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Full Length Research Paper

Mutation of the *Streptomyces clavuligerus* by traditional and molecular breeding to increase the biosynthesis of clavulanic acid

Zhihan Zuo[1], Yichen Liu[1], Yichen Zhang[1], Chen Wang[2] and Cai Heng[2]*

[1]College of Life Science, Tianjin Normal University, Tianjin Key Lab of Cyto-genetical and Molecular Regulation, 300387, China.
[2]College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, 211816, China.

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Streptomyces clavuligerus **produces a number of β-lactam compounds, including clavulanic acid. Here, we describe an efficient procedure for creating clavulanic acid high producing** ***S. clavuligerus*** **mutant by using traditional UV light treatment method and PCR-targeting system. After inducing** ***S. clavuligerus*** **B71 with UV light** ***S. clavuligerus*** **B71-14 was selected for its 1.91-fold clavulanic acid production to the original strain. Then we applied polymerase chain reaction (PCR)-targeting system to mutate the** ***lat*** **gene of** ***S. clavuligerus*** **B71-14 to obtain** ***lat*** **gene knocked-out, unmarked mutant** ***S. clavuligerus lat::scar*****. Through traditional UV light treatment and PCR-targeting system to knock out the** ***lat*** **gene of** ***S. clavuligerus*** **wild type strain, the clavulanic acid of the mutant** ***S. clavuligerus lat::scar*** **was 2.85-fold to the original strain** ***S. clavuligerus*** **B71. This procedure allows repeated use of the same resistance marker for making multiple knock-outs in the same strain.**

Key words: *Streptomyces clavuligerus*, mutant, clavulanic acid, increase.

INTRODUCTION

Streptomyces clavuligerus is an actinomycete well known for its ability to produce a variety of β-lactam antibiotics, including isopenicillin N, desacetoxycephalosporin C and cephamycin C. These compounds are all derived from the cephamycin C pathway. *S. clavuligerus* produces another group of structurally related β-lactam compounds, the clavams. Among the clavam molecules only clavulanic acid possesses β-lactamase inhibitory activity which is related to its unique 3R,5R stereochemistry. Clavulanic acid has poor anti-bacterial activity, however, it binds to irreversibly to the serine hydroxyl group at the active center of β-lactamases, producing a stable acylated intermediate and resulting in the inactivation of the enzyme (Hirakata et al., 2009). The most important mechanism for bacterial resistance to β-lactam antibiotics such as penicillins and cephalosporins is the production of β-lactamases (Bebrone et al., 2010). Because of its β-lactamase inhibitory properties, clavulanic acid is used clinically in combination with conventional β-lactam antibiotics to treat infections caused by bacteria that would otherwise be resistant to these antibiotics. The combination of CA with amoxicillin is the most common and efficient example with high levels of antibacterial activity used in the treatment of infectious diseases (Nagy et al., 2010). However, the other clavam metabolites produced by *S. clavuligerus* show weak antibacterial and antifungal activities (Reading and Cole, 1977). Since clavulanic acid is produced industrially by fermentation using *S. clavuligerus*, the regulation of clavulanic acid biosynthesis is a point of great interest.

Clavulanic acid is biosynthesized by a pathway that begins by condensation of 3-phosphoglyceraldehyde with L-arginine, and proceeds through a number of steps finally to form clavulanic acid. Although there are no biosynthetic enzymes shared by the cephamycin C and the clavulanic acid pathways, the genes encoding clavulanic acid biosynthetic enzymes are located in a cluster adjacent to the cephamycin C gene cluster (Ward and Hodgson, 1993), and the two biosynthetic pathways

*Corresponding author. E-mail: caihengmail@yahoo.com.cn.

are coregulated by the same transcription activation protein, CcaR (López-García et al., 2010). The cephamycin C pathway draws upon L-lysine, L-cysteine, and L-valine as precursors, and the earliest step of this pathway is L-lysine converts to piperideine-6-carboxylate, which is catalyzed by lysine-ε-amino transferase encoded by lat gene (Paradkar et al., 2001). Lat is the first gene in cephamycin C biosynthesis and is the top candidate gene for regulation (Liras, 1999).

Paradkar et al. (2001) have reported when cephamycin C production was blocked in wild-type *S. clavuligerus* by disruption of the *lat* gene, specific production of clavulanic acid would increase. Here, we describe the traditional UV light treatment method to increase the clavulanic acid production of *S. clavuligerus*, then a PCR targeting-based gene disruption protocol that permits the deletion of the *lat* gene for cephamycin C metabolism. Through these methods the clavulanic acid production of the obtained *S. clavuligerus* mutant was significantly increased. PCR-targeting system allows relative rapid generation of nonpolar, in-frame deletions. This procedure allows repeated use of the same resistance marker for making multiple knock-outs in the same plasmid or the same strain (Kieser et al., 2000).

MATERIALS AND METHODS

Strains, plasmids and culture conditions

Escherichia coli BW25113 and DH5α were grown in SOB or LB (Sambrook et al., 1989) respectively. *E. coli* DH5α was used as a host for plasmid constructions. Cephamycin C indicator organism *E. coli* ESS was maintained on trypticase soy broth supplemented with 1.8% agar. *S. clavuligerus* NRRL 3585 was maintained on YMGA medium. *S. lividans* TK24 was maintained on either R5 or MYM medium, *S. clavuligerus* and *S. lividans* were grown in TSB liquid culture to isolate plasmid DNA (Lynch and Yang, 2004). All *Streptomyces* spp. cultures were propagated at 28°C on a rotary shaker at 250 rpm. Strains are listed in Table 1.

Plasmid-bearing cultures were supplemented with Ampicillin (Amp, 100 μg/ml), Apramycin (Apra, 50 μg/ml), chloramphenicol (Cm, 25 μg/ml), carbenicillin (Carb, 100 μg/ml), Thiostrepton (Thio, 20 μg/ml for *S. lividans* and 10 μg/ml for *S. clavuligerus*) all from Sigma, were added to growth media when required. L-arabinose (10 mM final concentration; Sigma) was added as indicated to SOB medium from a 1M sterile filtered stock solution to induce genes under control of the pBAD promoter.

UV light treatment on *S. clavuligerus*

Spore suspension of wild type strain *S. clavuligerus* B71 was induced for 45 s by UV light and was spread on YMGA with 12% glycerol agar plates. After 5 days incubation at 28°C, tested clavulanic acid production of the UV light induced *S. clavuligerus* colonies, selected clavulanic acid high yield mutant strains.

Gene replacement on plasmids

Replacement of *lat* in the plasmid pUCm-T-*lat* (Table 1) was achieved by PCR-targeting system as described by Gust et al. (2002) except that the plasmid vector was used instead of cosmid vector. A 1010 bp fragment carrying the 5' end of *lat* and a 613 bp fragment carrying the 3' end of *lat* were positioned upstream and downstream, respectively, of an apramycin resistance gene cassette (*aac(3)IV-oriT-FRT*). This generated the plasmid pLATA. The disrupted *lat* gene was then subcloned into the high-copy-number *Streptomyces* vector pIJ486 as an *Eco*RI/*Hind*Ш fragment to create pEHA.

In-frame deletion mediated by FLP recombinase

Electro-competent *E. coli* DH5α/BT340 cells was transformed with the targeted plasmid pLATA. Then the central part of the *lat* disruption cassette was removed on FRT sites by the FLP recombinase expressed by BT340, leaving behind an 81bp "*scar*" sequence. This generated the plasmid pSCAR which containing the in-frame deletion. This was achieved by PCR-targeting system as described by Bebrone et al. (2010). The *lat* in-frame deletion cassette which containing 1010bp 5' moiety and 613bp 3' moiety of *lat* gene separated by 81bp *scar* sequence was then subcloned into pIJ486 as an *Eco*RI/*Hind*Ш fragment to create pEHS.

Transformation of streptomyces

Protoplasts of *S. lividans* and *S. clavuligerus* were obtained and transformed with plasmid DNA as described previously by García-domínguez et al. (1987) except that protoplasts were subjected to a heat shock (10 min at 42°C) before transformation. Protoplasts were regenerated on R2YEG (R2YE with 10 g glycerol per liter) (Kieser et al., 2000).

Allelics exchange in *S. clavuligerus*

pEHA was introduced into *S. clavuligerus* B71-14 by polyethylene glycerol (PEG)-mediated protoplast transformation. $Apra^R$ and $Thio^R$ colonies were obtained from the transformants following growth and sporulation in the absence of antibiotic selection, indicating a double-crossover exchange between pEHA and *S. clavuligerus* B71-14 chromosome. Then *S. clavuligerus lat::Apra*^{R} mutant was obtained.

pEHS which contains the in-frame deletion was transformed into *S. clavuligerus lat::Apra*^{R}. Selected for transformants with $Apra^R$ and $Thio^R$ and subsequent screening for the loss of both $Thio^R$ and $Apra^R$, indicated the successful replacement of the apramycin resistant marker with an unmarked, in-frame deletion. The unmarked *S. clavuligerus* mutant was named *S. clavuligerus lat::scar*.

Confirmation of *S. clavuligerus* lat mutants

S. clavuligerus lat::Apra^R was confirmed by southern blot analysis. For Southern hybridizations, 10 mg of digested DNA of *S. clavuligerus* B71-14 and *S. clavuligerus lat::Apra*^{R} were used. The probes were labelled with a North2South HRP Labeling and Detection Kit (PIERCE), using the conditions suggested by the manufacturer. $Apra^R$ cassette (*aac(3)IV-oriT-FRT* sequence) was used as the probe. *S. clavuligerus lat::scar* was confirmed by PCR analysis with primer P1 and P2.

Bioassay and HPLC analysis of culture supernatant

In an indicator bioassay for β-lactamase inhibitors, clavulanic acid was detected using *Klebsiella pneumoniae* ATCC29665 as the

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Description	Reference or source
Strains		
S. clavuligerus B71	*S. clavuligerus* NRRL3585 wild-type strain with comparatively high yield of clavulanic acid	This study
S. clavuligerus B71-14	Mutant strain obtained by treated *S. clavuligerus* B71 with UV light and its clavulanic acid production is 1.91-fold to the original strain	This study
S. clavuligerus lat::Apra^R	Mutant strain obtained by replaced *lat* gene of *S. clavuligerus* B71-14 with *aac(3)IV* ::*oriT* cassette	This study
S. clavuligerus lat::scar	Unmarked mutant strain obtained by knocked out *aac(3)IV*::*oriT* cassette of *S. clavuligerus lat::Apra*^{R}	This study
S. lividans TK24	*Str-6*; plasmidless cloning host (SLP2$^-$ SLP3$^-$)	Kindly provided by professor Zhu Chunbao. Shanghai Institute of pharmaceuticul industry
E. coli ESS	β-lactam-sensitive indicator organism	Kindly provided by professor Susan Jencen. Department of Biological Sciences, University of Alberta
E. coli BW25113	K12 derivative:*ΔaraBAD, ΔrhaBAD*	This study
Plasmids		
pIJ773	*aac(3)IV* (Apra^R) *oriT*	This study
pIJ790	λ-RED (*gam, bet, exo*), *cat, araC, rep101*^{ts}	This study
BT340	FLP recombination plasmid; *flp,bla,cat, rep101*^{ts}	This study
pUCm-T	T-vector for PCR cloning; Amp^R	Sangon company
pIJ486	High-copy-number *Streptomyces* promoter-probe plasmid; Thio^R	Kindly provided by professor Mervyn Bibb. John Innes Institute, Norwich, United Kingdom
pUCm-T-*lat*	pUCm-T vector containing a 1.8kb fragment of *lat* gene	This study
pLATA	*aac(3)IV* ::*oriT* replaced with part of *lat* gene in pUCm-T-*lat*	This study
pSCAR	*scar* replaced with *aac(3)IV* ::*oriT* in pLATA	This study
pEHA	pIJ486 containing a 3kb *Eco*RI/ *Hind*Ⅲ fragment of *lat* gene flanked *aac(3)IV* ::*oriT* cassette	This study
pEHS	pIJ486 containing a 1.7kb *Eco*RI/*Hind*Ⅲ fragment of *lat* gene flanked *scar* cassette	This study

indicator organism grown on LB plates containing 6 μg/ml ampicillin. Cephamycin C was detected in culture filtrates by bioassay using *E. coli* ESS (Aharonowitz and Demain, 1978) as the indicator organism grown on TSB plates. The production of clavulanic acid was followed by high-performance liquid chromatography (HPLC) analysis of culture supernatants after imidazole derivatization as described previously (Paradkar and Jensen, 1995), except that a Shim-pack VP-ODS (150×4.6) C18 column was used in the analysis. *S. clavuligerus* culture supernatants from both of the original strain and the *lat* mutant strain of *S. clavuligerus* were centrifuged and filtered through 0.45 μm membrane before derivatization.

RESULTS

Selection of UV light induced *S. clavuligerus* mutant

During selection of clavulanic acid high producing strain, using glycerol tolerant as selection pressure can obviate low producing strain and increase the successful rate of obtaining high producing strain, it is better than traditional screening approach. Experiment showed that the higher the glycerol tolerant, the higher clavulanic titre is (Li et al., 1999). Glycerol tolerance of *S. clavuligerus* B71 was tested. The highest glycerol tolerant concentration of *S. clavuligerus* B71 was 10%, and the minimum inhibitory concentration of glycerol is 12%. So YMGA with 12% glycerol was used as the selected medium for clavulanic acid high producing strain. 600 *S. clavuligerus* colonies grown on YMGA (12% glycerol) were tested for the clavulanic acid production. Mutant strain *S. clavuligerus* B71-14 was selected for its 1.91-fold clavulanic acid high yield to the original strain. *S. clavuligerus* B71-14 was used as the original strain for PCR-targeting disruption of the *lat* gene aimed to further increase the clavulanic acid production of this strain.

Adaptation of PCR targeting for *S. clavuligerus*

For the disruption of *lat* gene cloned in pUCm-T-*lat* and the efficient transfer of the mutant derivatives to *S. clavuligerus*, a gene replacement cassette containing *aac(3)IV* ($Apra^R$) and $oriT_{RK2}$ was constructed by PCR (Figure 1a). This $Apra^R$ cassette is flanked of both ends by FRT sequence to allow efficient removal of $Apra^R$ cassette by yeast FLP recombinase (Figure 1 b to h).

In designing primers P3 and P4 (Table 2) for PCR amplification of cassette, the 39bp of *lat* targeting sequence “tails” were supplied as the 5’ ends of long PCR primers, whose 3’ ends consisted of 19 or 20 bp priming sequences (Figure 1a). The 39 bp 5’ target sequence tails were designed to generate “*scar*” sequences that were in a reading frame that lacked in-frame stop codons.

Disruption of lat

The 1.8 kb lat gene which was obtained by PCR with primers P1 and P2 (Table 2) was subcloned into pUCm-T to obtain pUCm-T-lat. pLATA was achieved by replacement of lat in pUCm-T-lat with the tailed lat gene disruption ApraR cassette (obtained by PCR with primers P3 and P4). Primers were designed according to *lat* gene sequence of GenBank: M64834.1 and *aac(3)IV* sequence of pIJ773. In pLATA the $Apra^R$ cassette was flanked by 1010 and 613 bp *S. clavuligerus lat* gene sequence. The *lat* gene flanked $Apra^R$ cassette was digested from pLATA by *Eco*RI/*Hind*III and was ligated into *Streptomyces* vector pIJ486 to obtain recombinant plasmid pEHA. The next step is to introduce the mutant allele pEHA into *S. clavuligerus* by PEG-mediated protoplast transformation. $Apra^R$ and $Thio^R$ transformants were selected. Then $Apra^R$ and $Thio^S$ colonies were obtained from the transformants following growth and sporulation in the absence of antibiotic selection, indicating double-crossover gene replacement and loss of the pIJ486 vector (Figure 1d to e). The obtained mutant *S. clavuligerus lat::*$Apra^R$ was confirmed by southern blot analysis, indicating the $Apra^R$ cassette has been integrated into *S. clavuligerus* B71-14 *lat* gene (Figure 2).

The *aac(3)IV-oriT* sequence was removed by FLP-mediated recombination from the *lat* replacement plasmid pLATA to obtain pSCAR. The *lat::scar* sequence (*scar* sequence flanked by *lat* gene sequence) was digested by *Eco*RI/*Hind*III from pSCAR, and was ligated into the same restriction enzyme digested vector pIJ486. Thus *lat* gene deleted recombination plasmid pEHS was obtained.

pEHS containing the *scar*-marked *lat* deletion had only the $Thio^R$ encoded by *tsr* in pIJ486 for selection in *Streptomyces*. After constructed in *S. lividans*, the plasmid was introduced into *S. clavuligerus lat::*$Apra^R$ by PEG-mediated protoplast transformation. $Thio^R$ transformants were obtained. Nonselective propagation and screening for $Apra^S$ and $Thio^S$ transformants readily yielded an unmarked deletion of *lat* containing a “*scar*” sequence of 81 bp, *S. clavuligerus* mutants and *S. clavuligerus* B71-14 were confirmed by PCR analysis with primer P1 and P2 (Figure 3).

Analysis of the lat gene disrupted mutants

Growth and metabolites of three independent strains: *S. clavuligerus lat::*$Apra^R$, *S. clavuligerus lat::scar* and *S. clavuligerus* B71-14 were tested. No obvious difference of growth between these strains was observed on YMGA (data not shown) and TSB medium (Figure 4). While the yield of clavulanic acid and cyphamycin C of *S. clavuligerus* B71-14 and the *lat* gene disrupted mutants was different. *S. clavuligerus* B71-14 produced both cephamycin C and clavulanic acid, while all three of the *lat* gene disrupted mutants showed little bioactivity of cyphamycin C (Figure 5). Under the same conditions, the highest clavulanic acid production of the mutants was 1.49-fold to that seen in *S. clavuligerus* B71-14 in soy flour medium at 120 h incubation (Table 3). Each time point production was an average clavulanic acid productivity from two parallel fermentation for each strain (single assays from each fermentor). So the yield of clavulanic acid of the lat knocked out mutant was 2.85-fold to the wild type strain *S. clavuligerus* B71(Figure 6). Growth and the production of cephamycin C and clavulanic acid of *S. clavuligerus lat::*$Apra^R$ and *S. clavuligerus lat::scar* were almost the same.

DISCUSSION

In this paper, wild type strain *S. clavuligerus* B71 was

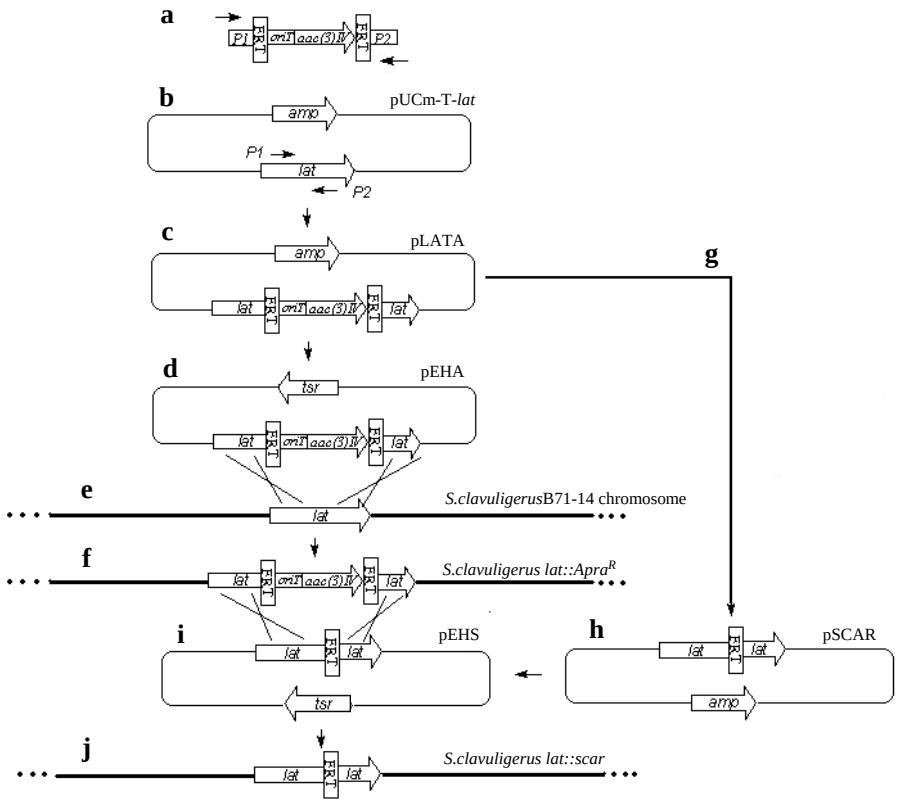

Figure 1. Strategy for gene replacement in *Streptomyces clavuligerus*. (a) A gene replacement cassette containing *aac(3)IV* (Apra^R), *oriT* of RK2, and two FRT sites were amplified by PCR by using primers containing 39-nt 5' homology extensions corresponding to regions in the *S. clavuligerus* target gene (*lat*) and 20-nt 3' homology to the unique priming sites P3 and P4 of the disruption cassette. (b) The PCR product from (a) was used to transform *E.coli*BW25113/pIJ790/pUCm-T-*lat*. (c) Apra^R transformants were selected, and the recombinant plasmid was identified. (d) The Apra^R cassette flanked by *lat* gene was digested from pLATA and was ligated into pIJ486 to obtain pEHA. (e) pEHA was constructed in *S. lividans* and from there into *S. clavuligerus*B71-14. (f) *S. clavuligerus lat::Apra^R* were selected, and the double cross-over allelic exchange was confirmed. (g) *E.coli*DH5α cells containing BT340 were transformed with the mutagenized plasmid pLATA, and FLP synthesis was induced to remove the *Apra^{R}oriT* sequence from the *lat* replacement plasmid pLATA to obtain pSCAR. (h) The *lat::scar* cassette was digested from pSCAR to ligate into pIJ486 to obtain pEHS. (i) pSCAR was introduced into *S. clavuligerus lat::Apra^R*. (j) Apra^R and Thio^R transformants arising from (f) were selected for the loss of both Apra^R and Thio^R, indicating a successful replacement by the in-frame deletion.

Table 2. PCR primers used in this study.

Primer	Sequence(5'-3')	Function
P1	GAATTCCCCTGAACACGAAGCTGAGCAACA	5' *lat* PCR primer
P2	CTCTCGGGTGCTTACTACAGTCGTGCCATG	3' *lat* PCR primer
P3	GGCGACAACCACCTCAGCGCGGAGTTCCTCCAGGCC ATGATTCCGGGGATCCGTCGACC	5' *aac(3)IV* ::*oriT* cassette PCR primer
P4	CGTTCTCGGGGACCTCGTCGATCCGGCCGCCGCCCA TCATGTAGG CTGGAGCTGCTTC	3' *aac(3)IV* ::*oriT* cassette PCR primer

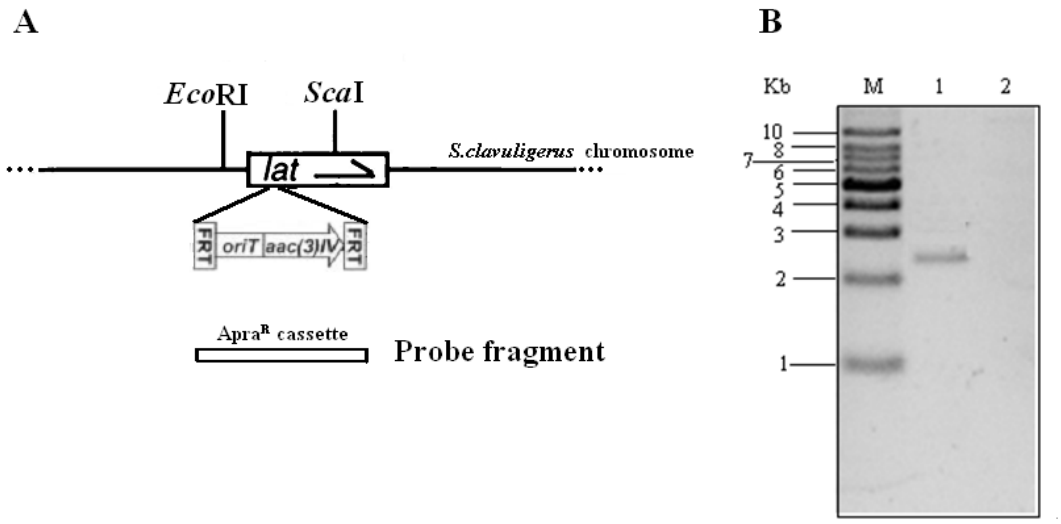

Figure 2. Disruption of the *lat* gene. (A) Diagram of the chromosome of a *S. clavuligerus lat::Apra*^{R} gene disruption mutant in the region of the *lat* gene. Open boxes represent the *lat* and Apra^R cassette. Arrows indicate the direction of transcription. （B）Southern analysis of one of the *S. clavuligerus lat::Apra*^{R} mutants(lane 1). Lanes 1 and 2, *Eco*RI-*Sca*I digested genomic DNA from *S. clavuligerus lat::Apra*^{R} mutant and *S. clavuligerus* B71-14 *lat*, respectively.

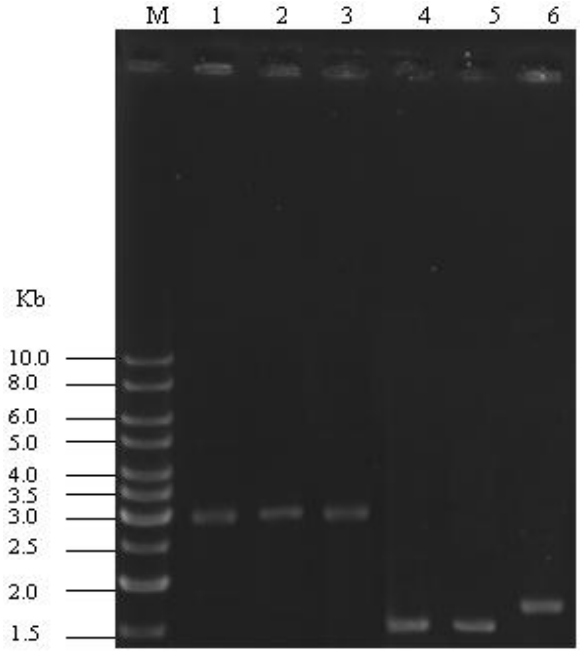

Figure 3. PCR analysis of the *S. clavuligerus* mutants. Lane M, 10.0 kb marker; lanes 1,2 and lane 3, PCR products of *S. clavuligerus lat::Apra*^{R}; lane 4 and lane 5, PCR products of *S. clavuligerus lat::scar*; lane 6, PCR products of *S. clavuligerus* B71-14.

used as the original strain for UV light treatment. After inducing *S. clavuligerus* B71 with UV light *S. clavuligerus* B71-14 was selected for its 1.91-fold clavulanic acid production to the original strain. During selection of clavulanic acid high producing strain, using glycerol tolerant as selection pressure can obviate low producing

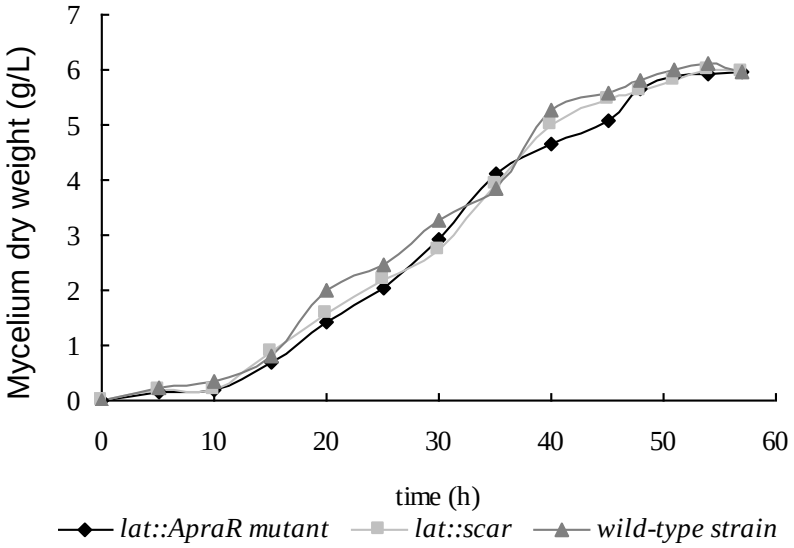

Figure 4. Mycelium dry weight of the *lat* gene disrupted mutant and the wild-type strain at different incubation times in TSB. Mycelium dry weight of *S. clavuligerus lat::Apra*^{R}(◆), *S. clavuligerus lat::scar* (■) and the parental *S. clavuligerus* B71-14 (▲) when fermented in 250 ml baffle flasks is shown. Each time point represents an average value from two parallel flasks from each strain.

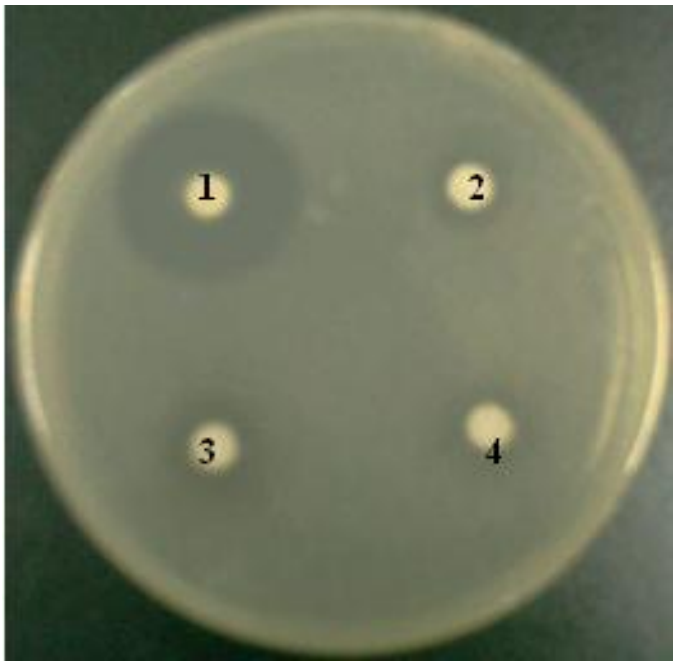

Figure 5. Production of cephamycin C in solid medium by *S. clavuligerus* B71-14 (1), *S. clavuligerus lat::Apra*^{R} (2) and *S. clavuligerus lat::scar* (3), (4). Note the lack of production in the disrupted mutants.

strain and increase the successful rate of obtaining high producing strain. Baltasar et al. (1992) have reported *S. clavuligerus* containing glycerol transport system (GTS) active and specifically induced by glycerol which is

Table 3. HPLC analysis of clavulanic acid production of *S. clavuligerus* B71-14 and *S. clavuligerus lat* mutants.

Strains[a]	96 h (μg/ml)[b]	Fold[c]	120 h (μg/ml)[b]	Fold[c]
O	468.62	1.0	530.66	1.0
M-1	542.94	1.16	682.92	1.28
M-2	529.77	1.13	715.33	1.35
M-3	543.92	1.16	690.76	1.30
M-4	552.08	1.18	790.68	1.49

[a]Mutant strains are designated with the letter M (M-1 and M-2 are *S. clavuligerus lat::Apra*^{R}, M-3 and M-4 are *S. clavuligerus lat::scar*), followed by a number to indicate the primary transformant which gave rise to the mutant, *S. clavuligerus* B71-14 is designated with the letter O. [b]Clavulanic acid titers were determined with supernatants of single cultures grown in Soy medium for 96h and 120h at 28°C. [c]*S. clavuligerus* mutants clavulanic acid production relative to *S. clavuligerus* B71-14 production.

responsible for the transport of more than 90% of the glycerol taken up to the cells. Possibly because in high glycerol tolerant strain the GTS activity is strong, it can efficiently convert glycerol to synthesize clavulanic acid. However, in low glycerol tolerant strain the GTS activity is weak, so the excessive glycerol limit growth of the strain contrarily.

Then we applied PCR-targeting system to mutate the *lat* gene of *S. clavuligerus* B71-14. *lat* gene is the first gene in the cephamycin C biosynthesis pathway. The cephamycin C pathway draws upon L-lysine, L-valine as precursors, and the earliest step of this pathway is L-lysine converts to piperideine-6-carboxylate, which is catalyzed by lysine-ε-amino transferase encoded by the *lat* gene (Paradkar et al., 2001). It is known that both the cephamycin C and the clavulanic acid biosynthetic pathways are controlled by the same regulatory gene *cca*R (López-García et al., 2010; Alexander and Jensen, 1998). Although, there are no biosynthetic enzymes shared by the clavulanic acid and the cephamycin C pathways, the clavulanic acid production was increased obviously when the *lat* gene of *S. clavuligerus* was disrupted. Possibly because when the *S. clavuligerus lat* gene was disrupted, L-α-aminoadipate, L-cysteine and L-valine were unable to be incorporated into the biosynthesis of cephamycin C, then the secondary metabolites inverted more to the clavulamic acid biosynthetic pathway (Paradkar et al., 2001).

Murphy et al. (2000) showed that induction of the λ–Red genes (*gam, bet* and *exo*) can significantly increase homologous recombination in *E. coli*. Datsenko and Wanner (2000) developed this strategy into a rapid and efficient protocol. Baltasar et al. (1992) described an efficient PCR-targeting system in *Streptomyces coelicolir*. They created precise gene replacements in the cosmid clones by using PCR targeting and λ–Red-mediated recombination. The cloned *Streptomyces* genes are replaced with a cassette containing a selectable antibiotic resistance and *oriT*$_{RK2}$ for efficient transfer to *Streptomyces* by RP4-mediated intergeneric conjugation. Through, double-crossover recombination between cosmid clones and *Streptomyces*, gene replacements were created. Because the antibiotic resistance cassette is flanked by yeast FLP recombinase target sequences, the antibiotic resistance cassette and *oriT*$_{RK2}$ can be removal to generate unmarked and nonpolar mutants.

Concentration of clavulanic acid was increased up to two-folds after UV mutagenesis of *Streptomyces clavuligerus* by Korbekandi et al. (2010). In this study, the PCR-targeting system has been applied to replace the *lat* gene of UV mutagenesis strain *S. clavuligerus* B71-14 whose clavulanic acid production was 1.91-fold to the wild type strain *S. clavuligerus* B71 aimed to obtain the clavulanic acid production further increased strain. The specific clavulanic acid production in the *lat* gene disrupted mutants was 1.49-fold to that seen in *S. clavuligerus* B71-14 (Table 3). Paradkar et al. (2001) used gene replacement technology to disrupt the *lat* gene of *S. clavuligerus* and the mutant strain increased 1.5-fold on clavulanic acid production. We have applied a single-crossover recombination method to disrupt the *lat* gene of wild type *S. clavuligerus* previously Zhihan and Yanping (2006) and the increased extent of *S. clavuligerus* B71-14 *lat* mutant was lower than the wild-type *S. clavuligerus lat* mutants. Possibly because after repeatedly treated with UV light and selected for several generations, some genes of *S.claculigerus*B71-14 were mutated (Colonies of *S. clavuligerus* B71-14 is smaller than the wild-type strain and it grows more slowly compared with the wild-type strain.) and the ability of cephamycin C production of *S.claculigerus* B71-14 had been reduced. Cephamycin C production of *S. claculigerus* B71-14 and the wild-type *S. clavuligerus* B71 had been determined and compared, cephamycin C production of the wild-type strain was more than *S. claculigerus* B71-14 in some degree (data not shown). The basis for the different phenomenons in the increased level of clavulanic acid production of *S. clavuligerus* B71-14 and the wild-type strain is difficult to analyze. It depends on the nature of the two kinds of strains.

Through, traditional UV light treatment method and PCR-targeting system to knock out the *lat* gene of *S. clavuligerus* B71, the clavulanic acid of the mutant *S. clavuligerus lat::scar* was 2.85-fold to the original wild

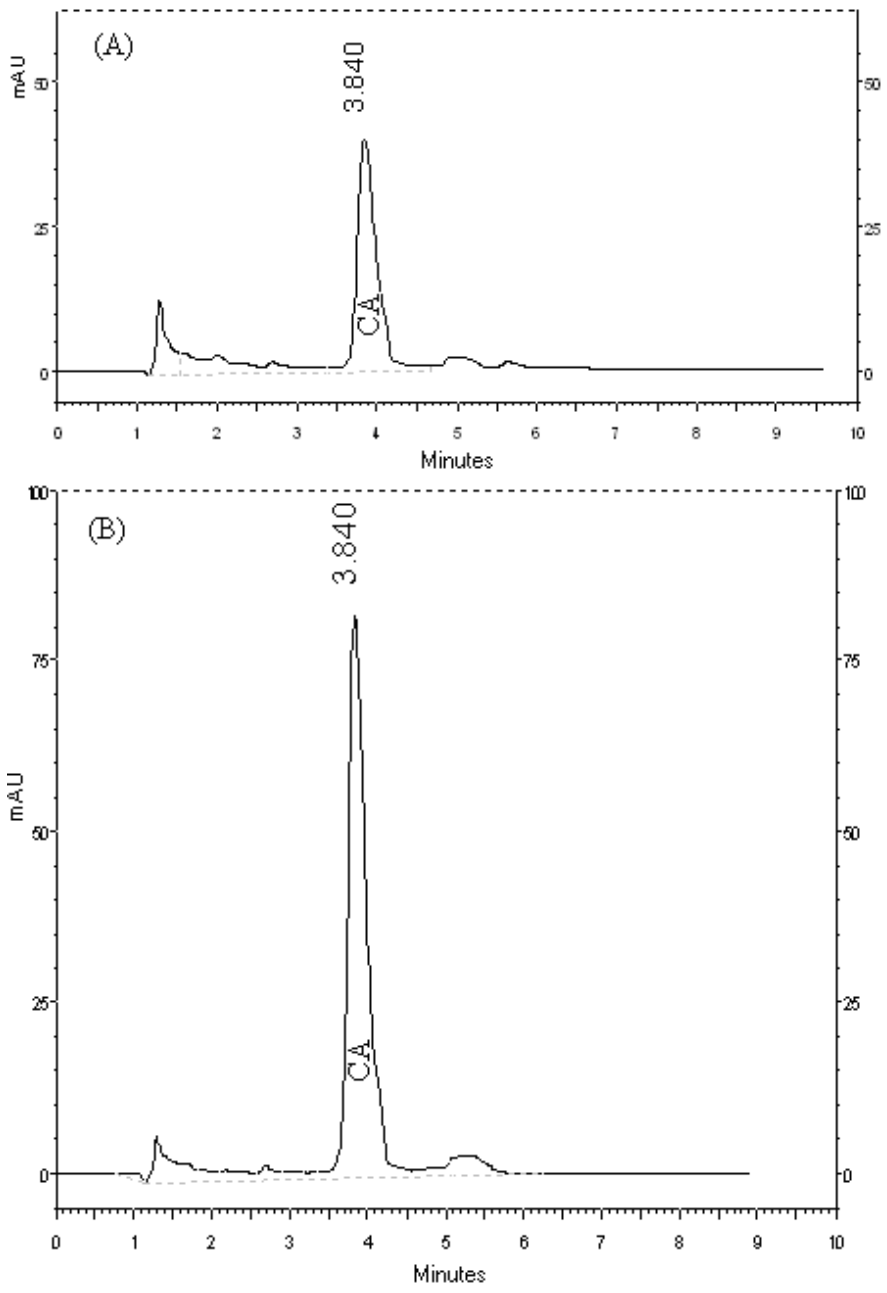

Figure 6. HPLC analysis of clavulanic acid production of (A) the original strain *S. clavuligerus* B71and (B) the mutant *S. clavuligerus lat::scar*.

type strain. And this unmarked *lat* mutant allows repeated use of the Apra^R marker for making multiple knock-outs negative regulatory genes or expressing positive regulatory genes of clavulanic acid in the same strain for further increase in yield of clavulanic acid.

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