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A NOVEL POINT MUTATION IN *rpsL* GENE OF *STREPTOMYCES COELICOLOR* WITH ENHANCED BLUE PIGMENT PRODUCTION

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ABSTRACT:

Three strains showing enhanced blue pigment production were screened among thirty streptomycin resistant mutants of *Streptomyces coelicolor* 100. Their yields of blue pigment were all improved, especially for mutant *S*-3. Analysis of the mutants revealed that a point mutation occurred in mutant *S*-3. In rpsL gene, A to G transition at the position 128 resulted in Lys-43 to Arg substitution in the ribosomal protein S12 encoded by *rpsL* gene. There was no mutation in *rpsL* gene of other two streptomycin resistant mutants. Compared with the wild type strain, the mutant *S*-3 showed increased blue pigment yield and slightly decreased growth rate. This strategy of streptomycin resistance induction could be especially effective for improving the blue pigment production from the wild type strain of *Streptomyces coelicolor*.

INTRODUCTION:

Streptomyces is one of the most important groups of industrial microorganisms since they are responsible for the production of the majority of secondary metabolites such as some antibiotics in the current clinical and agricultural use. It has been of many researchers' interest for last two decades to improve the production of the secondary metabolites from *Streptomyces*. ppGpp and pppGpp are believed to play a central role in triggering the onset of antibiotic production (Ochi, 1987; Takano and Bibb, 1994). Antibiotics synthesis would not be initiated until ppGpp accumulates to certain concentration *in vivo*. But acquisition of certain streptomycin resistance mutation in *Streptomyces* allows its antibiotics synthesis to be initiated without by its prerequisition. This offers a possible strategy for improving the antibiotic productivity of the wild type *Streptomyces* strains.

Streptomyces coelicolor 100 produces a blue pigment during the stationary phase of culture. The properties of the blue pigment have been minutely studied (Zhang *et al.*, 1999). In the work, we attempted to improve the blue pigment productivity of *Streptomyces coelicolor* 100 by the streptomycin resistant induction and expound some physiological aspects of the mutant strains.

MATERIALS AND MATHODS:

Strains and preparation of mutants: The wild type strain of *Streptomyces coelicolor* 100 was screened from soil and identified (Zhang *et al*., 1999). Spontaneous streptomycin-resistant (*str*^{r}) mutants were obtained from colonies that grew at 30°C for 7 days after spores (or cells) were spread on MSBFF agar containing various concentrations of streptomycin. Their characterization was performed without selective pressure of streptomycin.

Media and growth conditions: MSBF medium contained mannitol 20g, soy bean flour 20g, agar 20g in 1 liter tap water, pH 7.2~7.5. K1 medium contained soluble starch 20g, beef extract 2g, NaCl 0.5g, K_2HPO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g per

liter tap water, pH 7.2~7.5. K2 medium contained mannitol 20g, soy bean flour 20g, NaCl 0.5g, K_2HPO_4 2.28g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g per liter tap water, pH 7.2~7.5. All strains were stored as spore suspensions at -20°C. In each experiment, spore suspensions were spread onto MSBF agar plates and incubated for 7 to 10 days at 30°C to allow for sporulation. To prepare vegetative inoculum, two loops of spores from a plate culture on MSBF agar were added to 30 ml of K1 medium in a 250-ml flask. The culture was incubated for 24 h at 30°C on a rotary shaker (200 r/min). The washed mycelium, resuspended in the same volume of water, was used as the inoculum. K2 medium (30 ml) in a 250-ml flask was inoculated at 10 % (v/v) level with mycelial culture, incubated for 7 days at 30°C on a rotary shaker (200 r/min).

Mutation analyses of *rpsL* gene: The *rpsL* gene fragment of the strain was obtained by PCR using the strain genomic DNA as a template and the synthetic oligonucleotide primers P1 (forward: 5'-ATTCGGCACACA GAAAC-3') and P2 (reverse: 5'-AGAGGAG AACCGTAG ACC-3') which were designed from the sequence for *S. coelicolor* (Gene bank accession No. AL161691). Ex*Taq* (Takara) was used to perform PCR according to the manufacturer's instructions. A Perkin-Elmer Cetus thermal cycler was used, and reaction conditions were 5 min of incubation at 97°C, following 35 cycles of 94°C for 30s, 43°C for 1 min, and 72°C for 5 min, and a final step at 72°C for 10 min, 4°C for 10 min. The nucleotide sequences of the PCR products were directly determined (Model ABI377, PE Biosystems).

The definition of relative pigment titer unit: Pigment extracted from one-milliliter broth was determined at pH 9 and 588 nm. A 0.1 A_{588} is defined a relative pigment titer unit per ml.

Analysis: To measure extracellular blue pigment, the supernatant of culture was adjusted to pH 9, and A_{588} of the supernatants was determined. For the measurement of intracellular blue pigment, pellets were harvested after centrifugation (4000*g*, 15 min), washed twice, resuspended in 2 % SDS (pH 12), thoroughly mixed for 25 min, and centrifuged (4000*g*, 15 min). The supernatant was collected and adjusted to pH 9, and A_{588} was determined. A_{588} value of the total blue pigment is the sum of two A_{588} values of the extracellular and intracellular blue pigment. Growth was measured as the dry cell weight in a sample by centrifugation and drying.

RESULTS AND DISCUSSION:

Resistant streptomycin mutants were constructed. Thirty *str*^{r} mutants of *S. coelicolor* 100 under selective pressure of different streptomycin concentrations were obtained as colonies that grew at 30°C for 7 days. Three highly productive strains (*S*-1, *S*-2, and *S*-3) with different resistance level to streptomycin (Table 1) were screened among thirty *str*^{r} mutants of *S. coelicolor* 100. Their yields of blue pigment were all improved (Fig. 1), especially for *S*-3 with an increase of 18.6 %. It was evident that blue pigment production increased with the level of resistance to streptomycin. It should be stressed that the streptomycin resistance concentration of mutant *S*-3 was 100 μg/ml, while those of S-1 and S-2 were 5 μg/ml (Table 1).

In the identification of the mutations of *rpsL* gene, primers P1 and P2 were used to amplify the internal segment of the *rpsL* homolog from the wild type strain and its *str*^{r} mutants. Fragment of 466 bp was obtained in all PCR reactions (Fig. 2). Gene sequence analysis of *str*^{r} mutants revealed that a point mutation in *rpsL* gene occurred in mutant *S*-3, and the base A was changed to G at the position 128 (Fig. 3). This is a novel mutation point in *Streptomyces*. Correspondingly, Lys-43 was altered to Arg in ribosomal protein S12 encoded by *rpsL* gene (Table 1). The

mutation in *rpsL* gene of other two *str*^{r} resistant mutants has not been found. It was notable that the streptomycin resistance concentration of mutant *S*-3, 100 μg/ml, was much higher than that of 5 μg/ml for other two strains (Table 1).

Streptomycin is an aminocyclitol glycoside antibiotic that interferes with prokaryotic ribosomal protein synthesis by binding to the 30S ribosomal subunit. It mainly induces misreading of the genetic code and inhibits translational initiation (Finken *et al*., 1993). Streptomycin-induced miscoding is believed to be the result of interference with a proof reading step in translation, causing a decrease in translational accuracy and inhibiting translocation of the ribosome. By the mutations altering certain ribosomal proteins or 16S rRNA, such detrimental effects of aminoglycoside antibiotics can be circumvented (Moazed and Noller, 1987; Okamoto-Hosoya *et al*., 2000). These mutations have been found in either ribosomal protein S12 or the nucleotide 530 or 915 regions of 16S rRNA.

Amino acid alterations at the positions Lys-43, Arg-86, Lys-88, and Pro-91 in ribosomal protein S12 have been reported to confer streptomycin resistance in some microorganisms. In bacteria, the reported mutation of *rpsL* gene occurred at the position 271(C→T) in *Escherichia coli* (van Acken, 1975), 127(A→C), 128(A→G or C) and 129(G →T) in *Bacillus subtilis* (Hosoya *et al*., 1998), 128(A→G or C), 129(G→T), 262(A→G) and 263(A→G) in *Mycobacterium tuberculosis* (Finken *et al*., 1993). In Actinomyces, *S. coelicolor* (Okamoto-Hosoya *et al*., 2000; Hesketh and Ochi, 1997; Shima *et al*., 1996) has been investigated extensively, the reported mutation of *rpsL* gene appeared at the position 129(G→T), 258(G→A), 262(A→G), 263(A→G) and 271(C→T).

A high level of resistance to streptomycin has been previously shown to result in point mutation in *rpsL* gene (Finken *et al*., 1993; Hesketh and Ochi, 1997; Shima *et al*., 1996). Similarly, a point mutation at the position 128(A→G) of *rpsL* gene in the *str*^{r} mutant (*S*-3) occurred, which showed a high level of resistance. However, those *str*^{r} mutants with low levels of resistance (*S*-1 and *S*-2) showed no mutation in the *rpsL* gene. The mutations in other genes might exist or a different streptomycin resistance mechanism be implemented. It should be stressed that the point mutation occurring at the position 128(A →G) in *rpsL* gene had already been found as the sited mutations conferring streptomycin resistance in *B. subtili*s and *M. tuberculosis*, but it has not, so far, been reported in *Streptomyces*.

The fermentative features of mutant *S*-3 were different from the wild type strain. The characterization of mutant *S*-3 in shaker fermentation revealed that mutant *S*-3 had a somewhat reduced growth rate without selective pressure of streptomycin while it entered the stationary phase at the same time as the wild type strain (Fig. 4A). Although the blue pigment production of the wild type strain and mutant *S*-3 were triggered at the same time, both the pigment yield and specific production rate ($q_{p)}$ of *S*-3 were higher than that of the wild type strain (Fig. 4B, 4C).

In the present study, blue pigment production of *S. coelicolor* was successfully improved by inducing mutation for streptomycin resistance. Three highly productive strains were screened among thirty *str*^{r} mutants to inducing streptomycin resistant of *S. coelicolor* 100. Their abilities for producing blue pigment were all improved, especially for *S*-3 in which a point mutation in *rpsL* gene took place. This breeding strategy of streptomycin resistance induction could be especially effective for improving the blue pigment production by *S. coelicolor*.

1 attcggcaca cagaaaccgg agaagtagtg cctacgatcc agcagctggt ccggaagggc
61 cggcaggaca aggtcgagaa gaacaagacg cccgcactcg agggttcgcc ccagcgccgt
121 ggcgtctgca cgcgtgtgtt cacgaccacc ccgaagaagc cgaactcggc cctgcgtaag
181 gtcgcgcgtg tgcgtctgac cagtgggatc gaggtcaccg cttacattcc gggtgagggg
241 cacaacctgc aggagcactc catcgtgctc gtgcgcggcg gccgtgtgaa ggacctgccg
301 ggtgttcgct acaagatcat ccgcggttcg cttgacaccc agggtgtgaa gaaccgcaag
361 caggcccgca gccgctacgg cgccaagaag gagaagtaag aatgcctcgt aagggccccg
421 ccccgaagcg cccggtcatc atcgacccgg tctacggttc tcctct

Figure–3: Sequences of the *rpsL* gene of *S. coelicolor* 100 and its *str*^{r} mutant *S*-3. The *rpsL* gene sequence is set in grey and black letters, primer sequences in underlined. The *rpsL* gene of *str*^{r} mutants *S*-3 occurred a point mutation at position 128 (A→G) compared with that of the wild type strain. The mutation position 128 is boxed.

Table-1: Mutation position in *rpsL* gene and resulting amino acid exchange in ribosomal protein S12.

Strain	Resistance to streptomycin (μg/ml) [a]	Mutation position in *rpsL* gene	Exchange position in amino acid sequence
100	0.2	— [b]	
S-1	5	NF[c]	
S-2	5	NF	
S-3	100	128 A→G	43 (Lys→ Arg)

[a] **Determined after 7 days cultivation on MSBF agar;** [b] **—, wild-type *rpsL*;** [c] Mutation was not found (NF) in the *rpsL*.

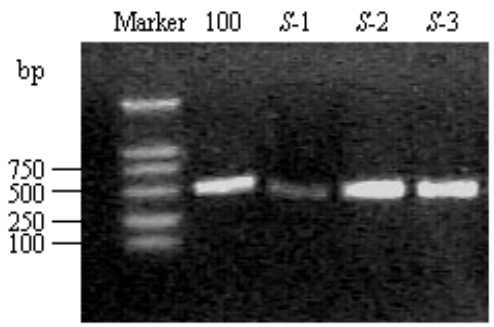


Figure -2: Electrophoresis of PCR products of *rpsL* gene of the wild type strain and its *str*^{r} mutants of *S. coelicolor* (*S*-1, *S*-2 and *S*-3)

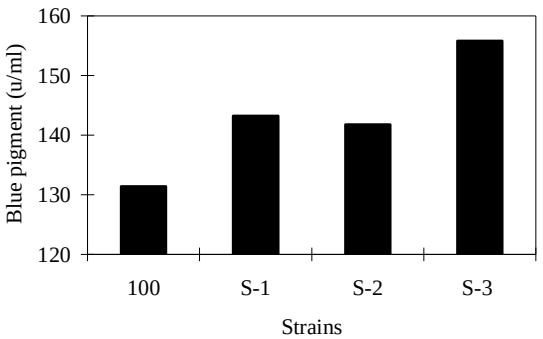
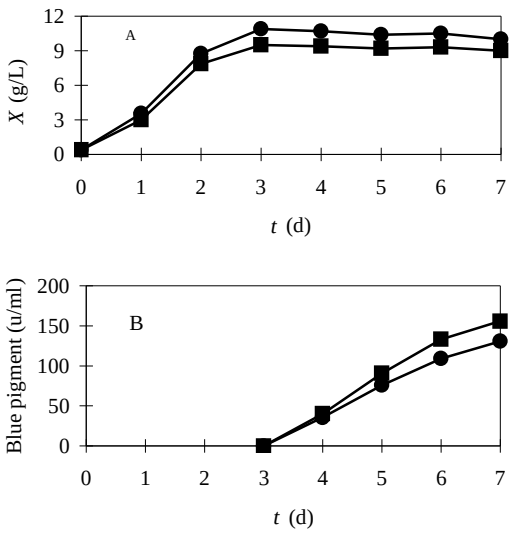


Figure-1: The improvement of blue pigment production of the *str*^{r} mutants of *S. coelicolor* as compared with that of the wild type strain



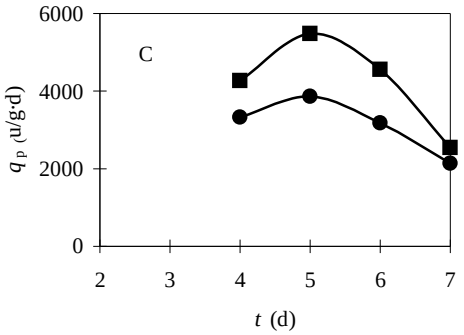


Figure 4: The *str*^{r} mutant (*S*-3, ■) reduced the growth (A) and improved blue pigment production (B) and specific production rate q_p (C) as compared with wild type strain (*S. coelicolor* 100, ●) when cultured in K2 medium

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