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Enhanced Clavulanic Acid Production in *Streptomyces clavuligerus* NRRL3585 by Overexpression of Regulatory Genes

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Abstract We constructed four recombinant plasmids to enhance the production of clavulanic acid (CA) in *Streptomyces clavuligerus* NRRL3585: (1) pIBRHL1, which includes *ccaR*, a pathway-specific regulatory gene involved in cephamycin C and CA biosynthesis; (2) pIBRHL2, containing *claR*, again a regulatory gene, which controls the late steps of CA biosynthesis; (3) pGIBR containing *afsR-p*, a global regulatory gene from *Streptomyces peucetius*; and (4) pKS, which harbors all of the genes (*ccaR*/*claR*/*afsR-p*). The plasmids were expressed in *S. clavuligerus* NRRL3585 along with the *ermE** promoter. All of them enhanced the production of CA; 2.5-fold overproduction for pIBRHL1, 1.5-fold for pIBRHL2, 1.6-fold for pGIBR, and 1.5-fold for pKS compared to the wild type.

Keywords: clavulanic acid, production, *Streptomyces clavuligerus*, regulatory gene, *ccaR*, *claR*, *afsR-p*

INTRODUCTION

Streptomyces clavuligerus is a Gram-positive filamentous bacterium that produces several secondary β-lactam metabolites such as cephamycin C (CC), clavulanic acid (CA), and other clavam derivatives [1]. Clavulanic acid is a potent inhibitor of serine β-lactamases belonging to class A, B, and D [2]. Because CA is commonly used in combination with broad-spectrum β-lactam antibiotics such as amoxicillin [3], a lot of research effort is dedicated to enhancing CA production in the cultured bacteria. Genetic manipulations are most commonly employed. In fact, almost studies employ manipulation of pathway-specific regulatory genes such as *ccaR* and *claR*, which directly regulate CA biosynthesis [4,5]. The genes encoding CC and CA biosynthesis are genomically concentrated in the so-called *β-lactam super cluster* [6,7], where the CC gene cluster is located upstream of the CA gene cluster. Previous studies have shown that the CC cluster regulatory gene *ccaR* controls the production of both, CC and CA [8]. Disruption of *ccaR* inhibits the biosynthesis of these antibiotics, which can be restored when the mutation is repaired [8]. In addition to being controlled by the *ccaR* gene, CA biosynthesis is also regulated by *claR* encoding a Lys-type transcriptional activator [5]. The *claR* gene plays a crucial role in the conversion of CA biosynthesis intermediates. Both, *ccaR* as well as *claR*, control CA biosynthesis through a cascade of regulatory proteins. While the genes involved in the biosynthesis for penicillin and CC are clearly identified, the genes participating in the production of CA are less well established. So far, nineteen CA cluster open reading frames have been linked to CA synthesis (Fig. 1) [9,10]. Experiments involving the ingestion of radiolabeled amino acids have been conducted, associating condensation of arginine and three-carbon glycolytic intermediates such as glyceraldehydes-3-phosphate with CA biosynthesis (Fig. 2) [11,12]. It was found that clavaminate synthase (CAS), a multifunctional enzyme, catalyzes three oxidative transformations resulting in the intermediate clavaminate [13,14]. Two locally separated isoenzymes, CAS1 and CAS2, exist in the genome of *S. clavuligerus* [15-17].

Secondary metabolite production in *Streptomyces* is controlled by a promiscuous serine/threonine kinase AfsK that phosphorylates serine and threonine residues of AfsR, a regulatory protein involved in the secondary metabolism in *S. coelicolor* A3 [18,19] and in the morphological differentiation of *S. griseus* [20]. Earlier work has demonstrated that AfsR can enhance secondary metabolite production by phosphorylating other transcriptional regulators. It could also be shown that AfsR activates transcription from the *afsS* gene, generating a 63-amino acid protein. Phosphorylation of AfsR greatly enhances its DNA binding activity.

The global regulatory gene *afsR2* was originally isolated from *S. lividans* and subsequently shown to induce

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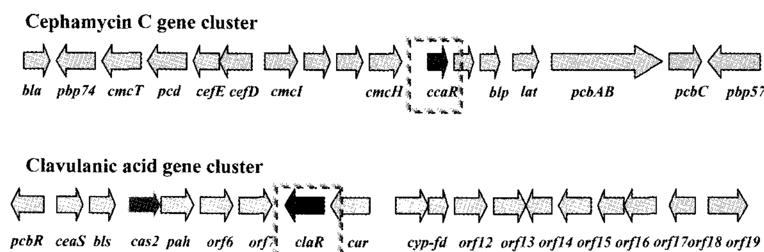

Fig. 1. Cephamycin C clavulanic acid super gene cluster.

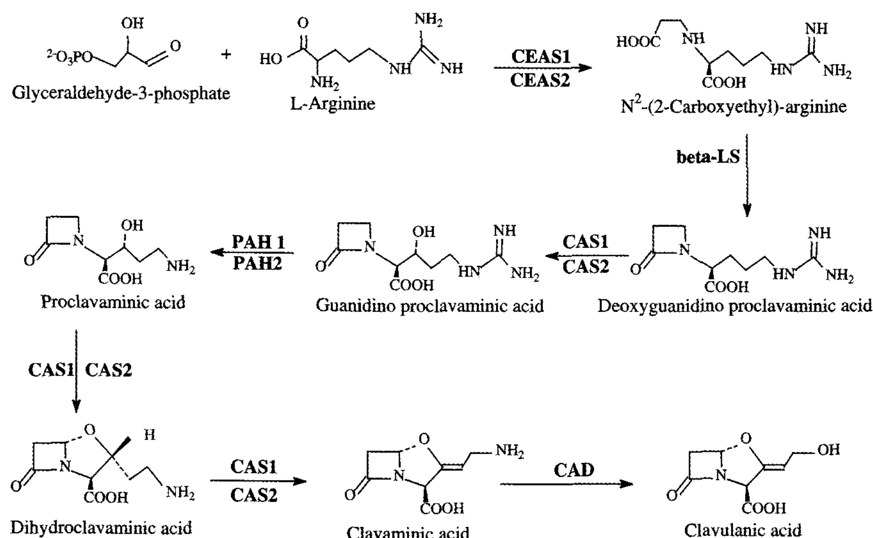

Fig. 2. Biosynthese of clavulanic acid.

the production of two structurally unrelated antibiotics, actinorhodin and undecylprodigiosin. We have isolated an *afsR* homologue (from hereon referred to as *afsR-p*) through genome analysis of *S. peucetius* ATCC 27952. At the amino acid level, AfsR-p is 60% identical to AfsR from *S. coelicolor* A3 (2) [21]. To identify the function and the effect of *afsR-p* on CA production, we amplified *afsR-p* from *S. peucetius* and expressed it heterologously in *S. clavuligerus*. Production of CA was monitored through culture broth sampling.

MATERIALS AND METHODS

Bacterial Strains and Vectors

The bacterial strains used in this study were described by Hung *et al.* [22]. All bacterial strains were stored in 30% glycerol at –70°C.

Medium and Culture Conditions

Escherichia coli (*E. coli*) strains were grown in Luria broth (LB) based liquid or on agar plates. *S. clavuligerus* NRRL3585 was grown in either ISP2 (Difco, Detroit, USA) or R2YE (103 g/L sucrose, 0.25 g/L K_2SO_4, 10.12 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L Difco casaminoacids, 2 mL trace element solution, 5 g/L Difco yeast extract and 5.73 g/L TES) media supplemented 1% maltose (w/v) [23]. All plasmid-containing cultures additionally had 5 μg/mL thiostrepton (Sigma St. Louis, MO, USA) added to the medium. For protoplast transformations, *S. clavuligerus* was inoculated in tryptic soy broth (TSB, Difco, Detroit, USA). Maltose was supplemented 1% (w/v) to the seed culture. After 48 h of shaking (250 rpm) at 28°C a 3-mL aliquot was withdrawn to inoculate R2YE media (maltose was used instead of glucose in R2YE) using the same conditions as in the seed culture. For CA production, production media medium (10 g/L glycerol, 15 g/L peptone, 27 g/L tryptic soy broth and 13 g/L MOPS) was used [22,24,25].

Recombinant DNA Work

Oligonucleotides used for the amplifications of *ccaR*, *claR*, and *afsR-p* have been described previously by Hung and Niranjan [21,22]. Polymerase chain reactions (PCRs) were performed in a total volume of 50 μL containing 0.2 mM deoxyribonucleotides, 10 pmol of each primer, 1.5 mM $MgCl_2$, 1 unit LA Taq polymerase (Takara, Japan), LA Taq buffer, and template DNA running cycles of 95°C for 1 min, 60~64°C for 30 sec, and 72°C for 1 min. The

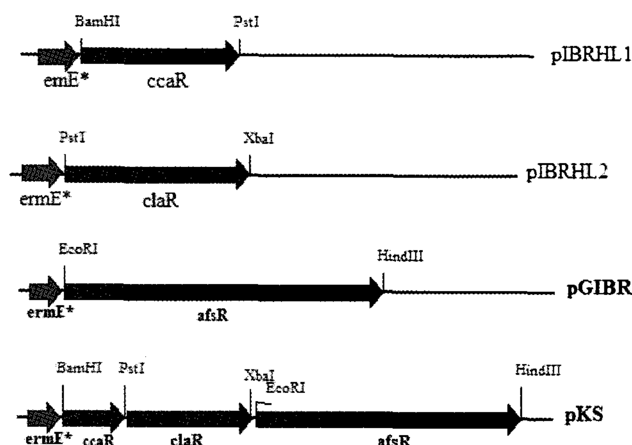

Fig. 3. Plasmid maps.

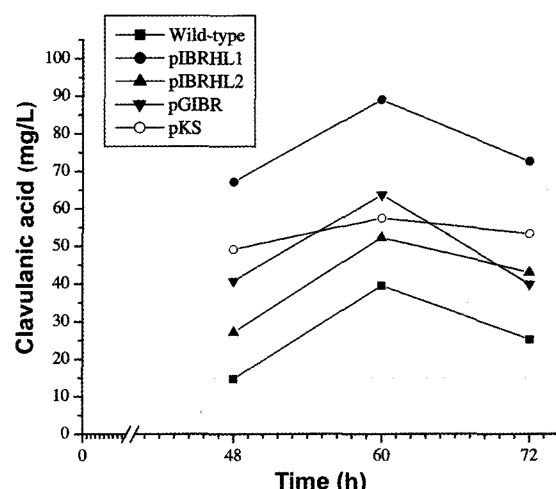

Fig. 4. Clavulanic acid production in wide type *S. clavuliregus* NRRL 3585 (■) and in transformants hosting pGIBR (▼), pIBRHL1 (●), pIBRHL2 (▲), or pKS (○).

PCR products were purified using PCR clean-up kit (GeneMed, Seoul, Korea). The purified PCR products were inserted into the pGEM-T-easy vector (Promega, Madison, WI, USA) and sequenced. Construction of pIBR25 for *Streptomyces* expression was done as reported elsewhere [26].

Construction of Recombinant Plasmids

Construction of the plasmids pIBRHL1 (*ccaR*), pIBRHL2 (*claR*), pGIBR (*afsR-p*), and pKS (*ccaR/claR/afsR-p*) followed standard procedures [10,26]. The purified PCR products were first inserted into pGEM-T-easy and then subcloned into their respective plasmid as follows: *ccaR* via *Bam*HI/*Pst*I restriction into pIBR25 to yield pIBRHL1, *claR* via *Pst*I/*Xba*I into pIBR25 to yield pIBRHL2. Construction pGIBR was described by Niranjan *et al.* [21]. The plasmid pKS was made by insertion of a 3.0-kb *Eco*RI/*Hin*dIII fragment into *Eco*RI/*Hin*dIII-digested pIBRHL12 (Fig. 3). Experimental conditions the plotoplast formation, regeneration, and DNA transformation were modified slightly from Kieser *et al.* [23,27].

High-pressure Liquid Chromatography Analysis of CA Production

High-pressure liquid chromatography (HPLC) analysis (220 nm) was used to measure the CA production. Culture broth supernatant was sampled and analyzed with a C-18 reversed phase column (4.6 × 250 mm, 50 μm, KANTO Reagents). The pH of the mobile phase was set to 4.4 with sodium phosphate buffer with 50 mM Na_2HPO_4.

RESULTS AND DISCUSSION

Over-expression of *ccaR* and *claR* in *S. clavuligerus* NRRL3585

The function of the *ccaR* gene product (CcaR) has been described earlier. It displays homology to several *Streptomyces* pathway-specific transcriptional activators [10,28] and gives rise to a 2- to 3-fold increase in CC and CA production during overexpression [8]. We amplified *ccaR* from *S. clavuligerus* NRRL 3585 and inserted it via *BamHI/PstI* double-digestion into the *ermE** promoter-containing pIBR25 vector (construct pIBRHL1). Transformants carrying multiple copies of *ccaR* showed a 2.25- to 2.5-fold increase in CA production (Fig. 4), which is similar to earlier reports [8]. We interpret this finding as *ccaR* being essential to CA biosynthesis, where it may be involved in early or intermediate steps during CA production, based on the fact that intermediate synthesis steps have already been found to be controlled by ClaR, another pathway-specific regulator [5].

It is known that ClaR is a pathway-specific gene, and its product take part in CA biosynthesis. To examine how CA production is affected by ClaR, we inserted a *Pst*I/*Xba*I fragment of the *claR* gene into *Pst*I/*Xba*I-digested pIBR25 (construct pIBRHL2). We then transformed wild type *S. clavuligerus* with this construct and quantified CA by HPLC. Cell harboring the *claR* gene showed a 1.5-fold increase in CA production over their parental wild type strain under identical growth conditions (Fig. 4). While similar effects of claR overexpression have been reported from a previous study, our experiments did not research the same extent of CA production increase [5]. This may be due to differences in the growth media: our incubations were done in glycerol-supplemented TSB medium instead of in synthetic (GSPG medium: Glucose Sucrose Proline Glutamic acid) or complex (MSF: MOPS Sucrose Soy trypticasein) media used elsewhere [5]. Our TSB media was additionally made from research-grade compounds.

Analysis of the ClaR protein suggests that ClaR is an LysR-type transcriptional factors. Several pathway-specific regulatory genes are now known in antibiotic-producing actinomycetes. There are two motif at the *N*- and the *C*-terminal end ClaR (amino acids residues 7–51 and 338–

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains & plasmid vectors	Relevant features	Sources or references
Bacterial strains		
S. clavuligerus	Wide type, CA producer	NRRL 3585
E. coli		
XL-1-Blue	Generic cloning host	Stratagene PBL company
ET12567	Generic cloning host	Stratagene Lajolla CA, USA
Plasmid vectors		
pGEM®-T easy vector system I	Generic cloning vector, Amp(R)	Promega, USA
pIBR25	*Streptomyces* expression vector with an *ermE** promoter	IBR laboratory, Sun Moon Univ.
pIBRHL1	pIBR25 with *ccaR* insert	This study
pIBRHL2	pIBR25 with *claR* insert	This study
pGIBR	pIBR25 with *afsR-p* insert	This study
pKS	pIBR25 with *ccaR, claR,* and *afsR*-p insert	This study

Table 2. Oligonucleotide primers used in this study

Oligonucleotide primers	Sequence	Function
ccaR for	CGTGGATCCTTCACAAGGGGGACCGCCA	5'-*ccaR* primer
ccaR rev	CGTTTCTGCAGCGTTGGTTCAGGGA	3'- *ccaR* primer
claR for	CGTCTGCAGGTGTCAGCCGATGCGA	5'- *claR* primer
claR rev	CGGTCTAGACCCGCTCAGCCGGACA	3- *claR* primer
afsR for	TCCGGAATTCCCGGCAGGGGGC	5'- *afsR* primer
afsR rev	CGAAGCTTCGGACCGAGCACGA	3'- *afsR* primer

393), where the similarity to other transcription factors is highest. The amino-terminal HTH (helix-turn-helix) domain resembles the highly conserved *N*-terminal amino acid in transcription activators usually starting between amino acids 4 and 12. The second HTH domain (HTH2) is located in the ClaR carboxy-terminus. The presence of these motifs indicates that ClaR may be involved in signal transduction in *S. clavuligerus*. As we could show, over-expression of *claR* had a stimulatory effect on the biosynthesis of CA. It is conceivable that ClaR somehow induced CA production by activating other genes involved in CA biosynthesis. We conclude that CA biosynthesis in *S. clavuligerus* is controlled by a cascade of regulatory proteins that include CcaR and ClaR.

Over-expression of *afsR-p* in *S. clavuligerus* NRRL3585

During a genomic analysis of *S. peucetius* ATCC 27952, we identified a homolog of *afsR* which we named *afsR-p*. The AfsR-p protein shares 60% of its amino acid residues with AfsR from *S. coelicolor* A3 [28]. We expressed *afsR-p* under the control of the *ermE** promoter (construct pGIBR) in *S. clavuligerus, S. peucetius, S. lividans* TK24, and *S. griseus*. All strains showed enhanced production of antibiotics in comparison to cells harboring only the empty vector. We also noted a 1.5- to 1.6-fold increase in the CA production in *S. clavuligerus* (Fig. 4). We speculate that phosphorylated AfsR-p binds to the promoter region of *afsS*, which activates downstream regulatory genes and induces the production of secondary metabolites.

Over-expression of *ccaR/claR/afsR-p* in *S. clavuligerus* NRRL 3585.

To assess the combined effect of *ccaR, claR,* and *afsR-p,* we generated a construct that incorporated all three genes. This was achieved by inserting a *Pst*I/*Xba*I fragment of *claR* gene into *Pst*I/*Xba*I-digested pIBRHL1 to generated plasmid pIBRHL12. Next, we subcloned an *Eco*RI/*Hin*dIII fragment of *afsR-p* into *Eco*RI/*Hin*dIII-digested pIBRHL12 to obtain the final construct pKS. Over-expression of pKS resulted only in a 1.45-fold increase in CA production, which was less than ether one of the three constructs (*i.e.*, pIBRHL1, pIBRHL2, and pGIBR) had generated by themselves (Fig. 4). In theory, *afsR-p* activates *afsS* in the AfsK/AfsR cascade, which in turn control the activity of other pathway-specific regulatory gene like *ccaR* and *claR*. While over-expression of combined *ccaR, claR,* and *afsR-p* was not additive, one cannot draw the conclusion that these regulatory genes only activated or downregulated CA production. A more complicated regulation by global and/or specific-pathway regulatory systems may underlie our observation, whose description requires more detailed experiments.

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