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Enhancement of Clavulanic Acid by Replicative and Integrative Expression of *ccaR* and *cas2* in *Streptomyces clavuligerus* NRRL3585

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Abstract Clavulanic acid (CA) is an inhibitor of β-lactamase that is produced from *Streptomyces clavuligerus* NRRL3585 and is used in combination with other antibiotics in clinical treatments. In order to increase the production of CA, the replicative and integrative expressions of *ccaR* (encoding for a specific regulator of the CA biosynthetic operon) and *cas2* (encoding for the rate-limiting enzyme in the CA biosynthetic pathway) were applied. Six recombinant plasmids were designed for this study. The pIBRHL1, pIBRHL3, and pIBRHL13 were constructed for overexpression, whereas pNQ3, pNQ2, and pNQ1 were constructed for chromosomal integration with *ccaR*, *cas2*, and *ccaR-cas2*, respectively. All of these plasmids were transformed into *S. clavuligerus* NRRL3585. CA production in transformants resulted in a significantly enhanced amount greater than that of the wild type, a 2.25-fold increase with pIBRHL1, a 9.28-fold increase with pNQ3, a 5.06-fold increase with pIBRHL3, a 2.93-fold increase with pNQ2 integration, a 5.79-fold increase with pIBRHL13, and a 23.8-fold increase with pNQ1. The integrative pNQ1 strain has been successfully applied to enhance production.

Keywords: *Streptomyces clavuligerus* NRRL 3585, clavulanic acid, overproduction, *cca*R, *cas*2

Streptomyces are Gram-positive filamentous bacteria, well known for producing a variety of secondary metabolites, including many clinically important antibiotics, antitumor agents, immunosupressors, enzyme inhibitors, etc. [6, 27, 28, 41]. *Streptomyces clavuligerus* is a soil bacterium with an unusual facility for the production of β-lactam metabolites such as isopenicillin N, desacetoxycephalosporin C, cephamycin C, clavulanic acid (CA), and certain other clavams [5, 11, 14]. Although clavulanic acid and the other clavams are structurally related to each other, only clavulanic acid is inhibitory to β-lactamases [3]. The structure for clavulanic acid (Fig. 1) and the relative stereochemistry have been unambiguously elucidated by X-ray crystallography. CA is unique in that it is the first reported naturally occurring fused β-lactam containing oxygen instead of sulfur, and it does not possess the acylamino side chain that is present in penicillins and cephalosporins [10]. CA is an important industrial product, because it irreversibly inactivates a wide range of β-lactamase enzymes. It is primarily administered in combination with other β-lactam antibiotics to combat infections, as in augmentin, which is a mixture of CA and amoxicillin [4, 31]. Enhancement of CA production, therefore, plays an important role for the commercial pharmaceutical market. However, CA produced by *S. clavuligerus* can be degraded during the bacterial cultivation. Inorganic ions, like ammonium salts (except ammonium hydroxide), and polar amino acids cause the instability of CA during cultivation [13, 16, 32]. There are three main ways to increase CA production: medium and condition optimizations for fermentation, reduction of degradation of CA during culture, and genetic enhancement of CA biosynthetic potentials such as the biosynthetic gene cluster and other regulatory genes [9, 21, 25]. In this study, we reported a large amount of CA production by genetic manipulation of the parental strain of *S. clavuligerus*.

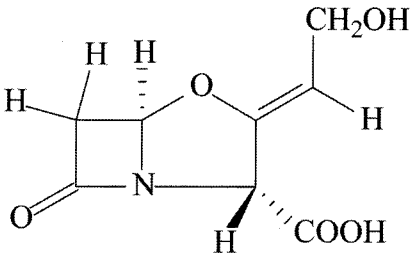

Fig. 1. Structure of clavulanic acid (CA).

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Thus far, 19 open-reading frames (ORFs) comprising a putative biosynthetic gene cluster of CA have been proposed [15, 18]. The biosynthesis of CA follows a pathway that is initiated by condensation of L-arginine with 3-phosphoglyceraldehyde and proceeds through a number of steps to finally form CA [39]. Among the enzymes of the CA biosynthetic pathway, clavaminate synthase (CAS) is one of the best understood with regard to its crystalline structure, mechanism, and activity [33, 40]. It is mainly responsible for three distinct oxidative transformations during the construction of intermediate clavaminate, including a stereochemical conformational alteration in the bicyclic β-lactam ring structure [38]. Sequence analysis in *S. clavuligerus* has revealed two isoenzymes, CAS1 and CAS2, that are highly similar but genomically separated into two genes, designated *cas*1 and *cas*2, respectively [20, 22, 24]. The *cas2* gene lies within the CA gene cluster, but the *cas1* gene does not. Despite the fact that CAS2 plays a crucial role in CA biosynthesis, there currently are no data on how this action may be enhanced. We, therefore, chose to genetically vary the number of copies of *cas*2 in an effort to improve CA production in this study. Furthermore, several problems have been surmounted by virtue of the manipulation of crucial genes operant in the biosynthetic pathway by overexpression in multicopy numbers of CA, particularly specific-pathway genes of the biosynthetic gene cluster such as *ccaR* and *claR* [29, 30]. Both *ccaR* and *claR* are specific regulatory genes. A *ccaR* gene that controls the productions of both cephamycin C and clavulanic acid has been found in the cephamycin gene cluster [29]. The production of CA is controlled by regulation of *ccaR*; it is also regulated by regulation of another specific-pathway regulatory gene, *claR*. However, *claR* encoding for a Lys-type transcriptional activator seems to regulate biosynthesis leading to CA at the late stages [30]. The function of *claR* is considered to have an important role in the regulation of conversions of biosynthetic intermediates. In fact, the mechanistic regulation of both *ccaR* and *claR* for the biosynthesis of CA is still far beyond our understanding. The biochemical and functional regulation of *ccaR* and *claR* are thought to likely modulate CA biosynthesis *via* a regulatory cascade.

Here, we report construction of the recombinant plasmids (*ccaR*, *cas2*, and *ccaR-cas2*) for their multicopy overexpression as well as their genomic integration in *S. clavuligerus* NRRL3585. A comparison of the effects on CA production between overexpression and genomic integration of the plasmids is presented.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli (*E. coli*) strains were grown at 37°C in Luria-Bertani (LB) media in both liquid and agar plates supplemented with the appropriate amount of antibiotics when necessary (ampicillin 100 μg/ml, apramycin 100 μg/ml, chloramphenicol 25 μg/ml, kanamycin 25 μg/ml, and tetracycline 12.5 μg/ml).

DNA manipulation was carried out in *E. coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.). Prior to transformation, *Streptomyces clavuligerus* NRRL3585 plasmids were propagated in *E. coli* ET12567/pUZ8002 [23], a nonmethylating (*dam dcm*$^-$ *hsdS*$^-$ Cm^r), to obtain unmethylated DNA. *Streptomyces clavuligerus* NRRL3585 was used as a clavulanic acid producer. Different media were used to culture *S. clavuligerus* for different purposes. *Streptomyces clavuligerus* NRRL3585 was grown in R2YE (per liter: sucrose, 103 g; K_2SO_4, 0.25 g; $MgCl_2 \cdot 6H_2O$, 10.12 g; Difco casamino acids, 0.1 g; trace element solution, 2 ml; Difco yeast extract, 5 g; and TES, 5.73 g) at 250 rpm and 28°C [17]. *S. clavuligerus* NRRL3585 was grown in 50 ml of R2YE medium in a 500-ml baffled flask at 28°C for the preparation of genomic DNA, whereas R2YE with 0.5% glycine was used for the preparation of protoplast. Five μg/ml of thiostrepton was added to the growth media to maintain plasmid-harboring *S. clavuligerus*. For CA production, the seed medium culture was composed of (per liter) glycerol, 20 ml; tryptic soy broth (Sigma Chemical Co), 20 g; and peptone, 5 g, at pH 7.0, and was referred to as TSB-P and cultured at 25 ml for 24 h. Then, 2.5 ml of growing seed culture was inoculated into the main CA production medium (50 ml), which was composed of (per liter) glycerol, 15 ml; tryptic soy broth, 30 g; peptone, 10 g; and MOPS, 10.5 g; and was cultured at pH 6.5 for up to 72 h.

Bacterial stocks were stored in 20%–40% glycerol at −70°C. All bacterial strains and a complete list of vectors and recombinant plasmids, including their relevant sources, are listed in Table 1.

DNA Isolation, Manipulation, and Analysis

Plasmids were isolated from *E. coli* by the alkaline lysis method and purified using Qiagen ion-exchange columns (Hilden, Germany). Standard methods were used for DNA cloning, plasmid isolation, and restriction enzyme digestion [17, 34]. Oligonucleotides used for the amplification of *ccaR* and *cas2* were identical to the ones described by Hung and colleagues [12]. Standard primers were used for DNA sequencing according to the dideoxynucleotide chain termination method using an automated DNA sequence analyzer. The computer-based analysis of nucleotide sequences was performed by the use of a BLAST database search. Primers used for the amplification of *acc(3)IV* were as follows: *acc(3)IV* forward: 5'-CAA TAC GAA TGG CGA AAA GCC GAG CT-3', and *acc(3)IV* reverse: 5'-CCT CTG GCG GAT GCA GGA AGA TCA A-3'. Polymerase chain reaction (PCR) was carried out in a total volume of 50 μl, containing 0.2 mmol deoxyribonucleotides,

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains & plasmid vectors	Relevant features	Sources or references
Bacterial strains		
S. clavuligerus		
NRRL3585	Wide type, cephamycin and clavulanic acid producer	ATCC
E. coli		
XL-1-Blue	General cloning host	Stratagene PBL
ET12567/pUZ8002	General cloning host	Strategene La Jolla, CA, U.S.A.
Plasmid vectors		
Cloning vectors		
pGEM®-T Easy		
Vector System I	*E. coli* general cloning vector, Amp^R	Promega, U.S.A.
pET-32a-c(+)	*E. coli* expression vector, Amp^R	Novagen
pGEM®-7Zf(−)	*E. coli* general cloning vector, Amp^R	Promega, U.S.A.
pIBR25	*Streptomyces* expression vector, contains thiostrepton resistance cassette and *ermE** promoter	Sthapit, 2004
pIBR27	*Streptomyces* expression vector, contains thiostrepton resistance cassette and *ermE** promoter	Hung, 2005
pSET152 *ermE**	*Streptomyces* integrative vector, apramycin and thiostrepton resistance	Parajuli *et al.*, 2005
Complimentation constructs		
pIBRHL1	*ccaR* gene inserted into pIBR25	Hung *et al.*, 2005
pIBRHL3	*cas2* gene inserted into pIBR27	Hung *et al.*, 2005
pIBRHL13	*ccaR-cas2* gene combination inserted into pIBR25	This study
pNQ2-1	*cas2* gene inserted into pET-32a-c(+)	This study
pNQ2-2	*cas2* gene inserted into pGEM®-7Zf(−)	This study
pNQ2	*cas2* gene inserted into pSET152 *ermE**	This study
pNQ3	*ccaR* gene inserted into pSET152 *ermE**	This study
pNQ1-1	*ccaR-cas2* gene inserted into pGEM®-7Zf(−)	This study
pNQ1	*ccaR-cas2* gene combination inserted into pSET152 *ermE**	This study

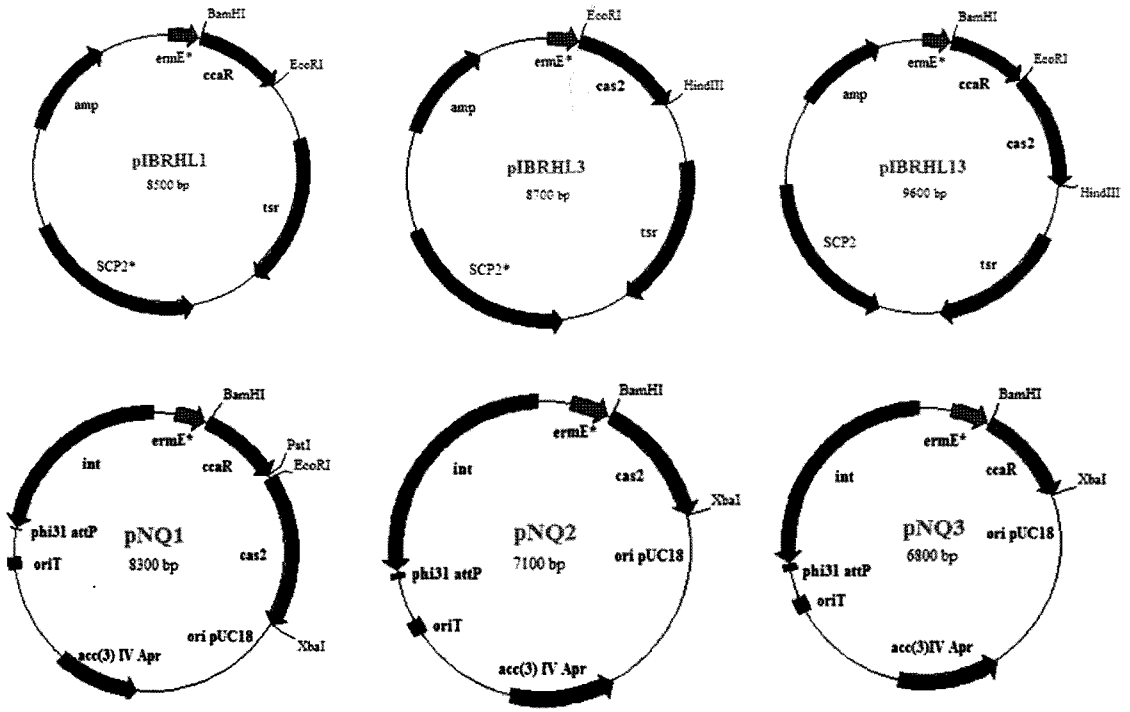

Fig. 2. The map of recombinant plasmids used for overexpression and integration in *S. clavuligerus* NRRL3585.

10 pmol of each primer, 1.5 mmol $MgCl_2$, and 1 unit of LA Taq polymerase (Takara, Japan), LA Taq buffer, and DNA template each. The PCR programs were subjected to 30 cycles of denaturation (95°C, 1 min), annealing (60°C to 64°C, 30 s), and elongation (72°C, 1 min). The purified PCR products were ligated into the pGEM-T-easy vector (Promega, U.S.A.) and sequenced.

Construction of Recombinant Plasmids

The pIBR25 [36] and pIBR27 vectors [12] were originally constructed for recombinant expression, whereas the pSET152 *ermE** vector [26] was created for genomic integration.

In brief, a 0.7-kb BamHI/PstI fragment of *ccaR* and a 1.1-kb EcoRI/HindIII fragment of *cas2* were cloned into BamHI/PstI-digested pIBR25 and EcoRI/HindIII-digested pIBR27, respectively, and they are referred to as pIBRHL1 and pIBRHL3 (Fig. 2). pIBRHL13 was formed by the insertion of a 1.1-kb EcoRI/HindIII-*cas2* gene into EcoRI/HindIII-digested pIBRHL1. The integration plasmids, pNQ3, pNQ2, and pNQ1, were constructed as follows. For the construction of pNQ2, a 1.1-kb EcoRI/HindIII fragment of *cas2* digested from pIBRHL3 was ligated into the digested EcoRI/HindIII pET-32a-c(+), resulting in the formation of pNQ2-1. The BamHI/HindIII fragment of *cas2* was then digested from pNQ2-1 and ligated into the digested BamHI/HindIII pGEM-7Zf(−), resulting in the generation of pNQ2-2. Finally, a BamHI/XbaI fragment of *cas2* from pNQ2-2 was inserted into BamHI/XbaI pSET152 *ermE**. For the construction of pNQ3, a BamHI/XbaI fragment of *ccaR* from pIBRHL1 was ligated into BamHI/XbaI pSET152 *ermE**. The plasmid pNQ1 was generated by a combination of *ccaR-cas2* into pSET152 *ermE**. To begin, a 1.9-kb BamHI/HindIII fragment of *ccaR-cas2* that was inserted into a digested BamHI/HindIII pGEM-7Zf(−) to form pNQ1-1 was then digested from pIBRHL13 with careful controlling of digestion. Next, the 1.9-kb BamHI/XbaI fragment of *ccaR-cas2* obtained from pNQ1-1 was cloned into a digested BamHI/XbaI pSET152 *ermE**, referred to as pNQ1 (Fig. 2).

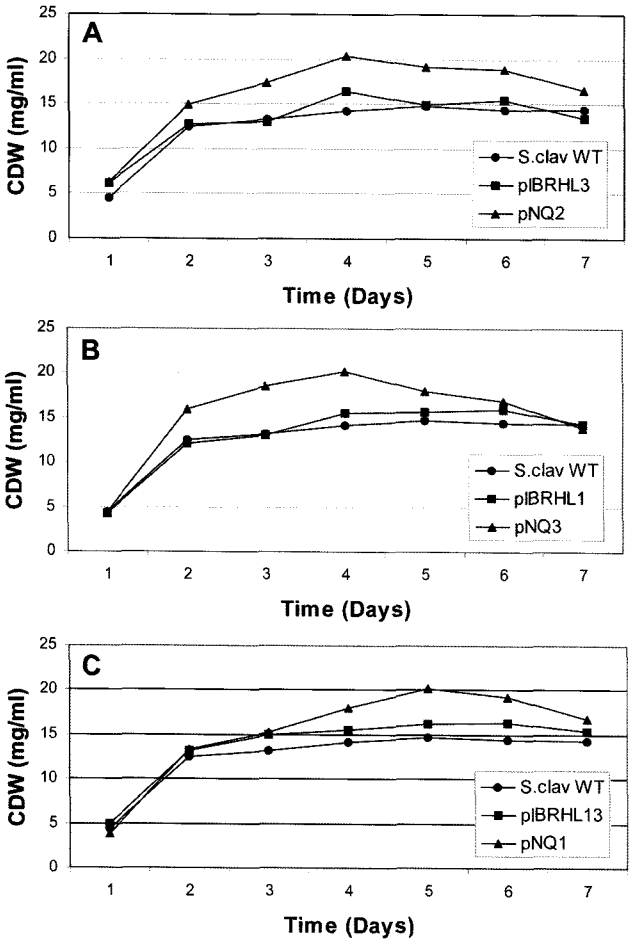

Fig. 3. Growth rate profiles. Growth curve obtained by overexpression and integration of (**A**) *cas2*, (**B**) *ccaR*, and (**C**) a *ccaR-cas2* combination in *S. clavuligerus* NRRL3585.
Growth curve: For chromosome integration strains (▲), overexpression strains (■), and wild type (●).

Protoplast Transformation, Analysis of Growth Rate, and CA Production

The conditions for protoplast formation, regeneration, and DNA transformation were slightly modified from the methods of Hopwood [8, 17]. After the formation of protoplasts, they were treated with 0.1 mM aurintricarboxylic acid (ATA) (Sigma) for 10 min before being mixed with the plasmids. Then, 200 μl of 40% (w/v) polyethylene glycol 1,000 (PEG, Merck-Shuchardt) solution was promptly added followed by a brief period of centrifugation to remove the PEG and resuspension in P-buffer. The transformed protoplasts were then plated on R2YE regeneration plates and incubated at 28°C for 24 h, at which time the plates were overlaid with a 0.3% agar solution containing 5 μg/ml of thiostrepton for the pIBRHL1, pIBRHL3, and pIBRHL13 transformants and 25 μg/ml of apramycin for the pNQ1, pNQ2, and pNQ3 transformants.

For the comparison of growth rates, *Streptomyces clavuligerus* NRRL3585 and transformant strains were activated in 50 ml of R2YE at 28°C and 230 rpm. After 48 h of incubation, 1 ml of seed medium culture was transferred to 50 ml of CA production media (TSB-P) (see Culture Conditions above). A 1-ml sample was taken every 24 h from each culture, and the cell pellet was used to determine the dry cell weight (DCW) as the biomass (Fig. 2).

Clavulanic acid production was measured for each transformed strain and its wild-type counterpart from individually cultured 50-ml samples of the incubation media (see above). After 48, 60, and 72 h of incubation, a 1-ml sample from each flask was centrifuged twice at 15,000 rpm to remove the mycelia and other insoluble ingredients. The supernatant was then immediately injected into a high-pressure liquid chromatography C_{18} reverse-

phase column (4.6×250 mm, 50 μm KANTO Reagents, Japan). The mobile phase was run with sodium phosphate buffer at pH 4.4. CA production was measured at 220 nm. Additional CA production measurements were performed as described by Foulstone and Reading [7]. All experimental procedures for analysis of growth rate and CA production were repeated three times.

Results and Discussion

Comparison of CA Production by Overexpression and Integration of *cas2* in *S. clavuligerus* NRRL3585

The biochemical function of *cas2* has been shown to catalyze three intermediate formation reactions within the CA biosynthetic pathway. Enzyme CAS2, encoded by *cas2*, is considered to be one of the most crucial enzymes in the CA biosynthetic pathway. Thus, expression of multicopy numbers of *cas2* was theoretically expected to augment CA production. To evaluate this theoretical prediction, CA production was elicited *via* overexpression in multicopy numbers and chromosomal integration. For that, the pIBRHL3 plasmid for overexpression and pNQ2 for the integrative chromosome carrying *cas2* were constructed. After transformation of the pIBRHL3 and pNQ2 plasmids, the transformants were selected for the measurement of CA production. Cell density was measured as dry weight. As shown in Fig. 3A, the biomass of pIBRHL3 transformants and wild-type strains were quite similar, but pNQ2 transformants showed higher biomass. For the pIBRHL3 transformants, a 5.06-fold increase in CA production was measured; pNQ2 transformants produced only a 3.0-fold increase compared with parental wild-type controls (Fig. 4A). In the case of integration, we confirmed these integrative chromosomes *via* the PCR of an *acc(3) IV* gene, which is located in the pSET152 *ermE**, from the total genome of pNQ2 transformants. The production of CA by CAS2 was more enhanced in the case of overexpression than in chromosome integration. A study to explain the mechanisms of this enhancement is still under way, but it has been found that the CAS2 protein may be expressed more strongly in terms of overexpression than in chromosomal integration.

Comparison of CA Production by Overexpression and Integration of *ccaR* in *S. clavuligerus* NRRL3585

The CcaR protein has been previously identified as a member of the *Streptomyces* antibiotic regulatory protein (SARP) family [35], which includes compounds such as actinorhodin [2], daunorubicin [19, 37], and cephamycin C [1]. This protein shows a high degree of homology with the protein encoding for Act-ORF4-like transcriptional regulator, which is well known to be essential for the biosynthesis of the antibiotics found in *S. coelicolor*. The

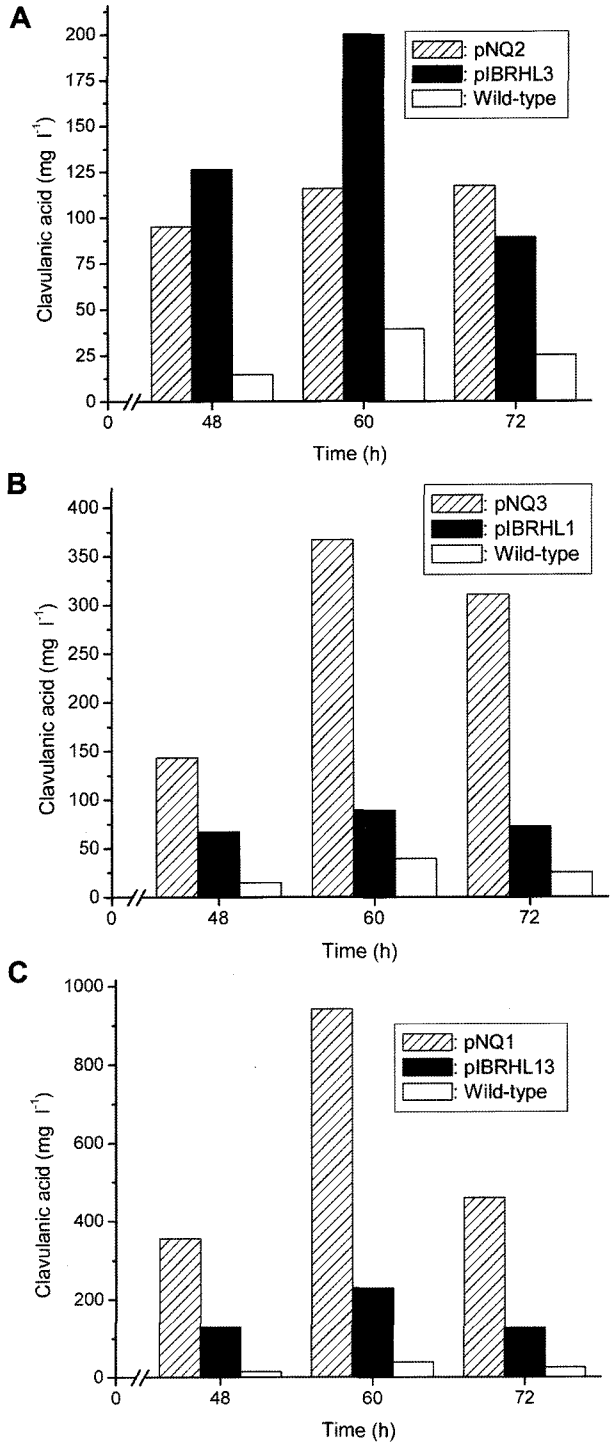

Fig. 4. Comparison of CA production obtained by overexpression or integration of (**A**) *cas2*, (**B**) *ccaR* , and (**C**) a *ccaR-cas2* combination in *S. clavuligerus* NRRL3585.
CA production obtained from chromosome integration strains (▨), CA production obtained from overexpression strains (■), and CA production obtained from wild-type strain (□).

ActII-ORF4-encoded protein activates the transcription of the *actIII-actI* and the *actVI-orf1-orfa* regions, which, in

turn, regulate actinorhodin biosynthesis [2]. The function of *ccaR* has been characterized relatively well in several previous studies [29, 35]. In this study, *ccaR* was amplified, and was either overexpressed in a high-copy number vector with a strong *ermE** promoter (pIBRHL1) or was integrated into the chromosome of *S. clavuligerus* (pNQ3). Improvements in CA production were assessed *via* HPLC by comparing the averaged results from pIBRHL1 and pNQ3 transformants to those of wild-type S. *clavuligerus* at 48, 60, and 72 h of culturing. The cell densities of transformants and wild-type strains are given in Fig. 3B. The cell density for the integration transformant was higher than those of the wild type and the expression transformants. The pIBRHL1 transformants presented with a 2.25-fold increase in CA production, whereas pNQ3 transformants improved CA production 9.4-fold over that of the controls (Fig. 4B). The result of increasing CA production from overexpression of *ccaR* (pIBRHL1) was similar to that of the previous report [29].

Several recent studies have attempted to address the manipulation of *ccaR*, as well as the mechanism operant in the activation of other genes within the biosynthetic gene cluster, in which *ccaR* plays a crucial role in the biosynthetic enzymatic pathway relevant to the production of CA. CcaR functions as an autoregulatory activator by binding with the *cefD-cmcI* bidirectional promoter. The *ccaR* sequence exhibits a high degree of homology with all β-lactam producers, all of which have been shown to possess a common mechanism for the modulation of biosynthetic pathways of β-lactam compounds in *Streptomyces* spp. In the super cephamycin C and CA gene clusters, the biochemical functions of *ccaR* take part as a specific-pathway regulator (CcaR) that controls the production of both cephamycin C and CA [35]. Our findings have suggested that CcaR may bind some promoters in the CA gene cluster to activate the cascade production of CA. To understand the regulatory mechanisms further, an investigation of protein-DNA interaction and detection of mRNA expression levels of all CA gene clusters from transformants are necessary.

Comparison of CA Production by Overexpression and Integration of *ccaR-cas2* in *S. clavuligerus* NRRL3585

Both the overexpression and integration of *cas2* and *ccaR* increase the production of CA as compared with that produced by wild-type strains. In addition, combinations of *ccaR* and *cas2* were also analyzed by either overexpression or integration in *S. clavuligerus*. Both genes were constructed into the pIBR25 expression vector and the pSET152 *ermE** integrative vector, which generated recombinant plasmids, pIBRHL13 and pNQ1, respectively. The pIBRHL13 transformants showed a 5.79-fold increase of CA production, whereas integrative pNQ1 transformants showed a 23.8-fold increase compared with the controls (Fig. 4C). The combination by overexpression of *ccaR-cas2* (5.79-fold increases) showed higher CA production than individual expression of *ccaR* (2.25-fold increases) or *cas2* (5.06-fold increases). The combination of *ccaR-cas2* by chromosomal integration also strongly increased CA production (23.8-fold), whereas integration of *ccaR* alone produced a 9.28-fold increase, and integration of *cas2* alone produced a 2.93-fold increase.

Overexpression and chromosome integration of pathway-specific regulators and key enzymes of a biosynthetic gene cluster are commonly used to enhance antibiotic production. The main disadvantage of overexpression is its limited applicability in industrial pharmaceutical engineering owing to its lack of stability over multiple generations. Chromosomal integration, by contrast, is inherently stable and, in our experiments, remains unchanged after twenty-five generations of pNQ1, pNQ2, or pNQ3 transformants. Therefore, we conclude that the chromosome integration approach is the preferred method to obtain high-titer CA production strains. Using this strategy, we have successfully generated an industrial pNQ1 transformant that could be applied in large-scale industrial production of CA. In addition, this model using the combination of a specific regulatory gene and a gene important in the biosynthetic pathway may have wide application to enhance the production of antibiotics.

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