One or Two Low Affinity Penicillin-binding Proteins May Be Responsible for the Range of Susceptibility of *Enterococcus faecium* to Benzylpenicillin

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Three benzylpenicillin-resistant, clinical isolates of *Enterococcus faecium* (MIC values 16–64 µg ml⁻¹) contained six penicillin-binding proteins (PBPs), of which PBP5 was the most abundant and had the lowest affinity for the antibiotic. Four benzylpenicillin-susceptible strains (MIC values 0.031–0.5 µg ml⁻¹) were obtained as spontaneous derivatives from these above organisms. There were significant decreases in the amounts of PBP5 in each of the derivatives, with the concomitant appearance of a new, higher affinity PBP (5*) in three strains. Increased amounts of PBP5, with no changes in PBP5*, were found in several mutants with intermediate-level benzylpenicillin-resistance (MIC values 1–8 µg ml⁻¹) selected from two of the susceptible strains. Examination of 18 other clinical isolates, with a wide range of susceptibilities to benzylpenicillin (MIC values 0.062–128 µg ml⁻¹), showed that PBP5* was present in 13 strains, and PBP5 in all of them, but in differing amounts. The results concerning the relative amounts and relative affinities of PBPs 5* and 5 allowed the categorization of the various strains into six groups, within which organisms had somewhat similar susceptibilities to benzylpenicillin.

INTRODUCTION

Most (70%) clinical isolates of *Enterococcus faecium* are currently multiply resistant to a variety of antibiotics including β -lactam antibiotics, tetracycline, erythromycin and other macrolides, aminoglycosides (high level) and chloramphenicol (Horodniceanu & Delbos, 1980). The natural resistance level of *E. faecium* to benzylpenicillin is assumed to be entirely due to the interactions of the antibiotic with the penicillin-binding proteins (PBPs) (Coyette *et al.*, 1980) since β -lactamase activity has not been detected (Le Bouguénec & Horodniceanu, 1982; Weinstein & Moellering, 1975) and the cell wall does not act as a permeability barrier (Williamson *et al.*, 1983). In a study of two strains of *E. faecium* selected *in vitro* which had become extraordinarily susceptible to a variety of β -lactam antibiotics, Eliopoulos *et al.* (1982) showed that there were no apparent changes in the PBPs of these strains when compared with those of the wild-type, parental strains. Moreover, the susceptibilities of these strains to a variety of other antibiotics were generally the same as the parental strains.

Previous results (Le Bouguénec & Horodniceanu, 1982) showed that resistance to tetracycline, erythromycin, aminoglycosides and chloramphenicol could be transferred by conjugation from *E. faecium* into *E. faecalis*, but that resistance to benzylpenicillin was not transferred. To extend these experiments, derivatives were obtained from three clinical isolates of *E. faecium* in which either single or multiple resistance characteristics were lost, either spontaneously or by treatment with ethidium bromide, in particular with a 32- to 2000-fold increase in susceptibility

Abbreviation: PBP, penicillin-binding protein.

to benzylpenicillin. In the present studies we have examined the PBPs of these strains to determine the cause of the loss of this resistance. We also examined the PBPs in an additional 18 clinical isolates of *E. faecium*, which also had a 2000-fold range in susceptibilities to benzylpenicillin, with a variety of additional resistance patterns, to determine if the results obtained with the derivatives selected *in vitro* were applicable to the natural isolates.

METHODS

Bacterial strains and growth conditions. The 21 strains of *E. faecium*, identified by the criteria of Facklam (1972), were from the collection of the Reference Centre of Streptococci, Institut Pasteur, Paris, France, and originally all were clinical isolates. Bacteria were grown without aeration at 37 °C in Brain-Heart Infusion broth (BHI; Difco), and growth was monitored with a Perkin-Elmer Junior III spectrophotometer at 650 nm.

Antibiotics and reagents. Benzylpenicillin and streptomycin were obtained from Specia, Paris, France, chloramphenicol and erythromycin from Roussel-Uclaf, Romainville, France, kanamycin from Bristol Laboratories, Paris, France, and tetracycline from Laboratoires Diamant, Puteaux, France. [³H]Benzylpenicillin (26 Ci mmol⁻¹; 0.66 TBq mmol⁻¹) was generously provided by Rhône-Poulenc Recherche, Vitry-sur-Seine, France, and synthesized at the Service des Molécules Marquées, Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. Lysozyme was purchased from Sigma, and M1-muramidase was the generous gift of K. Yokogawa, Dainippon Pharmaceutical Co., Suita/Osaka, Japan.

Susceptibility tests. Minimum inhibitory concentration (MIC) values were determined using twofold dilutions of antibiotics in BHI agar. Approximately 10⁴ colony-forming units were applied to the agar surface with a Steers replicator. Plates were incubated for 18 h at 37 °C.

Isolation of antibiotic-susceptible and benzylpenicillin-resistant strains. Antibiotic-susceptible strains were obtained either spontaneously or by treatment of resistant strains with ethidium bromide at 42 °C as previously described (Horodniceanu *et al.*, 1976). Spontaneous, benzylpenicillin-resistant mutants were obtained by plating approximately 10⁹ exponential-phase organisms onto BHI agar containing benzylpenicillin at twofold increasing concentrations from the MIC up to 64 × MIC. Colonies were picked from plates up to the highest concentration of antibiotic allowing growth after 48 h incubation at 37 °C, and streaked onto BHI agar containing appropriate amounts of benzylpenicillin.

Analysis of penicillin-binding proteins. The technique used was a modification of that used previously (Williamson *et al.*, 1983). Exponential-phase organisms (OD₆₅₀ of 0.25, approximately 2 × 10⁸ bacteria ml⁻¹) were rapidly chilled in ice, centrifuged (5000 *g* for 2 min at 2 °C), resuspended in ice-cold 50 mM-sodium phosphate buffer, pH 7.0 (0.04 × original volume), and samples (40 µl) were incubated with various amounts of [³H]benzylpenicillin (0.0002–20 µg) in a final volume of 50 µl for 30 min at 37 °C. All PBPs were saturated within 15 min at the highest concentration of antibiotic. The samples were then placed in ice, an excess of unlabelled benzylpenicillin was added (2 ml of 1 mg ml⁻¹), and the bacteria were recovered by further centrifugation as above. The supernates were removed by aspiration, and organisms were resuspended in 20 µl sodium phosphate buffer containing 10 µg lysozyme, 10 µg M1-muramidase and 0.1% (w/v) Triton X-100, and the samples were incubated at 37 °C for 20 min to cause complete lysis.

The PBPs in the lysates were analysed by SDS-PAGE and fluorography as previously described (Williamson *et al.*, 1983), except that the final concentrations of acrylamide and *N,N'*-bis-acrylamide in the separating gels were reduced to 6% (w/v) and 0.08% (w/v), respectively, to enable a significantly better resolution of the individual PBPs. Band densities of the PBPs on the fluorograms were quantified with a Helena Laboratories Cliniscan (Beaumont, Texas, USA) and were corrected for any variations in width. The data presented are the means of at least two experiments with each strain, and the variation was generally less than 7% of the mean.

RESULTS

Antibiotic susceptibilities and PBPs of parental and mutant strains

The antibiotic susceptibilities of the three wild-type clinical isolates and a variety of derivatives obtained from them are presented in Table 1. Strains D359, D371 and D344 were resistant to benzylpenicillin (MIC values 16–64 µg ml⁻¹), tetracycline (MIC values 64–256 µg ml⁻¹), erythromycin (MIC values > 1024 µg ml⁻¹) and to high levels of kanamycin (MIC values > 8000 µg ml⁻¹) and streptomycin (MIC values > 2000 µg ml⁻¹); strain D371 was also resistant to chloramphenicol (MIC 32 µg ml⁻¹). Resistance to erythromycin, as well as to other macrolides and related antibiotics (MLS phenotype), was lost spontaneously from strain D359 to give BM6223. This strain and D344 were cured of aminoglycoside resistance by treatment with ethidium bromide to give strains BM6224 and BM6219, respectively. In contrast, strains

Fig. 1. Penicillin-binding proteins (PBPs) of parental and derivative strains of *Enterococcus faecium*. The PBPs of strains BM6218 and D344 are shown in tracks A–E after incubation of whole organisms with 20, 2, 0.2, 0.02 and 0.002 μg [^3H]benzylpenicillin respectively. The PBPs of strains BM6225, D359 and BM6222 are shown after saturation with 20 μg [^3H]benzylpenicillin. The PBPs of D371 were identical to those of D344 and D359. Estimated molecular weights are shown, and PBPs 5* (*) and 5 (●) are indicated. PBP6 is not shown, since it co-electrophoresed with the [^3H]benzylpenicillin during SDS-PAGE.

Fig. 2. Penicillin-binding proteins 5* (*) and 5 (●) detected under saturating conditions.

BM6222 and BM6221, obtained as spontaneous benzylpenicillin-susceptible derivatives from D371, had also lost resistance to all six antibiotics tested. Strains BM6225 and BM6218 were both obtained as spontaneous benzylpenicillin-susceptible derivatives from BM6224 and D344 respectively, but in the former case tetracycline resistance was also lost, whereas in the latter case there were no changes in resistance to the other four antibiotics. The spontaneous resistant mutants (BM6225/1, BM6225/2, BM6225/4, BM6221/0.5 and BM6221/4) selected with benzylpenicillin, at frequencies between 10^{-8} and 10^{-9} , from strains BM6225 and BM6221 did not become resistant to other antibiotics.

The three resistant, clinical isolates D359 (Fig. 1), D371 and D344 (Fig. 1) had six PBPs, as did the erythromycin- or aminoglycoside-susceptible mutants (BM6223 and BM6224, and BM6219) isolated from D359 and D344, respectively. However, examination of the benzylpenicillin-susceptible mutants BM6225 (Fig. 1), BM6222 (Fig. 1) and BM6221 showed the presence of an extra band (PBP5*) and a significant reduction in the amount of PBP5, with a somewhat lower affinity in the two latter strains (Table 1). In contrast, there was no PBP5* in strain BM6218 (Fig. 1) derived from D344, and in this case the reduced quantity of PBP5 had a much higher affinity for benzylpenicillin than the parental strain. Examination of the PBPs in the resistant mutants selected *in vitro* (BM6225/1, BM6225/2, BM6225/4, BM6221/0.5 and BM6221/4; Fig. 2, Table 1) showed that the amounts of PBP5 were increased according to the

Table 1. *Antibiotic susceptibilities of parental and mutant strains of Enterococcus faecium, and characteristics of PBPs 5* and 5*

Strain	Selection procedure*	Resistance profile†	Pc MIC ($\mu\text{g ml}^{-1}$)	PBP5*‡		PBP5‡	
				Amount	S ₅₀	Amount	S ₅₀
D359	Wild-type	Pc Tc MLS Km Sm	16	—	—	10660	7.0
BM6223	Spont	Pc Tc Km Sm	16	—	—	8580	6.7
BM6224	EtBr	Pc Tc	16	—	—	9610	7.7
BM6225	Spont		0.5	3980	1.6	620	14
BM6225/1	Spont Pc		2	4080	ND	2320	ND
BM6225/2	Spont Pc		4	3740	ND	5090	ND
BM6225/4	Spont Pc	Pc	8	3790	ND	8950	ND
D371	Wild-type	Pc Tc MLS Km Sm Cm	32	—	—	11850	15
BM6222	Spont		0.5	2990	1.7	530	41
BM6221	Spont		0.125	2710	2.3	1060	50
BM6221/0.5	Spont Pc		1	2950	ND	4140	ND
BM6221/4	Spont Pc	Pc	8	2320	ND	8390	ND
D344	Wild-type	Pc Tc MLS Km Sm	64	—	—	8600	58
BM6219	EtBr	Pc Tc MLS	64	—	—	9000	54
BM6218	Spont	Tc MLS Km Sm	0.031	—	—	2050	1.6

ND, Not done; —, not present.

* Spont, isolated as spontaneous susceptible or resistant mutant; EtBr, isolated after treatment with ethidium bromide; Pc, benzylpenicillin.

† Pc, benzylpenicillin; Tc, tetracycline; MLS, macrolides (erythromycin), lincosamides, streptogramin B; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol.

‡ Amounts (arbitrary units) were calculated from densitometry of fluorograms, and are the means of at least two experiments. PBPs were detected under saturating conditions with [³H]benzylpenicillin. S₅₀ is the concentration ($\mu\text{g ml}^{-1}$) of [³H]benzylpenicillin required to give 50% saturation.

Table 2. *Antibiotic susceptibilities of clinical isolates of Enterococcus faecium, and characteristics of PBPs 5* and 5*

Abbreviations are as in Table 1. Strains D359, D371 and D344 were the parental strains in Table 1.

Category	Strain	Resistance profile	Pc MIC ($\mu\text{g ml}^{-1}$)	PBP5*		PBP5	
				Amount	S ₅₀	Amount	S ₅₀
1	D383		0.062	1930	1.1	770	23
	D378		0.25	2030	1.8	1540	60
	D65		0.5	3990	1.4	1270	37
2	D61		1	3550	2.7	4230	75
	D66		1	3860	0.9	4380	54
	D63		2	3040	1.5	4650	46
	9116		4	3780	ND	3860	ND
	9253		4	3550	ND	3810	ND
	9534	Tc MLS	4	4200	ND	4170	ND
	9547		4	4350	ND	4650	ND
3	D379	Pc Tc MLS Km Sm Cm	8	—	—	9950	12
	D370	Pc Tc Sm	8	—	—	13400	11
	D359	Pc Tc MLS Km Sm	16	—	—	10660	7
	D380	Pc Tc MLS Km Sm	16	—	—	9870	15
	D371	Pc Tc MLS Km Sm Cm	32	—	—	11850	15
4	D368	Pc	16	5530	7.1	8130	12
	D360	Pc Tc MLS Km Sm	16	4880	9.4	8050	15
5	D344	Pc Tc MLS Km Sm	64	—	—	8600	58
	D382	Pc Tc MLS Km Sm Cm	64	—	—	7790	ND
	D372	Pc Tc MLS Km Sm Cm	128	—	—	7950	ND
6	D331	Pc Tc MLS Km Sm	128	2140	30	4510	77

level of resistance, but that PBP5* remained at a level similar to that in the parental, susceptible strains BM6225 and BM6221 (Fig. 2).

The amounts of PBPs 1, 2, 3 and 4 differed by up to threefold between the various strains (data not shown), but there was no correlation with the resistance level. Moreover, the affinities of these PBPs for benzylpenicillin did not differ significantly in the strains. The mean (with standard deviation) concentrations of [^3H]benzylpenicillin required to give 50% saturation (S_{50} values) were ($\mu\text{g ml}^{-1}$): PBP1, 0.22 (0.04); PBP2, 0.020 (0.007); PBP3, 0.041 (0.021); and PBP4, 0.11 (0.032). The respective values for PBP6, measured in the three clinical isolates and the susceptible derivatives under conditions allowing resolution of this PBP, were 0.15 (0.049). Since all the PBPs had relatively high affinities for benzylpenicillin, and PBP5 in the wild-type strains had S_{50} values in the range $0.5\text{--}1 \times \text{MIC}$, it seemed possible that PBP5 could be the essential PBP in these resistant organisms. In support of this, growth of the wild-type strains in non-radioactive benzylpenicillin at $0.2 \times \text{MIC}$ for each strain resulted in almost complete saturation of PBPs 1, 2, 3, 4 and 6, but only slight saturation of PBP5 with no inhibition of the growth rate, suggesting that the other PBPs are non-essential targets. Subsequent experiments were done to examine whether PBP5* as well as PBP5 existed in other wild-type strains.

Antibiotic susceptibilities and PBPs in clinical isolates

An additional 18 clinical isolates were chosen on the basis of a wide range of susceptibility to benzylpenicillin, the MIC values being from 0.062 to $128 \mu\text{g ml}^{-1}$, as shown in Table 2 which also includes the three wild-type, clinical isolates presented in Table 1. Most of the relatively benzylpenicillin-susceptible strains (MIC values $< 8 \mu\text{g ml}^{-1}$) were susceptible to the other antibiotics tested. In contrast, 10 of the 11 benzylpenicillin-resistant strains (MIC values $> 8 \mu\text{g ml}^{-1}$) were multiply resistant.

PBPs 1 to 4 and 6 were found in all of the additional 18 strains in amounts and with affinities similar to those given earlier for strains D359, D371 and D344, except that in two strains (D360 and D368) the molecular weight of PBP2 differed slightly from that in other strains, and strain D66 had no PBP4. These differences could not be correlated with the resistance levels to benzylpenicillin. PBP5* was found in 13 of the 21 isolates (Table 2), and this PBP was present mostly in the relatively benzylpenicillin-susceptible strains. The results concerning the amounts and affinities obtained with PBPs 5* and 5 allowed the grouping of the organisms into six categories (Table 2), based primarily on the amounts of PBP5 and the presence or absence of PBP5*. Strains within each group had somewhat similar susceptibilities to benzylpenicillin, as well as similar amounts and S_{50} values for PBPs 5* and 5. Two categories (3 and 5) did not contain PBP5*. When present, the amounts of PBP5* remained similar (with a twofold range) in the various strains, but the PBP had lower affinities for the antibiotic in the more resistant categories (4 and 6). In contrast, the amounts of PBP5 differed significantly (Student's *t*-test) between each of the six categories, and the mean values compared with that in category 1 were 3.6, 9.4, 6.8, 6.8 and 3.8 times greater in categories 2, 3, 4, 5 and 6, respectively. The affinities of PBP5 for benzylpenicillin in categories 3 and 4 appeared higher than those of PBP5 in the other categories.

PBPs 5* and 5, or 5 alone if 5* was not present, were the most abundant PBPs in each strain, accounting for an average of 49% of the total under saturating conditions. The lowest amounts were found in strain D378 where PBPs 5* and 5 accounted for 37.5% of the total, and the greatest in strain D344 where PBP5 accounted for 68.9% of the total PBPs.

When whole cells or membranes obtained from the various clinical isolates of *E. faecium* were incubated for extended periods (up to 150 min) under saturating conditions, there were no changes in the amounts of PBPs, and the results were identical to those obtained after 30 min.

DISCUSSION

In the present studies, the loss of resistance to benzylpenicillin in two strains of *Enterococcus faecium* was associated with a significant decrease in the amounts of a low affinity PBP5, and a new PBP5* not present in the parental strains was detected in most of the spontaneous,

Fig. 3. Model of the primary growth and division site of *Enterococcus faecium* with localization of PBPs 5* and 5. Diagrams 1 and 2 represent categories 1 and 2 of the clinical isolates described in Table 2, and show the relative amounts of PBP5* (○) and PBP5 (●) adjacent to the septal wall. The total amounts of the PBPs are shown in proportion with that in category 1, and the area in which synthesis of peptidoglycan occurs for ingrowth of the septum is shaded. It is assumed that PBPs 5* and 5 outside this area are therefore not essential for this ingrowth. For the other categories of strains listed in Table 2, PBP5*, when present, and PBP5 would be included in the model in the amounts given.

susceptible derivatives. These results differ greatly from those of Eliopoulos *et al.* (1982), who found no changes in the PBPs of hypersusceptible derivatives of *E. faecium*, but this was probably due to insufficient saturation of PBP5 in the wild-type strains. Analysis of PBPs in the various clinical isolates in our study revealed that most organisms (64%) contained both PBP5* and PBP5, and that six categories of strains could be defined on the basis of the amounts of PBP5, the presence or absence of PBP5*, and the affinity of PBP5* for benzylpenicillin. It would therefore seem likely that the natural susceptibility (or resistance) level of *E. faecium* to benzylpenicillin is related to the relative amounts as well as the relative affinities of two essential PBPs (5* and 5). Presumably, each of these proteins must have transpeptidase activity, and they must be the sole functional PBPs allowing continued growth of the organisms in concentrations of benzylpenicillin up to the minimal inhibitory concentration.

A possible mechanism is that PBPs 5* and 5 could be primarily located at the primary growth and division site (septum) of the cocci, that only a limited number of enzyme molecules can be involved in peptidoglycan synthesis in a small area of the nascent wall, and that the relative abundance of one enzyme compared with the other could determine which would have the major role in synthesis (Fig. 3). For example, in category 1, PBP5* is present in double the amount of PBP5, which has the lower affinity. Therefore, when PBP5* becomes inhibited by benzylpenicillin, its function cannot be taken over by PBP5 since the acylated PBP5* would physically block access of PBP5 to the site of synthesis, or, alternatively, the low amount of PBP5 is not enough for continued peptidoglycan synthesis. Thus the high susceptibility of strains in this category is due to the inhibition of the higher affinity target even though the lower affinity PBP5 is present. Since the PBPs of *E. faecium* have extremely long half-lives when acylated with benzylpenicillin (Coyette *et al.*, 1980; Williamson *et al.*, 1983) it is unlikely that any great amounts of enzyme activity are regained by de-acylation of the complexes. Within category 2, the amount of PBP5 is increased about three- to fourfold, and more of this PBP could be present at the growth site. In this situation, a large proportion of PBP5* could be saturated but the lower affinity target would still have sufficient activity to allow continued growth at a greater concentration of the antibiotic than that occurring in category 1. Categories 3 and 5 contain strains having only PBP5, therefore the resistance level is dependent only on the amount and affinity of this PBP.

On the basis of this above model, it would be expected that overproduction of the lower affinity target (PBP5) would decrease the susceptibility of the organism, since the increased amount could displace some of, or take over from, the higher affinity target (PBP5*) at the site of synthesis. In the present studies, spontaneous resistant mutants were obtained with up to an eightfold increase in the quantity of PBP5, but with unchanged amounts of PBP5*. Although the PBPs involved in resistance of enterococci to benzylpenicillin have been described as 'slow-binding PBPs' (Fontana *et al.*, 1983), we would not define either PBP5* or PBP5 as such, since this apparent characteristic is due to the extremely low affinity for the antibiotic and very high concentrations of [3 H]benzylpenicillin can give relatively rapid saturation of these PBPs.

The occurrence of increased amounts of low affinity PBPs in resistant mutants, as described here for *E. faecium* and elsewhere for *E. durans*, *E. faecalis* and *E. faecium* (Daneo-Moore & Pucci, 1982; Daneo-Moore *et al.*, 1983; Fontana *et al.*, 1983), as well as their partial loss in susceptible derivatives of *E. faecium*, must indicate that the amounts of these proteins are regulated, either genetically or physiologically. In contrast with the report of Daneo-Moore *et al.* (1983), we did not observe multiple changes in the amounts of cytoplasmic membrane proteins stained with Coomassie Blue, except for the partial loss of a 79.5 kDal protein in the case of each of the susceptible derivatives (data not shown), and this protein had the same molecular weight as PBP5. The benzylpenicillin-susceptible mutants isolated by Eliopoulos *et al.* (1982) were also deficient in membrane proteins with molecular weights of 82 kDal and 108 kDal, but neither of these corresponded to the molecular weights of the PBPs.

The basis for the appearance of PBP5* with partial loss of PBP5 in some susceptible derivatives obtained from strains lacking PBP5* is unknown at present. It is interesting that these derivatives would be placed in category 1 of Table 2 on the basis of the amounts of PBP5, whereas both parental strains are in category 3. We speculate that *E. faecium* may be naturally relatively susceptible to benzylpenicillin, but that some mutation or other genetic change causes loss of PBP5* and overproduction of PBP5 resulting in resistance to the antibiotic. Thus the organisms in categories 3 and 5 may have been derived from those in categories 1 and 2. Since the benzylpenicillin-resistant mutants selected *in vitro* only had increased amounts of PBP5, it seems possible that the loss of PBP5* and overproduction of PBP5 may have been separate events during the evolution of resistance. Nevertheless, the benzylpenicillin-susceptible derivatives containing PBP5* were obtained spontaneously from three wild-type, resistant strains at a relatively high frequency (10^{-3} to 10^{-4}); therefore a single mutation or genetic change, different from that regulating only the amount of PBP5, may be responsible for the overall change. An alternative mechanism of resistance may be represented by the organisms in categories 4 and 6, in which the decreased affinities of PBP5* could be more important than the increased amounts of PBP5. Previous reports have shown that losses of PBPs and changes in affinities as well as amounts of PBPs, probably due to several different mutations, contribute to the resistance of some strains of *Streptococcus pneumoniae* to benzylpenicillin (Handwerger & Tomasz, 1984; Zighelboim & Tomasz, 1980). We are currently investigating whether PBPs 5* and 5 have any biochemical homology. It seems unlikely that one is a degradation product of the other, since the continued incubation of whole cells or membranes with the radioactive benzylpenicillin did not change the amounts of either PBP.

Although the occurrence of benzylpenicillin-susceptible derivatives was generally associated with loss of resistance to a variety of other antibiotics, the derivative BM6218 obtained from D344 retained the other resistance markers. The apparent reason for the susceptibility in this case was a fourfold decrease in the amount of PBP5 together with a significant increase (about 35-fold) in the affinity of this PBP for benzylpenicillin, and we have recently found virtually identical results with one of the pairs of resistant (4379) and hypersusceptible (4379-S) strains obtained from G. M. Eliopoulos and R. C. Moellering, Jr (data not shown). Therefore, the strains BM6218 and 4379-S may have had mutations in the structural genes coding for PBP5, revealing a basis for susceptibility in addition to the appearance of PBP5* and partial loss of PBP5.

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