ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1982, p. 295-301
0066-4804/82/080295-07$02.00/0 Vol. 22, No. 2

Resistance to β-Lactam Antibiotics in *Streptococcus faecium*

GEORGE M. ELIOPOULOS,[1]* CHRISTINE WENNERSTEN,[1] AND ROBERT C. MOELLERING, JR.[1,2]

Department of Medicine, Harvard Medical School[2] and Infectious Disease Section, Department of Medicine, New England Deaconess Hospital,[1] Boston, Massachusetts 02215

Received 8 February 1982/Accepted 26 February 1982

Clinical isolates of *Streptococcus faecium* are characteristically resistant to β-lactam antibiotics. Two strains, selected for hypersusceptibility to penicillin, were derived from normally resistant isolates treated with novobiocin. These strains were also found to be hypersusceptible to other β-lactams. Differences in β-lactam susceptibility between the original isolates and the hypersusceptible strains could not be attributed to alterations in penicillin-binding protein affinities, and no evidence of a relative permeability barrier was found in the resistant strains. Isolated cell membranes prepared from resistant strains were found to possess two protein bands which were absent or greatly diminished in the membranes of susceptible strains. Hypersusceptibility to β-lactam antibiotics in these strains may be due to the absence or alteration of one or more cell membrane proteins distinct from the penicillin-binding proteins of these organisms.

In comparison with other streptococci, the enterococci are intrinsically more resistant to the inhibitory effects of β-lactam antibiotics. In addition, they are characteristically tolerant of the lethal effects of several agents which act of inhibit steps involved in cell wall synthesis (7). Among the enterococci, *Streptococcus faecium* is even more resistant to β-lactams than are strains of *Streptococcus faecalis*, with minimum inhibitory concentrations (MICs) of penicillin G often exceeding levels which can be achieved easily in the serum (11). To date, the mechanisms of this resistance to β-lactams among the enterococci in general, and of the even greater resistance of *S. faecium* in particular, are still incompletely understood, although evidence has been presented to suggest that such resistance may be due to the relatively low affinity of enterococcal penicillin-binding proteins (PBPs) for β-lactam antibiotics (4; R. Williamson, S. Calderwood, A. Tomasz, and R. C. Moellering, Jr., Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 714, 1980). The availability of two laboratory strains of *S. faecium* which are extraordinarily susceptible to a variety of β-lactam antibiotics in comparison with the clinical isolates from which they were derived provided an opportunity to explore further the nature of antibiotic resistance in this species.

(This work was presented in part at the 21st Interscience Conference on Antimicrobial Agents and Chemotherapy, 4 to 6 November 1981, Chicago, Ill.)

MATERIALS AND METHODS

Bacterial strains. From two clinical isolates of *S. faecium* (strains 4379 and 1140), penicillin-susceptible derivatives were obtained as follows: Approximately 10^5 colony-forming units (CFU) of each strain were inoculated into dextrose phosphate broth (DPB; GIBCO Diagnostics, Madison, Wis.) containing serial twofold dilutions of novobiocin in an attempt to cure these strains of a penicillin resistance determinant. After 24 h of incubation at 37°C, samples from tubes containing concentrations of novobiocin near the MIC were spread onto antibiotic-free agar. The approximately 200 to 300 colonies which appeared were replica plated onto DPB-agar containing penicillin G (1 μg/ml), and those colonies which failed to produce growth on the penicillin plates were selected. These penicillin-susceptible strains, labeled 4379-S and 1140-S, were recovered at a rate of 0.3 and 0.5%, respectively, of the colonies plated. Both strains were identified as *S. faecium* by standard methods (3). The derived strains resembled the parent isolates in colony morphology, appearance of Gram stain, and doubling time in the log phase of growth. Neither the parent isolates nor the penicillin-susceptible strains produced detectable β-lactamase, as determined by the method of O'Callaghan et al. (12). Despite multiple passes on blood agar and freezing at −70°C, there was no tendency toward loss of penicillin susceptibility.

Strain 1140-B, which has been described previously (17), was derived from strain 1140 by curing it of a 40,000-molecular-weight plasmid with novobiocin, resulting in the loss of high-level resistance to streptomycin and kanamycin, with no change in penicillin susceptibility compared with the parent isolate.

Antibiotics and chemicals. Benzyl-[^{3}H]penicillin, ethylpiperidinium salt (53.5 mCi/mg), was a generous gift of the Merck Sharp & Dohme Research Labora-

TABLE 1. Antimicrobial susceptibility of *S. faecium* strains 4379, 4379-S, 1140, and 1140-S

Antibiotic	MIC (μg/ml) for strain:			
	4379	4379-S	1140	1140-S
Penicillin G	32	0.06	16	0.125
Ampicillin	16	0.06	16	0.125
Cloxacillin	>128	4	>128	4
Mecillinam	>1,000	8	>1,000	250
N-Formimidoyl-thienamycin	32	0.125	64	0.25
Cephalothin	64	0.125	250	2
Cephalexin	500	125	>128	32
Cefaclor	>128	64	>128	32
Cycloserine	500	64	1,000	125
Vancomycin	1	1	2	2
Bacitracin[a]	1	2	2	2
Erythromycin	0.25	0.125	250	250
Clindamycin	0.5	1	>128	>128
Chloramphenicol	8	8	8	4
Gentamicin	16	8	8	4
Tobramycin	1,000	250	1,250	1,250
Novobiocin	4	2	1	4
Coumermycin	125	64		
Oxolinic acid	125	250	125	125
Acriflavin	16	8	16	16
Nitrofurantoin	250	32	250	64

[a] MICs of bacitracin are given in units per milliliter.

tories, Rahway, N.J., which also supplied *N*-formimidoyl thienamycin. Other antibiotics were provided as follows: penicillin G, cephalexin, cefaclor, and cycloserine, Eli Lilly and Co., Indianapolis, Ind.; cloxacillin and ampicillin, Bristol Laboratories, Syracuse, N.Y.; clindamycin, bacitracin, and novobiocin, Upjohn Corp., Kalamazoo, Mich.; mecillinam, Hoffmann-LaRoche, Inc., Nutley, N.J.; oxolinic acid, Warner-Lambert Research Institute, Morris Plains, N.J.; chloramphenicol, Parke, Davis & Co., Detroit, Mich. Also, erythromycin lactobionate was obtained from Abbott Laboratories, Chicago, Ill.; tobramycin sulfate and cephalothin, Eli Lilly; gentamicin sulfate, Schering Corp., Bloomfield, N.J.; acriflavin, Sigma Chemical Co., St. Louis, Mo.; nalidixic acid, Calbiochem, La Jolla, Calif.; and nitrofurantoin, Norwich-Eaton Pharmaceuticals, Norwich, N.Y.

Chemicals for the preparation of polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Calif.; 2,5-diphenyloxazole was purchased from New England Nuclear Corp., Boston, Mass. Other laboratory reagents were purchased from several commercial sources.

Susceptibility testing. Antimicrobial susceptibility was determined in DPB by a standard broth dilution technique for most of the antibiotics (16). An inoculum (10^5 CFU/ml) of log-phase organisms was used except when testing novobiocin, when an inoculum of 10^3 CFU/ml was employed since the higher inoculum resulted in faint turbidity even in high concentrations of the drug. MICs were determined by visual inspection for lack of turbidity after 24 h of incubation at 37°C. Samples of 0.01 ml each from each clear tube were streaked onto blood agar (GIBCO), and the minimum bactericidal concentration was determined at the 99.9% killing level. Susceptibility to some antibiotics was determined by agar plate dilution (16) on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) with an inoculum of 10^4 CFU of each organism.

Membrane preparation. One-liter cultures of organisms in DPB were grown at 37°C with agitation until a cell density of 10^8/ml was reached, at which point the cells were recovered by centrifugation, washed in iced 10 mM potassium phosphate buffer (pH 7.0), and suspended in 5 ml of buffer. Cells were disrupted by fifteen 20-s cycles of sonication with a Branson S75 Sonifier, allowing 60 s for cooling between cycles. This and subsequent steps were carried out at 4°C. The remaining unbroken cells and large fragments were removed by centrifugation at 1,000 × *g* and 12,000 × *g*, respectively. The cell membranes were then pelleted and washed twice in buffer at 100,000 × *g* for 120 min in a Beckman model L ultracentrifuge. Membranes were suspended in 1 ml of buffer, and the protein concentration was determined by the method of Lowry et al. (9). Membrane suspensions were then stored at −70°C until used.

Studies of PBPs. (i) Isolated cell membranes. A volume of membrane suspension containing 150 to 200 μg of protein and 10 μl of various concentrations of [^{3}H]penicillin in 50 mM potassium phosphate buffer (pH 7.0) were added to glass tubes containing enough buffer to achieve a final volume of 50 μl. The tubes were incubated at 37°C for 10 min to allow the labeled penicillin to be bound. The reaction was then stopped by the addition of an excess of unlabeled penicillin (120 mg/ml in water) in a volume of 5 μl. Sample dilution buffer (30 μl) containing 0.2 M Tris buffer (pH 6.8), 3% sodium dodecyl sulfate, 30% glycerol, 16% mercaptoethanol, and 0.002% bromphenol blue was added, and the mixture was immediately heated to 100°C for 5 min. The sample was then applied to a polyacrylamide slab gel. Apparent molecular weights

of the PBPs were determined by comparison with molecular weight standards purchased from Bio-Rad Laboratories.

(ii) Intact cells. Bacteria were grown in 20 ml of DPB at 37°C with agitation to a density of 10^8 CFU/ml. The cell suspension (1 ml) was incubated with 10 μl of [^{3}H]penicillin at various concentrations at 37°C for 10 min, and the reaction was stopped by the addition of excess cold penicillin. The cells were then pelleted and suspended in 40 μl of 50 mM potassium phosphate buffer (pH 7.0). Lysis was accomplished by the addition of 5 μl of lysozyme (6 mg/ml) and 5 μl of Triton X-100 (0.1%) followed by incubation at 37°C for 20 min. Sample dilution buffer (30 μl) was added, and the specimen was heated to 100°C for 5 min and then applied to the gel.

(iii) Binding of other β-lactams to PBPs. Samples of membranes or intact cells were first incubated with 10 μl of various concentrations of β-lactam antibiotics at 37°C for 10 min. At that point, [^{3}H]penicillin was added to achieve a final concentration of 8 μg/ml (an amount shown to give good visualization of all PBPs), and the samples were incubated for an additional 10 min to allow binding to any PBPs not saturated with other β-lactams. Excess cold penicillin was then added, and the samples were processed as indicated above for membranes or intact cells.

(iv) Deacylation of PBPs. To study the rate of spontaneous loss of labeled penicillin from PBPs, we first incubated the membranes with [^{3}H]penicillin (8 μg/ml) at 37°C for 10 min. Excess unlabeled penicillin was added, and 50-μl samples containing approximately 200 μg of membrane protein were removed at intervals over 60 min to determine residual binding of labeled penicillin. Specimens were treated with sample dilution buffer and processed as indicated above.

Polyacrylamide gel electrophoresis and fluorography. A discontinuous sodium dodecyl sulfate-polyacrylamide slab gel system as described by Laemmli and Favre (8) was used to separate cell membrane proteins. Acrylamide concentrations of either 7.5 or 10% were used in the separating gel and 3.5% in the stacking gel. *N,N'*-methylenebisacrylamide was added to acrylamide in a ratio of 1:75. Electrophoresis was carried out at 50 V through the stacking gel and then at 90 V until the marker dye reached the end of the separating gel. Gels were stained for the inspection of membrane proteins with Coomassie brilliant blue dye and destained with 30% methanol–5% acetic acid. Fluorography was performed by the method of Bonner and Laskey (1). Kodak X-Omat AR-5 film was presensitized and exposed to the dried gel for 3 to 7 days at −70°C. Intensity of the resulting bands was measured with a scanning densitometer (E-C Apparatus Company).

RESULTS

Susceptibility studies. MICs of several antibiotics against the four strains of *S. faecium* are shown in Table 1. Strains 4379-S and 1140-S were several fold more susceptible than were the parent isolates to each of the β-lactam antibiotics tested. However, the penicillin-susceptible and penicillin-resistant strains did not differ in susceptibility to a number of the other antibiotics, including vancomycin, bacitracin, erythromycin, chloramphenicol, and gentamicin. Exposure of cultures to novobiocin during the selection process did not result in the acquisition of significant levels of resistance to this agent by the laboratory strains. The ratio of the minimal bactericidal concentration to the MIC for penicillin, cephalothin, and *N*-formimidoyl thienamycin were ≥32 for both of the susceptible strains, indicating that loss of intrinsic resistance to these drugs was not accompanied by loss of tolerance to their bactericidal effects.

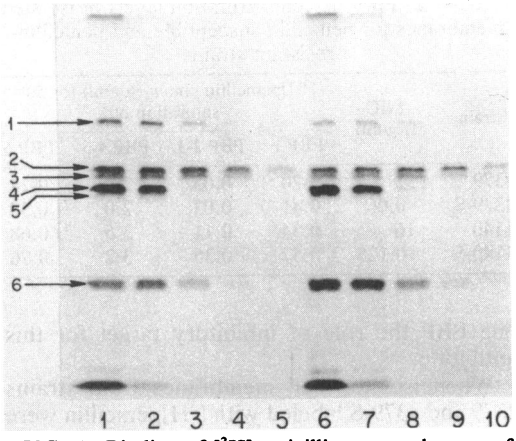

FIG. 1. Binding of [^{3}H]penicillin to membranes of penicillin-susceptible (4379-S, slots 1–5) and penicillin-resistant (4379, slots 6–10) strains. Penicillin concentrations: slots 1 and 6, 32 μg/ml; slots 2 and 7, 8.0 μg/ml; slots 3 and 8, 0.24 μg/ml; slots 4 and 9, 0.06 μg/ml; slots 5 and 10, 0.015 μg/ml.

Binding of [^{3}H]penicillin to isolated membranes. Six PBPs were identified in all strains, labeled 1 through 6, with apparent molecular weights of 130,000, 94,000, 86,000, 78,000, 76,000 and 47,000, respectively. Over a wide range of penicillin concentrations, similar patterns of binding were observed for both penicillin-susceptible and penicillin-resistant strains (Fig. 1). In analyzing densitometer tracings of fluorograph bands, it was not possible to separate consistently PBP 2 from 3 or PBP 4 from 5; therefore, each of these pairs was considered as a complex (PBP 2-3 and PBP 4-5). Concentrations of penicillin resulting in 50% saturation of each PBP complex relative to the amount bound at penicillin concentrations of 8 μg/ml were similar for the penicillin-susceptible and penicillin-resistant strains of each pair (Table 2). At penicillin concentrations near the MICs of the susceptible strains, PBP 2 accounted for the majority of bound [^{3}H]penicillin. However, since lower but still detectable binding by other PBPs occurred even at these low concentrations of penicillin, it was not possible to ascribe to any

TABLE 2. Fifty-percent saturation levels of isolated membranes for penicillin-susceptible and penicillin-resistant strains

Strain	MIC (μg/ml)	[^{3}H]penicillin concn (μg/ml) for 50% saturation of:			
		PBP 1	PBP 2-3	PBP 4-5	PBP 6
4379	32	0.20	0.05	2.0	0.24
4379-S	0.06	0.41	0.03	2.0	0.30
1140	16	0.54	0.11	3.5	0.68
1140-S	0.125	0.57	0.35	3.2	0.76

one PBP the role of inhibitory target for this antibiotic.

When isolated cell membranes from strains 4379 and 4379-S labeled with [^{3}H]penicillin were examined at intervals up to 60 min after the addition of excess cold penicillin, no significant spontaneous loss of bound penicillin from any PBP could be detected for either strain.

Binding of [^{3}H]penicillin to intact cells. PBP patterns obtained when intact cells of the penicillin-susceptible strains were incubated with various concentrations of [^{3}H]penicillin were nearly identical to those produced by the normally resistant parent isolates. Figure 2 shows the results obtained with strains 1140 and 1140-S. Moreover, the patterns noted upon labeling the intact cells were roughly comparable to those observed with membrane preparations exposed to the same concentration of penicillin.

For strains 4379 and 4379-S incubated with a concentration of labeled penicillin equal to the MIC of the latter strain, the total amounts of penicillin bound to the PBPs of each strain were almost identical (expressed in arbitrary units of band density: 19 versus 21, respectively). It was not possible to accurately measure by densitometry the faint binding to the PBPs of strains 1140 and 1140-S at the MIC of the latter, but by inspection PBP 2 produced the densest bands, with faint visualization of PBPs 1, 3, and 6 in both strains. Thus, no evidence for a major difference in the access of penicillin to the PBPs of susceptible and resistant strains was found. At high concentrations of penicillin (32 μg/ml), the total amount of penicillin bound by the cells of strain 4379 was approximately twice as high as that bound by 4379-S. At the same concentration, strains 1140 and 1140-S bound approximately equal amounts of penicillin as determined by measurement of absolute band density.

Competition studies. Lacking other radiolabeled β-lactam antibiotics, we assessed the binding of other penicillins and cephalosporins to the PBPs of our organisms by measuring the amount of [^{3}H]penicillin binding activity which remained after preincubation with these agents (Fig. 3).

Competition studies were performed with isolated cell membranes to determine whether the differences in β-lactam susceptibilities observed in the paired strains resulted from differences in PBP affinities. There was no evidence that any PBP of either susceptible strain demonstrated significantly increased affinity for these antibiotics as compared with those of the penicillin-resistant clinical isolates (Table 3). Visual inspection of fluorograms indicated that mecillinam bound preferentially to PBP 2 until high concentrations of the drug were reached. Ampicillin bound to PBPs 2 and 3 to the greatest extent, but even at low concentrations signifi-

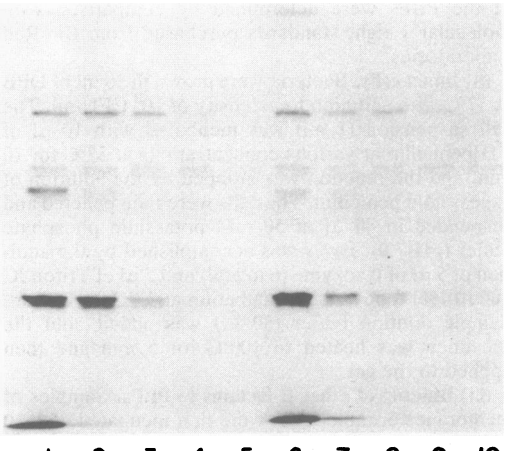

FIG. 2. Binding of [^{3}H]penicillin to intact cells of penicillin-resistant (1140, slots 1–5) and penicillin-susceptible (1140-S, slots 6–10) strains. Penicillin concentrations: slots 1 and 6, 32 μg/ml; slots 2 and 7, 8.0 μg/ml; slots 3 and 8, 2.0 μg/ml; slots 4 and 9, 0.5 μg/ml; slots 5 and 10, 0.125 μg/ml.

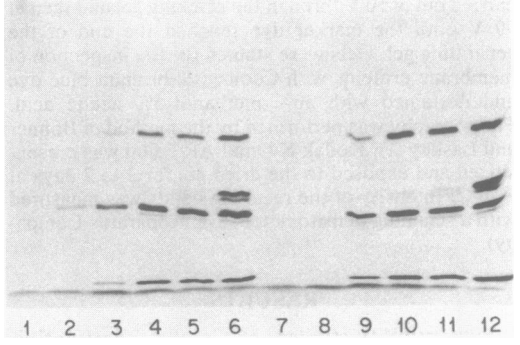

FIG. 3. Competition between ampicillin and [^{3}H]penicillin for PBPs of penicillin-resistant (1140, slots 1–6) and penicillin-susceptible (1140-S, slots 7–12) strains. Ampicillin concentrations: slots 1 and 7, 16 μg/ml; slots 2 and 8, 2.0 μg/ml; slots 3 and 9, 0.5 μg/ml; slots 4 and 10, 0.125 μg/ml; slots 5 and 11, 0.06 μg/ml; slots 6 and 12, no ampicillin. All samples were incubated with [^{3}H]penicillin (8 μg/ml).

TABLE 3. Concentrations of antibiotics resulting in 50% inhibition of subsequent binding by [^{3}H]penicillin in isolated membranes

Antibiotic	Strain	MIC (μg/ml)	I_{50} (μg/ml)			
			PBP 1	PBP 2-3	PBP 4-5	PBP 6
Mecillinam	4379	>1000	630	4.5	>2000	800
	4379-S	8	>2000	50	>2000	>2000
	1140	>1000	>1000	89	>1000	>1000
	1140-S	250	>1000	130	>1000	>1000
Ampicillin	1140	16	0.18	0.03	0.44	0.25
	1140-S	0.125	0.29	0.03	0.57	0.38
Cephalothin	4379	64	0.96	0.01	>1.3	>1.3
	4379-S	0.125	1.5	0.17	>1.3	>1.3
Cephalexin	1140	>128	130	1.6	560	400
	1140-S	32	420	1.6	400	250

cant binding to the remaining PBPs was evident. PBP 3 appeared to have the greatest affinity for cephalothin and cephalexin, whereas PBPs 4, 5, and 6 showed little binding except at high concentrations.

Results of competition studies with intact cells are shown in Table 4. Concentrations of the various antibiotics resulting in 50% inhibition of subsequent binding by [^{3}H]penicillin (I_{50} values) for PBP 2-3 were identical in penicillin-susceptible and penicillin-resistant strains for each drug. Although some differences in I_{50} values for the other PBPs were found, these were small in comparison with the differences in MICs between the susceptible and resistant strains. Concentrations of ampicillin, cephalothin, and cephalexin near the MICs of the resistant strains inhibited subsequent binding of labeled penicillin to PBPs 1 and 2-3 by >90%, with smaller but detectable degrees of inhibition of binding to the remaining PBPs. In contrast, at concentrations near the MICs of susceptible strains, substantial binding of these drugs was limited to the PBP 2-3 complex.

Membrane proteins of susceptible and resistant cells. Inspection of polyacrylamide gels stained with Coomassie brilliant blue revealed two distinct bands in isolated cell membranes of strains 4379 and 1140 which were absent or greatly diminished in preparations of the penicillin-susceptible strains (Fig. 4). These protein bands, with apparent molecular weights of 108,000 and 82,000, could not be distinguished on stained gels of lysed whole cell preparations. Absence of the high-molecular-weight band was also noted in membranes prepared from strain 1140-B. However, in that strain the intensity of staining of the lower-molecular-weight band was actually increased relative to that of the parent isolate. Strain 1140-B also differed from the parent strain and from 1140-S with respect to several additional membrane protein bands.

DISCUSSION

Although *S. faecium* accounts for a minority of enterococcal infections in humans (15), several properties of this species are relevant to the treatment of such infections. Since most clinical isolates are not killed by cell wall-active antibiotics at concentrations attainable in the serum (7), synergistic combinations of these agents with an aminoglycoside are required for treatment of serious infections against which bactericidal therapy is important. However, because of the characteristic resistance of these organisms to penicillin, high concentrations of this drug are frequently necessary to demonstrate synergism in vitro, suggesting that relatively high serum levels must be achieved to assure adequate killing (11).

The ability to select strains of *S. faecium*

TABLE 4. Concentrations of antibiotics resulting in 50% inhibition of subsequent binding by [^{3}H]penicillin in intact cells

Antibiotic	Strain	MIC (μg/ml)	I_{50} (μg/ml)			
			PBP 1	PBP 2-3	PBP 4-5	PBP 6
Ampicillin	1140	16	0.35	0.03	1.2	4.9
	1140-S	0.125	0.50	0.03	0.57	5.7
Cephalexin	1140	>128	71	1.6	400	>1000
	1140-S	32	250	1.6	71	>1000
Cephalothin	4379	64	0.30	0.01	110	>250
	4379-S	0.125	>0.50	0.01	22	126

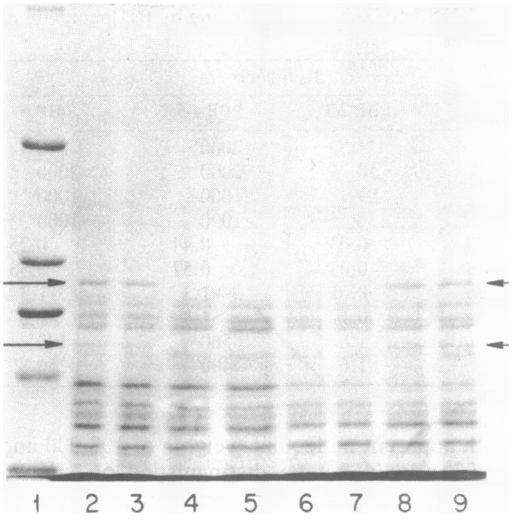

FIG. 4. Coomassie brilliant blue-stained gel of membrane proteins from penicillin-resistant (4379, slots 2 and 3; 1140, slots 8 and 9) and penicillin-susceptible (4379-S, slots 4 and 5; 1140-S, slots 6 and 7) strains of *S. faecium*. Portions of membrane preparations containing 300 μg of protein were treated as described in the text and applied to the gel in duplicate. Arrows indicate protein bands seen only in membranes of resistant cells. Molecular weight standards are shown in slot 1 (top to bottom: 200,000, 116,250, 92,500, 66,200, and 45,000).

which are hypersusceptible to β-lactam antibiotics provides a unique system for the study of the mechanisms of intrinsic β-lactam resistance in this species. We chose to begin this investigation by examining the PBPs of these strains, since alterations in the patterns or affinities of β-lactam binding to these cell membrane proteins have been demonstrated in both penicillin-resistant pneumococci (5, 13, 18) and methicillin-resistant *Staphylococcus aureus* (6) when compared with susceptible strains.

It was apparent that over a wide range of concentrations the patterns of binding of [^{3}H]penicillin to the PBPs of isolated membranes of the penicillin-susceptible strains of *S. faecium* were strikingly similar to those of the parent penicillin-resistant isolates. Despite the fact that densitometric measurement was limited by the inability to consistently separate PBP 2 from 3 and PBP 4 from 5 and by the potential inaccuracies in scanning low-intensity bands, numerical analysis supported the conclusion that the marked differences in the MICs of β-lactams between susceptible and resistant cells could not be attributed to differences in PBP affinities for the various antibiotics. Furthermore, the lack of any demonstrable loss of bound penicillin over 60 min in either the susceptible or resistant strain suggested that differences in susceptibility were not due to differences in rates of spontaneous deacylation.

The observation that MICs of several non-β-lactam antibiotics were identical for the penicillin-susceptible and penicillin-resistant members of each pair of strains provided evidence against the existence of nonspecific hyperpermeability in strains 4379-S and 1140-S at the cell membrane level. However, because at least some of the PBPs of bacteria are presumably located at the outer surface of the cell membrane (14), lack of difference in cell permeability would not prove the absence of altered access to individual PBPs in our strains. The finding that the [^{3}H]penicillin binding patterns of intact penicillin-resistant cells were similar to those of penicillin-susceptible cells does indicate, however, that no significant permeability barrier to the PBPs exists in the resistant strains. Further, this conclusion is supported by the results of competition studies which showed identical I_{50} values for several antibiotics for PBP 2-3 in susceptible and resistant strains. In some cases, the I_{50} values for PBPs of penicillin-susceptible cells were actually higher than those for the corresponding resistant strains. The significance of this observation is not clear.

The nature of the defects leading to hypersusceptibility to β-lactams in strains 4379-S and 1140-S remains obscure. Since these strains are not more susceptible to vancomycin and bacitracin in comparison with the parent strains, they do not appear to have a nonspecific defect in cell wall synthesis. We do not have a satisfactory explanation for the heightened susceptibility of the laboratory strains to cycloserine, but this finding does appear to point to an abnormality in the cell wall structure.

The marked differences in penicillin MICs in the absence of altered PBP affinities between penicillin-resistant and -susceptible strains of *S. faecium* recall the previous studies by Hartman and Tomasz (6), which showed that for methicillin-resistant *Staphylococcus aureus* growth conditions which permit markedly enhanced susceptibility to methicillin (i.e., pH 5.2) are not associated with alterations in PBP affinities for this drug. Those events which link the binding of β-lactams to PBPs of bacteria to the subsequent inhibition of growth are still not well understood. Therefore, we cannot explain at this time why strains 4979-S and 1140-S were inhibited by concentrations of penicillin which substantially saturated only the PBP 2-3 complex. It is tempting to speculate that the hypersusceptibility of these strains may be related to an alteration of one or more cell membrane proteins as observed on Coomassie blue-stained gels. The disappearance of the protein band of molecular weight

108,000 from the cell membranes of strains 4379-S and 1140-S does not appear to be specifically related to loss of penicillin resistance, since this band is also absent from the membranes of strain 1140-B, which is normally penicillin resistant. The lower-molecular-weight band was, however, diminished only in the penicillin-susceptible strains. Elucidation of the role of these protein bands in normal cells may provide valuable insight into the mechanisms of intrinsic β-lactam resistance in *S. faecium*.

Novobiocin was employed in the process of isolating the penicillin-susceptible strains described here because it had been shown to cure plasmids mediating high-level aminoglycoside resistance in *S. faecium* (17). Preliminary experiments in this laboratory suggest that penicillin susceptibility in strains 4379-S and 1140-S is not associated with the loss of plasmid bands (unpublished data). Penicillin susceptibility also does not appear to be due to the acquisition of resistance to novobiocin or to the related compound coumermycin A1. Although the role of novobiocin as a mutagen has not been clearly established, strains of *Escherichia coli* with mutations resulting in abnormal DNA gyrase (the enzyme inhibited by novobiocin) show increased mutation rates after exposure to UV light (2, 10). Thus, it seems possible that in these experiments novobiocin acted as a mutagen.

While the penicillin-susceptible strains we described here are almost certainly unnatural in that they are inhibited by concentrations of β-lactam antibiotics below which significant saturation of all PBPs occurs, their similarity to the original clinical isolates in terms of PBP affinities and accessibility will permit their future use in studies exploring the relationships between penicillin binding, inhibition of growth, and ultimate cell death.

ACKNOWLEDGMENT

This work was supported in part by Public Health Service training grant no. 5T-32-AI-07061-05 to G.M.E.

LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
2. **Crumplin, G. C.** 1981. The involvement of DNA topoisomerases in DNA repair and mutagenesis. Carcinogenesis **2**:157–160.
3. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. **23**:1131–1139.
4. **Georgopapadakou, N. H., and F. Y. Liu.** 1980. Binding of β-lactam antibiotics to penicillin-binding proteins of *Staphylococcus aureus* and *Streptococcus faecalis*: relation to antibacterial activity. Antimicrob. Agents Chemother. **18**:834–836.
5. **Hakenbeck, R., M. Tarpay, and A. Tomasz.** 1980. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17**:364–371.
6. **Hartman, B., and A. Tomasz.** 1981. Altered penicillin-binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **19**:726–735.
7. **Krogstad, D. J., and A. R. Parquette.** 1980. Defective killing of enterococci: a common property of antimicrobial agents acting on the cell wall. Antimicrob. Agents Chemother. **17**:965–968.
8. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–599.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **McCoy, E. C., L. A. Petrullo, and H. S. Rosenkranz.** 1980. Nonmutagenic genotoxicants: novobiocin and nalidixic acid, 2 inhibitors of DNA gyrase. Mutat. Res. **79**:33–43.
11. **Moellering, R. C., Jr., O. M. Korzeniowski, M. A. Sande, and C. B. Wennersten.** 1979. Species-specific resistance to antimicrobial synergism in *Streptococcus faecalis*. J. Infect. Dis. **140**:203–208.
12. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
13. **Percheson, P. B., and L. E. Bryan.** 1980. Penicillin-binding components of penicillin-susceptible and -resistant strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **18**:390–396.
14. **Storm, D. R., P. M. Blumberg, and J. L. Strominger.** 1974. Inhibition of the *Bacillus subtilis* membrane-bound D-alanine carboxypeptidase by 6-aminopenicillanic acid covalently coupled to Sepharose. J. Bacteriol. **117**:783–785.
15. **Toala, P., A. McDonald, C. Wilcox, and M. Finland.** 1969. Susceptibility of group D streptococcus (enterococcus) to 21 antibiotics in vitro, with special reference to species differences. Am. J. Med. Sci. **258**:416–430.
16. **Washington, J. A. II, and V. L. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453–458. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
17. **Wennersten, C. B., and R. C. Moellering, Jr.** 1980. Mechanism of resistance to penicillin-aminoglycoside synergism in *Streptococcus faecium*, p. 710–712. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease. American Society for Microbiology, Washington, D.C.
18. **Zighelboim, S., and A. Tomasz.** 1980. Penicillin-binding proteins of multiply antibiotic-resistant South African strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17**:434–442.