Presence of an Additional Penicillin-Binding Protein in Methicillin-Resistant \textit{Staphylococcus epidermidis}, \textit{Staphylococcus haemolyticus}, \textit{Staphylococcus hominis}, and \textit{Staphylococcus simulans} with a Low Affinity for Methicillin, Cephalothin, and Cefamandole

J. PIERRE, R. WILLIAMSON, M. BORNET, and L. GUTMANN

Laboratoire de Microbiologie Médicale, Université Pierre et Marie Curie, 15, rue de l'Ecole de Médecine, 75270 Paris Cedex 06, and Service de Microbiologie, Hôpital Bichat, 75018 Paris, France, and Glaxo Group Research Limited, Microbial Targets, Middlesex, UB6 0HE, United Kingdom

Received 13 November 1989/Accepted 6 June 1990

The presence of an additional penicillin-binding protein (PBP) was demonstrated in methicillin-resistant strains of \textit{Staphylococcus epidermidis}, \textit{S. haemolyticus}, \textit{S. hominis}, and \textit{S. simulans}. In these four species, the apparent molecular mass of this protein was analogous to that of PBP 2' of methicillin-resistant \textit{S. aureus} SR 1550-9. It exhibited a low affinity for methicillin, cephalothin, and cefamandole; and its synthesis was methicillin inducible. Peptide mapping of this PBP from the four species yielded identical results that were analogous to those obtained with \textit{S. aureus} SR 1550-9. These results suggest that this protein is similar to, if not the same as, PBP 2' of \textit{S. aureus} and that it is involved in methicillin resistance in the four species studied.

It has been demonstrated that methicillin resistance in \textit{Staphylococcus aureus} is due to the presence of an additional penicillin-binding protein (PBP) designated PBP 2' or PBP 2a (5, 11). This protein exhibits low affinity for most penicillins and cephalosporins, resulting in cross-resistance to \beta-lactam antibiotics, even if phenotypic resistance to cephalosporins is not consistently expressed.

The existence of the same type of resistance in coagulase-negative staphylococci has, until recently, remained controversial. The aim of this study was (i) to compare the PBPs and their affinities for methicillin, cefalothin, and cefamandole in methicillin-resistant (MR) and methicillin-susceptible (MS) strains of \textit{S. epidermidis}, \textit{S. haemolyticus}, \textit{S. hominis}, and \textit{S. simulans}, and (ii) to determine whether methicillin resistance in coagulase-negative staphylococci is caused by a mechanism analogous to that described for \textit{S. aureus} and whether a relationship exists between the biochemical mechanism and phenotypic expression.

MATERIALS AND METHODS

Bacterial strains. Among 136 strains of coagulase-negative staphylococci collected from routine specimens and identified by comparing the electrophoretic profiles of total proteins and patterns of PBPs with those of reference strains (10), 10 strains of MS and MR \textit{S. epidermidis}, \textit{S. haemolyticus}, \textit{S. hominis}, and \textit{S. simulans} were studied: one MS strain of each species, two MR strains each of \textit{S. epidermidis} and \textit{S. haemolyticus}, and one MR strain each of \textit{S. hominis} and \textit{S. simulans}. \textit{S. aureus} 209 P, an MS strain, and \textit{S. aureus} SR 1550-9, an MR strain deficient in PBP 2 and provided by K. Murakami (Shionogi Research Laboratories, Osaka, Japan) (8), were used for the comparison of the PBPs in \textit{S. aureus} and coagulase-negative staphylococci.

Antimicrobial agents. The following antibiotics were obtained as powders from the indicated companies: clavulanic acid, Beecham Laboratories, Paris, France; methicillin, Bristol Laboratories, Syracuse, N.Y.; and cefamandole and cephalothin, Eli Lilly & Co., Indianapolis, Ind.

In vitro susceptibility testing. \beta-Lactamase production was tested for each strain by using nitrocefin. MICs of methicillin, cefalothin, and cefamandole were determined after twofold serial dilution of the antibiotics in Mueller-Hinton agar. Plates were inoculated, using a multinjector, with a bacterial suspension of $10^4$ to $10^5$ CFU per spot and incubated at 30°C for 48 h. In addition, MICs were also determined after growth in the presence of methicillin ($10 \mu g/ml$ or at 0.25× the MIC).

Preparation of bacterial membrane fractions. Strains were grown to the late logarithmic phase in 500 ml of tryptic soy broth, with or without methicillin ($10 \mu g/ml$ or at 0.25× the MIC) as an inducer, in a shaking water bath at 30°C. Cells were harvested by centrifugation at 7,000 × g at 4°C for 10 min and washed twice in 10 ml of 50 mM Tris hydrochloride buffer (pH 7.5) containing 145 mM NaCl and 5 mM MgCl2. The cell pellet was suspended in 8 ml of the same buffer containing lysostaphin (50 μg/ml), DNase and RNase (10 μg/ml each), and 5 g of glass beads. Cells were mechanically disrupted in a cell disintegrator (Mickle Laboratories Engineering Co., Gomshau, United Kingdom) at 4°C for 1 h. After removal of the unbroken cells by centrifugation at 5,000 × g for 10 min, the membrane fractions were collected by centrifugation at 100,000 × g for 30 min at 4°C, washed twice, and suspended in 500 μl of 50 mM Tris hydrochloride (pH 7.5) (4).

PBP assays. To inhibit the \beta-lactamase activity, membranes of penicillinase-producing strains were first incubated for 5 min at 30°C with the lowest concentration of clavulanic acid that inhibited all enzymatic activity detectable by nitrocefin (2 to 30 μg/ml).

In direct PBP assays, samples of membrane proteins (about 120 μg) were incubated at 30°C for 10 min with [3H]benzylpenicillin (30 μg/ml). The reaction was stopped by the addition of an excess of nonradioactive penicillin. Pro
teins were solubilized by the addition of sample buffer and were boiled for 2 min (3, 11).

For competition experiments, samples were incubated with the nonradioactive antibiotic, which was tested at various concentrations (1, 10, 100, and 1,000 μg/ml), for 10 min at 30°C, before the addition of [3H]benzylpenicillin. Samples were then processed as described above.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% acrylamide, 0.067% bisacrylamide) (14). Gels were processed for fluorography for the detection of the PBPs (2).

The concentration of each antibiotic required for 50% saturation of the PBPs was estimated by measuring the amounts of labeled PBPs by using scanning densitometry.

Peptide mapping. Peptide mapping of isolated PBP 2’ was performed by using V8 protease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described previously (13). Briefly, after labeling of membrane fractions (100 to 200 μg), proteins were separated by gel electrophoresis as described above; gels were then stained and destained for a short time; and the bands corresponding to PBP 2’ were cut from the gel, washed five times in distilled water, and incubated for 20 min at 4°C in Tris hydrochloride buffer (0.1 M; pH 6.8) containing 2 μg of S. aureus V8 protease per ml. The fragments were placed on the top of a second gel (15% acrylamide, 0.2% bisacrylamide) with a stacking gel from which sodium dodecyl sulfate was omitted and that contained 2 μg of S. aureus V8 protease per ml. This gel was run at 70 V through the stacking gel and at 120 V through the separating gel and stained, destained, and processed for fluorography.

RESULTS

In vitro susceptibility studies. All MR strains exhibited β-lactamase activity, as did the two MS strains of S. haemolyticus and S. simulans. For MS strains, the MICs of methicillin, cephalothin, and cefamandole were below 4, 0.25, and 0.5 μg/ml, respectively (data not shown). Among the MR strains (Table 1), the level of resistance was variable. For strains S. epidermidis 44751, S. haemolyticus 52462, and S. simulans 47553, MICs of methicillin were equal to or greater than 128 μg/ml; these strains are referred to as high-level resistant strains. For the three other strains, which are designated low-level resistant strains, the MICs of methicillin did not exceed 32 μg/ml. For MR strains, MICs of the three antibiotics remained identical after the strains were grown in the presence of methicillin.

PBP patterns of MR and MS strains. A specific PBP profile was demonstrated for each species (Fig. 1 and 2) and was identical for MS and MR strains, except for one additional band that was present only in all MR strains (see, in particular, Fig. 1, lanes E and F, and Fig. 2, lanes F and G). The corresponding protein, which specifically bound [3H]benzylpenicillin, migrated at a position identical to that of PBP 2’ of S. aureus SR 1550-9. In our gel system, PBP 2’ in noninduced cells of MR coagulase-negative staphylococci could not always be easily separated from another PBP of approximately 78 kilodaltons that was also present in the susceptible strains. The additional PBP observed in MR strains is referred to PBP 2’, by analogy with that of S. aureus. After growth in the presence of 0.25× the MIC of methicillin, no such inducible protein was found in the MS strains.

The synthesis of this PBP was induced by growing the bacteria in the presence of methicillin. As has been observed previously (7), this was better seen on Coomassie blue-stained gels (Fig. 3 and 4), on which this protein could be located by using its molecular mass (ca. 78 kilodaltons) and its comigration with a protein of the control strain S. aureus SR 1550-9 that was perfectly superimposable with PBP 2’ on the fluorogram. Moreover, the amount of protein produced depended on the concentration of the inducer, as documented in Fig. 3, lanes F, G, and H.

Affinity of PBP 2’ of S. epidermidis, S. haemolyticus, S. hominis, and S. simulans for methicillin, cephalothin, and cefamandole. To determine the affinity of the PBPs for β-lactam antibiotics, competition experiments were performed using labeled methicillin, cephalothin, and cefamandole at concentrations of 128 μg/ml. Binding to the PBPs was expressed as a percentage of the total radioactivity added, as measured by a Beckman 1212 gamma counter.

<table>
<thead>
<tr>
<th>Strain and antibiotic</th>
<th>Low-level resistant strains</th>
<th>High-level resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC50 (μg/ml)</td>
<td>MIC (μg/ml)</td>
</tr>
<tr>
<td>S. epidermidis 45286</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>850</td>
<td>32</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>S. epidermidis 44751</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>550</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>275</td>
<td>4</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>213</td>
<td>16</td>
</tr>
<tr>
<td>S. haemolyticus 50768</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>55</td>
<td>16</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>S. haemolyticus 52462</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>40</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>40</td>
<td>128</td>
</tr>
<tr>
<td>Cefamandole</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>S. hominis 49682</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>600</td>
<td>32</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>125</td>
<td>1</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>S. simulans 47553</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>60</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>66</td>
<td>16</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>80</td>
<td>4</td>
</tr>
</tbody>
</table>

a SC50, 50% saturation concentration measured by competition experiments in uninduced strains.

b MICs were determined at 30°C.

FIG. 1. PBPs of S. simulans (lanes A, B, and C), S. haemolyticus (lanes E, F, and G), and S. aureus SR 1550-9 (lane D). MRI, MR induced strain.
formed with methicillin, cephalothin, and cefamandole. One example of a competition experiment is shown in Fig. 5.

In MR and MS strains of the four species, 50% saturation of normal PBPs was obtained with low concentrations of methicillin, cephalothin, and cefamandole (1 to 5 μg/ml; data not shown). In contrast, PBP 2' of MR strains remained unsaturated at high concentrations of the three antibiotics (Table 1). In particular, 50% saturation concentrations were above 1,000 μg/ml for all MR strains after induction.

**PBP 2' peptide mapping.** To ascertain that the protein considered as PBP 2' was similar in the different MR strains, peptide mapping was done with V8 protease by using membranes from strains grown with methicillin, to saturate the normal PBPs. The same pattern was found in the four strains with four main peptides that were similar to those of *S. aureus* SR 1550-9 (Fig. 6).

**DISCUSSION**

As described previously (4, 12, 14), we have shown that an additional inducible PBP can be revealed in MR strains of *S. epidermidis* and *S. haemolyticus*, and here we extended the observation to *S. hominis* and *S. simulans*. The 50% saturation values of PBP 2' for methicillin, cephalothin, and cefamandole found in our study were in the same range as those reported previously, although some variations exist in the values reported by the different investigators. These variations are probably due to differences in the experimental conditions (3–6, 15). However, in our study, the 50% saturation values were very different in experiments performed with induced and noninduced strains. This might be explained by the different amounts of protein present under the two conditions, although one cannot exclude the comigration of a normal PBP of 78 kilodaltons with PBP 2', for which rapid saturation would result in an apparent decrease of the corresponding band in noninduced strains, at least for *S. hominis* and *S. simulans*. This phenomenon would not be observed after induction since this normal PBP would be saturated by methicillin after growth in the presence of this drug. Growth in the presence of methicillin may also partially saturate PBP 2', so that additional binding may become apparently more difficult; however, induction was performed with concentrations of methicillin which were low (10 μg/ml) compared with those used in the competition experiments and, in any case, much lower than the 50% saturation concentrations observed in the absence of induction.

The protein corresponding to PBP 2' of *S. aureus* was present and inducible in resistant strains of all four species, but it was not detected in MS strains. It was thus considered to be responsible for the methicillin resistance, although no correlation was observed between the amount of the protein, its saturation by the different β-lactam antibiotics, and the phenotypic expression of resistance. It is noticeable that despite the low MICs, the affinity of this protein was not significantly better for cephalosporins than it was for methicillin, suggesting a cross resistance to these antibiotics. Moreover, the fact that the MICs of the cephalosporins did not vary after induction by methicillin eliminates the possibility that their apparent good activity was due to poor induction. Since there was such a dissociation between the affinity of PBP 2' for the different antibiotics and the MICs of these antibiotics, one could imagine, for coagulase-negative staphylococci, an additional factor that interferes with the expression of methicillin resistance, independent of PBP 2', as has been suggested for *S. aureus* (1, 9). In addition, the

![Image](http://aac.asm.org/Downloaded.from/http://aac.asm.org)
peptide mapping of PBP 2' of the different MR strains of coagulase-negative staphylococci was similar to that of *S. aureus*, and therefore confirmed and extended the results of previous studies (4, 11). Thus, a common gene could encode PBP 2' in all MR strains of staphylococci, and a very similar overall regulation of methicillin resistance could exist in the MR strains of *S. aureus* and coagulase-negative staphylococci.

**ACKNOWLEDGMENTS**

The [3H]benzylpenicillin was generously provided by Rhône-Poulenc Recherches and was synthesized at the Service des Molecules Marquées, Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

This work was supported by grants from the Caisse Nationale d'Assurance Maladie des Travailleurs Salarisés (87-3-22-03-E) and the Institut National de la Santé et de la Recherche Médicale (CIF 89-01).

**LITERATURE CITED**