase over the parent strain PA1201 (Fig. 2).

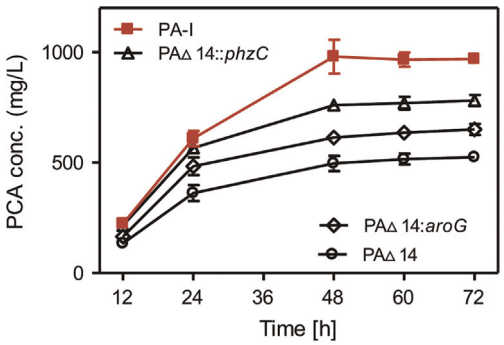

Fig. 6. PCA production of strains overexpressing *phzC1* and/or *aroG*. PAΔ14:*aroG*: PAΔ14 with *aroG* driven by promoter P_{RPO}. PA-I: PAΔ14 carrying *aroG* driven by promoter P_{RPO}, and *phzC* driven by promoter P_{RPO}. Three replications were performed for each strain, and error bars are standard deviations.

3.5. Engineering the promoters of two PCA biosynthetic gene clusters to improve PCA production

Almost all *P. aeruginosa* strains contain two nearly identical PCA biosynthetic gene clusters *phzA1-G1* and *phzA2-G2*, each with unique promoter and flanking sequences (Recinos et al., 2012). In *P. aeruginosa* strains PA14 and M18, *phzA1-G1* was found to be expressed at a higher level than *phzA2-G2* although the latter showed a greater contribution to phenazine production (Li et al., 2011; Recinos et al., 2012). Also in strain M18 a 5′-untranslated region (5′-UTR) in the promoter of *phzA1-G1* was found to negatively regulate post-transcriptional expression (Li et al., 2011; Du et al., 2014).

In this study, the 5′-UTR within the promoter of the gene cluster *phzA1-G1* was deleted and the resulting truncated promoter was named P_{UTR} (Figs. S6B and S7C). Strain PA1201 with the truncated promoter P_{UTR} was named as PAΔUTR. Strain PAΔUTR produced 351 mg/L PCA, 333% higher than its parent strain PA1201 (Fig. 1B). The resulting strain PA-IΔUTR produced 2468 mg/L PCA, 155% higher than its parent strain PA-I (Fig. 7B). The original promoter of *phzA2-G2* was replaced by promoter P_{UTR} in strains PA1201 and PA-I (Fig. S7D). The resulting strain PA:pUTR produced 169 mg/L PCA, 109% higher than its parent strain PA1201 (Fig. 1B). The resulting PA-I:P_{UTR} produced 1684 mg/L PCA, 73% higher than its parent strain PA-I (Fig. 7B). Deleting the 5′-UTR of the *phzA1-G1* promoter, or replacing the *phzA2-G2* promoter by promoter P_{UTR} had little effect on bacterial growth in PPM medium (Fig. 7A). Both modifications were combined in strain PA-I and the resulting strain PA-II produced 3015 mg/L PCA in PPM medium with a yield of 1479.3 mg/g DCW (Fig. 7B), which was a 37.6-fold increase over the parent strain PA1201 (Fig. 2). Using *phzC* as a representative of *phzA-G*, western blotting analysis confirmed that promoter engineering significantly increased the level of PCA biosynthetic enzymes (Fig. 7C).

3.6. Engineering the efflux pump to improve PCA production

The *mexGHI-opmD* gene cluster is adjacent to *phzA1-G1*, and its products MexH, MexI, and OpmD are highly conserved in relation to other components of *P. aeruginosa* antibiotic efflux pumps (Poole, 2002). Previously, the production of PYO was drastically reduced in the *mexI* and *opmD* mutants of PAO1 (Aendekerk et al., 2005), suggesting that the *mexGHI-opmD* gene products function as a PCA efflux pump.

Our preliminary results showed that overexpressing *mexGHI-opmD* via the expression vector pBBR-1-MCS significantly increased PCA production in strain PA1201. In this study, the original promoter of *mexGHI-opmD* was replaced by the highly

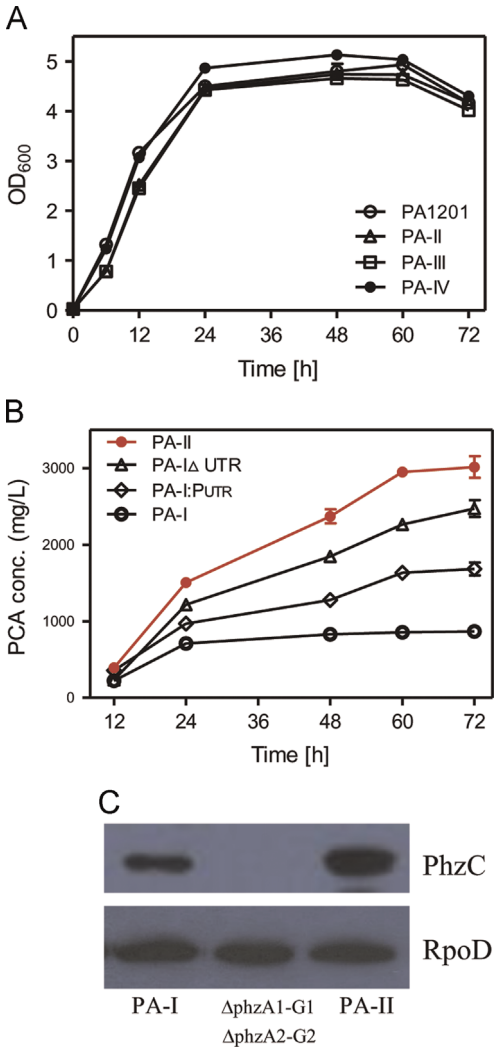

Fig. 7. Engineering the *phzA1-G1* and *phzA2-G2* promoters increased PCA production. (A) Growth time course of parent and engineered strains. (B) PCA production of PA-I-derived strains in PPM medium. (C) Western blotting analysis of *phzC* as an indicator of *phzA1-G1* and *phzA2-G2* expression. RpoD is used as an internal control unaffected by promoter engineering. PA-II: strain PA-I carrying two *phzA-G* clusters with engineered promoters. Three replications were performed with each strain, and the error bars indicate standard deviations.

expressed promoter P_{UTR} in strain PA1201 (Fig. S7E). The resulting strain PA:*mex* produced 315 mg/L PCA in PPM medium at 72 h, about 293% higher than strain PA1201 (Fig. 8A). The same modification was conducted in strain PA-II and the resulting strain PA-III produced 3638 mg/L PCA with a yield of 1785.0 mg/g DCW in PPM medium after 72 h (Fig. 8B), which was a 45.4-fold increase over the parent strain PA1201 (Fig. 2). Promoter replacement had little effect on bacterial growth (Fig. 7A).

3.7. Engineering secondary metabolism to improve PCA production

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Using the program antiSMASH (http://antismash.secondarymetabolites.org/), a total of 32 gene clusters were predicted to be responsible for secondary metabolite biosynthesis in PA1201. These gene clusters were individually deleted and the resulting mutants were evaluated for bacterial growth and PCA production. Our results showed that individual deletion of any of the

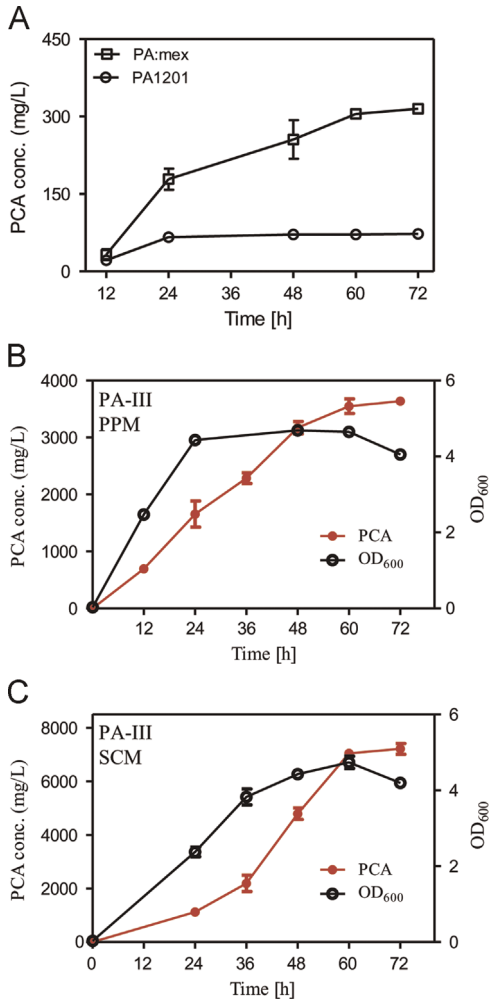

Fig. 8. Promoter engineering to increase the expression of *mexGHI-opmD*, which encode a PCA efflux system. (A) PCA production of strain PA:mex (PA1201 over-expressing *mexGHI-opmD* by P_{UTR} promoter) in PPM medium. (B) Growth and PCA production of strain PA-III in PPM medium. (C) Growth and PCA production of PA-III (PA-II overexpressing *mexGHI-opmD* by the P_{UTR} promoter) in SCM medium. Three replications were performed for each strain, and the error bars indicate standard deviation.

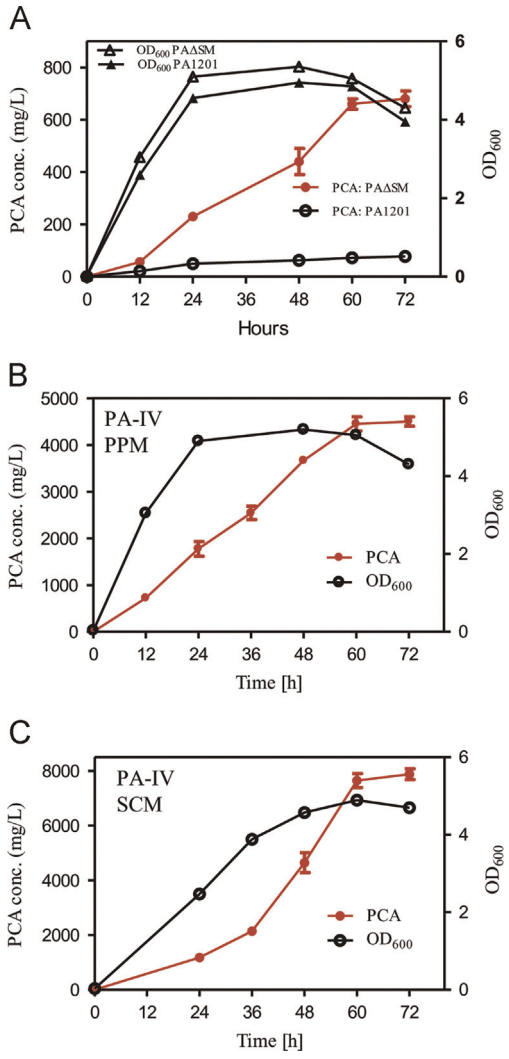

Fig. 9. The effects of altering secondary metabolism on PCA production. (A) PCA production of strains PA1201 and PAΔSM (PA1201 carrying deletions of 21 gene clusters). (B) Growth and PCA production of strain PA-IV (PA-III carrying deletions of 21 gene clusters) in PPM medium. (C) Growth and PCA production of strain PA-IV in SCM medium. Three replications were performed with each strain, and the error bars indicate standard deviations.

21 gene clusters had little effect on bacterial growth (Fig. S8A). Single deletion of *pch*, *hcn* or three other putative clusters led to increased PCA production ranging from 12.5% to 56.2% compared to strain PA1201 (Fig. S8B). Thus, all 21 gene clusters were deleted in strain PA1201, and the resulting strain PAΔ*SM* produced 680 mg/L PCA, 750% higher than strain PA1201 (Figs. 9A and 1A). These gene clusters were subsequently deleted in strain PA-III and the resulting strain PA-IV displayed slightly faster growth than strain PA-III during bacterial growth (Fig. 7A). Strain PA-IV produced 4503 mg/L PCA with a yield of 2106.6 g/mg DCW in PPM medium after 72 h (Fig. 9B), a 53.6-fold increase over strain PA1201 (Fig. 2).

The cytotoxicity of strain PA-IV to human cell line A549 was about half that of strain PAΔ6 and was comparable to that of *E. coli* (Fig. 3A). The survival rate of strain PA-IV-fed flies was also significantly higher than that of strain PAΔ6 (Fig. 3B). After being subcultured for 192 h at 48 h intervals in PPM medium supplemented with Sp (100 μg/mL), strain PA-IV was re-inoculated in PPM medium and cultured for 72 h. PCA at 4491 mg/L was detected in the culture (Fig. S9). This is comparable to the PCA level found in fresh 72 h culture (4503 mg/L).

3.8. PCA production of PA1201-derived strains in SCM medium

Medium SCM, containing soybean meal, corn steep liquor and ethanol as nitrogen and carbon sources, was optimized for PCA yield in shake flask culture of *P. aeruginosa* strain M18 (Zhou et al., 2010). Medium SCM was also used for industrial production of PCA. However, SCM medium is dark and thick, making it inconvenient for bacterial growth determination during fermentation. This is why PPM medium was used to evaluate bacterial growth and PCA production in this study. To further evaluate the application potential of the strains generated in this study, bacterial growth and PCA production was further investigated in shake flask SCM culture at 28 °C. The PCA production of strains PA1201, PA-I, PA-II, PA-III and PA-IV was 317 mg/L, 2261 mg/L, 6429 mg/L, 7211 mg/L and 7880 mg/L after 72 h with yields of 195.6 mg/g DCW, 1060.7 mg/g DCW, 3016.2 mg/g DCW, 3383.1 mg/g DCW and 3677.3 mg/g DCW, respectively (Figs. 2, 8, 9). Strain PA-IV grew slightly faster than strains PA-III and PA1201 in SCM medium (Figs. 8 and 9).

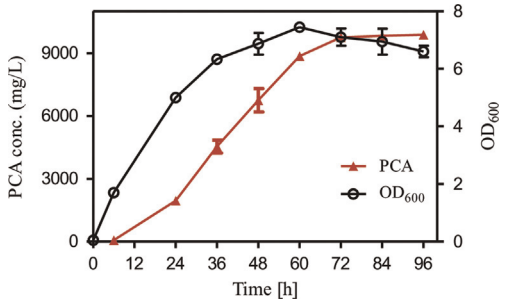

Fig. 10. Growth and PCA production of strain PA-IV during fed-batch fermentation. Line with circles indicates growth (OD_{600}), and line with triangles indicates PCA titer. Three replications were performed and the error bars indicate standard deviation.

3.9. Fed-batch fermentation of strain PA-IV for PCA production

In order to obtain higher PCA production, fed-batch fermentation of strain PA-IV was first performed in a 5 L fermentor containing 2.5 L PCM liquid medium. Bacterial growth reached stationary stage at 60 h after inoculation. Production of PCA continued to increase until 72 h, and the maximum production was 9882 mg/L with a yield of 3514.6 mg/g DCW (Fig. 10).

4. Discussion

4.1. Gene engineering methods

Gene deletion and plasmid-based gene amplification methods are commonly used in microbial genetic engineering. However, these two methods have clear disadvantages for essential genes or large gene complexes (Alper et al., 2005; Ajikumar et al., 2010; Zhao et al., 2013). Additionally, it is not feasible to use antibiotics for plasmid maintenance during industrial fermentations. In addition to the classic gene deletion method, the present study used the following gene engineering methods to improve PCA production. First we used two high strength promoters of PA1201-origin, P_{RPO} and P_{UTR}, to replace plasmid-based gene amplification. The resulting strains PAΔ14:*aroG* or PA:mex produced 28.2% or 293% higher PCA than their parent strains PAΔ14 or PA1201, respectively (Fig. 2B, 6 and 8). Second, the Mini-Tn7 transposon system (Choi and Schweizer, 2006) was used to integrate the fusion gene P_{UTR}-*phzC* into the chromosomal *att*Tn7 site to achieve stable expression. The resulting strain PAΔ14::*phzC* produced 34.5% higher PCA than the strain PAΔ14 (Fig. 6). Third, a point mutation ($W^{323} \rightarrow L^{323}$) was introduced in *PheA* to reduce chorismate mutase activity. This increased PCA production by 23% (Fig. 2B). Finally, the chorismate pyruvate-lyase encoding gene *ubiC* was replaced by *Rv2949c* from *M. tuberculosis,* and this increased PCA levels by 20%. After subculturing for 192 h, the strain PA-IV remains the same PCA production ability as the fresh culture (Fig. S9), suggesting that these modifications are genetically stable.

4.2. Systematic modifications for improving PCA production

Previous efforts to improve PCA production in *P. aeruginosa* M18 were mainly focused on modifying regulatory genes or plasmid-based amplification of the PCA biosynthetic gene cluster (Yuan et al., 2008; Zhou et al., 2010; Du et al., 2013). In the current study, a total of 478 genes were engineered, which accounts for 9.15% of the PA1201 genome. PCA production was additively increased using the following four strategies. First, PCA production was improved by blocking PCA conversion and by enhancing PCA efflux pumping (Figs. 4 and 8). Second, PCA production was improved by increasing metabolic flux towards the shikimate pathway through deleting 21 gene clusters for other secondary metabolites and overexpressing two DAHP synthase genes *phzC* and *aroG* (Figs.1, 6, and 9). Third, PCA production was improved by increasing supplies of the PCA precursor chorismate by engineering the genes which specified chorismate utilization (Figs. 2 and 6). Finally, PCA production was improved by increasing the expression of *phzA2-G2* and translation of *phzA1-G1* (Fig. 7). The resulting strain PA-IV exhibits a 54-fold PCA yield increase over the wild type in PPM media and produced 9882 mg/L PCA in SCM medium. This is about twice the highest yield observed with the M18 UMS strain (Du et al., 2013). To the best of our knowledge, this is the highest PCA titer and yield ever obtained among engineered PA-producing strains. Moreover, in view of its reduced cytotoxicity to human cell line A549 and reduced virulence to *Drosophila*, strain PA-IV is safer than the PCA-producing strains derived from strain M18. Furthermore, strain PA-IV is markerless and the SCM medium used in this study was originally optimized for strain *P. aeruginosa* M18. Thus, the PCA yield of strain PA-IV might be further enhanced through additional genetic and metabolic engineering as well as medium and process optimization.

4.3. Mechanism for PCA production improvement by secondary metabolism engineering

In nature, microbes produce a huge array of secondary metabolites with important ecological functions such as antibiotics, toxins, ionophores, bioregulators, and intra- and interspecific signals (Marinelli and Marcone, 2011). In laboratory and industrial conditions, that is, in liquid batch fermentations based on complex rich media, many secondary metabolites are not usually necessary (Marinelli and Marcone, 2011). The present study showed that deletion of 21 gene clusters for secondary metabolite production slightly increased bacterial growth in PPM medium (Fig. 7A), and significantly increased PCA production by 750% (Fig. 9A). The mechanisms underlying this phenomenon are not yet fully understood. Generally, the elimination of gene clusters unnecessary for cell growth increases PCA productivity by increasing genome stability, reducing the metabolic burden and improving the metabolic efficiency of cells (Lee et al., 2009). Specifically, pyochelin is synthesized using chorismate as a precursor (Zamri et al., 2003); thus, deleting the pyochelin biosynthetic gene cluster led to more chorismate being available for PCA biosynthesis (Fig. 2B). In *P. aeruginosa*, HCN is synthesized from glycine via HCN synthase which is encoded by the *hcnABC* gene cluster (Pessi and Haas, 2000). Lundgren et al., (2013) showed that the assimilation of glycine and PYO biosynthesis in PAO1 are both dependent on the PA2449 gene. Thus, it is likely that deletion of *hcnABC* significantly increased PCA production via glycine metabolism. In addition, deletion of gene clusters for secondary metabolites should reduce the production of unwanted by-products, and this should further improve PCA purity and Shenqinmycin quality.

5. Conclusions

Through the combined genetic engineering of virulence factors, PCA biosynthetic pathway, PCA efflux pumping system and secondary metabolism of *P. aeruginosa* strain PA1201, we obtained a genetically stable, cytotoxicity-reduced strain with a 54-fold PCA yield increase compared to the wild type. This strain produced 9882 mg/L PCA with a yield of 3514.6 mg/g DCW. This is the highest PCA titer and yield ever obtained so far for an engineered *Pseudomonas* strain.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ymben.2015.09.003.

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