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Functional Effects of Increased Copy Number of the Gene Encoding Proclavaminate Amidino Hydrolase on Clavulanic Acid Production in *Streptomyces clavuligerus* ATCC 27064

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The effect of increasing levels of proclavaminate amidino hydrolase (Pah) on the rate of clavulanic acid production in *Streptomyces clavuligerus* ATCC 27064 was evaluated by increasing dosoge of a gene (*pah2*) encoding Pah. A strain (SMF5703) harboring a multicopy plasmid containing the *pah2* gene showed significantly retarded cell growth and reduced clavulanic acid production, possibly attributable to the deleterious effects of the multicopy plasmid. In contrast, a strain (SMF5704) carrying a single additional copy of *pah2* introduced into chromosome *via* an integrative plasmid showed enhanced production of clavulanic acid and increased levels of *pah2* transcripts. Analysis of transcripts of other genes involved in the clavulanic acid biosynthetic pathway revealed a pattern similar to that seen in the parent. From these results, it appears that clavulanic acid production can be enhanced by duplication of *pah2* through integration of a second copy of the gene into chromosome. However, increasing the copy number of only one gene, such as *pah2*, does not affect the expression of other pathway genes, and so only modest improvements in clavulanic acid production can be expected. Flux controlled by Pah did increase when the copy number of *pah2* was doubled, suggesting that under these growth conditions, Pah levels may be a limiting factor regulating the rate of clavulanic acid biosynthesis in *S. clavuligerus*.

Keywords: *Streptomyces clavuligerus* ATCC 27064, proclavaminate amidino hydrolase, clavulanic acid

Streptomyces clavuligerus is an industrially important microorganism producing a variety of β-lactam antibiotics, including clavulanic acid. In combination with amoxicillin, clavulanic acid has been used effectively for the treatment of diseases caused by various pathogenic microorganisms that would otherwise be resistant to β-lactam antibiotics [4]. The world market for clavulanic acid is large and increasing, resulting in considerable research interest in ways to produce the compound more economically [33]. The biosynthetic pathway leading from the primary metabolites L-arginine and 3-phosphoglyceraldehyde to clavulanic acid, as shown in Fig. 1 [14, 35], is still under intensive investigation.

The first step in the biosynthetic pathway gives rise to N^2-(2-carboxyethyl)-arginine and is catalyzed by carboxyethylarginine synthase (Ceas) [14, 25]. β-lactam synthetase (Bls) catalyzes the second step converting carboxyethylarginine to deoxyguanidinoproclavaminate, which now contains the β-lactam ring [1]. Deoxyguanidinoproclavaminate is then hydroxylated to give guanidinoproclavaminate by clavaminate synthase (Cas) [2, 17]. Subsequently, proclavaminic acid amidino hydrolase (Pah) catalyzes the reaction from guanidinoproclavaminate to proclavaminic acid, where the guanidino group from the arginine-derived end of the molecule is removed [6]. Clavaminate synthase (Cas) then catalyzes the formation of the first bicyclic intermediate through oxidative ring closure of proclavaminic acid to give dihydroclavaminate followed by desaturation to form clavaminate [2, 30]. Clavaldehyde, the last known intermediate of the pathway, ultimately gets converted to clavulanic acid by clavulanic acid dehydrogenase (Cad) [19]. However, the mechanism by which clavaminate is converted to clavaldehyde is still not clear. Furthermore, the pathways leading from clavaminate to the four other clavam metabolites produced by *S. clavuligerus* are also largely unknown.

Identification of essential genes and determination of their functions in the biosynthetic pathways for clavulanic acid and the other clavams in *S. clavuligerus* are necessary first steps that then may make possible the metabolic engineering of the pathway for increased production of

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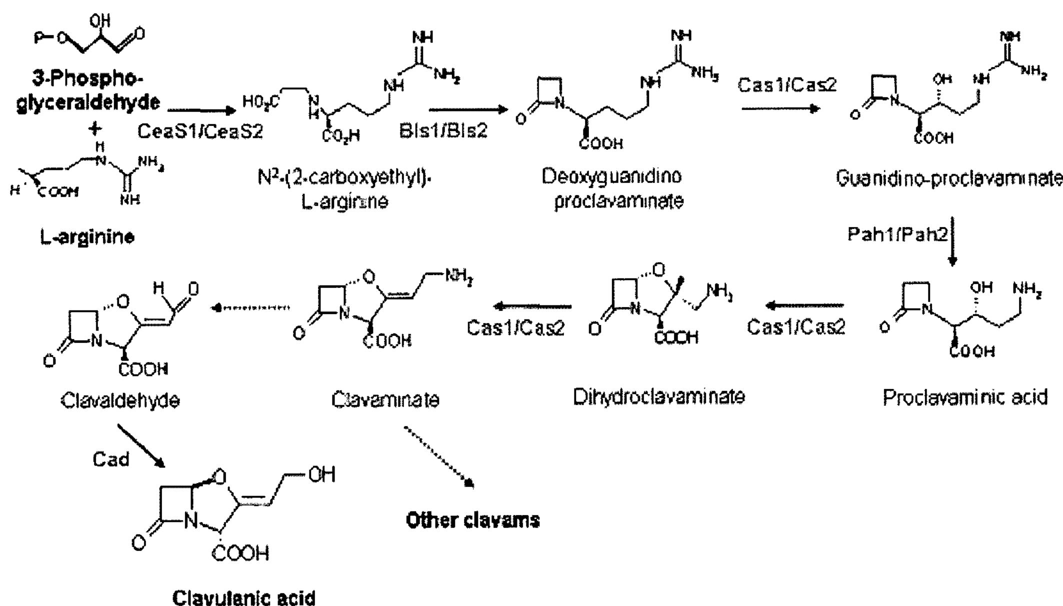

Fig. 1. Pathway for clavulanic acid biosynthesis in *S. clavuligerus*.

Ceas1/Ceas2 (*N*-carboxyethylarginine synthase), Bls1/Bls2 (β-lactam synthetase), Cas1/Cas2 (clavaminate synthase), and Pah1/Pah2 (proclavaminate amidino hydrolase) are enzymes critically involved in the early steps of production of clavulanic acid and clavams. Cad (clavulanate-9-aldehyde reductase) catalyzes the last step of clavulanic acid production.

desired products. For instance, the transcriptional regulator CcaR regulates expression of genes responsible for both clavulanic acid and cephamycin production, and overproduction of CcaR in *S. clavuligerus* resulted in yields of cephamycin and clavulanic acid that were increased by about three-fold, respectively [23]. On the other hand, clavaminic acid production can be increased by the mutation of ClaR, a regulator that was identified as controlling the expression of genes involved in the late stages of clavulanic acid biosynthesis [21]. Conversely, amplification of the *claR* gene on a multicopy plasmid resulted in a three-fold increase in clavulanic acid production and a five- to six-fold increase in alanylclavam biosynthesis, whereas cephamycin production was significantly reduced [25].

In addition to effects caused by manipulation of regulatory genes, deletion or amplification of genes encoding biosynthetic enzymes has also proven to affect clavulanic acid production. For instance, an earlier onset and higher production of clavulanic acid was made possible by the specific amplification of *ceas*, encoding carboxyethylarginine synthase, the first enzyme in the biosynthetic pathway. Amplification of *ceas* also resulted in reduced production of cephamycin, whereas deletion of *ceas* gave rise to increased cephamycin production [25]. Recently, it was reported that clavulanic acid production was increased about 24-fold through combinatorial expression of *ccaR* and *cas2* genes [10].

Disruption of genes related to formation of undesirable metabolites from a common intermediate is another possibility to consider for the overproduction of clavulanic acid or cephamycin. A specific deletion of the *lat* gene encoding lysine epsilon-aminotransferase, an essential enzyme involved in cephamycin biosynthesis, resulted in a 2- to 2.5-fold increase in clavulanic acid production [22]. Similarly, increasing the copy number of certain clavulanic acid biosynthetic genes such as *orf10*, *orf11*, *orf12*, and *orf14* by introduction on multiple copy expression plasmids showed beneficial effects for clavulanic acid production, although the functions of the *orfs* in clavulanic acid production are not well understood [18].

Since the introduction of various biosynthetic or regulatory genes at multiple copy has been shown to improve clavulanic acid production, the identity of the rate-limiting step is not clear. On the contrary, since several different genes have resulted in increased production when any one was introduced at multiple copy, this may suggest that some interaction between the genes or gene products controls the overall pathway throughput. In this regard, no investigation has yet been made of the effects of increasing the gene dosage of *pah*. Pah enzyme activity is required for clavulanic acid biosynthesis, and two paralogous *pah* genes, *pah1* and *pah2*, have been identified from the genome of *S. clavuligerus*. *pah2* and *pah1*, located in the clavulanic acid gene cluster and the paralog gene cluster, respectively, show 72% similarity [13]. The *pah* genes also show appreciable sequence similarity to agmatine ureohydrolase and other arginases [37], and so the Pah protein was classified as a member of the evolutionarily divergent arginase family, but was suggested to have evolved to bind β-lactams rather than the more typical amino acid substrate of the arginases [20]. The fact that both *pah1* and *pah2* encode functional Pah enzymes is evident from the fact that mutation of either gene alone causes

only a partial reduction of clavulanic acid production, whereas mutation of both genes causes a complete loss of production of both clavulanic acid and the other clavams [13].

In this study, we examined the effects of overexpression of *pah2* on the production of clavulanic acid. To achieve this aim, we cloned the *pah2* gene on seperately a multicopy and an integrative vector and introduced both into *S. clavuligerus* ATCC 27064. Profiles of the transcripts of *pah2* and of other clavulanic acid biosynthetic genes from cultures carrying the integrated *pah2* gene were compared by RT-PCR, and the fermentation kinetics were examined in order to demonstrate the importance of the *pah2* gene in clavulanic acid production.

MATERIALS AND METHODS

Microorganisms and Plasmids Used

S. clavuligerus ATCC 27064, the wild-type strain, was maintained on ISP4 agar medium for immediate use, and spores formed on ISP4 medium were suspended in 20% (w/v) glycerol and stored at −70°C for long-term preservation. Mutants (SMF5703 and SMF5704) of *S. clavuligerus* constructed were preserved on agar plates of medium ISP4 containing apramycin (25 µg/ml). Strains of *E. coli* carrying plasmids were grown in Luria-Bertani (LB) medium containing antibiotics as appropriate, for immediate use, and cells were suspended in 20% (w/v) glycerol and stored at −70°C for long-term preservation. All strains and plasmids used in this work are listed in Table 1.

General Gene Manipulation

DNA manipulation in *E. coli* and *S. clavuligerus* was carried out according to methods reported elsewhere [21]. Enzymes for DNA manipulation were used according to the manufacturer's recommendations (Koschem Co). DNA fragments were purified using PCR purification kits and gel extraction kits (QIAGEN Co.). Conjugation of *S. clavuligerus* with plasmids from *E. coli* was carried out as described by Kieser *et al.* [15]. For amplification of DNA by PCR, all primers used in this work are listed in Table 2. Ex-*Taq* PCR Premix (Takara Inc.) was used for PCR amplification under the following conditions: PCR mixture was prepared in a final

Table 1. Microorganisms, plasmids, and cosmids used in this study.

Microorganisms, plasmids, cosmids	Description[a]	Reference or source
Microorganisms		
S. clavuligerus ATCC 27064	Parent strain	American Type Culture Collection (ATCC) Centre
S. clavuligerus SMF5703	Harbors pSMF5703 (pWHM3 derivative plasmid) containing *pah2* gene regulated by *ermE*p	This study
S. clavuligerus SMF5704	Harbors pSMF5704 (pSET152 derivative plasmid) containing *pah2* gene regulated by *ermE*p; integrated at the ϕC31 *attB* site in the chromosome	This study
E. coli DH5α	F- φ*dlacZΔ*M15 *endA1 recA1 hsdR17*(rk- mk+) *supE44 thi-1 gyrA9Δ(lacZYA-agrF) U169λ-*	[9]
E. coli BW25113/pIJ790	*lacI*^{q} *rrnB*T14 *ΔlacZ*WJ16 *hsdR514* *ΔaraBAD*AH33 *ΔrhaBAD*LD78	[5]
E. coli ET12567/pUZ8002	*dam- dcm-* for methylation-deficient DNA manipulation *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173*	[16]
Plasmids and Cosmids		
pWHM3	*E. coli-Streptomyces* shuttle vector, used for expression experiments in *Streptomyces*; Amp^r, Tsr^r	[36]
pSET152	*E. coli* cloning vector; integrates into the *Streptomyces* chromosome; Apr^r	[3]
12B8	pWE15 derivative cosmid containing *pah2* gene	Jensen, S. E.
pSMF5701	Construct of *ermE*p; pWHM3 containing the 502 bp HindIII/PstI DNA fragment	This study
pSMF5702	Construct for *pah2* expression regulated by *ermE*p; pWHM3 containing the 502 bp HindIII/PstI fragment of *emrE*p and 945 bp PstI/EcoRI fragment of *pah2* (final 1,447 bp insert)	This study
pSMF5703	Construct for *pah2* expression regulated by *ermE*p; pSMF5702 but with Tsr^r replaced by *acc(3)IV* (apramycin resistance gene) and *oriT*	This study
pSMF5704	Construct for *pah2* expression regulated by *ermE*p; pSET152 containing the 1,444 bp EcoRI/EcoRV fragment	This study

[a]Amp^r, ampicillin resistance; Apr^r, apramycin resistance; Spc^r, spectinomycin resistance; Tsr^r, thiostrepton resistance.

volume of 50 μl containing 0.5 μM of each primer, 0.25 mM of each of the dNTPs, 1 μl of template DNA, 1 U of Pyrobest DNA polymerase (Takara Inc.) with its recommended reaction buffer, and 2 μl of DMSO. To amplify *ermE*p and *pah2*, an initial denaturation step of 2 min at 94°C, followed by 30 cycles of amplification (40 sec at 94°C, 40 sec at 62°C, 20–60 sec at 72°C), and a final extension period of 5 min at 72°C were carried out in a PCR Thermal Cycler (DICE Standard, Takara Inc.).

Cloning and Expression of the *pah2* Gene

Vectors with a strong promoter, *ermE*p, were used for expression of the *pah2* gene. The *ermE*p promoter DNA fragment was amplified with primers ermE1_F_HindIII and ermE1_R_PstI. Plasmid pHCG2, which contains *ermE*p together with a 1.7 kb fragment of the erythromycin resistance gene, was used as template DNA. The *ermE*p PCR product (504 bp) was digested with HindIII and PstI and then ligated to pWHM3, digested with the same enzymes, to generate pSMF5701. The *pah2* gene was amplified by PCR from cosmid 12B8 using primers pah2_F_PstI and pah2_R_EcoRI. The *pah2* PCR product (954 bp), digested with PstI and EcoRI, was inserted into pSMF5701 to give pSMF5702. The multicopy plasmid, pWHM3, on which pSMF5702 is based has a thiostrepton resistance gene (Tsr^r) and no *oriT* sequence for conjugation. In order to replace the Tsr^r gene with an apramycin resistance gene (*acc(3)IV*) and provide an *oriT* sequence to support plasmid conjugation from *E. coli* to *Streptomyces*, an *oriT*-containing apramycin resistance cassette was amplified from pIJ773 [8] using the PCR targeting primers, tsr_target_F and tsr_target_R. The amplified fragment was introduced into *E. coli* BW25113/pIJ790 carrying pSMF5702, whereupon the λRED functions encoded by pIJ790 promoted replacement of the Tsr^r gene of pSMF5702 with the apramycin *oriT* cassette, to yield pSMF5703. An alternative *pah2* expression vector, pSMF5704, was prepared to integrate an additional copy of *pah2* at the *ϕ*C31 *attB* site of the chromosome. Primers ermEp_F_EcoRV and pah2_R_EcoRI were selected to amplify *ermE*p-*pah2* using pSMF5703 as the template. The resulting PCR product (1,451 bp) was digested with EcoRI and EcoRV and cloned into the integrative plasmid, pSET152, to generate pSMF5704.

Introduction of the *pah2* Gene into *S. clavuligerus*

pSMF5703 and pSMF5704 were transferred by conjugation from *E. coli* ET12567/pUZ8002 to *S. clavuligerus* ATCC 27064 and transformants were selected by overlaying with apramycin (final concentration 25 μg/ml). To confirm the presence of the introduced plasmids, mycelia from the wild-type parent and the plasmid-bearing strains, grown on Trypticase Soy Broth containing 1% soluble starch (TSBS) medium, were resuspended in 200 μl of 10 mM Tris/HCl (pH 8.0) buffer containing 200 mg of glass

Table 2. Oligonucleotide primers for PCR experiments used in this study.

Designation	Nucleotide sequence (5'-3')	Description
ermE1_F_HindIII	GCCGGAAAGCTTCTCCCGCAACGACTTCGC	Forward primer for *ermE*p PCR to insert into pWHM3
ermE1_R_PstI	CTCCACCTGCAGCATGCGAGTGTCCGTTCG	Reverse primer for *ermE*p PCR to insert into pWHM3
pah2_F_PstI	CGCATGCTGCAGGTGGAGCGCATCGACT	Reverse primer for *pah2* PCR to insert into pSMF5701
pah2_R_EcoRI	TCTCCTGAATTCTCACAACTGGGTTCTGTGG	Reverse primer for *pah2* PCR to insert into pSMF5701 and pSMF5703
ermEp_F_EcoRV	AGCTTCGATATCTCCCGCAACGACTTCGC	Reverse primer for *ermE*p PCR to insert into pSMF5703
tsr_target_F	ATCGCGTCACTGAACACAGCAGCCGGTAG-GACGACCATGATTCCGGGGATCCGTCGACC	Forward primer for targeting Tsr^r deletion
tsr_target_R	AGGTCGAGGAACCGAGCGTCCGAGGAAC-AGAGGCGCTTATGTAGGCTGGAGCTGCTT	Reverse primer for targeting Tsr^r deletion
pah2_RT_F	CGCAGTGAGTCGGGCCTCAT	Forward primer for *pah2* RT-PCR
pah2_RT_R	CACGTCGATGTCGACCGAGA	Reverse primer for *pah2* RT-PCR
cas2_F	TACCGCGACGAGCTGCTC	Forward primer for *cas2* RT-PCR
cas2_R	CTGGAGGATGTGGTACGC	Reverse primer for *cas2* RT-PCR
bls2_F	ACCGGTTCTTCCGGCTGC	Forward primer for *bls2* RT-PCR
bls2_R	GTTCACCAGCCGGAAGGC	Reverse primer for *bls2* RT-PCR
ceaS2_F	AGCGGCAAGCCTACCGCC	Forward primer for *ceaS2* RT-PCR
ceaS2_R	GGCGACGATCGCCACCGA	Reverse primer for *ceaS2* RT-PCR
ccaR_F	GATACCCATCCGCGGGCA	Forward primer for *ccaR* RT-PCR
ccaR_R:	TCGACGAGCTCCGACAGC	Reverse primer for *ccaR* RT-PCR
claR_F	GGTGCTGTCGCTGGTCTC	Forward primer for *claR* RT-PCR
claR_R	GGCGAGCCGAGACCTATC	Reverse primer for *claR* RT-PCR
cad_F	TCATCACGGGCGCGAGCT	Forward primer for *cad* RT-PCR
cad_R	GCCCGCGATCGAGGACAT	Reverse primer for *cad* RT-PCR
pah1_F	GGCGACATCGACCTCAGC	Forward primer for *pah1* RT-PCR
pah1_R	ACGTCGAGCAGGGTCAGC	Reverse primer for *pah1* RT-PCR
hrdB RT-R	GGCCACCGCGACCTGCTGC	Reverse primer for *hrdB* RT-PCR [34]
hrdB RT-F	CGGCCAAGCGCACCACTACC	Forward primer for *hrdB* RT-PCR [34]
apra_F	TCGAAGATGGGCCACTTGGA	Forward primer for *aac(3)IV*
apra_R	CGGATGCAGGAAGATCAACG	Forward primer for *aac(3)IV*

beads by vortexing for 1 min. Samples were boiled for 10 min and then centrifuged at 13,000 rpm for 5 min to remove cell debris. One µl of supernatant was used as template in PCR reactions with apra_F and apra_R (Table 2) as primers to detect the *acc(3)IV* gene.

Assessment of Transcripts by Semiquantitative RT-PCR

To prepare RNA for analysis of transcripts, mycelia from the parent and plasmid-bearing strains that were grown in TSBS as seed culture, cultured in 2 l of GA fermentation medium, were harvested and resuspended in RNA protect bacterial reagent (QIAGEN Co.) for 1 h at 30°C. GA medium for production of antibiotics consisted of 10 g glycerol, 6 g arginine, 2 g KH_2PO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.05 g $MnCl_2 \cdot 4H_2O$, and 0.05 g $ZnCl_2$ per liter of distilled water. The initial pH of all the media was adjusted to 7.2 [29]. RNA was purified from the mycelial suspension as described in the RNeasy midi kit manual (QIAGEN Co.). First-strand cDNA was synthesized by Superscript II RT (Invitrogen Co.) according to the manufacturer's instructions, and then double-stranded DNA was synthesized using Ex-*Taq* premix (Takara Inc.). The PCR program started with a 95°C denaturation for 5 min, followed by 25 cycles of 94°C (40 sec), 57°C (40 sec), 72°C (30 sec), and a stop with post-polymerization at 72°C for 5 min. Primers pah2_RT_F and pah2_RT_R were used for RT-PCR to assess the levels of *pah2* transcripts. Primers hrdB RT-R and hrdB RT-F were used to amplify *hrdB* to assess RNA amounts. The primers for transcriptional analysis of *ceaS*, *bls2*, *cas2*, *ccaR*, *claR*, *cad*, and *pah1* are listed in Table 2. Abundance of amplified DNA in RT-PCR reactions was estimated from gel images using Multi Gauge version 3.0 (Fuji Film Co.).

Fermentation and Analysis of Fermentation Kinetic Parameters

Seed cultures were prepared by inoculating spores or mycelia formed on agar plates of ISP4 medium into TSBS media, where apramycin (25 µg/ml) was supplemented to the seed medium for the mutants of SMF5703 and SMF5704. The seed culture was incubated for 48 h at 30°C on a rotary shaking incubator (220 rpm). For shake flask cultures, 10 ml of the seed culture was inoculated into 100 ml of GA medium contained in baffled culture flasks and incubated at 30°C on a rotary shaking incubator (220 rpm). For growth in stirred jar fermentors, 200 ml of seed culture was inoculated into 1,800 ml of GA medium prepared in a jar fermentor where the temperature was maintained at 30°C and the pH was adjusted to 7.0 by automatic addition of 0.5 M HCl and 1 M NaOH. Agitation was fixed at 250 rpm and the aeration was controlled to 1.0 vvm. Batch cultures were utilized to evaluate various kinetic parameters, including specific growth rate (μ), specific glycerol uptake rate (q_{gly}), and specific clavulanic acid production rate (q_{CA}), which are the basic equations for the changes in the concentration of glycerol, biomass, clavulanic acid, and ammonia [26].

Analytical Methods

To measure cell growth, mycelium was collected on preweighed filters (Whatman GF/C) by vacuum filtration. After collection, the mycelium was dried at 80°C for 24 h for dry weight determination. The concentration of glycerol was determined using the periodate/acetyl acetone assay [27]. The concentration of ammonium ion was measured immediately after sampling, with a specific ion analyzer (Model EA940; Orion Research). The concentration of clavulanic acid was quantified by HPLC after derivatization with imidazole [7].

RESULTS

Construction of Strains Carrying the Amplified *pah2* Gene

The *pah2* gene and *ermE*p promoter were amplified by PCR and cloned into the plasmid pWHM3 to obtain plasmid pSMF5702 (Fig. 2A). The Tsr^r gene in pSMF5702 was then replaced with a cassette carrying *acc(3)IV* and *oriT*, to give plasmid pSMF5703. The DNA fragment containing *ermE*p and *pah2* in pSMF5703 was amplified and then inserted into an integration vector, pSET152, to give pSMF5704 (Fig. 2B). Plasmid pSMF5704 allows integration at single copy into the ϕC31 *attB* site on the chromosome.

The multicopy vector, pSMF5703, and the integration vector, pSMF5704, were introduced to the parent strain of *S. clavuligerus* by conjugation. Two apramycin-resistant

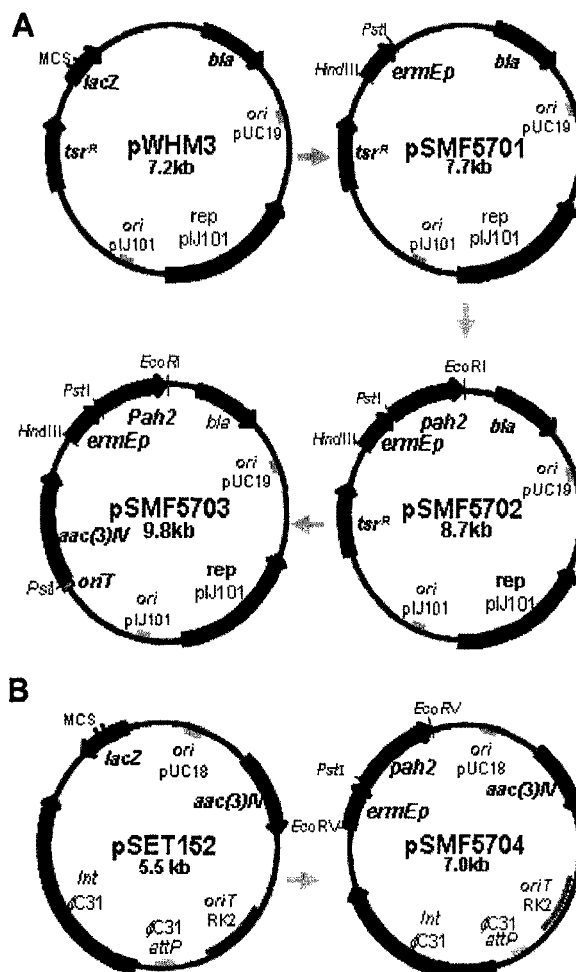

Fig. 2. Construction of expression plasmids for *pah2* regulated by *ermE*p promoter.

A. *ermE*p and *pah2* were inserted sequentially at the multicloning site of pWHM3 to obtain pSMF5702. The Tsr^r gene of pSMF5702 was replaced with an apramycin cassette containing *aac(3)IV* and *oriT*. **B.** Construction of an integration vector containing *pah2* regulated by *ermE*p. The DNA fragment carrying *ermE*p-*pah2* was cloned into pSET152 to generate pSMF5704.

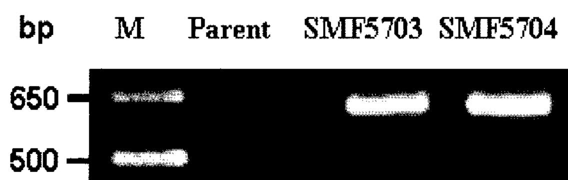

Fig. 3. Verification of the constructed strains by colony PCR.
M, 1 kb DNA plus ladder. No PCR product for *aac(3)IV* was observed from the parent strain. However, clear PCR products were detected from strains SMF5703 and SMF5704.

strains (SMF5703 and SMF5704) were screened by serially transferring candidate colonies on solid medium containing apramycin and finally selected as candidates where the cloned *pah2* gene had been successfully introduced into *S. clavuligerus*. The presence of the plasmid containing *pah2* in the mutants of SMF5703 was confirmed by extraction of the plasmid and detected by electrophoresis using 1% agarose (data not shown). The introduction of the plasmids into the strain was confirmed by colony PCR for the *acc(3)IV* gene, which was located in pSMF5703 and pSMF5704 (Fig. 3). The size of colony PCR product was 617 bp. From the verification, it was clear that the SMF5703 mutants carried the *pah2* gene in the multicopy plasmid pSMF5703 but not the chromosome, whereas SMF5704 carried the *pah2* gene in the chromosome. It was clear that the *acc(3)IV* gene was present only in the SMF5703 and SMF5704 strains but not in the wild-type parent (Fig. 3).

Batch Culture Kinetics in Shake Flasks

The effects of additional copies of *pah2* on production of clavulanic acid in batch culture were compared using the parent culture, strain SMF5703, in which the *pah2* gene is harbored on a multicopy plasmid, and strain SMF5704, in which the *pah2*-containing plasmid is integrated into the chromosome. Glycerol utilization in the parent and plasmid-bearing strains followed a similar pattern (Fig. 4A). However, whereas growth of the parent and strain SMF5704 was very similar, that of strain SMF5703 was very remarkably retarded, and the amount of mycelium in cultures of strain SMF5704 also declined very rapidly when glycerol was exhausted (Fig. 4B). Production of clavulanic acid in strain SMF5704 was apparently enhanced about 1.6-fold compared with the parent strain, whereas that of strain SMF5703 was profoundly reduced. Clavulanic acid production ceased simultaneously with the depletion of glycerol, and thereafter, rapid degradation of clavulanic acid followed (Fig. 4C). From these data, it appears that the *pah2* gene product, proclavaminate amidino hydrolase, plays a key role in the biosynthesis of clavulanic acid, and that clavulanic acid production can be enhanced by duplication of the *pah2* gene using an integrative plasmid vector, but not by introduction of *pah2* on a multicopy plasmid.

Batch Culture Kinetics Using Stirred Jar Fermentors

Since strain SMF5704 proved to produce increased clavulanic acid compared with the parent strain, more accurate batch culture kinetics were evaluated using jar fermentors, where the culture environment can be precisely controlled. Under these conditions, profiles for the utilization of glycerol by the parent and by strain SMF5704 were similar (Fig. 5A). Mycelial growth during the exponential phase was also similar for both strains, but the decrease in the amount of mycelium thereafter was more profound in the parent strain (Fig. 5B). Clavulanic acid production in strain SMF5704 was again higher compared with the parent strain (Fig. 5C), just as was seen in the shake flask cultures. Ammonia generated was minimal in both cultures until glycerol was exhausted, and then was higher in strain SMF5704 compared with that of the parent (Fig. 5D). The enhanced accumulation of ammonium ion in strain

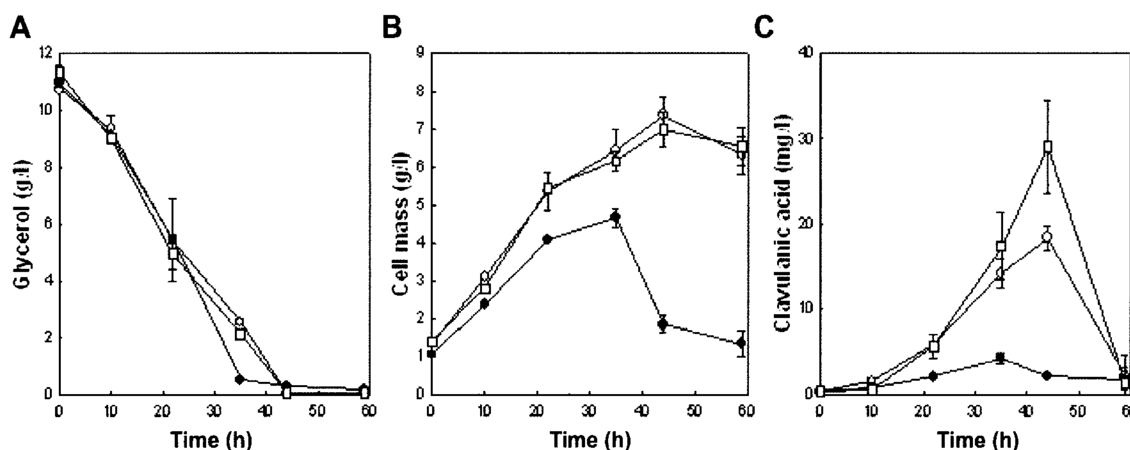

Fig. 4. Kinetic analysis of shake flask batch cultures of parent and plasmid-bearing strains of *S. clavuligerus*.
A. Glycerol utilization; **B.** Cell growth; and **C.** Clavulanic acid. Genotypes: parent *S. clavuligerus* ATCC 27064 (-○-), strain SMF5703 (-●-), and strain SMF5704 (-□-). Culture conditions: 100-ml culture in 500-ml baffled flask, 30°C, 220 rpm grown in GA medium.

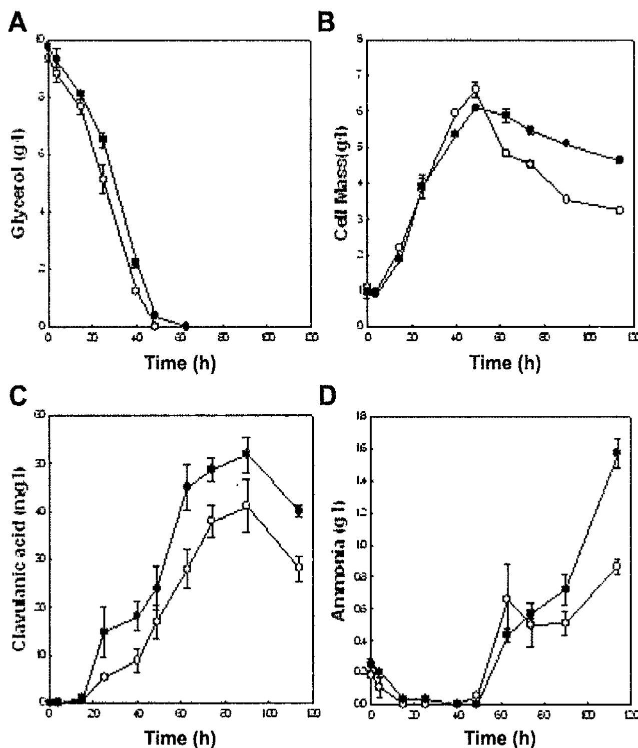

Fig. 5. Kinetic analysis of stirred jar fermentor batch cultures of parent and plasmid-bearing strains of *S. clavuligerus*.

A. Glycerol utilization; **B.** Cell growth; **C.** Clavulanic acid; and **D.** Ammonium ion. Genotypes: parent *S. clavuligerus* ATCC 27064 (-○-), strain SMF5704 (-●-). Culture conditions: 2 l culture in 2.5 l jar, 30°C, 250 rpm, 1 vvm.

SMF5704 in the later stages of the culture likely results from two different situations. On the one hand, deamination of arginine, once it was required as a carbon source following the depletion of glycerol, would cause release of ammonia. In addition, the increased production of urea and proclavaminic acid that accompanies the enhanced activity of Pah as it hydrolyzes guanidinoproclavaminate may also result in accumulation of ammonia if urease activity is also present.

From these results, it appears that proclavaminate amidino hydrolase plays a role in the determination of the biosynthetic rate of clavulanic acid. The kinetic parameters are shown in Table 3. The specific growth rate (μ_{max}) and maximum glycerol uptake rate (q_s^{max}) of the parent strain were higher than those of strain SMF5704 when kinetic parameters of both of the strains were analyzed. In the case

Table 3. Kinetic parameter analysis of parent strain and strain SMF5704 of *S. clavuligerus* in batch culture.

Kinetic parameters	Parent strain	SMF5704
μ_{max} (/h)	0.101	0.087
q_S^{max} (g/g/h)	0.135	0.108
q_{CA}^{max} (mg/g/h)	0.139	0.220
q_{NH4}^{max} (mg/g/h)	3.242×10^{-3}	6.646×10^{-3}

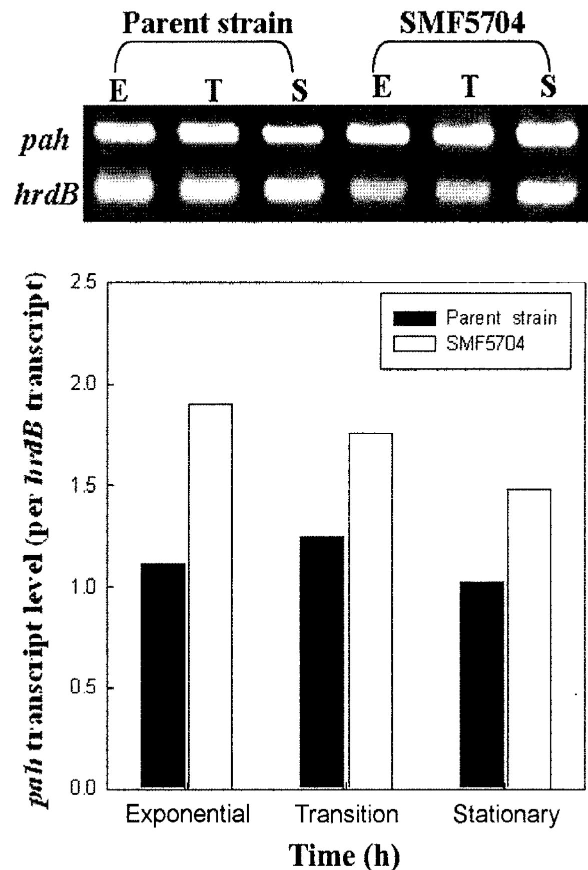

Fig. 6. *pah2* transcript levels in batch cultures of the *S. clavuligerus* parent, and strain SMF5704 carrying a second copy of the *pah2* gene integrated into the chromosome.

The levels of RNA transcripts from wild type and strain SMF5704, harboring the *pah2*-overexpressing plasmid (pSMF5704), were compared by RT-PCR analysis. *hrdB* was used as a control for RNA content. The samples for isolation of mRNA were harvested at the exponential phase (E), transition phase (T), and stationary phase (S).

of the clavulanic acid production rate (q_{CA}^{max}), strain SMF5704 showed a considerably higher rate in the early stages of growth, whereas the parent strain had a higher q_{CA}^{max} in the late stages. Moreover, the q_{CA}^{max} of strain SMF5704 was almost 1.6-fold higher than that of the parent strain. The excretion rate of ammonium ion, q_{NH4}, of strain SMF5704 increased continuously throughout the culture, and measurable q_{NH4}^{max} was 6.646×10^{-3} for strain SMF5704 whereas the q_{NH4}^{max} of the parent strain was 3.242×10^{-3} (Table 3).

Assessment of Transcript Levels of Genes Involved in Clavulanic Acid Biosynthesis

The transcript levels of the genes were estimated by semiquantitative RT-PCR, where the *hrdB* gene was used as a gene for comparison of the transcript level. The absence of DNA contamination in the purified RNA samples was confirmed by PCR amplification (data not shown). The results indicated that the transcript level measured by RT-PCR could represent the level of transcription of genes.

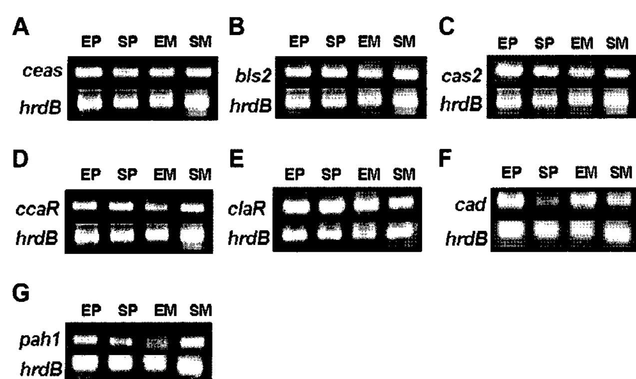

Fig. 7. Assessment of the transcription levels of clavulanic acid biosynthesis genes.

(A–G) RT-PCR analyses of the transcripts from *ceaS2*, *bls2*, *cas2*, *ccaR*, *claR*, *cad*, and *pah1* were carried out. The levels of transcripts from the parent strain (P) and strain SMF5704 (M) were compared for each gene using *hrdB* as a control for RNA content. The samples for purification of RNA were harvested at the exponential phase (E) and stationary phase (S).

The transcript profiles for *pah2* in the parent strain and strain SMF5704 were determined by RT-PCR (Fig. 6). Since only 25 cycles of amplification were used in the PCR step, the amounts of PCR product observed give a semiquantitative indication of the original amounts of *pah2* transcript present in the cells. On this basis, it appears that the level of *pah2* transcripts in strain SMF5704 was higher than that of the parent strain, regardless of culture time. These results suggest that the *pah2* gene integrated in the chromosome was being transcribed and that more Pah was being produced throughout the culture in strain SMF5704.

Transcript levels of other genes such as *ceaS2*, *bls2*, *cas2*, *cad*, and *pah1* and regulatory genes such as *ccaR* and *claR*, all essential genes from the clavulanic acid biosynthetic pathway, were also determined using mycelium harvested at both the exponential phase and stationary phase (Fig. 7). The transcript levels of these genes were not significantly different between the parent strain and the strain SMF5704 for both exponential phase cells (clavulanic acid production phase) and stationary phase cells (mycelium degradation phase). These data indicate that the duplication of *pah*2 did not markedly affect the expression of other genes involved in the clavulanic acid biosynthetic pathway, although the transcript levels of *pah2*, and clavulanic acid production, were enhanced by the duplication of the *pah2* gene in the chromosome of *S. clavuligerus*.

Discussion

In this study, we have constructed a strain based on a metabolic engineering scheme, and used it to examine the effect of gene dosage of *pah2*, encoding proclavaminate amidino hydrolase, on clavulanic acid production in *S. clavuligerus*. As the first step, the *pah2* gene and a strong promoter, *ermE*p, were cloned in a multicopy number plasmid. However, the strain (SMF5703) harboring the multicopy plasmid showed retarded cell growth followed by rapid cell death and remarkable reduction of clavulanic acid level. The results observed in strain SMF5703 presumably reflect the detrimental effects of multicopy plasmid on mycelial growth. To circumvent these difficulties, the *pah*2 gene, still under the control of the strong *ermE*p, was integrated into the chromosome of *S. clavuligerus*. The resulting plasmid-bearing strain (SMF5704) showed enhanced production of clavulanic acid accompanied by higher *pah2* transcript levels compared with the parent strain. These results indicate that increased levels of Pah can contribute to enhanced clavulanic acid biosynthesis.

The higher accumulation of ammonium ion in the strain SMF5704 culture might have resulted from enhanced activity of the Pah enzyme (Figs. 5C and 5D). From kinetic parameter analysis, the specific accumulation rate of ammonium ion (q_{NH4}) increased continuously in strain SMF5704, whereas the parent strain showed the maximum value of q_{NH4} at 80 h and then decreasing values thereafter. Moreover, the q_{NH4}^{max} value of strain SMF5704 was two-fold higher than that of the parent (Table 3). Even though strain SMF5704 showed a normal morphological phenotype and enhanced ability for clavulanic acid production, the accumulation of ammonia in this strain could still be having detrimental effects on cell growth and antibiotic production. Such detrimental effects of ammonia accumulation might also explain the phenotype of strain SMF5703, where Pah is overproduced from a multicopy plasmid and might be expected to result in even greater accumulation of ammonia. Cell growth, biosynthesis of clavulanic acid and cephamycin have been shown previously to be inhibited by increased concentration of ammonium ion in the culture medium, where clavulanic acid was apparently more affected [11, 12, 28]. Therefore, schemes for the removal or consumption of ammonium ion must be considered as a necessary adjunct to strain improvement by increasing Pah levels.

Analysis of transcripts of genes involved in the clavulanic acid biosynthetic pathway revealed that most genes were not appreciably affected, although the transcript level of *pah2* and the clavulanic acid production were enhanced. It has been reported that the K_m of Pah2 for deoxyguanidinoproclavaminic acid is higher than that of Cas2 for proclavaminic acid [30]. Because of complexity of biosynthetic pathways, clavulanic acid production might be expected to be enhanced by even duplication of *pah2*, although Cas2 activity is required for three separate reactions in clavulanic acid biosynthesis. From our current studies, the overproduction of Pah can better be achieved by integration of the gene in the chromosome of *S. clavuligerus*, rather than by cloning on a multicopy plasmid. Finally, flux control by Pah is surely not the only factor to affect the rate of clavulanic acid production, and

the enhancement of only one enzyme such as Pah may only give marginal improvement in production of clavulanic acid, since the availability of balanced levels of many enzymes is required for its synthesis.

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