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ORIGINAL PAPER

Improvement of chloramphenicol production in *Streptomyces venezuelae* ATCC 10712 by overexpression of the *aroB* and *aroK* genes catalysing steps in the shikimate pathway

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Abstract *Streptomyces venezuelae* ATCC 10712 produces chloramphenicol in small amounts. To enhance chloramphenicol production, two genes, *aroB* and *aroK*, encoding rate-limiting enzymes of the shikimate pathway were overexpressed using the expression vector pIJ86 under the control of the strong constitutive *ermE** promoter. The recombinant strains, *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK*, produced 2.5- and 4.3-fold greater amounts respectively of chloramphenicol than wild type at early stationary phase of growth. High transcriptional levels of *aroB* and *aroK* genes were detected at the early exponential growth of both recombinant strains and consistent with the enhanced expression of *pabB* gene encoding an early enzyme in chloramphenicol biosynthesis. The results suggested that the increment of carbon flux was directed towards intermediates in the shikimate pathway required for the production of chorismic acid, and consequently resulted in the enhancement of chloramphenicol production. This work is the first report of a convenient genetic approach to manipulate primary metabolite genes in *S. venezuelae* in order to increase chloramphenicol production.

Keywords Chloramphenicol · *aroB* · *aroK* · Overexpression · *Streptomyces venezuelae*

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Introduction

Chloramphenicol (Cm) is produced by actinomycetes including *Streptomyces venezuelae* ATCC 10712 (Vining and Stuttard 1995). It is a bacteriostatic antibiotic for the treatment of a number of bacterial infections including meningitis, typhoid, plague and cholera by specific binding to the 50S ribosomal subunit and thus inhibiting protein synthesis. The biosynthesis of Cm includes parts of glycolysis, the pentose phosphate pathway, and the shikimate pathway. Erythrose-4-phosphate (E4P) and phosphoenol pyruvate (PEP) are converted by seven sequential

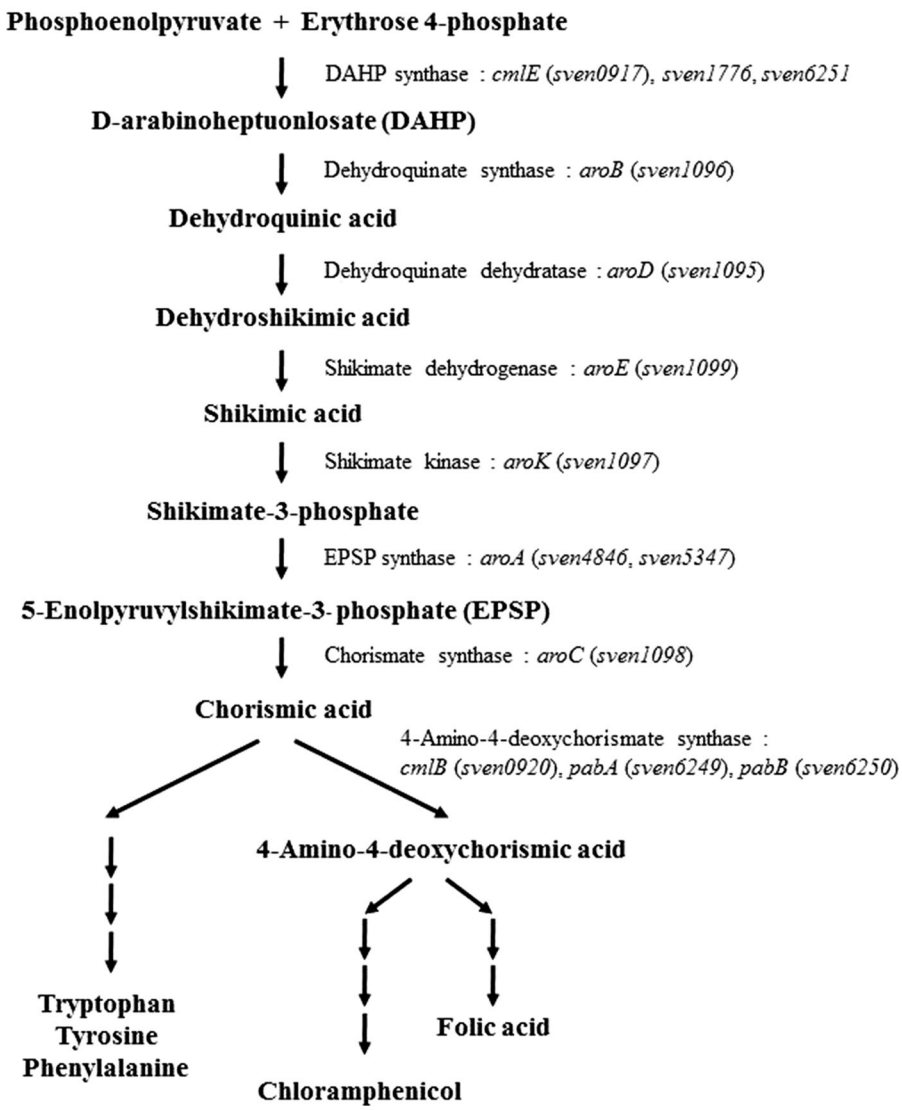

Fig. 1 Overview of the shikimate pathway, showing the early steps of the chloramphenicol biosynthesis in *S. venezuelae*. *sven*, genes designated from StrepDB (http://strepdb.streptomyces.org.uk/)

enzymes of the shikimate pathway to produce chorismic acid, a precursor of essential amino acids and secondary metabolites including Cm (Fig. 1). For Cm biosynthesis, chorismic acid is converted to the intermediate 4-amino-4-deoxychorismic acid (ADC) by ADC synthase gene products (*pabAB*; *sven6249*, *sven6250*; Brown et al. 1996; He et al. 2001) as well as *CmlB* (*sven0920*; Fernández-Martínez et al., 2014) and through a number of specific enzymatic steps to Cm (Fig. 1). The Cm biosynthetic gene cluster of *S. venezuelae* has been described (He et al. 2001; Fernández-Martínez et al. 2014).

The increase of antibiotic production by important microbes such as *Streptomyces* and related genera is a primary goal for the pharmaceutical industry. There have been several rational genetic approaches to enhance antibiotic production, including expression of global regulatory genes such as *afsR* (Matsumoto et al. 1995), constitutive expression of pathway-specific regulatory genes such as *SARP* (Streptomyces Antibiotic Regulatory Protein family) and *LAL* (Large ATP-binding regulators of the LuxR family) (Bibb 2005; Laureti et al. 2011), metabolic engineering of pathway-specific genes for precursors (Reeves et al. 2007; Thykaer et al. 2010; Chen et al. 2012), mutation of RNA polymerase and ribosomal protein genes (*rpoB*, *rpsL*, *rsmG*; Tamehiro et al. 2003; Tanaka et al. 2009; Ochi and Hosaka 2013) and rational engineering

of heterologous expression hosts (Gomez-Escribano and Bibb 2011; Thanapipatsiri et al. 2015). Moreover, manipulation of genes controlling primary metabolism to drive carbon flux to enhance secondary metabolites has been considered as an interesting approach (Kern et al. 2007). Primary metabolism significantly influences secondary metabolism by providing the precursors for secondary metabolites, mostly generated from central carbon metabolism (Hodgson 2000; Rokem et al. 2007). For example, metabolic engineering of key enzymes controlling carbon metabolism apparently increased the production of actinorhodin in *Streptomyces coelicolor* (Ryu et al. 2006), clavulanic acid in *Streptomyces clavuligerus* (Li and Townsend 2006) and oxytetracycline in *Streptomyces rimosus* (Tang et al. 2011). More strategies and approaches for the improvement of antibiotic production from actinomycetes have been extensively reviewed (Olano et al. 2008; Hwang et al. 2014).

S. venezuelae ATCC 10712 produces chloramphenicol in small amounts under a variety of growth conditions in the laboratory. It has been recently demonstrated that overexpression of a regulatory gene in the Cm biosynthetic gene cluster of *S. venezuelae* caused a large increase Cm production compared to that of wild-type (Fernández-Martínez et al. 2014). In this work, we attempted to overexpress the primary metabolic genes, *aroB* and *aroK*, encoding enzymes for rate-limiting steps in the shikimate pathway in *S. venezuelae* to increase the production of Cm. Transcription levels of these genes as well as the *pabB* gene of the Cm biosynthetic cluster were also determined.

Materials and methods

Bacterial strains, plasmids and culture conditions

Escherichia coli DH5α (Invitrogen, USA) was used as a cloning host and methylation-deficient *E. coli* ET12567 (*dam-13::Tn9*, *dcm-6*, *hsdM*, *hsdS*; MacNeil et al. 1992) containing pUZ8002 (Kan^r/*tra, neo, RP4*; Paget et al. 1999) was used as a donor for intergeneric conjugation. They were grown at 37 °C in Luria–Bertani (LB) broth or on solid medium. *S. venezuelae* ATCC 10712, a chloramphenicol producer, was used as a host for overexpression plasmids. pIJ86 (*ermE*p*, *aac(3)IV*, *oriT* (RK2), *ori* (pIJ101), *ori* (pUC18); Healy et al. 2009) was used for gene expression in *S. venezuelae*. Media for the growth of *S. venezuelae* were tryptic soy broth (TSB) and mannitol soya agar (MS: 20 g mannitol, 20 g soya flour, 20 g agar in 1 l of distilled water; Hobbs et al. 1989). The following antibiotics were used when required: 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} apramycin, 25 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} kanamycin and 25 μg ml^{-1} nalidixic acid. Cells or spores of all bacterial strains were kept in 20 % glycerol at −70 °C.

DNA manipulations and cloning procedure

Genomic and plasmid DNA isolation and purification of *E. coli* were performed according to standard procedures (Sambrook and Russell 2001) or using the Gene pH low^{TM} Gel/PCR kit (Geneaid, Taiwan) following the manufacturer's protocol. Genomic DNA isolation of *S. venezuelae*was according to Kieser et al. (2000). The dehydroquinate synthase (*aroB*, *sven1096*) and shikimate kinase (*aroK*, *sven1097*) genes were amplified from chromosomal DNA of *S. venezuelae* using primers aroBF-1 and aroBR-1, and aroKF-1 and aroKR-1, respectively (Table 1). The primers aroBF-1 and aroBR-1were designed to include restriction sites for *Bgl*II and *Hin*dIII, while aroKF-1 and aroKR-1 contained *Bam*HI and *Hin*dIII sites, respectively. The forward primers for both aroBF-1 and aroKF-1were designed to include the ribosome binding sites of *sven1096* and *sven1097* respectively. PCR was performed using Phusion® Hot Start II-High Fidelity DNA polymerase (Thermo Scientific, USA) with one denaturation cycle of 3 min at 98 °C and 30 cycles of 30 s at 98 °C, 30 s at an appropriate annealing temperature (Table 1) and 1 min at 72 °C, and a final cycle of 4 min at 72 °C. The PCR products were purified and digested with restriction enzymes and ligated into the corresponding sites on the expression plasmid, pIJ86, and confirmed by restriction enzyme digestion and DNA sequencing using primer pIJ86F1 (Table 1). The recombinant plasmids were designated pIJ86-*aroB* and pIJ86-*aroK*.

Intergeneric conjugation

The plasmids pIJ86-*aroB* and pIJ86-*aroK* were introduced into *E. coli* ET12567/pUZ8002 by transformation and transferred into *S. venezuelae* by intergeneric conjugation. The conjugation procedure was

Table 1 Primers used in this study

Primer	Sequences (5′-3′)	Description	References	T_a (°C)
aroBF-1	AAAAAAAGATCTCCCGAAGAGGTCGCGGACGC	PCR of *aroB* (*sven1096*) for cloning into pIJ86	This work	65
aroBR-1	AAAAAAAAGCTTTCACGCGGACACCTCGCCGTA			
aroKF-1	AAAAAAGGATCCCGACAGCGTCCCGGAGAC	PCR of *aroK*(*sven1097*) for cloning into pIJ86 and for qRT-PCR of *aroK*	This work	68
aroKR-1	AAAAAAAAGCTTTCATGCGTCCTTCTTCAGGTCCAG			
aroBF-2	TTTGAATTCGTCGGCGGCAAGACCGGTATCA	qRT-PCR of *aroB*	This work	68
aroBR-2	TTTAAGCTTGCTTGGCGAGCCCGTCGAGGA			
pabBF	CGCAACCCCTCGCCGCACAT	qRT-PCR of *pabB (sven6250)*	This work	68
pabBR	TCGYGCGGATGCACAGGGCC			
hrdBF	CCGAGTCTGTGATGGCGCTC	qRT-PCR of *hrdB*	Xie et al. (2012)	62
hrdBR	TTGGTGGCGGTGCGCTTGAC			
pIJ86F1	ACGCCTGGTCGATGTCGGAC	Sequencing primers for recombinant plasmids of pIJ86	Thanapipatsiri et al. (2015)	60
pIJ86R2	TGCGGTCAGTGCGTGTGTCG			

Note Restriction sites are underlined

conducted using the method described by Phornphisutthimas et al. (2010) using spores of *S. venezuelae* as recipient. Conjugation was performed on MS agar containing 10 mM $MgCl_2$. Exconjugants were selected for resistance to apramycin. The recombinant strains were designated as *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK*, respectively.

Analysis of growth rate and chloramphenicol production

Spore suspensions of *S. venezuelae* wild-type and recombinant strains were used to inoculate 10 ml of GNY seed medium (20 ml glycerol, 8 g nutrient broth, 3 g yeast extract, 5 g K_2HPO_4 in 1 l distilled water; Malik and Vining 1970) to give an initial 10^9 spores ml^{-1}. The culture was grown at 28 °C on a rotary shaker at 200 rpm for 24 h. 2 ml of each seed culture was transferred into 100 ml of GI production medium (20 ml glycerol, 30 g glucose, 8 g nutrient broth, 3 g yeast extract, 5 g K_2HPO_4, 7.5 g L-isoleusine in 1 l distilled water; Chatterjee et al. 1983) in a 500-ml flask containing a stainless steel spring coil and grown under the same conditions. The cells and supernatants were collected by centrifugation of culture broth for 30 min at 8000×*g* at 12, 24, 36, 48 and 60 h of growth for measurement of dry weight and Cm production.

The supernatant of each strain was extracted with an equal volume of ethyl acetate. The organic phase was collected and immediately concentrated under reduced pressure using a rotary evaporator. The solid residue was dissolved in methanol to reach a final concentration of 50 mg ml^{-1}. 20 µl aliquot of the extract was analysed by HPLC (Agilant, USA) using a reversed phase Luna 5u C18(2) 100A column (4.60 × 250 mm, 5 um; Phenomenex, USA) with a gradient elution system of acetonitrile at 20 % (v/v) 3 min, 20–35 % (v/v) 20 min, 35 % (v/v) 3 min, 35–100 % (v/v) 3 min, 100 % (v/v) 1 min and 20 % (v/v) 5 min. A peak at the exact retention time of Cm (Sigma, USA) was collected through HPLC and subjected to mass spectrometric analysis using micrOTOF-QIII (Bruker, USA) in ESI positive ion mode.

To assess Cm production from recombinant strains, antibiotic activity of the extracts and fractions were preliminarily determined by disk assay against *Micrococcus luteus* TISTR 883.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from mycelium of each strain of *S. venezuelae* collected at each time point from the above experiments. Mycelium was suspended in 500 µl lysis solution (0.3 M sucrose, 25 mM Tris–

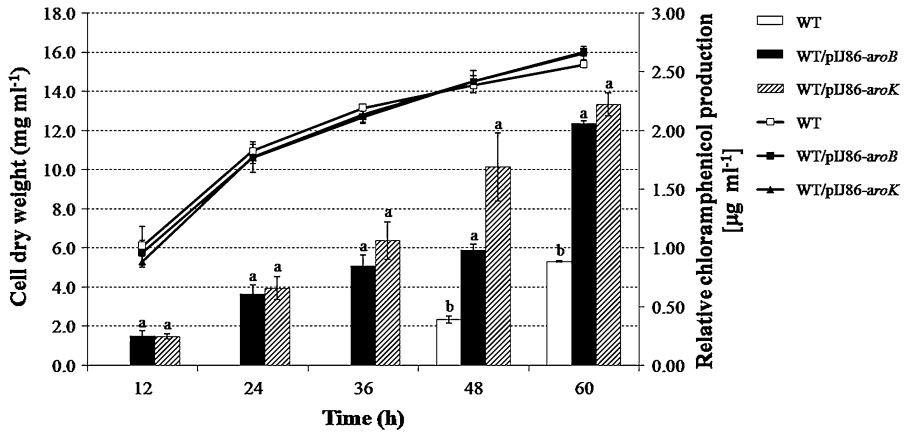

Fig. 2 Comparison of growth curves and relative chloramphenicol production by *S. venezuelae* ATCC 10712 (WT), *S. venezuelae*/pIJ86-*aroB* (WT/pIJ86-*aroB*) and *S. venezuelae*/pIJ86-*aroK* (WT/pIJ86-*aroK*) during flask fermentation at 12, 24, 36, 48, 60 h. Differences in Cm production between the three strains were statistically analysed by ONE-WAY ANOVA with Tukey test, and significant differences are indicated by different letter at a significance level $p < 0.05$. Error bars, SD from three replicate experiments. Data are mean ± standard deviation of data (N = 3)

HCl pH 8.0, 25 mM EDTA pH 8.0) containing 10 µg µl^{-1} lysozyme and incubated at 37 °C for 1 h. Then total RNA was extracted following the manufacture's protocol (Trizol® Reagent, Invitrogen, USA). RNA samples were treated with DNase I (Thermo Scientific, USA) to remove trace amounts of DNA according to the manufacture's protocol. cDNA was synthesised from equal amounts of RNA templates using the Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo Scientific, USA).

Semi-quantitative RT-PCR analysis was performed using cDNA products and the corresponding primer pairs listed in Table 1. PCR reactions were performed using Phusion® Hot Start II-High Fidelity DNA polymerase (Thermo Scientific, USA) with one denaturation cycle of 30 s at 98 °C and 30 cycles of 10 s at 98 °C, 30 s at an appropriate annealing temperature (Table 1) and 1 min at 72 °C, and a final cycle of 4 min at 72 °C. The expression level of each product was quantified by Gel Doc™ XR + with Image Lab™ Software (Biorad, USA) and normalized against the expression of the housekeeping gene, *hrdB* (Xie et al. 2012). RNA extraction, cDNA synthesis and qRT-PCR were performed in triplicate.

Statistical analysis

Statistical analysis was conducted using ONE-WAY ANOVA with Tukey test (SPSS16.0 software, IBM, USA). Data were expressed as mean ± SD. Statistical significant differences were expressed at a significance level $p < 0.05$.

Results

Construction of recombinant strains of *S. venezuelae*

Genes encoding rate-limiting enzymes in the shikimate pathway, namely dehydroquinate synthase (*aroB*, *sven1096*) and shikimate kinase (*aroK*, *sven1097*) of *S.venezuelae* ATCC 10712 were overexpressed in an attempt to increase Cm production in the wild-type strain. Both genes were cloned into the expression plasmid pIJ86 under the control of the strong constitutive *ermE** promoter. The recombinant plasmids were independently transferred into *S. venezuelae* by intergeneric conjugation and confirmed by PCR using primers pIJ86F1 and pIJ86R2 (Table 1), yielding *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK*, respectively. Both recombinant strains showed the same morphology and spore formation as wild-type. Their growth curves were similar to that of wild-type, in which exponential growth was observed before 36 h and early stationary phase between 36 and 60 h (Fig. 2; Table S1).

Effect of *aroB* and *aroK* overexpression on Cm production

Cm production was determined by HPLC analysis of ethyl acetate extracts from culture supernatants of wild-type and recombinant strains of *S. venezuelae* collected at 12, 24, 36, 46 and 60 h of growth (Fig. S1, Table S1). The identity of the compound was confirmed to be chloramphenicol by high-resolution mass spectrometry (HRMS (ESI-QTOF) *m/z* $[M + H]^+$ calculated for $C_{11}H_{12}C_{l2}N_2NaO_5$: 345.0015, found: 345.0031). Cm production was detected after 48 h of growth of the wild-type, while it was observed as early as 12 h of growth for both recombinant strains, *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* (Fig. 2). At 48 h, Cm production by *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* was significantly increased by 2.5- and 4.3-fold, respectively compared to that of wild-type (Fig. 2, Table S1). When the culture reached 60 h of growth, Cm production by *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* was about 2.2–2.5 fold higher than that of wild-type (Fig. 2, Table S1). A similar pattern of Cm production was observed in the vector-only control strain, *S. venezuelae*/pIJ86, as in the wild-type (data not shown).

Transcriptional analysis of *aroB*, *aroK* and *pabB* genes

As described above, *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* showed greater levels of Cm production compared to that of wild-type. qRT-PCR (Fig. S2) revealed that the transcription level of the *aroB* gene at every time point was significantly increased in *S. venezuelae*/pIJ86-*aroB* by about twofold compared to wild-type and *S. venezuelae*/pIJ86-*aroK* (Fig. 3a; Table S2). The transcription levels of *aroK* of *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* were essentially the same at every time point and about 1.7–2.1 fold higher than that of wild-type (Fig. 3b, Table S2).

To investigate the influence of overexpression of *aroB* and *aroK* on the expression level of Cm biosynthetic genes, *pabB* encoding part of ADC synthase was analysed. The qRT-PCR results (Fig. S2) revealed that *pabB* of wild-type was expressed at a low level during exponential growth (12–24 h) and increased after 36 h of growth (Fig. 3c, Table S2), which correlated with the beginning of Cm production (Fig. 2). On the other hand, *pabB* was highly expressed in *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* from 12 h of growth and about 3–3.5 fold greater than that of wild-type during exponential growth (Fig. 3c, Table S2).

The enhancement of Cm production and transcription level of *aroB*, *aroK* and *pabB* in *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* were reasonably steady although the recombinant strains were maintained for more than five generation (data not shown) which indicated that the expression of those genes are relatively stable under the control of replicative plasmid, pIJ86.

Discussion

The shikimate pathway is common to the biosynthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine, as well as folate and secondary metabolites such as chloramphenicol (Fig. 1). In *E. coli*, four rate-limiting steps were reported in the shikimate pathway catalysed by DHQ synthase (*aroB*), shikimate kinase (*aroK*), 5-enolpyruvoylshikimate 3-phosphate (EPSP) synthase (*aroA*) and chorismate synthase (*aroC*) (Dell and Frost 1993). We have described herein a successful improvement in the production of Cm in *S. venezuelae* ATCC 10712 by overexpression of *aroB* and *aroK* encoding the corresponding rate-limiting enzymes. *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* showed no visible effect on growth and cell mass compared to wild-type (Fig. 2). The results suggested that the manipulation of primary metabolism enhanced the pool of precursor, in this case chorismic acid, and consequently increased the production of Cm, while no effect of the cellular growth was observed. *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* showed enhanced production of Cm from 12 h of log phase growth, before the wild-type began antibiotic production, and yielded 2.5- to 4.3-fold more than the wild-type at early stationary phase (Fig. 2), suggesting an increment of carbon flow through the shikimate pathway, as previously evidenced by the increasing levels of L-phenylalanine (Dell and Frost, 1993) and L-tyrosine production in *E. coli* (Juminaga et al. 2012) when rate limiting genes were manipulated. Our results are also in agreement with a previous study

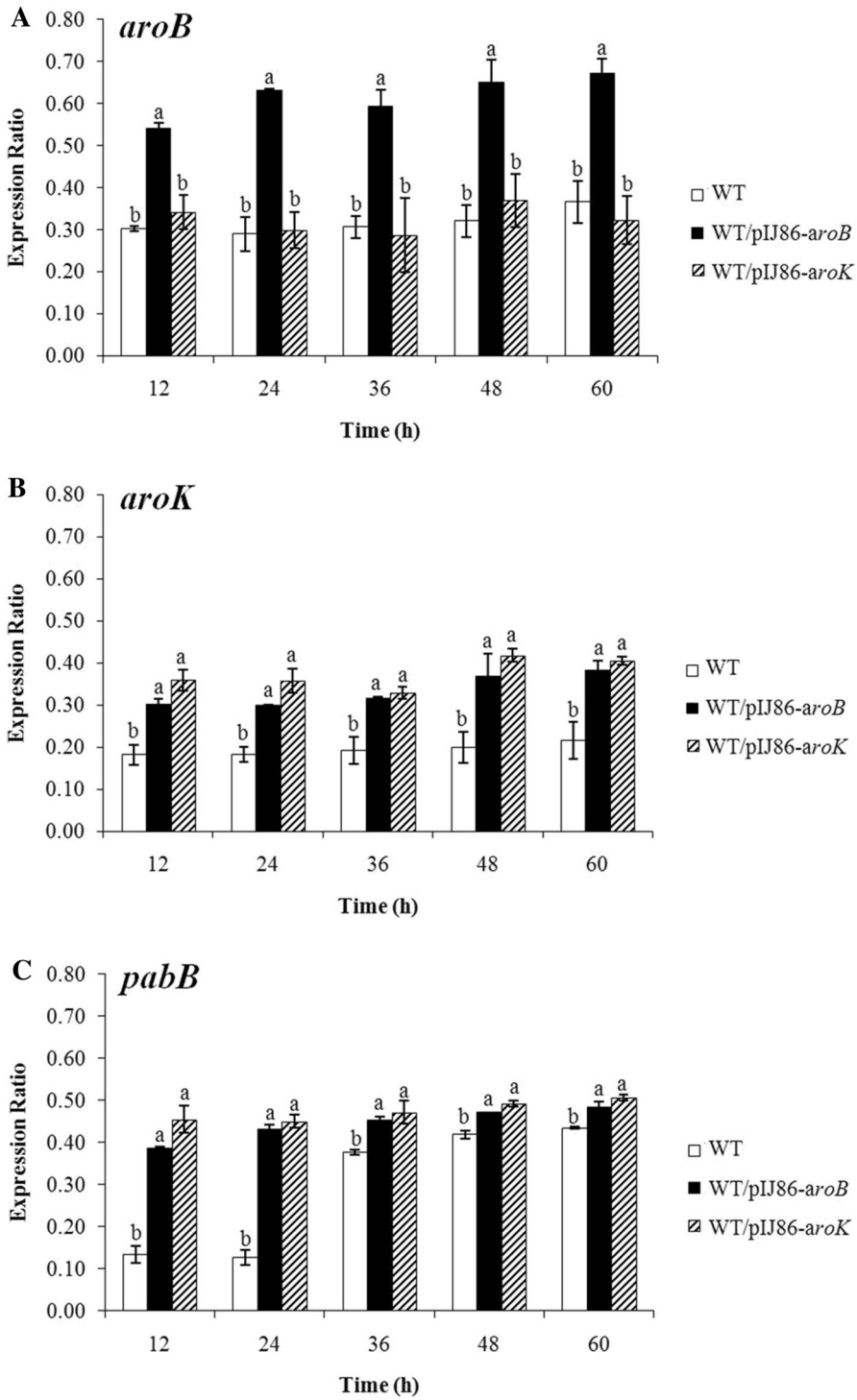

Fig. 3 Comparison of gene expression ratio from qRT-PCR analysis of *aroB* (A), *aroK* (B) and *pabB* (C) of *S. venezuelae* ATCC 10712 (WT), *S. venezuelae*/pIJ86-*aroB* (WT/pIJ86-*aroB*) and *S. venezuelae*/pIJ86-*aroK* (WT/pIJ86-*aroK*) at 12, 24, 36, 48, 60 h of growth. *hrdB* was used as an internal reference gene for normalisation. Differences of gene expression between the three strains were statistically analysed by ONE-WAY ANOVA with Tukey test, and significant differences are indicated by different letter at a significance level $p < 0.05$. *Error bars* SD from three replicate experiments

showing that AroB and AroK were promising metabolic engineering targets to alleviate flux control in L-phenylalanine-producing *E. coli* (Oldiges et al. 2004). Therefore, expression of *aroB* and *aroK* could increase the production of primary metabolites such as those of essential amino acids as well as secondary metabolites such as Cm.

The enhancement of Cm production was consistent with the significantly increased transcriptional levels of *aroB* at every time point of growth of *S. venezuelae*/pIJ86-*aroB* compared to those of wild-type and *S. venezuelae*/pIJ86-*aroK* (Fig. 3a). Interestingly, an increment in the transcription level of the intact *aroK* gene in *S. venezuelae*/pIJ86-*aroB* was also detected, suggesting that high levels of AroB might lead to desirable changes in metabolite levels of the shikimate pathway and subsequently up-regulation of AroK. The results implied that carbon flux from PEP and E4P was directed towards intermediates in the shikimate pathway, DHQ and shikimate-3-phosphate, and on to the final important precursor chorismic acid, leading to a positive effect on Cm production. When *aroK* was overexpressed, the transcription level detected in *S. venezuelae*/pIJ86-*aroK* was greater than that of wild-type and consistent with the increment of Cm production (Fig. 3b). The results corresponded to the previous report that overexpression of *aroK* could increase L-tyrosine production in *E. coli* (Lütke-Eversloh and Stephanopoulos 2008). Our results indicated that overexpression of *aroK* alone enhanced Cm production to a similar level as that caused by *aroB* overexpression in which transcription of the intact *aroK* gene was additionally increased. Thus, *aroB* and *aroK* genes contribute equally to carbon flux through chorismic acid and thus enhance Cm production in *S. venezuelae*.

For Cm biosynthesis, the precursor chorismic acid, the end product of the shikimate pathway, is first converted to 4-amino-4-deoxychorismic acid (ADC) by ADC synthase (encoded by *pabAB*). It was demonstrated that Cm production was substantially decreased when either *pabA* or *pabB* were disrupted (Brown et al. 1996; He et al. 2001). Therefore, ADC synthase is important to direct flux from the shikimate pathway toward Cm biosynthesis. We then investigated the transcription level of *pabB* when *aroB* and *aroK* were individually overexpressed. A high level of transcription of *pabB* in *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* was detected from 12 h of growth and greater than that of wild-type during exponential growth (Fig. 3c). The results indicated that the effects of AroB and AroK on Cm biosynthesis by *S. venezuelae*/pIJ86-*aroB* and *S. venezuelae*/pIJ86-*aroK* could increase more carbon flux through the corresponding intermediates in the shikimate pathway required for the production of chorismic acid and consequently resulted in the increment of transcription levels of the Cm biosynthetic gene, *pabB*. Such metabolic engineering of important enzymes controlling primary metabolism to drive carbon flux to enhance the production of antibiotics was successfully demonstrated in the production of actinorhodin, clavulanic acid and oxytetracycline in streptomycetes (Li and Townsend 2006; Ryu et al. 2006; Tang et al. 2011).

We conclude that the increment of carbon flux in the primary metabolic pathway, in this case the shikimate pathway, by overexpression of rate-limiting enzymes enhances Cm production. The results also confirmed the tight connection between primary and secondary metabolisms and suggested that systematic analysis of primary metabolism should be a useful strategy to manipulate secondary metabolite production (Hodgson 2000; Kern et al. 2007; Rokem et al. 2007). Recently, overexpression of a transcriptional activator gene, *sven0913*, in the Cm biosynthetic gene cluster of *S. venezuelae* led to enhanced production of Cm (Fernández-Martínez et al. 2014). A further increase in Cm production can, presumably, be achieved by combining overexpression of shikimate primary metabolic genes with other genes in the Cm biosynthetic gene cluster, including the regulatory gene.

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