Synergy and Resistance to Synergy between β-Lactam Antibiotics and Glycopeptides against Glycopeptide-Resistant Strains of *Enterococcus faecium*

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A synergistic effect between vancomycin and teicoplanin and different β-lactam antibiotics was found for two strains of *Enterococcus faecium*, EFM4 and EFM11, expressing resistance to glycopeptides and belonging to the VANA class. The MICs of penicillin for these two strains were 16 and 128 μg/ml, respectively. By using a penicillin-binding protein (PBP) competition assay, it was shown that the affinities of PBPs for different β-lactam antibiotics and the MICs of these antibiotics obtained in the presence of teicoplanin correlated with the substitution of two high-molecular-weight PBPs for the low-molecular-weight PBPs as the essential target. Mutants of EFM4 and EFM11 which had lost the synergistic effect between β-lactams and glycopeptides were selected on teicoplanin plus ceftriaxone at a frequency of 10⁻² and 10⁻³, respectively. The mechanism of the loss of synergy was explored. For the mutants derived from EFM4, it was associated with a change in PBPs, while for the mutants derived from EFM11, it was related to some unknown change on the conjugative plasmid responsible for the glycopeptide resistance. These combined observations reflect the relationship which seems to exist between the new β-lactate peptidoglycan precursor, synthesized when the vancomycin resistance is expressed, and the affinity of the different PBPs for this precursor.

Resistance to glycopeptides in *Enterococcus faecium* and *Enterococcus faecalis* has emerged in different countries (18). In the highly resistant strains of class VANA, as well as in the strains with low-level resistance of class VANB (3, 10, 22, 25, 27), replacement of normal by new precursors of peptidoglycan is involved in the resistance to glycopeptides (1, 5, 7, 17, 24). In the VANA strains, the presence of a β-lactate residue instead of D-alanine at the C-terminal position of the new precursor (1, 4, 17, 24) is thought to prevent binding of vancomycin (6) and thereby explain the resistance to glycopeptides.

We have previously shown for a class VANB strain that the synergistic effect which exists between β-lactam antibiotics and vancomycin could be explained by the functional replacement of PBPs, as the essential penicillin-binding protein (PBP), by some high-molecular-weight PBP(s) (2). In this work we have examined which PBP(s) is involved in the synergy between different β-lactam antibiotics and glycopeptides against two strains of *E. faecium* of the VANA class, EFM4 and EFM11, which showed different levels of resistance to penicillin. Since mutants resistant to synergy could be selected from the two strains, we also studied the mechanism involved in this type of resistance.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. faecium* EFM4 and EFM11 were isolated from the stools of patients at the Saint-Joseph hospital and found to belong to the VANA class. Mutants resistant to synergy between β-lactams and glycopeptides were isolated from these two strains on plates containing ceftriaxone (16 μg/ml) and teicoplanin (8 μg/ml). Growth was promoted at 37°C in brain heart infusion (BHI) broth or on BHI agar (Difco Laboratories, Detroit, Mich.).

**MIC determination and antibiotics.** MICs were determined on BHI agar containing serially twofold-diluted antibiotics and inocula of 10⁶ CFU per spot. MICs were read after 18 h at 37°C. Antimicrobial agents were kindly provided as follows: ceftriaxone, Roche S.A. (Paris, France); ceftazidime, Glaxo Pharmaceuticals Ltd. (Greenford, United Kingdom); aztreonam, E.R. Squibb and Sons (Princeton, N.J.); cefsoludin, Takeda (Paris, France); penicillin G, Specia (Paris, France); vancomycin, Eli Lilly and Co. (Saint-Cloud, France); and teicoplanin, Merrell Dow (Paris, France). [³H]benzylpenicillin (0.66 TBq/nmol) was generously provided by Rhône-Poulenc Recherche and synthesized at the Service des Molécules Marquées, Commissariat à l’Energie Atomique (Gif-sur-Yvette, France).

**Analysis of PBPs and cytoplasmic membranes.** PBPs of intact cells were analyzed as previously described (2) except that for competition experiments exposure to unlabeled antibiotic was followed by exposure to 20 or 100 μg of [³H]benzylpenicillin per ml. Analysis of PBPs was performed on sodium dodecyl sulfate (SDS)-polyacrylamide (6.5%) gels (28), and PBP bands were quantified with a Cliniscan densitometer (Helena Laboratories, Beaumont, Tex.). The total cytoplasmic membrane proteins isolated from intact cells (2) were analyzed by electrophoresis on SDS-polyacrylamide (10%) gels.

**Transfer of glycopeptide resistance.** Filter matings were performed as previously described (20, 23) except that all manipulations were performed on BHI agar plates without horse serum. Strains EFM1, which is resistant to rifampin and fusidic acid and susceptible to glycopeptides (penicillin MIC, 128 μg/ml; ceftriaxone MIC, >1,024 μg/ml), and D359 (28), which is also resistant to rifampin and fusidic acid and susceptible to glycopeptides (penicillin MIC, 8 μg/ml; ceftriaxone MIC, 512 μg/ml), were used as recipients. The concentrations...
for selection of transconjugants were as follows: rifampin, 50 μg/ml; fusidic acid, 25 μg/ml; and vancomycin, 10 μg/ml.

RESULTS

Susceptibility testing. The synergistic effect between β-lactam antibiotics and glycopeptides against EFM4 and EFM11 was assessed with vancomycin and teicoplanin. As shown in Table 1, a greater synergistic effect was obtained with teicoplanin at 8 μg/ml than with vancomycin at 32 μg/ml. This could be explained by the better induction of the glycopeptide resistance in the presence of teicoplanin, as shown for EFM4 and EFM11, in which the VANA protein was better expressed in the presence of teicoplanin (8 μg/ml) than in the presence of vancomycin (32 μg/ml) (Fig. 1). Therefore, teicoplanin was used for further studies. As shown in Table 2, synergy between teicoplanin and some third-generation cephalosporins was observed, while only a very moderate synergy was observed with aztreonam.

Assessment of PBPs involved in the synergy. The possible role of β-lactam antibiotics as inhibitors of glycopeptide resistance was first tested. Strains EFM4 and EFM11 were grown to an optical density at 650 nm of 0.1. Teicoplanin (8 μg/ml) was then added and left for a further 3 h, which was the time necessary to induce the resistance, either alone or in the presence of penicillin at 16 to 32 times the quantities necessary to obtain the synergy (Table 1 and Fig. 2). As shown in Fig. 2, when extracts from 1 ml of a 3-h cell culture, adjusted to an optical density of 0.2, were applied to a gel, equal quantities of the 39-kDa VANA protein were observed in the absence and in the presence of penicillin. Previous work has shown that low-molecular-weight PBPs, and PBP5 in particular, are responsible for the resistance of E. faecium to β-lactam antibiotics (11, 28). Since no differences in the PBP profiles of EFM4 and EFM11 were observed after growth in the absence and in the presence of teicoplanin (data not shown), we assessed which PBP(s) could be involved in the synergy. This was tested by direct labeling with [3H]benzylpenicillin and by competition experiments with several β-lactam antibiotics which showed different degrees of synergy when associated with 8 μg of teicoplanin per ml (Table 2). For both EFM4 and EFM11, the better correlation between the MICs of the β-lactams tested in the absence of teicoplanin and the 50% inhibition values for the PBPs was observed with the low-affinity PBP5. In contrast, when tested in the presence of teicoplanin, the MICs of the different β-lactam antibiotics correlated better with the half-saturation of high-molecular-weight PBPs. Among these, depending on the antibiotic tested, PBP2 and PBP3 appeared as

### TABLE 1. MICs of β-lactam antibiotics alone and in the presence of vancomycin or teicoplanin for E. faecium EFM4 and EFM11

| Antibiotic(s)* | MIC (μg/ml) for: |
|---------------|------------------|
|               | EFM4 | EFM11 |
| Vancomycin    | 512   | 512   |
| Teicoplanin   | 128   | 128   |
| Ampicillin    | 8     | 64    |
| Penicillin    | 16    | 128   |
| Penicillin + V32 | 0.12 | 4     |
| Penicillin + T8 | 0.03 | 0.12  |
| Ceftriaxone   | 256   | >1,024|
| Ceftriaxone + V32 | 1    | 2     |
| Ceftriaxone + T8 | 0.12 | 1     |

* V32, vancomycin at 32 μg/ml; T8, teicoplanin at 8 μg/ml.

### TABLE 2. Inhibition of the PBPs of E. faecium EFM4 and EFM11 by various β-lactam antibiotics

| Antibiotic and strain | 50% inhibitory concn (μg/ml) | MIC (μg/ml) |
|-----------------------|------------------------------|-------------|
|                       | PBP1 | PBP2 | PBP3 | PBP4 | PBP5 | Antibiotic alone | Antibiotic plus teicoplanin (8 μg/ml) |
| Ceftriaxone EFM4      | 5    | 0.06 | 0.12 | <0.03 | 256 | 256 | 0.12 |
| EFM11                 | 6.5  | 0.07 | 0.06 | <0.06 | >256 | >1,024 | 1 |
| Ceftazidime EFM4      | 27   | 1.5  | 5    | 0.6   | >256 | >1,024 | 1 |
| EFM11                 | 16   | 0.7  | 3    | <0.2  | >256 | >1,024 | 4 |
| Cefsulodin EFM4       | >128 | 8    | 1    | 4     | >256 | >1,024 | 4 |
| EFM11                 | >128 | 8    | 1.3  | 5     | >256 | >1,024 | 8 |
| Aztreonam EFM4        | >512 | 300  | 6.5  | <1    | >512 | >1,024 | 128 |
| EFM11                 | >512 | 512  | <2   | <2    | >512 | >1,024 | 512 |
| Penicillin EFM4       | 0.25 | 0.07 | 0.05 | 0.07  | 10   | 16   | 0.03 |
| EFM11                 | 0.2  | 0.06 | 0.04 | 0.03  | 90   | 128  | 0.12 |
likely candidates to take over the essential role of PBP5 when the glycopeptide resistance was expressed.

Resistance to synergy between glycopeptides and β-lactam antibiotics. During the assessment of the synergistic effect between β-lactams and teicoplanin, it was found that when high inocula were used, stable mutants resistant to synergy could be selected from either EFM4 or EFM11 on BHI agar plates containing teicoplanin (8 μg/ml) plus ceftriaxone (16 μg/ml). Mutants selected from EFM11, which expressed higher intrinsic resistance to β-lactams than EFM4, were obtained at higher frequencies (ca. 10⁻³ and 10⁻⁵, respectively). The MICs of penicillin and ceftriaxone, when combined with teicoplanin, were higher for the EFM11 mutant (EFM11-1) than for the EFM4 mutant (EFM4-1). However, in the presence of teicoplanin, the relative increases in MICs were similar for the mutants of both strains, i.e., 128-fold for penicillin and 1,024-fold for ceftriaxone (Table 3).

The mechanism of resistance to synergy could be expected to rely on a change in the PBPs of the mutants. EFM4-1 and EFM11-1, respectively, were chosen for further studies. Examination of the PBPs of EFM4-1 showed decreased quantities of PBP1 (−60%), PBP2 (−75%), and PBP3 (−85%) and an increased quantity of PBP5 (80%), which was best seen after only a short exposure of the fluorogram (Fig. 3). No obvious changes in the affinities of the PBPs were observed (data not shown). Since an increase in the MICs of β-lactam antibiotics was observed for EFM4-1 (Table 3), even in the absence of teicoplanin, we examined whether similar mutants resistant to synergy could be obtained after selection on penicillin (32 μg/ml) or ceftriaxone (512 μg/ml) in the absence of teicoplanin. Indeed, mutants with the same characteristics as EFM4-1 could be obtained at very similar frequencies (data not shown).

In contrast, for EFM11-1 no changes were observed in either the quantity or the affinity of the PBPs. Since there was an apparent increase in the glycopeptide resistance of this mutant, it was likely that a mechanism other than modification of PBPs was responsible for the resistance to synergy. To assess whether a relationship existed between the gene(s) involved in the resistance to synergy and the glycopeptide resistance genes, which are often transferable (22), we attempted to transfer the glycopeptide resistance from EFM11 and EFM11-1 to EFM1, which is glycopeptide susceptible and for which the MIC of penicillin is 128 μg/ml, close to that for EFM1. Glycopeptide-resistant transconjugants were obtained and were called EFM111 and EFM111-1, respectively. As shown in Table 4 for EFM11-1, the resistance to the synergistic effect between β-lactams and teicoplanin was cotransferred with glycopeptide resistance from EFM11-1 to EFM1. When similar experiments were performed with EFM4 and EFM4-1 as donors and EFM1 as the recipient, no resistance to synergy was observed in the transconjugant from EFM4-1 (data not shown), reinforcing the

![FIG. 2. Expression of the VANA protein (39 kDa) of E. faecium EFM4 and EFM11 in the presence of penicillin. T8, teicoplanin (8 μg/ml); T8 + P0.25, teicoplanin (8 μg/ml) plus penicillin (0.25 μg/ml); T8 + P2, teicoplanin (8 μg/ml) plus penicillin (2 μg/ml); C, control without antibiotic.]

![FIG. 3. PBPs of E. faecium EFM4, EFM11, and their derivatives EFM4-1 and EFM11-1 labeled with [3H]benzylpenicillin at 40 or 100 μg/ml. Exposure times of the fluorograms were 8 days (A) and 1 day (B).]

**TABLE 3. MICs for the mutants resistant to synergy**

| E. faecium strain | Frequency of mutation | Vancomycin (μg/ml) | Teicoplanin (μg/ml) | Penicillin (μg/ml) | Penicillin + T8a | Ceftriaxone (μg/ml) | Ceftriaxone + T8a (μg/ml) |
|------------------|----------------------|--------------------|--------------------|-------------------|----------------|-------------------|--------------------------|
| EFM4             |                      | 512                | 1,024              | 16                | 0.03           | 256               | 0.12                     |
| EFM4-1a          | 2 × 10⁻⁵             |                    |                    |                   |                |                   |                          |
| EFM11            |                      | 512                | 1,024              | 128               | 4              | 1,024             | 256                      |
| EFM11-1a         | 2 × 10⁻⁵             | 2,048              | 512                | 128               | 4              | 1,024             | 1,024                    |

* T8, teicoplanin at 8 μg/ml.

* Mutant selected on ceftriaxone (16 μg/ml) plus teicoplanin (8 μg/ml).
conclusion that in EFM4-1 only PBP modifications were involved in this phenomenon.

Finally, to define the respective roles of PBP modifications and glycopeptide resistance in bringing about the effect of synergy, another set of experiments was carried out. We used as recipients either *E. faecium* D359, a glycopeptide-susceptible strain for which MICs of penicillin and ceftriaxone are 8 and 512 µg/ml, respectively, or *E. faecium* D359P, selected from D359 on penicillin, for which MICs of penicillin and ceftriaxone are 32 and 2,048 µg/ml, respectively. The PBP patterns of *E. faecium* D359 and D359P were similar to those of EFM4 and EFM4-1, respectively, with a decrease in quantities of PBPs 1, 2, and 3 and an increase (40%) of PBP5 (Fig. 4). As presented in Table 4, similar results, although quantitatively less pronounced, were obtained when D359 and D359P were the recipients during conjugation with EFM11 and EFM11-1. Resistance to synergy between teicoplanin and ceftriaxone was observed for D359P11-1, with an eightfold increase in the MIC of ceftriaxone compared with that determined for D359P11. Similarly, a 16-fold loss of the synergistic effect between teicoplanin and ceftriaxone was observed for D359P11-1, to which the glycopeptide resistance of EFM11 had been transferred. When the change in PBPs of D359P was associated, in strain D359P11-1, with the glycopeptide resistance transferred from EFM11-1, an even more pronounced loss of the synergistic effect (256-fold) was observed. However, in no case did the MICs of ceftriaxone in the presence of teicoplanin reach those observed for EFM4-1 and EFM11-1.

**DISCUSSION**

The mechanism of synergy between β-lactam antibiotics and glycopeptides was examined with two VANA strains. In the absence of glycopeptides, they expressed different levels of resistance to β-lactam antibiotics, which could be related to the affinity of PBP5 for these compounds, as previously described for *E. faecium* (11, 14, 19, 28). Synergy between different β-lactam antibiotics and vancomycin or teicoplanin was demonstrated, but it was better expressed in the presence of teicoplanin.

As in our previous work with a VANB strain (2), no inhibition of VANA induction was observed in the presence of penicillin at concentrations 16-fold higher than those necessary for growth inhibition in the presence of teicoplanin. Since no direct effect of penicillin on the VANA ligase has been observed (6), it is very unlikely that penicillin inhibits the expression of vancomycin resistance. This suggests that the synergistic effect would, in fact, depend upon the expression of glycopeptide resistance. Studies of affinities of PBPs for different β-lactams with very low affinities for PBP5, the normally essential target of *E. faecium*, showed that the inhibition only of high-molecular-weight PBPs, which have high affinity for β-lactams, could explain the low quantities of β-lactam necessary to obtain synergy. Among these, PBP2 and PBP3 would be the most likely candidates to become the essential PBP when the glycopeptide resistance is expressed in the presence of teicoplanin. One simple explanation for this exchange of essential PBPs would be that the new precursor, which in VANA strains ends in D-alanine-D-lactate (1, 4, 17, 24) and has no affinity for glycopeptides, would be essentially processed in our strains by these high-molecular-weight PBPs. Thus, the effect of synergy could be explained by the fact that these new essential PBPs also have high affinity for the β-lactams. In
contrast, it is very likely that the low-molecular-weight PBPs, and PBP5 in particular, which has a low affinity for these β-lactams, would also have only low affinity for the new precursors and therefore be unable to process them. Allen et al. (1), using an in vitro polymerization assay, showed that the new precursor could be polymerized into peptidoglycan. However, these authors did not examine whether the reaction could be inhibited by β-lactams at a concentration lower than that necessary to inhibit polymerization in the presence of the normal precursor ending in D-alanyl-D-alanine.

In our previous work, using the *E. faecium* VANB strain D366, we suggested that after induction of vancomycin resistance, PBP1 could become the essential target of β-lactam antibiotics (2). This apparently contradicts our present results, which would indicate rather PBP2, and possibly PBP3, as the essential target when glycopeptide resistance is expressed. The reason for this is not entirely clear. We have demonstrated a residual synthesis of the normal pentapeptide precursor in *E. faecium* D366 but not in strains expressing the high-level VANA-type resistance (5). It might then not be impossible that in D366 the residual normal pentapeptide is quantitatively insufficient for processing by PBPS but is enough of a substrate for other PBPs, and PBP1 in particular. This might obscure the essential role of the other high-molecular-weight PBPs in the processing of the new precursor. It should be noted that the role of the high-molecular-weight PBPs as essential targets for β-lactam antibiotics, even in the absence of glycopeptide resistance, has previously been demonstrated in an *Enterococcus* mutant which had completely lost expression of PBPS (12). As for the mechanism of resistance to synergy in the mutants of VANA strains described here, two types were observed, which appeared to differ depending upon the intrinsic level of β-lactam resistance of the strains. Starting with strain EFM4, which has low-level β-lactam-resistance, there was an apparent link between the PBP modifications of the mutant EFM4-1 and the resistance to synergy, while such resistance was not cotransferred with glycopeptide resistance from EFM4-1 to EFM1. Our hypothesis is that since lesser amounts of high-molecular-weight PBPs, especially PBP2 and PBP3, are present in EFM4-1, more of the new precursor could be now processed by the other PBPs, and in particular PBP5, which is more abundant and has less affinity for the β-lactams than the high-molecular-weight PBPs. It may be worthwhile to note that a spontaneous mutation modified the production of multiple PBPs, which was previously observed in enterococci but only after multiple steps of selection for penicillin resistance (29).

In the case of EFM11-1, which derives from strain EFM11, which has an intrinsically higher level of resistance to penicillin, resistance to synergy was closely linked with glycopeptide resistance, since no change in PBPs was observed and cotransfer of resistance to synergy was obtained in the transconjugant EFM11-1. By using various other VANA strains with intrinsic levels of β-lactam resistance similar to that of EFM11, mutants resistant to synergy could be selected, and this resistance was again cotransferred with the glycopeptide resistance (data not shown). Compared with EFM11, and in contrast to what was observed for EFM4-1, the level of teicoplanin resistance was higher in EFM11-1 and its transconjugant EFM11-1. It is conceivable that in the latter two strains greater quantities of precursor were synthesized and that the higher substrate-to-enzyme ratios could allow its processing by PBPS. Preliminary experiments showed no significant changes in the production of the protein VANA between EFM-11 and EFM11-1 (data not shown). However, this does not exclude the possibility that expression of other genes involved in the resistance might be modified.

The effect of synergy between glycopeptides and β-lactam antibiotics against VANA strains remains subject to debate. In some studies synergy was observed (2, 8, 21, 26), while in others no synergy was found (9, 13, 15), although synergy with teicoplanin was not tested. However, it is not impossible that most of the strains which showed no synergy either had a background similar to that of our mutant EFM14-1 or resulted from a particular selection, as did EFM11-1. Interestingly, all these strains showed MICs of ampicillin (≥128 μg/ml) higher than those for our strains. This would suggest that either a different PBP5 with less affinity for β-lactams or another, unknown mechanism of β-lactam resistance was present (19), which would not allow expression of the synergy between glycopeptides and β-lactams which is observed in strains somewhat more susceptible to ampicillin (2, 8). It does not seem impossible that the PBPS differed from those of our strains and allowed the transpeptidation of the pentapeptide ending in D-lactate (16).

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