Altered Permeability and \( \beta \)-Lactam Resistance in a Mutant of \textit{Mycobacterium smegmatis}

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Beta-lactam resistance in mycobacteria results from an interplay between the following: (i) beta-lactamase production, (ii) affinity of the penicillin-binding proteins (PBPs) for the drugs, and (iii) permeation of the drugs. A laboratory mutant of \textit{Mycobacterium smegmatis} was studied in order to evaluate the roles of these factors in beta-lactam resistance. Mutant M13 was between 7- and 78-fold more resistant than the wild type to cephaloridine, cefoxitin, cefazolin, cefamandole, and cephalothin. Increased beta-lactamase activity toward these antibiotics was not observed in the mutant. The PBP profiles of the wild type and M13 were comparable. However, the affinities of PBP 1 for the beta-lactams tested were lower for the mutant than for the wild type. The permeation of the drugs measured in intact cells was lower for M13 than for the parent strain. The liposome swelling technique, which could be used for cephaloridine, also supported this view. Reduced permeation was not restricted to the beta-lactams alone. Glycine uptake was also lower in M13. Taken together, the results suggest that decreased affinities of PBP 1 for beta-lactams, combined with the decreased permeability of the cell wall of the mutant, lead to the development of high-level acquired beta-lactam resistance.

The genus \textit{Mycobacterium} includes a number of pathogens that cause tuberculosis (\textit{M. tuberculosis}, \textit{M. africanum}, and \textit{M. bovis}), leprosy (\textit{M. leprae}), and diseases affecting immunocompromised subjects (e.g., AIDS patients) and involving opportunistic pathogens such as \textit{M. avium} and \textit{M. fortuitum}. The treatment of mycobacterial infections has been complicated by the emergence of multidrug-resistant strains (2, 6, 9) and by the fact that only a limited number of antimicrobial agents are available for use, because mycobacteria are naturally resistant to a wide range of antibiotics (4, 8, 11). This has prompted reevaluation of the potential of existing chemotherapeutic agents, including the beta-lactam antibiotics (21, 23, 25). The resistance of mycobacteria to beta-lactams has been attributed to an interplay between the following factors: (i) permeability of these drugs, (ii) beta-lactamase production, and (iii) affinity of the penicillin-binding proteins (PBPs) for the drugs. The low level of mycobacterial cell wall permeability is attributed to the unusually high hydrophobicity of the cell wall, which contains large amounts of C\textsubscript{60} to C\textsubscript{90} fatty acids, the mycolic acids, which are covalently attached to arabinogalactan, which in turn is linked to the underlying peptidoglycan (3). Hydrophilic solutes traverse the mycobacterial cell wall, presumably through channels of recently discovered porins (18, 20). Beta-lactam antibiotics have been demonstrated to permeate through porin channels in \textit{M. smegmatis} (19). The present study describes a laboratory mutant of \textit{M. smegmatis} in which the enhanced resistance may be attributed to an interplay between decreased cell wall permeability and decreased affinities of PBP 1 for beta-lactams rather than the involvement of enhanced beta-lactamase activity.

**MATERIALS AND METHODS**

**Antibiotics and chemicals.** \[^{3}H\]Benzylenepicillin (18 Ci/mmoll) was purchased from Amersham, Buckinghamshire, United Kingdom; cefamandole, cephalothin, cefazolin, cephaloridine, cefotaxim, and benzylpenicillin were from Sigma Chemical Co., St. Louis, Mo. All other reagents used were of analytical grade.

**Bacterial strains.** \textit{M. smegmatis} SN\textsubscript{W} was obtained from the Indian Institute of Science, Bangalore, India. Mutant M13 was obtained from the parental strain by nitrosourea-induced mutagenesis and selection on cefoxitin plates. Briefly, the wild-type cells were pelleted from exponentially growing cultures, washed in 0.1 M sodium citrate buffer (pH 5.5) containing 1% (vol/vol) Tween 80, and suspended at an \( A_{600} \) of 1.0 in 0.05 M sodium phosphate buffer (pH 7) containing 100 mg of nitrosoguanidine per ml and 1% Tween 80. After incubation for 30 min at 37°C, the cells were washed in the same buffer without nitrosoguanidine. For the selection of cefoxitin-resistant mutants, the cells were spread onto Muller-Hinton agar plates containing cefoxitin (8 \mu g/ml). Colonies growing on cefoxitin plates were then adapted to twofold serially increasing concentrations of cefoxitin in Middlebrook 7H10 medium supplemented with Tween 80 (0.05%).

**Growth media.** Strains were routinely grown in the medium described by Youman and Karlson (24). The MICs of the antibiotics were determined by using twofold serial dilutions of the antibiotics on Muller-Hinton agar plates. A total of 10\textsuperscript{9} or 10\textsuperscript{8} CFU was spotted onto agar plates supplemented with the antibiotics, and the MICs were read visually after 48 h of incubation at 37°C. The concentration of antibiotic at which no visible growth occurred was taken as the MIC of that particular antibiotic.

**Preparation of bacterial membranes.** Membranes were prepared as described by Basu et al. (1). Briefly, the cell homogenate was centrifuged at 1,500 \times g for 10 min, and the supernatant was collected and centrifuged at 45,000 \times g for 30 min. The pellet was suspended in 10 mM Tris-HCl (pH 7.5) at a concentration of 10 to 20 mg/ml. Protein content was measured with the Pierce biocinchoninic acid protein assay reagent.

**Cell wall preparation.** Cell wall extracts were prepared as described by Trias and Benz (18). Briefly, the cell homogenate was centrifuged at 1,500 \times g for 10 min, and the supernatant was collected and centrifuged at 45,000 \times g for 30 min. The pellet was suspended in a small volume of 20 mM Tris-HCl (pH 8) and was applied to a stepwise sucrose gradient of 30, 40, and 70% (wt/vol) sucrose. It was centrifuged overnight at 100,000 \times g. The fraction containing the cell wall sedimented in the gradient between 40 and 70% sucrose. It was pelleted, washed, and stored at \(-20^\circ\mathrm{C} \). Analysis of PBPs and competition assays. Analysis of PBPs and competition assays were done as described by Basu et al. (1) with \[^{3}H\]Benzylenepicillin. To inhibit the beta-lactamase activity, membranes were first incubated with 2 \times 10\textsuperscript{-10} M \textit{β}-lactampenicillanic acid for 20 min at 30°C. Samples (100 \mu g) of membrane proteins were incubated with \[^{3}H\]Benzylenepicillin (10 \times 10\textsuperscript{-10} M; 5 \mu Ci/\mu mol) for 20 min at 30°C. The reaction was stopped by the addition of excess nonradioactive penicillin and sodium lauroyl sarcosinate (1%). After allowing the samples to stand at room temperature for 20 min, sodium deoxycholate (SDS)-gel denaturing buffer was added and the mixture was boiled immediately for 3 min. Samples were applied on 10% polyacrylamide-SDS gels, followed by fluorography. In competition experiments, membranes were incubated with different concentrations of the competing nonradioactive beta-lactam for 20 min at 30°C prior to incubation with \[^{3}H\]Benzylenepicillin as described above. For determination of...
50% inhibitory concentrations (IC_{50}), the intensities of the bands were analyzed by densitometric scanning of the fluorograms with Kodak X-Omat AR film. Lane a, control; lanes b, c, d, and e, supernatants of the cells grown with subinhibitory concentrations of the drugs. Band intensities were measured with a densitometer. The relative levels of the bands were calculated from each gel by normalizing the intensity of the band with respect to the densitometric area of the control sample (i.e., the ratio of the integrated area under the densitometric peak to the integrated area of the control peak). The increase in optical density due to cell aggregation during the measurement, was determined from the increase in cell size, and the V_{max} obtained for the cell suspension was used to determine the V_{max} of the beta-lactamase in the cell suspension.

Beta-lactam permeation assays in intact cells. The permeability coefficients of beta-lactam antibiotics were determined by a modification of the method of Jarlier and Nikaido (11) as described by Trias and Benz (19). M. smegmatis SN_{2} synthesized a beta-lactamase that was not secreted in the medium. Its expression was constitutive. Rates of hydrolysis of cephalosporins were determined spectrophotometrically at 260 nm for cephaloridine and cephalothin, at 266 nm for cefamandole, and at 273 nm for cefazolin, and the Michaelis constant (K_{m}) was determined by Lineweaver-Burk double-reciprocal plotting of the data. The K_{m} values of the antibiotics ranged from 11 to 120 μm. The rate of hydrolysis of the sonic extract of the cells was used to determine the V_{max} of the beta-lactamase contained in the sonicated cells, and the V_{max} obtained for the cell suspension supernatant was used to correct for any beta-lactamase that had leaked out of the cells. Cells were suspended to a turbidity of 100 Klett units. Hydrolysis by intact cells was measured with 0.2 mM cephalosporin by using a cuvette with a path length of 1 cm. Since some cell aggregation occurred during the measurement, this was corrected by recording the absorption at the isobestic point (i.e., the wavelength for each cephalosporin at which no change in absorption occurs) for each antibiotic. The isobestic points were 242 nm for cephaloridine, 250 nm for cefamandole, and 244 nm for cephalothin and cephalosporin. For each batch of cell suspension, the decreases in the A_{400} at the different isobestic points were determined in the absence of cephalosporin. The ratio, i.e., slope_{260}/slope_{isobestic}, was the correction factor used to correct for the decrease in optical density due to cell aggregation. The actual decrease related to cephalosporin hydrolysis was obtained by the following equation: slope_{calc} = slope_{260} 	imes (slope_{isobestic} 	imes correction factor). A portion of the initial cell suspension was dried and weighed to obtain the exact concentration of cells. The substrate was used at 1 mM for all the antibiotics.

RESULTS AND DISCUSSION

Susceptibility to beta-lactam antibiotics. The susceptibilities of the parent strain and mutant M13 to beta-lactam antibiotics are presented in Table 1. The mutant was between 7- and 78-fold more resistant to the beta-lactams tested. The mutant was stable in its resistance to beta-lactam antibiotics, showing no changes after repeated subcultures. M13 had a slower growth rate compared to that of the wild type. In Middlebrook 7H9 broth supplemented with Tween 80, M13 reached the mid-logarithmic phase after 36 h of growth, whereas the wild type reached the mid-logarithmic phase after 26 h. No apparent changes in colony morphology were observed. The mechanism of cefoxitin resistance could not be explored in the manner used for the other antibiotics because the rate of hydrolysis of this drug by the beta-lactamase was very low. The parent and the mutant showed comparable beta-lactamase activities with reference to the antibiotics studied (Table 1). The V_{max} values of all the antibiotics tested were not very different from each other. The substrate profile was in harmony with those reported by Kaneda and Yabu (12) and Kasik (13) for M. smegmatis or by Jarlier et al. (10) for M. chelonae. However, in contrast to their findings, the present study did not show significant variation in the V_{max} values of these antibiotics. This may be due to the variations in the species (M. chelonae) or strain (M. smegmatis).

PBPs of the parent and the mutant strain. [3H]benzylpenicillin bound to five major PBPs of both the parent and the mutant strain. These had molecular masses of 94, 84, 67, 50, and 43 kDa and were designated PBPs 1, 2, 3, 4, and 5, respectively. No apparent change in the relative quantities of the PBPs of the two strains was observed. Competition assays were performed with the antibiotics cefamandole, cefazolin, cefoxitin, and cephaloridine. A representative photograph of the fluorogram obtained following competition with cefalothin is provided in Fig. 1. The 67-kDa PBP often appeared as a faint band, and it was not possible to determine the IC_{50} of most of the antibiotics. In Escherichia coli, all the antibiotics tested are

TABLE 1. Susceptibility of M. smegmatis SN_{2} and its mutant M13 to beta-lactams

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml)</th>
<th>β-Lactamase activity (V_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>M13</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>32</td>
<td>700</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>62</td>
<td>1,500</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>125</td>
<td>850</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>32</td>
<td>2,500</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>5</td>
<td>150</td>
</tr>
</tbody>
</table>

* Results represent the means ± standard deviations (three determinations).
known to interact with PBP 1. By analogy, it was reasoned that PBP 1 of \textit{M. smegmatis} would be one of the main targets of these antibiotics. It was evident from the IC$_{50}$R (Table 2) that PBP 1 of M13 was more resistant to the beta-lactams tested.

\textbf{Permeability to beta-lactams.} The permeabilities of intact cells were measured (Table 3) by using the antibiotics cephaloridine, cephalothin, cefazolin, and cefamandole, which have different hydrophobicities (5) and charges (19). As reported by others (19), the zwitterionic cephalosporin, cephaloridine permeated through the cell wall 8- to 13-fold faster than the monoanionic compounds cefamandole and cephalothin, in spite of its relatively high hydrophobicity. The rates of penetration of the other beta-lactams were comparable, as observed in the case of \textit{M. tuberculosis} (5). The rate of penetration of beta-lactams into the cells was slower in M13 than in the wild type.

The liposome swelling technique with the cell wall protein extract of the parent and the mutant could be used with reliability only for the zwitterionic compound cephaloridine, since the changes in absorbance in the case of the other antibiotics were too low to be measured accurately. By this assay the permeability of the mutant to cephaloridine was again found to be lower than that of the wild type.

\textbf{Permeation of glycine.} In order to confirm the lower permeability of M13, the uptake of the amino acid glycine was measured. The mutant showed lower permeability, with $V_{\text{max}}$ values for glycine uptake of 0.4 ± 0.01 and 0.1 ± 0.005 nmol/min/mg (dry weight) of cells for the wild type and the mutant M13, respectively. Permeability differences were therefore not restricted to the beta-lactams alone.

\textbf{Concluding comments.} Mycobacterial species are resistant to many antibiotics, including the beta-lactams. It has been proposed that the cell wall slows down the permeation of the beta-lactams in \textit{M. smegmatis}, \textit{M. phlei}, and \textit{M. fortuitum} (11). Resistance to beta-lactams results from a combination of factors, namely, permeability to the drugs, beta-lactamase production, and the affinities of the PBPs for the drugs (5, 7). The present report describes a laboratory mutant of \textit{M. smegmatis} which is 7- to 78-fold more resistant than the parent strain to the beta-lactams tested. The PBPs of the two strains were identical, as seen by SDS-polyacrylamide gel electrophoresis and fluorography after binding of labeled benzylpenicillin to membranes. The affinity of the PBP 1 of M13 for the beta-lactams was lower than that observed in the case of the PBP 1 of the wild type. The cell-associated beta-lactamase activity was similar in both strains with reference to the antibiotics tested. Permeability measurements with intact cells demonstrated that the mutant is less permeable than the wild type to the beta-lactams. Liposome swelling assays also showed that the permeation of cephaloridine was lower in the mutant than in the wild type. The cell wall barrier has earlier been proposed to contribute to resistance in beta-lactamase-producing bacteria. The present findings suggest that in beta-lactamase-producing strains of mycobacteria, alterations in the cell wall permeability can contribute significantly toward the development of resistance by limiting the entry of beta-lactams, resulting in hydrolysis of the antibiotic at a rate faster than it can enter the cell. Moreover, in the case of the fast-growing mycobacterial species, slower half-equilibration times across the cell wall are likely to exert a greater effect on the MICs than in the case of slowly growing strains, in which the long generation times are sufficient for the drug to reach inhibitory concentrations at the target PBPs even with slow penetration rates. This study presents evidence of acquired beta-lactam resistance in mycobacteria involving an interplay between the lowered affinities of at least one high-molecular-mass PBP for cephalosporin, as well as reduced permeability of the cell wall, rather than enhanced beta-lactamase production.

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\textbf{REFERENCES}


