Studies on the Mechanism of Intrinsic Resistance to β-Lactam Antibiotics in Group D Streptococci

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Six penicillin-binding proteins (PBPs) were detected in clinical isolates of each one of three group D streptococci: *Streptococcus bovis*, *S. faecalis* and *S. faecium*. When examined in whole organisms, the PBPs of *S. faecium*, the most penicillin-resistant species of group D streptococci, generally had lower affinities for the antibiotic than those of *S. faecalis* (intermediate penicillin resistance), which in turn were of lower affinity than those of *S. bovis* (penicillin-sensitive). On the other hand, no quantitative correlation could be established between the binding of penicillin to any one PBP or group of PBPs, and the penicillin MIC value for the corresponding micro-organism. Examination of the amounts of antibiotic bound and the rates of binding to PBPs of equal numbers of protoplasts and whole bacteria of *S. faecalis* and *S. faecium*, indicated that there was no permeability barrier to benzylpenicillin in the cell walls of these species. The lower antibacterial effectiveness of cephalothin compared with ampicillin in group D streptococci was paralleled by the higher concentrations of cephalothin needed in competition assays to inhibit the lower molecular size PBPs of these bacteria.

INTRODUCTION

Among streptococci, enterococci are uniquely resistant to β-lactam antibiotics, both in terms of the relatively high MIC values of the antibiotics (Toala *et al.*, 1969) and also in the poor killing of the organisms after inhibition of growth has been achieved (Krogstad & Parquette, 1980; Moellering *et al.*, 1974). The mechanism of this natural resistance is unknown. Previous investigations (Weinstein & Moellering, 1975) have indicated that the bacteria do not produce a β-lactamase. Alternative explanations of resistance include low affinities of the target sites of the antibiotics in comparison with those of other streptococci or diminished penetration to such sites.

The β-lactam antibiotics bind to membrane-associated proteins (penicillin-binding proteins, PBPs) of bacteria, and these PBPs are presumably the target sites of action, corresponding to the β-lactam antibiotic-sensitive enzymes involved in the terminal stages of peptidoglycan synthesis. The purpose of this study was to characterize the PBPs of three representative strains of group D streptococci isolated from blood cultures at the Massachusetts General Hospital. The strains were a penicillin-sensitive, non-enterococcal *S. bovis*, and two penicillin-resistant enterococci, *S. faecalis* and an *S. faecium*. The ultimate aim of the study was to determine whether the penicillin resistance of enterococci was associated with differences in PBP affinity for β-lactam antibiotics or with reduced permeability of the cell wall in comparison with the non-enterococcal group D *S. bovis*. The interactions of ampicillin and cephalothin with the PBPs were also examined since enterococci are substantially more resistant to cephalosporins than to ampicillin (Thornsberry *et al.*, 1974; Moellering, 1981).

METHODS

Bacterial strains and growth conditions. Streptococcus bovis 4054, *S. faecalis* 447, and *S. faecium* 4379 were all β-lactamase-negative clinical isolates obtained from the Massachusetts General Hospital, Boston. Identification of isolates as group D streptococci was by standard bacteriological techniques, and identification to the species level was according to the recommendations of Facklam (1972) and Gross *et al.* (1975). Identification of *S. faecalis* and *S. faecium* strains was confirmed by the Department of Clinical Microbiology, The New York Hospital. Stock cultures were maintained on Brucella agar slants; single colony isolates from blood agar plates, containing tryptose blood agar base (Difco) and 3% (v/v) defibrinated sheep blood (Wilfer Laboratory, Stillwater, Minn.), were used for subsequent procedures. Bacteria were also grown without aeration at 37 °C in dextrose phosphate broth (GIBCO Diagnostics, Madison, Wis.). Growth was monitored with a Coleman nephelometer.

Antibiotics and reagents. Ampicillin was obtained from Bristol Laboratories, Syracuse, N.Y., benzylpenicillin and cephalothin from Eli Lilly & Co., Indianapolis, Ind., and [*para*-³H]benzylpenicillin, ethylpiperidinium salt (26 Ci mmol⁻¹, 0·66 TBq mmol⁻¹) from E. O. Stapley, Merck, Sharp & Dohme, Rahway, N.J. Lysozyme was purchased from Millipore. M1-muramidase was the generous gift of K. Yokogawa, Dainippon Pharmaceutical Co. Ltd., Suita/Osaka, Japan, and was purified to homogeneity (Siegel *et al.*, 1981). Protein molecular weight standards were purchased from Bio-Rad.

Susceptibility tests. MIC values were determined by the standard broth dilution method in dextrose phosphate broth, pH 7·4. The inoculum was 10^5 to 10^6 c.f.u. ml⁻¹. The lowest concentration of antibiotic preventing visible growth after overnight incubation at 37 °C was recorded as the MIC.

Preparation of membranes. Exponential phase organisms were disrupted by sonication, and membranes recovered as previously described (Williamson *et al.*, 1980). Protein concentrations were estimated by the Lowry method with bovine serum albumin as standard.

Analysis of penicillin-binding proteins (PBPs). Membranes (about 100 μg protein) were incubated with increasing amounts (0·03 ng to 3·0 μg) of [³H]benzylpenicillin in a total volume of 50 μl 50 mM-sodium phosphate buffer, pH 7·0, at 37 °C for 10 min. An excess of unlabelled benzylpenicillin (600 μg) and 1% (w/v, final concentration) Sarkosyl NL-97 were then immediately added to each sample. In some experiments, membranes were incubated (10 min at 37 °C) with various amounts of either ampicillin or cephalothin, before further incubation with saturating amounts of the radioactive benzylpenicillin (0·32 μg for *S. bovis*, and 3·2 μg for *S. faecalis* and *S. faecium*). The samples were then analysed by slab SDS–PAGE (Williamson *et al.*, 1980), except that the final concentrations (w/v) of acrylamide and *N,N′*-bis-acrylamide in the stacking and separating gels were 3·0% and 0·036%, and 6·75% and 0·081%, respectively. The PBPs were detected by fluorography with exposure for 1–5 d, as described earlier (Williamson *et al.*, 1980) and quantitated by densitometry. The quantitative data tabulated in Tables 1 to 5 represent the mean values of three to five identical experiments.

The extent of breakdown of the benzylpenicillin-associated PBPs was measured using membranes exposed to [³H]benzylpenicillin for 10 min at 37 °C, 0·32 μg for *S. bovis* and 3·2 μg for *S. faecalis* and *S. faecium*; however, reaction mixtures were incubated for increasing lengths of time after addition of the unlabelled benzylpenicillin before the addition of Sarkosyl to terminate the reaction. In some experiments, either 0·14 M-2-mercaptoethanol or 0·2 M-hydroxylamine, pH 7·0, were added with the unlabelled benzylpenicillin.

Exponential phase organisms (1 ml, about 2×10^8 organisms ml⁻¹) were incubated with [³H]benzylpenicillin for 10 min at 37 °C. An excess of unlabelled benzylpenicillin (600 μg) was then added, and the samples immediately chilled in ice. Organisms were recovered by centrifugation (1100 *g* for 2 min at 2 °C) suspended in 50 μl of 50 mM-sodium phosphate buffer, pH 7·0, containing 10 μg lysozyme and 10 μg M1-muramidase, and incubated at 37 °C for 10 min. This resulted in complete lysis and samples were analysed as above. Lysis was not caused by either enzyme added alone. In some experiments, organisms were pretreated with various amounts of either ampicillin or cephalothin at 37 °C for 10 min before the addition of saturating amounts of [³H]benzyl-penicillin (3·6 μg for *S. bovis*, 4·8 μg for *S. faecalis*, and 11·2 μg for *S. faecium*).

To determine the availability of PBPs in whole organisms and lysed bacteria, samples (10 ml) of exponential phase cultures (about 2×10^8 organisms ml⁻¹) were rapidly chilled in ice and recovered by centrifugation (12000 *g* for 5 min at 2 °C). Supernates were removed by aspiration, and the bacteria resuspended in 350 μl ice-cold 50 mM-sodium phosphate buffer, pH 7·0, containing 2 M-sucrose, 5 mM-MgCl₂, and 5 μg DNAase. One set of samples of cell suspension (each 35 μl) received 20 μl sodium phosphate buffer and was incubated at 37 °C for 10 min, and for a further period of 15 min following addition to each sample of 15 μl of one of several concentrations of [³H]-benzylpenicillin; after addition of unlabelled benzylpenicillin (600 μg in 5 μl) to each sample together with lysozyme (10 μg in 10 μl) and M1-muramidase (10 μg in 10 μl) reaction mixtures were incubated at 37 °C for 10 min to lyse the organisms.

To produce protoplasts, duplicate samples of cell suspension (35 μl) received lysozyme (10 μg in 10 μl) and M1-muramidase (10 μg in 10 μl) and were incubated at 37 °C for 10 min. Virtually no whole organisms were seen by phase-contrast microscopy after this procedure. The protoplast samples (55 μl) were incubated at 37 °C for 15 min with various amounts of [³H]benzylpenicillin (15 μl), were then treated with an excess of unlabelled benzyl-

penicillin and incubation was continued at 37 °C for 10 min. All samples were immediately mixed with sample dilution buffer, without glycerol, after the final incubation period, and processed for SDS–PAGE.

The rate of binding of the [³H]benzylpenicillin to the PBPs in whole organisms and protoplasts was determined using the conditions described above except that the final concentration of the radioactive antibiotic was 8·6 µg ml⁻¹. The excess of the unlabelled benzylpenicillin (600 µg, 5 µl) was added to identical samples after 2, 4, 6, 8, 10 and 15 min incubation.

RESULTS

Six PBPs were detected in membranes from each of the three streptococcal strains, and the patterns of the PBPs were clearly different in each case (Fig. 1). When the membranes were incubated with a range of concentrations of the [³H]benzylpenicillin, the PBPs of *S. bovis* were detected at lower concentrations than those of *S. faecalis* or *S. faecium*. The MIC values (µg ml⁻¹) of benzylpenicillin for the three strains were: *S. bovis*, 0·036; *S. faecalis*, 4·8 and *S. faecium*, 37·2; and when the total amount of [³H]benzylpenicillin bound to the membranes was estimated (Fig. 2) there was a general trend indicating that the PBPs of *S. bovis* appeared to have higher affinities than those of *S. faecalis*, which in turn were higher than those of *S. faecium*. The interactions of [³H]benzylpenicillin were also examined with whole organisms and the concentration of the antibiotic required for 50% saturation (S_{50}) was calculated for each of the PBPs. As indicated in Table 1, the S_{50} values of individual PBPs (either in membranes or whole organisms) did not appear to correlate quantitatively with the relative resistance of each strain to the antibiotic. Only two PBPs (3 and 6) of *S. bovis* were detected in whole organisms of this
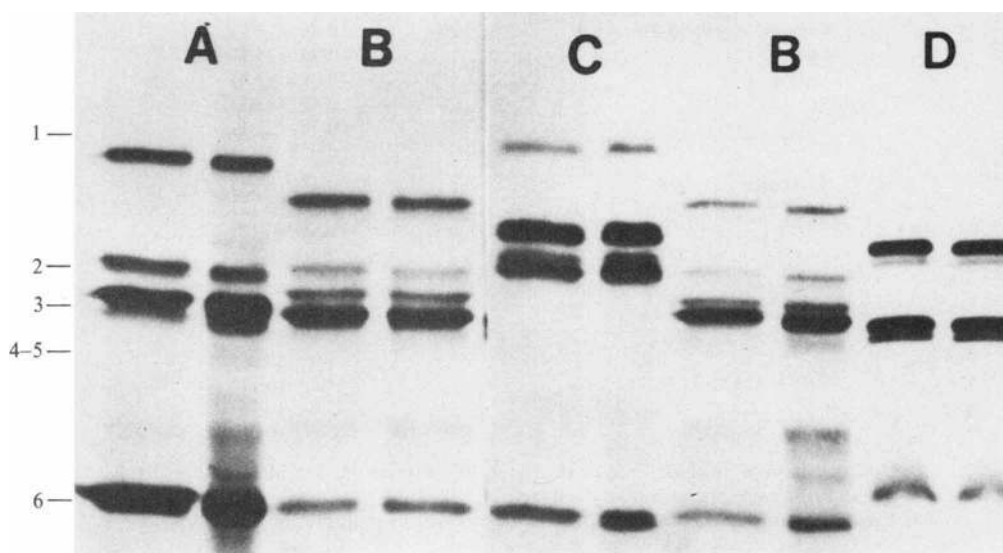

Fig. 1. Penicillin-binding proteins of group D streptococci. PBPs were labelled in whole organisms as described in Methods and applied to the gels in pairs. The left and right side sample in each pair was exposed to 1 ng and 100 ng [³H]penicillin ml⁻¹, respectively. Samples of the *S. faecalis* PBPs (B) were applied in duplicate. It is hoped that this geometric arrangement helps to compare the different relative positions of PBPs. A, *S. faecium* (4379); B, *S. faecalis* (447); C, *S. bovis* (4054). As reference, the PBPs of *S. pneumoniae* are also shown in D. Numbers 1 to 6 refer to the PBPs in the order of their decreasing molecular size. Molecular weight standards (Bio-Rad) were used to estimate the molecular sizes of the six PBPs of *S. faecalis*, which were as follows (in the order of PBP 1 to 6): 106000; 88000; 80000; 77000; 75000 and 42000. The molecular sizes of *S. faecium* PBPs were 126000; 91000; 86000; 79000; 77000 and 48000. PBP 2 (91000) is not well visible in the Figure. The molecular size of PBP 1 of *S. bovis* was estimated at about 130000. The fluorograms represent typical results obtained in a very large number (over 50) different experiments.
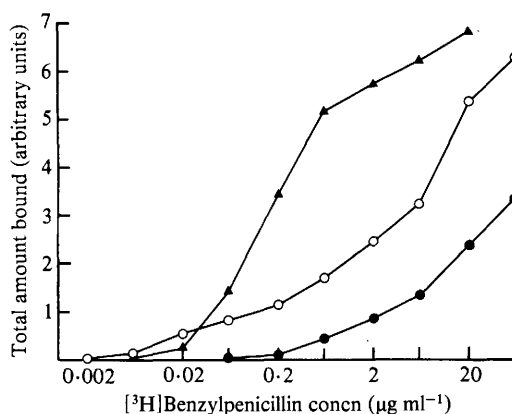
Fig. 2. Binding of [³H]benzylpenicillin to membranes. Membranes from each strain were exposed to various concentrations of [³H]benzylpenicillin as indicated in Methods. The data were derived from densitometric scanning of fluorograms. Data are from a single experiment representative of a number (10–15) of separate runs.

Table 1. *Affinities of PBPs for [³H]benzylpenicillin, examined in isolated membranes and whole organisms*

Organism	MIC (μg ml^{-1})	PBP no.	S_{50} (μg ml^{-1})* Membranes	S_{50} (μg ml^{-1})* Whole organisms
S. bovis	0·036	1	0·27	0·45 (12·5)‡
		2	0·105†	0·96 (26·7)
		3		0·085 (2·4)
		4	0·25†	0·47 (13·1)
		5		0·76 (21·1)
		6	0·35	0·053 (1·5)
S. faecalis	4·8	1	8·8	0·83 (0·17)
		2	2·1	0·56 (0·12)
		3	8·0	0·55 (0·11)
		4/5†	6·2	2·3 (0·48)
		6	0·017	0·62 (0·13)
S. faecium	37·2	1	1·5	1·1 (0·03)
		2	7·8	0·15 (0·00)
		3	1·7	1·0 (0·03)
		4	11·2†	4·2 (0·11)
		5		5·6 (0·15)
		6	4·3	0·9 (0·02)

* Concentration of antibiotic required to give 50% saturation. See Methods for details.
† These PBPs were not sufficiently resolved.
‡ Values in parentheses indicate S_{50} values expressed as multiples of the MIC.

strain when exposed to the [³H]benzylpenicillin at the MIC, whereas all of the PBPs were evident at the MIC in *S. faecalis* and *S. faecium*.

The loss of [³H]benzylpenicillin from the PBP complexes in membranes was also measured in each strain, and the calculated half-lives are presented in Table 2. All of the PBP complexes had observable, but relatively low, rates of breakdown. The addition of neutral hydroxylamine (nucleophilic donor) or 2-mercaptoethanol significantly increased the rates of loss of the antibiotic from all of the PBPs.

The interactions of unlabelled ampicillin and cephalothin with the PBPs were measured by pretreatment of either membranes or whole organisms with the antibiotics, followed by labelling of unbound PBPs with an excess of [³H]benzylpenicillin (Table 3). The lower antibacterial

Table 2. *Release of [³H]benzylpenicillin from the PBPs*

Organism	PBP no.	No addition	2-Mercaptoethanol	Hydroxylamine
		Time for 50% release of [³H]benzylpenicillin (min)		
S. bovis	1	117	28 (4·2)†	37 (3·2)†
	2/3*	119	57 (2·1)	89 (1·3)
	4/5*	189	71 (2·7)	99 (1·9)
	6	108	51 (2·1)	70 (1·5)
S. faecalis	1	189	23 (7·9)	18 (10·5)
	2	209	34 (6·1)	38 (5·5)
	3	107	17 (6·3)	30 (3·6)
	4/5*	593	149 (4·0)	158 (3·8)
	6	255	83 (3·1)	123 (2·1)
S. faecium	1	179	42 (4·3)	57 (3·1)
	2/3*	102	49 (2·1)	54 (1·9)
	4/5*	716	212 (3·4)	181 (4·0)
	6	236	ND	ND

ND, Not determined.

* These PBPs were not sufficiently resolved.

† Values in parentheses indicate extent of increase of rate of release of [³H]benzylpenicillin following treatment with 2-mercaptoethanol or hydroxylamine. See Methods for details.

Table 3. *Affinities of the PBPs of group D streptococci, examined in isolated membranes and whole organisms, for ampicillin and cephalothin*

Strain	PBP no.	MIC (nmol ml⁻¹)	I_{50} Membranes	I_{50} Whole organisms	MIC (nmol ml⁻¹)	I_{50} Membranes	I_{50} Whole organisms	Ratio Membranes	Ratio Whole organisms
		Ampicillin			Cephalothin			Ratio of I_{50} Cephalothin:I_{50} Ampicillin	
S. bovis		0·17			0·62 (3·6)‡				
	1		0·018	0·22		0·019	0·07	1·1	0·3
	2 ⎱		0·073†	0·31		0·17†	0·075	2·3	0·2
	3 ⎰			0·43			0·4		0·9
	4 ⎱		0·048†	0·19		0·59†	0·11	12·3	0·6
	5 ⎰			0·30			4·2		14·0
	6		0·023	0·21		1·40	7·8	60·9	37·0
S. faecalis		2·8			153·0 (55·0)‡				
	1		0·47	1·5		4·4	13·0	9·4	8·7
	2		0·19	0·09		0·12	0·35	0·6	3·9
	3		0·19	0·92		1·1	8·0	5·8	8·7
	4/5†		0·39	0·67		10·8	48·0	27·7	72·0
	6		254·0	22·0		19·7	54·0	0·1	2·5
S. faecium		22·5			1230·0 (55·0)				
	1		0·19	0·42		0·31	2·9	1·6	6·9
	2 ⎱		0·19†	0·064		0·17†	<0·05	0·9	<0·8
	3 ⎰			0·11			0·064		0·6
	4		1·4	1·9		111·0	160·0	79·3	84·0
	5		0·19	0·76		18·7	100·0	98·4	131·0
	6		1·7	7·2		123·0	1400·0	72·4	194·0

* Concentration of antibiotic required to give 50% inhibition of the subsequent binding of [³H]benzylpenicillin.

† These PBPs were not sufficiently resolved.

‡ Ratio of MIC of cephalothin to MIC of ampicillin.

effectiveness (higher MIC) of cephalothin in comparison with ampicillin for each organism was reflected by decreased affinities (higher I_{50} values) to one or more of the lower molecular weight PBPs; this decrease was observed whether the interactions were measured in isolated membranes or whole organisms.

Table 4. *Comparison of the binding of* [^{3}H]*benzylpenicillin to the PBPs of whole organisms and protoplasts*

| | | S_{50} (μg ml^{-1})* | |
| | PBP | Whole | |
Organism	no.	organisms	Protoplasts
S. bovis	1	0·78	0·82
	2	1·6	1·7
	3	0·17	0·19
	4	0·86	0·73
	5	1·3	1·4
	6	0·10	0·12
S. faecalis	1	1·1	0·95
	2	0·64	0·55
	3	0·80	0·91
	4/5†	2·8	3·2
	6	0·97	0·91
S. faecium	1	1·7	1·9
	2	0·24	0·23
	3	1·4	1·5
	4	7·5	6·0
	5	9·3	7·7
	6	1·4	1·6

* Concentration of [^{3}H]benzylpenicillin required to give 50% saturation. See Methods for details.
† These PBPs were not sufficiently resolved.

Table 5. *Rate of binding of* [^{3}H]*benzylpenicillin to the PBPs of whole organisms and protoplasts*

| | | Time for 50% saturation (min) | |
| | PBP | Whole | |
Organism	no.	organisms	Protoplasts
S. faecalis	1	3·9	3·6
	2/3*	2·7	2·5
	4/5*	3·5	3·5
	6	0·8	0·8
S. faecium	1	0·3	0·3
	2	0·3	0·3
	3	0·3	0·3
	4/5*	6·4	5·7
	6	1·0	0·8

* These PBPs were not sufficiently resolved.

In another series of experiments the accessibilities of the PBPs in whole organisms and protoplasts were compared in parallel. Titration of the [^{3}H]benzylpenicillin with samples of whole organisms or protoplasts derived from the same number of bacteria showed that equivalent amounts became bound to the PBPs at each concentration tested (Table 4). Moreover, the total amount bound under saturating conditions was essentially the same in each case (data not shown). The rates of binding of the [^{3}H]benzylpenicillin to the various PBPs of each organism under saturating conditions were also compared in whole organisms and protoplasts (Table 5), and again were virtually identical.

DISCUSSION

Several distinct mechanisms may be responsible for penicillin resistance in bacteria. The first mechanism, production of a β-lactamase, has not been identified in enterococci (Weinstein &

Moellering, 1975). A second mechanism of penicillin resistance involves the presence of a permeability barrier between the β-lactam antibiotic in the growth medium and the target enzymes in the bacterial cell membrane. Such a mechanism has recently been shown to be of major importance in the resistance of *Pseudomonas aeruginosa* to β-lactam antibiotics (Zimmerman, 1980). A third mechanism of penicillin resistance involves alteration of the affinities of the target enzymes. This mechanism of resistance is found in penicillin-resistant clinical isolates of *S. pneumoniae* (Hakenbeck *et al.*, 1980; Zighelboim & Tomasz, 1980), intrinsically penicillin-resistant *Neisseria gonorrhoeae* (Dougherty *et al.*, 1980), and methicillin-resistant *Staphylococcus aureus* (Hartman & Tomasz, 1981). We wished to distinguish decreased permeability to β-lactam antibiotics from diminished affinity of the target enzymes as the mechanism of penicillin resistance in enterococci.

The use of high specific activity [^{3}H]benzylpenicillin allowed sensitive labelling of PBPs both in isolated cell membranes and in actively growing bacterial cells. Using this reagent we characterized the PBPs of three strains of group D streptococci. Though each strain had six major PBPs, the pattern differed in each strain. The possibility of using PBP patterns for taxonomic purposes has already been suggested (Georgopapadakou & Liu, 1980*a*).

Comparisons of the PBP patterns of our *S. faecalis* and *S. faecium* strains with the PBP patterns of group D streptococci reported in the literature require comment because of taxonomic confusion. The strain of *S. faecalis* (447) used in this study showed the fermentation reactions typical of this species and exhibited the expected difference from *S. faecium* in taxonomic tests. The molecular weights of the PBPs in strain 447 of *S. faecalis* as well as in two other independent clinical isolates from the Veterans Administration Hospital, New York (un-published data) corresponded closely to those previously reported by Georgopapadakou & Liu (1980*b*), with the exceptions that we detected two PBPs (4 and 5) in the vicinity of the PBP 4 of *S. faecalis* SC 9011, and we did not detect the 35000 molecular weight protein. On the other hand, the molecular weights of PBPs from *S. faecalis* (447) or *S. faecalis* SC 9011 are clearly different from those described by Coyette *et al.* (1978) and Fontana *et al.* (1980) in *S. faecalis* ATCC 9790, which was very similar to the PBP pattern from *S. faecium* (4379) and another clinical isolate of this species (unpublished data). Strain ATCC 9790 was designated *S. faecium* var. *durans* by Smith (1970) and is listed in current editions of the American Type Culture Collection as *S. faecium* ATCC 9790; the PBP patterns are consistent with such a designation.

However, the MIC for benzylpenicillin of strain ATCC 9790 is low by comparison with most clinical isolates of *S. faecium*. Penicillin MIC values (μg ml^{-1}) for ATCC 9790 have been reported as 0·04 (at 45 °C in synthetic medium), 0·7 (at 37 °C in rich medium), and 4·0 (at 32 °C in synthetic medium). By comparison, *S. faecium* (4379) had a penicillin MIC of 37·2 μg ml^{-1} which is well within the range reported for most clinical isolates of this species (Moellering *et al.*, 1979). The MIC values of *S. faecalis* (447) and two strains of *S. faecalis* from the Veterans Administration Hospital (New York) were 4·8, 2·7 and 2·7 μg ml^{-1}, respectively, again within the range characteristic of isolates of this species. The anomalously low penicillin MIC value of strain ATCC 9790 is reminiscent of the behaviour of a penicillin-hypersensitive *S. faecium* mutant (G. Eliopoulos, C. Wennersten & R. C. Moellering, Jr, unpublished findings), in which the penicillin MIC was 0·06 μg ml^{-1} compared with 32 μg ml^{-1} for the parental strain.

In the present study, the molecular weights of PBPs observed in isolated cell membranes and in actively growing bacterial cells of the same strain were identical. On the other hand, significant differences were noted between the penicillin 'affinities' of PBPs as labelled *in vitro* (in membranes) versus *in vivo* (in growing cells) particularly in the case of the *S. faecalis* isolate. In general, substantially higher concentrations of [^{3}H]penicillin were needed to achieve half saturation of a PBP *in vitro* as compared to *in vivo*. This anomaly may be related to some alteration of the PBPs during membrane preparation or to the substantially higher concentration of membrane protein (20 μg ml^{-1}) used in the *in vitro* assays, relative to the corresponding membrane protein concentration in the *in vivo* labelling experiments (2 × 10^8 cells, or about 5–10 μg membrane protein ml^{-1}). A similar anomaly has been noted in other bacteria (Chase *et al.*, 1981; Gutmann *et al.*, 1981). In sharp contrast to the discrepancies described above, virtually identical PBP titration profiles were obtained when the PBPs were labelled in a parallel experiment utilizing equal numbers of intact cells and protoplasts.

All of the PBPs formed stable complexes with benzylpenicillin, with relatively long half-times of dissociation. The value of PBP 6 in *S. faecium* (4379) (236 min) correlates well with the value of 265 min for this PBP previously reported by Coyette *et al.* (1978). Mercaptoethanol and hydroxylamine stimulated the release of [^{3}H]penicillin from the PBP complexes.

Although there was a roughly inverse correlation between the MIC value and the total amount of benzylpenicillin bound to the streptococcal membranes exposed to the same concentration of [^{3}H]penicillin (Fig. 2), the S_{50} values of individual PBPs showed poor correlation with the MIC and there was variation both within a strain and between strains. In *S. bovis* (4054), for example, only 2 PBPs were detected at benzylpenicillin concentrations equal to the MIC. In *S. faecium* (447), on the other hand, all of the PBPs showed 50% binding at concentrations of benzyl-penicillin well below the MIC. Even in cultures of actively dividing whole cells there was no satisfactory correlation between S_{50} and MIC values of the strains.

Fontana *et al.* (1980) recently showed that the MIC of benzylpenicillin for ATCC 9790 (*S. faecium* var. *durans*) was 100-fold less when the strain was grown at 45 °C as compared to 32 °C. At this higher temperature only PBP 3 bound benzylpenicillin when incubated with a concen-tration equal to the lowered MIC. It was postulated, therefore, that PBP 3 may be the 'killing target' of this bacterium when grown at 45 °C. A unique killing site of benzylpenicillin for enterococci was not found in the present study. Indeed, the S_{50} values of all six PBPs of *S. faecium* were dramatically lower than the corresponding MIC. It seems likely that measurement of such S_{50} values, even in whole bacteria, does not yield a quantitative correlation with inhibition of cell growth by the antibiotic.

Recently, a strain of *S. faecium* sensitive to benzylpenicillin has been derived by novobiocin treatment of the same *S. faecium* strain used in these experiments (G. Eliopoulos, C. Wennersten & R. C. Moellering Jr, unpublished findings). Interestingly, the PBPs of the penicillin sensitive strain showed nearly identical penicillin binding to those of the penicillin resistant parent. In other words, the same degree of PBP saturation that was associated with inhibition of cell growth in one strain, had no measurable effect on the other. Still another example for the lack of direct, quantitative correlation between antibiotic binding to PBPs and the antibiotic sensitivity of the corresponding bacterium has been noted in studies with methicillin-resistant staphylococci (Hartman & Tomasz, 1981). The 1000-fold increase in the phenotypic methicillin sensitivity (MIC change from 3200 to 0·4 µg ml^{-1}) observable during growth of the methicillin resistant *S. aureus* in media with a low pH value, was not accompanied by the expected parallel change (increase) in PBP affinity for methicillin.

To further examine potential differences in PBP affinities between the three strains of group D streptococci, we investigated the effects of pretreatment of either isolated membranes or intact bacteria with either ampicillin or cephalothin; PBPs remaining unbound after the pre-treatment were detected by adding a saturating amount of [^{3}H]benzylpenicillin. These antibi-otics were chosen because the MIC values of cephalosporins for enterococci are much higher than those of ampicillin. Again there was poor correlation of the I_{50} values of individual PBPs with the ampicillin and cephalothin MIC values. For example, PBP 2 of *S. faecalis* showed high cephalothin affinity (as reflected in a low I_{50} value), despite the very high cephalothin MIC; this discrepancy has been previously noted by Georgopapadakou & Liu (1980 b). For other PBPs, however, particularly the lower molecular weight PBPs of the enterococci, cephalothin showed much poorer affinity. We expressed PBP binding for cephalothin in relation to ampicillin as the ratio of the respective I_{50} values. As seen in the last two columns of Table 3, the value of this ratio for certain lower molecular weight PBPs correlated roughly with the ratio of cephalothin to ampicillin MIC values. For example, in *S. faecium*, the I_{50} ratios for PBPs 4, 5 and 6 correlate roughly with the MIC ratio of 55. These data suggest the possibility that diminished cephalothin binding to selected lower molecular weight PBPs in group D streptococci may relate in a general way to its diminished antibacterial effectiveness in comparison with ampicillin.

When the same numbers of whole organisms and protoplasts were exposed to various concen-trations of [^{3}H]benzylpenicillin there was no difference in binding to individual PBPs at the con-centrations of penicillin tested. Furthermore, there were no differences in the total amount of [^{3}H]benzylpenicillin bound to each PBP at saturation (data not shown), nor in the half-times of

the rates of penicillin binding. These findings conclusively demonstrated that the bacterial cell wall of group D streptococci imposed no permeability barrier to the penetration of penicillin G. Gutmann *et al.* (1981) have recently documented the similar absence of a permeability barrier to penicillin in group A streptococci using parallel labelling of the PBPs in whole organisms and in crude lysates.

In summary, the experiments described seem to explode the possibility that variations in a permeability barrier of the bacterial wall are responsible for the high and varying MIC values of β-lactam antibiotics for group D streptococci. Our data indicate that the sensitivity to these antibiotics is related to the antibiotic affinities of the PBPs. However, a quantitative correlation between MIC values and corresponding PBP binding was not demonstrable in membrane preparations or during labelling of the PBPs in live bacteria.

While labelling of PBPs *in vivo* clearly represents a more realistic experimental design than labelling of isolated membrane preparations, it should be remembered that the MIC value is a function of antibiotic dose (i.e. concentration multiplied by time of exposure) rather than antibiotic concentration alone. It is conceivable that the establishment of a quantitative correlation between MIC and PBP saturation would require studies in which binding to the PBPs was determined during exposure times long enough to cause a morphological effect (Chase *et al.*, 1981), or inhibition of growth and peptidoglycan synthesis (R. Williamson & A. Tomasz, unpublished findings).

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