Effects of Asp-179 Mutations in TEM$_{pUC19}$ β-Lactamase on Susceptibility to β-Lactams

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To examine the effect of disruption of the salt bridge (between Arg-164 and Asp-179 [numbering of Ambler et al. (Biochem J. 267:269–272, 1991)]) that anchors the conserved Ω-loop in class A β-lactamases, we obtained mutant enzymes with each of the 19 other amino acid residues replacing Asp-179 in the TEM β-lactamase encoded by pUC19 and studied the level of resistance to various β-lactams conferred by each enzyme. All mutations of Asp-179 compromised the level of resistance to ampicillin, but most of them enhanced resistance to ceftazidime. In contrast, mutations of Asp-179 generally impaired the low levels of resistance to cefepime and aztreonam. One might expect to find clinical isolates with mutant TEM β-lactamases with replacements of Asp-179 that express an expanded spectrum of resistance to β-lactams including ceftazidime.

Novel extended-spectrum β-lactamases in enterobacteria have been found to arise by mutation from class A β-lactamases, such as TEM and SHV. Several of these new β-lactamases have contained multiple mutations, including replacement of the conserved arginine-164 by serine or histidine (3, 8, 11, 19). The presence of the Ser-164 or His-164 replacement is sufficient to produce clinically relevant levels of resistance to ceftazidime. (The numbering of residues in class A β-lactamases in this article is that of Ambler et al. [1].)

Crystallographic determination of the structures of several class A β-lactamases, including TEM-1, has shown that Arg-164 forms a salt bridge with the invariant Asp-179 to anchor the base of the Ω-loop, a conserved feature which consists of residues 164 to 179 (4, 6, 9, 10, 16). The mutations of Arg-164 that confer resistance to ceftazidime are expected to disrupt the salt bridge, and this disruption in turn may alter active-site topology by restructuring the conformation of the Ω-loop. The observed alteration of the activity profile of the enzyme (7, 15) might reflect such a structural change.

Mutation of Asp-179 would be expected similarly to interrupt the salt bridge to Arg-164. It should be noted that Herzberg et al. determined the crystallographic structure of an Asp-179→Asn mutant of another class A β-lactamase, the enzyme from *Staphylococcus aureus* PC1, and found indeed, in comparison with the crystallographic structure of the parental enzyme, that the salt bridge was disrupted and the Ω-loop was distorted (5). Although they reported that the activity of the mutant enzyme against penicillin G was compromised, they did not test any other possible β-lactam substrates, nor did they examine the effects of the mutation on resistance to various β-lactams. Therefore, we carried out site-directed mutagenesis of Asp-179 in the TEM β-lactamase from pUC19 (TEM$_{pUC19}$) to all other 19 amino acids and examined the consequences of each mutation on resistance conferred to various β-lactams, including ceftazidime.

(This work was presented in part at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fla., 1994 [18].)

Escherichia coli JM83 [ara Δ(*lac-proAB*) *rspL* φ*80lacZ*Δ M15] (20) was used as the host for all plasmids. Plasmid pUC19::kan was constructed by cloning the kanamycin resistance gene, *aphA1*, from Tn*903* (12) into the *Bam*HI site in the polylinker of pUC19 (20). This plasmid encodes the parental TEM pUC19 (TEM$_{pUC19}$) β-lactamase, which differs from the TEM-1 enzyme (17) by two mutations, Val-84→Ile and Ala-184→Val, which are considered to be enzymologically inconsequential (15).

Ampicillin and kanamycin were obtained from Sigma. Other antibiotics were gifts from the following: ceftazidime, Glaxo; cefotaxime, Hoechst-Roussel; cefepime and aztreonam, Bristol-Myers Squibb; imipenem, Merck and Co.

Susceptibilities to antibiotics were determined by microbroth dilution in Mueller-Hinton broth with inocula of 10^5 CFU/ml and 18-h incubation at 37°C. Each MIC is the result of at least five determinations.

Isolation of bacterial DNA, restriction endonuclease digestion, ligation, and transformation were performed as described elsewhere (13).

Mutagenesis of Asp-179 in the parental TEM$_{pUC19}$ β-lactamase was carried out with a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). Random site-directed mutagenesis employed a mutagenic oligonucleotide mixture with all four bases at each of the three positions corresponding to the codon for residue 179. After mutagenesis, *E. coli* JM83 was transformed with mutated DNA by electroporation, and transformants were selected by growth on Mueller-Hinton agar plates containing 30 μg of kanamycin per ml.

DNA for nucleotide sequencing was isolated by an alkaline method (2). The nucleotide sequence of the β-lactamase gene and its promoter region on each putative mutant plasmid was determined by the method of Sanger et al. (14) using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio) and α-^{35}S-dATP (DuPont NEN, Boston, Mass.).

Mutant β-lactamases were identified according to the sequence of the codon in the *bla* gene corresponding to residue 179 of the TEM$_{pUC19}$ β-lactamase. Following site-directed

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TABLE 1. Susceptibilities to β-lactams of *E. coli* JM83 alone and producing parental TEM$_{pUC19}$ β-lactamase or mutant enzymes with replacements of Asp-179

Amino acid 179	MIC (μg/ml)[a]			
	AMP	CAZ	CEP	AZTRE
None[b]	4	0.125	0.015	0.06
Asp	16,000	0.25	0.12	0.25
Asn	1,024	8.0	0.12	0.25
Cys	512	2.0	0.06	0.06
Gly	512	4.0	0.12	0.25
Glu	128	0.5	0.03	0.12
Ala	64	1.0	0.03	0.12
Ser	64	2.0	0.03	0.12
His	32	1.0	0.03	0.12
Gln	32	1.0	0.03	0.12
Trp	32	2.0	0.015	0.12
Phe	16	4.0	0.03	0.06
Tyr	16	4.0	0.03	0.12
Leu	16	1.0	0.03	0.06
Ile	16	0.5	0.03	0.06
Val	16	0.25	0.015	0.12
Met	16	4.0	0.06	0.12
Thr	16	0.25	0.015	0.12
Arg	8	0.5	0.03	0.12
Lys	8	0.25	0.015	0.06
Pro	8	0.5	0.06	0.06

[a] AMP, ampicillin; CAZ, ceftazidime; CEP, cefepime; AZTRE, aztreonam. All cefotaxime MICs except those for the Gly and Tyr mutants (0.06 μg/ml) were 0.03 μg/ml. All imipenem MICs were 0.25 μg/ml.
[b] *E. coli* JM83.

random mutagenesis, sequencing of DNA from 126 individual transformant colonies was required to obtain clones with 18 of the 19 possible amino acid substitutions at position 179. Only 25 clones had no mutation. The last mutant, with glutamine 179, was obtained by subsequent glutamine-specific site-directed mutagenesis. Mutants with one of each of the 19 amino acid replacements of Asp-179 were chosen for further study. The entire *bla* gene and its promoter region from each of these 19 mutants were sequenced to ensure that no other mutations were present. One mutant with an Asp-179→Arg replacement also had a single-base mutation leading to an Ala-11→Thr replacement in the leader peptide region, and another mutant with an Asp-179→Lys replacement had several other mutations in the region corresponding to residues 30 to 36. The extraneous mutations were removed from these two mutants by replacing the larger *Xmn*I-*Alw*NI fragment of the mutated plasmid, encoding the N terminus of the TEM$_{pUC19}$ β-lactamase through residue 65, with the equivalent fragment from an unmutated plasmid. The *bla* genes from the resulting recombinant plasmids were sequenced to confirm the presence of only the desired mutation, Arg-179 or Lys-179.

Table 1 presents the susceptibilities of the background strain, *E. coli* JM83, alone or bearing plasmids encoding the parental TEM$_{pUC19}$ β-lactamase or each of the 19 mutant enzymes resulting from replacement of Asp-179. We have listed the mutant strains in decreasing order of ampicillin MICs. Replacement of Asp-179 by any other amino acid residue dramatically compromised resistance to ampicillin from the MIC of 16,000 μg/ml conferred by the parental enzyme. Nonetheless, residual levels of resistance to ampicillin remained high (MICs, 512 to 1,024 μg/ml) for strains producing mutant enzymes with Asn, Cys, or Gly at position 179. The MICs of ampicillin conferred by the Glu-, Ala-, and Ser-179 mutant enzymes were in the 64- to 128-μg/ml range. The other

mutant enzymes conferred minimal levels of ampicillin resistance, especially when one considers the multicopy nature of the plasmid encoding the parental and mutant β-lactamases.

For ceftazidime, the parental enzyme conferred only a two-fold rise in MIC over the MIC for the plasmidless strain. In contrast to the deleterious effects of mutations of Asp-179 on ampicillin resistance, most of the mutations enhanced resistance to ceftazidime. In fact, only three replacements of Asp-179 (Lys, Thr, and Val) failed to elevate the MICs of ceftazidime at all. Furthermore, 12 of the 19 mutant enzymes raised the MIC of ceftazidime at least fourfold above that conferred by the parental plasmid, and 5 of them produced 16- to 32-fold increases, i.e., to the range of 4 to 8 μg/ml. One might therefore expect that ceftazidime resistance in a clinical enterobacterial isolate might be found to result from a mutation of Asp-179 in a TEM β-lactamase, alone or in combination with other mutations affecting this enzyme or outer membrane permeability. Clinical microbiology laboratories should be alert for strains of *E. coli* that exhibit low-level resistance to ceftazidime or other broad-spectrum cephalosporins, since such resistance may reflect novel β-lactamase mutations such as replacements of Asp-179.

Cefepime, which was developed as an alternative to ceftazidime against bacteria with class C β-lactamases, also showed an advantage over ceftazidime against these mutants of a class A β-lactamase. Cefepime was active not only against the strain bearing the parental TEM β-lactamase but also against all of the mutant strains. In fact, for all mutants but those with Asn-179 and Gly-179 the MIC of cefepime was lower than that conferred by the parental enzyme. As with cefepime, the minimal level of resistance to aztreonam conferred by the parental enzyme was compromised by most of the mutations of Asp-179, and no mutation enhanced the MIC of aztreonam over that conferred by the parental β-lactamase. The production of parental or mutant enzymes had no effect on susceptibility to either cefotaxime or imipenem.

In conclusion, we have shown that alteration of the highly conserved Asp-179 of the TEM$_{pUC19}$ β-lactamase markedly compromised the resistance to ampicillin which this enzyme confers. Similarly, the modest levels of resistance to cefepime and to aztreonam are also compromised by such mutations. In contrast, most of the substitutions of Asp-179 increase the level of resistance to ceftazidime.

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