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Enhancement of Clavulanic Acid Production by Expressing Regulatory Genes in *gap* Gene Deletion Mutant of *Streptomyces clavuligerus* NRRL3585

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***Streptomyces clavuligerus* NRRL3585 produces a clinically important β-lactamase inhibitor, clavulanic acid (CA). In order to increase the production of CA, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*gap*) was deleted in *S. clavuligerus* NRRL3585 to overcome the limited glyceraldehyde-3-phosphate pool; the replicative and integrative expressions of *cca*R (specific regulator of the CA biosynthetic operon) and *cla*R (Lys-type transcriptional activator) genes were transformed together into a deletion mutant to improve clavulanic acid production. We constructed two recombinant plasmids to enhance the production of CA in the *gap*1 deletion mutant of *S. clavuligerus* NRRL3585: pHN11 was constructed for overexpression of *cca*R–*cla*R, whereas pHN12 was constructed for their chromosomal integration. Both pHN11 and pHN12 transformants enhanced the production of CA by 2.59-fold and 5.85-fold, respectively, compared with the *gap*1 deletion mutant. For further enhancement of CA, we fed the pHN11 and pHN12 transformants ornithine and glycerol. Compared with the *gap*1 deletion mutant, ornithine increased CA production by 3.24- and 6.51-fold in the pHN11 and pHN12 transformants, respectively, glycerol increased CA by 2.96- and 6.21-fold, respectively, and ornithine and glycerol together increased CA by 3.72- and 7.02-fold, respectively.**

Keywords: *Streptomyces clavuligerus* NRRL3585, clavulanic acid, regulatory gene, glyceraldehyde-3-phosphate dehydrogenase, fed-batch fermentation, metabolic engineering

Clavulanic acid (CA) is a potent β-lactamase inhibitor produced by *Streptomyces clavuligerus* NRRL3585, a strain that also synthesizes cephamycin C (CC) [22] and a few other clavam structures [21]. Clavulanic acid is an important industrial product because it irreversibly inactivates a wide range of β-lactamases. When used in combination with conventional β-lactam antibiotics, it restores their effectiveness against antibiotic-resistant microbes. As a result, the biosynthesis of clavulanic acid and the genes associated with its production have come under intense investigation in recent years. Specific genes thought to be involved in rate-limiting steps can be duplicated by inserting the desired gene(s) into a chromosome by homologous recombination or by site-specific integration. In *S. clavuligerus*, gene dosage constructs of the biosynthetic genes *cea*S and *cas*2 resulted in recombinant strains with 60% and 100% higher clavulanic acid production, respectively, compared with the wild-type strain [20].

Enhancement of CA production is important for the commercial pharmaceutical market. There are three main ways to increase CA production: optimization of medium and conditions for fermentation, reduction of CA degradation during culture, and genetic enhancement of CA biosynthetic principals such as the biosynthetic gene cluster and other regulatory genes [9, 14, 17]. In this study, we increased the amount of CA production through genetic manipulation of the parental strain of *S. clavuligerus*. In fact, most studies involve manipulation of pathway-specific regulatory genes such as *cca*R and *cla*R, which directly regulate CA biosynthesis [16, 19]. The genes encoding CC and CA biosynthesis are genomically concentrated in the so-called β-lactam super cluster [1, 29], where the CC gene cluster is located upstream of the CA gene cluster. Previous studies have shown that the regulatory gene *cca*R controls the production of both CC and CA [18]. Disruption of *cca*R inhibits the biosynthesis of these antibiotics, which can be restored when the mutation is repaired [18]. In addition to being controlled by the *cca*R gene, CA biosynthesis is also regulated by *cla*R, which encodes a Lys-type transcriptional activator [19].

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Fed-batch fermentation is an approach aimed at efficiently carrying out fermentation for production of biomolecules. Industrial fermentation of most amino acids is accomplished with this method. Fed-batch culture has been used extensively to increase the productivity of microbial processes. Glycerol is one of the best carbon sources for clavulanic acid fermentation [4, 7, 10].

The primary metabolic precursors of clavulanic acid are D-glyceraldehyde-3-phosphate (G3P) [11] and L-arginine [28]. G3P is converted into 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glycolytic pathway and then enters the Krebs cycle through pyruvate. Thus, increasing the intracellular pool of G3P could enhance clavulanic acid production in *S. clavuligerus*. To achieve this, a new strategy has been to manipulate the primary metabolism of this organism through targeted inactivation of the gene encoding GAPDH to channel the carbon flux of G3P towards the clavulanic acid pathway [13]. Li and Townsend. [13] reported the identification of two new GAPDH (*gap*) genes in *S. clavuligerus* and constructed gap disruption mutants. They found a doubled production of CA in *gap*1 mutant but the *gap*2 disruptant produced a similar level of CA as the wild-type strain. They also fed arginine to the cultured mutant for the improvement of CA production, which resulted in a 2-fold increase of CA production over wild type. In this paper, the GAPDH (*gap*1) gene was disrupted in *S. clavuligerus* NRRL3585, and we report the construction of recombinant plasmids (*cca*R–*cla*R) for multicopy overexpression as well as genomic integration in the Δ*gap*1 mutant. A comparison of the effects of overexpression and genomic integration of the plasmids on CA production is presented. Here, we also focus on the effect of the interaction between glycerol and ornithine to enhance production of clavulanic acid in fermentation. An intermittent fed-batch operation was employed for individual or simultaneous feeding of glycerol and ornithine to the fermentation with different transformants.

MATERIALS AND METHODS

Bacterial Strains, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria–Bertani (LB) broth and maintained on LB agar medium at 37°C. DNA manipulation was carried out in *E. coli* XL1-Blue (Stratagene). Plasmids were propagated in *E. coli* ET12567 to obtain unmethylated DNA for transformation into *S. clavuligerus* NRRL3585. *S. clavuligerus* was cultured on different media according to the desired purpose. For protoplast transformation, 25 ml of TSB (1.7% tryptone, 0.3% soytone, 0.25% dextrose, 0.5% sodium chloride, and 0.25% potassium phosphate) supplemented with 1% (w/v) maltose was inoculated with a seed culture of *S. clavuligerus* at 28°C for 24 h. The growing seed culture was then transferred to 50 ml of R2YE medium (5% sucrose, 0.02% potassium sulfate, 1% magnesium chloride, 1% glucose, 0.5% yeast extract, and 0.01% Difco casamino acid) at 250 rpm and 28°C for 36 h. *Streptomyces* transformants were supplemented with apramycin (25 μg/ml) or neomycin (20 μg/ml). For CA production, transformants were cultured in medium composed of 2.0% glycerol, 3% tryptic soy broth, 1% peptone, and 1.05% MOPS at pH 6.5 for up to 120 h. All bacterial stocks were stored in 30% glycerol at −70°C.

DNA Isolation, Manipulation, and Analysis

The pGEM-T Easy and pGEM-3Zf(+) (Promega) vectors were the routine cloning vectors used for DNA manipulation. pKC1139 [12] was used for the disruption of *gap*1. The pIBR25 vector [27] was used for overexpression of pHN11 (harboring *ccaR/claR*) and the

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Description	Source or References
Streptomyces clavuligerus	Wild-type clavulanic acid and 5S clavam producer	NRRL3585
S. clavuligerus/Δ*gap*1	*gap*1 insertional disruption mutant	This study
S. clavuligerus/Δ*gap*1/pIBR25	pIBR25 vector overexpressed into *S. clavuligerus*/Δ*gap*1	This study
S. clavuligerus/Δ*gap*1/pSET152	Integration of pSET152 vector into *S. clavuligerus*/Δ*gap*1	This study
S. clavuligerus/Δ*gap*1/pHN11	pIBR25 with *cca*R and *cla*R genes overexpressed into *S. clavuligerus*/Δ*gap*1	This study
S. clavuligerus/Δ*gap*1/pHN12	pSET152 with *cca*R and *cla*R genes integrated into *S. clavuligerus*/Δ*gap*1	This study
E. coli XL1 Blue	General cloning host	Stratagene
E. coli ET 12456	General cloning host	Stratagene
pGEM-T easy vector	*E. coli* general cloning vector, *amp*^{r}	Promega, U.S.A.
pHN9	Disruption construct carrying *gap*1 disrupted with *neo*^{r}	This study
pKC1139	*Streptomyces*–*E. coli* bifunctional vector, *apr*^{r}	Stratagene
pSET152	*E. coli* cloning vector, integrates into *Streptomyces* chromosome, *apr*^{r}	[5]
pIBR25	*E. coli*–*Streptomcyes* shuttle vector	[27]
pHN11	pIBR25 with *cca*R and *cla*R	This study
pHN12	pSET152 with *cca*R and *cla*R	This study

pSET152 vector [5] was used for integration of pHN12 (harboring *ccaR/claR*) in the *gap*1 mutant. 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 [12, 25]. Oligonucleotide primers were synthesized at Geno-Tech (Korea), and the enzymes were obtained from Takara (Japan). All chemicals were of molecular biology grade and commercially available. Southern hybridization was performed with Hybond N nylon membranes (Amersham). Probes were labeled with digoxigenin using a DIG labeling and detection kit (Boehringer Mannheim).

Disruption of Gap1

The *gap*1 gene was disrupted using a homologous recombination approach with pKC1139. For the inactivation of *gap*1 in *S. clavuligerus*, the upstream fragment, *gap*1-U (682 bp), and the downstream fragment, *gap*1-D (671 bp), were amplified by PCR. The primer pairs are shown in Table 2. The amplified DNA fragment of *gap*1-U was digested with HindIII and XbaI and cloned into the corresponding sites of pKC1139, resulting in pUC1. The PCR fragment *gap*1-D was digested with XbaI and EcoRI and ligated into the same sites of pUC1 to yield pUD. pUD was digested with XbaI and ligated with the fragment of the neomycin-resistance gene, resulting in pHN9. In this final construct, pHN9, *gap*1 was replaced by *neo*^{r} in-frame. pHN9 was finally transformed into the *E. coli* ET12567 demethylation host and then transformed into wild-type *S. clavuligerus* NRRL3585 for deletion by replicative plasmid-mediated homologous recombination. The conditions for protoplast formation, regeneration, and DNA transformation were slightly modified from the methods of Hopwood [8, 12]. After formation, protoplasts 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 brief 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. After 24 h, the plates were overlaid with 0.3% agar solution containing 20 μg/ml of neomycin to obtain the pHN9 transformants.

Screening for Disruption Mutants *S. clavuligerus*/Δ*gap1* and Southern Blot Hybridization

After the fifteenth generation of selection at 37°C, more then 3,500 colonies were picked at random, and each colony was patched out on separate R2YE plates containing neomycin (20 μg/ml) or apramycin (25 μg/ml). One colony displayed the double-crossover phenotype of neomycin resistance and apramycin sensitivity. The disrupted *gap*1 was confirmed by Southern blot hybridization analysis *via* standard protocols [12]. Hybridization was performed under high stringency conditions (0.5×SSC, 68°C) as described elsewhere (Roche) by using probe labeled with the Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Boehringer) and Southern blot analysis on Hybond-N nylon membranes (Amersham). A chromogenic method was used for the detection of probes on a blot.

Construction of Recombinant Plasmids and Transformation into *S. clavuligerus*/Δ*gap1*

The plasmids pHN11 and pHN12 were generated by combining *cca*R–*cla*R into pIBR25 and pSET152 *ermE**, respectively. Oligonucleotides used for the amplification are shown in Table 2. The purified PCR products were first inserted into the pGEM-T-Easy vector (Promega) and then subcloned into their respective plasmids. A 771 bp BamHI/PstI fragment of *cca*R was cloned into BamHI/PstI-digested pIBR25 and designated as pHN10. A 1.29 kb PstI/XbaI fragment of *cla*R was cloned into the same sites of pHN10 and referred to as pHN11. Next, the 2.07 kb BamHI/XbaI fragment of *cca*R–*cla*R obtained from pHN11 was cloned into BamHI/XbaI-digested pSET152 *ermE** and referred to as pHN12. pHN11, pHN12, pIBR25, and pSET152 were transformed into the *E. coli* ET12567 demethylation host and then again transformed into *S. clavuligerus*/Δ*gap*1. These transformants were designated *S. clavuligerus*/Δ*gap*1/pHN11, *S. clavuligerus*/Δ*gap*1/pHN12, *S. clavuligerus*/Δ*gap*1/pIBR25, and *clavuligerus*/Δ*gap*1/pSET152, respectively. Transformants were obtained by selection with 5 μg/ml thiostrepton and 25 μg/ml apramycin.

Fed-Batch Culture

Feeding experiments were started after 40 h of batch culture. Two ml of feed solution was added each time; that is 2% glycerol and 10 mM ornithine, respectively, were added every 12 h. Samples were withdrawn at a 12 h interval until the end of the cultivation.

Analysis of CA Production

A 2 ml sample was taken from each culture once every 12 h during the fermentation process (48 h), and the supernatant was used for CA assays [2, 21, 23]. CA was analyzed by reaction with imidazole [6] in which the filtered fermentation supernatant (90 ml) was reacted with 30 ml of 3 M imidazole solution (pH 6.8) at 37°C for 40 min. The imidazole derivative was diluted 15-fold with ddH_2O, and its absorbance was measured at 312 nm [13]. CA yields in *S. clavuligerus* strains were determined by comparison with a calibration curve generated from a pure CA standard. CA was also analyzed by high-performance liquid chromatography (HPLC) using a C-18

Table 2. Oligonucleotide primers used in this study.

PCR products	Primer sequence (5'-3')	Purpose
*gap*1-U	F: AT**AAGCTT**CGCGTATGTCACCGACTCGGG R: AG**TCTAGA**ATGGTGTCGTACTTCAGCAG	Amplification of *gap*1 upstream region
*gap*1-D	F: AT**TCTAGA**ACCAGGACACCTACGACCCG R: AT**GAATTC**CCCTTTCGCCCTTCACTGAG	Amplification of *gap*1 downstream region
*cca*R	F: CT**GGATCC**TTCACAAGGGGGACCGCCA R: AT**CTGCAG**TCAGGCCGGGGTACCGACCC	Amplification of *cca*R
*cla*R	F: TA**CTGCAG**GTGTCAGCCGATGCGATCTG R: G**TCTAGA**TCAGCCGGACATCCGGGCCCCG	Amplification of *cla*R

reversed-phase column (4.6×250 mm, 50 μm KANTO Reagents, Japan) with detection at 312 nm. The mobile phase was consisted of 0.1 M KH_2PO_4 (pH 3.3 adjusted with H_3PO_4) and 6% methanol (flow rate 1 ml/min). The mycelium dry weights of *S. clavuligerus*/Δ*gap1*/pHN11, *S. clavuligerus*/Δ*gap1*/pHN12, *S. clavuligerus*/Δ*gap1*/pIBR25, and *clavuligerus*/Δ*gap1*/pSET152 were determined at 12 h intervals in CA production media. Mycelia in a 1 ml culture were harvested by centrifugation at 12,000 ×*g* for 15 min. After two washes with sterile ddH_2O, the mycelia were dried at 100°C to constant weight.

RESULTS AND DISCUSSION

Construction of Recombinant Plasmid pHN9, and Transformation and Generation of *S. clavuligerus*/Δ*gap1*

To construct the *gap*1 disruption plasmid, we used pKC1139, which has a temperature-sensitive replication origin. The plasmid (pHN9) was constructed as described in Materials and Methods. The genetic organization and the restriction endonuclease map are shown in Fig. 1A. pHN9 was introduced into *S. clavuligerus* by PEG-mediated protoplast transformation. After plasmid curing, one putative mutant (*S. clavuligerus*/Δ*gap*1) that was apramycin-sensitive and neomycin-resistant was selected after the fifteenth generation. For this selection process, more then 3,500 colonies were picked at random. Insertional inactivation of *gap*1 *via* double-crossover was verified by Southern blot hybridization using a DNA fragment from the upstream region of *gap*1 as the probe (Fig. 1B). Restriction with NcoI resulted in a hybridization band that was detected at 1.4 kb when probing wild-type DNA or a 1.35 kb signal from the mutants (Fig. 1B). The genetic exchange was further confirmed by PCR amplification of the neomycin marker gene from the genomic DNA of the apramycin-sensitive colonies (data not shown).

Transformation of pHN11 and pHN12 into *S. clavuligerus*/Δ*gap1*

In order to determine the effects of pHN11 and pHN12 harboring *cca*R and *cla*R, these plasmids were introduced into *S. clavuligerus*/Δ*gap*1 by protoplast transformation. Transformants were confirmed by both enzyme digestion and PCR amplification of the respective genes from recombinant plasmids after isolation of plasmid from transformants. The cell densities of transformants and mutant strains are given in Fig. 2A. The cell density of the integration transformant was higher than those of the mutant and the expression transformant (Fig. 2A).

Comparison of CA Production by Overexpression and Integration of *ccaR* and *claR* together in *S. clavuligerus*/Δ*gap1*

CcaR is a positive-acting transcriptional regulator involved in cephamycin C and clavulanic acid biosynthesis in *Streptomyces clavuligerus* [1]. This protein shows a high degree of homology with the protein encoding an Act-ORF4-like transcriptional regulator, which is known to be essential for the biosynthesis of the antibiotics found in *S. coelicolor*. The ActII-ORF4-encoded protein activates the transcription of the *act*III-*act*I and the *act*VI-*orf*1-*orf*a regions, which in turn regulate actinorhodin biosynthesis

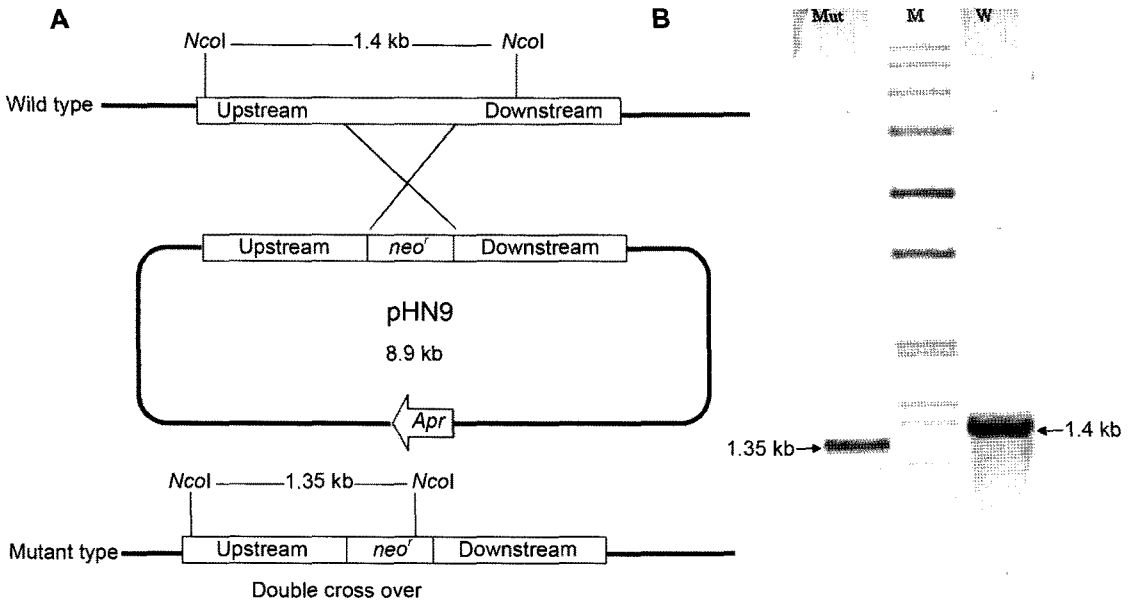

Fig. 1. Generation of *gap*1-deleted mutants (*S. clavuligerus*/Δ*gap*1) *via* double-crossover integration of pHN9 (**A**) and Southern analysis of genomic DNA from wild-type and *gap*1-deleted mutants after NcoI digestion (**B**).
Lane Mut, Δ*gap1* mutants; lane M, molecular size marker; and lane W, wild-type.

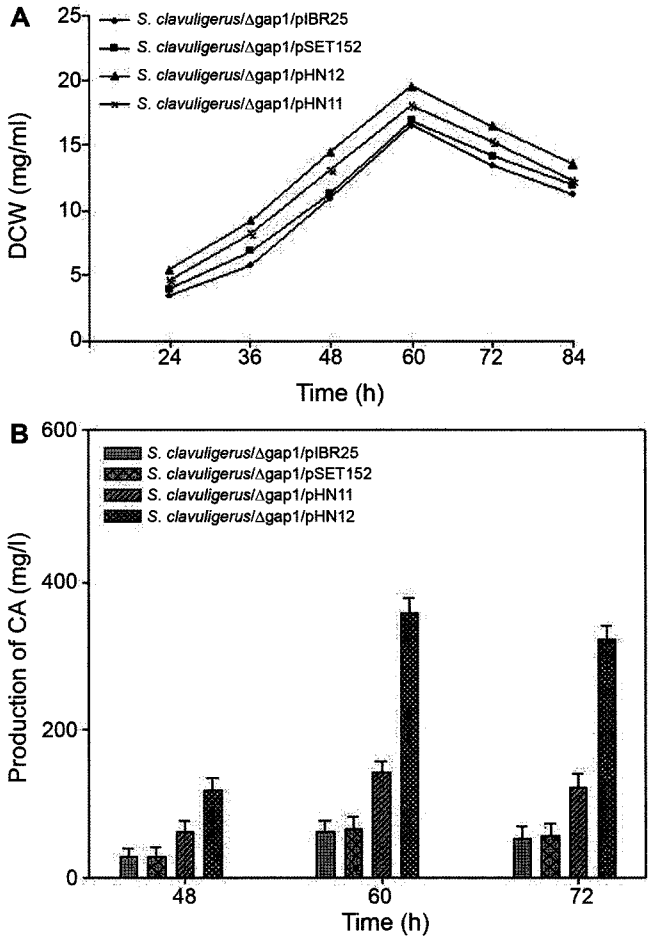

Fig. 2. Comparison of dry weights (**A**) and CA products (**B**) from control and transformants.

Measurement of CA products at different growth stages (exponential phase, 48 h; stationary phase, 60 h; phase of decline, 72 h). Symbols: ▒, from *S. clavuligerus*/Δ*gap*1/pIBR25; ▒, from *S. clavuligerus*/Δ*gap*1/pSET152; ▒, from *S. clavuligerus*/Δ*gap*1/pHN11 harboring *ccaR* and *claR*; and ▒, from *S. clavuligerus*/Δ*gap*1/pHN12 harboring *ccaR* and *claR*.

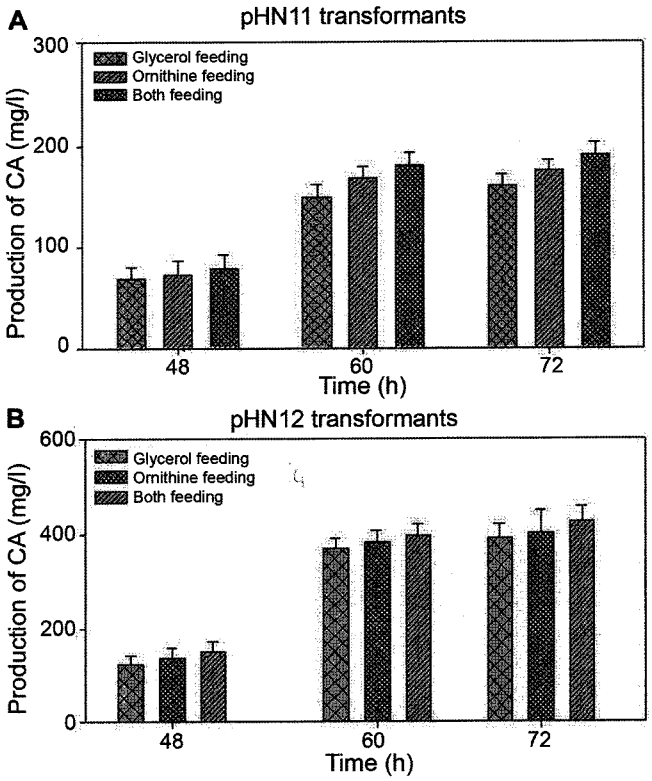

Fig. 3. Effects of glycerol, ornithine, and both combined on clavulanic acid production in long-term cultures of (**A**) *S. clavuligerus*/Δ*gap*1/pHN11 (▒, glycerol; ▒, ornithine; and ▒, both); and (**B**) *S. clavuligerus*/Δ*gap*1/pHN12 (▒, glycerol; ▒, ornithine; and ▒, both).

[3]. It is known that *cla*R is a pathway-specific gene, and its product is involved in CA biosynthesis. *cca*R controls the expression of the early stage of CA biosynthetic genes whereas *cla*R transcriptional regulator is essential for expression of the late genes of clavulanic acid biosynthesis. Considering this, when *cca*R and *cla*R are expressed together, the early stage and late stage of CA biosynthesis genes will transcribe with additional stimulatory effect and activate other genes involved in CA biosynthesis, resulting in the higher titer of CA production. To assess the combined effect of *cca*R and *cla*R, we generated a construct that incorporated both genes. For this, both genes were inserted into the pIBR25 expression vector and the pSET152 *ermE** integrative vector to generate the recombinant plasmids, pHN11 and pHN12, respectively. We expressed and integrated pHN11 and pHN12, respectively, into *S. clavuligerus*/Δ*gap*1 under the control of the *ermE** promoter. We found that CA production was increased by 1.9-fold in *S. clavuligerus*/Δ*gap*1 compared with that in the parental strains. Compared with the mutant, the pHN11 transformants showed a 2.59-fold increase of CA production, whereas integrative pHN12 transformants showed a 5.85-fold increase (Fig. 2B).

Several recent studies have attempted to address the manipulation of *cca*R, as well as the operant mechanism in the activation of other genes within the biosynthetic gene cluster in which *cca*R 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 *cef*D–*cmc*I bidirectional promoter. The *cca*R sequence exhibits a high degree of homology with the 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, *cca*R functions as a specific pathway regulator (CcaR) that controls the production of both cephamycin C and CA [26]. Analysis of the ClaR protein suggests that ClaR is a LysR-type transcriptional factor. Several pathway-specific regulatory genes have been identified in antibiotic-producing actinomycetes. There are two motifs at the N-

and the C-terminal ends of ClaR (amino acids residues 7–51 and 338–393) in which the similarity to other transcription factors is highest. The N-terminal HTH (helix-turn-helix) domain resembles the highly conserved N-terminal amino acids of transcription activators, which usually start between amino acids 4 and 12. The second HTH domain (HTH2) is located in the ClaR C-terminus. The presence of these motifs indicates that ClaR may be involved in signal transduction in *S. clavuligerus*. As we demonstrated here, overexpression and integration of CcaR and ClaR had a stimulatory effect on the biosynthesis of CA. It is conceivable that CcaR and 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 including CcaR and ClaR. Our findings suggest that CcaR and ClaR may bind to promoters in the CA gene cluster to activate the cascade of CA production. To further understand the regulatory mechanisms, an investigation of protein–DNA interactions and the detection of mRNA expression levels from all CA gene clusters in the transformants are necessary. The regulatory network is very complicated, and thus a thorough analysis of the regulatory network structure is essential for a complete understanding of the gene regulatory pattern and morphology, including physiological development, in *Streptomyces* and other complex microorganisms. Ultimately, it will provide new strategies for manipulating secondary metabolism and for increasing the production of valuable biologically active natural products. Generally, overexpression and chromosomal integration of pathway-specific regulators and key enzymes in a biosynthetic gene cluster are used to enhance antibiotic production. The main disadvantage of overexpression is its limited applicability due to the lack of stability over multiple generations. In contrast, chromosomal integration is inherently stable and, in our experiments, remains unchanged through fifteen generations of pHN12 transformants. It can be described in the sense that it may directly integrate into promoters of the clavulanic acid gene cluster. The integrated regulatory genes may strongly activate the genes involved in the CA biosynthesis to transcribe with additional stimulatory effect. A little contribution to increase CA production to a smaller extent may have been supported by the growth rate also, because the integrated strains showed little higher growth rate as compared with that of the replicated one. (Fig. 2A). Therefore, we conclude that chromosomal integration is the preferred method for obtaining high-titer CA production strains.

Effects of Ornithine and Glycerol Feeding in Shake-Flask Culture

Clavulanic acid biosynthesis can be stimulated by glycerol and ornithine, considering the fact that the feeding of glycerol during fermentation could maintain cell metabolism, and thereby enhance effectively the clavulanic acid production. In addition to this, when ornithine was added, the unusual presence of the urea cycle in prokaryotes would provide an abundant supply of arginine, the C5 precursor to clavulanic acid. Not only did ornithine reduce the anaplerotic carbon flux of C3 to the synthesis of C5-amino acid, but it was also required for removal of ammonia from cells resulting in toxic intracellular concentration [15]. Furthermore, Romero *et al.* [24] reported that ornithine strongly inhibits cephamycin biosynthesis, another β-lactam antibiotic, produced by the same strain. Therefore, the feeding of ornithine could allow more C3 precursor for the clavulanic acid biosynthesis. Considering this fact, we came to a conclusion to feed glycerol and ornithine into transformants to achieve the higher yield of clavulanic acid production. Addition of 10 mM ornithine to the culture increased the CA production by about 3.24- and 6.51-fold in the pHN11 and pHN12 transformants, respectively, compared with that without addition of amino acids. In the fed-batch experiments, after feeding 2% glycerol to the culture, the CA production was increased by about 2.96- and 6.21-fold in the pHN11 and pHN12 transformants, respectively, compared with that of no addition of glycerol. Feeding glycerol and ornithine resulted in a further increase in clavulanic acid production to 3.72- and 7.02-fold in the pHN11 and pHN12 transformants, respectively. Ornithine provided a sufficient supply of arginine for clavulanic acid production. It is well documented that pyruvate acts as a C3 precursor, whereas arginine acts as the C5 precursor for clavulanic acid production [10]. In fermenter batch culture, degradation of clavulanic acid began after 72 h. In fed-batch cultures fed glycerol and ornithine, clavulanic acid production was not only increased, but also remained stable for up to 120 h. Triplicate cultures were always used and all samples were analyzed in duplicate.

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