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# Mutagenesis of the Rapamycin Producer *Streptomyces hygroscopicus* FC904

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Rapamycin (RPM) is produced by *Streptomyces hygroscopicus* FC904 isolated from soil in Fuzhou, China. It is a triene macrolide antibiotic with potential application as an immunosuppressant and drug for human gene therapy. In an attempt to improve rapamycin production, mutation and screening of the parent culture have been carried out. Thousands of survivors were obtained after mutagenesis by NTG (3 mg/ml) and UV (30 W, 15 cm, 30 seconds) of spore suspensions. None showed improved production of RPM. We determined the susceptibility to antibiotics of *S. hygroscopicus* FC904 by two fold dilutions of antibiotics in oatmeal agar plates. It was found that the strain was resistant to penicillin, erythromycin, RPM, tetracycline and chloramphenicol, but susceptible to mitomycin C (MIC, 10 $\mu$g/ml) and aminoglycosides such as gentamicin (MIC, 0.1 $\mu$g/ml), kanamycin (MIC, 0.1 $\mu$g/ml) and streptomycin (MIC, 0.3 $\mu$g/ml). Protoplasts of strain FC904 were prepared after finding the best conditions for their formation. They were treated with gentamicin, erythromycin, mitomycin C and NTG. Surprisingly, gentamicin was especially effective for obtaining higher RPM-producing mutants. Mutant C14 was selected by exposing the protoplasts of the parent strain FC904 to 1 $\mu$g/ml of gentamicin at 28°C for 2 hours. A higher RPM-producing mutant (C14-1) was obtained from the protoplasts of mutant C14 treated with gentamicin, and its titer was 60% higher than that of the parent strain FC904 by HPLC analysis. Another improved mutant (C14-2) was obtained from the spores of mutant C14 treated with 1 $\mu$g/ml of gentamicin plus 2 mg/ml of NTG at 28°C for 2 hours. Mutant C14-2 had a titer 124% higher than FC904. The possible mechanism for the effect of gentamicin by using protoplasts or spore suspensions will be discussed, *i.e.* the possibility of gentamicin being a mutagen or a selective agent.

Rapamycin (RPM) is an unusual nitrogen-containing triene macrolide with potential application as an immunosuppressant and drug for human gene therapy[1,2] (Fig. 1). We recently found it to be produced by *S. hygroscopicus* FC904 isolated from soil in Fuzhou, China. Many reports on the RPM biosynthesis have been published[3~9], however few have been concerned with the mutagenesis of the producing microorganism for improvement of RPM production. In this paper, we report on the mutagenesis of *S. hygroscopicus* FC904.

## Materials and Methods

### Microorganism

*Streptomyces hygroscopicus* FC904 used as starting strain was isolated from soil in Fuzhou, China. *Candida albicans* ATCC 11651 was used as test microorganism for bioassay.

### Fermentation

RPM producers were incubated on 2% oatmeal agar slants at 28°C for 15 days. Three ml of 20% glycerol were added to each tube, and the resulting spore suspension was

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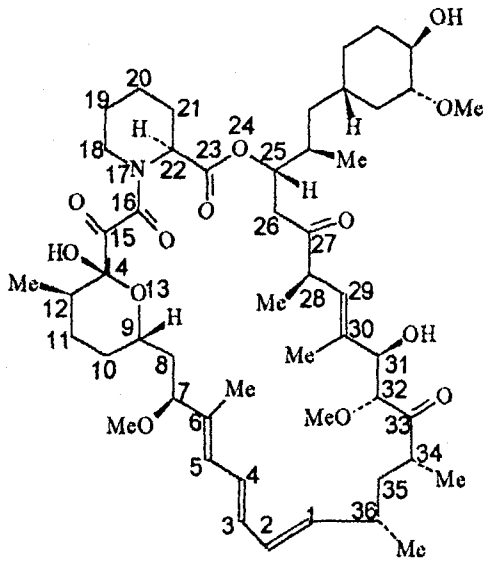
Fig. 1. Structure of rapamycin.



filtered to discard the mycelial debris and stored at −20°C. One ml of spore suspension was inoculated into a 500 ml flask containing 80 ml of fermentation medium consisting of (g/liter) glucose 4.0, glycerol 1.0, oatmeal 3.0, L-lysine 0.1, dry yeast 0.2, peptone 0.1, soybean meal 2.0, NaCl 0.5, pH 6.0. Incubation was carried out at 28°C for 5 days on a rotary shaker operating at 200 rpm.

### Extraction and Bioassay

Extraction and bioassay were carried out as described in our previous paper[6].

### HPLC Analysis

HPLC was performed on a column of ODS-C18 at 40°C with an eluant of MeOH - $H_2O$, 75 : 25 (v/v). The flow rate was 1 ml per minute, and UV absorption of the effluent was monitored at 277 nm. The retention time for RPM was 20.5 minutes.

### Determination of Minimum Inhibitory Concentration

MICs were determined by the two fold-dilution method, applying to discs which were plated oatmeal agar medium, and incubating at 28°C for 10 days.

### Mutagenesis

#### By Ultraviolet Light

A spore suspension was diluted with sterile water and placed in an uncovered Petri dish. It was irradiated under a UV lamp with constant agitation for an appropriate length of time (killing rate 99%).

#### By *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)

Mutagenesis of spore suspensions or protoplasts by NTG was performed as described by HOPWOOD[10] except for the use of 0.1 M Tris-HCl (pH 8.0) in place of TM buffer (0.05 M Tris and 0.05 M maleic acid, pH 8.0 or 9.0), and incubation at 37°C for 2~3 hours instead of 30°C for 1~2 hours.

### Protoplast Formation and Regeneration

Mycelium was harvested from cultures grown on a rotary shaker (200 rpm) for 40 hours, at 28°C in GYP medium (glucose 1%, dry yeast 0.6%, polypeptone 0.1%, $K_2HPO_4 \cdot 3H_2O$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0), supplemented with 10% sucrose and 0.7% glycine. The protoplasts were obtained by the method of HOPWOOD *et al.*[11], except for the use of lysozyme (2 mg/ml) digestion at 30°C for 1 hour. Protoplasts were plated on $R_2$YE regeneration medium[11] and incubated at 28°C for 10 days.

### Treatment of Protoplasts or Spore Suspension with Antibiotics

A stock solution of antibiotic was added into a suitable measured volume of the protoplasts in P buffer or to a spore suspension in normal saline to give the correct final antibiotic concentration. After mixing completely, the mixture was incubated at 28°C for 3 hours, and then diluted to give the appropriate dilution. A 0.1 ml of the dilution was plated onto the regeneration medium, $R_2$YE or oatmeal agar medium (for spore dilution), and incubated at 28°C for 10~15 days.

## Results

### Susceptibility of *S. hygroscopicus* FC904 to Antibiotics

Table 1 shows the susceptibility to antibiotics of the starting strain *S. hygroscopicus* FC904. It was very sensitive to aminoglycosides such as gentamicin, kanamycin and streptomycin, and to mitomycin C; less susceptible to tetracycline, chloramphenicol and penicillin; and resistant to rapamycin, erythromycin and candicidin.

### Protoplast Formation

Protoplasts of the *Streptomyces* genus are usually effectively prepared with normal procedures. However, the optimum conditions for various species and strains are

Table 1. Susceptibility of *S. hygroscopicus* FC904 to antibiotics.

| Antibiotic | MIC (μ g/ml) |
|---|---|
| Gentamicin | 0.1 |
| Kanamycin | 0.1 |
| Streptomycin | 3 |
| Mitomycin C | 10 |
| Tetracycline | 65 |
| Chloramphenicol | 100 |
| Penicillin G | 100 |
| Rapamycin | 450 |
| Erythromycin | >1000 |
| Candicidin | >1000 |

Table 2. Optimal condition for protoplasting.

| Component | Concentration (g/liter) |
|---|---|
| Sucrose | 100 |
| Glycine | 7 |
| Lysozyme | 2 |
| Temperature rang | 30~37℃ |

Table 3. Preliminary screening of 1571 regenerating strains.

| "Mutagen" | Dose (μ g/ml) | Amount | Positive-mutation (%) | Negative-mutation (%) |
|---|---|---|---|---|
| Gentamicin | 1.0 | 363 | 6.1 | 13.2 |
| Mitomycin C | 2.5 | 563 | 7.6 | 20.8 |
| Erthyromycin | 1000 | 345 | 1.2 | 7.2 |
| NTG | 2000 | 300 | 5.3 | 27.0 |

Bioassay: *Candida albicans* ATCC 11651

Positive-mutation: Inhibition zone>2mm, compared with the starting strain.

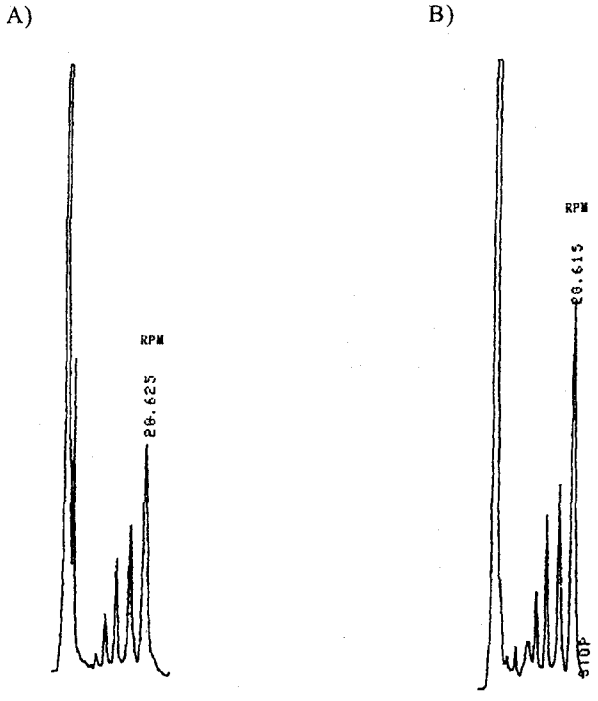
Negative-mutation: Inhibition zone<2mm, compared with the starting strain.

different. Under optimum condition as shown in Table 2, protoplast conversion was almost complete, *i.e.* after lysozyme treatment, colony forming ability decreased by $10^8$.

### Treatment of Protoplasts with Antibiotic and/or NTG

The protoplasts of *S. hygroscopicus* FC904 were exposed to gentamicin (GM), erythromycin (EM), mitomycin C (MC) or NTG at 28°C for 2 hours. The dosages of GM (1 μg/ml), MC (2 μg/ml) and NTG (2 mg/ml) giving a killing rate of 90.0% to 95.0% were adopted. In a series of choosing mutagens or selective agents for the protoplasts of the starting strain, the superiority of GM as compared to MC and NTG respectively can be seen in Table 3. However, as expected, EM could not be used as a selective agent in this experiment.

Fig. 2. HPLC analysis of the starting strain (A) and its first generation mutant C14 (B).




### Higher RPM-producing Mutants by Exposing the Protoplasts to GM

In an attempt to obtain strains producing higher titers of RPM, mutant C14, which is among the 25 positive-mutation strains shown in Table 3, was chosen. Its titer (118 μg/ml) was 36% higher than that of the starting strain FC904 (87 μg/ml) by HPLC analysis (Fig. 2). Changes in its cultural characteristic are described in Table 4, and depicted in Fig. 3. A higher RPM producing mutant C14-1 was obtained from the protoplasts of mutant C14 treated with 1 μg/ml of GM at 28°C for 2 hours, and its titer (139 μg/ml) was 60% higher than that of the starting strain FC904 by HPLC analysis (Fig. 4).

### Another Higher RPM-producing Mutant by Exposing the Spores to GM Plus NTG

In the course of selecting better RPM producing mutants, an improved C14-2 was obtained from spores (not protoplasts) of mutant C14 treated with 1 μg/ml of GM and 2 mg/ml of NTG at 28°C for 2 hours. The titer of mutant C14-2 (195 μg/ml) increased by 124% as compared with

Fig. 3. Colonies of *Streptomyces hygroscopicus* FC904 (A) and mutant C14 growing on oatmeal agar plates (B).

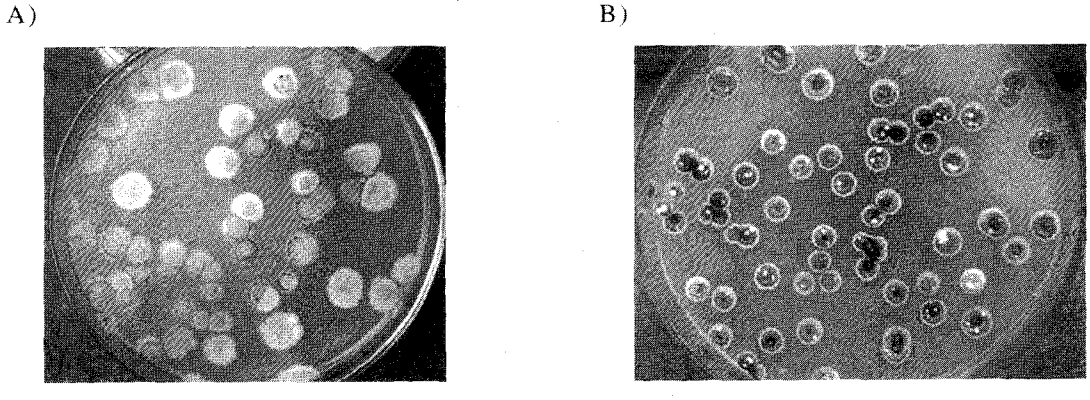


Table 4. Comparison of cultural characteristics between *S. hygroscopicus* FC904 and mutant C14.

| | *S. hygroscopicus* FC904 | Mutant C14 |
|---|---|---|
| Growth rate | Slow | Rapid |
| Colony | Diameter 5-6mm, round with many circles, flat with wrinkles; bald colony appearance | Diameter 3-4mm, round with elevation in center and wrinkled edges; not bald |
| Aerial mycelium | Scant, snow-white | Abundant, white with light yellowish tint |
| Spore mass | Gray, hygroscopic water at the 18th day | Deep gray, hygroscopic water at the 12th day |

Fig. 4. HPLC analysis of the starting strain (A) and its second generation mutant C14-1 (B).

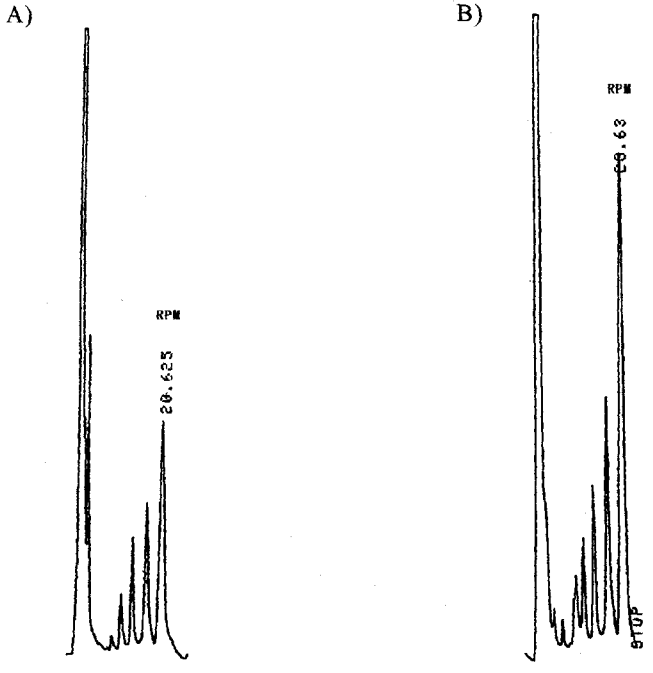
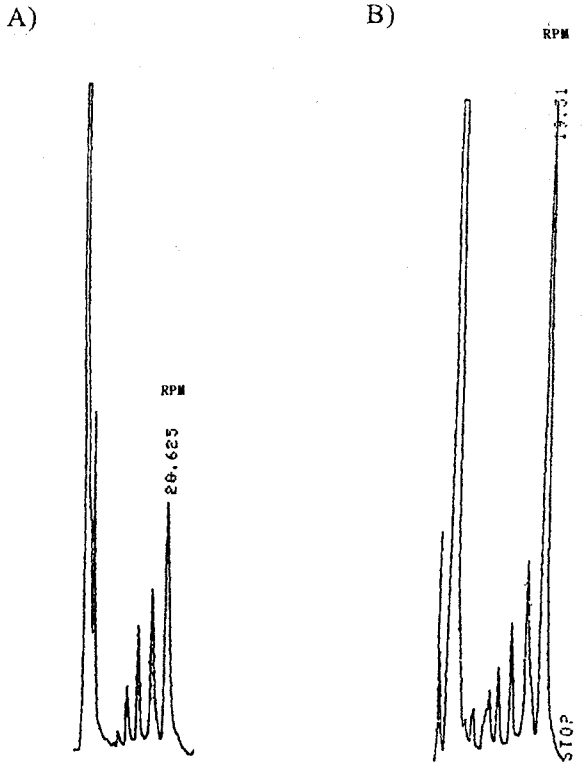



Fig. 5. HPLC analysis of the starting strain (A) and its third generation mutant C14-2 (B).




the starting strain (Fig. 5). There are very few differences between mutant C14-2 and mutant C14 in cultural characteristics.

## Discussion

Although thousands of survivors were obtained by the treatment of spore suspensions with NTG, EMS and UV

light respectively, no higher RPM producing mutant was obtained (data not show). It is interesting the starting strain was very susceptible to aminoglycosides and MC. Surprisingly, GM was very effective for obtaining higher-RPM producing strains by exposing either protoplasts or spore suspensions to this antibiotic.

GM is an aminoglycoside antibiotic which inhibits bacterial growth by interfering with protein synthesis. Its action appears to interrupt the "ribosome cycle" at the initiation of protein synthesis, *i.e.* it causes misreading of an m-RNA codon and a wrong amino acid is incorporated into the growing peptide chain[12]. On the contrary, MC is an antitumor antibiotic which is also active against bacteria and is considered a rapidly acting selective inhibitor of DNA synthesis[13]. It is possible that GM is acting as a selective agent and not a mutagen.

Acknowledgement

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