Novel Assay for Screening Rifamycin B-Producing Mutants†

RAKESH M. VOHRA

Institute of Microbial Technology, Post Box no. 1304, Sector 39A, Chandigarh 160 014, India

Received 24 June 1991/Accepted 25 October 1991

A simple method that allows easy identification of rifamycin B-producing strains is described. This method involves the use of an enzyme, rifamycin oxidase, which converts inactive rifamycin B to active rifamycin S. In this method, colonies to be tested are grown in pairs. The two colonies are then transferred to two plates seeded with a sensitive strain of Staphylococcus aureus, one plate of which contains the enzyme rifamycin oxidase. All paired colonies which show a larger inhibition zone diameter on the enzyme-containing plate are identified as rifamycin B producers.

Rifamycins belong to the ansamycin group of antibiotics. Their derivatives have been used successfully over the years for the treatment of tuberculosis and leprosy (1, 5, 8). The precursor for these clinically useful drugs is rifamycin B, a fermentative product of Nocardia mediterranei (7). This organism has been known to produce a battery of other rifamycins, some of which though produced in lower quantities have greater antibiotic activity (3). This characteristic of the organism poses a problem when classical mutation methods are applied for screening overproducing mutants. Many mutants produce rifamycins other than or in addition to rifamycin B and, when a single test organism is used for screening, the identification of a true B producer becomes difficult. The reported methods for identification of rifamycin B-producing organisms involve growing randomly selected mutants in shake flasks and then running a thin-layer chromatogram of their extracted broth for identification of the various rifamycins (2, 3, 6). The procedure is time-consuming as well as labor-intensive. Therefore, a simple test procedure which definitively identifies rifamycin B producers is desirable. This paper describes such a procedure.

We have isolated a strain of Curvularia lunata var. aeria which produces an extracellular enzyme, rifamycin oxidase (9). This enzyme converts rifamycin B to rifamycin S. This study shows the application of this enzyme in selective screening for rifamycin B-producing mutants in a background of other mutants which produce rifamycins with greater antibacterial activity but are of little interest for fermentative production of rifamycin B. Different strains of N. mediterranei, viz., ATCC 13685, ATCC 21789, ATCC 21271, an industrial strain (R-8), and UV-induced mutants of R-8 developed in our laboratory, were used in this study. The strains were grown on Bennett’s agar (1 g of yeast extract, 1 g of beef extract, 2 g of NZAmine, 10 g of glucose, and 15 g of agar per liter [pH 7.3]). The enzyme rifamycin oxidase was prepared from Curvularia lunata var. aeria grown on YPD medium (10 g of yeast extract, 10 g of peptone, and 10 g of glucose per liter [pH 6.5]) as described previously (9).

The organisms to be tested were picked from the master plates with toothpicks and seeded on Bennett’s agar in duplicate. The agar plates were incubated at 30°C for 5 to 6 days to allow for production of the antibiotic. The paired colonies were then punched out with a stainless steel cylin-

der. One colony of each pair was put onto a nutrient agar plate seeded with the sensitive strain of Staphylococcus aureus NCTC 6571 (1% of a 12-h-old culture in nutrient broth). The other colony of the pair was placed onto a similar plate treated with a filter-sterilized solution of the enzyme rifamycin oxidase. Quantitatively, a 0.5-mL solution of the enzyme (10 U/mL in 0.1 M phosphate buffer, pH 7.0) is spread on a plate (90 mm diameter) containing 20 mL of nutrient agar. The two plates were incubated at 4°C for an hour to allow for the diffusion of the antibiotics from the colony plugs. After incubation at 37°C for 16 to 18 h, the growth inhibition zone diameters of the paired colonies were measured and compared.

The data in Table 1 show that in the cases of strains ATCC 21789 and ATCC 13685, both rifamycin B producers, the inhibition zone diameters in the rifamycin oxidase-containing plates were larger by 35 and 20% respectively, than those in the control plates. In contrast, in the case of strain ATCC 21271, which is a rifamycin SV producer (4), the inhibition zone diameters were the same in control and rifamycin oxidase-containing plates. Similar experiments were done by using the industrial strain R-8 and three UV-induced random mutants derived from R-8. We found that the R-8 strain and the rifamycin B-producing mutant number 3 showed larger inhibition zone diameters, whereas the rifamycin SV-producing mutants number 1 and number 2, which produced an uncharacterized rifamycin derivative, showed no differential response in the two types of plates. The nature of the rifamycins produced by all the strains in

<table>
<thead>
<tr>
<th>N. mediterranei strain</th>
<th>Type of rifamycin produced</th>
<th>Inhibition zone diam² (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>ATCC 21789</td>
<td>B (2.0)</td>
<td>16.3 ± 0.2</td>
</tr>
<tr>
<td>ATCC 13685</td>
<td>B (1.0)</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>ATCC 21271</td>
<td>SV (1.2)</td>
<td>25.0 ± 0.7</td>
</tr>
<tr>
<td>R-8</td>
<td>B (3.5)</td>
<td>31.2 ± 0.7</td>
</tr>
<tr>
<td>Mutant no. 1</td>
<td>SV (1.6)</td>
<td>31.7 ± 0.8</td>
</tr>
<tr>
<td>Mutant no. 2</td>
<td>Unknown</td>
<td>29.0 ± 0.0</td>
</tr>
<tr>
<td>Mutant no. 3</td>
<td>B (2.5)</td>
<td>26.1 ± 0.2</td>
</tr>
</tbody>
</table>

* Production carried out in shake flasks. Values in parentheses indicate the amount produced (in grams per liter).
† Results represent the mean of three experiments.
‡ Treated with rifamycin oxidase.

† This is communication number IMTECH 005/91 from the Institute of Microbial Technology, Chandigarh, India.
shake flasks was determined by high-performance liquid chromatography analysis (10) of the fermented broths.

These results indicate that the plate assay reported in this paper will provide a rapid, simple, and unequivocal method for screening large numbers of colonies for rifamycin B-producing strains. The identification of unusual rifamycin producers in a background of predominantly B-producing strains is also made possible by this method.

I am grateful to S. K. Basu for suggesting the idea and for critical evaluation of the manuscript.

REFERENCES