Mutations altering substrate specificity in OHIO-1, an SHV-1 family β -lactamase

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The OHIO-1 β -lactamase does not normally hydrolyse oxyimino- β -lactam substrates like cefotaxime, ceftriaxone, ceftazidime or aztreonam. We were able to select spontaneous mutants of an OHIO-1-bearing strain of *Escherichia coli* using the antibiotic substrates listed above by enrichment methods at frequencies of 10^{-8} – 10^{-10} for all antibiotics except ceftazidime (frequency $< 10^{-10}$). Most mutants with increased resistance to the other β -lactams were also more resistant to ceftazidime. Mutations identified by DNA sequencing included a Gly²³⁸ $\rightarrow$ Ser²³⁸ substitution identical with the SHV-2 mutation previously described, cysteine and valine substitutions at the identical site, and a Gly²⁴² $\rightarrow$ Cys²⁴² substitution. The Cys²³⁸ and Cys²⁴² mutant enzymes had less affinity for aztreonam than had the other mutant enzymes. Hydrolysis of cefotaxime, but not cephaloridine, by the cysteine-substituted enzymes was inhibited by *p*-chloromercuribenzoate. The mutant enzymes had, in general, greater affinity for the mechanism-based inhibitors sulbactam, clavulanic acid and tazobactam. These results suggest two non-mutually exclusive hypotheses for the structural role of substitutions in this area of the enzyme. Either potential hydrogen-bond donors, such as serine and cysteine, interact directly with the β -lactam molecules, or the steric bulk of these substitutions distorts the β -pleated sheet such that the β -lactam is held in a position favourable for stable binding and catalysis. Finally, our data raise questions about a strategy relying on oligonucleotide-probe technology to detect such mutations, because of the variety of substitutions that give rise to similar phenotypes.

INTRODUCTION

The OHIO-1 β -lactamase shares 97% amino-acid-sequence similarity with SHV-1, a common enzyme among *Klebsiella pneumoniae* strains [1,2]. The OHIO-1 gene, although geographically restricted to Ohio, has been found on a variety of conjugative plasmids and on one non-conjugative plasmid, always linked to *aadB* (encoding resistance to gentamicin and other aminoglycosides) [3,4].

A number of reports of clinical isolates of Gram-negative bacilli bearing mutant SHV-1 enzymes (SHV-2–5) have recently appeared [5–8]. The mutant enzymes are characterized by an increased affinity for the new cephalosporin antibiotics and render their host bacteria more resistant to these agents. They are thought to have been selected during therapy with newer cephalosporin antibiotics. Other mutants have been described after site-directed mutagenesis using oligonucleotides [10]. The latter approach might be biased by preconceived ideas of the sites to be examined. The former approach depends on examining many clinical specimens. We undertook our studies to allow a detailed examination of the active site of SHV-1-family β -lactamases in an unbiased way. We also wanted to have some idea as to which β -lactams might be most or least likely to select resistance during therapy. In addition, a number of workers have suggested the use of specific oligonucleotide probes for the detection of mutant β -lactamases, where the oligonucleotide sequence would be determined by known sequence changes leading to changes in enzyme substrate specificity. However, if unexpected substitutions give rise to this phenotype, resistance to new cephalosporins, this approach might not be useful. Our study design would allow the detection of unexpected mutations. We chose OHIO-1 as a model because of its close relationship to SHV-1 and its location on a relatively low-copy-number non-conjugative plasmid with linked gentamicin resistance.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strains HB101 and HB101 (pDS075) were previously described [1,2].

Antibiotics

The antibiotics used for this study and their sources have been described previously [1].

Selection of mutants

A 100 ml portion of Luria–Bertani Broth supplemented with 1, 2, 4 or 8 times the minimum inhibitory concentration (MIC) of the β -lactam of interest was inoculated with 10 ml of an overnight culture of HB101 (pDS075). The culture was grown overnight with agitation at 37 °C. A 10 ml sample was inoculated into 100 ml of LB broth without antibiotic and again grown overnight. A 10 ml aliquot of this culture was reinoculated into 100 ml of LB broth supplemented with the same concentration of antibiotic as previously used. Samples were taken at each step and plated on LB agar with 0–8 times the MIC of the selecting β -lactam.

Susceptibility testing

The MICs of antibiotics for the HB101 (pDS075) parent and mutant derivatives were performed using antibiotics diluted serially through Mueller–Hinton agar supplemented with thiamin (0.001%). Clavulanic acid was used at 2 μ g/ml and was always freshly prepared.

Enzyme extraction and measurement of kinetic parameters

These methods have been described previously [1], except that, in the present study, inhibitor kinetics were measured against cephaloridine instead of nitrocefin. The β -lactamase inhibitors

Abbreviations used: LB, Luria–Bertani; MIC, minimum inhibitory concentration; PCMB, *p*-chloromercuribenzoate; IC₅₀, concentration causing 50% inhibition.

were always prepared within 72 h of use and were stored at -20°C .

Bacterial transformation

Plasmids were purified as previously described [1]. DNA was used to transform HB101 using electroporation as previously described [11]. Transformants were selected on LB agar containing gentamicin ($4\text{ }\mu\text{g/ml}$) and were screened for the mutant β -lactamase phenotype [resistance to the newer cephalosporins inhibited by clavulanic acid ($2\text{ }\mu\text{g/ml}$)] by the agar/dilution/MIC method described above.

DNA sequencing

The nucleotide sequence of mutant OHIO-1 genes was performed using plasmid DNA, purified as described above, as a template for double-strand sequencing [12]. A set of three 'custom-built' oligonucleotides spanning the gene were used as primers [1]. The deduced amino acid sequence is expressed using the consensus numbering system proposed by Ambler *et al.* [13].

RESULTS

Selection of mutants resistant to various cephalosporins

At least one cycle of enrichment was necessary to isolate mutants resistant to any of the β -lactams examined. Thus we estimate the frequency with which mutants existed in our cultures at the time of plating to be between 10^{-8} and 10^{-10} . We were able to select resistant mutants with cefotaxime (mutant TAX-1), ceftriaxone (mutants TRI-1 and TRI-3) and aztreonam (mutant AZT-5), but not with ceftazidime. The susceptibility of HB101 (pDS075) and its mutant derivatives is shown in Table 1. In all cases the increase in resistance is inhibited by the β -lactamase inhibitor clavulanic acid at a concentration of $2\text{ }\mu\text{g/ml}$. Mutants

Fig. 1. Nucleotide sequence (NS) and predicted amino acid sequence (AAS) of OHIO-1 and its spontaneous mutant derivative β -lactamases

The numbers above and below represent nucleotide and amino acid positions respectively. Note the deletion of position 239 in the (amino acid) consensus sequence numbering system [13].

were chosen for characterization on the basis of differences in susceptibility profiles or on selection by different antibiotics. Even though no mutant β -lactamase-bearing clones were isolated after selection with ceftazidime, all mutants, with the possible exception of TAX-1, had increased resistance to ceftazidime and aztreonam compared with the OHIO-1 parent.

DNA sequence of mutant OHIO-1 genes

Nucleotide sequencing of the mutant OHIO-1 genes from the four classes defined above revealed the changes shown in Fig. 1. The predicted Gly²³⁸ $\rightarrow$ Ser²³⁸ change has been previously reported to be responsible for substrate specificity alterations in SHV-2, a derivative of SHV-1 first found in a clinical isolate [5,14]. This change results in a higher level of resistance to cefotaxime and ceftriaxone than the other mutations. Our data suggests that all mutations led to similar levels of resistance to aztreonam and ceftazidime. The predicted Gly²³⁸ $\rightarrow$ Cys²³⁸, Gly²³⁸ $\rightarrow$ Val²³⁸ and Gly²⁴² $\rightarrow$ Cys²⁴² changes resulted in similar levels of resistance to the agents tested. These data already

Table 1. MICs of β -lactams for OHIO-1 mutants

Abbreviations used: APC, ampicillin; CLA, clavulanic acid ($2\text{ }\mu\text{g/ml}$); TAX, cefotaxime; TRI, ceftriaxone; TAZ, ceftazidime; AZT, aztreonam.

Enzyme	Substitution	MIC ($\mu\text{g/ml}$)									
		APC	APC+ CLA	TAX	TAX+ CLA	TRI	TRI+ CLA	TAZ	TAZ+ CLA	AZT	AZT+ CLA
OHIO-1	None	> 2048	16	0.126	0.060	0.125	0.060	0.500	0.250	0.125	0.125
TRI-1	Ser238	> 2048	8	16	< 0.015	8	0.125	8	0.500	4	0.125
TRI-3	Cys238	> 2048	64	2	0.060	2	0.060	8	1	2	0.250
AZT-5	Val238	> 2048	16	2	0.060	2	0.060	4	0.500	4	0.250
TAX-1	Cys242	> 2048	8	2	0.060	2	0.015	1	0.250	0.500	0.060

Table 2. Michaelis constants for β -lactamases

β -Lactam	Enzyme ... Substitution ...	Michaelis constant [K_m or K_i (μM)]				
		OHIO-1 None	TRI-1 Ser ²³⁸	TRI-3 Cys ²³⁸	AZT-5 Val ²³⁸	TAX-1 Cys ²⁴²
Penicillin		15	15	2	2	1
Piperacillin		44	15	16	5	14
Mezlocillin		75	12	100	19	61
Cephaloridine		348	22	62	148	63
Cefoperazone		76	12	41	23	41
Cefotaxime		> 1000	26	97	168	45
Aztreonam		> 1000	108	557	151	> 1000
Sulbactam		6.4	3.50	0.86	0.43	0.23
Tazobactam		0.11	0.62	0.88	0.80	0.27
Clavulanate		0.15	0.01	0.016	0.10	0.027

Table 3. Relative velocity of hydrolysis of β -lactams by OHIO-1 and derivative β -lactamases

Relative velocities are shown with that for penicillin set at 100. The values in parentheses are actual $V_{\max}$ values (nmol hydrolysed/min) rounded up to the nearest integer.

β -Lactam	Enzyme ... Substitution ...	Relative velocity (actual $V_{\max}$)				
		OHIO-1 None	TRI-1 Ser ²³⁸	TRI-3 Cys ²³⁸	AZT-5 Val ²³⁸	TAX-1 Cys ²⁴²
Penicillin		100 (11)	100 (25)	100 (21)	100 (9)	100 (12)
Piperacillin		12 (1)	4 (1)	3 (0.6)	11 (1)	3 (0.4)
Mezlocillin		25 (3)	12 (3)	20 (4)	36 (3)	18 (2)
Cephaloridine		385 (44)	36 (9)	49 (10)	448 (39)	113 (14)
Cefoperazone		34 (4)	16 (4)	49 (11)	56 (5)	15 (2)
Cefotaxime		< 0.1	12 (3)	13 (3)	44 (4)	12 (1)

Table 4. Relative catalytic efficiencies of OHIO-1 and derivative β -lactamases

Relative catalytic efficiencies are shown with that for penicillin set at 100. Values in parentheses in the first row are actual catalytic-efficiency values ($V_{\max}/K_m$) expressed in units of min^{-1} .

β -Lactam	Enzyme ... Substitution ...	Relative catalytic efficiency				
		OHIO-1 None	TRI-1 Ser ²³⁸	TRI-3 Cys ²³⁸	AZT-5 Val ²³⁸	TAX-1 Cys ²⁴²
Penicillin		100 (0.77)	100 (1.5)	100 (11.2)	100 (43)	100 (11.2)
Piperacillin		4.0	3.0	0.3	1.1	0.1
Mezlocillin		5.0	11.0	0.3	0.9	0.1
Cephaloridine		16.0	19.0	1.2	1.4	0.8
Cefoperazone		7.0	16.0	1.9	1.2	0.2
Cefotaxime		—	5.0	0.2	0.1	0.1

suggested that a cysteine substitution would be less efficient than a serine substitution at position 238.

Increase in resistance to cephalosporins is due to altered substrate specificity of the β -lactamase

The kinetic parameters of the β -lactamase in extracts from HB101 (pDS075) and its mutant derivatives is summarized in Tables 2–4. The structures of selected antibiotics are presented in Fig. 2. It is clear that, for all mutant enzymes, there has been an overall increase in the affinity of the enzyme for a variety of β -lactam substrates. This appears most marked for penicillin and clavulanic acid, but is also apparent for the newer cephalosporins and aztreonam, a monobactam. The Gly²³⁸ → Ser²³⁸ mutation resembles the parent enzyme in its affinity and hydrolysis rates for penicillin and sulbactam. The other mutants have an increased affinity for penicillin, but maintain a stable $V_{\max}$, as can be deduced from the data in Tables 2 and 4.

The kinetic data confirmed what was suspected on the basis of MIC testing. A cysteine residue at position 238 resulted in an enzyme with a higher K_m for cefotaxime and aztreonam than a serine residue at the same position. A valine substitution at the same position resulted in a higher K_m for cefotaxime, but the affinity for aztreonam was retained compared with the serine substitution. On the other hand, a change from a glycine to a cysteine residue at position 242 resulted in a loss of affinity for aztreonam compared with the serine or cysteine substitutions at position 238. This observation is consistent with the small to negligible increase in resistance to ceftazidime and aztreonam demonstrated by this mutant (Table 1).

In general, all mutants had lower K_m values (measured as apparent K_i) for the β -lactamase inhibitors than the parent enzyme, consistent with their overall increased affinities for β -

lactams in general. This was most striking for clavulanic acid. On the other hand, the Gly²³⁸ → Ser²³⁸ mutation resulted in little change in affinity for sulbactam, consistent with the observations of others [15], whereas the Gly²³⁸ → Cys²³⁸, Gly²³⁸ → Val²³⁸ and Gly²⁴² → Cys²⁴² mutations resulted in much higher affinities for sulbactam. The affinity of the mutant enzymes for tazobactam was lower compared with the parent enzyme, with the possible exception of the Gly → Cys change at position 242.

p-Chloromercuribenzoate inhibits the hydrolysis of cefotaxime by Cys-substituted enzymes

Cell extracts were incubated with 0, 10 and 100 μM -PCMB for 10 min before the addition of cephaloridine (100 μM). An approximate IC_{50} (concn. causing 50% inhibition) value was calculated from the percentage inhibition of cephaloridine hydrolysis. The data is shown in Table 5 and indicates that PCMB, a thiol-group blocking agent, is a potent inhibitor of the mutant enzymes with the Cys²³⁸ or Cys²⁴² substitutions when inhibition of cefotaxime is measured. Using the same method we also attempted to measure inhibition of cephaloridine hydrolysis, but found little or no inhibition of either OHIO-1 or any of the mutant enzymes. These observations are consistent with a direct interaction of the cysteine, for example, as a hydrogen-bond donor, with the oxyimino group, as a hydrogen-bond acceptor, which is present in cefotaxime but not cephaloridine. They do not necessarily rule out a steric bulk effect of cysteine.

DISCUSSION

A number of mutant SHV genes have been detected, by virtue of their phenotype (resistance to newer cephalosporins inhibited by β -lactamase inhibitors) as having arisen in bacteria colonizing

Fig. 2. Structures of some oxyimino-cephalosporins and aztreonam

(a) Cefotaxime; (b) ceftriaxone; (c) ceftazidime; (d) aztreonam.

Table 5. Inhibition of OHIO-1 β -lactamase by PCMB

For OHIO-1, inhibition of cephaloridine hydrolysis is shown. For the mutant enzymes, inhibition of cefotaxime hydrolysis is shown.

Enzyme	Substitution	IC ₅₀ (μ M)
OHIO-1	None	> 100
TRI-1	Ser ²³⁸	> 100
TRI-3	Cys ²³⁸	< 10
AZT-5	Val ²³⁸	> 100
TAX-1	Cys ²⁴²	28

or infecting patients. Of these, SHV-2 was the first such mutant described and is explained by a single amino acid substitution, Gly²³⁸ $\rightarrow$ Ser²³⁸ [5,14]. Arg²⁰² $\rightarrow$ Leu²⁰² or Lys²⁰² changes are not thought to be responsible for changes in the substrate specificities of SHV, but do change the isoelectric point of the enzymes SHV-3 and SHV-5 respectively [6–8]. SHV-4 and -5, in addition to the Ser²³⁸ substitution, have a Glu²⁴⁰ $\rightarrow$ Lys²⁴⁰ substitution, which is thought to mediate efficient hydrolysis of ceftazidime and aztreonam and results in high levels of resistance ($\geq 128 \mu\text{g/ml}$) to these agents [7,8]. This is thought to be a second mutation, only arising in the background of a previous Ser substitution at

position 238 [7,8]. Analogous results have been reported for a related β -lactamase, TEM [15].

The only prior information on the ability of the thiol group of cysteine to substitute for the hydroxy group of serine in these enzymes comes from site-directed mutagenesis of the active-site serine residue at position 70 of TEM. Here, cysteine can replace serine, although the resulting enzyme is less efficient [16]. The amino acid at position 70 performs a catalytic function, whereas those around positions 236–242 are probably involved in substrate binding. Thus a comparison of function for similar substitutions at these different sites may not be valid. Nevertheless, to our knowledge, the mutations described in the present study are the first reporting a role for cysteine in mediating binding of newer cephalosporins by substituting at positions 238 and 242 of these enzymes.

Our data suggest that a variety of amino acid substitutions at position 238 and a substitution at a site not previously reported to be involved in substrate binding, 242, can result in enzymes capable of mediating increased resistance to the new cephalosporins. In fact, since several of our mutants had similar phenotypes (Gly²³⁸ $\rightarrow$ Cys²³⁸, Val²³⁸), one might predict that there may be additional substitutions not yet identified that could yield this phenotype. This suggests that strategies to detect these mutations that rely, for example, on oligonucleotide probes [17], may underestimate the prevalence of such mutant enzymes by failing to detect unpredicted mutations.

In general terms, even though the mutant enzymes result in resistance to β -lactams normally active against strains producing the parent enzyme, the enzyme 'pays a price'. This is best seen by examining the Michaelis constants and the relative maximum velocities of hydrolysis of various substrates by the enzymes. In general, the mutant enzymes were less efficient than the parent, except for the newer cephalosporins, represented by cefotaxime in our data.

Hydrogen-bonding has been proposed as the mechanism by which substitutions at position 238 lead to binding and hydrolysis of oxyimino cephalosporins [18]. All the mutations we noted were the result of substitutions of amino acids capable of hydrogen-bonding with substrates, with the important exception of the valine at position 238. The β -pleated strand in which the 238 and 242 substitutions are occurring is thought to be involved in binding the R groups of β -lactams [19]. However, the amino acid at position 242 appears to point away from the substrate and to be farther removed than the amino acid at 238 [19]. The Val²³⁸ and Cys²⁴² substitutions raise the alternate hypothesis that it is actually steric bulk leading to distortion of this β -pleated strand that results in more stable binding of the oxyimino cephalosporins with a more favourable position related to the active-site residue, serine-70. This hypothesis might also be consistent with the improved binding of β -lactams without large R groups, such as sulbactam and the other inhibitors. Alternatively, both hypotheses may be correct. The data showing that PCMB strongly inhibits cefotaxime, but not cephaloridine, hydrolysis only by the enzymes with cysteine substitutions at positions 238 or 242 are intriguing. This observation might be more consistent with the hydrogen-bonding model, since distortion of the β -pleated sheet might be expected to effect the binding of all β -lactams, and PCMB might have been expected to inhibit hydrolysis of cephaloridine under these circumstances. The cysteine-for-glycine substitutions result in enzymes with a weak affinity for aztreonam. This might suggest either that the hydrogen-bonding properties of serine are more likely to yield stable interactions with aztreonam than those with the thiol group of cysteine or that differing strand-distortion effects explain these observations. Clearly, more data on the structure of the enzyme-substrate complex is required.

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