Overproduction of Rifamycin B by *Amycolatopsis mediterranei* and Its Relationship with the Toxic Effect of Barbital on Growth

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(Received for publication August 11, 1997)

A novel method for selecting overproducing strains of rifamycin B was developed. This technique involves the use of lysozyme and the effect of barbital on the growth of *A. mediterranei*. Complete medium added with glycine and barbital was inoculated with mutagenized mycelium, incubated for 48 hours and treated with lysozyme. The lysozyme resistant mycelium was washed with dilute detergent. Complete medium with glycine but without barbital was inoculated with the washed mycelium. Protoplasts were obtained and regenerated and the colonies were picked and seeded on Bennet agar plates with and without barbital. Two selected mutants were sensitive to 0.5% barbital producing 200% more rifamycin than the parental strain. In addition, 30 barbital resistant mutants were isolated and their production level was lower than the one observed with the parental strain. These results suggest that the effect of barbital on secondary metabolism (rifamycin production) is related to its effect on primary metabolism.

Rifamycin B is an important antibiotic produced by *Amycolatopsis mediterranei*, originally described by Sensi and coworkers. Semisynthetic derivatives are prepared from this fermentation product. This family of antibiotics has activity against *Mycobacterium tuberculosis* and *Mycobacterium avium*. The former is the causative agent of an infection common in patients with Acquired Immune Deficiency Syndrome (AIDS).

Several attempts have been made to improve rifamycin production strains of *A. mediterranei*, using recombinant DNA techniques and classical mutation methods. But none has been based on a compound that gives a marked stimulatory response on rifamycin B biosynthesis, such as barbital (5,5-diethylbarbituric acid). Margalith and Pagani in their classical work showed that addition of 11 mM barbital to the culture medium caused a shift towards production of rifamycin B and away from other rifamycins.

On the other hand, barbital has a toxic effect upon *A. mediterranei*. Mycelial growth is 20 to 25% more abundant in a medium without barbital than in its presence. In addition, the presence of 0.2% barbital reveals inhibition in colonial growth in surface culture. In this paper, we demonstrate that there is a relationship between the toxic effect of barbital and biosynthesis of rifamycin B in *A. mediterranei*. We developed a novel method to isolate high rifamycin-producing mutants, based on their sensitivity to barbital. A comparison was also made between sensitive and resistant mutants and the parental strain, in relation to their production of rifamycin B.

**Materials and Methods**

**Microorganism**

*A. mediterranei* type strain (ATCC 13685), *A. mediterranei* M18 (ATCC 21789) and mutants strains derived from M18 (ATCC 21789) were used for these studies. They were stored lyophilized and maintained in Bennet agar medium.

**Media and Culture Conditions**

Agar Bennet was composed of (g/liter): glucose, 10; meat extract, 1; NZ-amine A, 2; yeast extract, 1 and agar, 20.

Bacteria were usually grown in seed medium containing the following components, in grams per liter: glucose, 20; soybean meal, 20; CaCO₃, 2.5; MgSO₄·7H₂O, 0.4; FeSO₄·7H₂O, 0.01; ZnSO₄·7H₂O, 0.05 and CoCl₂·6H₂O, 0.003. The cultures were grown in a
shaking incubator at 25°C for 84 hours. The production medium was composed of glucose, 120; soybean meal, 15; (NH₄)₂SO₄, 5; MgSO₄·7H₂O, 1; KH₂PO₄, 1; CaCO₃, 6; CuSO₄·5H₂O, 0.0033; FeSO₄·7H₂O, 0.01; ZnSO₄·7H₂O, 0.05; MnSO₄·7H₂O, 0.004; CoCl₂·6H₂O, 0.002; (NH₄)₂MoO₄, 0.001 and 5,5-diethylbarbiturate 0.7. This medium was inoculated with 20% of the seed culture and was incubated at 25°C and 250 rpm for 144 hours.

For the determination of specific production, liquid Bennet medium was used as the seed culture and production medium as it was described by LEE et al.⁶).

Assays
Rifamycin B production was measured by reversed-phase high-performance liquid chromatography⁷). The biomass concentration was measured on the basis of its dry cell weight. MILLER’s method was used for measurement of glucose in the fermentation broth.

Mutagenesis
A culture in Bennet medium was incubated for 48 hours at 25°C and 150 rpm. Mycelium was collected by centrifugation, resuspended in water and placed in a Petri dish. It was irradiated under the UV lamp with constant agitation for an appropriate time (40% of survival).

Protoplast Formation and Regeneration
Mycelium was collected from cultures grown 36 hours, 250 rpm at 25°C in CRM medium (glucose 1%, sucrose 10.3%, MgCl·6H₂O 1.012%, soybean tryptone 1.5% and yeast extract 0.5%) supplemented with 2.5% of glycine. The protoplasts were obtained by the method described by SHUPP and DIVERS⁸) and LAL et al.⁹), but we used P buffer⁹) instead of R2L, and the incubation with lysozyme (5 mg/ml) was done at 30°C by 3 hours. Protoplasts were plated on CRM (with agar Difco) and then the plates were overlaid with 3 ml of soft agar (0.5%).

Selection of Sensitive Mutants to Barbital
CRM medium with glycine (2.5%) and barbital (0.4%) was inoculated with mutagenized mycelium. The incubation was done at 25°C and 150 rpm for 48 hours. The culture was treated with lysozyme as it was described previously. The resistant mycelium to lysozyme was washed with diluted detergent (0.01% SDS). CRM medium with glycine and without barbital was inoculated with this washed mycelium. Protoplasts were obtained and the regenerated colonies were seeded on Bennet agar plates with 0.5% barbital and without barbital.

Estimation of Unknown Parameters
The non-linear regression program (Solver, Microsoft Windows 95) fits a mathematical model to experimental data in order to determine a number of unknown parameters.

Models used: \[ \frac{dx}{dt} = \mu(l - x/x_{\text{max}})x ; \]
\[ -\frac{dS}{dt} = \mu dx/dt + mx ; \]
\[ \frac{dP}{dt} = \alpha dx/dt + \beta x , \]
where: \( t \) = fermentation time, \( x \) = biomass, \( P \) = rifamycin production, \( S \) = residual substrate, \( \mu \) = specific growth rate, \( m \) = substrate required for cell maintenance, \( \beta \) = constant of proportionality for a batch process, \( \alpha \) = constant of proportionality for a batch process.

Reproducibility or Results
Experiments were performed in duplicate and repeated at least once. The results are mean values.

Results
Barbital Sensitivity of \textit{A. mediterranei}

The toxic effect of barbital on growth was determined in plates with Bennet medium supplemented with barbital. The data in Table 1 shows that the M18 strain could not grow with 0.5% barbital, and it was more sensitive than the type strain (ATCC 13685).

Growth of the M18 strain was observed when Bennet medium without barbital was inoculated with mycelium from a young culture (96 hours) with 0.75% barbital. In accordance with Table 1, in this concentration the strain could not grow. However, this preincubation did not result in the death of mycelium.

Effect of the Age of Mycelium on Lysozyme Activity

The effect of the age of mycelium on lysozyme activity was studied with a view to develop an efficient procedure to select sensitive mutants to barbital. The results of these experiments are shown in Table 2.

Selection of Resistant Mutants

Thirty mutants were selected for their ability to grow with 0.5% barbital (at a frequency of \( 8 \times 10^{-5} \)). Eighteen of these mutants grew with 0.75% barbital and 9 with 1%. Five, out of 30 isolated mutants, were chosen at random for further characterization.
Table 1. Effect of barbital on the growth of \textit{A. mediterranei} in plates with Bennet medium (The results are expressed in total milligrams of dry weight).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Barbital (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Type (ATCC 13685)</td>
<td>180.2</td>
</tr>
<tr>
<td>M18 (ATCC 21789)</td>
<td>178.6</td>
</tr>
</tbody>
</table>

Table 2. Effect of the age of mycelium on lysozyme activity.

<table>
<thead>
<tr>
<th>Time of growth in liquid CRM + glycine (hour)</th>
<th>Growth on plates with CRM medium (cfu/ml)*</th>
<th>Lysozyme treatment and protoplast count by hemocytometer (protoplast/ml)</th>
<th>Growth on plates with CRM medium after lysozyme treatment (col./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>$1 \times 10^6$</td>
<td>$1.4 \times 10^4$</td>
<td>$1.3 \times 10^2$</td>
</tr>
<tr>
<td>72</td>
<td>$2.3 \times 10^6$</td>
<td>$3.5 \times 10^2$</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>$8 \times 10^6$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>$1.2 \times 10^7$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>144</td>
<td>$1 \times 10^7$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The mycelium of \textit{A. mediterranei} (ATCC21789) was obtained from cultures (CRM with 2.5% glycine) incubated from 48 hours to 144 hours. Then the mycelium was treated with lysozyme. Plates with CRM medium were inoculated before and after lysozyme treatment.

Fig. 1. Time course of rifamycin B production (---) and glucose consumption (--------) in the parental strain (M18) and the mutants resistant to barbital (Mrb).

The time course of rifamycin B production by the parental strain (M18) and five selected mutants is presented in Figure 1. These mutants showed less rifamycin production and glucose consumption than the parental strain.

It is important to mention that the colonies of all resistant mutants (30 isolates) showed less intense color than the parental strain, which indicates that secondary metabolism is altered.

Selection of Sensitive Mutants
Ten mutants were selected and two of them were sensitive to 0.5% barbital. The time course of antibiotic production of these mutants is shown in Figure 2.
Fig. 2. Time course of rifamycin B production (——) and glucose consumption (--------) in the parental strain (M18) and the mutants sensitive to barbital (Msb).

Table 3. Characteristics of rifamycin B production of the parental strain and mutants.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Strain</th>
<th>Yield P/X (mg/g)</th>
<th>Yield P/S (g/g)</th>
<th>$\mu_m$ (h$^{-1}$)</th>
<th>$X_{max}$ (g)</th>
<th>m (g/ml)</th>
<th>$\beta$ (mg g$^{-1}$ l$^{-1}$ h$^{-1}$)</th>
<th>$\alpha$ (mg g$^{-1}$ l$^{-1}$)</th>
<th>Sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>M18</td>
<td>259</td>
<td>9.8</td>
<td>0.053</td>
<td>4.08</td>
<td>0.009</td>
<td>0</td>
<td>5941</td>
<td>9.6 E-02</td>
</tr>
<tr>
<td>BAR$^R$</td>
<td>Mrb2</td>
<td>86</td>
<td>6.4</td>
<td>0.050</td>
<td>4.36</td>
<td>0.001</td>
<td>0</td>
<td>1547</td>
<td>1.6 E-01</td>
</tr>
<tr>
<td></td>
<td>Mrb3</td>
<td>65</td>
<td>5.1</td>
<td>0.060</td>
<td>3.98</td>
<td>0.005</td>
<td>0</td>
<td>1055</td>
<td>8.4 E-02</td>
</tr>
<tr>
<td></td>
<td>Mrb10</td>
<td>45</td>
<td>3.5</td>
<td>0.034</td>
<td>3.98</td>
<td>0.002</td>
<td>0</td>
<td>1356</td>
<td>6.4 E-01</td>
</tr>
<tr>
<td></td>
<td>Mrb11</td>
<td>6</td>
<td>1.0</td>
<td>0.038</td>
<td>3.98</td>
<td>0.002</td>
<td>0</td>
<td>171</td>
<td>6.1 E-01</td>
</tr>
<tr>
<td></td>
<td>Mrb27</td>
<td>56</td>
<td>3.2</td>
<td>0.034</td>
<td>3.98</td>
<td>0.004</td>
<td>0</td>
<td>1739</td>
<td>8.9 E-01</td>
</tr>
<tr>
<td></td>
<td>(mean):</td>
<td>(51.6)</td>
<td>(3.8)</td>
<td>(0.043)</td>
<td>(4.06)</td>
<td>(0.003)</td>
<td>(0)</td>
<td>(1174)</td>
<td>(4.8 E-01)</td>
</tr>
<tr>
<td>BAR$^S$</td>
<td>Msb1</td>
<td>453</td>
<td>20.4</td>
<td>0.059</td>
<td>5.18</td>
<td>0.009</td>
<td>0</td>
<td>7674</td>
<td>2.1 E-01</td>
</tr>
<tr>
<td></td>
<td>Msb2</td>
<td>599</td>
<td>27.0</td>
<td>0.058</td>
<td>5.18</td>
<td>0.007</td>
<td>0</td>
<td>10512</td>
<td>7.8 E-01</td>
</tr>
<tr>
<td></td>
<td>(mean):</td>
<td>(526)</td>
<td>(23.7)</td>
<td>(0.058)</td>
<td>(5.18)</td>
<td>(0.008)</td>
<td>(0)</td>
<td>(9093)</td>
<td>(4.9 E-01)</td>
</tr>
</tbody>
</table>

$\mu_m$ = maximum specific growth rate, $X_{max}$ = maximum biomass, m = substrate required for cell maintenance, $\beta$ = constant of proportionality for a batch process, $\alpha$ = constant of proportionality for a batch process.

Both sensitive mutants (Msb1 and Msb2) produced more rifamycin than the parental strain (M18) and consumed similar amounts of glucose.

Table 3 gives a comparison in specific production ($Y_{P,X}$), of the different strains. The resistant mutants to barbital (BAR$^R$) reduced their $Y_{P,X}$ mean value (50 mg/g) to less than half of the $Y_{P,X}$ value (315 mg/g) of the parental strain, and 10 times less than sensitive mutants (525 mg/g). Also the mean $Y_{P,X}$ value for the two BAR$^S$ mutants was 67% higher than the $Y_{P,X}$ of the parental strain. The values of substrate yields ($Y_{P,S}$) changed in a similar way to $Y_{P,X}$. They were the lowest (3.8 mg/g) for BAR$^R$ strains, the highest (47 mg/g) for BAR$^S$ strains and intermediate for the parental strain.

Biomass production measured as $Y_{X,S}$ was very similar for all strains except for Mrb11, although BAR$^R$ strains had slightly higher mean value (0.09 mg/g) than the parental strain (0.04 mg/g).

BAR$^S$ strains had lower substrate yields ($Y_{P,S}$) and very low productivity ($Y_{P,X}$). Whereas, sensitive mutants nearly doubled the $Y_{P,X}$ and $Y_{P,S}$ values as compared to the parental M18 strain.

The specific growth rate ($\mu$) of BAR$^S$ strains was slightly higher than $\mu$ of the parental strain. This is a very good trait, because these strains had similar production times than the parental strain.
Biomass level ($X_{\text{max}}$) was higher for BAR$^S$ strains and lower for BAR$^R$ strains than the parental strain. This observation is in agreement with the pattern of $\mu$ values mention above.

Finally, it is important to mention that the concentration of rifamycin W and V (indicators of the more than 20 rifamycins) produced by the mutants or the parental strain was very low (5 $\mu$g/ml or less), practically undetectable by the analytic method used (HPLC).

Production curves were followed by the Model described in Materials and Methods. In all cases $\beta$ value was approximately zero and $\alpha$ coefficient positive. This correspond to production model closely associated to bacterial growth. Mean $\alpha$ coefficient of BAR$^S$ strains (9.035 mg h g$^{-1}$ l$^{-1}$) was clearly higher than $\alpha$ coefficient of BAR$^R$ strains (1.174 mg h g$^{-1}$ l$^{-1}$) and also higher than the parental strain (5.941 mg h g$^{-1}$ l$^{-1}$).

These experiments were done in a medium without insoluble solids, because we were interested in specific antibiotic production. However, it is interesting to note that the mutant Msb2 was able to produce up to 6 mg/ml of rifamycin B when in an industrial type medium (unpublished results).

**Discussion**

As mentioned in the Introduction, barbital at sublethal concentrations has a positive effect on rifamycin B biosynthesis. In addition, the presence of high concentrations of this compound has a toxic effect on the growth of *A. mediterranei*, which was demonstrated by Margalith and Pagni$^5$ in 1961. However, the relationship between barbital sensitivity and high rifamycin production had not been studied previously.

Our results show that all (30) the isolated barbital-resistant mutants produced less rifamycin than the parental strain. On the other hand, the two sensitive mutants isolated showed an important increase in rifamycin B production (around 200%). This suggests that there is a relationship between the sensitivity to barbital and the overproduction of rifamycin B. This idea is further supported by the fact that the parental strain (M18) and the type strain (ATCC 13685) showed the same pattern: the M18 strain was more sensitive to barbital and higher rifamycin B producer (1200 $\mu$g/ml) than the type strain (350 $\mu$g/ml).

Another interesting finding was the observation that the specific growth rate of sensitive mutants was greater than the parental strain in fermentation (Bennet) medium. This was an unexpected result, but when we compared M18 against the type strain, the growth of M18 was greater. In other words, the higher producing strains were more sensitive to barbital and they grew faster in the fermentation medium.

With respect to the selection of sensitive mutants, the classical enrichment methods involve the use of penicillin or successive filtering. However these techniques were not available for this organism. This was mainly due to the fact that *A. mediterranei* is resistant to penicillins and these strains do not produce spores. These characteristics of the organism pose a problem when we wanted to isolate a recessive mutant. The random selection has been the only technique available so far and it is a very long procedure.

Our results indicate that barbital does not provoke the death of mycelium when it is incubated for 96 hours. This means that barbital can be used in the enrichment technique. On the other hand, the older mycelium (72 hours) is resistant to lysozyme activity. With these data, we developed an enrichment technique. This is based on the hypothesis that sensitive mycelium can not grow in a medium with barbital, and after incubation for 48 hours it will be resistant to lysozyme activity. In accordance with the hypothesis, we isolated two mutants sensitive to barbital. One of these mutants showed 270% greater yield, higher productivity (207%), and was able to produce 230% more rifamycin B than the parental strain. These results show that selection of barbital sensitive mutants is an effective way to improve the production of rifamycin B by *A. mediterranei*.

All these results support the use of BAR$^S$ phenotype as a good selection criterion. This is, to our knowledge, the first time that the selection of BAR$^S$ phenotype is suggested as a practical way to improve production of this antibiotic.

Future work should focus on identifying the genes responsible for barbital sensitivity in order to examine whether barbital sensitivity is related to rifamycin production.

Finally, it is important to mention that barbital gives a marked stimulatory response on production of other secondary metabolites. Addition of 8 mM solution of this compound to the culture medium increased the yield of streptomycin$^{10}$ by 29%. Also in a different system, 22 mM barbital stimulated yields of galirubins$^{11}$ as much as 285%. Therefore, we think it is interesting to isolate sensitive and resistant mutants to barbital of *Streptomyces griseus* and *S. galilaeus*. 
Acknowledgments

The authors wish to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) for financial support (Grant 2115-30902). Thanks are also due to Dr. Sergio Sánchez and Dr. Edgaro Escamilla for helpful advice.

References