Evolutionary engineering of a β-Lactamase activity on a D-Ala D-Ala transpeptidase fold

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The β-Lactamase hydrolytic activity has arisen several times from DD-transpeptidases. We have been able to replicate the evolutionary process of β-Lactamase activity emergence on a PBP2X DD-transpeptidase. Some of the most interesting changes, like modifying the catalytic properties of an enzyme, may require several mutations in concert; therefore it is essential to explore efficiently sequence space by generating the right diversity. We designed a biased combinatorial library in which biochemical and structural information were incorporated by site directed mutagenesis on relevant residues and then subjected to random mutagenesis to allow for mutations in unforeseen positions. We isolated mutants from this library conferring 10-fold higher cefotaxime resistance levels than the background wild-type through mutations exclusively in the coding sequence. We demonstrate that only three substitutions in the DD-transpeptidase active site, two produced by the directed and one by the random mutagenesis, are sufficient to acquire this activity. The purified product of one mutant (MutE) had a $10^5$-fold increase in cefotaxime deacylation rate allowing it to hydrolyze β-Lactams yet it has apparently conserved DD-peptidase activity. This work is the first to show a possible evolutionary intermediate between a β-Lactamase and a DD-transpeptidase necessary for the development of antibiotic resistance.

Keywords: β-Lactamase/combinatorial mutagenesis/directed evolution/evolutionary engineering/PBP2X

Introduction

The number of unique protein folds has been estimated to be no more than a few thousand (Chothia, 1992), much lower than the number of proteins encoded by nature. Thus, extant folds should be reused by divergent evolution to provide the functional range represented across the entire protein universe. Recent systematic studies show that enzymes with a common ancestor can have different biochemical and physicochemical properties and can even catalyze overall unrelated reactions (Gerlt and Babitt, 2001).

Fold versatility has been proved by both rational and evolutionary protein engineering: just a few amino acid substitutions have allowed impressive changes in protein function: a histidine biosynthesis isomerase was engineered into a tryptophan biosynthesis isomerase by broadening its substrate specificity (Jurgens et al., 2000); a cyclophilin isomerase was converted into an endopeptidase by grafting a catalytic triad (Quemener et al., 1998), and thioredoxin was transformed into a histidine hydrolase by the design of an active site cleft and inclusion of the catalytic residue (Bolon and Mayo, 2001).

The DD-peptidase/β-Lactamase superfamily is an example of a fold with functional diversity; it is composed mainly of hydrolases and transferases, although a carboxyesterase has been found recently to belong to this superfamily (Wagner et al., 2002). Although members of the family exhibit less than 20% sequence identity, all of them bind β-Lactam antibiotics by the same mechanism. DD-peptidases and β-Lactamases evolved from a common ancestor, they share the same fold, three short signature sequences in the active site, and the acylation mechanism. The DD-peptidase/β-Lactamase structural superfamily has an antiparallel β-sheet, flanked by α-helices on both sides and an α-helical domain (Todd et al., 2001). Its catalytic mechanism involves two steps and proceeds through two tetrahedral intermediates; in the first step an invariant nucleophilic Ser residue attacks the labile peptide bond of the substrate to form an acyl–enzyme intermediate; the second step involves the hydrolysis or transfer of this intermediate (Cohen and Pratt, 1980; Fisher et al., 1981).

DD-peptidases are enzymes involved in the biosynthesis and remodelling of the bacterial cell wall, and are often referred to as Penicillin Binding Proteins (PBP). These proteins can act as carboxypeptidases or as transpeptidases. They catalyze the cleavage of the terminal β-alanyl–β-alanine bond of the stem peptide and the subsequent formation of the peptide bridge to an amino acceptor on an adjacent peptidoglycan branch. β-Lactam antibiotics acylate both DD-peptidases and β-Lactamases; nevertheless the deacylation step in DD-peptidases is much less efficient than in β-Lactamases (over 10$^8$ fold lower) (Guillaume et al., 1997; Lu et al., 2001). This extremely slow reaction is responsible for the covalent inhibition of DD-peptidases, resulting in the synthesis of non-functional peptidoglycan and eventually cell death (Ghuysen, 1994; Goffin and Ghuysen, 1998).

β-Lactamases are the most common mechanism of resistance to β-Lactam antibiotics, such as penicillins and cephalosporins. These enzymes catalyze the hydrolysis of the β-Lactam ring amide bond thereby protecting bacteria from the action of the drug. β-Lactamases have been grouped in four classes: classes A, C and D are active site serine β-Lactamases, while class B is a zinc-dependent metalloenzyme (Bush et al., 1995). Sequence, structure and catalytic behavior analyses show that the DD-peptidases gave rise to at least two of the various classes of serine β-Lactamases independently (Knox et al., 1996; Massova and Mobashery, 1998). This suggests that both catalytic activities are close in catalytic space and as such it should be feasible to interconvert one into another.

We wanted to explore whether this change in catalysis could be gradual, with both catalysts coexisting or if they were exclusive one of another. Directed evolution studies carried out on β-Lactamases have shown that the gain of a new specificity implies the gradual loss of the previous one (Zaccolo

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and Gherardi, 1999; Wang et al., 2002), and recent studies on the directed evolution of a β-glucuronidase into a β-galactosidase show that the change proceeds through an evolutionary intermediate which has a broadened substrate range (Matsumura and Ellington, 2001).

We have used a rational evolutionary strategy to elicit a β-Lactamase activity on a dδ-peptidase of known structure. This method has allowed us to obtain libraries with an effective high mutant multiplicity. Our starting point was a wild-type Streptococcus pneumoniae PBP2X dδ-transpeptidase (MX1). With just a few amino acid substitutions we were able to obtain a cephalosporinase thus recreating the evolutionary process of β-Lactamase activity appearance. We have done the biochemical analysis of one mutant which does not seem to have lost its original dδ-transpeptidase activity yet has gained the capacity to hydrolyze β-Lactams, thus behaving as an evolutionary intermediate.

Materials and methods

Construction of the Escherichia coli expression plasmid pBC-PBP2X

The gene encoding for the PBP2X was cloned by PCR amplification from a penicillin-sensitive clinical isolate of Streptococcus pneumoniae using primers: T 5′(CGG AAT TCG CCG AGT AAG ATA TGA AGT G)3′, and H 5′(GTC AGC TCT AGA TTA GTG ATG GTG ATG GTC TCC TAA AGT TAA TGT AAT TT)3′. The amplified DNA fragment was treated with Klenow fragment, digested with EcoRI, and cloned into pBC SK(+), a chloramphenicol-resistant pUC19 derivative (Stratagene, La Jolla, CA). A PsiI site was created by site directed mutagenesis at position encoding Leu269, the restriction site does not modify the amino acid sequence and was created to facilitate the recloning of the transpeptidase-domain coding region. The PBP2X gene is under the lac promoter and the protein has a C-terminal His 6 flag. This gene has been called MX1, and has been submitted to GenBank with accession number AF 468152.

The constructions were sequenced to confirm gene identity. Protein expression and localization were detected by western blot, using an anti-His IgG (Roche, Mannheim, Germany).

Library construction

The C library was constructed by recursive PCR. The degenerate oligonucleotides used as primers were: M1 5′(AAC CAA GGT AGC CCC CAT ATA CTT G)3′, M2 5′(AAC TAT GAG CCG RYG TCA ACC ATG AA)3′, M3 5′(GAG GGT CAT CCC AGC GTT ATC TGA CRK TGC AAA ACC TTG)3′, M4 5′(GCA GTT AAA TCC GGT RSC RGC GRS ATC GCT GAT GAG AA)3′, M5 5′(AAC TGA GAA ATT AVY ATT GGT AGA AC)3′. The primers were incorporated as follows: M1, M3, M5, M4, and M2. After each PCR, DNA fragments were purified by gel extraction (Qiagen, Hilden, Germany), and amplifications were carried out using the previous product as megaprimer and the next oligonucleotide (Merino et al., 1992). Each reaction was also performed without its megaprimer to allow for the wild-type residues. Error prone PCR (Cadwell and Joyce, 1992) was carried out on the DNA fragment corresponding to the transpeptidase domain.

For recombination libraries shuffling mutagenesis was performed as described by Stemmer (Stemmer, 1994). Fragments of 100–300 bp were recovered after DNase I digestion and reassembled by PCR without primers. The rate of point mutations per shuffling cycle was 0.5%.

The SM library was constructed by recursive PCR using the following degenerate oligonucleotides: SM312 5′(TT CTT AGT ATC SNN ATC AAA GGT TG)3′, SM336 5′(AAC CTT CAT GGT TGA SNN CCG CTC ATA G)3′, SM450-452 5′(CAC TGA ATT CCC SNN CNT TGA GCT TTG C)3′.

To clone the libraries, 2 µg of PsiI/BsrEI digested PCR product was ligated to 2 µg of PsiI/BsrEI digested pBC-PBP2X at 16 °C overnight, using 3U of T4 ligase (Roche). Ligation mixtures were precipitated with butanol, washed with 70% ethanol, dried, and dissolved in 15 µl of water. These were transformed in E.coli MC1061 electrophoretic cells.

PCR

All PCR reactions were performed with 5U Taq DNA polymerase (Gibco-BRL, Rockville, MD), 1.5 mM MgCl2, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 0.25 mM each dNTP and 1 µM of each primer. Cycling was done according to the following program: 94 °C, 5 min (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min) × 25, 72 °C, 5 min.

Selection

A library of E.coli MC1061 transformants, with a 10-fold size coverage, was enriched for 48 h at 30 °C in 2 ml LB medium with chloramphenicol and cefotaxime. Plasmid DNA was isolated and used to retransform E.coli MC1061; cells were selected on LB agar plates with chloramphenicol and cefotaxime at 37 °C. The minimum inhibitory concentrations (MIC) for the selected colonies were determined by a dilution method on LB agar plates at 37 °C, with inocula of 10–30 colony-forming units dispensed in 5 µl drops (Viudi et al., 1995).

Protein purification

pBC-Δ19-48 was constructed by the removal of the hydrophobic transmembranal-domain coding region (amino acids 19–48) to create PBP2X soluble forms (Jamin et al., 1993). E.coli XL1-Blue (Stratagene) carrying plasmid pBC-Δ19-48 cells were grown in a 1.5 liter fermentor containing LB medium supplemented with chloramphenicol and induced with 1 mM IPTG. The cell pellet was suspended in 15 ml of buffer A (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole pH 8.0) and sonicated. The lysate was centrifuged at 14 000 g for 30 min at 4 °C. The supernatant was filtered (0.45 µm) and loaded into a His-Trap column (Pharmacia) that was previously equilibrated with buffer A. The column was washed with 5 volumes of buffer A, and the protein was eluted with a linear gradient of 20–200 mM imidazole. The fractions containing PBP2X were pooled and dialyzed against 100 mM sodium phosphate buffer pH 7.0. Protein was quantified by the Bradford method using a Bio-Rad kit, with bovine serum albumin as standard. The homogeneity of protein samples was determined by SDS–PAGE.

Enzyme kinetics

All kinetic assays were performed at 37 °C in 100 mM sodium phosphate buffer pH 7.0, using a Beckman DU650 spectrophotometer for cefotaxime and Bz-(D)-Ala2-Gly thioester (S2d) hydrolysis determination, and a Bio-Rad EIA Reader 2550 microplate spectrophotometer for the nitrocefin hydrolysis assay. All points were determined at least in triplicate.

β-Lactamase activities were determined by standard steady state kinetics methods. Hydrolysis of cefotaxime was monitored by loss of absorbance at 262 nm (Δε262 = 7250 M⁻¹ cm⁻¹). Hydrolysis of the chromogenic cephalosporin, nitrocefin, was monitored by the increase of absorbance at 492 nm.
Transpeptidase activity was determined by the hydrolysis of S2d, a thioester analog of cell wall stem peptides (Adam et al., 1990). The thio-group release, that was coupled to 4,4′-dithiodipyridine, was measured by monitoring the increase of optical density at 325 nm (Δε₃₂₅ = 19,800 M⁻¹ cm⁻¹) as described before (Zhao et al., 1997), with minor modifications. Reaction mixtures (0.5 ml each) contained 0.1–2 mM of S2d, 0.4 mM of 4,4′-dithiodipyridine, and 16 µg of wild-type protein or 20 µg of MutE. The increase in absorbance at 325 nm was monitored for 60 min.

The MX1 wild-type PBP2X cefotaxime deacylation rate constant (k_cat), was determined by following the recovery of activity against S2d as a function of time. 46 µM of PBP2X (~4 mg/ml) was incubated with 0.5 mM cefotaxime for 5 min at 37 °C. Unbound β-Lactam was removed by centrifuging the reaction mixture at ~1000 g for 2 min in a Penefsky column (Penefsky, 1977). The effluent containing the PBP2X bound to β-Lactam was diluted (1 mg/ml) and incubated at 37 °C. At various time intervals an aliquot of the sample was removed to measure activity against S2d. Assay buffer contained 2.0 mM S2d, 0.4 mM 4,4′-dithiodipyridine, and 150 mg/ml of PBP2X. At t₀, inhibition of wild-type PBP2X was complete. As less than 20% of activity was recovered after 48 h (when less than half of the free enzyme remains active), only apparent k_cat was determined. We have verified the complete removal of free cefotaxime by centrifuging 0.5 mM cefotaxime in a Penefsky column and pre-incubating PBP2X with the effluent. Under those conditions the wild-type PBP2X remains fully active as indicated by S2d hydrolysis.

Chemicals

Ampicillin, penicillin, cefuroxime, ceftriaxone and cefotaxime were purchased from Sigma Chem, St Louis, MO. Nitrocefin was purchased from Becton Dickinson & Co, Franklin Lakes, NJ. Ceftazidime was supplied by Glaxo, Brentford, UK. S2d was purchased from Absolute Science (MA, USA).

Results and discussion

Combinatorial library design and construction

We followed a rational evolutionary strategy which combines rational design with directed evolution to elicit a β-Lactamase activity from a dd-transpeptidase. In a rational strategy, mutations are directed to positions that probably are going to produce relevant phenotypes yet their scope will be limited by the a priori information available. In a classical directed evolution approach, for instance, a 10¹⁰ clone library would be needed to be able to screen for all possible triple mutants, many of which would be inconsequential. A hybrid approach allows us to increase the odds of obtaining new mutations—produced by the mutagenic PCR—in combination with previously chosen positions. Thus a greater return can be made with the same number of mutants than with a random whole gene mutagenesis scheme (Voigt et al., 2000). We constructed a combinatorial library (C library) based on relevant information, by promoting mutations at sequence positions that are expected to show improvements.

The most promising targets for rational mutagenesis are the positions that distinguish β-Lactamase from dd-transpeptidase active sites as they must be the ones conferring the differences between both catalysts. Both active sites recognize β-Lactams and covalently bind them to the catalytic serine; a stable acyl-enzyme complex is formed in dd-transpeptidases while this intermediary is hydrolyzed in β-Lactamases, therefore, an activated water molecule is needed in the active site to promote the hydrolysis.

A significant difference between the β-Lactamase and dd-peptidase active sites is that the former are more exposed to solvent. The PBP2X crystal structure analysis (Pares et al., 1996; Gordon et al., 2000) shows that the active site is protected from water mainly by Phe450 and Trp374. As water is necessary for acyl–enzyme hydrolysis, these positions were mutated to saturation to increase water access to the active site. Moreover, Phe450 is in a position analogous to that of Glu166 in the class A β-Lactamases, where this amino acid is catalytic and activates water for deacylation (Adachi et al., 1991; Strynadka et al., 1992), and thus a charged amino acid in this position could activate a water molecule. Trp374 is equivalent to the class C Leu293, mutations in this residue increase activity for some cephalosporins (Zhang et al., 2001). As residues 450 and 374 are situated in variable size loops, residue correspondence to other structures is not apparent in sequence alignments (Figure 1).

Residues involved in broadening specificity were also mutated, as changes in specificity could result in the improvement of binding interactions or in transition state stabilization (Viadiu et al., 1995). A better catalytic efficiency could be reached by the substrate proper positioning. Residues Thr550, Ala551, Gln552 are situated in the β3, an element of the β-Lactamase active site; mutations in this strand produce extended spectrum enzymes, which confer resistance to third generation cephalosporins (Mantagne et al., 1998). Gly336 is located just next to the nucleophilic serine and is buried within the hydrophobic interface between the two domains, β-Lactamases mutated in an analogous position are resistant to clavulanate (Delaire et al., 1992).

Several residues have been shown to be conserved and essential for function in β-Lactamases (Huang et al., 1996), we mutated those which are near the active site and different from those found in dd-peptidases. Although the function of these residues is not completely clear, we assume that they must be involved in determining β-Lactamase catalytic properties as they are conserved in the active site. Those residues conserved away from the active site might be important for stability or folding but both properties are already present in dd-peptidases. Residues Leu394, Ser396, Val398 form a wall of the active site and are located next to Ser395, which is involved in the acylation mechanism (through a hydrogen bonding network) (Imitaz et al., 1994); the equivalent residues in class A β-Lactamases are conserved, in particular Ser396 (Asp131 in β-Lactamases) which is invariant. Tyr568 is located on the β4 pointing towards the active site; the equivalent residue in β-Lactamases is a glycine (Figure 1).

Based on the previous analysis, the C library was designed and constructed by site directed mutagenesis of 10 PBP2X residues as follows: Gly336MetAla, Trp374X, Leu394MetThr, Ser396Asp, Val398Ala, Phe450X, Thr550AlaGlySerThr, Ala51GlySer, Gln552GlyGluSer and Tyr568Gly (Figure 2). The wild-type residue was allowed for all positions. After the combinatorial site directed mutagenesis, the transpeptidase domain coding region was amplified by error prone PCR, at a 0.8% mutagenesis rate, to allow the appearance of advantageous substitutions in unforeseen positions. The amplified material was cloned into the pBC-PBP2X vector, by exchange
Fig. 1. Sequence alignment of the β-Lactamase/DD-peptidase superfamily. The secondary structure elements are shown. Strictly conserved residues (shaded letters), and homologous residues (black boxes) are indicated. Triangles mark the residues mutated in C library. The alignment was produced by Dali server (Dietmann and Holm, 2001), using the structural alignment of class A, C and D β-Lactamase representatives (1btl, 1fof and 1gce respectively) with a high molecular weight and low molecular weight (LMW) DD-peptidases (1qme and 3pte). The 1qme sequence was replaced by the MX1 sequence to produce the alignment shown here.
β-Lactamase/DD-peptidase catalytic migration

Fig. 2. Diagram of the C library mutagenesis scheme. Secondary structure and catalytic serine (337) are also indicated. The positions and type of the mutated residues around the active site are depicted.

Table I. Amino acid substitutions in cefotaxime-resistant clones

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Ala312</th>
<th>Gly336</th>
<th>Trp374</th>
<th>Phe450</th>
<th>Gln452</th>
<th>Ala554</th>
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</thead>
<tbody>
<tr>
<td>C library MutA</td>
<td>Thr</td>
<td>Ala</td>
<td>Leu</td>
<td>His</td>
<td>Thr</td>
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<tr>
<td>MutB</td>
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<td>Arg</td>
<td>Thr</td>
<td></td>
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<tr>
<td>MutC</td>
<td>Thr</td>
<td>Ala</td>
<td></td>
<td>Leu</td>
<td>His</td>
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<tr>
<td>MutD</td>
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<td>Ala</td>
<td></td>
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<tr>
<td>SM library MutE</td>
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<td>Leu</td>
<td>His</td>
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<tr>
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<td>Met</td>
<td>His</td>
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<tr>
<td>MutG</td>
<td>Pro</td>
<td>Ala</td>
<td></td>
<td>Leu</td>
<td>His</td>
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</tr>
<tr>
<td>MutH</td>
<td>Ser</td>
<td>Ala</td>
<td></td>
<td>Leu</td>
<td>His</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of the transpeptidase coding region. We obtained a library of \(5 \times 10^6\) transformants.

The C library transformant cells were selected for their ability to grow on LB with cefotaxime (200 ng/ml). Selection was performed first in liquid medium to enrich for resistant clones before further selection on solid medium to isolate the more resistant ones. Seven clones were selected and their plasmid DNA was isolated. Fresh cells were transformed with these plasmids to confirm the phenotype. The transpeptidase-domain region was sequenced and four different clones were identified: MutA, B, C and D; their sequences include both random and site directed mutations (Table I). The Gly336Ala, Trp374Arg and Phe450Leu substitutions are the product of the site directed mutagenesis, while Ala312Thr, Gln452His and Ala554Thr arose from the error prone PCR. Mutations at 312, 336, 450 and 452 were found in several clones; it is interesting that the leucine codons at position 450 are different, indicating that MutA and MutC are independent clones. E.coli transformed with the wild-type β-Lactamase or with the empty plasmid have the same background β-Lactam resistance level, while MutA, B, C and D clones conferred 4- to 10-fold higher cefotaxime resistance levels (Figure 3).

**Error prone and shuffling mutageneses**

We undertook further directed evolution rounds looking for higher levels of resistance, and thus higher catalytic efficiencies. We constructed two error prone libraries and four libraries by gene shuffling. Library sizes varied between \(1.2 \times 10^6\) and \(8.7 \times 10^6\) clones. The effectiveness of the mutageneses performed for each library was verified by sequencing of unselected clones. We identified a clone exhibiting a 100-fold higher cefotaxime resistance level than the wild-type from one of the shuffling libraries intended to eliminate neutral and deleterious mutations. The increase in resistance was conferred by a deletion between the promoter and the start codon that induced protein expression (detected by western blot, data not shown). When the transpeptidase coding region was subcloned into a fresh vector, the transformed cells were again only 10-fold more resistant than the wild-type.

**Saturation mutagenesis library**

As mutations at residues 312, 336, 450 and 452 were found at least twice in the clones selected from the C library, we presumed that these positions were important for β-Lactamase activity. The structure of the genetic code and the C library design reduce the accessibility to numerous portions of the protein sequence space, including non-conservative substitutions that require multiple base changes within a single codon. The exploration of further mutations on positions previously shown to be important for the desired properties have yielded greatly improved variants which otherwise would not have been obtained (Miyazaki and Arnold, 1999). We constructed a new library (SM library) by saturation mutagenesis to find the best substitution combination at these positions. We did not find any increase in resistance levels but achieved the segregation of mutations, allowing us to evaluate the individual contribution of each substitution to the overall clone fitness. Positions 312, 336, 450 and 452 were subjected to saturation mutagenesis resulting in a \(1.2 \times 10^7\) library, covering 10-fold all the combinatorial possibilities \((1.05 \times 10^6)\). The SM library was selected as previously described (cefotaxime 200 ng/ml). After selection, 17 transformants were isolated; their analysis showed that they were grouped into four different clones (Table I). We found alanine at position 336, histidine at position...
452 and leucine or methionine at position 450; however we found various amino acids at position 312 (even the wild-type), indicating that this residue is not involved in antibiotic resistance. Residues 336, 450 and 452 are in the vicinity of the active site, 336 is next to the catalytic Ser (337), whereas 450 and 452 reside in the omega loop (see below).

As we have not observed any increase of β-Lactamase activity in these libraries, it appears that we have reached a local maximum in the β-Lactamase PBP2X transpeptidase landscape. Further improvements in the catalytic efficiency may require several mutations acting synergistically. Either a higher mutagenic rate—therefore larger libraries—or the design of a new combinatorial library that includes more information would be necessary to obtain these mutations. The detection of small additive increases in β-Lactamase activity could also be hindered by the lack of resolution in our selection procedures.

### Resistance to β-Lactams

The β-Lactamases belonging to the different classes exhibit very different resistance spectra. Penicillinases tend to have very poor cephalosporinase resistance levels and vice versa, when class D imipenemases tend exhibit a broader resistance spectrum, albeit at low levels. As the mutants were selected only for cefotaxime resistance we wanted to know if the resistance encompassed other β-Lactam antibiotics. We determined the MIC of ampicillin, penicillin, amoxycillin, imipenem, cefuroxime, ceftriaxone, ceftazidime and cefotaxime (Figure 3). The mutants with highest cefotaxime resistance levels, which have a leucine at residue 450, were also resistant to cefotaxime and ceftriaxone; none showed resistance to cefuroxime, ampicillin, penicillin, amoxycillin or imipenem. Although the MIC is not proportional to the enzymatic activity, Figure 3 suggested that these mutants have a broadened resistance range. The resistance spectrum observed resembles that of naturally occurring β-Lactamases in that they only confer resistance to certain β-Lactam antibiotics (Mantagne et al., 1998; Nagai et al., 2002). We decided to use MutE for further characterization as it has only three substitutions and confers the highest resistance level to cefotaxime.

### Kinetics of PBP2X and MutE purified proteins

To corroborate that the resistance phenotype was produced by the β-Lactamase activity, wild-type PBP2X and MutE proteins were purified to homogeneity to measure their kinetic parameters. The N-terminal membrane-anchoring domain of both enzymes was removed to confer solubility without compromising enzyme activity (Chittock et al., 1999). The wild-type enzyme did not show any detectable β-Lactamase activity by the direct spectrometric method, therefore deacylation (kcat) was measured by recovery of the peptidase activity (Table II).

The MX1 PBP2X deacylation rate is in agreement to the rates measured for R6 PBP2X by S2d hydrolytic activity recovery (Jamin et al., 1993). The MutE activity kinetic parameters for β-Lactamase were determined by standard steady state methods using cefotaxime, and nitrocefin as substrates. The MutE Kcat values were 33 μM for cefotaxime and 321 μM for nitrocefin, and the kcat values were 1.49×10⁻² s⁻¹ for cefotaxime and 1.46×10⁻³ s⁻¹ for nitrocefin (Table II). When compared to natural β-Lactamases, the turnover is slow for both substrates (Matagne et al., 1998); nevertheless when compared to the MX1 wild-type PBP the turnover is 10²-fold greater, which is enough to confer a clearly detectable β-Lactam resistance level. The R6 PBP2X deacylation rate has been determined by various methods including recovery of peptidase activity (Jamin et al., 1993), fluorography of radioactive inhibitors (Mouz et al., 1999), infrared spectrometry (Chittock et al., 1999), and electrospray mass spectrometry (Di Guilmi et al., 2000; Lu et al., 2001); these methods are not consistent and the reason for this discrepancy is unknown. The MutE and wild-type PBP2X deacylation rates are so different that they could not be determined by the same method; therefore the increment in deacylation rate could be an approximation.

To evaluate the specificity of MutE, the catalytic constants were also determined against ampicillin, a semisynthetic penicillin, and ceftazidime, another cephalosporin; in contrast to the wild-type, these activities are detectable by direct spectrophotometric methods. The activity for ampicillin is 10-fold lower than that for cefotaxime (Kcat 1240 μM, and kcat 5.22×10⁻² s⁻¹), and is not sufficient to confer resistance to this antibiotic. The catalytic activity against ceftazidime is similar to that of cefotaxime, yet the Kcat and kcat are higher (Kcat 1310 μM, and kcat 5.68×10⁻¹ s⁻¹).

Transpeptidase activity was evaluated by the hydrolysis of Bz-(D)-Ala-Gly (S2d), a thioester that mimics the (D)Ala bond recognized by PBP on its natural substrate (Jamin et al., 1993; Zhao et al., 1997). The hydrolytic activity values (kcat/Km) obtained for MutE and wild-type were 69 (M⁻¹ s⁻¹) and 46 (M⁻¹ s⁻¹), respectively (Table II).

Apparent β-Lactamase activity arose in MutE without compromising its original Dd-peptidase activity. Both activities might only be incompatible in highly specialized enzymes. Considering that PBP2X appears to be a transpeptidase without compromising its original DD-peptidase activity. Both activities might only be incompatible in highly specialized enzymes. Considering that PBP2X appears to be a transpeptidase without

### Table II. Kinetic parameters of MutE compared to the wild-type PBP2X

<table>
<thead>
<tr>
<th>β-Lactamase activity</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>kcat/Km (M⁻¹ s⁻¹)</th>
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<tr>
<td>Cefotaxime</td>
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<tr>
<td>Mut E</td>
<td>1.49 [± 0.15]×10⁻²</td>
<td>33.6 [± 3.5]</td>
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<td>Wild-type</td>
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<tr>
<td>Nitrocefin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut E</td>
<td>1.46 [± 0.14]×10⁻¹</td>
<td>321 [± 67]</td>
<td>4.55</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Nd</td>
<td>Nd</td>
<td></td>
</tr>
</tbody>
</table>

²Nd, not detected.

Some S.pneumoniae strains are naturally resistant to β-Lactam antibiotics and this capacity is conferred by altered PBP2X and PBP2B enzymes (Grebe and Hakenbeck, 1996). These enzymes have evolved to be less susceptible to β-Lactam acylation by changing their substrate specificity (Zhao et al., 1997; Di Guilmi et al., 2000), resulting in a different cell wall composition (Severin et al., 1996). However there is a documented case in which an 80-fold increase of deacylation efficiency was detected in a resistant PBP2X (Lu et al., 2001). Changes in penicillin-resistant S.pneumoniae PBP2X only protect the mutated enzyme and not the other existing types.

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of PBPs which are also targets of the antibiotic. Although transformation of \textit{S.pneumoniae} with a resistant form of PBP2X can confer a certain degree of protection, the level of protection is only up to the antibiotic threshold concentration that inactivates another essential PBP (Laible and Hakenbeck, 1991). In our case, the PBP2X mutant we obtained is capable of conferring resistance to cefotaxime to the strains harboring it. In this heterologous system, the \textit{S.pneumoniae} PBP2X seems not to have any direct participation in the \textit{E.coli} cell wall synthesis, as shown by the lack of phenotype in strains carrying the wild-type PBP2X, and therefore the protective effect must be caused by its increased hydrolytic capacity.

MX1 PBP2X was isolated from a β-Lactam-sensitive strain, classified into \textit{S.pneumoniae} strain group II based on nucleotide substitutions within conserved amino acid motifs (Asahi et al., 1999). The kinetic parameters for a sensitive PBP (R6 PBP2X) most recently determined were a $k_{\text{cat}}/K_m$ for S2d of 4900 M$^{-1}$ s$^{-1}$ (Thomas et al., 2001), and a $k_{\text{cat}}$ for cefotaxime of 3.5×10$^{-6}$ s$^{-1}$ (Lu et al., 2001); these values are higher than the ones we determined. These differences are not surprising as 27 out of the 351 residues of the transpeptidase domain are the ones we determined. These differences are not surprising as 27 out of the 351 residues of the transpeptidase domain are different between MX1 PBP2X and R6 PBP2X.

The MutE active site has acquired the necessary functional properties for β-Lactam hydrolysis without losing its original activity and as such this enzyme may be an evolutionary intermediate between the two fully specialized enzymes (Matsumura and Ellington, 2001). Nevertheless, considering that S2d hydrolysis is not a direct measure of PBP2X DD-transpeptidase catalytic process, we may not be detecting a drop in the MutE DD-transpeptidase capacity caused by the rise of the β-Lactam activity.

**Residues 336, 450 and 452**

The minimal set of altered residues necessary for cefotaxime resistance is Ala336, Leu450 and His452 as seen in the clones selected from the SM library. In naturally occurring PBP2X, residues Gly336, Phe450 and Gln452 are absolutely conserved, even in the resistant clones. Vernet and co-workers (Chesnel et al., 2002) have recently replaced Phe450 by an aspartic acid in the R6 PBP2X. This mutant deacylates penicillin 110-fold faster, and they propose that this aspartic acid activates the water molecule for hydrolysis. Since we selected both leucine and methionine in this position (both hydrophobic amino acids), the deacylation mechanism must proceed otherwise.

A possible role for the substitutions Gly336Ala, Phe450Leu and Gln452His can be suggested by the analysis of the \textit{S.pneumoniae} R6 PBP2X X-ray crystal structure (Gordon et al., 2000). Residue 336 lies next to the catalytic Ser337 on a short turn and is buried in the hydrophobic interface between the two transpeptidase domains, constituting the hinge of the active site. The substitution of Gly336 for a bulkier amino acid may change the tilt of the β3 strand, favoring the initial binding and positioning of the β-Lactam for further hydrolysis (Knox et al., 1996). Residues 450 and 452, in the omega loop, are pointing to the active site. It is well known that substitutions in the β-Lactamase omega loop result in structural changes, reflected in the active site shape, $k_{\text{cat}}$, specificity and/or folding kinetics (Venkatachalam et al., 1994; Maveyraud et al., 1996; Petrosino and Palzkill, 1996; Guillaume et al., 1997; Wheeler et al., 1998; Ibuka et al., 1999; Vanwestwinkel et al., 2000). Substitutions Phe450Leu and Gln452His may result in the destabilization of the entire loop through the loss of some interactions. In summary, the three substitutions distort the active site conformation, probably by changing the angle between the domains and restructuring the Ω loop (Figure 4).

Deacylation has been solved by different functional groups located in different positions of the active site in the DD-peptidase/β-Lactamase superfamily (Figure 5). In class A β-Lactamases, water is activated by Glu166 (Lamotte et al., 1992), in class B β-Lactamases by Tyr150 (Powers et al., 1999), while in class D β-Lactamases Lys70 is responsible for it (Golemi et al., 2001). As seen in Figure 5, these residues are located in different secondary structure elements and also in different orientations in regard to the substrate. The simplest hypothesis for the mechanism of acyl MutE hydrolysis would...
be that Gln452His is responsible for water activation as it is the only substitution with a new functional group that could act as a potential proton acceptor, this histidine could act as in a serine protease catalytic triad. This hypothesis is also supported by the Glu166Gln:Asn170Asp PC1 mutant. In this class A β-Lactamase mutant the general base has been moved to a different position of the omega loop without losing its deacylation capacity, proving that water could be activated by another residue of the omega loop (Chen and Herzberg, 1999).

Deacylation could also be promoted by the reorientation of some other residue(s) already present in wild-type PBP2X. Nevertheless only a crystalllographic structure would allow us to discriminate among these possibilities and assign the proper role to each of the substituted amino acids.

Directed evolution has allowed the generation of new functional proteins. This engineering strategy consists on the production of multiple variants and their later selection. It is an extremely powerful idea, however it can be limited in practice by the number of variants that we can produce and select. Some of the most interesting changes may require several mutations in concert; therefore it is essential to explore efficiently sequence space by generating the right diversity.

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References


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