Overproduced β-Lactamase and the Outer-membrane Barrier as Resistance Factors in Serratia marcescens Highly Resistant to β-Lactamase-stable β-Lactam Antibiotics

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In a clinical isolate of Serratia marcescens different states of low and high resistance to different β-lactam antibiotics considered to be β-lactamase-stable, viz. cefotaxime, ceftizoxime, ceftazidime, aztreonam, cefoxitin and imipenem, were found to be connected with the presence of constitutively overproduced, chromosomally encoded β-lactamase at concentrations in the bacterial periplasm of 0.4 and 0.9 mM, respectively. All the antibiotics were degraded by the β-lactamase. However, kinetic constants varied widely: $K_m$ from 92 to 0.012 μM, and $k_{cat}$ from 3.4 to $2 \times 10^{-4}$ s⁻¹. The relative contributions to resistance by the functioning of periplasmic β-lactamase, resynthesis of this enzyme, and limitation of antibiotic penetration by the bacterial outer membrane were analysed by computer simulations according to steady-state and non-steady-state models of interactions in the periplasm. Results for cefotaxime, ceftizoxime, ceftazidime, aztreonam and latamoxef revealed overproduced β-lactamase as the sole cause of the state of low resistance while antibiotic permeability was the same as in non-resistant S. marcescens strains. In contrast, high resistance was due to β-lactamase action and decreased permeability of antibiotics. For resistance to aztreonam, only, immobilization of the antibiotic as covalent acyl–enzyme by newly synthesized β-lactamase was essential. For cefoxitin, ampicillin and imipenem the analyses indicated that additional resistance factors may play a role, e.g. induction of β-lactamase.

INTRODUCTION

The appearance of high resistance to β-lactam antibiotics in clinically important Gram-negative bacteria has been recognized as the result of a concerted interplay of two main resistance factors, viz. restriction of antibiotic penetration into the bacteria by the permeability barrier of the outer membrane and inactivation of sparsely penetrating antibiotic by β-lactamase in the bacterial periplasm (Vu & Nikaido, 1985; Livermore et al., 1986; Nikaido & Normark, 1987; Waley, 1987; Frère & Joris, 1988).

It has been shown further that in specific Gram-negative bacteria such as Enterobacter cloacae and related enterobacteria the efficiency of the same resistance factors can be greatly enhanced by mutation, resulting in the quite unexpected extension of resistance to the newer β-lactam compounds, which are known for their greatly increased stability to inactivation by β-lactamases. Changes in outer membrane composition were found here to cause further

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Abbreviation: IEF, isoelectric focusing.
restriction of antibiotic penetration (Bush et al., 1985; Marchou et al., 1987). Simultaneously or alternatively, the resistant bacteria were seen to overproduce the typical chromosomally coded class C β-lactamase and accumulate it in high concentration in their periplasm whereas in normal bacteria this enzyme is expressed constitutively at a low level only, and synthesis at higher concentration requires induction (Sanders & Sanders, 1979; Beckwith & Jahre, 1980; Lampe et al., 1982; Gootz et al., 1982, 1984; Seeberg et al., 1983; Curtis et al., 1986; Lindberg & Normark, 1986).

Much of this seeming paradox was resolved when recent studies demonstrated that representative members of the family of structurally different β-lactam antibiotics with apparent β-lactamase stability interact with class C β-lactamases with high affinity and suffer hydrolytic inactivation, albeit at a low rate (Bush et al., 1982; Vu & Nikaido, 1985; Joris et al., 1986; Livermore et al., 1986; Nikaido & Normark, 1987; Galleni et al., 1988).

It was thus plausible to conclude that following their slow penetration into resistant bacteria the β-lactam antibiotics, including the β-lactamase-stable ones, are hydrolytically degraded by the overproduced periplasmic β-lactamase and are thereby prevented from reaching their target sites, the essential bacterial penicillin-binding proteins (PBP).

The present study was undertaken to verify the validity of this resistance mechanism in β-lactamase overproducing clinical strains of Serratia marcescens with high resistance to cefotaxime and a varied pattern of high and low resistance to structurally different β-lactamase-stable β-lactam compounds.

In order to define the conditions leading to the postulated elimination of the antibiotics from the target sites, the interplay of relevant events in the bacterial periplasm, including outer membrane penetration by the antibiotics, interaction of antibiotics with periplasmic β-lactamase and continued synthesis of β-lactamase was analysed according to the steady state and non-steady state models of interaction which have been reported recently by Frère (1989) and by Frère et al. (1989).

**METHODS**

*Bacteria.* The clinical strain *Serratia marcescens* 921/79 was received from Professor W. Stille, Zentrum für Innere Medizin, Universität Frankfurt/Main, FRG; it exhibited high resistance to cefotaxime (MIC 256 µg ml⁻¹). Taxonomic classification was confirmed with the Enterotube II (Roche), API 20E and Rapid 20E (bioMérieux) systems, with additional determination of exo-DNAses activity and colistin sensitivity. Gradual loss of high resistance occurred during serial subculture in broth or on solid medium in the absence of antibiotic to reach a stable state of lower resistance to cefotaxime (LR, MIC 16–32 µg ml⁻¹) within 3–6 months. On the other hand, a rise to even higher cefotaxime resistance (HR, MIC 1024 µg ml⁻¹) was obtained in subcultures of the clinical isolate 921/79 after 27 serial transfers of inocula of 10⁶ bacteria in 2 ml batches of Mueller–Hinton broth containing 256 µg cefotaxime ml⁻¹ with 48 h incubation periods of 30°C. *S. marcescens* 921/79 HR and LR with high and low resistance to cefotaxime were preserved in the lyophilized state at −20°C for further experiments. Non-resistant reference strains of *S. marcescens*, CCM 303 (type strain) and HIM 307-2 (clinical isolate) were kindly supplied by Professor W. Mannheim, Med Zentrum für Hygiene, Universität Marburg/Lahn, FRG.

*Susceptibility tests.* MICs of β-lactam antibiotics were determined by standard macro broth dilution assay in Mueller–Hinton medium.

*Antibiotics.* β-Lactam compounds were donated by the following companies: imipenem and cefoxitin (MSD Sharp & Dohme, München); cefotizoxime (Dr Vömel, Boehringer, Mannheim); latamoxef, cephalothin and cephaloridine (Eli Lilly, Bad Homburg); aztreonam (Squibb-Von Heyden, München), cefazidime (Cascan, Wiesbaden); cefotiam (Ciba-Geigy, Frankfurt/Main), cefotaxime (Hoechst, Frankfurt/Main); nitrocefin (Glaxo, Greenford, UK). Benzylpenicillin, ampicillin and nalidixic acid were commercial products.

*β-Lactamase techniques.* Cultivation and induction of *S. marcescens* for β-lactamase production. Exponential-phase cultures of *S. marcescens* grown to an OD₅₇₈ of 0.8–1.0 in 250 ml Penassay broth (Difco) in 1 litre conical flasks on a rotary shaker at 180 r.p.m. and at 37°C were supplemented with 10 µg cefoxitin ml⁻¹ as β-lactamase inducer and incubation was continued for 2 h. Then cultures were cooled to 4°C, cells harvested by centrifugation and washed by resedimentation from suspension in 200 ml potassium phosphate buffer (0.05 M, pH 7.0).

Isolation and purification of β-lactamase. Crude extracts of soluble β-lactamase were prepared from a suspension of 240 g (wet wt) of induced cells of *S. marcescens* 921/79 HR in 1 litre potassium phosphate buffer with batchwise fragmentation of bacteria by sonication. Insoluble cell fragments were removed by sedimentation at 165000 g for 1 h at 4°C. Steps of β-lactamase purification from the crude extract were as follows:
(i) Precipitation of nucleic acids by addition of 1.5% (w/v) streptomycin.
(ii) Change to sodium acetate buffer (0.05 M, pH 4.5) by exhaustive dialysis, removal of precipitated material by centrifugation, adsorption of \( \beta \)-lactamase from solution to 100 ml carboxymethyl–trisacryl M cation exchange resin (Pharmacia), washing of resin-bound \( \beta \)-lactamase with sodium acetate buffer, desorption of \( \beta \)-lactamase from the ion exchange resin with 150 ml sodium acetate buffer (0.3 M-NaCl) and removal of NaCl from the enzyme solution by dialysis in sodium acetate buffer (0.05 M, pH 7.0).
(iii) Ion exchange chromatography on a column of 13 ml carboxymethyl–trisacryl M resin eluted with a linear gradient of 0–0.3 M-NaCl in sodium acetate buffer (0.05 M, pH 4.5) at a flow rate of 52 ml h\(^{-1}\), concentration of the enzyme peak volume by ultrafiltration on Amicon Diaflo membranes, type PM 10 and transfer to phosphate buffer (0.05 M, pH 7.0).
(iv) Gel filtration on a 1 x 85 cm column of Ultrogel AcA 54 (Pharmacia) eluted with phosphate buffer (0.05 M, pH 7.0)/0.05 M-NaCl, at a flow rate of 0.5 ml min\(^{-1}\) and monitoring of the eluate at 280 nm.

**Isoelectric point of \( \beta \)-lactamase.** Determinations were made by isoelectric focusing for 2 h on Servalyt-Precotes, pH 3–10 (Serva). \( \beta \)-Lactamase bands were identified by flooding gels with nitrocefin solution (Matthew et al., 1975). Reference proteins were cytochrome c, ribonuclease, myoglobin (whale) and myoglobin (horse) (pI values of 10.68, 9.4, 8.3 and 7.73, respectively).

\( M_0 \) of \( \beta \)-lactamase. This was determined by SDS-PAGE (Laemmli & Favre, 1973). Standard proteins were bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c (\( M_0 \), values 67000, 45000, 25000 and 125000, respectively).

**Assays of \( \beta \)-lactamase activity.** Assays of the hydrolysis of \( \beta \)-lactam compounds were done with recording spectrophotometers (Perkin-Elmer lambda 3, or Beckman DU 8 for measurements at highest sensitivities) at the following wavelengths (nm) for the different substrates: cephalothin (262), cephaloridine (260), cefotiam (276), cefotaxime (264), ceftizoxime (250), cefazedone (257), cefoxitin (265), latamoxef (275), aztreonam (315), imipenem (297) and nitrocefin (482) (Minami et al., 1981). \( K_m \) for the quantification of \( \beta \)-lactamase activity, initial velocities were determined with 0.3 mM-cephalothin or 0.1 mM-nitrocefin at about 3 x the \( K_m \) of these good substrates. In contrast to nitrocefin, cephalothin caused slow substrate-induced inactivation of *Serratia* \( \beta \)-lactamase as described previously (Joris et al., 1986). However, this did not impede short time assays of 60–120 s duration.

Hydrolysis of penicillins was measured by alcalimetric pH-stat titration (Citri & Zykg, 1965) with a combititrator (Deutsche Methrom). One unit (U) of \( \beta \)-lactamase hydrolysates 1 \( \mu \)mol of substrate min\(^{-1}\) in phosphate buffer (0.05 M, pH 7.0) at 25 °C.

**Enzyme kinetic constants.** Values of \( K_m \) and \( V \) were derived from direct linear graphs of initial velocities (Eisenthal & Cornish-Bowden, 1974) with an enzyme concentration of either 4 nM or 0.4–2 \( \mu \)M for good and poor substrates, respectively. Alternatively, \( K_m \) and \( V \) came from computerized analyses of complete time courses of substrate hydrolysis monitored spectrophotometrically in the Beckman DU 8 (De Meester et al., 1987). \( K_i \) constants for competitive inhibition were obtained as intercepts on the abscissa of secondary plots of \( k_2 \) vs [I], whose value is given by equation (1)

\[
EI + P \xrightarrow{K_m} EI* \xrightarrow{V} E + P
\]

where \( E \) is the enzyme, \( I \) aztreonam or imipenem, \( K \) the dissociation constant of \( EI \) and \( EI* \) the acyl–enzyme intermediate.

Under those conditions, the decrease of the rate of hydrolysis of the reporter substrate was characterized by a pseudo-first-order rate constant, \( k_2 \), whose value is given by equation (1)

\[
k_2 = k_3 + k_4 = k_3 + \frac{k_2[I]}{[I] + K_m + [S]} \quad (1)
\]

where the \( K_m \) is that for the reporter substrate \( S \).

The rate of hydrolysis of the reporter substrate at the steady state (\( v_{es} \)) could be compared to that observed in the absence of inactivator (\( v_{0} \))

\[
v_{es} = 1 + \frac{K_m}{[S] + K_m} \cdot \frac{k_2 + k_3}{k_2 + k_3} \quad (2)
\]
If \( I \ll K \) and \( k_3 \ll k_2 \), equations (1) and (2) simplify to

\[
k_a = k_3 + \frac{K_m}{[S] + K_m} \frac{k_2}{K} [I]
\]

and

\[
\frac{v_0}{v_{ns}} = 1 + \frac{K_m}{[S] + K_m} \frac{k_2}{k_3} \frac{[I]}{K}
\]

and the values of \( k_2/K \) and \( k_3 \) can easily be obtained. Also, under these conditions minimal values of \( k_a \) and \( K \) can be derived (Galleni & Frère, 1989).

Alternatively, \( k_3 \) was determined as described by Marquet et al. (1974) as the rate constant of the recovery of enzyme activity in \( \beta \)-lactamase samples after complete inhibition by imipenem or aztreonam and subsequent initiation of the decay of the acyl–enzyme formed by 20-fold dilution of samples with phosphate buffer.

Application of models of periplasmic interactions and computation of the relative permeability factors. The steady-state model of Frère (1988) was used when the \( k_{cat} = k_3 \) value for the \( \beta \)-lactam was larger than 0.01 s\(^{-1}\). This method relies on the hypothesis that the concentration of antibiotic rapidly reaches a steady state in the periplasmic space.

For the comparison of antibiotic permeabilities between sensitive bacterial strains (sb) with a low \( \beta \)-lactamase content and resistant bacteria with higher concentrations of periplasmic \( \beta \)-lactamase and for the determination of the rate constant of antibiotic diffusion into the bacteria, \( k_D \), the model uses a rearrangement of the well-known steady-state equation of Zimmermann & Rosselet (1977)

\[
\frac{k_D}{(k_{cat} E_0)_{sb}} = \frac{R_{I_{pl}}}{(K_m + I_p)(MIC - I_p)}
\]

where experimentally obtained values are \( E_0 \) and \( (k_{cat} E_0)_{sb} \), the initial periplasmic concentration and the specific activity of \( \beta \)-lactamase with a standard substrate, respectively, in the sensitive strain, \( R \), the ratio of specific \( \beta \)-lactamase activities of resistant to sensitive strains, \( K_m \), pertaining to the penetrating antibiotic as \( \beta \)-lactamase substrate, and \( MIC \), the minimal inhibitory concentration of the antibiotic for growth of the resistant strain.

The remaining unknown in the right-hand side of the equation is \( I_{pl} \), the lethal value of the periplasmic concentration of the antibiotic, \( I_p \).

Determination of \( I_{pl} \) is then possible by calculating the curves

\[
Z = \frac{R_{I_{pl}}}{(MIC - I_p)(K_m + I_p)}
\]

for each sensitive and resistant strain, with \( I_p \) as the independent variable. Curves from strains with identical target sensitivities and permeabilities but different \( \beta \)-lactamase contents intersect at the coordinates \( I_{pl} \) and \( Z \). From these values, \( k_D \) and recalculated values \( MIC_{calc} \) can be computed; also for strains were curves \( Z \) do not converge at \( I_{pl} \), \( Z \) can be derived, indicating an altered permeability.

With aztreonam, the value of \( k_3 = 2 \times 10^{-4} \) s\(^{-1}\) (see Results) was too low to verify the steady-state hypothesis. Therefore, the non-steady-state model of Frère et al. (1989) was applied. This method enables the simulation of the periplasmic interaction by choosing values for the initial concentrations of the reaction partners and for the rate constants and using a program of numerical integration of the unsolved differential equations for the calculation of values of periplasmic concentrations of \( \beta \)-lactamase \( E \), antibiotic \( I_p \) and acyl–enzyme \( EI^* \) as a function of time. Values of \( k_D \) are obtained directly. This method also takes into account the production of new \( \beta \)-lactamase and the increase in the total periplasmic volume due to bacterial growth.

The model assumes that the increasing periplasmic volume is exactly matched by the increasing transmembrane antibiotic flux and enzyme synthesis. At first sight one might imagine that this should result in a situation identical to that observed with the steady-state model. However, the situation in the periplasm can be visualized as follows. All the new \( \beta \)-lactamase that is produced is active enzyme, \( E \), while the volume increase results in the dilution of a mixture of free and acylated enzyme. Thus, the periplasmic concentrations of \( E \) and \( EI^* \) will be larger and smaller, respectively, than predicted by the steady-state model.

The \textit{a priori} choice of a model was difficult for imipenem, which has an intermediate value of \( k_3 = 1.7 \times 10^{-3} \) s\(^{-1}\). However, application of both models yielded similar results.

According to Frère (1989) the rate constant \( k_0 \) can be related to the well-known ‘permeability coefficient’ \( P \) (cm s\(^{-1}\)) described by Nikaido (1979) and the ‘diffusion parameter’ \( C \) (cm\(^3\) s\(^{-1}\)) of Zimmermann & Rosselet (1977) by showing that

\[
k_D = P \frac{A}{Vol}
\]

where \( A \) and \( Vol \) are the surface area and the periplasmic volume corresponding to
RESULTS

Sensitivity of S. marcescens strains 921/79 with high (HR) and low (LR) cefotaxime resistance to structurally different β-lactam antibiotics

The origin of the highly cefotaxime-resistant strain HR and of the less resistant, spontaneous revertant LR from the clinical isolate 921/79 is described in Methods. Table 1 presents MIC values of β-lactamase-sensitive and β-lactamase-stable β-lactam antibiotics for these strains in comparison to those for the non-isogenic, cefotaxime-susceptible reference strains CCM 303 and HIM 307-2. A typically large increase of MICs from sensitive to moderately and highly resistant strains was observed for the majority of β-lactamase-stable antibiotics.

In contrast, differences between MICs for sensitive and resistant strains were moderate or small for cefoxitin, ampicillin, cephalothin and cefotiam, in accordance with the known lower sensitivity of non-resistant S. marcescens for these antibiotics (Atkinson, 1981). In agreement with similar observations in other resistant Gram-negative bacteria (Bush et al., 1985; Cullmann et al., 1982; Vu & Nikaido, 1985), the growth inhibitory activity of imipenem was largely exempt from resistance and a small increase in the MIC was determined in connection with the highest state of resistance in strain 921/79 HR only.

Resistance and bacterial growth rate

Generation time determined by viable counts of bacteria growing at 37 °C in rotary shake cultures of Penassay broth were 60 min for strain 921/79 HR and 30 min for strain 921/79 LR, CCM 303 and HIM 307-2. Thus, the state of high resistance of the HR strain was connected with a considerable decrease in growth rate.

Properties of β-lactamase in the cefotaxime-resistant S. marcescens strains

Previous reports have described the presence of inducible chromosomally coded class C β-lactamase with the substrate specificity of a cephalosporinase in S. marcescens (Tajima et al., 1981; Sawai et al., 1982; Joris et al., 1986). In the non-resistant strains CCM 303 and HIM 307-2 constitutive β-lactamase activities were typically low [0.13 and 0.21 U (mg protein)-1] but increased 30-40-fold to 5-2 and 6-2 U (mg protein)-1 upon induction with 10 µg cefoxitin ml-1 according to Minami et al. (1980). In contrast, both the HR and LR strain of resistant S. marcescens 921/79 contained high amounts of constitutive β-lactamase [14 and 8 U (mg protein)-1, respectively] and were further inducible to a 2- and 3-fold higher content of the

Table 1. MIC of β-lactam antibiotics for S. marcescens

The highly cefotaxime-resistant strain 921/79 HR, the less-resistant revertant strain 921/79 LR, and the sensitive strains, CCM 303 (type strain) and HIM 307-2 (clinical strain) were tested.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Cefotaxime</td>
<td>1024</td>
<td>32</td>
<td>1</td>
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<tr>
<td>Ceftazidime</td>
<td>64</td>
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<td>0.25</td>
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<tr>
<td>Aztreonam</td>
<td>128</td>
<td>8</td>
<td>0.25</td>
<td>0.12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cefotizoxime</td>
<td>256</td>
<td>8</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>Latamoxef</td>
<td>512</td>
<td>32</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1024</td>
<td>256</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Cefotiam</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Cephalothin</td>
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<td>&gt;1024</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1024</td>
<td>512</td>
<td>128</td>
<td>16</td>
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</table>
enzyme. In the resistant strains it was therefore necessary to elucidate whether the β-lactamase present was a single enzyme or a mixture of different β-lactamases, possibly of inducible chromosomal and constitutive plasmid-coded enzymes.

**Substrate profile.** The β-lactamase in crude extracts from sonicated 921/79 HR bacteria had the typical substrate profile of a class C chromosomal β-lactamase with rapid hydrolysis of the classical cephalosporins and slower degradation of penicillins when measurements were made at identical (100 μM) concentrations of different substrates regardless of their greatly different $K_m$ values. Hydrolysis of β-lactamase-stable compounds by the low enzyme concentration of the crude extract was quite slow or unmeasurable. Specific activities of the crude enzyme with representative β-lactams were as follows [μU (mg protein)$^{-1}$]: cephaplatin, 3540; cephaloridine, 653; cefotiam, 365; cefotaxime, 2.4; ceftizoxime, 23; benzylpenicillin, 154; and ampicillin, 19. Hydrolysis of cefoxitin and latamoxef was not detected under these conditions.

**Isoelectric focusing (IEF).** In concentrated crude extracts from induced and non-induced cells of strains 921/79 HR and LR, IEF revealed a major β-lactamase band at pI > 9 and three minor bands at pI slightly below 9. Upon tenfold dilution only the pI > 9 enzyme remained visible. There was no evidence of β-lactamase bands in the pI 5.3–7.7 range, typical for the majority of plasmid-coded enzymes in Gram-negative bacteria (Matthew, 1979). Identical bands of pI > 9 β-lactamase were also focused from extracts of cefoxitin-induced, non-resistant strains CCM 303 and HIM 307-2. This was in agreement with the pI values reported previously for chromosomal β-lactamase from *S. marcescens* (Tajima *et al.*, 1979; Sawai *et al.*, 1982; Joris *et al.*, 1986). It was therefore concluded that *S. marcescens* 921/79 HR and LR contained mainly, if not exclusively, the typical chromosomal β-lactamase of the species, but with constitutive synthesis and overproduction.

**Purification of β-lactamase.** β-Lactamase from *S. marcescens* 921/79 HR was isolated and highly purified in order to obtain values of $M_r$ and of kinetic parameters of the enzyme with different substrates (Table 2) for calculations of the concentration of periplasmic β-lactamase and analysis of interaction with β-lactamase-stable antibiotics.

An enzyme preparation with a specific activity of 1030 U (mg protein)$^{-1}$ (determined at $V$ with the substrate cephaplatin) was obtained at purification step (iv), gel filtration on Ultra-gel AcA 54; SDS-PAGE and IEF of this enzyme revealed a single protein band of $M_r$ 36000 and pI > 9. No further purification and increase of specific activity was achieved by subsequent high performance liquid chromatography.

**Concentration of constitutive β-lactamase in the periplasmic space of resistant and sensitive strains of *S. marcescens***

The number of β-lactamase molecules per cell was determined by the procedure of Bush *et al.* (1985). The total β-lactamase activity at $V$ was measured with cephaplatin in crude extracts obtained by sonication from known numbers of exponential-phase bacteria grown in Penassay broth without induction. Calculations were made using the $K_m$ and $V$ values for cephaplatin and the $M_r$ of the β-lactamase. Values obtained were $5.7 \times 10^4$ and $2.4 \times 10^4$ molecules of β-lactamase per cell in *S. marcescens* 921/79 HR and LR strains, respectively. For the calculation of the periplasmic concentration of β-lactamase it was assumed that the periplasmic space has a volume of $10^{-15}$ l, i.e. 10% of a total cell volume of $10^{-14}$ l [mean of values published by Hazelbauer (1979), Nikaido (1979) and Hobot *et al.* (1984)]. In that case the periplasmic concentrations of β-lactamase were 950 μM in HR bacteria and 400 μM in LR bacteria. Thus, remarkably, HR and LR strains both contained high, and only 2.4-fold different concentrations, of constitutive β-lactamase, although the LR strain was from 16- to 64-fold more sensitive to most of the β-lactamase-stable antibiotics. Clearly, also, in the HR strain and even more pronouncedly in the LR strain periplasmic concentrations of β-lactamase were of the same order of magnitude or, in most cases, manyfold higher than the external concentrations of the β-lactamase-stable antibiotics to which the bacteria were exposed at the MICs.

With the same method periplasmic β-lactamase concentrations for sensitive strains CCM 303 and HIM 307-2 were found to be 8.8 and 14 μM, respectively.
Table 2. Kinetic constants of interaction of β-lactam antibiotics with β-lactamase of S. marcescens 921/79 HR

Values were obtained from measurements of (a) direct hydrolysis, (b) competitive inhibition, (c) inhibition of hydrolysis of reporter substrate, (d) rate of decay of acyl-enzyme $E*P$, (e) computed as $k_3/K$ (see Table 3) and (f) $k_{cat}$. Kinetic parameters measured with different methods are listed in Table 2.

<table>
<thead>
<tr>
<th>Cephalothin</th>
<th>Ceftizoxime</th>
<th>Cefotaxime</th>
<th>Ceftazidime</th>
<th>Imipenem†</th>
<th>Aztreonam†</th>
</tr>
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<tbody>
<tr>
<td>$K_m$ (μM)</td>
<td>102a</td>
<td>61c</td>
<td>14a, 4-5a</td>
<td>92a</td>
<td>0.2e</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>621a</td>
<td>3-4c</td>
<td>0.84a</td>
<td>0.09a</td>
<td>2 x 10$^{-3c}$</td>
</tr>
<tr>
<td>$K_i$ (μM)</td>
<td>$6.1 \times 10^{10}$</td>
<td>$5 \times 10^{4c}$</td>
<td>$6 \times 10^{3a}$</td>
<td>$1.9 \times 10^{5r}$</td>
<td>$10^{2a}$</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}$</td>
<td>$k_{2c}$</td>
<td>$K_m$</td>
<td>$K_i$</td>
<td>$K$</td>
</tr>
</tbody>
</table>

† In the case of imipenem and aztreonam $k_{cat} = k_{2c}$.

Kinetic parameters of interaction of S. marcescens β-lactam with β-lactamase-stable β-lactam antibiotics

As compounds of key interest the structurally different cefotaxime, ceftizoxime, ceftazidime, aztreonam and imipenem were chosen because the resistance of the S. marcescens HR strain to these antibiotics varied widely in spite of their common property of β-lactamase stability. Kinetic parameters measured with different methods are listed in Table 2.

Cefotaxime, ceftizoxime and ceftazidime. Whereas hydrolysis of cefotaxime was barely measurable at the low concentration of 4 nM-β-lactamase used for good substrates, it proceeded rapidly and went to completion at 400 nM-β-lactamase and at molar ratios of cefotaxime to enzyme >25. New batches of cefotaxime were again hydrolysed at the same rate when added repeatedly after the exhaustion of the previous amount of this substrate. Also, the capacity of the β-lactamase for rapid hydrolysis of cephalothin remained unimpaired by previous interactions with cefotaxime, excluding the possibility of formation of a long-lived enzyme–cefotaxime intermediate complex. Limits of sensitivity of the photometric assay allowed precise measurements upward from 10 μM-cefotaxime only. Within these limits saturation kinetics without evidence of substrate-induced enzyme inhibition up to 200 μM-cefotaxime were observed and values of $K_m$ and $k_{cat}$ similar to those reported previously (Joris et al., 1986) were derived from initial velocities (Fig. 1a, Table 2).

When assayed for β-lactamase inhibition in a ternary mixture (Frère et al., 1982; De Meester et al., 1987) and added without preincubation to the reporter substrates cephalothin or nitrocefin ($K_m = 40$ μM), cefotaxime caused instant inhibition of hydrolysis of these good substrates to lower constant rates. This indicated the establishment within seconds of a steady state between enzyme and inhibitor. Parameters of inhibition kinetics (Fig. 1b) and of hydrolysis characterized cefotaxime as a conventional substrate with moderately high affinity for S. marcescens β-lactamase. The apparent competitive inhibition could thus be more accurately called ‘substrate competition’.

Ceftizoxime and ceftazidime differed considerably from cefotaxime in their kinetic parameters but behaved similarly as substrates which established steady states rapidly with the β-lactamase.

Imipenem and aztreonam. These antibiotics had common features as substrates and inhibitors of Serratia β-lactamase quite different from those of cefotaxime.
**Direct hydrolysis.** Even at the high β-lactamase concentration of 2 μM hydrolysis of imipenem was very slow. However, rates of degradation of 17 μM-imipenem could be measured reproducibly from time-courses of the linear decrease of UV-absorbance at 297 nm, yielding a value of $k_{cat} = k_3$ of $1.7 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$. Hydrolysis of aztreonam concentration up to 140 μM by 2 μM-β-lactamase was not detectable during continuous monitoring of decrease of absorbance at 315 nm indicating an even slower rate-determining step in the interaction of this antibiotic with the enzyme.

**Inactivation of β-lactamase.** Time-courses of progressive inactivation of 1 nM-β-lactamase by imipenem (10–28 μM) and aztreonam (2–8 μM), respectively, were recorded in ternary mixtures with nitrocefin as reporter substrate. Both imipenem and aztreonam were typical progressive inactivators of the β-lactamase.

After prolonged phases of deceleration steady states of small, but significant residual enzyme activities remained at all inhibitor concentrations, showing that no permanent inactivation of enzyme occurred even at the highest concentration of inhibitors. Thus, interactions of imipenem and aztreonam with the β-lactamase could be assumed to proceed according to the reaction scheme given in Fig. 2. With the method of De Meester et al. (1987) and Galleni & Frère (1988) pseudo-first-order rate constants $k_a$ of the progressive enzyme inactivation could be determined by continuous computerized monitoring of the decreased rates of hydrolysis of the reporter substrate.

From the data shown in Table 3, the following conclusions can be drawn.

1. With both compounds, $k_a$ increased linearly with $[I]$, indicating that the range of inactivator concentrations was well below $K$. This explains why the values of $K$ and $k_2$ for imipenem and aztreonam given in Table 2 are minimum values.

2. Extrapolation of those lines at $[I] = 0$ indicated that $k_3$ was much smaller than the smallest measured $k_a$ which showed that $k_3$ was $<< k_2$.

3. This was confirmed by the direct measurement of $k_{cat}$ for imipenem and by the rate of reactivation of the fully inactivated enzyme for both compounds (Table 2).

4. The values of $k_3$ which were computed from the $v_{cat}/v_0$ ratio using equation (4) were in excellent agreement with those obtained by the other methods.

5. The values of $k_i/[I]$ thus corresponded to $\frac{k_2}{Km} \frac{Km}{[S]+Km}$ and the final values of $k_2/K$ were thus 9100 M⁻¹ s⁻¹ and 16000 M⁻¹ s⁻¹, respectively, for imipenem and aztreonam.
Fig. 2. Model of interaction of β-lactamase ($E_o$) and β-lactam antibiotic ($I_o$) in the periplasm of Gram-negative bacteria. $I_o$, concentration of antibiotic in the external solution; $k_{D}$, first-order rate constant of antibiotic diffusion through the outer membrane; $v_{0}$, rate of synthesis and excretion of β-lactamase into the periplasm; $E_l$, $E_l^*$, $E$ and $P$, periplasmic concentration of Henri–Michaelis complex, acyl–enzyme, reactivated enzyme and antibiotic metabolite, respectively, from hydrolysis of the acyl–enzyme; $K$, dissociation constant of Henri–Michaelis complex; $k_2$, first-order rate constant, and $k_{D/K}$, second-order rate constant of acyl–enzyme formation; $k_3$, first-order rate constant of hydrolysis of acyl–enzyme; $T$, essential penicillin-binding proteins as targets of β-lactam antibiotics. For more details see Frère (1989) and Frère et al. (1989).

Table 3. Kinetic constants of interaction of aztreonam and imipenem with β-lactamase of S. marcescens 921/79 HR

Values were derived from the kinetics of progressive inhibition by the antibiotics of hydrolysis of the reporter substrate nitrocefin by the β-lactamase. Definitions of parameters and experimental conditions are given in Methods and Results.

<table>
<thead>
<tr>
<th>$I$ (μM)</th>
<th>$10^{-3} \times k_a$ (s$^{-1}$)</th>
<th>$10^{-2} \times \frac{v_{0x}}{v_{0}}$</th>
<th>$10^{-3} \times k_3$ (s$^{-1}$)</th>
<th>$10^{-3} \times k_i$ (s$^{-1}$)</th>
<th>$k_i$ / $I$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25.8 ± 1.4</td>
<td>8.4 ± 0.1</td>
<td>2.15 ± 0.1</td>
<td>23.6 ± 1.4</td>
<td>2360 ± 140</td>
</tr>
<tr>
<td>14.3</td>
<td>39.4 ± 1.4</td>
<td>5.0 ± 0.6</td>
<td>2.0 ± 0.2</td>
<td>37.4 ± 1.4</td>
<td>2620 ± 100</td>
</tr>
<tr>
<td>20</td>
<td>58.2 ± 3.6</td>
<td>3.8 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>56.2 ± 3.6</td>
<td>2810 ± 180</td>
</tr>
<tr>
<td>28.5</td>
<td>78.0 ± 10</td>
<td>2.3 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>76.0 ± 10</td>
<td>2670 ± 350</td>
</tr>
<tr>
<td>Mean</td>
<td>2.04 ± 0.18</td>
<td></td>
<td>2.04 ± 0.18</td>
<td>2610 ± 200</td>
<td></td>
</tr>
<tr>
<td>Aztreonam</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.9 ± 1.6</td>
<td>1.5 ± 0.1</td>
<td>0.17 ± 0.02</td>
<td>10.7 ± 1.6</td>
<td>5300 ± 800</td>
</tr>
<tr>
<td>4</td>
<td>16.9 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>0.21 ± 0.02</td>
<td>16.7 ± 0.3</td>
<td>4200 ± 75</td>
</tr>
<tr>
<td>6</td>
<td>25.1 ± 1.6</td>
<td>0.81 ± 0.07</td>
<td>0.21 ± 0.02</td>
<td>24.9 ± 1.6</td>
<td>4200 ± 300</td>
</tr>
<tr>
<td>8</td>
<td>41.1 ± 3.2</td>
<td>0.62 ± 0.05</td>
<td>0.25 ± 0.03</td>
<td>40.8 ± 3.2</td>
<td>5100 ± 400</td>
</tr>
<tr>
<td>Mean</td>
<td>0.21 ± 0.03</td>
<td></td>
<td>0.21 ± 0.03</td>
<td>4700 ± 600</td>
<td></td>
</tr>
</tbody>
</table>

† Residual β-lactamase activity at steady state.

Analysis of periplasmic interactions of antibiotics and β-lactamase

Events in the periplasm were assumed to proceed as depicted in Fig. 2. Analysis of this process in the four strains of S. marcescens was done on the basis of the determined values of rate constants and concentrations of reactants. With respect to the periplasmic concentrations of β-lactamase $E_o$, a choice had to be made between the values present during constitutive production or overproduction of the enzyme and those of the moderately or highly elevated enzyme concentrations elicited by induction. Use of values in uninduced bacteria seemed to be more valid for the study of antibiotics of greatest interest such as cefotaxime, ceftizoxime, ceftazidime and aztreonam, because for them the largest increases of MICs from susceptible to resistant S. marcescens were recorded whereas the same compounds were also known as ineffective β-lactamase inducers in Gram-negative bacteria (Bush et al., 1985; Martin et al., 1988).
Fig 3. Simulation of the penetration of imipenem (with $k_D = 0.1 \text{ s}^{-1}$) with strain 921/79 HR. $E_0 = 950 \text{ µM}; I_p = 13.4 \text{ µM}$. For the other parameters, see Table 4. $I_p$; $E_0$; $EI^\ast$. The arrows indicate the time at which $EI^\ast$ and $I_p$ reach 90% of their stabilized values ($t_{0.9}$). Note the rapid increase of $I_p$ at $t < 10 \text{ s}$.

Table 4. Comparison of the steady-state and non-steady-state models in the analysis of the interaction of imipenem with periplasmic $\beta$-lactamase in S. marcescens 921/79 LR and HR

A $10^3$-fold variation of permeability ($k_D$) was assumed. The kinetic parameters were taken from Table 2: $k_2/K = 0.0091 \text{ µM}^{-1} \text{ s}^{-1}$ and $k_4 = 0.0017 \text{ s}^{-1}$.

<table>
<thead>
<tr>
<th>Strain 921/79 HR†</th>
<th>Strain 921/79 LR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_D$ (s$^{-1}$)</td>
<td>$(I_p)_{ss}^|$</td>
</tr>
<tr>
<td>1</td>
<td>11.8</td>
</tr>
<tr>
<td>0.2</td>
<td>5.58</td>
</tr>
<tr>
<td>0.1</td>
<td>0.69</td>
</tr>
<tr>
<td>0.01</td>
<td>0.016</td>
</tr>
<tr>
<td>0.001</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

† For the HR strain, $E_0 = 950 \text{ µM}$, $I_p = 13.4 \text{ µM}$ and generation time = 3600 s.
‡ For the LR strain, $E_0 = 400 \text{ µM}$, $I_p = 3.36 \text{ µM}$ and generation time = 1800 s.
§ $(I_p)_{ss}$ and $(EI^\ast)_{ss}$ are the values computed with the steady-state model.
¶ $(I_p)_{all}$ and $(EI^\ast)_{all}$ are the stabilized values at $t = \infty$ computed with the non-steady-state model.
‖ $t_{0.9}$ is the time (expressed in s) required for the variable to reach 90% of its stabilized value in the non-steady-state model.

Of possible changes of the remaining potential resistance factors, outer-membrane permeability and sensitivity of antibiotic targets, no information is available on the latter phenomenon in S. marcescens. Therefore, target sensitivities were assumed to be identical in resistant and non-resistant bacteria, so that for a given antibiotic the required lethal periplasmic concentration $I_{pl}$ was the same in all strains. Sensitive strain CCM 303, with the lowest periplasmic concentration of constitutive $\beta$-lactamase (8.8 µM) was used as a reference. In this strain, values of $I_{pl}$ were expected to be not much lower than the MICs (Nikaido & Normark, 1987; Frère, 1989). As described in Methods, characterization of the different permeabilities connected with the varying stages of resistance was done by computer simulations with the steady-state model (Frère, 1989) for the determination of the parameters $I_{pl}$ and $Z_e$, and subsequent calculation of $k_D$ values on the basis of the various values of $K_m$ and MIC for the different antibiotics.

Simulations with the non-steady-state model (Frère et al., 1989) yielded values of $k_D$ directly. As outlined by these authors and summarized in Methods the applicability of the simple or complete model for each antibiotic depended on the half-life of the acyl–enzyme $EI^\ast$ in relation to the bacterial generation time.

(i) The steady-state model can be applied to imipenem. For imipenem with $k_{cat} = 1.7 \times 10^{-3} \text{ s}^{-1}$ the value of the half-life of the acyl–enzyme (408 s) was somewhat too high to allow the a priori
Resistance to ß-lactams in Serratia marcescens

Table 5. Analysis of the MIC values for the non-resistant and resistant strains of S. marcescens based on the steady-state model

$I_{pl}$ represents the periplasmic concentration of ß-lactam which is needed to kill the bacterium. It is assumed to be the same for all strains. R is the ratio of ß-lactamase concentration in a given strain to that observed in the poorest enzyme producer (strain CCM 303) taken as a reference. $Z$ represents $Z = k_D(k_{cat} E_o)_{CM303}$ at $I_{pl}$, and is the same if the different MICs can be explained by the sole variation of ß-lactamase concentration (Frère, 1989). The calculated MICs (MIC$_{calc}$) were computed as described by Frère (1989) and are compared to the observed values (MIC$_{obs}$). The $K_m$ values used in the calculation are taken from Table 2 for cefotaxime and ceftazidime, from the measurement of $K_m$ with a reporter substrate for ceftizoxime, or from the data of Joris et al. (1986) with a similar enzyme from S. marcescens for latamoxef (60 µM).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>CCM 303</th>
<th>HIM 307-2</th>
<th>921/79 LR</th>
<th>921/79 HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{pl}$ (µM)</td>
<td>$Z$</td>
<td>MIC (µM)</td>
<td>$Z$</td>
<td>MIC (µM)</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>calc.</td>
<td>obs.</td>
<td>calc.</td>
<td>obs.</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.5</td>
<td>0.035</td>
<td>1.86</td>
<td>2.28</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.475</td>
<td>0.22</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>0.05</td>
<td>0.0035</td>
<td>0.29</td>
<td>0.16</td>
</tr>
<tr>
<td>Latamoxef</td>
<td>0.12</td>
<td>0.001</td>
<td>2.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Table 6. Values of $k_D$ for strain CCM 303 and relative permeability factors for the other strains of S. marcescens

For aztreonam, all $k_D$ values were obtained directly from the simulations with the non-steady-state model (see text). For the other ß-lactams, they were calculated from $k_D = Z(k_{cat} E_o)_{CM303}$ (see Table 5) using $k_{cat}$ values obtained from Table 2 (cefotaxime, ceftazidime and ceftizoxime) or from Joris et al. (1986) for latamoxef.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>$k_D$ (s$^{-1}$) for strain CCM 303</th>
<th>Relative permeability factors of strains (strain CCM 303 = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIM 307-2</td>
<td>921/79 LR</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.26</td>
<td>3-6</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.17</td>
<td>1</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>0.10</td>
<td>3</td>
</tr>
<tr>
<td>Latamoxef</td>
<td>0.001</td>
<td>9</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.013</td>
<td>10</td>
</tr>
</tbody>
</table>

assumption that the steady-state model could be used. Therefore, simulations were done with both models and with parameters derived from the values presented in Tables 1 and 2 and in the text (see footnote to Table 4). As shown in Table 4 and Fig. 3, the periplasmic concentrations $I_{pl}$ computed by the two methods for a 10³-fold range of $k_D$ values were not significantly different and there was no reason to use the non-steady-state model.

(ii) Utilization of the steady-state model. Antibiotics which S. marcescens ß-lactamase hydrolysed with $k_{cat}$ values suitable for this model were cefotaxime, ceftazidime, ceftizoxime, latamoxef, cefoxitin, ampicillin and imipenem, as demonstrated above. Results with the four first mentioned antibiotics presented in Tables 5 and 6 could be coherently interpreted by assuming that non-resistant reference strain CCM 303 and moderately resistant strain 921/79 LR had similar permeabilities because they yielded unique values of $I_{pl}$ and $Z$, indicating identical $k_D$ values. The sensitive clinical strain HIM 307-2 was 3- to 10-fold more permeable, and the highly resistant strain 921/79 HR 5- to 50-fold less permeable than the reference strain. Calculation of MICs (MIC$_{calc}$) from the parameters of the model according to Frère (1989) yielded values in very good agreement with the measured MICs of the antibiotics (Table 5). Surprisingly, that relative scale of permeabilities did not apply to cefoxitin, ampicillin and
Fig. 4. Determination of the $k_D$ value for penetration of aztreonam in strain 921/79 HR. (a) and (b) show the variation of $E_p$ and $I_p$, respectively. The parameters were as follows: $E_0 = 950 \mu M$; $k_2/K = 0.016 \mu M^{-1} s^{-1}$; $k_3 = 2 \times 10^{-4} s^{-1}$; $I_e = 256 \mu M$; generation time = 60 min $k = 1.92 \times 10^{-4} s^{-1}$). It is interesting to note that since $I_p$ is much smaller than $I_e$, this latter factor and $k_D$ will only influence the simulation as their product, the rate of entry, $k_D \times I_e$. Thus, if $I_e$ is decreased to MIC/2, the product (with $k_D = 0.0013 s^{-1}$) is 0.166 $\mu M s^{-1}$ instead of 0.333 $\mu M s^{-1}$ at the MIC (256 $\mu M$). There is thus no chance of reaching $I_p \geq 0.2 \mu M$ since that value is not reached when $k_D = 0.0010$ and $I_e = 256 \mu M (k_D I_e = 0.256 \mu M s^{-1})$. The $I_p$ value which must be reached is about 0.2 $\mu M$ (see text).

Table 7. Relative permeability factors for cefoxitin, ampicillin and imipenem in non-resistant and resistant strains of *S. marcescens*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>CCM 303</th>
<th>HIM 307-2</th>
<th>921/79 LR</th>
<th>921/79 HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>2–3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1</td>
<td>10–100</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
<td>$\geq 1.6$</td>
<td>$\geq 50$</td>
<td>2.7–26</td>
</tr>
</tbody>
</table>

The values were obtained from the ratios of the $Z$ values calculated according to Frère (1989) using the $K_m$ and $K_{app}$ values from Table 2 (imipenem) or from Joris et al. (1986) for cefoxitin and ampicillin. If one assumes an $I_p$ value for imipenem not much smaller than 3.36 $\mu M$, the permeabilities of strain HIM 307-2 and 921/79 LR can be assumed to have any value above 1.6 or 50, respectively.

The only antibiotic for which treatment with this model was appropriate. Starting out from the lowest MIC of 0.25 $\mu M$-aztreonam in sensitive strain HIM 307-2 and considering that here $k_3$ was quite low and the periplasmic concentration of $\beta$-lactamase not very high ($E_p = 14 \mu M$), it was assumed that the $I_p$ value was not much lower than the MIC for this strain. In simulations with a $k_D$ value of 0.13 $s^{-1}$, $I_p$ stabilized at 0.196 $\mu M$ after 2100 s, while $E_p$ became 2.19 $\mu M$.

After 1000 s, the periplasmic concentration of aztreonam was already 0.176 $\mu M$, i.e. 90% of the stabilized value. To reach a similar stabilized $I_p$ value, the $k_D$ values for the other strains were
Resistance to \(\beta\)-lactams in \textit{Serratia marcescens}

as follows: strain CCM 303, 0.013 s\(^{-1}\); strain \(LR\), 0.0125 s\(^{-1}\); strain \(HR\), 0.0013 s\(^{-1}\) (Fig. 4). When given as relative permeabilities (Table 6), these results are in remarkable agreement with those obtained with the four other \(\beta\)-lactams whose properties are summarized in the same Table.

It was interesting to compute how aztreonam, which penetrates into the periplasm when the periplasmic concentrations have stabilized, is partitioned between immobilization as acyl-enzyme by newly synthesized \(\beta\)-lactamase and hydrolysis. In fact, between 13500 and 14000 s, about 50\% of the entering aztreonam is hydrolysed and 50\% is immobilized by the newly synthesized \(\beta\)-lactamase. During the same period of time, the quantity of synthesized \(\beta\)-lactamase is equivalent to about 60\% of the quantity of aztreonam which penetrates the cell. Most of the newly synthesized \(\beta\)-lactamase thus reacts with aztreonam to yield acyl-enzyme.

Our \(k_D\) values can be compared to those derived from the data of Nikaido & Normark (1987) for cefotaxime, ceftazidime and aztreonam. Assuming, as has been done in the present work, that the \(\beta\)-lactam only penetrates into the periplasm, the following \(k_D\) values were computed for \textit{E. coli}: cefotaxime, 1-1-18 s\(^{-1}\); ceftazidime, 0-15-1 s\(^{-1}\); aztreonam; 0-05-0-33 s\(^{-1}\). In all cases, the lower value is that computed by Frère (1989) on the basis of the MIC values and the larger one is that reported by Nikaido & Normark (1987) and corresponds to a directly measured permeability factor. In all cases, the range of variation of the \textit{S. marcescens} permeabilities is below that of \textit{E. coli}.

**DISCUSSION**

Sufficient quantitative data are now on hand from preceding reports (Bush et al., 1985; Vu & Nikaido, 1985) and from the present comparative study of resistant and sensitive \textit{S. marcescens} to establish that high resistance to \(\beta\)-lactamase-stable \(\beta\)-lactam antibiotics in important Gram-negative pathogens goes hand-in-hand with the presence of chromosomally coded, constitutively overproduced class C \(\beta\)-lactamase in the range 6 \(\times\) 10\(^4\) to 2 \(\times\) 10\(^5\) enzyme molecules per bacterial cell. In the highly resistant strain \textit{S. marcescens} 921/79 \(HR\) this amounted to 100 times the concentration of constitutive enzyme and three times the concentration of fully induced enzyme present in sensitive \textit{S. marcescens}.

The quantities of overproduced \(\beta\)-lactamase also correspond to periplasmic enzyme concentrations in the millimolar range. At the upper limit they represent the major portion of the total quantity of protein normally encountered in the periplasmic fluid (Brass, 1986). It appears that the accumulation of such high amounts of \(\beta\)-lactamase could not occur without unfavourable effects on the physiological state of the cell. This was indeed shown by the high generation time of 60 min of the highly resistant \textit{S. marcescens} strain 921/79 \(HR\), in contrast to the generation time of 30 min of the less resistant strain 921/79 \(LR\), to which strain 921/79 reverted spontaneously in the absence of selection for resistance to highly concentrated cefotaxime. Thus, although strain \(LR\) is still a constitutive \(\beta\)-lactamase overproducer, its periplasmic concentration of the enzyme (0.4 mM) may constitute the maximal \(\beta\)-lactamase content which is compatible with the normal generation time of 30 min of this bacterium, the same as that of sensitive \textit{S. marcescens} strains CCM 303 and HIM 307-2.

Information on the main topic of our study, definition of the causes of the different degrees of resistance in \textit{S. marcescens}, resulted from the application of the steady-state and non-steady-state models of Frère (1989) and Frère et al. (1989). This revealed distinct differences in the contributions by recognized resistance factors, viz. limited penetration of antibiotics, degradation of antibiotics by overproduced \(\beta\)-lactamase and continued synthesis of this enzyme. A remaining uncertainty comes from the fact that the moderately and highly resistant \textit{S. marcescens} 921/79 \(LR\) and \(HR\) strains could not be compared with their isogenic, sensitive parent strain from which they descended originally. The unrelated, sensitive strains included in our study, \textit{S. marcescens} type strain CCM 303 and clinical isolate HIM 307-2, had similar susceptibilities to the majority of antibiotics but differed significantly in their permeabilities and content of constitutive \(\beta\)-lactamase. Strain CCM 303 was chosen for comparison with the resistant strains because it contained the lowest concentration of constitutive \(\beta\)-lactamase.
With these reservations, results of the analyses allowed the coherent interpretation of the different degrees of resistance to cefotaxime, ceftizoxime, ceftazidime, latamoxef and aztreonam, a representative group of structurally different β-lactams subject to slow or very slow hydrolysis by S. marcescens β-lactamase.

The moderate resistance of S. marcescens 921/79 LR, characterized by MIC values of the five antibiotics from 4- to 128-fold higher than those in reference strain CCM 303, could be consistently understood as the exclusive result of degradation of the antibiotics to concentrations below their lethal periplasmic concentrations \( I_{pl} \) by the observed quantities of overproduced β-lactamase. Strain LR and reference strain CCM 303 were equally permeable to all antibiotics and no additional resistance factor was required.

In the highly resistant S. marcescens strain 921/79 HR, on the other hand, analyses indicated a complex origin of resistance. Although periplasmic β-lactamase concentration was increased further in this strain (to 2.4- and 100-fold the values present in strains LR and CCM 303, respectively), this could not account for the highly elevated MICs of all antibiotics. As an additional factor in resistance a 5- to 50-fold decrease of antibiotic permeabilities was computed from the models.

The decreased permeability of strain HR could be suspected to result from changes in the porin components of the outer membrane. However, comparative inspection of outer-membrane proteins by SDS-PAGE did not reveal significant differences between strain 921/79 HR and the other S. marcescens strains under study (U. Hechler, unpublished results). Obviously, a more detailed study of the restrictive penetration barrier of strain HR is necessary. Alternatively, other and so far unsuspected resistance factors, including changes in target sensitivity, may yet be found to play an important role.

It remains now to consider the exceptional cases of ampicillin, cefoxitin and imipenem for which the analyses could not verify the established correlation of resistance with β-lactamase overproduction and permeability restriction, and where the application of the models unexpectedly demonstrated the disappearance or even reversal of differences in permeability between resistant and non-resistant S. marcescens strains. In the case of imipenem, this may be connected with the existence of a specific penetration mechanism of this antibiotic, which is unrelated to that of other β-lactam compounds (Bücher et al., 1987; Frère et al., 1989). Other evidence points to the selective induction of β-lactamase as possible cause for this aberrant behaviour. Ampicillin, cefoxitin and imipenem, even at low concentrations, were shown to be good or excellent inducers of class C β-lactamase in several Gram-negative bacteria while cefotaxime, ceftazidime and aztreonam, in particular, had low or negligible inducing potency (Bush et al., 1985; Martin et al., 1988).

Our analyses also permitted quantitative estimates to be made of the relative importance of hydrolysis of the antibiotics and their immobilization as covalent acyl–enzymes (Waley, 1987; Frère et al., 1989) (formerly thought to be non-covalent ‘trapping’) for the maintenance of low β-lactam concentrations in the periplasm. For all studied antibiotics, with the single exception of aztreonam, hydrolysis was the exclusive mechanism of elimination. This applied even to imipenem with \( k_{cu} \) as low as \( 2 \times 10^{-3} \) s\(^{-1}\), i.e. \( 10^{-6} \) times the typical value for a good substrate. With aztreonam, the penetrating antibiotic was divided equally between hydrolysis and immobilization. But immobilization was only possible because new β-lactamase molecules were synthesized. In the absence of enzyme synthesis, the periplasmic concentration \( I_{p} \) of aztreonam would have reached a value similar to the external antibiotic concentration \( I_{e} \) within a bacterial generation time. Thus, for immobilization of antibiotic as acyl–enzyme to be a relevant phenomenon, the following conditions must be fulfilled.

(i) High periplasmic concentration of β-lactamase and consequently rapid synthesis of new enzyme.

(ii) Low \( k_{j} \) value (< \( 5 \times 10^{-4} \) s\(^{-1}\)).

(iii) Low \( k_{D} \) value. It is difficult, however, to set a strict upper limit for the last factor since a larger \( k_{D} \) value will result in the reaching of the lethal periplasmic concentration \( I_{pl} \) with a lower \( I_{e} \), even if most of the antibiotic is immobilized or hydrolysed upon entering the periplasm.
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