Role of residues 104, 164, 166, 238 and 240 in the substrate profile of PER-1 β -lactamase hydrolysing third-generation cephalosporins

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The class A β -lactamase PER-1, which displays 26% identity with the TEM-type extended-spectrum β -lactamases (ESBLs), catalyses the hydrolysis of oxymino- β -lactams such as cefotaxime (CTX), ceftazidime (CAZ) and aztreonam (AZT). Molecular modelling was used to identify in PER-1 the amino acid residues corresponding to those found at positions 104, 164, 238 and 240 in the TEM-type ESBLs, which are critical for hydrolysis of oxymino- β -lactams. The function of these residues in PER-1 was assessed by site-directed mutagenesis. In this enzyme, residue 104 could be either a glutamine, an asparagine or a threonine. The Gln $\rightarrow$ Gly mutation did not significantly affect the catalytic efficiency, while Asn $\rightarrow$ Gly and Thr $\rightarrow$ Glu resulted in a marked decrease in catalytic activity, probably due to the alteration of a

hydrogen bond network connecting the putative Asn-104 residue to Asn-132 and Glu-166. Replacement of Ala-164 by Arg in PER-1 resulted in a mutant with no detectable activity, thus suggesting that Ala-164 is important for catalysis and stability of PER-1. Conversely, Ser-238 $\rightarrow$ Gly and Gly-240 $\rightarrow$ Glu had little effect on k_{cat} and K_m values. Finally, the replacement of the catalytic residue Glu-166 by an alanine resulted in a complete loss of activity for CTX and a marked decrease of k_{cat} for CAZ and AZT. These results suggest that Glu-166 is an important residue in PER-1. However, residues other than Glu-166 could contribute in maintaining residual activity towards oxymino- β -lactams in the Ala-166 mutant.

INTRODUCTION

Production of β -lactamases is the main mechanism of acquired bacterial resistance to β -lactam antibiotics. Most of the β -lactamases described so far are active-site serine enzymes which cleave the amide bond in the β -lactam ring via an acyl-enzyme intermediate. Class A β -lactamases, which are mainly plasmid mediated, are the most widespread serine β -lactamases in Gram-negative bacteria. This group include the broad-spectrum β -lactamases TEM-1, TEM-2 and SHV-1 which are inhibited by clavulanic acid and are capable of hydrolysing penicillins and first- and second-generation β -lactam antibiotics such as cephalothin (Figure 1) [1]. However, they cannot hydrolyse either third-generation cephalosporins such as cefotaxime (CTX) and ceftazidime (CAZ) or the monobactam aztreonam (AZT) (Figure 1). Class A also encompasses the so-called extended-spectrum β -lactamases (ESBLs) which are derived from the parental TEM and SHV enzymes by a few amino-acid substitutions leading to increased catalytic activity for CTX, CAZ and AZT. In TEM variants, the amino-acid modifications associated with the extension of the substrate spectrum are located either within or near the active site [2,3], and the most frequently encountered substitutions, which can be found alone or in combination, are: Glu-104 $\rightarrow$ Lys, Arg-164 $\rightarrow$ Ser or His, Gly-238 $\rightarrow$ Ser, and Glu-240 $\rightarrow$ Lys [4], according to Ambler's numbering system [5].

PER-1, the first ESBL found in *Pseudomonas aeruginosa*, confers resistance to penicillins, first-, second-, and third-generation cephalosporins, and is efficiently inhibited by clavulanic acid. Therefore, PER-1 is characterized by a substrate spectrum

very similar to that displayed by various extended spectrum TEM-type β -lactamases [6]. Strikingly, the analysis of the nucleotide sequence of the *bla*_{PER-1} gene revealed a low identity at the amino-acid level between PER-1 and the other class A β -lactamases, e.g. 26% with TEM-3 and 27% with SHV-2 [7]. The fact that genetically non-related β -lactamases have similar substrate profiles justifies biochemical investigation to understand the reasons for PER-1 activity against third-generation cephalosporins. For this purpose, a three-dimensional model of the active site of the wild-type PER-1 enzyme was constructed. This model was used to identify the amino-acid residues in the enzyme that correspond to those found at positions 104, 164, 238 and 240 in the TEM-type ESBLs. These residues were modified by site-directed mutagenesis, and a kinetic study of the mutant enzymes obtained was performed.

MATERIALS AND METHODS

Antibiotics and chemicals

The antimicrobial agents used in this study were provided by the following suppliers: penicillin G, Laboratoires de Thérapeutique Moderne (Suresnes, France); cephaloridine, cephalothin and kanamycin, Sigma Chemical Co. (St. Louis, MO, U.S.A.); cefotaxime, Laboratoires Roussel (Paris, France); nitrocefin and ceftazidime, Glaxo (Paris, France); aztreonam, Bristol Myers Squibb (Paris-La Défense, France); and clavulanic acid, SmithKline Beecham (Nanterre, France).

Restriction endonucleases were obtained from Boehringer-Mannheim (Meylan, France), T4 DNA ligase from Promega

Abbreviations used: CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; ESBL, extended-spectrum β -lactamase (mutant β -lactamases are designated using the one-letter code for amino acids, e.g. S238G for Ser-238 $\rightarrow$ Gly).

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Figure 1 Structures of the β -lactam antibiotics used in the present study

(Madison, MI, U.S.A.), and [^{33} P]dCTP from Isotopchim (Ganagobie, France).

Bacterial strains, plasmids and media

Escherichia coli CJ236 [8] and MV1190 [9] were used in site-directed mutagenesis experiments according to the manufacturer's instructions (Bio-Rad). *E. coli* JM109 (Promega, Madison, U.S.A.) was used for expression of the wild-type and mutant PER enzymes. Bacteriophage M13mp19 [10] was used as a vector for site-directed mutagenesis. Plasmid pK19 (kanamycin^R) [11] was used in cloning experiments. The recombinant plasmid pRAZ1, encoding PER-1, has been described previously [6]. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth (Difco, Detroit, MI, U.S.A.) or on LB plates containing the appropriate antibiotics, when necessary. For β -lactamase purification, brain-heart infusion (BHI) broth (Difco, Detroit, MI, U.S.A.) was used.

Table 1 Sequence of oligonucleotides used in site-directed mutagenesis

Specific base changes are underlined.

Amino-acid modification	Oligonucleotide sequence
Gln-104 $\rightarrow$ Gly	5'-CGGAGCCCAGGTATT <u>CC</u> CTAAACCTTAGCCC-3'
Asn-104 $\rightarrow$ Gly	5'-TATCGGAGCCCAGGT <u>ACC</u> TGTAAACCTTAG-3'
Thr-104 $\rightarrow$ Glu	5'-CATATCGGAGCCCA <u>TT</u> CATTCTGTAAACCT-3'
Ala-164 $\rightarrow$ Ser	5'-CATCTGCGCTTCATT <u>TG</u> AGACCACAGCGGTCTC-3'
Ala-164 $\rightarrow$ Arg	5'-CATCTGCGCTTCATT <u>TCG</u> ACCACAGCGGTCTC-3'
Glu-166 $\rightarrow$ Ala	5'-GGCGTGATCTGCGC <u>TGC</u> ATTTCGACCACAG-3'
Ser-238 $\rightarrow$ Ala	5'-CCGGCTTTGATACC <u>CGC</u> AGTACCAGTTTATG-3'
Ser-238 $\rightarrow$ Gly	5'-CCGGCTTTGATACC <u>CCC</u> AGTACCAGTTTATG-3'
Gly-240 $\rightarrow$ Glu	5'-TTCCGGCTTTGAT <u>TT</u> CCGAAGTACCAGTTT-3'

Nucleic acid techniques

Plasmid DNA was purified using the alkaline lysis method for mini-preparations [12] and the Qiagen plasmid kit for maxi-preparations (Qiagen, Hilden, Germany). Isolation of single-stranded DNA and other standard DNA manipulations were carried out according to Sambrook et al. [12]. Double- and single-stranded DNA sequencing was carried out by the dideoxynucleotide chain termination method using the T7 Sequencing kit (Pharmacia Biotech S.A., Saint Quentin-en-Yvelines, France).

Site-directed mutagenesis

Mutagenesis was performed according to the uracil template procedure of Kunkel et al. [8] using the Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad S.A., Ivry-sur-Seine, France). The phosphorylated synthetic oligonucleotides (Eurogentec, Seraing, Belgium) designed to introduce different mutations are shown in Table 1. After mutagenesis, the mutant genes were cloned into plasmid pK19. The recombinant plasmids obtained were introduced by transformation [12] into *E. coli* JM109 cells which were plated on LB agar containing 25 μ g/ml of kanamycin. All the mutant genes were sequenced in their entirety on both strands.

Protein purification

Proteins were prepared from 1 l cultures of *E. coli* cells producing wild-type or mutant PER enzymes, grown in BHI broth supplemented with kanamycin (25 μ g/ml). Cells were harvested by centrifugation of 5800 *g* for 10 min. The pellet (4 g) was resuspended in 20 ml of ice-cold buffer A (20 mM Tris, 0.5 mM dithiothreitol, and 0.06 % sodium azide, pH 7.6), and the bacterial cells were lysed by ultrasonic treatment. The crude extract obtained was cleared by centrifugation (48 000 *g* for 30 min at 4 °C). Nucleic acids were precipitated by addition of spermine 0.2 M (7 % v/v) and centrifugation (27 000 *g* for 20 min at 4 °C). The supernatant was further clarified by ultracentrifugation (100 000 *g* for 60 min at 4 °C) and dialysed for 5 h against 5 l of buffer A at 4 °C. The clarified supernatant was applied to a 1 $\times$ 8 cm Bio-Scale Q-column (Bio-Rad S.A., Ivry-sur-Seine, France) previously equilibrated in the dialysis buffer. β -Lactamase activity was eluted at 0.5 ml/min with a 12.5 ml linear gradient of Tris (20–400 mM). Active fractions, which were detected with the chromogenic cephalosporin nitrocefin [13], were pooled, concentrated by ultrafiltration and loaded onto a Superose 12 HR 10/30 gel filtration column (Pharmacia Biotech S.A., Saint Quentin-en-Yvelines, France), previously equilibrated in 100 mM Tris buffer containing 0.5 mM dithiothreitol and 0.06 % sodium azide at pH 7.6. β -Lactamase activity was eluted

after 60 min with a flow rate of 0.2 ml/min. In order to avoid concerns about enzyme stability, the fractions containing pure β -lactamase were pooled and concentrated, and kinetic studies were performed immediately after the final purification step. Purity was assessed by electrophoresis on 12% SDS/polyacrylamide gels [14] stained with Coomassie Blue R-250 (Sigma Chemical Co., St. Louis, MO, U.S.A.). Protein concentrations were determined by spectrophotometry using C (mg/ml) = $A_{280\text{nm}} \times \epsilon_{280\text{nm}} / M_r$, with a calculated absorption coefficient of $34850 \text{ M}^{-1} \cdot \text{cm}^{-1}$. For the mutants exhibiting more than one protein band on SDS/PAGE analysis, the intensity of the β -lactamase band was evaluated with a densitometer (Densylab, Bioprobe), and the enzyme concentration was determined with reference to a standard BSA scale analysed under the same conditions.

Evaluation of kinetic parameters

The steady-state kinetic parameters (K_m , k_{cat} , k_{cat}/K_m) were determined spectrophotometrically at 30 °C in 0.1 M phosphate buffer (pH 7.0) on an Uvikon 940 spectrophotometer. The absorption coefficients used were: penicillin G, 232 nm, $\Delta\epsilon = 1100 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cephalothin, 262 nm, $\Delta\epsilon = 7960 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cephaloridine, 255 nm, $\Delta\epsilon = 9360 \text{ M}^{-1} \cdot \text{cm}^{-1}$; cefotaxime, 260 nm, $\Delta\epsilon = 6710 \text{ M}^{-1} \cdot \text{cm}^{-1}$; ceftazidime, 260 nm, $\Delta\epsilon = 8660 \text{ M}^{-1} \cdot \text{cm}^{-1}$; and aztreonam, 318 nm, $\Delta\epsilon = 650 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Initial rates were measured for a minimum of six different substrate concentrations. Kinetic parameters were determined by fitting the Michaelis–Menten equation to the experimental data using the regression analysis program LEONORA written by Cornish-Bowden [15]. The values of k_{cat} and K_m were estimated using a nonlinear least-squares regression method with dynamic weights [15].

Estimation of the three-dimensional structures of the wild-type and mutant PER β -lactamases

The three-dimensional model of the active site of PER-1 was constructed by homology modelling using the computer program SWISS-MODEL [16] and the reference crystal structures of three related class A β -lactamases (*Staphylococcus aureus* PC1, *Bacillus licheniformis* 749/C and TEM-1, protein databank entries 4BLC, 4BLM and 4BLT, respectively). Modelling of the amino-acid residues modified in PER-1 was made with the molecular graphics program O [17].

RESULTS AND DISCUSSION

Construction of the three-dimensional structure of the PER-1 active site

In order to identify the amino acid residues in PER-1 that correspond to those found specifically at positions 104, 164, 238 and 240 in the TEM- and SHV-type ESBLs, a model of the active-site of PER-1 was constructed on the basis of the crystallographic structures of the class A β -lactamases from *Staphylococcus aureus* PC1, *Bacillus licheniformis* 749/C and TEM-1. The α -carbon coordinates of the model structure obtained were superimposed on the crystal structure of the penicillin-binding site of TEM-1 (Figure 2A). Comparison of both structures suggests that the folding of the polypeptide chain in the catalytic cleft of PER-1 is very similar to that of TEM-1. However, significant differences are found when the two structures are compared (Figure 2A). The loop connecting strands β 3 and β 4 encompasses six amino acids in PER-1 (Ile-Lys-Ala-Gly-Lys-Thr) but only one in TEM-1 (Arg-241). Further, according to the

amino-acid sequence alignment shown in Figure 3, the region encompassing residues 102–107 in TEM-1 (Leu-102, Val-103, Glu-104, Tyr-105, Ser-106 and Pro-107) would encompass eight residues in PER-1 (Val-102, Leu-103, [Gln-Asn-Thr]-104, Trp-105, Ala-106 and Pro-107). Therefore, Lys-104, which is found in many TEM-type ESBLs such as TEM-3 [18], could correspond to either a Gln, an Asn or a Thr in PER-1 (see Figure 2A and Figure 3). The three-dimensional superimposition shown in Figure 2A also suggests that the alanine residue found at position 164 in PER-1 might be the counterpart of the serine residue found at the same position in a large number of TEM variants. Furthermore, the lysine residue found at position 240 in several TEM-type enzymes, e.g. in TEM-5 [2], could correspond to a glycine in PER-1 (Figure 2A). Finally, the serine residue identified in PER-1 at position 238 is one of the amino-acid substitutions frequently encountered in the TEM- and SHV-type ESBLs (Figure 2A) [4].

Construction, purification and characterization of mutant enzymes derived from PER-1

By using site-directed mutagenesis, the residues located at positions 104, 164, 238 and 240 in PER-1 were replaced by amino acids found at the same positions in various class A β -lactamases devoid of activity against third-generation cephalosporins, such as the *Bacillus licheniformis* 749/C enzyme or TEM-1 [5]. Thus, mutants N104G, Q104G, T104E, A164R, A164S, S238G, S238A, and G240E were constructed. In addition, the glutamic acid found at position 166 in PER-1 was replaced by Ala in order to confirm that Glu-166 is an important residue for the catalytic activity of PER-1, as has been previously demonstrated for many other class A β -lactamases [19–21]. The purification of the wild-type and mutant enzymes was performed by a two-step procedure based on an anion exchanger column and gel filtration. Analysis by SDS/PAGE of the purified enzymes showed a single band protein, except for the E166A mutant which yielded three bands (data not shown).

Kinetic study of the PER enzymes

The catalytic activity of PER-1 and the mutant enzymes was evaluated by determining the steady-state kinetic parameters k_{cat} and K_m of the purified β -lactamases towards the β -lactam antibiotics shown in Figure 1. The values obtained are shown in Table 2.

Wild-type enzyme

The wild-type PER-1 β -lactamase displayed low K_m values for penicillin G, cephalothin and AZT, whereas the values found for cephaloridine and third-generation cephalosporins (CTX and CAZ) were high, particularly for CAZ (K_m value, 3.5 mM) (Table 2). In contrast, the latter two drugs were characterized by high k_{cat} values (43 and 70 s^{-1} , respectively) compared to those of penicillins (7.2 s^{-1}), cephalothin or cephaloridine (12.4 and 13 s^{-1} , respectively), and AZT (4.3 s^{-1}) (Table 2). Therefore, PER-1 is characterized by catalytic properties very similar to those of the TEM-derived ESBLs [22]. These results explain the fact that the resistance pattern conferred *in vivo* by PER-1 is not distinguishable from those conferred by a large number of ESBLs, despite the relatively low similarity found at the amino acid level between the sequences of the former and the latter enzymes [6,7]. However, it must be pointed out that the high catalytic efficiency of PER-1 for CAZ ($k_{\text{cat}}/K_m = 19.9 \text{ s}^{-1} \cdot \text{mM}^{-1}$), which is comparable to other previously reported ESBLs such as TEM-3 ($k_{\text{cat}}/K_m = 40 \text{ s}^{-1} \cdot \text{mM}^{-1}$) [22], is due to high catalytic activity ($k_{\text{cat}} = 70 \text{ vs.}$

Figure 2 Three-dimensional models showing the substrate binding site of PER-1 in the wild-type and mutant enzymes

(A) Stereoscopic view of the superimposition of the active sites of PER-1 and TEM-1. Blue and green, C- α chain tracing of the computer model of PER-1 and the crystal structure of TEM-1 (PDB entry code 1BTL), respectively. Red, TEM-1 residues determining the specificity profile and which are substituted in the extended spectrum TEM-variants. Black letters, residues shared by PER-1 and TEM-1. Thin lines, side-chains of Ser-70 and Glu-166 which both play a major role in the catalytic process. $\beta 3$ and $\beta 4$, β -strands connected by a loop and numbered according to Joris et al. [31]. (B) Stereo view describing the active site of the T104E mutant. Red, residue Glu-104. Dark dotted lines, hydrogen bonds. (C) Stereoscopic view describing residues Ser-238, Arg-164 and Glu-240 found in PER-1, A164R and G240E β -lactamases, respectively. Red, residues Arg-164 and Glu-240. Dark dotted lines, as in (B). Green dotted line, electrostatic interaction. Figure generated using RASMOL [32].

	102		104		107	
TEM-1	L	V	-	E	-	Y S P
TEM-3	L	V	-	K	-	Y S P
PER-1	V	L	Q	N	T	W A P

Figure 3 Multiple sequence alignment of the amino-acid residues found between positions 102 and 107 in TEM-1, TEM-3 and PER-1

Upper-case letters represent the one-letter code of the residues found between positions 102 and 107 in the amino-acid sequences of PER-1 [7], TEM-1 [23] and TEM-3 [18]. Position of residues, indicated above the amino-acid alignment, is given according to the numbering system of Ambler et al. [5]. Dashes indicate gaps inserted to optimize the alignment. The bold letter shows the lysine residue found at position 104 in the ESBL TEM-3. Arrows indicate the three consecutive residues of PER-1 that could correspond to position 104 in TEM-1 and TEM-3, respectively.

7 s⁻¹ for TEM-3) rather than high affinity (K_m = 3.5 mM vs. 175 μ M for TEM-3).

[QNT]104 mutants

In PER-1, an insertion of three residues (Gln, Asn and Thr, see Figures 2A and 3) is found at position 104 which is occupied by a glutamate in TEM-1 and a glycine in the *B. licheniformis* 749/C enzyme [23,24]. The steady-state kinetic parameters determined from the Q104G mutant were nearly identical to those measured from PER-1. According to the PER-1 model, Gln-104 is located outside the penicillin-binding site (Figure 2B), therefore the Gln side-chain cannot easily contact other amino-acid residues in either the active site or the R₁ side-chain of the β -lactam. These results strongly suggest that Gln-104 does not play a significant role in PER-1.

By contrast, the k_{cat} values yielded by the N104G mutant were significantly decreased for penicillin G (10-fold), CTX (8-fold), CAZ (3-fold) and AZT (2.5-fold), with a concomitant decrease in the corresponding K_m values, thus resulting in k_{cat}/K_m ratios very similar to those yielded by the wild-type enzyme (Table 2). For cephalothin and cephaloridine, k_{cat} and k_{cat}/K_m values were not significantly affected. In the PER-1 active site, Asn-104 and Asn-132 could form a hydrogen bond (Figure 2B). Interestingly, Asn-132 could also form a hydrogen bond with the key catalytic residue Glu-166 (Figure 2B). Consequently, the replacement of Asn-104 by a glycine is likely to modify the orientation of the side-chain of Glu-166 in the active site, a modification which could reduce the catalytic efficiency of the enzyme.

Of the three mutations introduced at the level of position 104 in PER-1, the Thr-104 $\rightarrow$ Glu substitution gave rise to the most marked modifications in catalytic activity. Indeed, the T104E mutant had no detectable activity against penicillin G. In addition, the k_{cat} values for CTX (0.06 s⁻¹), CAZ (0.16 s⁻¹) and AZT (0.1 s⁻¹) were 43- to 716-fold lower than those found for PER-1, leading to markedly decreased k_{cat}/K_m (15- to 507-fold). For cephalothin and cephaloridine, the T104E mutation resulted in a marked but similar decrease of both k_{cat} and K_m values, therefore the corresponding k_{cat}/K_m values remained similar to those of PER-1. As suggested in Figure 2B, the side-chain of Glu-104 in this mutant would be adequately oriented to allow the formation of a strong ionic bond with the positively charged side-chain of Lys-110. Such an interaction could displace the QNE region and consequently perturb the orientation of Glu-166 via

Table 2 Kinetic parameters for hydrolysis of β -lactams by PER-1 and mutant enzymes

Units for K_m , k_{cat} and k_{cat}/K_m are μ M, s⁻¹ and s⁻¹·mM⁻¹ respectively. N.D., not determinable. Values of S.E.M. are indicated below the kinetic values.

Enzyme	Penicillin G			Cephalothin			Cephaloridine			Cefotaxime			Ceftazidime			Aztreonam		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
PER-1	31.3 ±4.7	7.2 ±0.35	230 ±25	46 ±3.1	12.4 ±0.3	269 ±13	237 ±30	13 ±0.8	55 ±4	652 ±31	43 ±1.5	66 ±1	3519 ±410	70 ±7.2	19.9 ±0.34	44.2 ±5.4	4.3 ±0.15	97.3 ±8.7
Q104G	41.5 ±3.9	7 ±0.2	168 ±11.5	62 ±0.3	12 ±0.02	193.5 ±0.66	259 ±12	10 ±0.2	38 ±1.2	1220 ±111	70 ±5	57.4 ±1.2	7673 ±977	148 ±17.6	19.3 ±0.18	16.1 ±1.2	5.9 ±0.1	366 ±22.7
N104G	5.3 ±0.7	0.7 ±0.01	132 ±15.6	147 ±3.7	18 ±0.24	122 ±1.5	599 ±2	19.5 ±0.03	32.5 ±0.05	101 ±6	5.6 ±0.07	55.4 ±2.6	1811 ±102	25.1 ±1.2	13.8 ±0.17	14 ±0.2	1.7 ±0.1	121.4 ±1.4
T104E	N.D.	N.D.	N.D.	3 ±0.1	0.44 ±0.003	146 ±5.3	±0.1	2.5 ±0.001	56 ±2	448 ±68	0.06 ±0.004	0.13 ±0.01	121 ±23	0.16 ±0.008	1.3 ±0.2	32 ±6	0.1 ±0.005	3.1 ±0.4
A164R	N.D.	N.D.	N.D.	±0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A164S	30 ±2.5	3.2 ±0.08	106 ±6.2	41.5 ±1.3	6.1 ±0.1	147 ±2.6	151 ±16	6 ±0.3	39.7 ±2.3	306 ±20	16.8 ±0.5	55 ±2	2213 ±201	37 ±2.6	16.7 ±0.4	35 ±2.9	2.2 ±0.06	62.8 ±3.7
E166A	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	40 ±7	0.1 ±0.004	2.5 ±0.4	N.D.	N.D.	N.D.	609 ±70	1.1 ±0.09	1.8 ±0.07	171 ±14	0.5 ±0.02	2.9 ±0.1
S238G	7.7 ±0.6	3.5 ±0.05	454 ±29	83 ±4.7	11.6 ±0.4	140 ±4	124 ±15	4.5 ±0.2	36.3 ±3.3	880 ±64	46.2 ±2.5	52.5 ±0.6	5604 ±1018	76.8 ±12	13.7 ±0.3	21 ±0.4	2.9 ±0.02	138 ±2
G240E	28 ±2	7 ±0.2	250 ±13	21.7 ±0.25	6.7 ±0.04	309 ±2.4	120 ±17.6	9.3 ±0.7	77.5 ±6.3	499 ±104	24.5 ±2.8	49 ±4.8	3007 ±173	24.8 ±1.1	8.2 ±0.09	170 ±11.7	6.3 ±0.25	37 ±1.1

the N104-N132-E166 hydrogen-bond network (Figure 2B), resulting in a large decrease of the catalytic efficiency of the mutant enzyme. Recent results obtained with various class A β -lactamases support these conclusions. Indeed, in TEM-1, the amino-acid residue found in position 104 is in close contact with the side-chain of Asn-132 and would contribute to the precise positioning of the 130–132 region, which plays a key role in transition state stabilization [25,26].

A164 mutants

Arg-164 is a residue highly conserved among class A β -lactamases. In PER-1, an alanine is found at this position. It was first replaced by an arginine, a residue strictly conserved by class A penicillinases [5]. Surprisingly, no enzymatic activity was detected with the A164R mutant against any of the β -lactam compounds included in the study. As shown in Figure 2C, such a result could be interpreted by assuming that the bulky side-chain of Arg-164 contacts the neighbouring amino-acid residues constituting the Ω -loop in PER-1. One of these residues, Asp-172, could form a salt-bridge with the side-chain of Arg-164 in the A164R mutant. Such an interaction could drastically perturb the structure of the Ω -loop carrying Glu-166 and would lead to a loss of enzyme activity. It should be noted here that the A164R protein was hardly detectable in the cytoplasm fraction (data not shown). Therefore, it is very likely that the Ala-164 $\rightarrow$ Arg substitution in the Ω -loop of PER-1 is not compatible with a normal level of expression of the mutant protein which could be improperly folded and rapidly proteolysed. Similar observations have been recently reported by Palzkill et al. [27] who used random-replacement mutagenesis to introduce various substitutions in the 161–170 region contained in the Ω -loop of TEM-1.

We also constructed a second mutant in which Ala-164 was replaced by a serine which is one of the two residues (Ser or His) found specifically at position 164 in various ESBLs [4]. The β -lactamase A164S was characterized by k_{cat} values 2-fold lower than those of the wild-type enzyme, the K_m values remaining mostly unchanged (Table 2). The data suggest that the short side-chain of Ser-164 is rather well accommodated in PER-1, when compared to the more bulky side-chain of Arg-164. Moreover, the hydrogen bond found in the A164S mutant between the side-chain hydroxyl of Ser-164 and the side-chain carbonyl-oxygen atom of Asn-179 might stabilize the Ω -loop structure. Taken together, these results strongly suggest that a short side-chain is required in PER-1 to reach high catalytic activity against third-generation cephalosporins, but a more detailed interpretation of the role played by Ala-164 in PER-1 requires further structural data on the enzyme.

S238 mutants

Previous studies have shown that Ser-238 is one of the residues specifically involved in the catalytic activity of ESBLs against third-generation cephalosporins [18,22]. The serine residue found at position 238 in PER-1 was first replaced by a glycine which is the amino acid found at the same position in TEM-1 [23]. The K_m and k_{cat} values of the S238G mutant were very close to those of PER-1 (Table 2). Very similar results were obtained when Ser-238 was substituted by an alanine, which is found at the same position in the *B. licheniformis* and *S. aureus* penicillinases [5]. In contrast, in TEM and SHV ESBLs, Ser-238 is involved in CTX, CAZ and AZT hydrolysis, either by deformation of the active site resulting from direct interactions between Ser-238 and residues 69 and 170 [28], or by formation of a hydrogen bond between the OH of Ser-238 and the oxime group carried by the

latter three drugs [22]. Since the loss of such interactions has no effect in PER-1, the role of Ser-238 in this enzyme remains to be elucidated.

G240E mutant

Position 240, corresponding to a glutamate in TEM-1 [23], is occupied by a glycine in PER-1. The G240E mutant retained most of the catalytic activity of PER-1 (Table 2), except against AZT for which K_m increased 4-fold (Table 2). This result is consistent with our three-dimensional model of the active site of PER-1, suggesting that the side-chain of Glu-240 lies outside the β -lactam binding site and makes no contact with the R_1 side-chain of β -lactams (Figure 2C). However, the 4-fold increase in K_m observed for AZT cannot be given a satisfactory explanation at this stage of the study.

E166A mutant

Glu-166, which is found in PER-1 and in every other class A enzyme described so far, is known to play a major role in the acylation-deacylation process [20,29,30]. The Glu-166 $\rightarrow$ Ala substitution in PER-1 (E166A mutant) resulted in a complete loss of activity against penicillin G, cephalothin and CTX (Table 2). These results underline the major role played by Glu-166 in PER-1, a role which is now generally recognized in class A β -lactamases [21,29,30]. However, this interpretation suffers from the kinetic results obtained with cephaloridine, CAZ and AZT. Indeed, a significant catalytic activity was detected against cephaloridine, CAZ and AZT ($k_{\text{cat}} = 0.1, 1.1, \text{ and } 0.5 \text{ s}^{-1}$, respectively). Moreover, for cephaloridine and CAZ, the K_m values of the E166A mutant (40 and $609 \mu\text{M}$, respectively) were 6-fold lower than those determined for PER-1 ($237 \mu\text{M}$ and 3.5 mM , respectively), so that the E166A mutant displayed significant k_{cat}/K_m values for cephaloridine, CAZ and AZT. In contrast, several authors have previously shown that conversion of Glu-166 to Ala or Asn in the *B. licheniformis*, *Streptomyces albus* G and TEM-1 β -lactamases can lead to more than a million-fold decrease in catalytic efficiency [9,30]. One can hypothesize that the unexpected residual activity of the E166A mutant against cephaloridine, CAZ and AZT might be due to residues located in the vicinity of position 166 in PER-1, which could contribute in maintaining a low level of activity in the E166A mutant. Interestingly, a histidine residue is found at position 170 in PER-1 instead of an asparagine which is highly conserved within class A β -lactamases [5]. Further site-directed mutagenesis experiments are in progress to assess the function of this residue in the catalytic activity of PER-1.

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