State of penicillin-binding proteins and requirements for their bactericidal interaction with β -lactam antibiotics in *Serratia marcescens* highly resistant to extended-spectrum β -lactams

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The quantities of penicillin-binding proteins (PBPs), and sensitivity to extended-spectrum β -lactams, were measured in isogenic strains of *Serratia marcescens* with high (HR) and low (LR) resistance to extended-spectrum β -lactam antibiotics and with constitutively overproduced chromosomal β -lactamase in the periplasm. The binding of structurally different β -lactams to PBPs in growing resistant bacteria was determined quantitatively. In *S. marcescens* HR, the amounts of PBPs 3 and 6 were, respectively, 1.5 and 2 times those in strain LR and in sensitive reference strains. Sensitivities of the essential PBPs in *S. marcescens* LR and HR to the tested β -lactams were identical. Only a single target, PBP 3, was highly sensitive to cefotaxime, ceftazidime and aztreonam. In contrast, three PBPs (2, 1A and 3) were highly sensitive to imipenem. In growing *S. marcescens* HR and LR, all antibiotics, even at fractions of their minimal growth inhibitory concentrations (MICs), bound extensively to those PBPs which were highly sensitive to them. Thus, overproduced β -lactamase did not prevent PBP- β -lactam interaction. Only at or above their (high) MICs did cefotaxime, ceftazidime and aztreonam bind to multiple targets. Growth inhibition of the otherwise highly resistant *S. marcescens* HR at the lower MIC of imipenem was correlated with the binding of this antibiotic to multiple, highly sensitive targets in the bacteria. Killing of the bacteria by inactivation of multiple targets was suggested. This assumption was supported by the synergistic killing of HR bacteria by combinations of the PBP-2-specific mecillinam with PBP-3-specific β -lactams.

Introduction

In various opportunistic pathogens among the Gram-negative bacteria, high resistance to β -lactam antibiotics as well as to the newer extended-spectrum compounds with increased stability to β -lactamases is encountered. This has been found to be related to the limitation of antibiotic penetration by the outer membrane barrier and the degradation of the β -lactams by overproduced, chromosomally encoded β -lactamase in the bacterial periplasm (Vu & Nikaido, 1985; Bush *et al.*, 1985; Marchou *et al.*, 1987; Nikaido & Normark, 1987; Frère, 1989; Hechler *et al.*, 1989; Nikaido, 1989). The

combined functions of both resistance factors are thought to block the access of the antibiotics to their targets, the essential penicillin-binding proteins (PBPs), on the bacterial cytoplasmic membrane.

In a previous study we demonstrated the validity of this concept by comparison of sensitive and β -lactam-resistant strains of *Serratia marcescens* (Hechler *et al.*, 1989). Mathematical models of the interaction of penetrating antibiotics with the permeability barrier and periplasmic β -lactamase (Frère, 1989; Frère *et al.*, 1989) were used to determine the contribution of the two resistance factors to the high and low resistance to extended-spectrum β -lactam antibiotics in the isogenic β -lactamase-overproducing strains *S. marcescens* 921/79 HR and LR. For some representative newer antibiotics, the action of β -lactamase alone accounted for the lower resistance of *S. marcescens* LR. For instance, the β -lactamase degraded cefotaxime, ceftazidime and aztreonam to concentrations below the required lethal periplasmic concentration (I_{pl} ; Frère, 1989) when these β -lactams were applied at external concentrations below

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Abbreviations: HR, high resistance; LR, low resistance; PBP, penicillin-binding protein; MIC, minimal growth-inhibitory concentration; EC_{50} , concentration of β -lactam reducing the binding of [3H]-benzylpenicillin to PBP by 50%; I_{pl} , lethal periplasmic concentration of antibiotic.

This paper is dedicated to Professor Otto Kandler on the occasion of his 70th birthday.

their minimal growth inhibitory concentrations (MICs) of 32, 1 and 8 $\mu\text{g ml}^{-1}$, respectively. The antibiotics could then be assumed to penetrate at the same rate into cells of the LR strain and of sensitive reference strains of *S. marcescens*.

For the highly resistant *S. marcescens* HR, with MICs of 1024, 64 and 128 $\mu\text{g ml}^{-1}$ for the antibiotics, the models revealed the requirement for a further resistance factor in addition to β -lactamase. Decreased outer membrane penetrability was tentatively assumed. However, increased amounts or decreased sensitivity of essential PBP targets were also possible causes of the high resistance.

It is not known which target(s) or what percentage of target molecules are inactivated in the resistant bacteria at the I_{PI} and respective MIC of a given β -lactam.

In the present study, we determined the quantities of PBPs in sensitive and resistant strains of *S. marcescens*. Sensitivities of PBPs to extended-spectrum β -lactams in the differentially resistant *S. marcescens* HR and LR were compared. Binding of β -lactams to PBPs in growing cells of *S. marcescens* HR and LR was measured in order to assess the degree of inactivation of PBPs required for bactericidal action and to characterize the ability of the individual antibiotics to accomplish this task.

Methods

Bacteria. The resistant strains *Serratia marcescens* 921/79 LR and HR, their different states of resistance, the MICs of extended-spectrum β -lactam antibiotics for these bacteria, and their constitutive overproduction of chromosomally encoded β -lactamase have been described (Hechler *et al.*, 1989). The β -lactam-sensitive reference strains of *S. marcescens* were the type strain CCM 303 and clinical strains HIM 307-2, HIM 337-5 and HIM 697-6 (Professor Dr W. Mannheim, Med. Zentrum für Hygiene, Universität Marburg/Lahn, FRG).

Susceptibility tests. Determinations of MICs by standard macro broth dilution assay and checkerboard tests for the detection of antibacterial synergism of combinations of β -lactam antibiotics (Krogstad & Moellering, 1980) were performed in Mueller-Hinton broth.

Measurements of bactericidal kinetics were carried out in 20 ml LB-broth (Miller, 1972) at 37 °C with slow agitation at 90 r.p.m. on a rotary shaker, in order to avoid mechanical damage of the cells. Initial inocula were 10^5 – 10^6 bacteria ml^{-1} . The media were supplemented with individual β -lactam antibiotics or combinations thereof immediately before inoculation. Surviving bacteria were counted as c.f.u. on LB-agar.

β -Lactamase. Assays of β -lactamase activity were performed as described previously (Hechler *et al.*, 1989).

Assays of PBPs. PBPs in cell envelopes of *S. marcescens* were determined quantitatively by a modification of the technique of Spratt (1977). Isolated cell envelopes were prepared by sonication of bacteria harvested at an OD_{578} of 0.8 from cultures in 250 ml Penassay broth (Difco) in 1 litre conical flasks incubated at 37 °C on a rotary shaker at 180 r.p.m. The envelopes were freed from adherent β -lactamase, as far as possible, by repeated washings in 0.05 M-sodium phosphate buffer,

pH 7.0. Suspensions of envelopes in phosphate buffer containing 25 mg protein ml^{-1} were stored at –80 °C. For radioactive labelling of PBPs, envelope samples (650 μg protein) were mixed rapidly and simultaneously with cloxacillin at 1 μM final concentration, to inactivate envelope-bound β -lactamase, and with 5 nmol (10 μCi , 370 kBq) [^3H]-benzylpenicillin (Amersham Buchler, Braunschweig, FRG) with a specific activity of 74 GBq mmol^{-1} in a total volume of 55 μl 0.05 M-sodium phosphate buffer, pH 7.0. After incubation for 20 min at 30 °C, the binding of labelled penicillin to the PBPs was stopped by the addition of unlabelled penicillin at a final concentration of 6 mM. The following steps, solubilization of labelled PBPs by Sarkosyl, separation of PBPs by SDS-PAGE and detection of the PBPs on gels by fluorography, were carried out according to published procedures (Spratt, 1977). For SDS-PAGE, samples containing 50–200 μg protein were applied to lanes of 12 mm width on the stacking gel. Quantities of separated labelled PBPs were determined by locating them on the gels from their positions on the fluorograms. The PBP bands were cut out and the gel segments were allowed to swell by incubation with 0.2 ml H_2O in counting vials for 4 h at 50 °C. The radioactive material was then extracted by treatment with 1.5 ml Protosol (NEN-DuPont) for 20 h at 37 °C with agitation. After neutralization of the samples with 4 M-HCl and addition of 4 ml Scintigel (C. Roth, KG) the radioactivity was counted by scintillation spectrometry at 29% counting efficiency.

Binding of extended-spectrum β -lactams to PBPs in cell envelopes. Identical samples of envelopes as described above were incubated for 10 min at 30 °C with a range of twofold increasing concentrations of unlabelled extended-spectrum β -lactams, in the presence of 1 μM -cloxacillin. Thereafter, PBPs to which the unlabelled β -lactams had not bound were labelled with [^3H]benzylpenicillin, as described. Quantities of PBPs with bound extended-spectrum β -lactams were determined by subtracting the amounts of labelled PBPs in samples preincubated with the unlabelled β -lactams from those of control samples which were preincubated identically, except for the absence of extended-spectrum β -lactams. Saturation curves obtained from these values yielded concentrations (EC_{50}) of extended-spectrum β -lactams which prevented the binding of [^3H]benzylpenicillin to 50% of the total amount of the PBPs (Spratt, 1977).

Binding of β -lactam antibiotics to PBPs in growing cells of *S. marcescens* 921/79 LR and HR. The bacteria were grown in shake cultures of 250 ml Penassay medium (Difco) in 1 litre conical flasks at 37 °C. When the cultures had reached the exponential phase of growth, at an OD_{578} of 0.8, β -lactam antibiotics at different concentrations were added and incubation was continued for 30 min, during which period rotary agitation was reduced from 180 to 80 r.p.m., in order to avoid binding of β -lactams to PBPs in mechanically damaged cells. The cultures were then chilled in an ice bath. Low temperature was maintained during the subsequent isolation of cell envelopes, in order to avoid the decay of existing complexes of PBPs with β -lactams. The cells were harvested rapidly by centrifugation and cell sediments were freed of antibiotic-containing growth medium by washing them twice in sodium phosphate buffer by centrifugation. Isolated cell envelopes were prepared as described above. PBPs in the isolated envelopes which had not previously bound unlabelled β -lactam penetrating into the living bacteria were labelled with [^3H]benzylpenicillin and counted. As controls, quantities of PBPs were determined in LR and HR strains taken through the same procedure, but without incubation of the cells with unlabelled β -lactams.

Antibiotics. β -Lactam antibiotics were kindly provided by the following companies: Merck, Sharp & Dohme (imipenem); Squibb-Von Heyden (aztreonam); Cascan (ceftazidime); Hoechst (cefotaxime); Eli Lilly (cephalexin); Rorer (mecillinam); Pfizer Central Research (6- β -iodopenicillanic acid); Beecham-Wülfig (cloxacillin).

Fig. 1. Effect of the inactivation of envelope-bound β -lactamase by various cloxacillin concentrations on the covalent binding of [^3H]benzylpenicillin to PBPs in isolated envelopes of resistant *S. marcescens*, strains LR (a) and HR (b). As a control, competition of cloxacillin with [^3H]benzylpenicillin for PBP binding was measured in envelopes of sensitive *S. marcescens* CCM 303 (c), which contained only a negligible amount of bound β -lactamase. For curves marked ' $\times 10$ ' the actual values measured were 10 times those plotted.

Results and Discussion

Quantitative determination of PBPs in the β -lactamase-overproducing strains of *S. marcescens*

Envelopes prepared from the resistant, β -lactamase-overproducing *S. marcescens* 921/79 strains HR and LR contained firmly attached β -lactamase which could not be removed by repeated washing with buffer. Specific activities of β -lactamase in such envelopes were 57 and 30 mU (mg protein) $^{-1}$, respectively, in comparison to 3 to 5 mU (mg protein) $^{-1}$ in envelopes of four sensitive reference strains. As a consequence, several PBPs, mainly 1B, 1C, 5 and 6, were greatly underestimated by the standard PBP assay in envelopes of strains HR and LR, due to degradation by the envelope-bound β -lactamase of the [^3H]benzylpenicillin used for PBP labelling.

Cloxacillin (Jack & Richmond, 1970) and 6- β -iodopenicillanic acid (De Meester *et al.*, 1986) were tested for their ability to inactivate the envelope-bound β -lactamase without simultaneous binding to PBPs. At concentrations required for complete inactivation of β -lactamase, 6- β -iodopenicillanic acid also bound extensively to different PBPs. Cloxacillin inhibited the *S. marcescens* β -lactamase instantly and completely under PBP-assay conditions. The amounts of PBPs labelled by [^3H]benzylpenicillin in the presence of different concentrations of cloxacillin were determined in envelopes of the resistant strains HR and LR and of the sensitive strain CCM 303. As shown in Fig. 1, inactivation of β -lactamase greatly increased the measurable quantities of PBPs 1B, 1C and 5/6 in strains HR and LR, yielding a plateau at cloxacillin concentrations of 1–5 μM . In strain CCM 303, on the other hand, cloxacillin at 1 μM concentration bound to 10% of PBP 3 and 25% of PBP 4, and to 40%

Fig. 2. SDS-PAGE of PBPs in β -lactam-resistant and -sensitive *S. marcescens* labelled with [3 H]benzylpenicillin. Resistant strains: HR (lane A), LR (lane B). Sensitive strains: CCM 303 (lane C), HIM 307-2 (lane D), HIM 337-5 (lane E), HIM 697-6 (lane F). Samples contained 200 μ g (a) and 50 μ g (b) protein.

and more of these PBPs at higher concentrations. Therefore, radioactive labelling of PBPs was generally performed in the presence of 1 μ M-cloxacillin.

Amounts of PBPs in resistant and sensitive strains of S. marcescens

For quantitative comparison of PBPs in different strains of *S. marcescens*, equal amounts of isolated envelopes from identical numbers of bacteria were labelled with [3 H]benzylpenicillin of high specific activity. PBPs in identical amounts of solubilized envelope protein were separated by SDS-PAGE. The resistant strains HR and LR and the four sensitive strains agreed in the pattern and electrophoretic mobilities of the PBPs (Fig. 2). The numbering of the PBPs relied on the observed selective binding of mecillinam to PBP 2, and of cephalixin, aztreonam and ceftazidime to PBP 3, as known from *Escherichia coli* and other enterobacteria (Spratt, 1977; Curtis *et al.*, 1979; Georgopadakou, 1988). On PAGE analysis of larger samples, an additional minor PBP 3A was consistently found (Fig. 2). However, this PBP showed no selectivity or high affinity for any β -lactam and was therefore not considered further.

Representative counts of radioactively labelled PBPs in equal quantities of envelopes from resistant and sensitive *S. marcescens* strains are given in Table 1. Numbers of molecules of PBPs per cell were calculated from the amounts of radioactivity in known numbers of bacteria, assuming the formation of equimolar covalent complexes from [3 H]benzylpenicillin and PBPs. Most of

the eight PBPs were found in similar quantities in strain LR and in the four unrelated, sensitive strains. We conclude that the low resistance of *S. marcescens* strain LR is not connected with the increase of any PBP. In contrast, the highly resistant strain HR differed from the related strain LR and the sensitive strains by a more than twofold higher content of PBP 6. The augmentation of a low molecular mass PBP in highly β -lactam-resistant *Pseudomonas aeruginosa* (Godfrey *et al.*, 1981), and of PBP 6 in resistant *S. marcescens* (Gutmann & Chabbert, 1984) has been reported previously. It is not clear how the doubling of the amount of PBP 6, a non-essential PBP in enterobacteria, should contribute to the high β -lactam resistance of strain HR. Also, the significance of the observed smaller increase of PBP 3 in cells of strain HR remains uncertain.

Comparison of sensitivities of PBPs for extended-spectrum β -lactams in S. marcescens strains HR and LR

Sensitivities of the essential PBPs 1A, B, C, 2 and 3 in isolated cell envelopes of strains HR and LR were determined as concentrations (EC_{50}) of different extended-spectrum β -lactams preventing the binding of [3 H]benzylpenicillin to 50% of the amounts of individual PBPs, as described in Methods. The results did not reveal significantly different sensitivities of PBPs in *S. marcescens* strains LR and HR (Table 2). This agrees with our previous, tentative conclusions from model calculations that target sensitivities are the same in the high and low states of resistance of *S. marcescens* strains

Table 1. Quantities of PBPs in resistant (LR, HR) and sensitive (CCM 303, HIM 307-2, HIM 697-6) strains of *S. marcescens*

Values of triplicate labelling experiments were determined as amounts of PBP-bound [^3H]benzylpenicillin (c.p.m.: counts min^{-1} at 29% counting efficiency) in samples of 200 μg of Sarkosyl-solubilized envelope protein, and are shown $\pm$ standard deviations. Numbers of molecules of PBPs per cell were calculated from the counts of radioactivity and numbers of bacteria corresponding to the samples. The numbers for PBPs 3 and 4 were corrected for 10 and 25% loss, respectively, due to binding to the PBPs of cloxacillin added to the labelling mixtures as inactivator of envelope-bound β -lactamase. Relative amounts of PBPs in highly resistant strain HR are ratios of numbers of PBPs in HR to those in the related, less resistant strain LR.

PBB	Resistant strains				Sensitive strains						
	HR		LR		CCM 303		HIM 307-2		HIM 697-6		
	Quantity (c.p.m.)	Molecules per cell	Relative amounts	Quantity (c.p.m.)	Molecules per cell	Quantity (c.p.m.)	Molecules per cell	Quantity (c.p.m.)	Molecules per cell	Quantity (c.p.m.)	Molecules per cell
1A	3454 ± 338	161	1.2	2736 ± 214	131	2573 ± 328	95	2563 ± 272	122	2967 ± 261	160
1B	1109 ± 613	52	1.1	1021 ± 98	49	1049 ± 15	50	957 ± 71	46	948 ± 59	51
1C	1347 ± 49	63	0.8	1614 ± 88	77	1373 ± 86	70	1446 ± 51	69	2007 ± 80	109
2	1045 ± 75	49	1.3	773 ± 54	37	965 ± 161	46	858 ± 30	41	1038 ± 106	56
3	1447 ± 50	76	1.5	957 ± 25	51	1031 ± 126	54	1016 ± 14	53	1077 ± 55	64
4	3020 ± 163	188	1.1	2808 ± 201	179	2869 ± 178	183	2497 ± 136	159	2429 ± 118	175
5	5796 ± 464	272	1.0	5720 ± 624	273	6704 ± 218	320	5964 ± 456	285	5800 ± 776	314
6	14723 ± 660	688	2.2	6436 ± 512	313	6484 ± 412	309	5304 ± 264	253	5264 ± 472	285
5 + 6	22524 ± 931	1052	1.7	12610 ± 717	602	14016 ± 204	667	11686 ± 582	558	11268 ± 700	610
Total	33945 ± 399			22519 ± 692		24038 ± 660		21023 ± 401		21738 ± 483	

Table 2. EC_{50} values of antibiotics for essential PBPs in isolated envelopes of *S. marcescens* LR and HR

The EC_{50} is defined as the concentration ($\mu\text{g ml}^{-1}$) required to prevent the binding of [^3H]benzylpenicillin to 50% of the amounts of individual essential PBPs (see Methods).

PBP	Cefotaxime		Aztreonam		Ceftazidime		Imipenem	
	LR	HR	LR	HR	LR	HR	LR	HR
1A	1	<1	2	4	8	8	0.25	0.25
1B	4	2	>8	>8	32	32	>1	>1
1C	4	4	>8	>8	32	32	>1	>1
2	4	4	>8	>8	>32	32	0.06	0.06
3	<1	<1	<<0.5	<<0.5	<<2	<<2	<1	<1

HR and LR, and are presumably also identical with those in sensitive *S. marcescens* strains (Hechler *et al.*, 1989). Confirmation of this concept will require detailed analysis of the kinetic parameters of PBP- β -lactam interaction, as described by Frère & Joris (1985) and by Ghuysen *et al.* (1986). The observation of identical target sensitivities, if confirmed, will also support our previous assumption that the required lethal periplasmic concentrations (I_{pl}) (Frère, 1989) of the antibiotics are the same in sensitive and resistant *S. marcescens* (Hechler *et al.*, 1989).

The EC_{50} values also showed that sensitivities of essential PBPs for the individual antibiotics differed considerably. Sensitivity to cefotaxime, ceftazidime and aztreonam was high ($EC_{50} < 1 \mu\text{g ml}^{-1}$) for only one target, PBP 3, and moderately high for PBP 1A. In contrast, sensitivity to imipenem was high for PBPs 2 and 1A, and also moderately high for PBP 3. With the exception of high sensitivity of PBP 4 to imipenem (EC_{50} , $0.06 \mu\text{g ml}^{-1}$), the non-essential PBPs 4, 5 and 6 had low sensitivities for the tested β -lactams (data not shown). High sensitivities of multiple PBPs to imipenem have been reported previously for non-resistant *E. coli* (Spratt *et al.*, 1977; Georgopapadakou, 1988). We will discuss below why this exceptional feature may be a major cause of the so far unexplained low resistance of both LR and HR strains to imipenem, with MICs of 1 and $4 \mu\text{g ml}^{-1}$, respectively (Hechler *et al.*, 1989).

Binding of β -lactams to PBPs in growing cells of *S. marcescens* strains LR and HR

Typical defects in cell shape were observed microscopically in *S. marcescens* strains LR and HR growing at subinhibitory concentrations of different antibiotics in the MIC assay. The amidinopenicillin mecillinam was not growth-inhibitory for HR or LR strains even at $1024 \mu\text{g ml}^{-1}$. However, it caused formation of round cells at the much lower concentration of $4 \mu\text{g ml}^{-1}$. This

was in keeping with its known specific binding to enterobacterial PBP 2 and the ensuing inhibition of the formation of rod-shaped cells. In the presence of cefotaxime, ceftazidime or aztreonam, at one-quarter and less of their MICs (1024 , 64 and $128 \mu\text{g ml}^{-1}$ for strain HR, and 32 , 1 and $8 \mu\text{g ml}^{-1}$ for strain LR, respectively), the bacteria grew as filaments. This indicated binding of these antibiotics to PBP 3 and inactivation of its specific function for cell division (Spratt, 1975). Therefore, the interaction of β -lactams with targets in intact cells of the resistant bacteria was measured. The growing bacteria were exposed to different antibiotics over a range of concentrations from $0.25 \times \text{MIC}$ for strain LR to $2 \times \text{MIC}$ for strain HR. Exposure time was 30 min, corresponding to one generation time of LR-cells. The binding data are presented in Fig. 3.

It is obvious that the quantification of the PBP- β -lactam interaction in growing bacteria suffers from unavoidable imperfections and technical difficulties. MICs of antibiotics cannot be strictly correlated with the measured extent of their binding to PBPs in bacteria because 100-fold higher cell densities were necessary to obtain sufficient yields of membranes for PBP assays. At this higher cell density, β -lactams will be more extensively degraded by the periplasmic β -lactamase than at the low cell densities (10^5 – 10^6 cells ml^{-1}) of the standard MIC assay. The measured binding of extended spectrum β -lactams includes only the covalent PBP- β -lactam complexes which formed sufficiently rapidly and remained stable under the chosen conditions of preincubation and post-labelling of free PBPs. Therefore, our data probably underestimate the degree of PBP inactivation at the MICs. With this in mind, we report the following observations and tentative conclusions.

1. In both LR and HR strains, binding of cefotaxime, ceftazidime, aztreonam and imipenem to PBPs took place even at concentrations of 8 , 0.25 , 2 , and $0.25 \mu\text{g ml}^{-1}$, respectively. These values corresponded to one-quarter of the MICs of LR, and to fractions of the

Fig. 3. Binding of different extended-spectrum β -lactam antibiotics to PBPs in growing cells of *S. marcescens* LR (○) and HR (●). The bacteria were incubated with β -lactams for 30 min, i.e. one generation time of strain LR. Concentrations of antibiotics were from $0.25 \times \text{MIC}$ for strain LR to $2 \times \text{MIC}$ for strain HR. Subsequently, envelopes were isolated and those fractions of PBPs which had not bound β -lactams in whole cells were labelled with [^3H]benzylpenicillin.

MICs for HR ranging from $1/32$ to $1/256$. The binding involved a minimum of 40%, and in most cases much more, of at least one of the PBPs thought to be essential in enterobacteria. Thus, even far below their MICs, β -lactams were not excluded from their targets by the outer-membrane- β -lactamase barrier of resistant *S. marcescens*.

2. In HR cells, cefotaxime, ceftazidime and aztreonam at subinhibitory concentrations up to 64, 16 and $2 \mu\text{g ml}^{-1}$, respectively, bound only to PBP 3, the single target with high sensitivity ($\text{EC}_{50} \leq 1 \mu\text{g ml}^{-1}$) to these antibiotics. Under these conditions, 60–80% of this PBP was bound. However, this extensive inactivation of a single essential PBP was obviously not lethal for resistant *S. marcescens*.

Binding of mecillinam to PBPs in growing HR cells was measured at 2, 4 and $8 \mu\text{g ml}^{-1}$. These values represent 0.5, 1 and 2 times the minimal concentration of the antibiotic for uniform formation of round cells. Binding took place to PBP 2 only, to the extent of 12, 25 and 31%, respectively.

3. Maximal binding of cefotaxime and ceftazidime to PBP 3 in strain HR required eight-fold higher concentrations of the antibiotics than in strain LR. At 64 and $2 \mu\text{g ml}^{-1}$, respectively, these concentrations were still well below the MICs for strain HR. This requirement may be due to a lower outer membrane permeability of HR cells, as previously determined with the mathematical models (Hechler *et al.*, 1989). A defective permeability barrier in HR cells was also suggested by

our recent observation that outer membranes of strain HR are distinguished from those of strain LR by the reduced content of a 42 kDa protein. This protein resembled the OmpF porin of *E. coli* (Lugtenberg & Van Alphen, 1983) in peptidoglycan association and osmo-regulation of expression (H. Weindorf & H. H. Martin, unpublished data).

4. At and above their MICs, cefotaxime, ceftazidime and aztreonam bound to additional, less sensitive targets, predominantly to PBP 1A, in addition to extensive binding to PBP 3. Thus, inactivation of multiple essential PBPs, specifically PBPs 3 and 1A, may be necessary to kill the bacteria. The same conclusion was reached previously for non-resistant *E. coli* and *Salmonella typhimurium* (Chase *et al.*, 1981). The I_{pl} and respective MIC should then be those concentrations of a β -lactam which inactivate all lethal targets, including the least sensitive essential PBP. By rigorous quantitative analysis, the high MICs of some extended-spectrum β -lactams in resistant Gram-negative bacteria have been explained as those externally applied concentrations which still allow the antibiotics to reach the I_{pl} in spite of their extensive degradation during their passage through the outer membrane- β -lactamase barrier (Nikaido & Normark, 1987; Frère, 1989; Hechler *et al.*, 1989). Kinetic binding parameters of the essential multiple targets to individual β -lactams, once established, should contribute further to these resistance models.

5. Imipenem differed from the other antibiotics by binding to multiple targets in whole cells at concentrations below the MIC. At $0.25 \mu\text{g ml}^{-1}$, i.e. $1/4$ and $1/32$ of the MICs for strains LR and HR (1 and $4 \mu\text{g ml}^{-1}$, respectively), imipenem bound to more than 50% of PBPs 2, 4 and 1A. With increasing concentration up to the MICs, significant binding to all other PBPs (3, 1B, 1C, 5 and 6) took place.

An exceptional ability of imipenem to remain growth-inhibitory at low concentration has been reported frequently for β -lactamase-overproducing and penetration-restricted *Enterobacter cloacae* highly resistant to other extended-spectrum β -lactams (Bush *et al.*, 1985; Werner *et al.*, 1985; Then & Angehrn, 1986; Marchou *et al.*, 1987). For *P. aeruginosa* it has been proposed that imipenem uses specific penetration channels which are impermeable for other β -lactams (Büscher *et al.*, 1987; Nikaido, 1989). Such evidence is not yet available for enterobacteria.

Our present data suggest the alternative explanation that the high sensitivity to imipenem of several essential PBPs, especially of PBP 2, 1A and 3 at $EC_{50} \leq 1 \mu\text{g ml}^{-1}$, enables this antibiotic to simultaneously inactivate these lethal targets in growing bacteria at relatively low concentration. This antibiotic may thus be able to kill bacteria at a low I_{pl} and MIC in spite of its

Fig. 4. Synergistic growth inhibition of highly resistant *S. marcescens* HR by combinations of different extended-spectrum β -lactams with mecillinam. The points indicate the growth-inhibitory concentrations of the respective mixtures of antibiotics. Individual MICs of the antibiotics in $\mu\text{g ml}^{-1}$ are given in parentheses.

appreciable degradation by the highly concentrated periplasmic concentrations of β -lactamase in resistant *S. marcescens* (Hechler *et al.*, 1989). The moderate elevation of the MIC of imipenem to $4 \mu\text{g ml}^{-1}$ in strain HR could then be caused by the decreased permeability of HR cells.

Bactericidal synergism of β -lactam antibiotics by multiple target inactivation in the highly resistant S. marcescens strain HR

If bactericidal action depends on the inactivation of more than one essential PBP, synergistic killing of the bacteria could be expected from combinations of β -

Fig. 5. Bactericidal kinetics of combinations of extended-spectrum β -lactams with mecillinam in highly resistant *S. marcescens* HR. The following concentrations of antibiotics ($\mu\text{g ml}^{-1}$) were present individually or in combinations at the time of inoculation of the cultures: mecillinam (Me) 4, cefotaxime (Ct) 64, ceftazidime (Cf) 2, aztreonam (Az) 16. Absence of bactericidal synergism by inactivation of the bacterial β -lactamase was demonstrated by the growth curve with the combination of cefotaxime with β -lactamase-inactivating aztreonam.

lactams with different binding selectivities. At concentrations below their individual MICs, these should achieve simultaneous binding to two or more essential PBPs having high sensitivity to the respective antibiotics. Combinations of the PBP-2-binding mecillinam with different PBP-3-binding antibiotics were therefore tested.

Alternatively, support of the growth inhibitory action of one β -lactam could result from the function of a second β -lactam as inactivator of the periplasmic β -lactamase of the bacteria. This was tested by combinations with aztreonam. As shown previously (Hechler *et al.*, 1989), aztreonam is a highly effective inactivator of chromosomally encoded β -lactamase of *S. marcescens* because of its very high affinity for the enzyme (K_m 0.012 μM), the rapid formation of enzymically inactive acyl-enzyme (k_2/K $1.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$), and the effective immobilization as acyl-enzyme with a very low decay rate (k_3 $1.5 \times 10^{-4} \text{ s}^{-1}$).

Results of checkerboard assays of antibiotic combinations (Fig. 4) and of bactericidal kinetics (Fig. 5) supported the first alternative. For strain HR, MIC values of cefotaxime, aztreonam and ceftazidime were reduced from 1024 to 64, from 128 to 16, and from 64 to 1 $\mu\text{g ml}^{-1}$, respectively, in the presence of 4 $\mu\text{g ml}^{-1}$ mecillinam. In contrast, the MIC of cefotaxime remained unchanged at 1024 $\mu\text{g ml}^{-1}$ when aztreonam was added up to 32 $\mu\text{g ml}^{-1}$, i.e. 1/4 of the MIC of this antibiotic (Fig. 4).

Experiments on bactericidal kinetics of the same antibiotic combinations with strain HR were carried out as described in Methods. As shown in Fig. 5, combinations of mecillinam (4 $\mu\text{g ml}^{-1}$) with cefotaxime, aztreonam or ceftazidime, at 64, 16 and 2 $\mu\text{g ml}^{-1}$, respectively, were bactericidal. On the other hand, the combination of cefotaxime with 16 or 32 $\mu\text{g ml}^{-1}$ aztreonam was not bactericidal, and retarded the growth of HR cells only slightly more than the single antibiotics. Synergism of mecillinam with other β -lactams has been reported frequently in enterobacteria (Tybring & Melchior, 1975; Baltimore *et al.*, 1976; Neu, 1976; Gutmann *et al.*, 1986). In the highly β -lactamase-overproducing *S. marcescens* strain HR it was the more remarkable since *Serratia* β -lactamase hydrolysed mecillinam much more effectively than the extended-spectrum β -lactams. The enzyme was also not inactivated by mecillinam (M. Van den Weghe, unpublished results).

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