In *Streptococcus faecium* Penicillin-binding Protein 5 Alone Is Sufficient for Growth at Sub-maximal But Not at Maximal Rate

By PIETRO CANEPARI,¹ MARIA DEL MAR LLEÒ,¹ GIUSEPPE CORNAGLIA,² ROBERTA FONTANA^{3*} AND GIUSEPPE SATTA²

¹Istituto di Microbiologia, Università di Genova, Genova, Italy

²Istituto di Microbiologia e Virologia, Università di Cagliari, Cagliari, Italy

³Istituto di Microbiologia, Università di Padova, Padova, Italy

(Received 6 August 1985)

In *Streptococcus faecium* inhibition by both benzylpenicillin and cefotaxime of cells growing at maximal and at reduced rates was associated with saturation of different penicillin-binding proteins. Cells growing at reduced rates were not inhibited by benzylpenicillin concentrations that saturated all penicillin-binding proteins except penicillin-binding protein 5, but did stop growing when this protein was saturated.

INTRODUCTION

An important step towards understanding the specific functions that penicillin-binding proteins (PBPs) perform in bacteria (Spratt, 1975; Tomasz, 1979; Matsushashi *et al.*, 1982; Waxman & Strominger, 1983) would be the clarification of interactions between them and regulation of their activity in relation to the physiological status of the cells. From studies with β -lactam resistant mutants, several authors have suggested that certain PBPs can compensate for the loss of activity of others (Suzuki *et al.*, 1978; Giles & Reynolds, 1979; Brown & Reynolds, 1980; Fontana *et al.*, 1980, 1983*a, b*; Wyke *et al.*, 1982; Hartman & Tomasz, 1984) and the hypothesis has been proposed that function and activity of PBPs can vary depending on the physiological status of the cells (Fontana *et al.*, 1983*a*). In this study we examine growth inhibition of *Streptococcus faecium* by both benzylpenicillin and cefotaxime.

METHODS

Bacterial strains and growth conditions. *Streptococcus faecium* PS is a penicillin susceptible strain used in previous studies (Fontana *et al.*, 1980, 1983*a, b*). *Streptococcus faecium* R5, R20 and R40 are penicillin resistant mutants isolated from strain PS by serial culture in a medium containing increasing concentrations of penicillin (Fontana *et al.*, 1983*b*). *Streptococcus faecium* Rev 14 is a penicillin hypersusceptible strain, derived from strain R40, isolated after growth in the presence of novobiocin according to the procedure described by Eliopoulos *et al.* (1982) (Fontana *et al.*, 1985).

Bacteria were grown in a chemically defined medium (CDM; Shockman, 1963) or in a yeast extract/peptone medium (SB; Fontana *et al.*, 1983*a*). Growth was monitored in a Perkin–Elmer spectrophotometer.

Antibiotic and chemicals. [³H]Benzylpenicillin, ethylpiperidinium salt (53.5 mCi mg⁻¹; 1.98 GBq mg⁻¹) was a generous gift of the Merck, Sharp & Dohme Research Laboratories, Rahway, NJ, USA. Penicillin G was provided by Eli Lilly and cefotaxime by Hoechst.

Chemicals for the preparation of polyacrylamide gels were obtained from Serva. All other chemicals were of analytical grade from Merck. Lysozyme, DNAase and RNAase were purchased from Sigma. For fluorography we used Kodak X-Omat SO 282 5 X-ray film.

Susceptibility testing. Antimicrobial susceptibility was determined in CDM by a standard broth dilution technique. For a better correlation between growth inhibition and PBP binding experiments, a relatively high

Abbreviations: CDM, chemically defined medium; PBP, penicillin-binding protein.

inoculum (10^7 bacteria ml^{-1} , final density) of exponential phase organisms was used. MICs were determined by visual inspection for lack of turbidity after 18 h incubation at 30 or 45 °C without shaking.

Studies of PBPs. Bacteria in early exponential growth phase were inoculated into batches of CDM (10^7 bacteria ml^{-1} , final density) containing an appropriate β -lactam. Cultures were incubated for 60 min at 30 or 45 °C without shaking. A sample containing approximately 4 mg bacteria (dry weight) was taken, centrifuged (3000 g , 10 min), suspended in 100 μl 10 mM-sodium phosphate buffer containing [^3H]benzylpenicillin (48.5 $\mu\text{g ml}^{-1}$, final concentration) and incubated at the same temperature as was the culture before suspension. Control samples were preincubated in medium containing no antibiotic before exposure to the radioactive penicillin. Lysozyme, DNAase and RNAase were then added to the samples at final concentrations of 100 $\mu\text{g ml}^{-1}$, 5 $\mu\text{g ml}^{-1}$ and 2.5 $\mu\text{g ml}^{-1}$, respectively. Cell suspensions were incubated at the same temperature as used for growth until cell lysis occurred (30 min). The lysed cells were then resuspended in 10 ml cold sodium phosphate buffer and membranes were pelleted at 100000 g for 60 min in a Beckman model L ultracentrifuge. Membranes were suspended in 50 μl sample dilution buffer containing 0.2 M-Tris/HCl buffer (pH 6.8), 3% (w/v) SDS, 30% (v/v) glycerol, 16% (v/v) mercaptoethanol and 0.002% (v/v) bromophenol blue and immediately heated to 100 °C for 2 min. The sample was then applied to a polyacrylamide slab gel. Electrophoresis and PBP detection were done as described previously (Fontana *et al.*, 1980, 1983a, b).

RESULTS

Fig. 1 and Table 1 show results of a typical competition experiment where growing cells were treated first with increasing concentrations of non-radioactive benzylpenicillin and then with a saturating concentration of the radioactive antibiotic. It was evident that in cells growing at the maximal rate (in CDM at 45 °C), a concentration of non-radioactive benzylpenicillin equal to the minimum inhibitory concentration (MIC) bound PBPs 1, 2 and 3, but completely saturated only PBP 3. This PBP was the only one that did not bind the radioactive benzylpenicillin added afterwards and did not appear in the fluorogram. In contrast, in cells growing in CDM at the sub-optimal temperature of 30 °C, concentrations of non-radioactive benzylpenicillin (4 $\mu\text{g ml}^{-1}$), which caused virtually complete saturation of all PBPs except PBP 5, were not inhibitory and growth was inhibited only in the presence of the minimal antibiotic concentration needed to saturate completely PBP 5 (8 $\mu\text{g ml}^{-1}$).

Previous studies with isolated membranes had shown that cefotaxime had a very low affinity for PBPs 4, 5 and 6 while showing an affinity similar to that of benzylpenicillin for all other PBPs (Coyette *et al.*, 1983). Fig. 2 shows results of a typical competition experiment in which growing cells of *S. faecium* PS were treated first with increasing concentrations of non-radioactive cefotaxime and then with a saturating concentration of radioactive benzylpenicillin. As expected, in whole cells grown both at the maximal rate (45 °C) and at a reduced rate (30 °C) cefotaxime showed a very low affinity for PBPs 4, 5 and 6 (which it saturated at a concentration of 2000 $\mu\text{g ml}^{-1}$) and an affinity for the other PBPs that was close to that of benzylpenicillin. The MIC of cefotaxime was very close to that of benzylpenicillin in cells growing at the maximal rate (0.02 versus 0.04 $\mu\text{g ml}^{-1}$), but in cells growing at the reduced rate it was 250-fold higher (2000 versus 8 $\mu\text{g ml}^{-1}$) (Table 2). At both temperatures PBPs 2 and 3 and PBPs 4, 5 and 6 appeared to have the highest and the lowest affinity, respectively, for this antibiotic. PBPs 2 and 3 were saturated by a cefotaxime concentration equal to the MIC of the cells grown at the maximal rate, while PBPs 4, 5 and 6 were saturated by a concentration equal to the MIC for cells grown at a reduced rate (Table 2).

We have previously shown (Fontana *et al.*, 1983b) that *S. faecium* strains resistant to benzylpenicillin overproduce PBP 5 and that in these strains this PBP is the only one necessary for cell growth. Table 2 shows that in these mutants resistance to benzylpenicillin and cefotaxime was equal in cells growing at 30 and 45 °C. The MIC of cefotaxime was much higher than that of benzylpenicillin and, in both cases, corresponded to the amount of antibiotic necessary to saturate PBP 5, while PBPs 4 and 6 were saturated at lower concentrations.

We have recently isolated and investigated the characteristics of the *S. faecium* Rev 14 mutant that is hypersusceptible to benzylpenicillin and which did not appear to synthesize any PBP 5 (Fontana *et al.*, 1985). This strain showed a generation time very close to that of the parent at both 30 and 45 °C. Table 2 shows that the MICs of benzylpenicillin and cefotaxime for strain Rev 14 were the same at both 30 and 45 °C. In addition the MICs were very close to that of strain

Fig. 1. Competition between non-radioactive and radioactive benzylpenicillin for binding to PBPs in whole cells of *S. faecium* grown at 30 °C (a) and 45 °C (b). Samples (10 ml) of two exponential cultures, one growing at 30 °C and the other at 45 °C, were treated for 60 min with non-radioactive benzylpenicillin at several concentrations. An additional sample was left untreated as a control (C). Cells were immediately harvested by centrifugation and PBPs were labelled and visualized as described.

Table 1. *Benzylpenicillin MICs, generation times and extent of saturation of the various PBPs by non-radioactive benzylpenicillin in S. faecium PS grown in CDM at two different temperatures*

The results are from the experiments represented in Fig. 1 and are the mean of three separate experiments. The amount of radioactivity bound to PBPs was estimated by microdensitometry of the fluorograms with a Joyce Loebel MK3CS densitometer.

Growth temp. (°C)	Generation time (min)	MIC (µg ml ⁻¹)	Benzylpenicillin concn (µg ml ⁻¹)	Percentage saturation of PBP:					
				1	2	3	4	5	6
30	50	8	0.5	95	95	100	80	0	40
			1	100	100	100	90	0	60
			2	100	100	100	100	20	90
			4	100	100	100	100	60	100
			8	100	100	100	100	100	100
45	31	0.04	0.04	70	80	100	0	0	0
			0.08	80	95	100	30	0	10
			0.16	90	100	100	80	0	30
			2	100	100	100	90	50	80
			4	100	100	100	100	70	95
			8	100	100	100	100	100	100

PS growing at 45 °C and corresponded to an antibiotic concentration that saturated PBP 3 alone in all strains at both temperatures (see also Fontana *et al.*, 1980). This finding suggested that in the penicillin hypersusceptible strain the target for growth inhibition was the same under all the experimental conditions tested. Hence PBP 5 appeared to be necessary for *S. faecium* cells to become more resistant to β -lactams when growing at a reduced rate.

Fig. 2. Competition between cefotaxime and radioactive benzylpenicillin for binding to PBPs of *S. faecium* grown at 30 °C (a) and 45 °C (b). Experiments were done as described in the legend to Fig. 1 except that cefotaxime at various concentrations was used instead of non-radioactive benzylpenicillin.

Table 2. MICs for benzylpenicillin and cefotaxime, and concentrations required to saturate PBPs of *S. faecium* PS and penicillin resistant and hypersusceptible mutants grown at two different temperatures

The results are the mean of three separate experiments.

Strain	Growth temp. (°C)	Generation time (min)	MIC (µg ml ⁻¹) for:		Concn (µg ml ⁻¹) of benzylpenicillin saturating PBP:*		Concn (µg ml ⁻¹) of cefotaxime saturating PBP:†		
			Benzyl-penicillin	Cefotaxime	3	5	2 and 3	4 and 6	5
PS	30	50	8	2000	0.04	8	0.02	2000	2000
	45	31	0.04	0.02	0.04	8	0.02	2000	2000
R5	30	52	40	3000	0.04	40	0.02	2000	3000
	45	35	40	3000	0.04	40	0.02	2000	3000
R20	30	60	60	4000	0.04	60	0.02	2000	4000
	45	40	60	4000	0.04	60	0.02	2000	4000
R40	30	60	80	4000	0.04	80	0.02	2000	4000
	45	40	80	4000	0.04	80	0.02	2000	4000
Rev 14	30	54	0.01	0.01	0.04	—	0.02	2000	—
	45	36	0.01	0.01	0.04	—	0.02	2000	—

* Determined *in vivo* by binding with non-radioactive antibiotic and saturating with labelled penicillin as described in the legend to Fig. 1. The concentration is the minimum that caused complete disappearance of PBP from the fluorograms. All other PBPs of the mutants were saturated by the same benzylpenicillin concentrations that saturated the parent PBPs. PBP 5 was not synthesized by strain Rev 14.

† PBP 1 was saturated by the same concentration of cefotaxime in strain PS and in the mutants.

In the *in vivo* PBP binding experiments described above bacteria were incubated with β -lactams for 60 min, a time that in some micro-organisms might be sufficient to cause cell lysis particularly when concentrations above the MIC are used. However, *S. faecium* has proved to be rather resistant to lysis by both benzylpenicillin and cefotaxime. In fact concentrations of

Fig. 3. Effect of cefotaxime and benzylpenicillin on the growth of *S. faecium*. Cefotaxime (a, b) and benzylpenicillin (c, d) were added at various concentrations to exponentially growing cultures at zero time and the cultures were incubated at 30 °C (a, c) and 45 °C (b, d). The optical density of the cultures was monitored at 550 nm. Untreated cultures were used as controls. The numbers by the curves are the antibiotic concentrations ($\mu\text{g ml}^{-1}$).

Table 3. Susceptibility to benzylpenicillin and PBP saturation of *S. faecium* PS incubated at 37 °C in different media

Medium	Generation time (min)	MIC ($\mu\text{g ml}^{-1}$)	PBPs saturated by penicillin at:	
			MIC	$\frac{1}{2}$ MIC
SB	26	0.25	2 and 3	2 and 3
SB diluted sixfold	61	8	All	All, except 5

benzylpenicillin up to 8 $\mu\text{g ml}^{-1}$ and concentrations of cefotaxime up to 2000 $\mu\text{g ml}^{-1}$ at both 30 and 45 °C allowed the optical density of the culture to increase for at least 90 min (Fig. 3). The rate of increase in the optical density decreased as the antibiotic concentration increased, but was, in all cases, clearly evident (Fig. 3).

In the experiments described above different growth rates were obtained by changing the temperature of incubation. To exclude the possibility that the different susceptibilities observed under the different conditions of growth could be direct effect of temperature and not related to the growth rate, we evaluated both the penicillin MIC and the binding of the antibiotic to PBPs in *S. faecium* growing in different media at the same temperature. In SB medium incubated at 37 °C the generation time of *S. faecium* was 26 min, whereas in diluted SB incubated at the same temperature it was 61 min (Table 3). The susceptibility to β -lactams was higher in fast growing cells than in slow growing cells whereas the affinity of PBPs for β -lactams did not change. These findings also show that when *S. faecium* cells are incubated at the same temperature in media that permit growth at different rates, different concentrations of β -lactams are required to inhibit growth, which is consistent with the interpretation that the inhibition of different PBPs is responsible for growth inhibition at fast and slow rates.

DISCUSSION

These studies show that the PBPs which must bind and be saturated, either by benzylpenicillin or by cefotaxime, to cause growth inhibition are different in fast and slow growing cells. This finding demonstrates that in wild-type *S. faecium* different PBPs are essential in fast growing cells and in cells growing at a reduced rate and indicates that a PBP does not necessarily fulfil the same role under all conditions but that its function depends on the physiological status of the cells, as previously suggested (Fontana *et al.*, 1983*a*). Such a finding has implications for understanding the functions of PBPs in cell physiology and the way in which their activity is regulated. It is possible also that in other micro-organisms PBPs that are indispensable under some growth conditions may become dispensable under other growth conditions.

The competition experiments with non-radioactive benzylpenicillin have shown that PBP 3 (and possibly 1 and 2) activity is necessary in fast growing cells, while PBP 5 alone is sufficient for cell growth at a reduced rate. Previously (Fontana *et al.*, 1983*b*) we showed that enhanced amounts of PBP 5 can take over the functions of all other PBPs, even at the maximal growth rate (Table 2). Other authors have shown that β -lactam resistant *Staphylococcus aureus* strains overproduce a PBP which allows the cells to grow in the presence of β -lactam concentrations which saturate all other PBPs (Wyke *et al.*, 1982; Hartman & Tomasz, 1984). However it must be stressed that the case we describe in this paper is rather different since it involves a normal wild-type strain in which one of the PBPs becomes the only essential one during growth at sub-maximal rates. Since this PBP has a much lower affinity for β -lactams than the other PBPs, the growth conditions under which PBP 5 alone is essential for cell growth cause a shift in the target for the inhibitory action of β -lactams. This may explain the fact that susceptibility of *S. faecium* to β -lactams is greatly influenced by changes in growth conditions (Fontana *et al.*, 1983*a*; Hinks *et al.*, 1977). A similar explanation could also apply to other bacteria whose sensitivity to β -lactams is influenced by their growth rate (Annear, 1968; Thrupp, 1980).

We proposed previously (Fontana *et al.*, 1983*a*) that the mechanism of growth inhibition by β -lactams is indirect and not constant and depends on which PBP is indispensable and the affinity of this protein for these antibiotics. The results reported here that the PBP target for β -lactam inhibitory activity changes according to the physiological status of the cells and that this change is associated with a change in sensitivity to such antibiotics strongly support our previous proposal.

We thank Stephen Raffanti for his help in preparing the manuscript and Pietro Cavo, whose technical assistance has been greatly appreciated. We are very much indebted to Patrick J. Cassidy of Merck & Co., Inc., Rahway, NY, USA, for the generous gift of [3 H]benzylpenicillin.

This work was supported by grants 83.00375.04 and 83.00694.52 from the Consiglio Nazionale delle Ricerche (Italy).

REFERENCES

- ANNEAR, D. J. (1968). The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some other antibiotics. *Medical Journal of Australia* **52**, 444-446.
- BROWN, F. J. & REYNOLDS, P. E. (1980). Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *FEBS Letters* **122**, 275-278.
- COYETTE, J., SOMZÉ, A., BRIQUET, J. J., GHUYSEN, J. M. & FONTANA, R. (1983). Function of penicillin binding protein 3 in *Streptococcus faecium*. In *The Target of Penicillin*, pp. 523-530. Edited by R. Hakenbeck, J. V. Holtje & H. Labishinski. Berlin: Walter De Gruyter & Co.
- ELIOPOULOS, G. M., WENNERSTEN, G. & MOELLERING, R. C. (1982). Resistance to beta-lactam antibiotics in *Streptococcus faecium*. *Antimicrobial Agents and Chemotherapy* **22**, 295-301.
- FONTANA, R., CANEPARI, P., SATTA, G. & COYETTE, J. (1980). Identification of the lethal target of benzylpenicillin in *Streptococcus faecalis* by in vivo penicillin binding studies. *Nature, London* **287**, 70-72.
- FONTANA, R., CANEPARI, P., SATTA, G. & COYETTE, J. (1983*a*). *Streptococcus faecium* ATCC 9790 penicillin binding proteins and penicillin sensitivity are heavily influenced by growth conditions; proposal for an indirect mechanism of growth inhibition by beta-lactams. *Journal of Bacteriology* **154**, 916-923.
- FONTANA, R., CERINI, R., LONGONI, P., GROSSATO, A. & CANEPARI, P. (1983*b*). Identification of a streptococcal penicillin binding protein that reacts very slowly with penicillin. *Journal of Bacteriology* **155**, 1343-1350.
- FONTANA, R., GROSSATO, A., ROSSI, L., CHENG, Y. R. & SATTA, G. (1985). Transition from resistance

- to hypersusceptibility to beta-lactam antibiotics associated with loss of low affinity penicillin-binding protein in a *Streptococcus faecium* mutant highly resistant to penicillin. *Antimicrobial Agents and Chemotherapy* **28**, 678–683.
- GILES, A. F. & REYNOLDS, P. E. (1979). *Bacillus megaterium* resistance to cloxacillin accompanied by a compensatory change in penicillin binding proteins. *Nature, London* **280**, 167–168.
- HARTMAN, B. J. & TOMASZ, A. (1984). Low-affinity penicillin binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology* **158**, 513–516.
- HINKS, E. T., DANE-MOORE, L. & BRAVERMAN, S. (1977). Temperature effects on minimum inhibitory and bactericidal concentrations of cell wall antibiotics in *Streptococcus faecalis*. *Antimicrobial Agents and Chemotherapy* **12**, 281–283.
- MATSUHASHI, M., ISHINO, F., TAMAKI, S., NAKAJIAMA-IJIMA, S., TOMIOKA, S., NAGAKAWA, J., HIRATA, A., SPRATT, B. G., TSURUOKA, T., INOUE, S. & YAMADA Y. (1982). Mechanism of action of beta-lactam antibiotics. Inhibition of peptidoglycan transpeptidases and novel mechanisms of action. In *Trends in Antibiotics Research: Genetics, Biosyntheses, Actions and New Substances*, pp. 99–114. Edited by H. Umezawa, A. Demain, T. Hata & C. R. Hutchinson. Tokyo: Japan Antibiotics Research Association.
- SHOCKMAN, G. D. (1963). Aminoacids. In *Analytical Microbiology*, pp. 567–573. Edited by F. Kavanagh. New York: Academic Press.
- SPRATT, B. G. (1975). Distinct penicillin binding proteins involved in the division, elongation and shape of *Escherichia coli* K12. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2999–3003.
- SUZUKI, A., NISHIMURA, Y. & HIROTA, Y. (1978). On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 664–668.
- THRUPP, L. D. (1980). Susceptibility testing of antibiotics in liquid media. In *Antibiotics in Laboratory Medicine*, pp. 73–113. Edited by V. Lorian. Baltimore: Williams & Wilkins.
- TOMASZ, A. (1979). The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annual Review of Microbiology* **33**, 113–137.
- WAXMAN, D. J. & STROMINGER, J. L. (1983). Penicillin binding proteins and the mechanism of action of beta-lactam antibiotics. *Annual Review of Biochemistry* **52**, 825–869.
- WYKE, A. W., WARD, J. B. & HAYES, M. W. (1982). Synthesis of peptidoglycan in vivo in methicillin resistant *Staphylococcus aureus*. *European Journal of Biochemistry* **127**, 553–558.