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Penicillin-binding proteins in *Streptococcus faecalis* and *S. faecium*

H. Y. CHEN and J. D. WILLIAMS

Department of Medical Microbiology, The London Hospital Medical College, London E1 2AD

Summary. Penicillin-binding proteins (PBPs) of *Streptococcus faecalis* NCTC 775, *S. faecium* NCTC 7171 and an isolate of *S. faecium* (strain 37) highly resistant to β-lactam antibiotics were visualised by autoradiography. Five PBPs were detected in *S. faecalis* NCTC 775 and six in *S. faecium* NCTC 7171. Additional PBPs could not be found in the resistant isolate of *S. faecium*.

The PBP affinities of β lactams were compared with MIC values. The affinities of PBPs 3 and 4 of *S. faecalis* NCTC 775 for penicillin G, ampicillin, cefathiamidine, cephaloridine and cephazolin were related to the sensitivity of the strain to these antibiotics as were the affinities of PBPs 4 and 5 in each *S. faecium* strain for the β lactams. It is postulated that PBPs 3 and 4 of *S. faecalis* NCTC 775 and PBPs 4 and 5 of *S. faecium* are the relevant target enzymes of the test antibiotics. PBPs 4 and 5 of the highly β-lactam-resistant *S. faecium* strain 37 showed proportionally low affinities for the five β lactams compared to that of the less resistant strain *S. faecium* NCTC 7171. Decreased affinities of PBPs 4 and 5 may account for the resistance in *S. faecium* strain 37 to β lactams. The affinities for PBP 1, 2 and 5 in *S. faecalis* NCTC 775 and PBPs 1, 2, 3 and 6 in *S. faecium* were not related to the antibiotic sensitivities.

Introduction

Three basic enzymes that are involved in synthesis of the cell wall are known to be sensitive to the action of penicillin. These are transpeptidases, carboxypeptidases and endopeptidases (Blumberg and Strominger, 1974; Strominger *et al.*, 1974). Penicillin-binding proteins (PBPs) can be detected and quantified by an autoradiographic method (Spratt and Pardee, 1975; Georgopapadakou and Liu, 1980*a*), but their precise functions in cell growth and β-lactam activity remain unclear.

The role of PBPs of *Escherichia coli* and some other gram-negative species in growth, morphogenesis and β-lactam activity has been described to some extent (Spratt and Pardee, 1975; Spratt, 1983). PBPs of gram-positive bacteria have been studied less often than those of gram-negative bacteria. Alteration of PBPs has been reported in methicillin-resistant strains of *Staphylococcus aureus* (Hayes *et al.*, 1981; Wyke *et al.*, 1982; Ubukata *et al.*, 1985) and in penicillin-resistant *Streptococcus pneumoniae* (Hakenbeck *et al.*, 1980; Zighelboim and Tomasz, 1980).

The common mechanisms of resistance of bacteria to β-lactam antibiotics are the production of β lactamases that destroy the β lactams, the reduced penetration of β lactams through the outer membrane of gram-negative species (Nikaido, 1981), and alteration of affinity of PBPs for β lactams. Enterococci rarely produce β lactamase (Matthew and Harris, 1976; Murray and Mederski-Samaroj, 1983); there is no outer-membrane barrier, and no evidence that the peptidoglycan layer is a significant barrier to β lactams (Williamson *et al.*, 1983). Therefore, changes in the affinity of PBPs could have an important role in the mechanism of resistance to β lactams.

Five PBPs have been found in *Streptococcus faecalis* and six in *S. faecium* (Georgopapadakou and Liu, 1980*b*; Eliopoulos *et al.*, 1982; Williamson *et al.*, 1983). The PBP affinities for β lactams have been examined and the results are conflicting. Eliopoulos *et al.* (1982) showed nearly identical penicillin binding in two hypersensitive mutants of *S. faecium* and their parent strains. Williamson *et al.* (1983) examined the PBP affinities of one isolate each of *S. faecalis* and *S. faecium* for ampicillin and cephalothin and found that the affinities of low-mol. wt PBPs correlated broadly with the MICs. Fontana *et al.* (1983; 1985) found a low-affinity PBP in *S. faecium* mutants highly resistant to β lactams and the resistance correlated with the quantity of the PBPs. In the present study, the

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affinities of PBPs of *S. faecalis* and *S. faecium* for two penicillins and three cephalosporins were compared with their susceptibilities to these β lactams. One highly β-lactam-resistant isolate of *S. faecium* was examined in parallel with the relatively susceptible strain of *S. faecium*, NCTC 7171.

Materials and methods

Bacterial strains

S. faecalis NCTC 775, *S. faecium* NCTC 7171 and a highly β-lactam-resistant clinical isolate of *S. faecium*, strain 37, were used. MICs were determined in Antibiotic Medium 3 (Bacto-penassay broth, Difco) by the broth dilution method. The inoculum was *c.* 10^6 cfu/ml.

Antibiotics and protein mol. wt standards

Benzyl [^{14}C] penicillin potassium (specific activity 54 Ci/mol; Amersham International plc, Buckinghamshire) was used for labelling PBPs. Penicillin G (Glaxo Laboratories Ltd, Middlesex), ampicillin (Beecham Research Laboratories, Middlesex), cefathiamidine (Shanghai Third Pharmaceutical Co., Shanghai, China), cephaloridine (Glaxo) and cephazolin (Eli Lilly and Co. Ltd, Basingstoke) were used in the competition binding experiments. The mol. wt marker mixture included Low Molecular Weight Protein Standards (BIO-RAD Laboratories, CA, USA) and β-galactosidase (Sigma Chemical Co., St Louis, MO, USA).

Preparation of bacterial membranes

Antibiotic Medium 3 broth (10 L) was seeded with 100 ml of an overnight culture and incubated at 37°C with shaking. Organisms at the late log phase of growth were harvested by centrifugation at 4000*g* for 15 min and suspended in 60 ml of cold 0·05M phosphate buffer, *p*H 7·0, containing 0·01M $MgCl_2$. Thirty ml of the cell suspension was mixed with 25 cm^3 of glass beads (0·10–0·11 mm diameter) and subjected to violent shaking for 5 min in a Braun disintegrator (B. Braun Melsungen Appratebau, Melsungen, W. Germany). Cooling was achieved by spraying the apparatus with liquid CO_2. Disruption of cells was confirmed by examination of gram-stained smears and viable counts; unbroken cells and debris were removed by centrifugation at 4000*g* for 15 min at 4°C. The supernate was retained and the membrane particles were deposited by ultracentrifugation at 100 000*g* for 30 min at 4°C. The pellet was homogenised in 5 ml of 0·01M phosphate buffer, *p*H 7·0, and ultracentrifuged at 100 000*g* for 30 min at 4°C. The final pellet was resuspended in 5 ml of 0·01M phosphate buffer, *p*H 7·0, dispensed into small volumes and stored at −70°C until used. Concentrations of protein in membrane preparations were estimated by the Lowry method with graded concentrations of bovine serum albumin (Sigma) as standards.

Reaction of PBPs with ^{14}C-penicillin G

(a) *Direct PBP labelling.* A mixture of 90 μl of membrane preparation, 10 μl of distilled water and 10 μl of ^{14}C-penicillin G (*c.* 350 mg/L, specific activity 54 Ci/mol) was prepared and incubated at 30°C for 10, 20, 40 or 60 min; 10 μl of unlabelled penicillin G at a concentration of 60 g/L was then added, followed 10 min later by 10 μl of sodium lauryl sarkosinate 20%, the mixtures being vortex mixed briefly after each addition. After 20 min at room temperature, the mixture was ultracentrifuged at 100 000*g* for 30 min at 10°C. The supernate was mixed with 2-mercaptoethanol and sample buffer containing bromophenol blue 0·0075% sodium dodecyl sulphate (SDS) 3%, and glycerol 30% v/v in 0·2M Tris-HCl buffer (*p*H 6·8) in the proportion of 6:3:1 v:v:v. After heating at 100°C for 5 min the mixture was ready for electrophoresis.

(b) *Competition binding experiments with other β lactams.* Serial two-fold dilutions of the test antibiotic were prepared at 10 times the final concentration required and 10 μl of these dilutions were pipetted into Eppendorf centrifuge tubes. A control tube containing 10 μl of water instead of the antibiotic solution was also prepared. To each tube was then added 90 μl of the membrane preparation and after holding the mixture at 30°C for 10 min, 10 μl of ^{14}C-penicillin G (*c.* 350 mg/L, 54 Ci/mol) was added to each tube. After incubation at 30°C for a further 10 min, 10 μl of unlabelled penicillin G (60 g/L) was added. The other procedures were the same as described as the direct labelling.

Electrophoresis of soluble membrane proteins

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) was used for analysis of the sarkosyl-soluble membrane proteins (Boulton and Orr, 1983). A discontinuous slab gel system with a gel thickness of 1·5 mm was prepared in a 'Protean' slab gel apparatus (Bio-Rad Ltd, Herts). The separating gel contained 10% acrylamide 10%, N,N′-methylene-bis-acrylamide 0·1%, SDS 0·1%, freshly prepared ammonium persulphate 0·05% and N,N,N′,N′-tetramethylethylenediamine (TEMED) 0·05% v/v in 0·375M Tris [(hydroxymethyl)methylamine]-HCl buffer (*p*H 8·8). The stacking gel consisted of acrylamide 6%, N,N′-methylene-bis-acrylamide 0·06%, SDS 0·1%, ammonium persulphate 0·05% and TEMED 0·1% v/v in 0·375M Tris HCl buffer, *p*H 6·8.

Appropriate volumes of the membrane-proteins preparations (10 μl for *S. faecalis* and 15 μl for *S. faecium*) and standard protein markers were loaded into the wells of the gel. Electrophoresis was performed at a constant current of 20 mA. The running buffer contained 0·05 M Tris, 0·38M glycine and SDS 0·1%. The gel was stained with Coomassie Brilliant Blue R (Boulton and Orr, 1983). The profiles of proteins separated in the gel were recorded by photography and compared. The relative mobilities of the standard proteins were measured. The gels were then prepared for fluorographic detection of the radiolabelled PBPs.

Fluorography

The gels were prepared for fluorography as described by Boulton and Orr (1983). The X-ray plates were exposed to the gels for 100 days at −70°C. The PBPs were developed as dark bands and the amount of β lactams required to inhibit 50% of binding of radiolabelled penicillin G was judged visually.

Results

Electrophoresis of membrane proteins

The protein concentrations of the membrane preparations were determined as 6–9 mg/ml.

Staining of electrophoresed membrane proteins from enterococci with Coomassie Brilliant Blue R showed a range of protein species (fig. 1) and the protein profile of *S. faecalis* NCTC 775 was substantially different from those of the *S. faecium* strains. The two *S. faecium* strains, resistant and moderately resistant to β lactams, showed very similar protein profiles, but two more dense bands appeared in the resistant isolate of *S. faecium* strain 37 than in *S. faecium* NCTC 7171. The approximate mol. wts of the bands were 81 000 and 56 000 (fig. 1).

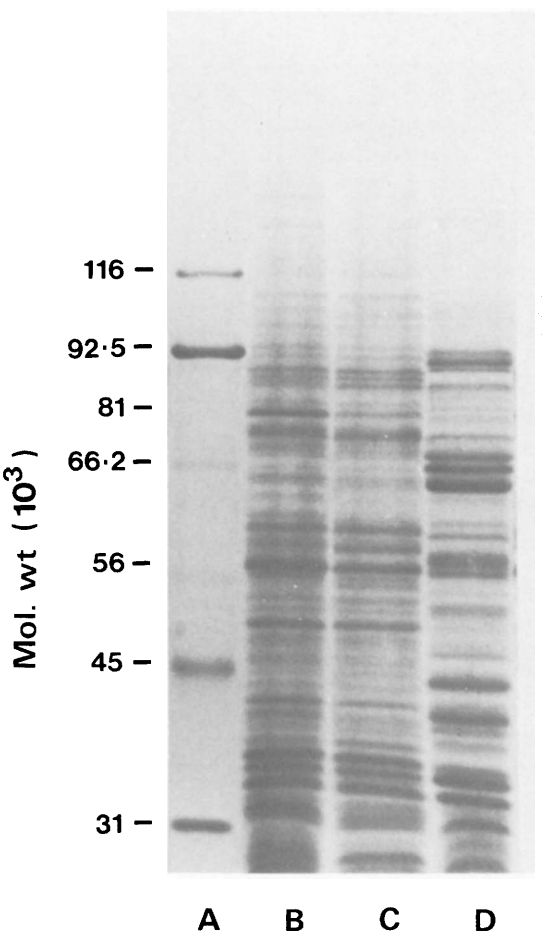

Fig. 1. Comparison of membrane protein profiles of enterococci. Tracks A: Protein mol. wt standards; B: *S. faecium* strain 37; C: *S. faecium* NCTC 7171; D: *S. faecalis* NCTC 775. Two arrows indicate two proteins increased in amounts in resistant *S. faecium* strain 37 compared with *S. faecium* NCTC 7171.

Direct binding of PBPs to ^{14}C-penicillin G

Five distinct PBPs were found in *S. faecalis* NCTC 775 whereas six PBPs were present in both *S. faecium* strains. The approximate mol. wts of PBPs 1–5 of *S. faecalis* NCTC 775 were 100 000, 80 000, 78 000, 74 000 and 46 000, and those of PBPs 1–6 of *S. faecium* NCTC 7171 were 110 000, 86 000, 84 000, 80 000, 76 000 and 46 000; those of *S. faecium* strain 37 were 110 000, 86 000, 83 000, 77 000, 74 000 and 44 000.

Membrane proteins of the test strains were exposed directly to radioactive penicillin G for different periods up to 60 min but no extra PBPs could be found in any of the test enterococci. Fig. 2 shows the PBPs of the resistant *S. faecium* strain 37 after different periods of exposure to ^{14}C-penicillin G (fig. 2).

Comparison of affinities of PBPs for β lactams in relation to MICs

MICs of *S. faecalis* NCTC 775 and *S. faecium* strains NCTC 7171 and 37 are shown in tables I and II in comparison with the PBP affinities for the antibiotics. *S. faecalis* NCTC 775 and *S. faecium* NCTC 7171 were relatively susceptible to some β lactams whereas *S. faecium* strain 37 was resistant.

The affinity of a PBP for individual unlabelled β lactams was expressed as the concentration of the antibiotic required to reduce ^{14}C-penicillin G binding to the PBP by 50% (I50). The affinities of PBPs of *S. faecalis* NCTC 775, *S. faecium* NCTC

Table I. Inhibition of binding ^{14}C-penicillin G to PBPs of *S. faecalis* NCTC 775 by five β lactams

Antibiotic (MIC, mg/L)		Concentration (mg/L) required to inhibit by 50% binding to PBP				
		1	2	3	4	5
Penicillin G	(4)	0·5	0·5	16	8	<0·25
Ampicillin	(2)	4	4	4	2	>128
Cefathiamidine	(2)	4	2	4	2	>128
Cephaloridine	(32)	2	128	16	64	<1
Cephazolin	(64)	128	64	512	64	512

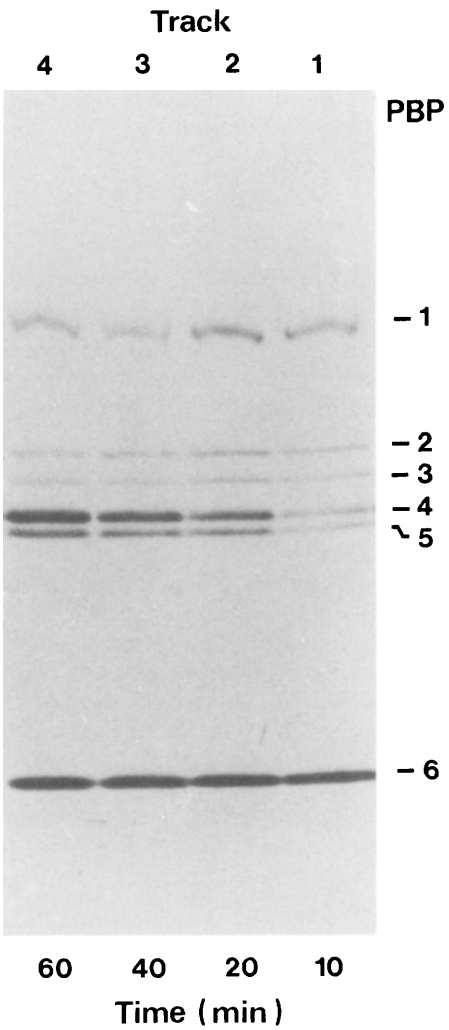

Fig. 2. Direct binding of radiolabelled penicillin G to PBPs of *S. faecium* strain 37. No extra PBPs were detected when the exposure time was increased from 10 to 60 min (tracks 1–4).

7171 and *S. faecium* strain 37 for five β lactams are shown in tables I and II in comparison with MICs.

PBPs 1 and 2 of *S. faecalis* NCTC 775 (table I) showed 4–8-fold greater affinity for penicillin G than ampicillin and cefathiamidine, and PBP 5 had even greater affinity for penicillin G compared to ampicillin and cefathiamidine, although ampicillin and cefathiamidine showed two-fold greater activity than penicillin G. PBPs 3 and 4 showed a more direct association between the affinity for the β lactams and the antibacterial activity of these compounds.

The affinities of PBPs 4 and 5 of *S. faecium* for β lactams were also proportionally related to respective MIC values. In the comparison of two strains of *S. faecium*, the affinities for PBPs 1, 2, 3 and 6 were similar, even though *S. faecium* strain 37 was far more resistant to the test β lactams than was *S. faecium* NCTC 7171 (table II). PBPs 4 and 5 of the resistant *S. faecium* strain 37 showed lower affinities in comparison with those of *S. faecium* NCTC 7171. PBP 6 showed relatively high affinity for cephaloridine although cephaloridine was not active against *S. faecium* strain 37. Figs. 3 and 4 show fluorographic results of the competition-binding experiments on *S. faecalis* NCTC 775, *S. faecium* NCTC 7171 and *S. faecium* strain 37.

Discussion

The ultrasonic treatment normally used for disruption of gram-negative bacteria appeared to be ineffective for enterococci. The reduction of viable counts after 5 min showed that the Braun disintegrator was as satisfactory with enterococci as with *Staph. aureus* (Huff *et al.*, 1964).

There was a distinct difference in sarkosyl-soluble protein components between *S. faecalis* and *S. faecium* strains (fig. 1). Our PBP profiles of *S. faecalis* and *S. faecium* (fig. 2) are similar to those of Georgopapadakou and Liu (1980*b*), Eliopoulos *et al.* (1982) and Williamson *et al.* (1983) Two *S. faecium* strains possessed six PBPs of nearly identical mol. wts despite the difference in their susceptibility to β lactams. In a few experiments PBP 2 of *S. faecium* strains separated to form two close bands, almost identical in their binding characteristics; the reason for this phenomenon was obscure. It has been suggested that PBP patterns could be used for taxonomic purpose (Georgopapadakou and Liu, 1980*a*). Eliopoulos *et al.* (1982) selected two mutants that were highly susceptible to penicillin G from normally resistant isolates of *S. faecium*. Cell membranes prepared from resistant strains were found to possess a protein (mol. wt 82 000) absent or less apparent in the membranes of susceptible strains. No alteration of PBP affinity was found in these mutants, therefore the protein was considered to be associated with the β-lactam resistance. In the present study, two proteins were found to be more dense in the resistant than in the susceptible *S. faecium* strain. One of the two protein bands had a mol. wt of 81 000 (fig. 1) similar to the protein of mol. wt 82 000 reported by Eliopoulos *et al.* (1982). This protein may be associated with β-lactam resistance or may be merely a phenomenon of strain variation. Detailed biochemical analysis is needed and it would be useful to see how widely it is distributed in β-lactam-resistant strains.

Table II. Inhibition of binding of ^{14}C-penicillin G to PBPs of resistant *S. faecium* strain 37 and of susceptible *S. faecium* NCTC 7171 by five β lactams

Antibiotic (MIC, mg/L)	Concentration required to inhibit by 50% binding to PBP													
	1		2a		2b		3		4		5		6	
	37	N	37	N	37	N	37	N	37	N	37	N	37	N
Penicillin G (37:125; N:4)	0·25	0·25	0·063	0·031	0·063	0·031	0·125	0·125	128	2	16	2	1	1
Ampicillin (37:63; N:2)	1	0·5	0·031	0·031	0·031	0·031	0·125	0·125	64	0·5	8	0·5	2	4
Cefathiamidine (37:256; N:8)	<1	<0·5	<1	<0·5	<1	<0·5	<1	<0·5	512	32	128	16	512	64
Cephaloridine (37:256; N:8)	<1	<0·25	<1	<0·25			<1	<0·25	1024	32	512	16	4	4
Cephazolin (37: >1024; N:256)	4	4	0·125	0·125	0·25	0·125	0·063	0·063	1024	512	1024	512	64	64

37 = strain 37; N = strain NCTC 7171.

Fontana *et al.* (1983*b*; 1985) showed that penicillin-resistant mutants of *S. faecium* strains carried a 'low-affinity' PBP which only became labelled after incubation for 20 min with radiolabelled penicillin G 48.5 mg/L and the amount of the PBP altered substantially with the extent of resistance. In this study, although the density of PBPs 4 and 5 of each *S. faecium* strain in the direct binding experiments showed an increase with the reactive time from 10 to 60 min when the membrane was exposed to ^{14}C-penicillin G (54 Ci/mol): 35 mg/L, no extra PBPs were observed with the increase in exposure time. Two distinct *S. faecium* strains were examined in this study, whereas resistant mutants or further

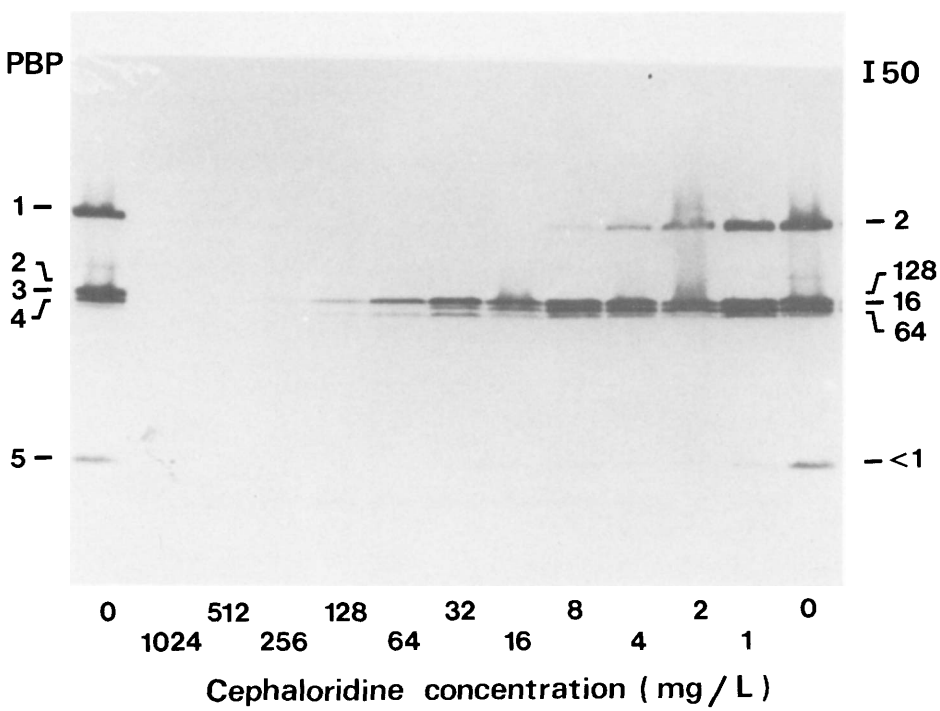

Fig. 3. Fluorogram of the competition binding experiment with *S. faecalis* NCTC 775 showing that the binding of radiolabelled penicillin G to individual PBPs was inhibited by different concentrations of cephaloridine.

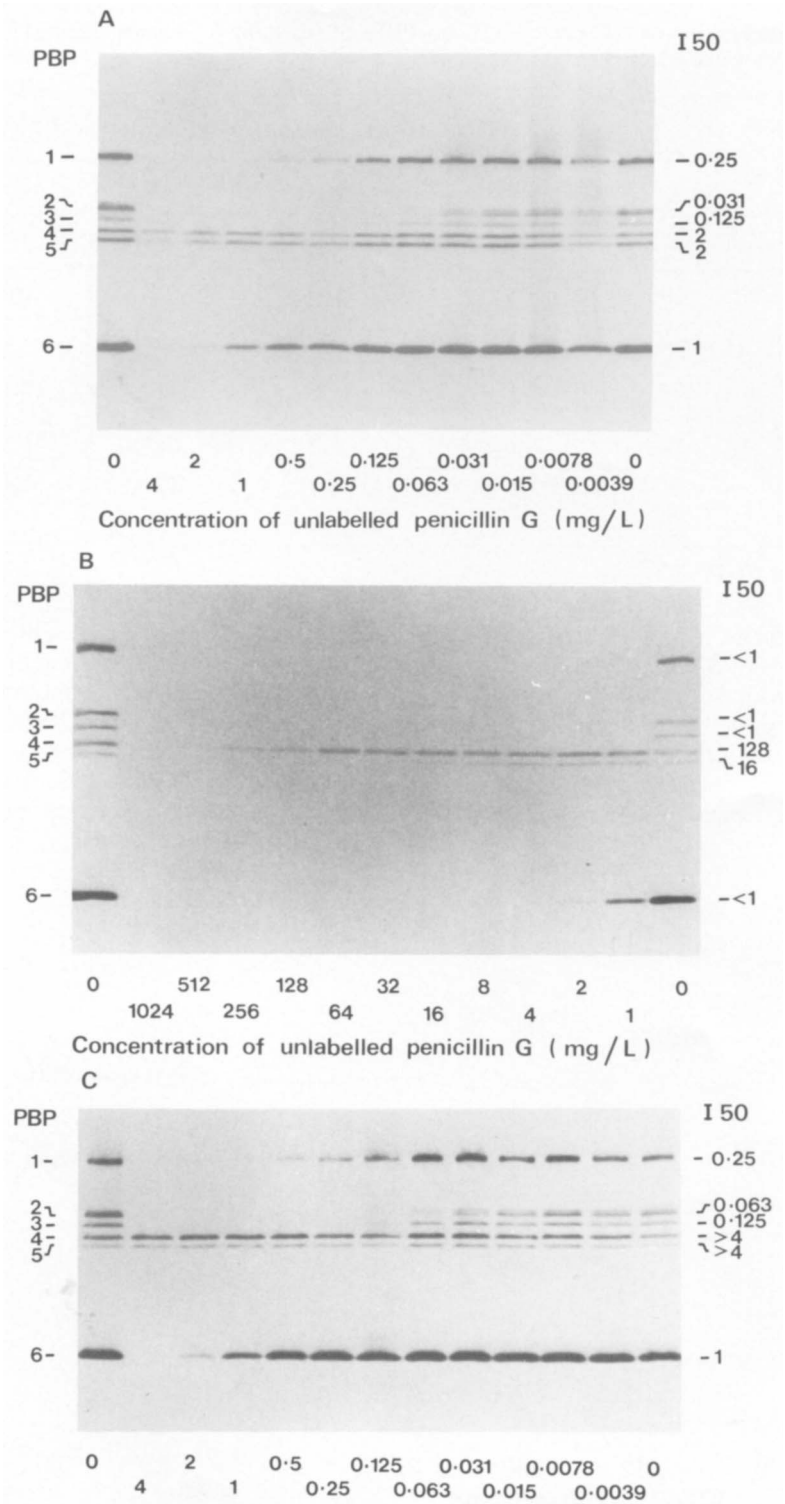

Fig. 4. Comparison of fluorograms of competition experiments with unlabelled penicillin G on *S. faecium* NCTC 7171 (gel A) and *S. faecium* strain 37; gels B and C are two experiments including an extended range of unlabelled penicillin G concentrations. I50 values are listed on the right of the fluorograms.

cured strains and their parental strains were used in the studies of Fontana *et al.* (1983*b*; 1985). Strain difference might account for the discrepant results.

In the competition experiments we noted that individual PBPs showed different affinities for different β lactams. In *S. faecalis* NCTC 775, high affinity of PBPs 3 and 4 for penicillin G, ampicillin and cefathiamidine (Chen and Williams, 1983) was associated with low MICs of the antibiotics; the relative affinities of PBPs 4 and 5 in the two *S. faecium* strains for the β lactams were proportionate to their sensitivities to the antibiotics. It is presumed therefore that PBPs 3 and 4 of *S. faecalis* NCTC 775 and PBPs 4 and 5 of *S. faecium* are the relevant target sites of the test β-lactam antibiotics. Moreover, PBPs 4 and 5 of the highly β-lactam-resistant *S. faecium* strain 37 showed proportionally low affinities for the five β lactams when compared with those of relatively susceptible *S. faecium* NCTC 7171. Decreased affinities of PBPs 4 and 5 might account for the β-lactam resistance in *S. faecium* strain 37.

The affinities of PBPs 1, 2 and 5 of *S. faecalis* NCTC 775 were not proportionally associated with MICs. The affinities of PBPs 1, 2, 3 and 6 of two *S. faecium* strains were very similar, whereas the difference in susceptibilities was remarkable for those two strains, e.g., PBP 5 of *S. faecalis* NCTC 775 had high affinity for cephaloridine but the strain was resistant to cephaloridine.

The experiments suggest that PBPs 1, 2 and 5 of *S. faecalis* NCTC 775 and PBPs 1, 2, 3 and 6 of *S. faecium* are unlikely to be relevant to the action of the β lactams. However, one β lactam binds to multiple PBPs and it is likely that PBPs are functionally intercomplementary.

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