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Improvement of industry-applied rifamycin B-producing strain, *Amycolatopsis mediterranei*, by rational screening

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An industrially applied rifamycin B-producing strain, *Amycolatopsis mediterranei* XC 1-02, was used for further screening. A special mutation and screening procedure was adopted to select a strain, which can alleviate the inhibition caused by both aromatic amino acid and *p*-hydroxybenzoic acid in the pathway of rifamycin B biosynthesis as well as enhance the production of propionate, one of the precursors of rifamycin B biosynthesis. By the above methods, a strain *A. mediterranei* XC 9-25 was obtained, and its rifamycin B productivity in shaking flask reaches 10 g/L, which is 2.38 times higher than that of the ancestral strain XC 1-02. The productivity of rifamycin B fed-batch fermentation in 60,000 L fermentor with *A. mediterranei* XC 9-25 reached 19.11 g/L.

Key Words—*Amycolatopsis mediterranei*; *p*-hydroxybenzoic acid; propionate; rational screening; rifamycin B; tryptophan

Introduction

The rifamycins (Oppolzer and Prelog, 1973), exemplified by rifamycin B, are a family of ansamycin antibiotics (Rinehart and Shield, 1976) with pronounced anti-mycobacterial activity that are extensively used in the clinical treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections (Sepkowitz et al., 1995). The rifamycins are produced by *Amycolatopsis mediterranei* (Sensi et al., 1959), and their biosynthesis involves a polyketide intermediate synthesized with chain extension of an unusual starter unit, 3-amino-5-hydroxybenzoic acid (AHBA) (Ghisalba and Nüesch, 1981; Kibby et al., 1980), and its assembly with two acetate and eight propionate units on a type I polyketide synthase (PKS) (Lancini and Carve-

lieri, 1997). AHBA is in turn synthesized via a variant of the shikimate pathway of aromatic amino acid biosynthesis (Ghisalba and Nüesch, 1978a,b; White and Martinelli, 1974). 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase is the first enzyme of the shikimate pathway, and its activity is inhibited by three aromatic amino acids. Among the inhibitions caused by the three aromatic amino acids, the inhibition caused by tryptophan is the strongest, and the inhibition by tyrosine and phenylalanine is relatively weak (Xia and Chao, 1989). The biosynthesis of AHBA is also inhibited by AHBA's analogues: *p*-aminobenzoic acid (PABA), *p*-hydroxybenzoic acid (PHBA), and anthranilate (ANA) (Gu et al., 1988).

Based on the above analysis on the pathway and regulation of rifamycin B biosynthesis, strain improvement of a rifamycin B producer *Amycolatopsis mediterranei* XC 1-02 was performed by rational screening strategy to increase its production.

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Materials and Methods

Microorganism. *Amycolatopsis mediterranei* XC 1-02, provided by Xinchang Pharmaceutical Factory, Zhejiang Medicine Co., Ltd., P. R. China, is the ancestor of all mutant strains described in this study.

Media and cultural conditions. Slant medium (g/L): yeast extract, 4; malt extract, 4; glucose, 4; oatmeal, 20; agar, 20; and adding distilled water to 1 L. Slant culture was incubated for 7–8 days at 28°C and 30–60% relative humidity.

Plate medium (g/L): starch, 20; KNO₃, 0.1; K₂HPO₄, 0.5; MgSO₄, 0.5; NaCl, 0.5; FeSO₄, 0.01; agar, 20; and adding distilled water to 1 L. The pH value of the medium was adjusted to pH 7.0–7.2 before autoclaving. Plate culture was incubated under the same conditions as the slant culture.

The mycelium from slant culture was inoculated to a 250 ml Erlenmeyer flask containing 25 ml of seed medium (2% starch, 1.5% glucose, 1% soybean meal, 1% peptone, 0.05% KNO₃, 0.03% KH₂PO₄, 0.3% CaCO₃). After incubation at 28°C for 48 h on a rotary shaker at 220 rpm, a 2 ml portion of the seed culture was used to inoculate 25 ml of production medium in 250 ml Erlenmeyer flask.

The production medium comprised: 12% glucose, 2% soybean meal, 1% peptone, 0.5% fish meal, 0.8% KNO₃, 0.05% KH₂PO₄, 0.0001% CoCl₂, 0.5% CaCO₃. The production culture was incubated under the same conditions as the seed culture, except the cultivation periods extended to 7 days.

Rifamycin B fed-batch fermentation in 60,000 L fermentor. The vegetative fermentor (400 L) containing 220 L of seed medium was inoculated with 480 ml of broth cultured in flask. After 72 h of incubation at 28°C, the vegetative broth was used to inoculate the preculture fermentor (4,000 L) containing 2,400 L of seed medium. After 48 h of incubation at 28°C, the seed broth was transferred into the productive fermentor (60,000 L) containing 28 m³ of production medium. During the fermentation, glucose and ammonia were fed continuously in order to maintain the reduced sugar concentration at 1.0–1.5% and pH value at 6.4–6.6. After 9 days' cultivation at 28°C, the fermentation was ended.

Mutation. We took 5 ml of mycelium suspension from a slant culture of *A. mediterranei* XC 1-02, and transferred it to an aseptic plate. The plate, with cover removed, was exposed to UV irradiation for 100 s at a

distance of 30 cm from UV lamp with a wavelength of 3,537 Å and power of 30 W.

Screening agents and screening for agent tolerant mutants. Tryptophan (trp), tyrosine (tyr), phenylalanine (phe), *p*-hydroxybenzoic acid (PHBA) and propionate were used as screening agents. The mycelium suspension of the parent strains after UV irradiation (the survival ratio was about 9.0%) was spread on the medium containing one of above agents, and the agent tolerant mutants were screened from above medium. The mycelium suspension with or without UV irradiation was also spread on agar medium without screening agent for comparison. A total of 30 colonies was isolated from each group, and after shake bottle fermentation, rifamycin B concentration was measured and compared. The standard deviation was calculated.

Analytical method. Reduced sugar was measured by Fehling's reagent method (Chen and Xu, 1991). Amino nitrogen was analyzed by the formaldehyde titration method (Chen and Xu, 1991). The dried cell weight (DCW) was determined according to the method described by Leblihi et al. (1987). Rifamycin B was assayed by the spectrophotometric method (Sensi and Thiemann, 1967).

Results

Rifamycin B was produced by *A. mediterranei* XC 1-02, which was an industry-applied strain and its rifamycin B productivity was about 4.5 g/L. In order to increase its productivity, a new screening procedure should be developed.

Because three aromatic amino acids (tryptophan, tyrosine and phenylalanine) and *p*-hydroxybenzoic acid are inhibitors and propionate is an important precursor for rifamycin synthesis according to the metabolic pathway, we examined the tolerance of the original strain, *A. mediterranei* XC 1-02, toward these agents for cell growth. The results indicated that when the initial concentration of these aromatic amino acids in the medium is 0.1%, the inhibitory ratio for cell growth is 99.6%, 92.2% and 87.6%, respectively. Similarly, when *p*-hydroxybenzoic acid and propionate concentration in the medium is 0.1% and 0.1%, respectively, the inhibitory ratio for cell growth is 98.9% and 97.6%. The above experimental results explained that a high concentration of these agents in the medium was harmful for the cell growth; therefore, rifamycin synthesis would certainly be affected. It is necessary to screen

tolerant strains for these agents.

Aromatic amino acid tolerant strain

After UV mutation, the aromatic amino acids' tolerant strains were screened. The abilities of these strains to produce rifamycin B were examined, and the results are listed in Table 1. The rifamycin productivities of strains without or with UV mutation grown on the aromatic amino acid-free medium are listed for comparison.

From Table 1 it was obvious that the average rifamycin B productivities of the aromatic amino acid-tolerant mutants were higher than those of natural isolates and UV mutants, and that the rifamycin B productivities of L-tryptophan-tolerant mutants were higher than those of L-tyrosine- and L-phenylalanine-tolerant mutants. Among L-tryptophan-tolerant mutants, a *trp*^t mutant XC 2-10, with a maximum potency increase of 25% compared to the highest of the natural isolates, was obtained.

In order to further examine the effect of L-tryptophan concentration on the rifamycin B productivity, 0.5% tryptophan-tolerant mutants were screened from *A. mediterranei* XC 2-10. The experimental data are listed in Table 2. The results indicated that the rifamycin B productivities of the 0.5% *trp*^t mutants were higher than those of the 0.1% *trp*^t mutants, and that the rifamycin B productivity of the 0.5% *trp*^t mutant XC 3-11 was increased 14% compared to the strain XC 2-10. *A. mediterranei* XC 4-22 was further screened by natural selection from strain XC 3-11, with an additional potency increase of 5.3%.

p-Hydroxybenzoic acid tolerant strain

After UV irradiation, 0.1% *p*-hydroxybenzoic acid-tolerant mutants were selected. The rifamycin productivities of the UV mutants grown on the medium with or without *p*-hydroxybenzoic acid were evaluated and the results are shown in Table 3. The results show that the productivities of rifamycin B of the *p*-hydroxybenzoic acid-tolerant mutants were higher than those of the natural isolates and UV mutants without *p*-hydroxybenzoic acid tolerance. Among them, a *phb*^t mutant XC 5-28, with a potency increase of 16% compared to the highest of the natural isolates, was obtained. A natural selection of *A. mediterranei* XC 5-28, which is named strain XC 6-12, further increased its potency up to 12%.

Table 1. Comparison of rifamycin B productivity among the natural isolates, UV mutants and the aromatic amino acid-tolerant mutant strains.

	Relative rifamycin B productivity		Standard deviation
	Average	The highest	
NS	100	100	5.91
UV	90	105	8.54
UV+0.1% <i>trp</i> ^t	120	125	7.75
UV+0.1% <i>tyr</i> ^t	110	112	8.06
UV+0.1% <i>phe</i> ^t	105	108	8.31

Table 2. Comparison of rifamycin B productivity of *trp*^t mutants with different tryptophan concentration.

	Relative rifamycin B productivity		Standard deviation
	Average	The highest	
NS+0.1% <i>trp</i> ^t	100	100	5.74
UV+0.1% <i>trp</i> ^t	102	106	8.06
UV+0.5% <i>trp</i> ^t	112	114	7.48

Table 3. Comparison of rifamycin B productivity among the natural isolates, UV mutants and the *p*-hydroxybenzoic acid-tolerant mutants.

	Relative rifamycin B productivity		Standard deviation
	Average	The highest	
NS	100	100	5.83
UV	95	102	8.37
UV+0.1% <i>phb</i> ^t	113	116	7.55

Propionate-tolerant mutant

The rifamycin B productivities of strains without or with propionate tolerance were evaluated and the results are listed in Table 4. Table 4 shows that the rifamycin B productivities of the propionate-tolerant mutants were higher compared with those of the natural isolates and UV mutants without propionate tolerance. Among them, a 0.1% *prp*^t mutant XC 7-24, with a potency increase of 8% compared to the strain XC 6-12, was obtained. *A. mediterranei* XC 9-25 was obtained

Table 4. Comparison of rifamycin B productivity among the natural isolates, UV mutants and the propionate-tolerant mutant strains.

	Relative rifamycin B productivity		Standard deviation
	Average	The highest	
NS	100	100	5.66
UV	96	103	8.25
UV+0.1% <i>prp</i> ^t	105	108	7.42

Fig. 1. The genealogical tree of *A. mediterranei* XC 9-25.

by two continuous natural selections with strain XC 7-24; its potency increased 10%.

As described above, the strain *A. mediterranei* XC 9-25 was characterized by 0.1% *trp*^t, 0.5% *trp*^t, 0.1% *phb*^t and 0.1% *prp*^t, respectively. The genealogical tree of *A. mediterranei* XC 9-25 is shown in Fig. 1. The rifamycin B productivity of *A. mediterranei* XC 9-25 reached 10 g/L, which is 2.38 times higher than that of the ancestral strain, *A. mediterranei* XC 102.

Hereditary stability of *A. mediterranei* XC 9-25

The hereditary stability of *A. mediterranei* XC 9-25 was examined with a colonial subculture method.

Table 5. Productivity in each generation of *A. mediterranei* XC 9-25.

Generation	Productivity (g/L)
1	10.01
2	10.50
3	10.02
4	9.98
5	9.92

There was little fluctuation in productivity among five generations (Table 5). This illustrated that the genetic stability of the high rifamycin B-producing strain *A. mediterranei* XC 9-25 was very good.

Scale up of rifamycin B fermentation with *A. mediterranei* XC 9-25

Fed-batch fermentation with *A. mediterranei* XC 9-25 for rifamycin B production was performed in a 60,000 L fermentor in Xinchang Pharmaceutical Factory, Zhejiang Medicine Co., Ltd., P. R. China. The time courses of fed-batch fermentation with *A. mediterranei* XC 9-25 are shown in Fig. 2.

From Fig. 2, it is observed that rifamycin B is only produced at the later stage of exponential growth phase and increases fast at stationary phase. It seems the fermentation is a biphasic process. This observation is different from previous report (Gisalba et al., 1984). In their research work, the rifamycin B production is growth-related; therefore, to increase the rifamycin B production, the cell density should be very high, which will cause an increase in both oxygen mass transfer resistance and viscosity of fermentation broth.

Because rifamycin B production with *A. mediterranei* XC 9-25 is a two-phase process, the mycelium concentration in the fermentation broth can be controlled, which is favorable not only for the oxygen mass transfer during the fermentation process but also for rifamycin B separation and purification. The main reasons behind the difference between *A. mediterranei* XC 9-25 and other strains might be the strain improvement and production medium applied.

Reducing sugar was still consumed rapidly in the stationary phase although the mycelium growth almost stopped in this phase, which meant that the consumed sugar was primarily used for rifamycin B biosynthesis and mycelium maintenance. During the fed-batch fer-

A

B

Fig. 2. Fed-batch fermentation of rifamycin B in 60 m³ fermentor with *A. mediterranei* XC 9-25.

(A) ◇, reduced sugar; △, amino nitrogen; □, pH. (B) ◇, dried cell weight; □, product.

mentation, glucose was supplied in order to maintain the reduced sugar at 1.0–1.5%.

The amino nitrogen was consumed slowly in the first day, which corresponded to the increase in the pH value of the fermentation broth. After that the amino nitrogen was consumed vigorously, accompanied with the pH value decrease in and synthesis of rifamycin B in large amounts. During the fed-batch fermentation, ammonia was provided in order to maintain the pH value at pH 6.4–6.6.

The productivity of rifamycin B in 60,000 L fermentor with *A. mediterranei* XC 9-25 can reach as high as 19.11 g/L after 9-day fed-batch fermentation, while the productivity of the original *A. mediterranei* XC 1-02 in 60,000 L fermentor is only 6.25 g/L with the same fermentation technique.

Discussion

Generally, auxotrophs and analog-resistant mutants were used for screening the high producing strain. For

Fig. 3. The feedback inhibition of aromatic compounds on rifamycin B biosynthesis.

E-4-P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DQA, dehydroquinate; DSA, dehydroshikimate; SA, shikimate; CHA, chorismate; phe, phenylalanine; tyr, tyrosine; trp, tryptophan; ANA, anthranilate; PABA, *p*-aminobenzoic acid; PHBA, *p*-hydroxybenzoic acid; AHBA, 3-amino-5-hydroxybenzoic acid.

industrially applied strains, which had been treated by various mutagens and screening procedures, how to further increase their productivities is still a question and little has been exploited.

According to the rifamycin B biosynthetic pathway, AHBA is the key precursor for rifamycin B synthesis. AHBA is synthesized via a variant of the shikimate pathway of aromatic biosynthesis (Ghisalpa and Nüesch, 1978a, b; White and Martinelli, 1974). DAHP synthase is the first enzyme of the shikimate pathway, and its activity is inhibited by the three aromatic amino acids (Fig. 3). Although the elimination of inhibition caused by these aromatic amino acids, such as aromatic amino acid auxotrophs, can increase the DAHP synthase activity, they are still required for mycelium growth and metabolism. So if the aromatic amino acid auxotrophs have been screened, we must add these amino acids to the medium to meet the requirement for mycelium growth, in the meantime, to prevent their inhibitory effects. This will lead to high costs and operation trouble.

In this work, the aromatic amino acid-tolerant mutants were screened. The aromatic amino acid-tolerant mutants brought higher productivity of rifamycin B, which suggested that the inhibition of DAHP synthase caused by the aromatic amino acids might be relieved so that more DAHP could be produced. The abundant supply of DAHP would result in higher rifamycin B productivity. The *trp*^r mutants produce more rifamycin B than the *tyr*^r mutants and the *phe*^r mutants. The reason is that tryptophan is the strongest inhibitor of DAHP

synthase. The higher the tryptophan concentration is, the more rifamycin B can be produced by *trp^t* mutants.

The biosynthesis of AHBA is also inhibited by the AHBA's analogues, *p*-aminobenzoic acid (PABA), *p*-hydroxybenzoic acid (PHBA), and anthranilate (ANA) (Fig. 3) (Gu et al., 1988). The *p*-hydroxybenzoic acid-tolerant mutants can eliminate or relieve the inhibitory effect on ABHA biosynthesis by *p*-hydroxybenzoic acid, so that more ABHA and rifamycin B will be synthesized.

There are eight propionate units involved in rifamycin B biosynthesis, so propionate is an important precursor of rifamycin B. The increase of propionate concentration will certainly enhance rifamycin B production. The propionate-tolerant mutants can live in the medium with high propionate concentration, which means the inhibition caused by propionate in the propionate biosynthesis pathway is weakened and more propionate will be produced to meet the requirement in the rifamycin B biosynthesis. The abundant supply of propionate will certainly result in higher productivity of rifamycin B.

For industry-applied strains, in order to increase their productivity further, selecting an agent-tolerant mutant, based on the metabolic pathway, might be a good choice as demonstrated in this work. After a series of screening procedures, *A. mediterranei* XC 9-25 (*trp^tphb^tprp^t*) can produce 10 g/L rifamycin B, which is 2.38 times higher than that of the ancestral strain, *A. mediterranei* XC 102.

In this work, the mutation was only examined at the cell level, and the results showed that the mutation and screening procedure was effective to raise the rifamycin productivity. Further work is required to understand the real reason at the gene level.

The growth pattern of *A. mediterranei* XC 9-25 (*trp^tphb^tprp^t*) is a biphasic process. With this feature, the maximum mycelium concentration at the end of the first phase can be controlled at an appropriate level, which is favorable to enhance the oxygen mass transfer and to reduce the viscosity of fermentation broth. At second phase, with fed-batch operation strategy, the rifamycin B production period is prolonged and the final rifamycin B productivity can reach as high as 19.11 g/L.

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