Relationship between $\beta$-Lactamase Activity and Resistance to $\beta$-Lactam Antibiotics in *Mycobacterium smegmatis*

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(Accepted for publication, May 22, 1985)

**Abstract**  Penicillin-susceptible mutants and $\beta$-lactamase-negative mutants were isolated from *Mycobacterium smegmatis* after nitrosoguanidine mutagenesis. Both the mutants were found to be susceptible to low levels of penicillin and cephalosporins by twofold dilution testing. Clavulanic acid reduced the minimal inhibitory concentrations of $\beta$-lactamase-labile $\beta$-lactams for the penicillin-susceptible mutants and the parent strain, but had no effect on the susceptibility of the $\beta$-lactamase-negative mutants. Comparison of the $\beta$-lactamase activities found in these mutants and the parent strain indicated that there was a rough correlation between the $\beta$-lactamase level in these organisms and their susceptibility to $\beta$-lactams.

*Mycobacterium smegmatis*, as well as other species of mycobacteria including *M. tuberculosis*, has been shown to have a constitutive $\beta$-lactamase (1, 4, 7, 9). However, little is known about the precise role of this enzyme in the resistance of these organisms to $\beta$-lactams, although it has been reported that there is a general correlation between $\beta$-lactam susceptibility and $\beta$-lactamase activity in many other species of bacteria. Other members of the Actinomycetes family (*Nocardia* and *Streptomyces*), which are usually resistant to $\beta$-lactam agents, are known to produce $\beta$-lactamase. In many *Streptomyces* strains there is no linear relationship between $\beta$-lactamase production and the minimal inhibitory concentration (MIC) of benzylpenicillin (10). In *Nocardia* a poor correlation was found between $\beta$-lactamase production and susceptibility to $\beta$-lactams (14). It has been shown that mechanisms of resistance other than or in addition to $\beta$-lactamase production may be responsible for resistance of *Nocardia* to $\beta$-lactams (14). In the previous paper (16), we suggested the possibility that characteristics other than $\beta$-lactamase production may be involved in a cefmetazole-resistant mutant of *M. smegmatis*.

One approach in studying the role of $\beta$-lactamase in resistance is to work with systems in which the enzyme plays no part. This can be achieved by the use of either $\beta$-lactamase-stable $\beta$-lactams or compounds which inhibit the enzyme activity. Cefmetazole, a $\beta$-lactamase-stable cephalosporin, has been reported to show potent antimicrobial activity against *M. smegmatis* (5). Recently, Cynamon and Palmer demonstrated that $\beta$-lactamase-labile $\beta$-lactams given with clavulanic acid, a $\beta$-lactamase inhibitor with weak antimicrobial activity, has synergistic activity...
against *M. fortuitum* (2) and *M. tuberculosis* (3). These findings provide indirect evidence that β-lactamase has a role in determining the *in vitro* response of these organisms to β-lactam agents. A more direct approach to the study of the resistance to β-lactams would be to isolate mutants not forming β-lactamase. Such mutants can be expected to have high susceptibility to β-lactams. In the present study, we isolated a series of penicillin-susceptible mutants with different degrees of susceptibility to penicillin, and β-lactamase-negative mutants of *M. smegmatis* and have attempted to relate the presence and activity of β-lactamase to the patterns of susceptibility to β-lactam agents. In addition, the effect of clavulanic acid on the susceptibility of these mutants to β-lactamase-labile β-lactams was studied.

**MATERIALS AND METHODS**

*Organisms.* Three penicillin-susceptible mutants and two β-lactamase-negative mutants of *M. smegmatis* ATCC607 were isolated. Selection of the penicillin-susceptible mutants has been described (16). The β-lactamase-negative mutants were isolated as follows. Exponentially growing cultures of *M. smegmatis* were treated with 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml as described previously (15). The surviving cells were suspended in saline containing 0.05 % Tween 80 and spread on plates containing Sauton's medium (5) solidified with 1.5 % agar. After incubation at 37°C for 3 days, individual colonies were transferred to duplicate plates for selection. The method of Sherratt and Collins (13) was used for detecting β-lactamase activity on plates, except that the plates were flooded with iodine solution after the reaction of β-lactamase with penicillin. Polyvinyl alcohol (0.75 %) was added to plates containing the test colonies. The plates were inspected daily. Clear halos were seen around the colonies of the parent strain. Colonies which failed to form a halo on the test plate were selected from the replica plate and cultured for further testing.

*Susceptibility test.* A modified Dubos liquid medium (15) was used for growth and determination of the MIC. The MICs of β-lactam agents were determined by twofold serial dilution in the modified Dubos liquid medium. Exponentially growing bacteria (2 to 6 x 10⁴ colony-forming units/ml) were inoculated into tubes containing 1 ml of the medium and incubated at 37°C for a week. The lowest concentration of antibiotic that inhibited visible growth was taken as the MIC.

*Preparation of cell-free extracts.* Samples for β-lactamase assays were prepared from exponential-phase cells grown on the Sauton medium as previously described (5). The harvested cells were suspended in 10 ml of 0.05 M Tris-HCl buffer (pH 7.5) and sonicated at 20 kHz for 10 min with 5-min cooling intervals every 3.3 min. Cellular debris was removed by centrifugation at 18,000 x g for 30 min at 0°C. The protein content of the supernatants was determined by the method of Lowry et al (8).

*Measurement of β-lactam hydrolysis.* The specific β-lactamase activity of the cell-free extracts against β-lactam substrates was measured by the iodometric assay of Sawai et al (12). The reaction mixture consisted of 1.45 ml of 0.1 M phosphate
buffer (pH 7.0) containing 10 μmol of β-lactam antibiotic and 50 μl of enzyme solution (200 to 500 μg of protein).

RESULTS

*M. smegmatis* and its mutants were tested for their susceptibility to benzylpenicillin, cefazolin, cephaloridine, cephalothin, and cefuroxime. The MICs of these agents are shown in Table 1. The parent strain was inhibited only at extremely high concentrations of all the β-lactams. A significant increase in susceptibility to each β-lactam was observed in all of the penicillin-susceptible mutants (A29, H11, K11). As expected, the acquisition of susceptibility to benzylpenicillin was accompanied by increased susceptibility to other β-lactams. The β-lactamase-negative mutants (M1, M4) were also susceptible to all the agents, with a level of susceptibility similar to that of the penicillin-susceptible mutants.

Cell-free extracts from the mutants and the parent strain described above were examined for β-lactamase activity by the iodometric assay (12). The specific

<table>
<thead>
<tr>
<th>Strain</th>
<th>Benzylpenicillin</th>
<th>Cefazolin</th>
<th>Cephaloridine</th>
<th>Cephalothin</th>
<th>Cefuroxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent strain</td>
<td>ATCC607</td>
<td>512</td>
<td>4,096</td>
<td>200</td>
<td>2,048</td>
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<tr>
<td>Penicillin-susceptible mutant</td>
<td>A29</td>
<td>128</td>
<td>512</td>
<td>20</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>H11</td>
<td>8</td>
<td>128</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>K11</td>
<td>16</td>
<td>512</td>
<td>20</td>
<td>256</td>
</tr>
<tr>
<td>β-Lactamase-negative mutant</td>
<td>M1</td>
<td>16</td>
<td>128</td>
<td>2.5</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>8</td>
<td>128</td>
<td>5</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 2. β-Lactamase activity of cell-free extracts from *M. smegmatis* and its mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Benzylpenicillin</th>
<th>Cefazolin</th>
<th>Cephaloridine</th>
<th>Cephalothin</th>
<th>Cefuroxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent strain</td>
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<td>40.0</td>
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<td>13.0</td>
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<tr>
<td>Penicillin-susceptible mutant</td>
<td>A29</td>
<td>5.3</td>
<td>19.9</td>
<td>14.2</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>H11</td>
<td>6.7</td>
<td>21.4</td>
<td>16.4</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>K11</td>
<td>5.5</td>
<td>21.4</td>
<td>16.0</td>
<td>7.0</td>
</tr>
<tr>
<td>β-Lactamase-negative mutant</td>
<td>M1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>0.7</td>
<td>1.2</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>
hydrolysis rates of the five β-lactams are shown in Table 2. The parent strain produced large quantities of β-lactamase; however, the specific activity of the enzyme against cefuroxime was low. All the penicillin-susceptible mutants produced an enzyme with activity against all substrates half that of the parent strain. No obvious differences in substrate profile seemed to exist between the enzymes from these mutants and the parent strain. These data suggest that the penicillin-susceptible mutants produce decreased amounts of β-lactamase and that this enzyme does not appear to be qualitatively different from that of the parent strain. The cell-free extracts from the β-lactamase-negative mutants had little or no detectable hydrolytic activity against cefuroxime, and the overall enzymatic activity of these mutants was fairly low.

All of the penicillin-susceptible mutants, one of the β-lactamase-negative mutants (M1), and the parent strain were tested for susceptibility to twofold dilutions of benzylpenicillin, cephazolin, or cefuroxime alone and in combination with 0.25, 1, and 4 µg of clavulanic acid per ml by the serial dilution method. Figure 1 shows the changes in the MICs of three different β-lactams when given with clavulanic acid. For the penicillin-susceptible mutants (A29, H11, K11) and the parent strain, the MICs of benzylpenicillin and cefazolin were reduced fourfold or more in the presence of 1 and 4 µg of clavulanic acid per ml, although clavulanic acid at these concentrations had no antimicrobial activity against any of these

![Diagram](image-url)
organisms. However, their susceptibility to cefuroxime was only slightly, if at all, reduced in contrast with the profound changes with cefazolin. For the \( \beta \)-lactamase-negative mutant, the addition of clavulanic acid brought no change in the susceptibility to any \( \beta \)-lactam agents.

**DISCUSSION**

*M. smegmatis*, *M. fortuitum*, and *M. tuberculosis* produce a constitutive \( \beta \)-lactamase which is active against penicillins and cephalosporins (4, 5, 7, 9). These species of mycobacteria are resistant to all of the \( \beta \)-lactamase-labile \( \beta \)-lactams. In the present study, two types of mutants were selected from *M. smegmatis* according to their susceptibility to benzylpenicillin or lack of extracellular \( \beta \)-lactamase. One was the penicillin-susceptible mutant which was susceptible to all the \( \beta \)-lactams tested and had a reduced level of \( \beta \)-lactamase. The other was the \( \beta \)-lactamase-negative mutant which produced little or no extracellular \( \beta \)-lactamase, and consequently was highly susceptible to \( \beta \)-lactams. The results obtained with these mutants agree with those reported by other workers in that certain nonpigmented, slow-growing mycobacteria, *M. avium-intracellulare* complex, do not have a constitutive \( \beta \)-lactamase and are more susceptible than *M. tuberculosis* to \( \beta \)-lactams (6).

The addition of clavulanic acid to \( \beta \)-lactams had interesting effects. A marked decrease in the MICs of benzylpenicillin and cefazolin was seen particularly for the penicillin-susceptible mutants (Fig. 1A and B). The effect of clavulanic acid was also seen with aminobenzylpenicillin, cephaloridine, and cephalothin (data not shown). This phenomenon cannot be attributed solely to an additive effect between the two agents, since clavulanic acid at the concentrations used showed no antimicrobial activity against these mutants and the parent strain. In contrast, clavulanic acid did not reduce the MICs of these agents for the \( \beta \)-lactamase-negative mutant. It seems that the effect of clavulanic acid is related to the amount of \( \beta \)-lactamase found but within certain limits.

In the study with cefuroxime and clavulanic acid, the MIC of cefuroxime was reduced to a small extent in the penicillin-susceptible mutants but no effect on the susceptibility of the parent strain was observed (Fig. 1C). These findings are probably related to the relatively high stability of cefuroxime to hydrolysis by the \( \beta \)-lactamase found in these organisms.

These observations suggest that the mechanism for the increased activity of the \( \beta \)-lactamase-labile \( \beta \)-lactams with the addition of clavulanic acid is likely due to the inhibition of \( \beta \)-lactamase activity of *M. smegmatis* by clavulanic acid. Cynamon and Palmer (3) previously reported that the addition of clavulanic acid increases the susceptibility to amoxicillin and they suggested that the \( \beta \)-lactamase of *M. tuberculosis* probably plays a role in determining the *in vitro* response to \( \beta \)-lactamase-labile \( \beta \)-lactams.

The parent strain showed a high MIC of cefuroxime despite the relatively high stability of this agent to \( \beta \)-lactamase. This fact does not support the view that the presence of constitutive \( \beta \)-lactamase is the sole explanation for the \( \beta \)-lactam
resistance in mycobacteria. Our results suggest that the mechanism of antimicrobial resistance in addition to β-lactamase production may be responsible for resistance of *M. smegmatis* to cefuroxime, although the lack of effect of clavulanic acid on its susceptibility to cefuroxime requires further investigations. Two mechanisms which are frequently proposed to explain non-β-lactamase-mediated resistance are increased intrinsic resistance and decreased permeability. The previous work with the cefmetazole-resistant mutant of *M. smegmatis* has provided evidence for another mechanism that is neither β-lactamase-mediated nor associated with penicillin-binding proteins (16). It has been reported that drug resistance in *M. avium* is probably due to a permeability barrier at the cell wall level (11).

In summary, the β-lactamase of *M. smegmatis* seems to play an important role in the susceptibility of this species to β-lactam agents, and factors other than, or in addition to, β-lactamase may be responsible for resistance to β-lactam agents, but further studies on particular characteristics of β-lactamase, permeability, and penicillin-binding proteins are needed before a conclusion can be drawn.

REFERENCES


(Received for publication, February 27, 1985)