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Application of *lat* gene disruption to increase the clavulanic acid production of *Streptomyces clavuligerus*

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Abstract

A 1.7 kb fragment of *lat* was obtained from *Streptomyces clavuligerus* NRRL 3585, and recombinant plasmid pKC1139-*lat*, which was used to disrupt the *lat* gene was constructed. pKC1139-*lat* was introduced into *S. clavuligerus* by bi-parental conjugation from *Escherichia coli* ET12567 to *S. clavuligerus*. The apramcin-resistant transformants were obtained and through homogeneous single-crossover between recombinant plasmid pKC1139-*lat* and the *S. clavuligerus* chromosome *lat* disrupted mutant strains were obtained. The genome of *S. clavuligerus* NRRL 3585 and the *lat* disrupted mutants were analyzed by PCR technique, the bioactivity of cephamycin C in the two kinds of strains were also tested. Both results proved that *lat* was disrupted by the insertion of pKC1139 in the *lat* disrupted mutants. And the production of clavulanic acid of these two kinds of strains were analyzed by HPLC with different incubation time interval (96 and 120 h), and the yield in the *lat* mutants was approximately 2.6 fold higher at their highest production point.

Keywords: *Streptomyces clavuligerus*; Clavulanic acid; *Lat*; Gene disruption

1. Introduction

Streptomyces clavuligerus produces a number of β-lactam compounds, including cephamycin C, clavulanic acid and at least four other known clavam metabolites [1]. Clavulanic acid and the other clavams differ from cephamycin C in that, their bicyclic nuclease contains an oxygen atom instead of the sulfer atom found in the more conventional cephamycin-type antibiotics [1]. Among the clavam molecules, only clavulanic acid possesses β-lactamase inhibitory activity which is related to its unique 3 R, 5 R stereochemistry. Clavulanic acid has poor antibacterial 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 [2]. So it is the species used industrially for the production of clavulanic acid, a multi-billion-dollar/annum product useful for its β-lactamase inhibitory activity [3]. Therefore, it is of great interest to determine if elimination of the pathway for the biosynthetically unrelated metabolite would have beneficial effects on clavulanic acid productivity in the industrial strain.

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 clavulanic acid pathways, the genes encoding clavulanic acid biosynthetic enzymes are located in a cluster adjacent to the cephamycin C gene cluster [4], and the two biosynthetic pathways are coregulated by the same transcription activation protein, CcaR [5]. The cephamycin C pathway draws upon L-lysine, L-cysteine, and L-valine as precursors (Fig. 1), 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* [6]. *lat* is the first gene in cephamycin biosynthesis and is the top candidate gene for regulation [2].

In *S. clavuligerus*, block of one kind of metabolite may result in the increasing of another biosynthetic pathway metabolite pathway. In this paper, we report the construction of *lat* gene disrupted mutants of wild-type *S. clavuligerus* blocked in the earliest step of the cephamycin C biosynthetic pathway.

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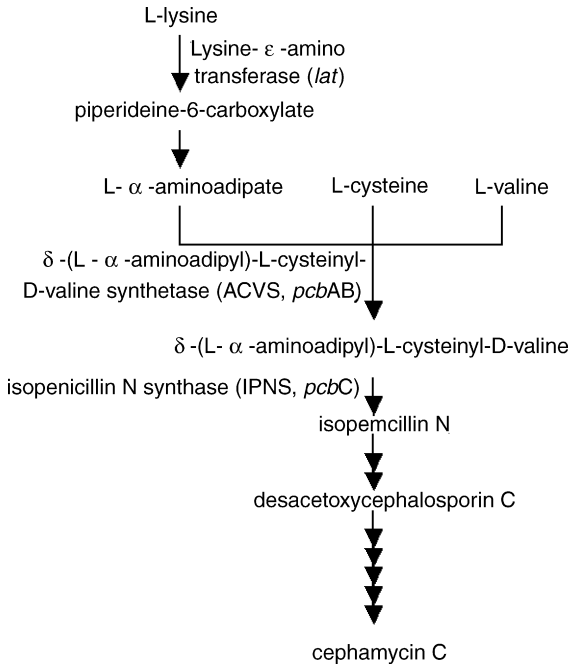

Fig. 1. Cephamycin C biosynthesis pathway. Abbreviated names of biosynthetic enzymes and their corresponding genes are given in parentheses [6].

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and culture conditions

S. clavuligerus NRRL 3585, the clavulanic acid indicator organism *Klabsiella pneumoniae* ATCC29665, *Escherichia coli* ET12567/pUZ 8002 for conjugation with *S. clavuligerus*, and *E. coli* DH5α were maintained in our laboratory. Cephamycin C indicator organism *E. coli* ESS was very kindly provided by Dr. Susan Jensen, Department of Biological Sciences, University of Alberta. Vector pUCm-T was obtained from Sangon Company, and the *E. coli-Streptomycete* conjugal transfer vector pKC1139 was a gift from Dr. Wang Yiguang, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences.

E. coli strains were grown on LB media as described by Sambrook et al. [7], while Cephamycin C indicator organism *E. coli* ESS was maintained on trypticase soy broth supplemented with 1.8% agar. *S. clavuligerus* wild-type and mutant strains were maintained on YMGA medium. YMGA medium [8] was consisted of the following compositions (per liter): yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 20 g. The medium was adjusted to pH 7.3 with 1 mol/L NaOH.

Spores of *S. clavuligerus* were inoculated into seed medium which was consisted of trypticase soy broth supplemented with 1% starch, and the cultures were grown at 28 °C on a rotary shaker (220 rpm) for 48 h. Mycelia from the seed cultures were washed twice with sterile water and used to inoculate soy medium at 1.5% (v/v) for clavulanic acid production. Then samples were removed at 72 and 96 h for analysis. These cultures were grown under the same conditions as the seed cultures. Soy medium [9] was consisted of the following compositions (per liter): soybean flour, 15 g; soluble starch, 4.7 g; KH_2PO_4, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.2 g. The medium was adjusted to pH 6.8 with 1 mol/L NaOH.

Representative mutants were grown in duplicate cultures. Plasmid-containing cultures were supplemented with ampicillin (100 μg/mL for *E. coli*), apramycin (25 μg/mL for all species), kanamycin (50 μg/mL for all species), or Chloramphenicol (25 μg/mL for *E. coli*) as appropriate.

2.2. Recombinant DNA procedure

Plasmid DNA isolation from *E. coli* cultures, restriction endonuclease digestions, ligations, PCRs and transformations of *E. coli* were all performed using standard techniques [7].

Plasmid and genomic DNA preparation from *S. clavuligerus* were isolated using standard techniques [10].

2.3. Disruption of lat

The 1.7 kb *lat* gene was generated by PCR using chromosomal DNA from *S. clavuligerus* as the template and the primers oligonucleotide sequence were as follows—*lat*-1: 5′-CGACGGCGATTTCTCGGACGTGGGAAACCT-3′; *lat*-2: 5′-CTCATGTGGCGAGACTTCCTGCGCGACGCG-3′. The 1.7 kb PCR product was subcloned into pUCm-T vector, this generated plasmid pUCm-T-*lat*. As a consequence, the mutant upstream-targeted *lat* gene lacked the carboxyl termini, thus, resulting in non-functional *lat* gene product. The resulting plasmid pUCm-T-*lat* was linearized at the *Eco*RI and *Hin*dIII, sites located upstream and downstream of the *lat* gene, respectively. And the 1.7 kb *lat* gene was then subcloned into pKC1139 as an *Eco*RI–*Hin*dIII fragment to create a *lat* gene truncated at both ends, *E. coli-Streptomycete* shuttle plasmid pKC1139-*lat*.

2.4. Introduction of recombinant plasmid into S.clavuligerus

Plasmid pKC1139-*lat* was introduced into *S. clavuligerus* spores by using the conjugation procedure as described by Kieser et al. [10] with *E. coli* ET12567/pUZ8002 as the donor strain. Exconjugants were isolated on AS-1 [11] supplemented with tryptone and glucose rather than on MS medium or AS-1 medium (all the media used for conjugation were supplemented with 10 mM $MgCl_2$ before use). The medium used for our conjugation experiment had the following composition (per liter): yeast extract, 1 g; L-alanine, 0.2 g; L-asparaginate, 0.5 g; L-arginine, 0.2 g; soluble starch, 5 g; NaCl, 2.5 g; Na_2SO_4, 10 g; tryptone, 5 g; glucose, 0.5 g, agar, 20 g. The medium was adjusted to pH 7.2 with 1 mol/L NaOH.

Replicated each single clonies onto DNA (Difco nutrient agar, DNA gives fast but non-sporulating growth) plates containing nalidixic acid (25 μg/mL) and apramycin (25 μg/mL) with and without kanamycin (50 μg/mL). Double-crossover exconjugants were kanamycin^S and apramycin^R.

2.5. Bioassay and HPLC analysis of culture supernatants

In an indicator bioassay for β-lactamase inhibitors, clavulanic acid was detected using *klebsiella pneumoniae* ATCC29665 as the indicator organism growing on LB plates containing 6 μg/mL ampicillin. Cephamycin C was detected in culture filtrates by bioassay using *E. coli* ESS [13] as the indicator organism.

The production of clavulanic acid was followed by high-performance liquid chromatography (HPLC) analysis of culture supernatants after imidazole derivatization as described previously [12], except that a Shim-pack VP-ODS (150 × 4.6) C18 column was used in the analysis. Culture supernatants from both wild-type and the *lat* gene disrupted mutant *S. clavuligerus* were centrifuged and filtered through 0.45 μm membrane before derivatization.

2.6. Determination of mycelium dry weight

Mycelium dry weight of the *lat* gene disrupted mutants and the wild-type strains were determined at different incubation times in TSB. Mycelium in 10 mL culture was harvested by centrifugation at 3500 rpm for 15 min. After washed twice with sterile water, the mycelium was dried at 85 °C to invariable weight.

3. Results

3.1. Construction of recombinant plasmid pKC1139-lat and its introducing into S. clavuligerus

The 1.7 kb truncated *lat* gene that obtained by PCR was subcloned into pUCm-T vector to generate pUCm-T-*lat*. pUCm-T-*lat* was then digested with *Eco*RI and *Hin*dIII, and the 1.7 kb *lat* gene was then subcloned into pKC1139 as an *Eco*RI–*Hin*dIII fragment to create a *lat* gene truncated at one end, *E. coli*-*Streptomycete* shuttle plasmid pKC1139-*lat* (Fig. 2.). pKC1139 carrying apramycin-resistant gene which can be expressed in both *E. coli* and *streptomyces*, and pKC1139 was a conjugative plasmid. Therefore, pKC1139-*lat* was a recombinant plasmid with conjugative function.

Recombinant plasmid pKC1139-*lat* were introduced into *S. clavuligerus* spores through the use of conjugation procedure. Three kinds of medium: MS, AS-1 and AS-1 supplemented with tryptone and glucose (all the media used for conjugation were supplemented with 10 mM $MgCl_2$ before use) were tested during the conjugation experiment. None exconjugants were obtained on MS medium, and only few of apramycin-resistant strains were obtained on AS-1 medium. But more exconjugants can be obtained when the AS-1 medium was supplemented with glucose and tryptone. Transconjugants were grown on YMGA medium under apramycin (25 μg/mL) selective conditions. Replicated each single clonies first onto DNA plates containing nalidixic acid (25 μg/mL) and apramycin (25 μg/mL), then onto plates with and without kanamycin (50 μg/mL). Finally, single-crossover exconjugants with kanamycin^S and apramycin^R were obtained. Plasmid extracted from the transconjugants was identical with the recombinant plasmid pKC1139-*lat* which was obtained from *E. coli* DH5α.

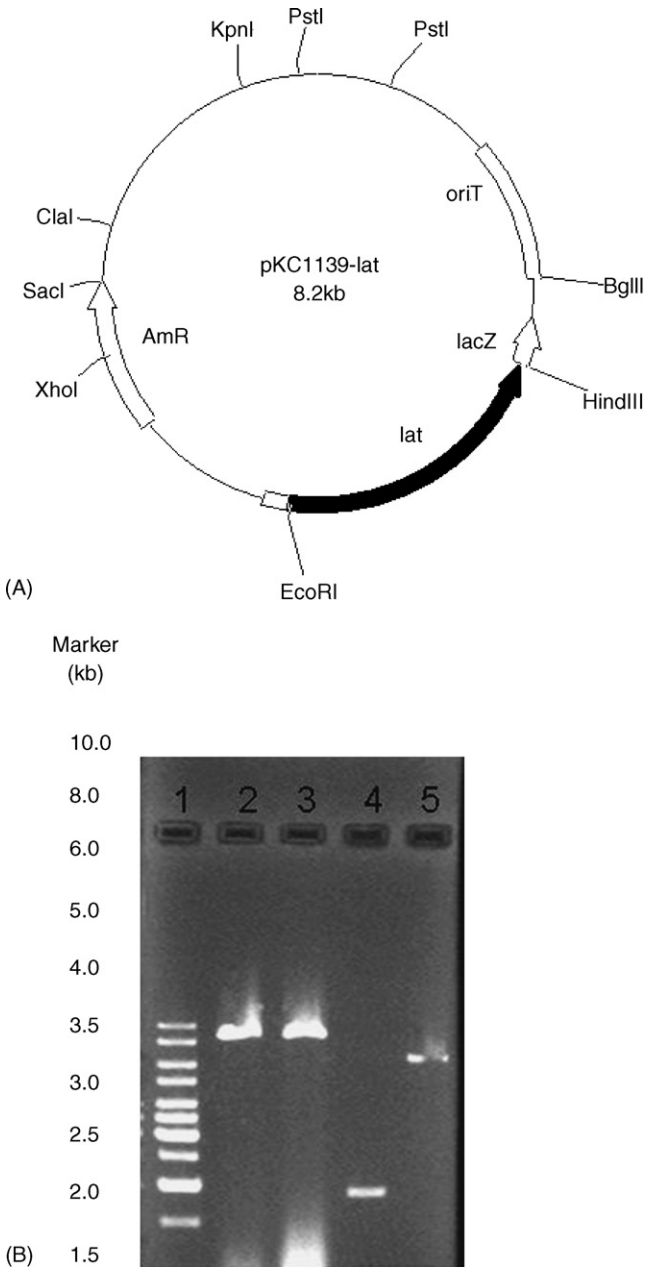

Fig. 2. (A) Restriction maps of pKC1139-*lat*. The 1.7 kb *lat* fragment was subcloned into the *Eco*RI and *Hin*dIII sites of the *lacZα* MCS in plasmid pKC1139. The 2.9 kb region of pKC1139 between the *Cla*I site and *ori*T containing the temperature-sensitive replicon from pSW344E. (B) Restriction analysis of the recombinant plasmidpKC113-*lat*. Lane 1 10 kb marker; lanes 2 and 3, *Eco*RI-digested and *Hin*dIII-digested plasmid pKC1139-*lat*, respectively; lane 4, *lat* gene fragment; lane 5, *Eco*RI-digested plasmid pKC1139.

3.2. Selection of lat gene disrupted mutants

The confirmed transconjugants were grown on YMGA plates containing apramycin (25 μg/mL) at 28 °C. Spores of the transconjugants were plated on YMGA medium supplemented with appropriate antibiotic. Approximately 100 spores were

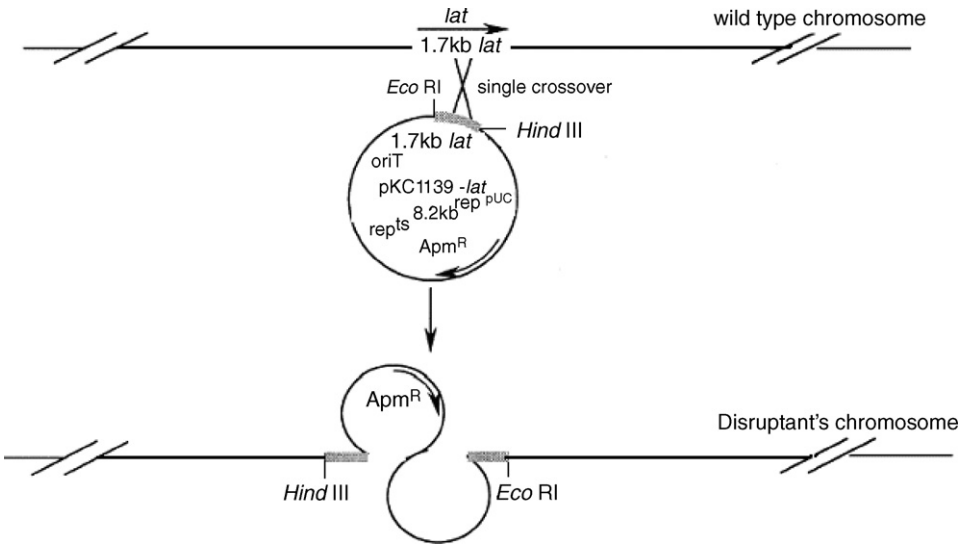

Fig. 3. The single-crossover process between recombinant plasmid pKC1139-*lat* and chromosome of *S. clavuligerus*.

plated on each plate. The plates were then incubated at 28 °C for 48–72 h until pinpoint-size colonies were visible, just before the aerial mycelia was formed, and then were shifted to 37 °C to continue incubation 7–10 days. Mutants resulting from single-crossover homologous recombination grew out of the original pinpoint-size colonies as easily distinguishable selectors in 7–10 days. Because pKC1139 bears a temperature-sensitive *Streptomycins* replication origin [14] which is unable to replicate at temperature above 34 °C, while the *S. clavuligerus* wild-type strain grows normally up to 37 °C. Thus, when spores of transconjugants were incubated at 37 °C, *lat* gene disrupted mutants that resulted from single-crossover homologous recombination between recombinant plasmid pKC1139-*lat* and the *S. clavuligerus* chromosome were obtained (Fig. 3).

3.3. Confirm of S. clavuligerus lat gene disrupted mutants

Inoculated three mutants randomly into ISP-liquid culture, supplemented with 25 μg/mL apramycin, incubated at 37 °C for 72 h. At the same time, inoculate wild-type *S. clavuligerus* into ISP medium without apramycin, incubated at 28 °C for 72 h. However, unable to isolate plasmid from the *lat* gene disrupted mutants, this proved the recombinant plasmid pKC1139-*lat* has integrated to the *S. clavuligerus* chromosome through single-crossover homologous recombination. To confirm the single-crossover homologous recombination, chromosome of the *lat* gene disrupted mutants and the wild-type *S. clavuligerus* were isolated, respectively. Consulted with the sequence of *aac*(3)IV gene (apramycin resistance gene of pKC1139), we designed the following oligonucleotides as PCR primers to test whether pKC1139 had inserted into *lat* (pKC1139 bears 785 bp *aac*(3)IV gene)—*aac*(3)IV-1: 5′-GAATTCGCATCGCATTCTTCGCATCC-3′; *aac*(3)IV-2: 5′-AAGCTTCGAATGGCGAAAAGCCGAGC-3′. Chromosome of mutants and wild-type *S. clavuligerus* were used as templates, respectively, to PCR, and the PCR products were electrophoretic analyzed (Fig. 4). The result showed that the PCR products of mutant strains chromosome template were 785 bp, which were identical to the *aac*(3)IV gene, while the wild strain chromosome was unable to PCR any DNA fragment. This proved that the recombinant plasmid pKC1139-*lat* has integrated into the *S. clavuligerus* chromosome through single-crossover recombination. The obtained mutants were the *lat* gene disrupted *S. clavuligerus*.

3.4. Analysis of the lat gene disrupted mutants

Growth of the mutants were comparable to that of the wild-type strain on YMGA and TSB media, and growth of the two kinds of strains were tested by determination of mycelium dry

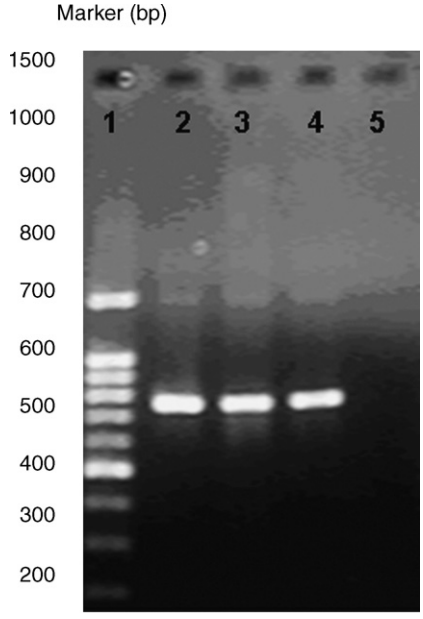

Fig. 4. PCR analysis of *lat* disrupted mutants and the wild *S. clavuligerus*. Lane 1, 100 bp marker; lanes 2–4 were the PCR results of *lat* disrupted mutants, lane 5 was the PCR results of the wild strain.

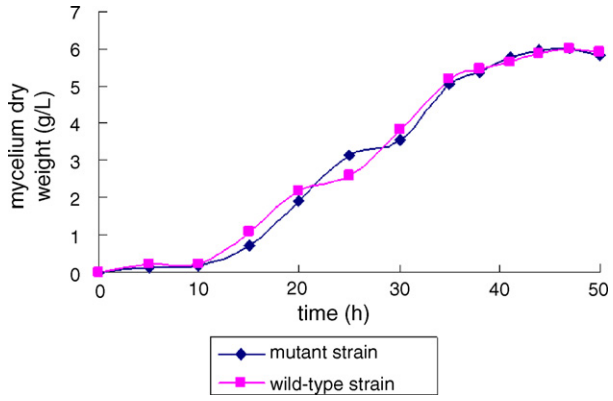

Fig. 5. Mycelium dry weight of the *lat* gene disrupted mutant and the wild-type strain at different incubation times in TSB, mycelium dry weight of both lat gene disrupted mutant (♦) and the parental wild-type strain (■) when fermented in 250 mL baffle flasks is shown. Each time point represents an average value from two parallel flasks from each strain.

weight (Fig. 5). From the results, conclusion can be made that no obvious differences between the two kinds of strains were observed.

As expected, the wild-type *S. clavuligerus* produce both cephamycin C and clavulanic acid, while all four of the *lat* gene disrupted mutants showed little bioactivity of cephamycin C (Fig. 6), whereas under the same conditions, the wild-type strain showed normal amounts of clavulanic acid production. The specific clavulanic acid production in the *lat* gene disrupted mutants was approximately 1.8–2.6 times to that seen in the wild-type strain (Table 1) in soy flour medium at 96 and 120 h of incubation. Each time point production was an average clavulanic acid productivity from two parallel fermentations for each strain (single assays from each fermentor). While growth of

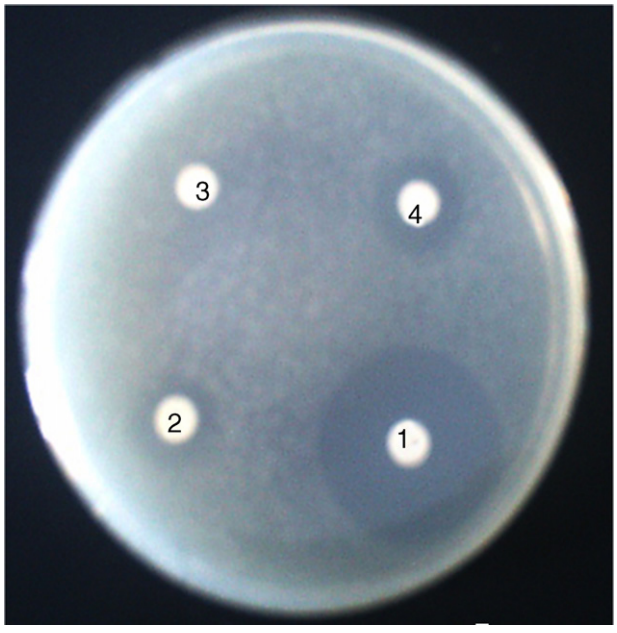

Fig. 6. Production of cephamycin C in solid medium by the wild-type strain (1) the *lat* gene disrupted mutants (2–4). Note the lack of production in the disrupted mutants.

Table 1
HPLC analysis of clavulanic acid production of wild-type *S. clavuligerus* and the *lat* gene disrupted mutants

Strains[a]	96 h (μg/mL)[b]	Times[c]	120 h (μg/mL)[b]	Times[c]
W	132.8	1.0	97.1	1.0
M-1	239.8	1.8	257.2	2.6
M-2	234.9	1.8	222.1	2.3
M-3	239.4	1.8	218.1	2.2
M-4	269.8	2.0	190.9	2.0

[a] Mutant strains are designated with the letter M, followed by a number to indicate the primary transformant which gave rise to the mutant, wild-type strains are designated with the letter W.

[b] Clavulanic acid titers were determined with supernatants of single cultures grown in soy medium for 96 and 120 h at 28 °C.

[c] *lat* gene disrupted mutants clavulanic acid production relative to wild-type *S. clavuligerus* production.

the *lat* gene disrupted mutants and the wild-type strain were similar in the fermentation cultures. To assay growth, viscosity measurements were taken throughout the fermentations, and no differences between the two kinds of strains were observed.

4. Conclusions

Now in the antibiotic production industry, improvements in yield in antibiotic fermentations have been achieved by traditional methods, such as classical mutagenesis and high-throughput screening of the antibiotic-producing microorganisms. The genetics and biochemistry of many antibiotics commonly used are partially known. So we can apply genetic engineering techniques to increase antibiotics production based on this knowledge. In this paper, we have demonstrated a simple procedure by using gene disruption technology to eliminate competing pathway of clavulanic acid biosynthesis of *S. clavuligerus*, and it is possible to achieve a high-titer industrial strain from a wild-type strain. In addition to the increase of clavulanic acid production benefit that already noted, elimination of cephamycin C production from the *S. clavuligerus* strains may also simplify the subsequent extraction and purification of clavulanic acid from the fermentation broth.

Although there are no biosynthetic enzymes shared by the cephamycin C and clavulanic acid pathways, the clavulanic acid production was increased obviously when the *lat* gene was disrupted. Possibly because when the *S. clavuligerus lat* gene was disrupted, L-α-aminoadipate, L-cysteine and L-valine were unable to incorporated into biosynthesis of cephamycin C, then the secondary metabolites inverted more to the clavulanic acid biosynthetic pathway. Furthermore, since the biosynthetic pathways for clavulanic acid and cephamycin C are biochemically distinct, the effect must be indirect. It is known that both the clavulanic acid and the cephamycin C biosynthetic pathways are controlled by the same regulatory gene, *cca*R [5,15]. Elimination of metabolites arising from the cephamycin C pathway may have a regulatory influence upon expression of the clavulanic acid biosynthetic genes.

No obvious differences between the *lat* gene disrupted mutants and the wild-type strains were observed during each phase of their growth. However, studies have also shown that

mutations that impair cephamycin C production have variable effects on clavulanic acid production in *S. clavuligerus* strain. Some mutants abolished production of cephamycin C showed no change in clavulanic acid production compared to the wild-type strains, in at least one case, clavulanic acid production was improved. The basis for this phenomenon in productivity is difficult to analyze, since the nature of the mutations were not determined. Answers to these questions could depend on the nature of the interactions between these two pathways.

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