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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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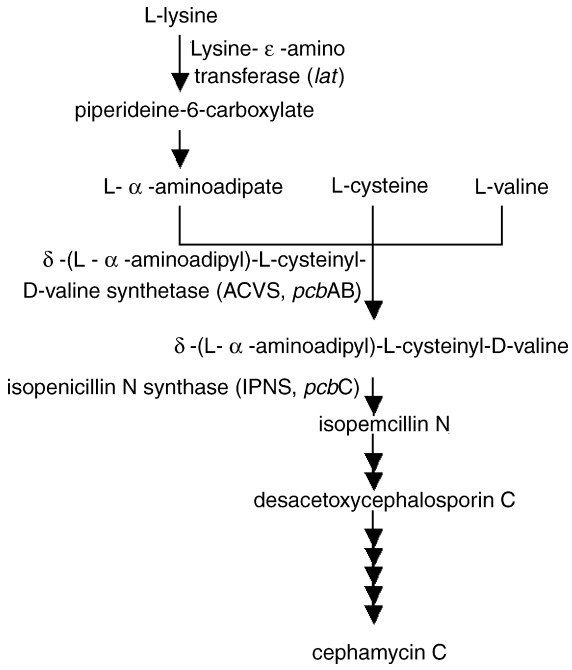

Fig. 1. Cephamycin C biosynthesis pathway. Abbreviated names of biosynthetic enzymes and their corresponding genes are given in parentheses [6].

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.