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BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Homologous expression of *lysA* encoding diaminopimelic acid (DAP) decarboxylase reveals increased antibiotic production in *Streptomyces clavuligerus*

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Abstract

lysA gene encoding meso-diaminopimelic acid (DAP) decarboxylase enzyme that catalyzes L-lysine biosynthesis in the aspartate pathway in *Streptomyces clavuligerus* was overexpressed, and its effects on cephamycin C (CephC), clavulanic acid (CA), and tunicamycin productions were investigated. Multicopy expression of *lysA* gene under the control of *glpF* promoter (*glpFp*) in *S. clavuligerus* pCOlysA led to higher expression levels ranging from 2- to 6-fold increase at both *lysA* gene and CephC biosynthetic gene cluster at T_{36} and T_{48} of TSBG fermentation. These results accorded well with CephC production. Thus, 1.86- and 3.14-fold higher volumetric as well as 1.26- and 1.71-fold increased specific CephC yields were recorded in *S. clavuligerus* pCOlysA in comparison with the wild-type and its control strain, respectively, at 48th h. Increasing the expression of *lysA* provided 4.3 times more tunicamycin yields in the recombinant strain. These findings suggested that *lysA* overexpression in *S. clavuligerus* made the strain more productive for CephC and tunicamycin. The results also supported the presence of complex interactions among antibiotic biosynthesis pathways in *S. clavuligerus*.

Keywords Streptomyces clavuligerus · lysA · Overexpression · Cephamycin C · Clavulanic acid · Tunicamycin

Introduction

Streptomyces clavuligerus produces several medically important secondary metabolites such as the highly potent β-lactam antibiotic cephamycin C (CephC); clavulanic acid (CA), a β-lactamase inhibitor; and tunicamycin, a glycosylation inhibitor. Of these secondary metabolites, CephC is a naturally occurring cephamycin antibiotic and potent against penicillin-resistant bacteria via a methoxyl group at the C-7 position of the cephalosporin core. CephC is highly stable at neutral pH and used as a raw material to synthesize semisynthetic antibiotics such as cefoxitin, cefmetazole, temocillin, and cefotetan [1, 2]. CA is a β-lactamase inhibitor used in combination with β-lactam antibiotics such as amoxicillin (Augmentin™) and ticarcillin (Timentin™) against β-lactamase-producing bacteria [3]. Non-β-lactam compounds such as holomycin with pyrrolidine structure and tunicamycin with a glucosamine-containing structure are also produced by *S. clavuligerus* [2, 4]. Tunicamycin is a fatty acyl nucleoside-type antibiotic and composed of a 14-membered gene cluster lacking cluster-situated regulator (CSR) [5].

CephC biosynthesis pathway starts with the non-ribosomal condensation of L-α-aminoadipic acid (α-AAA), L-cysteine, and L-valine precursors to form delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). α-AAA is a catabolic product of L-lysine produced from the lysine branch of the aspartate metabolic pathway (Fig. 1) [7, 8]. L-Lysine is produced through decarboxylation of the cell wall precursor meso-diaminopimelate, catalyzed by the diaminopimelate (DAP) decarboxylase (E.C.4.1.1.20). This enzyme is encoded by *lysA* gene in the aspartate pathway. Then, L-lysine is converted to α-AAA in two steps of the CephC pathway by the enzymes encoded by *lat* and *pcd* genes [9, 10]. Therefore, availability of α-AAA is a limiting step for precursor flux from the aspartate pathway and for β-lactam biosynthesis [8, 11].

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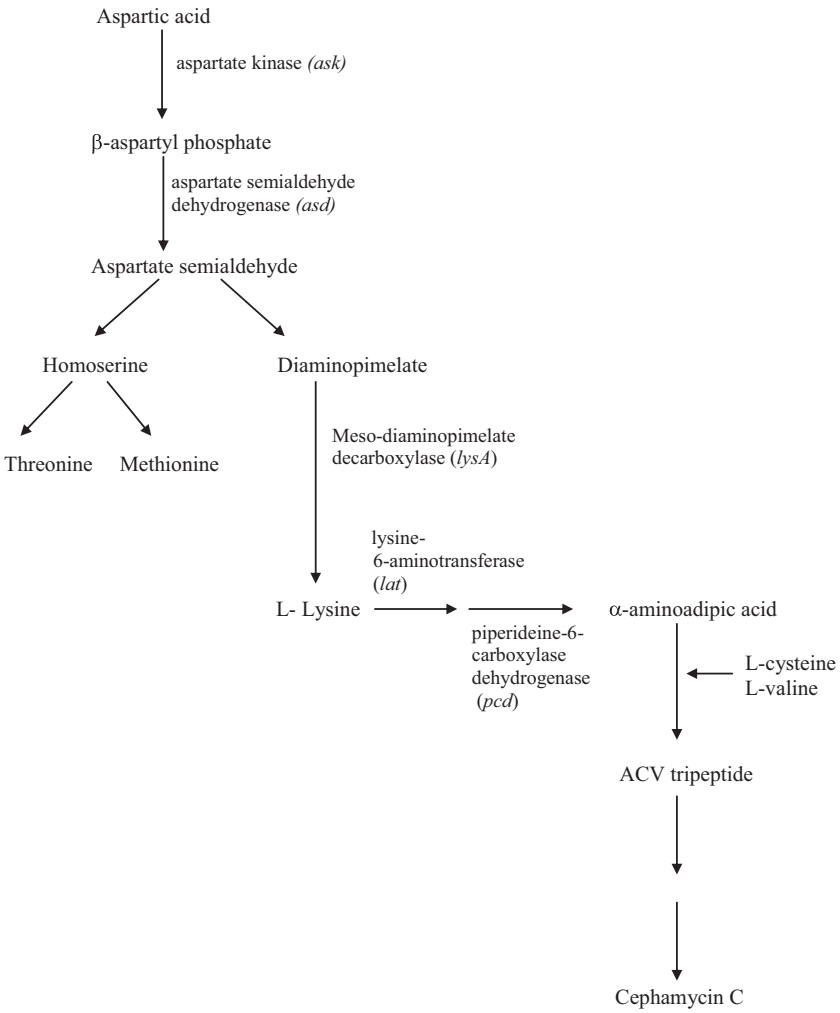

Fig. 1 Aspartate pathway and its link to CephC pathway in *S. clavuligerus* [6]

Fermentation optimization or metabolic engineering approaches, or both, were used to overproduce CephC in the cell. The addition of L-lysine, its product, i.e., α-AAA, or its precursor, i.e., diaminopimelate, stimulated the production of CephC in *S. clavuligerus* [2, 11, 12]. Similarly, supplementation of the fermentation medium with L-lysine or diamines putrescine, cadaverine, and diaminopropane also enhanced cephamycin production in *Nocardia lactamdurans*, another cephamycin producer actinomycete [13, 14].

In addition to precursor supply to increase CephC yield in *S. clavuligerus*, there are some genetic engineering studies targeting biosynthetic or regulatory genes to improve the CephC production. For instance, Özcengiz et al. [15] introduced multicopy *ask* (aspartokinase) gene in the aspartate pathway into *S. clavuligerus*, leading to 3.3-fold higher specific CephC production in the recombinant strain. In addition, *ccaR* overproduction in *S. clavuligerus* resulted in 6.1-fold higher production of specific CephC [16]. Recently, it has been reported that overexpression of *pimM* gene from *Streptomyces natalensis* in *S. clavuligerus* resulted in a remarkable increase in CephC as well as CA and tunicamycin yields [17].

In *S. clavuligerus*, CephC and CA are synthesized simultaneously through different metabolic pathways [18]. Several reports indicated the positive impact of inducers and precursors as well as carbon or nitrogen sources in the fermentation media for the production of either compound, CephC or CA [19]. For instance, the culture supplemented with soluble starch as carbon source or cottonseed extract as complex nitrogen source enhanced CephC production while the glycerol feeding did not cause an increase in maximum CephC production [20]. In contrast, the glycerol feeding strongly enhanced CA as previously reported [21]. Interestingly, the variable concentration of 1,3-diaminopropane in the culture changes that connected production in the favor of either compound [13, 22].

The *lysA* gene of *S. clavuligerus* NRRL 3585 was previously PCR-amplified and characterized, and its sequence was deposited to the GenBank with the accession number of DQ078790.1 [6]. This study aimed at homologous multicopy expression of *lysA* gene in *S. clavuligerus* to determine its effect in the

expression of CephC gene cluster and in the CephC yield. In order to see the effect of this manipulation more extensively, CA and tunicamycin productions were also evaluated in the *lysA*-overexpressed *S. clavuligerus* cells.

Materials and methods

Bacterial strains, plasmids, media, and culture conditions

Bacterial strains and plasmids used are given in Table 1. *S. clavuligerus* strains were grown in tryptic soy broth (TSB) medium at 28 °C and 220 rpm until the 1/10 diluted culture reached an optical density (OD_{600}) of approximately 0.6. These seed cultures were used to inoculate (5%, v/v) 500-ml triple-baffled flasks containing 100 ml of TSB medium supplemented with 0.5% glycerol (to induce the pSPG *glpF* promoter, *glpFp*). The cultures were grown in triplicate, and duplicate samples were taken to quantify growth and antibiotic concentration. Cultures of *S. clavuligerus* pCOlysA and *S. clavuligerus* pSPG were supplemented with apramycin (50 μg/ml) to maintain the multicopy plasmids. To propagate *Escherichia coli* (*E. coli*), Luria broth (LB) or agar plates (LA) were used, and the strains were incubated at 37 °C. Antibiotics (ampicillin [100 μg/ml], kanamycin [25 μg/ml], chloramphenicol [25 μg/ml]) were added when needed. *E. coli* ESS 3235, *Klebsiella pneumoniae* ATCC 29665, and *Bacillus subtilis* ATCC 6633 strains were grown in LB at 30 °C and 200 rpm to an OD_{600} value of 0.9–1.0 before use in the CephC, CA, and tunicamycin bioassays, respectively. Mannitol soy flour (MS) agar [27] was used to grow exconjugants after conjugation.

Construction of *S. clavuligerus* pCOlysA and *S. clavuligerus* pSPG

The promoterless *lysA* gene was isolated by PCR as 1400-bp fragment from *S. clavuligerus* genomic DNA using the specific primers listed in Table 2. The *lysA* amplicon was subcloned to pGEM-T® Easy to give pCO5. Next, the *lysA* gene was released from pCO5 with *NdeI-SpeI* and ligated to downstream of *glpF* promoter in pSPG. The constructions were verified by restriction digestion, PCR, and sequencing of the PCR-amplified fragment. pSPG and pCOlysA were introduced by transformation into the methylation-deficient *E. coli* ET12567/pUZ8002 to bypass *Streptomyces* restriction barrier. Intergeneric conjugation between *Streptomyces* and *E. coli* was carried out as described by Flett et al. [28]. Exconjugants grown on MS agar were transferred to TSA containing appropriate antibiotics, and they were allowed to grow for up to 4 days at 28 °C. The presence of pSPG and pCOlysA in *S. clavuligerus* exconjugants was confirmed by PCR using Apr primers (Table 2) designed to amplify the apramycin resistance gene. The strain *S. clavuligerus* carrying the empty plasmid pSPG was the control strain in this work.

Nucleotide sequencing

The DNA sequencing was provided by BGI (Europe) through the Genoks Company using BigDye Cycle Sequencing Kit

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source or reference
Strains		
S. clavuligerus		
NRRL 3585	Wild type, CephC, and CA producer	Agricultural Research Service (ARS)
S. clavuligerus pCOlysA	Recombinant strain of *S. clavuligerus* containing the recombinant pSPG expression vector carrying the *lysA* gene	This study
S. clavuligerus pSPG	*S. clavuligerus* NRRL 3585carrying pSPG (control strain)	This study
E. coli		
DH5α	*F'/dlacZD* (*lacZYA-argF*) *U169 supE44k-thi-1 gyrA recA1 relA1 endA1 hsdR17*	*E. coli* Genetic Stock Center
ET12567/pUZ8002	*F dam 13::Tn9 dcm-6 hsdM hsdR lacYI/oriT*⁻ plasmid	Kieser et al. [23]
ESS 22-35	β-lactam supersensitive strain	Hu et al. [24]
Bacillus subtilis ATCC 6633	Tunicamycin indicator	Bignell et al. [25]
Klebsiella pneumoniae ATCC 29665	CA indicator	Romero et al. [26]
Plasmids		
pGEM-T® Easy	Amp^R, *lacZ*	Promega Inc. (Madison, WI, ABD)
pCO5	*lysA gene* carrying recombinant pGEM-T® Easy	This study
pSPG	Amp^R, Ap^R, *glpFp*	Kurt et al. [16]
pCOlysA	*lysA* gene carrying pSPG expression vector	This study

Table 2 PCR primers used in cloning and verification experiments designed for this study as well as primer sequences used in qRT-PCR [16]

Name	5′ to 3′ primer sequence	Product size (bp)	Utility
lysA-F lysA-R	CATATGATGAGCCGTTCCGCACACCC ACTAGTTCACCCGACGTCGAGACGCA G	1400	To clone *lysA*
Apr-F Apr-R	ATTCCGGGGATCCGTCGA TGTAGGCTGGAGCTCCTT	1352	To confirm insertion of pSPG in *S. clavuligerus*
hrdB-FP hrdB-RP	CGCGGCATGCTCTTCCT AGGTGGCGTACGTGGAGAAC	109	To amplify by qRT-PCR a *hrdB* internal sequence
ccaR-FP ccaR-RP	CACCTGGAATGATGTGACGA GCTTCCTTGGAGATGACCTG	144	To amplify by qRT-PCR a *ccaR* internal sequence
orf10 FP orf10 RP	TGCGCTGGCTGGGGGTCTC CGCAGGGGCAGCCGTGAAT	133	To amplify by qRT-PCR a *orf10* internal sequence
blp-FP	CGCAGGGCCACTTCTTCTTCAAC	130	To amplify by qRT-PCR a *blp* internal sequence
blp-RP	GCCTCCGTCATGCCCGTCTG		
lat-FP lat-RP	TCACCCAGAAGCGGTATCTC CGTACGGCACCGAATAAAGA	159	To amplify by qRT-PCR a *lat* internal sequence
pcbAB-FP pcbAB-RP	CACGCTCGGCATCTGGAAGG GTGGTGCCGGTTGGTGACGA	124	To amplify by qRT-PCR a *pcbAB* internal sequence
pcbC-FP pcbC-RP	CGCGGCTCGGGCTTCTTCTAC GGATCGCCAGGTCGTGCTTCTC	111	To amplify by qRT-PCR a *pcbC* internal sequence
pcbR FP pcbR RP	GTGGGGCTCGGCTATTGGGGTTAC CCAGGCGCCGAGGAAGGTGT	111	To amplify by qRT-PCR a *pcbR* internal sequence
cefD-FP cefD-RP	GCTGTGGCAGGCGCGGGAGAG GCAGTGACGACGCGACGAGGTTGA	110	To amplify by qRT-PCR a *cefD* internal sequence
cefE-FP cefE-RP	ACCTCGCCCGTCCCCACCA GTCCAGATCCGCTCGAAGTCACCG	161	To amplify by qRT-PCR a *cefE* internal sequence
pcd-FP pcd-RP	CAGCAATCAGTGGTACCGACGAGA CGCGCAGGCCGAACAGAT	138	To amplify by qRT-PCR a *pcd* internal sequence
cmcT-FP cmcT-RP	GGCGGTCATGCTGCTGGTCT CGCTGTCGGGGGTGATGG	137	To amplify by qRT-PCR a *cmcT* internal sequence
pbpA-FP pbpA-RP	GGCGCTGCTGCTCGTCAT TGTCGCGCAGGGTGAGGA	116	To amplify by qRT-PCR a *pbpA* internal sequence
bla-FP bla-RP	CATCTGGAGCGGCGGGTCA CGGATGGCGGCGTCACAGA	116	To amplify by qRT-PCR a *bla* internal sequence
cmcI-FP cmcI-RP	CTGTTCCGGGGTCTGGGTGAG GGAGAAGTCCGAGTAGCCGAGGTC	123	To amplify by qRT-PCR a *cmcI* internal sequence
cmcJ-FP cmcJ-RP	CGCTTCAGCCGGTTCCAGAT CCATCAGCTTGCCCGCGTCGTTC	176	To amplify by qRT-PCR a *cmcJ* internal sequence
cefF-FP cefF-RP	GGATGGCCCCGCACTACGACCTG CGCGCCGCACATCACGACG	149	To amplify by qRT-PCR a *cefF* internal sequence
cmcH-FP cmcH-RP	CGTGGGCGCGTTCTACCTCGTCG CCATCAGCTTGCCCGCGTCGTTC	164	To amplify by qRT-PCR a *cmcH* internal sequence

V3.1 (Applied Biosystems) of the Applied Biosystem and the chain termination method.

Fermentation and growth determination

Fermentation experiments were performed by growing three biological replicates of *S. clavuligerus* pSPG, *S. clavuligerus* pCOlysA, and *S. clavuligerus* NRRL3585 in the TSBG medium for 72 h. Two milliliters of samples taken from cultures with 12-h intervals was used for DNA quantification [29] and CephC and CA bioassays [16]. In the case of tunicamycin, 5 ml of the cultures were taken at every 12 h.

Antibiotic bioassays

CephC and CA concentrations were determined using culture supernatants while tunicamycin was extracted directly from the cultures prior to bioassay experiments [30, 31]. CephC,

CA, and tunicamycin were quantified using the agar plate diffusion method [26]. CephC concentrations were calculated as cephalosporin C equivalents using a standard of cephalosporin C (Sigma). Potassium clavulanate (Sigma) and tunicamycin (Sigma) were used as standards to calculate the related antibiotic concentration in the samples.

RNA isolation and gene expression analyses

RNA was isolated from samples taken from cultures at 24th and 36th h of growth. RNA was purified using GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. In addition, Phase Lock Gel Heavy (5 PRIME) columns were used to reduce sample loss during phenol extraction. DNase treatment of the isolated RNA samples was performed using a DNA-free™ kit (Thermo Scientific). RNA amount was determined by the NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, Wilmington). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. Reactions with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) for qRT-PCR analysis were performed on CFX96 Real-Time PCR (Bio-Rad). The primers designed previously [16] were used for qRT-PCR analysis. A standard qRT-PCR reaction included the following components: reaction buffer (5×) 4 μl; forward primer (10 μM), 0.4 μl; reverse primer (10 μM), 0.4 μl; cDNA, 1 μl; dH_2O, 14.2 μl, in a final volume of 20 μl. The PCR reactions started with an initial denaturation step (30 s at 95 °C) and proceeded with 40 cycles of amplification (5 s at 95 °C and 30 s at 60 °C). In the qRT-PCR experiments, three biological and two technical replicates were used for each sample. *hrdB* (RNA polymerase sigma factor A) was used as the internal reference gene [16, 32]. The ΔΔCt method was chosen for calculation of the relative expression value of the genes [33].

Statistical analysis of qRT-PCR data

Two-way repeated ANOVA with Bonferroni's post hoc testing was performed on the normalized gene expression to check whether expression data were statistically different between *S. clavuligerus* NRRL3585 (wild strain) and *S. clavuligerus* pCOlysA or between *S. clavuligerus* pCOlysA and *S. clavuligerus* pSPG. The GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for statistical evaluation of qRT-PCR data. Error bars represent 95% confidence intervals. The statistical significance value in the graphs is indicated as $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$.

Results

Construction of *S. clavuligerus* pCOlysA

In this study, we constructed the *S. clavuligerus* pCOlysA strain for *lysA* overexpression. The presence of pCOlysA plasmid (Fig. 2a) in the recombinant strain was verified by PCR (Fig. 2b). The phenotypic observation of the recombinant strain together with the control strains is also provided in Fig. 3. As shown, the recombinant pCOlysA strain had similar mycelium formation and sporulation pattern as the wild type.

Analysis of the effect of *lysA* in the CephC biosynthesis genes

Multiple expressions of the *lysA* gene encoding meso-diaminopimelate decarboxylase under the control of *glpFp* in the pSPG replication vector in *S. clavuligerus* pCOlysA led to 1.5-fold ($p < 0.001$) increase in its own relative expression at T_{36}. On the other hand, the relative expression values of *lat* gene, involved in the conversion of L-lysine to α-AAA in the CephC biosynthesis step, were 2.41 and 2 (T_{24} and T_{36}), respectively, and were statistically significant ($p < 0.001$ and $p < 0.01$). At both sampling hours, the expression levels of *pcbAB*, *cefD*, and *cefF* biosynthetic genes showed a statistically significant increase ranging from 4 to 6 in *S. clavuligerus* pCOlysA compared with those of the control strain. The expression levels of other biosynthetic genes, i.e., *pcbC*, *cefE*, *cmcH*, and *cmcI*, showed 2- to 4-fold increase in T_{36} compared with those of the control strain ($p < 0.01$ or $p < 0.001$). Expression of the *ccaR* regulatory gene located in the pathway increased 2.85-fold, especially in T_{24}, as well ($p < 0.001$).

Interestingly, the relative expression value of *orf10*, a gene of unknown function, compared with that of the control strain was determined as 2.85 (at T_{36}). The *orf10* gene is expressed in the *ccaR–orf10–blp–lat–pcbAB–pcbC* polycistronic transcript [34], and its higher expression might also be related to CcaR regulation. The *pbpA*, a gene encoding putative low molecular weight penicillin-binding protein (PBP), plays a role with *pcbR* providing autoresistance to β-lactam antibiotics. Therefore, in *S. clavuligerus* pCOlysA, relative expression values of *pbpA* and *pcbR* were found to be 2.15 ($p < 0.05$) and 1.60 ($p < 0.001$) at 36 h compared with those of the control strain. Another gene of resistance, the *bla*, was expressed 3.64 times more in *S. clavuligerus* pCOlysA than that in the control strain ($p < 0.001$). The *blp* gene, probably coding for a β-lactamase inhibitor protein, showed 1.32-fold higher expression value at 36th h. However, there was no statistically significant difference in the relative expression value of *pcd*, *cmcJ*, and *cmcT* genes in both hours (Fig. 4).

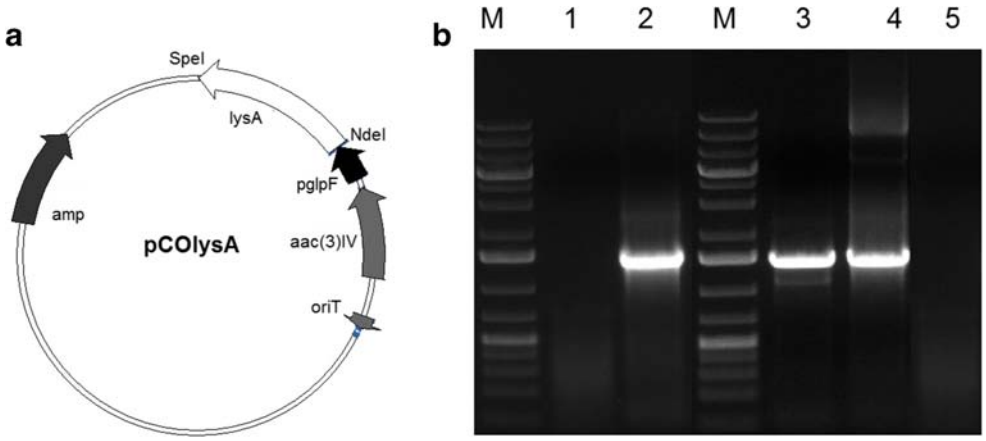

Fig. 2 Vector map of recombinant pCOlysA constructed using Vector NTI Advance® 11.5 (**a**). PCR verification of *S. clavuligerus* pCOlysA (**b**). M, O'GeneRuler 1 kb plus DNA ladder (#SM1343); 1, negative control PCR in which *S. clavuligerus* genomic DNA was used as template for amplification of *aac(3)IV-oriT* amplicon; 2, *aac(3)IV-oriT* amplicon obtained by using *S. clavuligerus* pCOlysA DNA and AprF-R primer pair; 3, the positive control PCR using *S. clavuligerus* DNA as template for amplification of the *lysA* gene; 4, the *lysA* amplicon obtained using *S. clavuligerus* pCOlysA DNA as template and lysAF-R primer pair; 5, the negative control PCR using pSPG plasmid as template for amplification of the *lysA* gene

Comparison of antibiotic (CephC, CA, and tunicamycin) production

Growth of *S. clavuligerus* pCOlysA was slightly higher at T_{72} than those of the other strains, and then gradually decreased depending on time (Fig. 5). When CephC production was compared, the highest volumetric CephC production belonged to *S. clavuligerus* pCOlysA as 100.75 μg/ml at 48th h. At the same time, the production values of *S. clavuligerus* NRRL 3585 and *S. clavuligerus* pSPG were 54.04 and 32.05 μg/ml, respectively. Thus, the multiple expressions of the *lysA* gene in *S. clavuligerus* pCOlysA were 1.86-fold more compared with those of the wild-type strain and 3.14 times more compared with those of the control strain. However, when the specific CephC productions were compared, the highest value was reached by *S. clavuligerus* pCOlysA at 48th h as in volumetric production. *S. clavuligerus* pCOlysA produced 1.29- and 1.71-fold higher specific CephC in comparison with those of wild-type and control strains (Fig. 6).

Regarding CA yields, *S. clavuligerus* pCOlysA provided the highest volumetric CA production as 114.87 μg/ml at T_{48}, corresponding to 1.72-fold more yield compared with that of *S. clavuligerus* NRRL 3585. However, multiple expressions of the *lysA* gene under the control of *glpFp* in the recombinant strain did not have a positive effect on specific CA production as being almost the same in both strains.

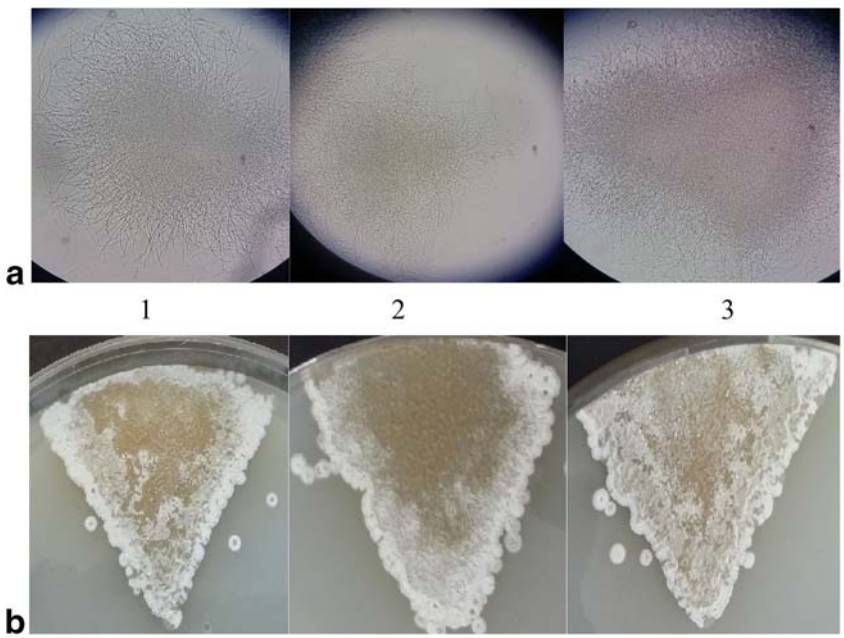

Fig. 3 Phenotypic observation of the wild-type *S. clavuligerus* (1), *S. clavuligerus* pSPG (2), and *S. clavuligerus* pCOlysA (3). **a** Microscopic observation. **b** Spore formation in ISP4 agar

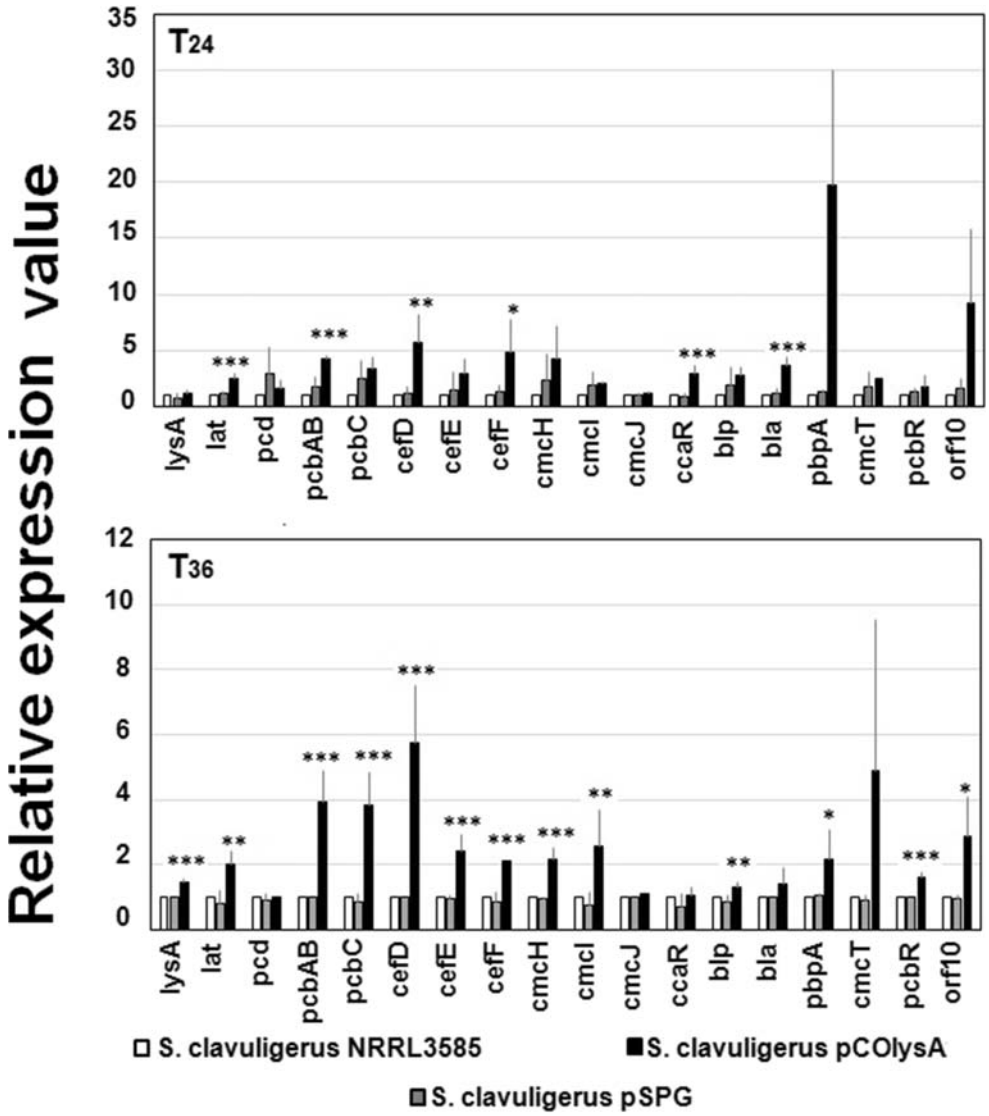

Fig. 4 qRT-PCR results of the *lysA* and CephC gene cluster in *S. clavuligerus* pCOlysA and *S. clavuligerus* pSPG control strain compared with those in *S. clavuligerus* NRRL 3585. The gene expression values in the wild strain were taken as 1 (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). The genes that have $p > 0.05$ do not have any asterisk

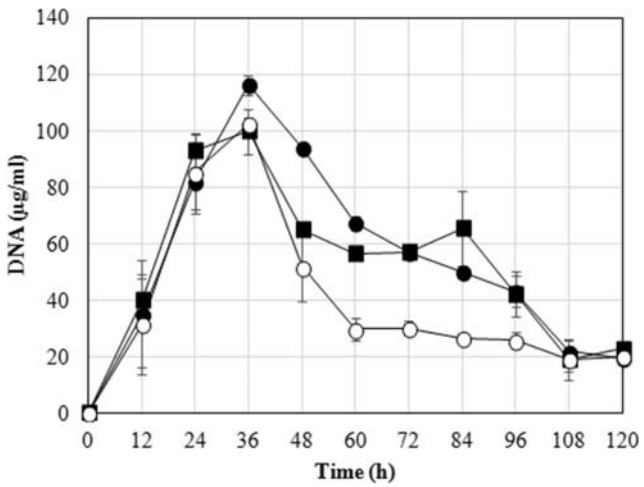

Fig. 5 Growth of *S. clavuligerus* pCOlysA (closed circles) and pSPG control strain (open circles) in TSBG compared with that of *S. clavuligerus* NRRL3585 (closed squares)

In the case of tunicamycin, its production delayed to late hours of incubation, especially after T_{84}. The highest production of tunicamycin was provided by *S. clavuligerus* pCOlysA at 120th h as 451.7 μg/ml, and this value corresponded to at least 4.5 times more volumetric tunicamycin than those of the controls. Similarly, the specific antibiotic yield of *S. clavuligerus* pCOlysA was very high at T_{108} and T_{120}, as well. The multiple expressions of *lysA* in *S. clavuligerus* resulted in a very productive tunicamycin overproducer strain.

Discussion

There have been many examples of strain improvement strategies applied to *S. clavuligerus* aiming high levels of antibiotic production. Such strategies range from media optimization to genetic engineering approaches. However, there are limited reports in regard to enhancing CephC yields through the

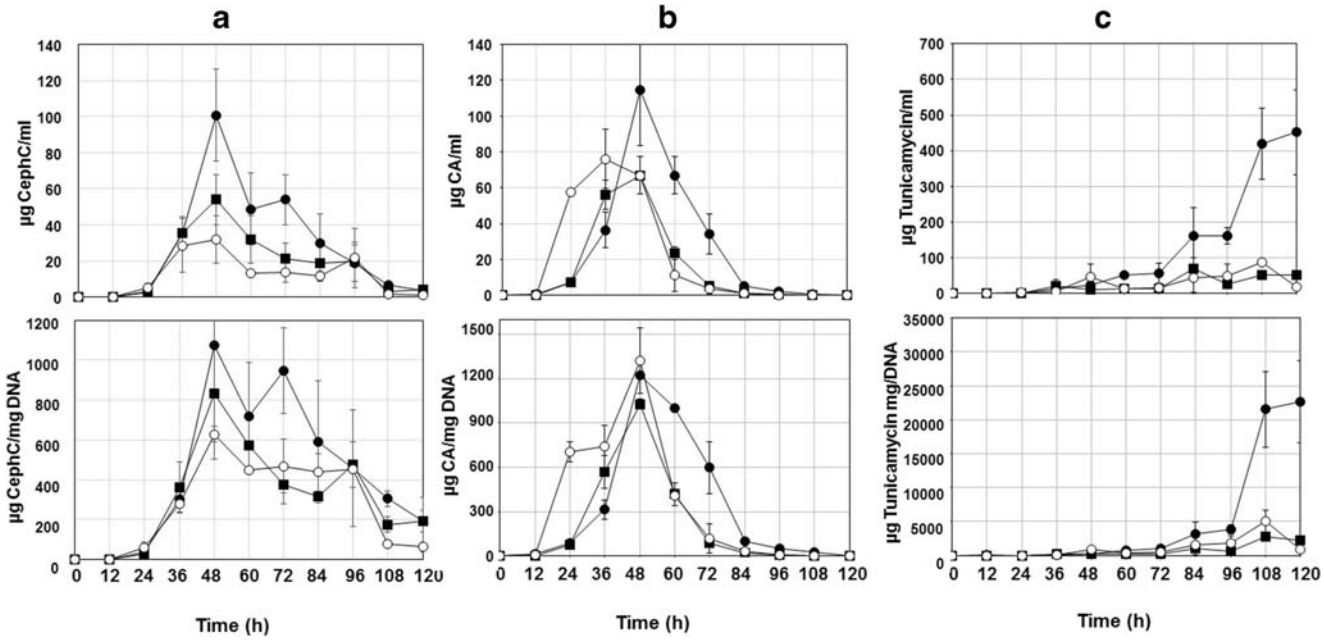

Fig. 6 Comparison of volumetric and specific antibiotic productions in *S. clavuligerus* pCOlysA (closed circles), *S. clavuligerus* NRRL 3585 (closed squares), and *S. clavuligerus* pSPG (open circles) strains depending on time. Panel **a** for CephC, panel **b** for CA, and panel **c** for tunicamycin

manipulation of aspartate pathway in *S. clavuligerus*. Yılmaz et al. [35] disrupted *hom* gene encoding homoserine dehydrogenase in the aspartate pathway in *S. clavuligerus* to see its effect in CephC yields. They showed that the *hom* mutant exerted 4.3-fold specific CephC production in chemically defined media. Later, the same group overexpressed multicopy of the *ask* gene, encodes aspartokinase involved in the first step of aspartate pathway in CephC biosynthesis, in *S. clavuligerus* NRRL 3585 and its *hom* mutant [15]. They reported that *ask* overexpression in the wild type resulted in an average of 3- and 1.8-fold increase in specific and volumetric CephC production in TSB and modified chemically defined medium, respectively, while *hom*-minus background significantly decreased CephC yields in both media probably due to enzyme overproduction or metabolic burden.

The deduced amino acid sequence of *S. clavuligerus lysA* gene exhibited significant homology to known DAP decarboxylases, especially those of actinobacteria such as *Streptomyces coelicolor*, *Corynebacterium efficiens*, and *Mycobacterium tuberculosis* [6]. LysR encoded by the *lysR* gene is located immediately upstream from *lysA* and was found to be a positive regulator in *E. coli* [36]. The *lysR* gene has also been determined by homology in *S. coelicolor* (U37580, GenBank). LysR-type transcriptional regulators (LTTRs) act on gene expressions in a similar way to the model regulator *lysR* that controls *lysA* expression in Enterobacteriaceae. Co-inducers are important for the function of LTTRs and probably contribute to a feedback loop. In that regulation, a product or intermediate of a given metabolic/synthesis pathway which is generally activated by an LTTR acts as the co-inducer required for transcriptional activation or repression [37, 38]. In our study, we applied a rational approach by homologous expression of the *lysA* gene under the control of *glpFp* in TSBG in *S. clavuligerus*. The *lysA* overexpression in the recombinant *S. clavuligerus* pCOlysA strain led to only 1.5-fold upregulation in its own expression level while the CephC gene cluster expression levels increased by 2- to 6-fold, indicating such a feedback loop could be possible in the control of *lysA* expression in the recombinant strain. Regarding increased CephC cluster expression levels, the expression results were in agreement with the production of CephC, which was almost 2-fold increase in the recombinant strain compared with that of the wild type.

CcaR is a common CSR of CephC and CA located at the CephC gene cluster. Several studies indicated a high correlation between CephC and CA. In addition to CSR regulation, pleiotropic and nutritional regulation mechanisms are also effective in the production of both metabolites. *S. clavuligerus* CcaR activator functions as a positive regulator in the CephC gene cluster by binding to *cefD-cmcI* bidirectional promoter, its own promoter, *lat* and *cefF* promoter regions [34]. The *ccaR* overexpression under the control of *pglpF* in TSBG in *S. clavuligerus* resulted in 6.1-fold increased specific CephC production compared with the wild type [16]. In addition, multicopy expression of *ccaR* under *glpFp* resulted in significantly improved industrial high-titer CA producer *S. clavuligerus* strains [39]. Thus, the increased expression of biosynthetic genes in *S. clavuligerus* pCOlysA might be

indirectly associated with positive effect of the *lysA* overexpression on CcaR.

Cavallieri et al. [19] showed by flux balance analysis that lysine and maltose addition to chemically defined media increased both CephC and CA productions in *S. clavuligerus*. It was also shown that the addition of glycerol or other vegetable oils promotes CA production [40, 41]. In our study, the *lysA* overexpression and glycerol addition did not affect CA yields in the recombinant strain.

Tunicamycin is composed of a uracil, an *N*-acetylglucosamine (GlcNac), an unusual 11-carbon 2 aminoaldose sugar called tunicamine, and an amid-linked fatty acid. The unique tunicamine 11-carbon dialdose sugar backbone arises from a 5-carbon furanose precursor derived from uridine and a 6-carbon *N*-acetylamino-pyranose precursor derived from UDP-GlcNac. The α,β-1″,11′-glycosidic linkage between tunicamine and GlcNac is specific for this metabolite [31, 42]. Therefore, tunicamycin biosynthesis might improve with the supplementation of 5- and 6-carbon carbohydrate precursors or uridine, which might considered bottlenecks for tunicamycin biosynthesis as stated by Martínez-Burgo et al. [17]. They reported that tunicamycin overproduction in the *pimM*-overexpressed *S. clavuligerus* was not due to overexpression of tunicamycin biosynthetic gene cluster but might be a result of an increase in the supply of tunicamycin precursors.

Apart from this study [17], the tunicamycin-related studies mostly focused on structural and functional elucidation. Its regulation and interaction with other antibiotic metabolic pathways are still unknown. The absence of a CSR in the tunicamycin gene cluster makes its regulation mechanism more attractive [5, 43–46]. However, we have recently proposed a possible role of BldG pleiotropic regulator for tunicamycin in *S. clavuligerus* through qRT-PCR and bioassay studies by using *bldG* mutant [25] and overexpressed strains [47]. Consequently, this manipulation in *S. clavuligerus* through multicopy expression of the *lysA* gene under the control of inducible glycerol promoter led to a significant increase in the tunicamycin titer. This is the first report indicating that a manipulation of the aspartate pathway resulted in an elevated level of tunicamycin in the recombinant strain although the exact underlying mechanism has not known yet.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Brites LM, Oliveira LM, Barboza M (2013) Kinetic study on cephamycin C degradation. Appl Biochem Biotechnol 171:2121–2128. https://doi.org/10.1007/s12010-013-0502-x
2. Leite CA, Cavallieri AP, Araujo ML (2013) Enhancing effect of lysine combined with other compounds on cephamycin C production in *Streptomyces clavuligerus*. BMC Microbiol 13(1):296
3. Toro L, Pinilla L, Avignone-Rossa C, Ríos-Estepa R (2018) An enhanced genome-scale metabolic reconstruction of *Streptomyces clavuligerus* identifies novel strain improvement strategies. Bioprocess Biosyst Eng 41(5):657–669. https://doi.org/10.1007/s00449-018-1900-9
4. De La Fuente A, Lorenzana LM, Martin JM, Liras P (2002) Mutants of *Streptomyces clavuligerus* with disruptions in different genes for clavulanic acid biosynthesis produce large amounts of holomycin: possible cross-regulation of two unrelated secondary metabolic pathways. J Bacteriol 184(23):6559–6565. https://doi.org/10.1128/JB.184.23.6559-6565.2002
5. Wyszynski FJ, Hesketh AR, Bibb MJ, Davis BG (2010) Dissecting tunicamycin biosynthesis by genome mining: cloning and heterologous expression of a minimal gene cluster. Chem Sci 1(5):581–589
6. Yağcıoğlu Ç (2004) Cloning and characterization of *Streptomyces clavuligerus* meso-diaminopimelate decarboxylase (*lysA*) gene. Dissertation, Middle East Technical University
7. Kern BA, Hendlin D, Inamine E (1980) L-lysine-ε-aminotransferase involved in cephamycin C synthesis in *Streptomyces lactamdurans*. Antimicrob Agents Chemother 17:679–685
8. Tunca S, Yılmaz EI, Piret J, Liras P, Özcengiz G (2004) Cloning, characterization and heterologous expression of the aspartokinase and aspartate semialdehyde dehydrogenase genes of cephamycin C-producer *Streptomyces clavuligerus*. Res Microbiol 155(7):525–534
9. Romero J, Martín JF, Liras P, Demain AL, Rius N (1997) Partial purification, characterization and nitrogen regulation of the lysine epsilon-aminotransferase of *Streptomyces clavuligerus*. J Ind Microbiol Biotechnol 18(4):241–246
10. Pérez-Llarena FJ, Rodríguez-García A, Enguita FJ, Martín JF, Liras P (1998) The *pcd* gene encoding piperideine-6-carboxylate dehydrogenase involved in biosynthesis of α-aminoadipic acid is located in the cephamycin cluster of *Streptomyces clavuligerus*. J Bacteriol 180(17):4753–4756
11. Rius N, Maeda K, Demain AL (1996) Induction of L-lysine-ε-aminotransferase by L-lysine in *Streptomyces clavuligerus*, producer of cephalosporins. FEMS Microbiol Lett 144:207–211
12. Özcengiz G, Demain AL (2013) Recent advances in the biosynthesis of penicillins, cephalosporins and clavams and its regulation. Biotechnol Adv 31(2):287–311. https://doi.org/10.1016/j.biotechadv.2012.12.001
13. Leitão AL, Enguita FJ, De La Fuente JL, Liras P, Martin JF (1999) Inducing effect of diamines on transcription of the cephamycin C genes from the *lat* and *pcbAB* promoters in *Nocardia lactamdurans*. J Bacteriol 181(8):2379–2384
14. Leitão AL, Enguita FJ, Martín JF, Santos Oliveira JF (2001) Effect of exogenous lysine on the expression of early cephamycin C biosynthetic genes and antibiotic production in Nocardia lactamdurans MA4213. Appl Microbiol Biotechnol 56:670–675. https://doi.org/10.1007/s002530100715
15. Özcengiz G, Okay S, Ünsaldı E, Taşkın B, Liras P, Piret J (2010) Homologous expression of aspartokinase (*ask*) gene in *Streptomyces clavuligerus* and its *hom*-deleted mutant: effects on cephamycin C production. Bioengineered Bugs 1(3):191–197

16. Kurt A, Álvarez-Álvarez R, Liras P, Özcengiz G (2013) Role of the *cmcH–ccaR* intergenic region and *ccaR* overexpression in cephamycin C biosynthesis in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 97(13):5869–5880
17. Martínez-Burgo Y, Santos-Aberturas J, Rodríguez-García A, Barreales EG, Tormo JR, Truman AW, Reyes F, Aparicio JF, Liras P (2019) Activation of secondary metabolite gene clusters in *Streptomyces clavuligerus* by the PimM regulator of *Streptomyces natalensis*. Front Microbiol 10:580. https://doi.org/10.3389/fmicb.2019.00580
18. Alvarez-Álvarez R, Rodríguez-García A, Santamarta I, Pérez-Redondo R, Prieto-Domínguez A, Martínez-Burgo Y, Liras P (2014) Transcriptomic analysis of *Streptomyces clavuligerus* ΔccaR::tsr: effects of the cephamycin C-clavulanic acid cluster regulator CcaR on global regulation. Microb Biotechnol 7:221–231
19. Cavallieri AP, Baptista AS, Leite CA, da Costa Araujo MLG (2016) A case study in flux balance analysis: lysine, a cephamycin C precursor, can also increase clavulanic acid production. Biochem Eng J 112:42–53
20. Bellão C, Antonio T, Araujo MLGC, Badino AC (2013) Production of clavulanic acid and cephamycin C by *Streptomyces clavuligerus* under different fed-batch conditions. Braz J Chem Eng 30:257–266. https://doi.org/10.1590/S0104-66322013000200004
21. Saudagar PS, Singhal RS (2007) Optimization of nutritional requirements and feeding strategies for clavulanic acid production by *Streptomyces clavuligerus*. Bioresour Technol 98:2010–2017
22. Leite CA, Cavallieri AP, Baptista AS, Araujo ML (2016) Dissociation of cephamycin C and clavulanic acid biosynthesis by 1,3-diaminopropane in *Streptomyces clavuligerus*. FEMS Microbiol Lett 363(1):fnv215. https://doi.org/10.1093/femsle/fnv215
23. Kieser T, Bibb M, Buttner M, Chater K, Hopwood D (2000) Practical *Streptomyces* genetics. The John Innes Foundation, John Innes Centre, Norwich
24. Hu WS, Braña AF, Demain AL (1984) Carbon source regulation of cephem antibiotic production by resting cells of *Streptomyces clavuligerus* and its reversal by protein synthesis inhibitors. Enzyme Microb Tech 6(4):155–160. https://doi.org/10.1016/0141-0229(84)90023-1
25. Bignell DR, Tahlan K, Colvin KR, Jensen SE, Leskiw BK (2005) Expression of CcaR, encoding the positive activator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus*, is dependent on BldG. Antimicrob Agents Chemother 49(4):1529–1541
26. Romero J, Liras P, Martín JF (1984) Dissociation of cephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 20:318–325
27. Hobbs G, Frazer CM, Gardner DCJ, Cullum JA, Oliver SG (1989) Dispersed growth of *Streptomyces* in liquid culture. Appl Microbiol Biotechnol 31:272–277. https://doi.org/10.1007/s00253-018-8748-4
28. Flett F, Mersinias V, Smith CP (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett 155:223–229
29. Burton K (1968) Determination of DNA concentration with diphenylamine. Methods Enzymol 12:163–166
30. Tsvetanova BC, Price NP (2001) Liquid chromatography-electrospray mass spectrometry of tunicamycin-type antibiotics. Anal Biochem 289(2):147–156. https://doi.org/10.1006/abio.2000.4952
31. Tsvetanova BC, Kiemle DJ, Price NP (2002) Biosynthesis of tunicamycin and metabolic origin of the 11-carbon dialdose sugar, tunicamine. J Biol Chem 277(38):35289–35296
32. Aigle B, Wietzorrek A, Takano E, Bibb MJ (2000) A single amino acid substitution in region of the principal sigma factor of *Streptomyces coelicolor* A3(2) results in pleiotropic loss of antibiotic production. Mol Microbiol 37:995–1004
33. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408
34. Santamarta I, López-García MT, Kurt A, Nárdiz N, Alvarez-Álvarez R, Pérez-Redondo R, Martín JF, Liras P (2011) Characterization of DNA-binding sequences for CcaR in the cephamycin-clavulanic acid supercluster of *Streptomyces clavuligerus*. Mol Microbiol 81(4):968–981. https://doi.org/10.1111/j.1365-2958.2011.07743.x
35. Yılmaz EI, Çaydasi AK, Özcengiz G (2008) Targeted disruption of homoserine dehydrogenase gene and its effect on cephamycin C production in *Streptomyces clavuligerus*. J Ind Microbiol Biotechnol 35:1–7
36. Stragier P, Richaud F, Borne F, Patte JC (1983) Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. I. Identification of a *lysR* gene encoding an activator of the *lysA* gene. J Mol Biol 168:307–320
37. Tropel D, van der Meer JR (2004) Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol Mol Biol Rev 68:474–500. https://doi.org/10.1128/MMBR.68.3.474-500.2004
38. Maddocks SE, Oyston PC (2008) Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology 154:3609–3623. https://doi.org/10.1099/mic.0.2008/022772-0
39. Kurt-Kızıldoğan A, Vanlı-Jaccard G, Mutlu A, Sertdemir G, Özcengiz G (2017) Genetic engineering of an industrial strain of *Streptomyces clavuligerus* for further enhancement of clavulanic acid production. Turk J Biol 41:342–353. https://doi.org/10.3906/biy-1608-17
40. Ser HL, Law JWF, Chaiyakunapruk N, Jacob SA, Jacob SA, Palanisamy UD, Chan KG, Goh BH, Lee LH (2016) Fermentation conditions that affect clavulanic acid production in *Streptomyces clavuligerus*: a systematic review. Front Microbiol 7:522. https://doi.org/10.3389/fmicb.2016.00522
41. Kurt Kızıldoğan A (2017) The effect of different vegetable oils on clavulanic acid production in *Streptomyces clavuligerus*. Anadolu J Agr Sci 32(2):223–228. https://doi.org/10.7161/omuanajas.287668
42. Price NP, Tsvetanova B (2007) Biosynthesis of the tunicamycins: a review. J Antibiot (Tokyo) 60(8):485–491
43. Winn M, Goss RJ, Kimura KI, Bugg TD (2010) Antimicrobial nucleoside antibiotics targeting cell wall assembly: recent advances in structure– function studies and nucleoside biosynthesis. Nat Prod Rep 27(2):279–304
44. Karki S, Kwon SY, Kwon HJ (2011) Cloning of tunicamycin biosynthetic gene cluster from *Streptomyces chartreusis* NRRL3882. J Korean Soc Appl Biol Chem 54(1):136–140
45. Wyszynski FJ, Lee SS, Yabe T, Wang H, Gomez-Escribano JP, Bibb MJ, Lee SJ, Davies GJ, Davis BG (2012) Biosynthesis of the tunicamycin antibiotics proceeds via unique exo-glycal intermediates. Nat Chem 4(7):539–546
46. Widdick D, Royer SF, Wang H, Vior NM, Gomez-Escribano JP, Davis BG, Bibb MJ (2018) Analysis of the tunicamycin biosynthetic gene cluster of *Streptomyces chartreusis* reveals new insights into tunicamycin production and immunity. Antimicrob Agents Chemother 62:e00130–e00118. https://doi.org/10.1128/AAC.00130-18
47. Baş L, Otur Ç, Kurt-Kızıldoğan A Enhanced tunicamycin biosynthesis in BldG overexpressed *Streptomyces clavuligerus*. Appl Biochem Microbiol (in press)