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Enhanced Tunicamycin Biosynthesis in BldG Overexpressed *Streptomyces clavuligerus*

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Abstract—Tunicamycin is a nucleoside type antibiotic with a potent antibacterial activity. Tunicamycin gene cluster in *Streptomyces clavuligerus* lacks a cluster-situated regulator (CSR). Therefore, there is no information about its regulation in the cell. To have an insight about the regulation of tunicamycin biosynthesis, the possible effects of BldG pleiotropic regulator involved in the control of secondary metabolite production in *S. clavuligerus* were investigated. To overexpress *bldG* in the cell, strains containing multiple copies of the gene expressed from P_{glpF} promoter of *S. clavuligerus* pLB1, and an additional *bldG* integrated in the chromosome of *S. clavuligerus* pLB2, were constructed. *S. clavuligerus* pLB1 and *S. clavuligerus* pLB2 fermentations resulted in 16.4- and 13.8-fold higher specific tunicamycin titers, respectively, in comparison to wild type by confirming quantitative reverse-transcription PCR (qRT-PCR) data. However, qRT-PCR expression analysis of tunicamycin genes in *S. clavuligerus* Δ*bldG* constructed by Bignell with coworkers [1] showed that gene expressions at T_{36} (except for *SCLAV_4274* and *SCLAV_4275*) were from 3.6- to 57.9-fold reduced compared to wild type. The tunicamycin titers were lower in *S. clavuligerus* Δ*bldG* than in wild type, as well. Consequently, the data presented here is the first report indicating a positive role of BldG on tunicamycin.

Keywords: tunicamycin, *Streptomyces clavuligerus*, *bldG*, qRT-PCR, overexpression

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The members of genus *Streptomyces* are versatile producers of a vast array of bioactive secondary metabolites including antibiotics, immunosuppressants and anticancer agents such as cephamycin C, clavulanic acid, tunicamycin and holomycin [2]. Secondary metabolite gene clusters are controlled by cluster situated regulators (CSRs) at the lowest level, and by higher-level global/pleiotropic regulators, in a complex cascade system. Thus, CSRs might have different functions as mentioned by Makitrynskyy et al. [3] and could be (i) an ultimate regulator, (ii) an ultimate regulator having cross-talk function [4], (iii) a true pleiotropic regulator [5], or (iv) regulator of a distant gene cluster [6]. In turn, global/pleiotropic regulators exert their effects on the gene clusters by controlling CSRs in response to different stimuli such as environmental signals, physiological conditions and developmental stage [7].

Tunicamycin is a fatty acyl nucleoside-type antibiotic produced by several *Streptomyces* species including *Streptomyces lysosuperificus, Streptomyces chartreusis* [8] and *Streptomyces clavuligerus* [9]. Also, there have been other studies showing different species having tunicamycin gene cluster resembling to that of *Streptomyces* [10, 11]. It has great potency against early stage of bacterial cell wall synthesis by targeting MraY (translocase I) that catalyzes the formation of peptidoglycan precursor typically referred to as lipid I [12]. In a recent study, it was shown that a marine-derived strain *Streptomyces* sp. DUT11 produces tunicamycin I, IV and VII showing superior anticomplement activity [13]. Since tunicamycin inhibits eukaryotic protein N-glycosylation its clinical use as antibacterial agent is not feasible yet [14]. 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 [15]. The α,β-1",11'-glycosidic linkage between tunicamine and GlcNac is also unique for this metabolite. The length of N-linked acyl chains is different among tunicamycin variants [16].

Although its structure and function are well known, biosynthetic gene cluster and metabolic pathway studies are limited [16, 17]. A total of 14 genes (*tun*1(A)-*tun*14(N)-*SCLAV_4287-4274*) were identified by genome mining approach coupled with cloning and heterologous expression studies, in *S. chartreusis* and *S. clavuligerus*, lacking a CSR within the cluster [18, 19] (Figs. 1a, 1b). Recently, Widdick et al. [20] have reported mutational analysis and transcriptional characterization study of tunicamycin gene cluster in *S. chartreusis*.

Since there have been no regulatory genes found in the tunicamycin cluster, a possible regulation at global level needs to be elucidate to get more information for

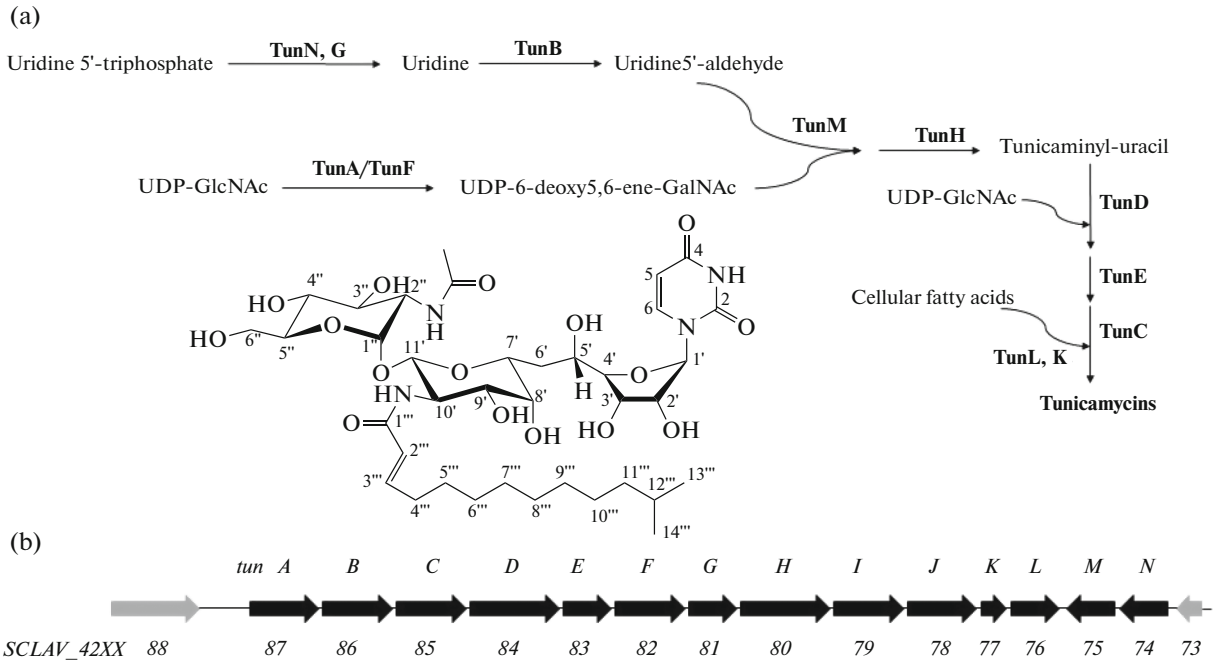

Fig. 1. Biochemical pathway (a) and gene cluster (b) of tunicamycin in *S. clavuligerus* [12, 14].

design of new tunicamycins for treatment of diseases and for strain improvement studies. In *S. clavuligerus*, BldG is an important pleiotropic regulator positively affecting antibiotic production and morphological differentiation [1]. It is used as an anti-anti σ-factor involved in posttranslational regulation and has positive effect on CcaR CSR and other positive modulators such as AdpA acting on antibitoic biosynthesis such as cephamycin C and clavulanic acid in *S. clavuligerus* [21]. The present study aims at deciphering the putative regulatory role of BldG in tunicamycin biosynthesis in *S. clavuligerus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The microorganisms and plasmids used in this study are listed in Table 1. *S. clavuligerus* strains were grown in TSB medium (Thermo Fisher Scientific, USA) at 28°C and 220 rpm to get 24 h old pre-cultures. Five ml of bacterial suspension were used to inoculate TSBY [22] supplemented with 0.5% glycerol [23]. Fermentation experiments were carried out for 4 days under the same conditions, repeated twice and two biological replicates were used. Triplicate samples were taken from the cultures to determine growth and antibiotic production. Cultures of recombinant *S. clavuligerus* strains were supplemented with apramycin (50 μg/mL) to maintain the plasmids.

Escherichia coli DH5α and *E. coli* ET12567/pUZ8002 were grown in LB broth or on LB agar at 37°C with appropriate antibiotics when necessary [ampicillin (100 μg/mL), apramycin (50 μg/mL), chloramphenicol (25 μg/mL), kanamycin (50 μg/mL) and nalidixic acid (25 μg/mL)]. MS agar [24] was used to grow exconjugants following conjugation. The tunicamycin supersensitive strain *Bacillus subtilis* 6633 was grown in TSB medium at 28°C and 200 rpm to OD_{600} of 0.9–1.0 prior to bioassay studies.

Construction of plasmids and strains. *S. clavuligerus bldG* null mutant was kindly provided from Prof. Kapil Tahlan [1]. *S. clavuligerus* LB1, *S. clavuligerus* LB2 and *S. clavuligerus* pSETermE* were constructed in the present study. The *bldG* gene was isolated by PCR from *S. clavuligerus* genomic DNA using the primers indicated in Table 2. The *bldG* gene was subcloned to the pGEM-T® easy vector (Promega, USA) as a fragment of 452 bp to give pLB14 plasmid. The construct was verified by restriction digestion, PCR and nucleotide sequencing. (i) The *bldG* gene was subcloned to downstream of the promoter of *glpF,* a gene involved in glycerol transport [25], in pSPG, a pIJ699-derived vector. Both pLB14 and pSPG were digested with *Nde*I-*Spe*I enzymes and ligated to generate pLB1, a multicopy vector expressing *bldG* from the *glpF* promoter. (ii) The *Eco*RI fragment carrying *bldG* was ligated to *Eco*RI linearized pSET152*ermE** to generate pLB2, an integrative single copy plasmid in which *bldG* is expressed from *ermE** constitutive promoter [26].

The constructions were verified by restriction digestion, PCR and sequencing. pLB1, pLB2 and pSET152*ermE** were introduced into the methylation-deficient *E. coli* ET12567/pUZ8002 strain to obtain non-methylated DNA. Intergeneric conjugation between *Streptomyces* and *E. coli* was carried out

Table 1. Microorganisms and plasmids used or constructed in the study

	Description	Source or Reference
Strains		
S. clavuligerus ATCC27064	Wild type, cephamycin C, and clavulanic acid producer	ATCC
S. clavuligerus Δ*bldG*	*bldG* null mutant, internal *bldG* sequence was replaced with a *aac(3)IV-oriT* cassette	[1]
S. clavuligerus pLB1	Recombinant with *bldG* inserted in pLB1 multicopy expression vector	This study
S. clavuligerus pSPG	pSPG carrying strain. Control strain for *S. clavuligerus* pAK23 and pLB1	[23]
S. clavuligerus pLB2	Recombinant with *bldG* inserted in pLB2 integration vector	This study
S. clavuligerus pSETermE*	pSET152ermE* integrated strain. Control strain for *S. clavuligerus* pLB2	This study
E. coli DH5α	*F′ ϕdlacZM15 (lacZYA argF), U169, supE44λ−, thi-1, gyrA, recA1, relA1, endA1, hsdR17*	*E. coli* Genetic Stock Center ATCC
E. coli ET12567/pUZ8002	*dam 13::Tn9 dcm-6 hsdM hsdR, oriT-RP4*	[34]
Bacillus subtilis 6633	Tunicamycin indicator strain	[1]
Plasmids		
pGEM-T®Easy	*AmpR, lacZ′*	Promega
pLB14	*bldG* containing pGEM-T® Easy	This study
pSPG	Ampicillin and apramycin resistant (Amp^R, Apr^R), *Streptomyces–E. coli* multicopy vector. It contains the promoter of the *glpF* gene, and *aac(3)IV-oriT*	[23]
pLB1	*bldG* containing pSPG multicopy plasmid at its *NdeI–SpeI* site. Ampicillin and apramycin resistant (Amp^R, Apr^R)	This study
pSET152*ermE**	*lacZ, rep^{puc}, att^{ΦC31}, oriT, ermE** promoter	Combinature Biopharm AG, [26]
pLB2	*bldG* inserted in *EcoRI* site of pSET152ermE*	This study

as described by Flett et al. [27]. Exconjugants grown on MS agar were transferred to TSB agar containing apramycin and allowed to grow for up to 4 days at 28°C. The presence of pLB1 and pLB2 in *S. clavuligerus* exconjugants was confirmed by PCR using the *bldG* reverse primer and a primer internal to the apramycin resistance gene (Table 2).

Nucleotide sequencing. DNA sequencing was carried out at BGI (Europe) through Genoks (Ankara, Turkey). Deduced nucleotide sequence was compared with the National Center for Biotechnology Information (USA) database using the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

Growth determination. The growth of the cultures was determined by DNA quantification according to Burton [28]. DNA concentrations were calculated using herring sperm DNA as standard and expressed as μg of DNA per mL of culture.

Tunicamycin extraction and bioassay for tunicamycin production. For tunicamycin extraction from culture broths a modified procedure adapted from Tsvetanova and Price [29] and Tsvetanova et al. [15] was used. TSBYG cultures were acidified using HCl at a final concentration of 1%. Acid insoluble tunicamycin complex was centrifuged at 4000×*g* for 10 min and washed in 0.2 M HCl, and this step was repeated twice. The pellets were mixed with methanol and vortexed in ice to dissolve tunicamycin. Following centrifugation (4000×*g*, 10 min. 4°C), the methanol containing supernatants were evaporated at 40°C, and the tunicamycin extracts were dissolved in appropriate amount of methanol prior to bioassay.

Tunicamycin was quantified using the agar well diffusion method with the use of *B. subtilis* 6633 as indicator organism [30]. Tunicamycin concentrations were calculated based on a standard curve generated using commercial tunicamycin (Sigma-Aldrich, USA).

RNA isolation and gene expression analyses. RNA was isolated from samples taken from cultures at 36 h of growth. RNA was purified using GeneJET RNA isolation kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions with some modifications. (i) lysozyme treatment for cell wall lysis, (ii) phenol/chlorophorm/isoamylalcohol treatment with the use of phase lock gel heavy columns (5 PRIME) and (iii) DNase treatment using DNA-free™ kit (Thermo Fisher Scientific, USA) at final step. The integrity, purity and amount of the RNA were determined in a NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific, USA).

RNA samples (2 μg) were converted to cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystems, USA). qRT-PCR reactions were carried out on CFX96 real time PCR robotics

Table 2. Primers used in study

Name	5' to 3' primer sequence	Product size (bp)	Utility
*bldG-FP**	CATATGGTGGACCTGTCCCTGTCGACTC	452	To clone *bldG* gene
*bldG-RP***	ACTAGTAAGAGCCGTGCCCGCCAC		
pSETD-FP	TAGTCCTGTCGGGTTTCGCCAC	1217	To confirm insertion of *bldG*
bldGR1	ACTAGTAAGAGCCGTGCCCGCCAC		into pSET152ermE*
pSPGD-FP	TGCCTTTGCTCGGTTGATCC	999	To confirm insertion of *bldG*
bldGR1	ACTAGTAAGAGCCGTGCCCGCCAC		into pSPG
hrdB-FP	CGCGGCATGCTCTTCCT	109	To amplify by qRT-PCR a
hrdB-RP	AGGTGGCGTACGTGGAGAAC		*hrdB* internal probe
SCLAV_4274-FP	TGTGCTGGTGATCCTTGGCTGCTA	144	To amplify by qRT-PCR a
SCLAV_4274-RP	GGGCGGCTGATGTCCTGCTTCC		*SCLAV_4274* internal probe
SCLAV_4275-FP	GTGAGGAGGAAGGGAACGGC	81	To amplify by qRT-PCR a
SCLAV_4275-RP	ATTGAAGGCGACACAGGTCG		*SCLAV_4275* internal probe
SCLAV_4276-FP	TCGGACTCGCCCAGGACA	101	To amplify by qRT-PCR a
SCLAV_4276-RP	ATTGCCCCAGGAGGTGATGAA		*SCLAV_4276* internal probe
SCLAV_4277-FP	CTTTCGGCGATCTGGATGTGGAC	146	To amplify by qRT-PCR a
SCLAV_4277-RP	GCTGACGCCGGCGATGTATTC		*SCLAV_4277* internal probe
SCLAV_4278-FP	GCGCAGCACCGGAGCCTTTGAGTA	102	To amplify by qRT-PCR a
SCLAV_4278-RP	GCGGGCAGCGAGAGCAGTGAGAA		*SCLAV_4278* internal probe
SCLAV_4279-FP	CCGCATCGAGGAGGGGGAGTT	148	To amplify by qRT-PCR a
SCLAV_4279-RP	GTACCGGGAGTGGGCGAAGACAT		*SCLAV_4279* internal probe
SCLAV_4280-FP	GGCCGGCcTTCCACCCCTACGAGA	122	To amplify by qRT-PCR a
SCLAV_4280-RP	CGGCGGAACGGGAGAAAGTGTGC		*SCLAV_4280* internal probe
SCLAV_4281-FP	GGCGGAGTCTGGCACGATTTCAT	147	To amplify by qRT-PCR a
SCLAV_4281-RP	CTCCCGCCACCGTTCAGCAGTTCT		*SCLAV_4281* internal probe
SCLAV_4282-FP	CCTGCGCCGGGTCTTTC	104	To amplify by qRT-PCR a
SCLAV_4282-RP	CCCGGTAGTACGTGGTGATGTC		*SCLAV_4282* internal probe
SCLAV_4283-FP	CGGGAGAACTGGGTGCGAAGGAGA	150	To amplify by qRT-PCR a
SCLAV_4283-RP	CCGGGTGGTGGGTGAGGACGAGT		*SCLAV_4283* internal probe
SCLAV_4284-FP	GGCCTTGTCGCGCACGGTCACTC	114	To amplify by qRT-PCR a
SCLAV_4284-RP	TCGGCGAAAGGCGGCACACTCAC		*SCLAV_4284* internal probe
SCLAV_4285-FP	ATCTCCTCGGTCATCGTCGTG	90	To amplify by qRT-PCR a
SCLAV_4285-RP	GAGCCGTGCGGTGTCGTA		*SCLAV_4285* internal probe
SCLAV_4286-FP	CCGTCCGCAAGGGGTTCTGG	88	To amplify by qRT-PCR a
SCLAV_4286-RP	TGGTTGGCCGCGTTGGTGATG		*SCLAV_4286* internal probe
SCLAV_4287-FP	CCGGAGGGGACAGGTAAAT	98	To amplify by qRT-PCR a
SCLAV_4287-RP	TTGTGCGAGGCTAGATGGTAAA		*SCLAV_4287* internal probe

* FP: Forward primer, ** RP: reverse primer.

(BioRad, USA) using GoTaq® qPCR master mix (Promega, USA) as the specific reagent. A standard reaction included the following components: 10 μL 2×GoTaq® qPCR master mix, 0.4 μL 10 μM forward primer, 0.4 μL 10 μM reverse primer, 1 μL cDNA and 8.2 μL dH_2O in a final volume of 20 μL. The qRT-PCR reactions were started with an initial denaturation step (2 min at 95°C) and proceded with 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). Melting curve analyses were performed to check for specificity of the amplifications. Two independent qRT-PCR runs were performed. No template control (NTC) containing dH_2O instead of cDNA were run to detect background signal and unwanted primer dimer formation. The *hrdB*-like gene encoding the major σ-factor in *S. coelicolor* A3(2) was chosen as reference gene in relative quantification of gene expression [31]. $2^{-\Delta\Delta Ct}$ method was used to analyze the data [32].

Statistical analysis of qRT-PCR data. One way ANOVA with Bonferroni *post hoc* testing was performed on the normalized gene expression to check whether expression data were statistically different between *S. clavuligerus* ATCC 27064 (wild type strain) and modified strains. Graphpad Prism Software (USA) was used for statistical evaluation of qRT-PCR data. Error bars represent 95% confidence intervals. Significance was stated as p values (*<0.05; **<0.01; ***<0.001).

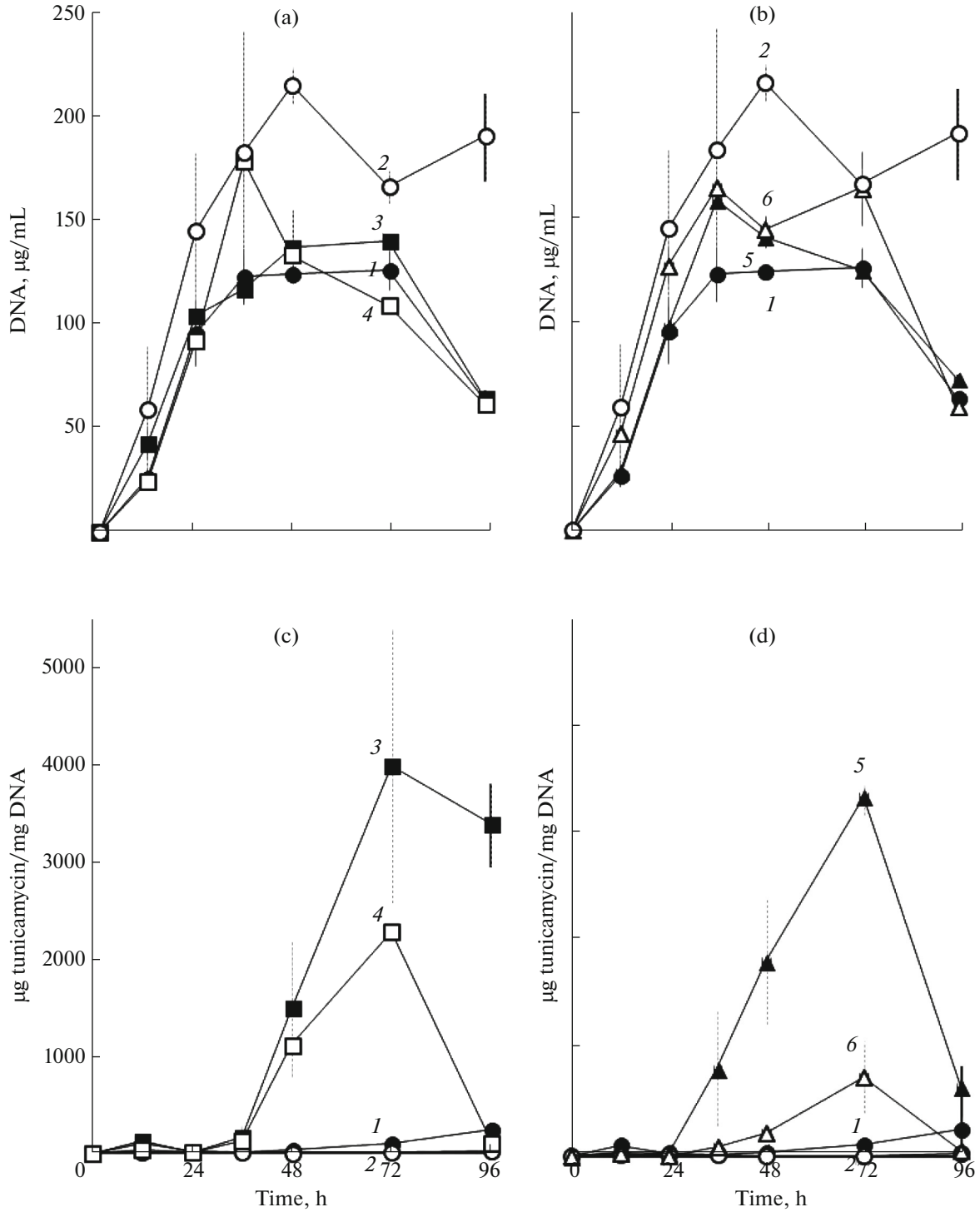

Fig. 2. The effect of *bldG* on tunicamycin production. Fermentation profiles of *S. clavuligerus* strains grown in TSBYG medium (a, b) and specific tunicamycin production (c, d). *1—S. clavuligerus* ATCC27064, *2—S. clavuligerus* Δ*bldG*, *3—S. clavuligerus* pLB1, *4—S. clavuligerus* pSPG, *5—S. clavuligerus* pLB2, *6—S. clavuligerus* pSETermE*.

RESULTS

The effect of BldG regulator in tunicamycin biosynthesis. In *S. clavuligerus* Δ*bldG* mutant, the growth was higher relative to that for wild type strain throughout the fermentation (Fig. 2). The growth of *S. clavuligerus* pLB1 was almost similar compared to the wild type strain during the time course of fermentation, while its growth was lower at 36 h of growth in comparison to the vector control strain (Fig. 2a). *S. clavuligerus* pLB2

exerted a faster growth than the wild type especially at 36 and 48 h of fermentation. However, its vector control, *S. clavuligerus* pSETermE* showed a higher growth pattern than *S. clavuligerus* pLB2 and the wild type till 96 h of incubation (Fig. 2b). The growth kinetics of *bldG* mutant was always higher than the manipulated strains.

In contrast to its high level of growth, *S. clavuligerus* Δ*bldG* produced at most 24.2 μg/mg tunicamycin throughout the fermentation corresponding to 10% specific titer of the wild type strain. Introduction of non-integrated multicopy *bldG* gene or integration of its extra copy into the genome of *S. clavuligerus* resulted in elevated tunicamycin levels. *S. clavuligerus* pLB1 reached 3968.3 μg/mg specific tunicamycin production corresponding to 16.4- and 1.8-fold higher tunicamycin titer than those of the wild type strain (242 μg/mg) and the vector control *S. clavuligerus* pSPG (2272.9 μg/mg) (Fig. 2c). *S. clavuligerus* pLB2 having extra copy of *bldG* along with *ermE** promoter produced as much as 3329.3 μg/mg specific tunicamycin and this production was 13.8- and 4.5-fold more than the wild type strain and *S. clavuligerus* pSETermE* (731.9 μg/mg), respectively (Fig. 2d).

Transcriptional analysis of the effect of *bldG* in tunicamycin C gene cluster. Expression of genes in the tunicamycin gene cluster of *S. clavuligerus* Δ*bldG* grown in TSBYG medium was compared with that of *S. clavuligerus* ATCC27064 by qRT-PCR (Fig. 3a). All genes studied showed significant expression levels with p values (<0.001, <0.01 or $p < 0.05$) with the exception of *SCLAV_4274* ($p > 0.05$). *SCLAV_4275* was the only gene that exerted 1.86-fold increased expression level in the mutant strain compared to the wild type *S. clavuligerus* at T36. All other tunicamycin genes were found to have lower expression values than the wild type strain ranging from 3.6- to 58-fold. The expression level of *SCLAV_4287*, encoding the first enzyme of tunicamycin biosynthesis pathway, was 57.9-fold lower than in the wild type. *SCLAV_4286* expression also 52-fold reduced in *S. clavuligerus* Δ*bldG* after 36 h of incubation. The expression of *SCLAV_4285-4283* was 30-36.7-fold lower in the *bldG* mutant of *S. clavuligerus*. The remaining genes of tunicamycin gene cluster were also downregulated with 3.6-20.9-fold decreased expression levels in the absence of *bldG* gene in *S. clavuligerus* (Fig. 3a).

In contrast to expression profiles of 14 tunicamycin genes in *S. clavuligerus* Δ*bldG* after 36 h of incubation, a totally different expression levels were observed at 72 h incubation. All genes showed statistically significant and higher expression levels in the range 5.4–54.2-fold at this time point in the Δ*bldG* mutant in comparison to the wild type. The highest expression ratio was obtained for *SCLAV_4275*.

The effect of extra copy/ies of *bldG* on the expression levels of tunicamycin genes was also determined by qRT-PCR. In *S. clavuligerus* pLB1, no significant increases for *SCLAV_4274, SCLAV_4275, SCLAV_4286* and *SCLAV_4287* gene expression were observed at both incubation times. However, *SCLAV_4276* and *SCLAV_4282* genes gave statistically significant expression levels with maximum 2.7- and 4.3-fold increases, respectively, over the wild type strain at 36 h (Fig. 3b).

In *S. clavuligerus* pLB2, the increase in the expression levels of *SCLAV_4275, SCLAV_4276, SCLAV_4280, SCLAV_4282, SCLAV_4284, SCLAV_4285* and *SCLAV_4286* genes was statistically significant at 36 h of growth while all the genes did not exert such a significant expression change at the later fermentation time (Fig. 3c).

DISCUSSION

S. clavuligerus Δ*bldG* mutant is unable to form aerial hyphae and cannot produce clavulanic acid and cephamycin C as CSR-CcaR transcription is dependent on BldG [1]. Recently it has been shown that BldG has a wider regulatory effect in the control of clavulanic acid production in *S. clavuligerus* at a level above of CcaR by acting on other proteins such as BldN/AdsA, AfsR-like regulator and a TetR family protein [33]. Makitrynskyy et al. [3] showed that AdpA and BldA pleiotropic regulators directly control moenomycin antibiotic production in *Streptomyces ghanaensis*. Like moenomycin, tunicamycin also have a CSR-free gene cluster. In our study, tunicamycin production in the *S. clavuligerus* Δ*bldG* mutant was very low compared to the wild type at all sampling times studied. Except for *SCLAV_4275*, the other tunicamycin genes had lower expression value after 36 h of incubation in the *bldG* mutant strain. The most drastic decrease was examined in *SCLAV_4286* and *SCLAV_4287* expressions. All tunicamycin genes were upregulated at later incubation time in the mutant strain. Interestingly, the most upregulated gene by the absence of *bldG* was *SCLAV_4275* with 53.2-fold increased expression level after 72 h of incubation. The present data showed that *SCLAV_4275* and *SCLAV_4286*, the genes that have rare TTA codon in the tunicamycin gene cluster exerted diverse expression changes in the *S. clavuligerus* Δ*bldG* mutant. *SCLAV_4275* (putative muT-like protein encoding gene) is involved in formation of tunicaminyluracil core together with *SCLAV_4286* encoding radical SAM binding protein [18]. *SCLAV_4286* involves TGGCCGGCTA, highly resembling to type II consensus sequence (TGGCCGGATT) for binding of AdpA located at the *ccaR* promoter region in the cephamycin C gene cluster [21]. In a recent study of Widdick et al. [20] it was reported that tunicamycin gene cluster is transcribed as a single operon from two promoters (tunp1 and tunp2) located upstream of *SCLAV_4287* in *S. chartreusis*. Although DNA binding of AdpA to upstream region of *SCLAV_4286* and *SCLAV_4287* was tested in our study, no binding was obtained probably due to poor experimental condi-

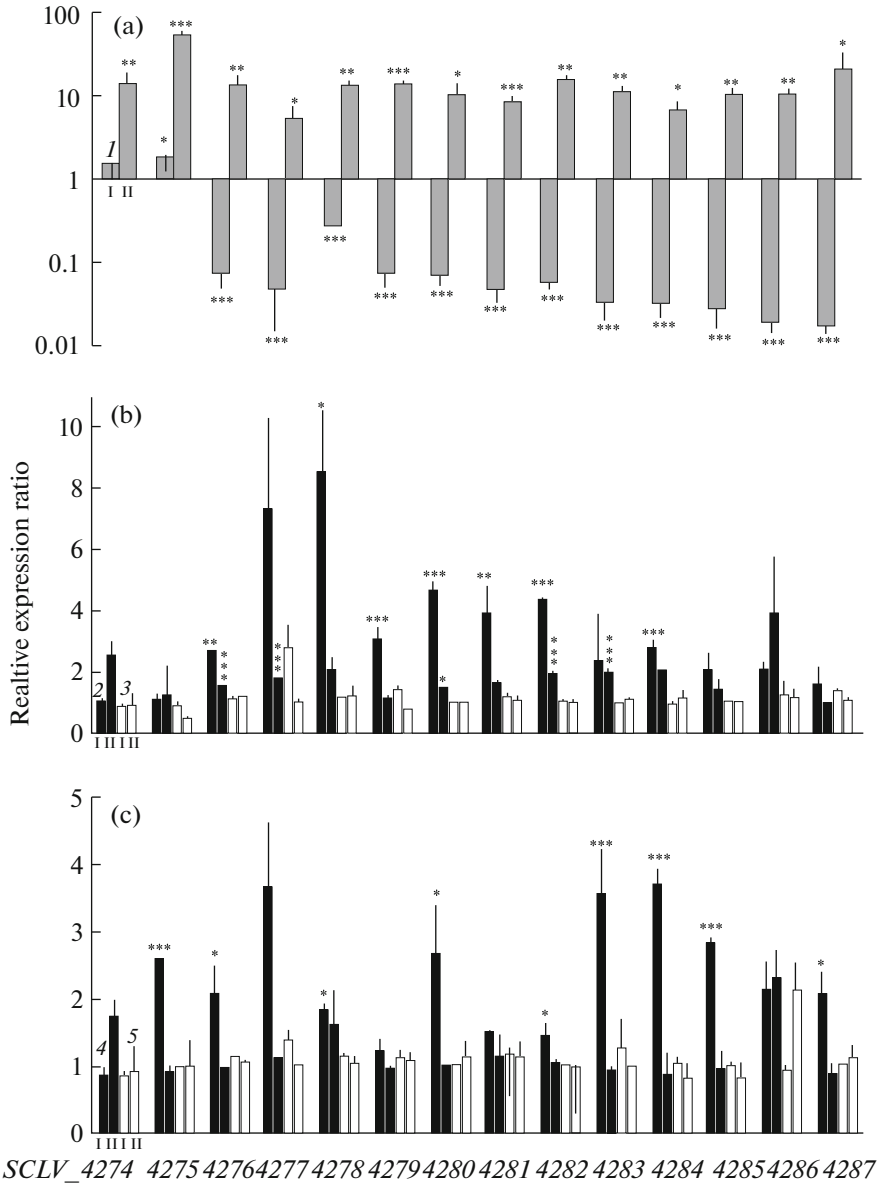

Fig. 3. The effect of *bldG* in the expression of tunicamycin gene cluster in *S. clavuligerus*. Expression changes of tunicamycin genes (a) in *S. clavuligerus* Δ*bldG*, (b) in *S. clavuligerus* pLB1 and *S. clavuligerus* pSPG, (c) in *S. clavuligerus* pLB2 and *S. clavuligerus* pSET152ermE*, compared to the *S. clavuligerus* ATCC27064 (the expression was taken as 1). *1*—*S. clavuligerus* Δ*bldG*, *2*—*S. clavuligerus* pLB1 (black bars), *3*—*S. clavuligerus* pSPG (white bars), *4*—*S. clavuligerus* pLB2 (dark grey bars), *5*—*S. clavuligerus* pSET152ermE* (white bars). I—36, II—72 h. Significance was stated as *p* values (*<0.05; **<0.01; ***<0.001).

tions (data not shown). Therefore, tunicamycin biosynthesis might be directly controlled by BldG or it might exert regulatory effect on tunicamycin through mediating AdpA expression that was shown to be underrepresented in the absence of BldG [33]. However, further studies are needed to elucidate the possible regulation mechanism of BldG in tunicamycin biosynthesis.

The results obtained by *bldG* mutation in tunicamycin production were confirmed by *bldG* overexpressed *S. clavuligerus* pLB1 and *S. clavuligerus* pLB2 recombinant strains. Furthermore, the introduction of integrated copy of *bldG* in the cell provided more tunicamycin production than that in *S. clavuligerus* pLB1, multicopy *bldG*-containing recombinant strain, with respect to the vector control, most probably due to the use of different promoters [23]. Concomitantly, tunicamycin gene expressions in *S. clavuligerus* pLB1 and *S. clavuligerus* pLB2 were differently regulated but a general upregulation was observed in only exception that at T72, the expression differences of most tunicamycin genes in *S. clavuligerus* pLB2 were statistically insignificant.

* * *

The study presented here is the first report regarding to examine possible regulation role acting on expression of tunicamycin genes and production of tunicamycin. It was shown that BldG pleiotropic regulator affects tunicamycin biosynthesis at transcriptional and translational level in *S. clavuligerus*. However, further studies are needed to elucidate the exact mechanism.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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