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Effect of substitution of Asn for Arg-276 in the cefotaxime-hydrolyzing class A β-lactamase CTX-M-4

Maria Gazouli, Nicholas J. Legakis, Leonidas S. Tzouvelekis *

Laboratory of Antimicrobial Agents, Department of Microbiology, Medical School, University of Athens, Athens, Greece

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Abstract

The effect of substitution of asparagine for arginine at position 276 (Ambler's numbering) on the properties of the extended-spectrum β-lactamase CTX-M-4 was studied. Compared with CTX-M-4, the mutant β-lactamase CTX-M-4(R276N) conferred lower levels of resistance to cefotaxime, ceftriaxone and aztreonam while the levels of resistance to penicillins and penicillin-inhibitor combinations were similar. Arg-276→Asn substitution rendered CTX-M-4 slightly less susceptible to inhibition by clavulanate and tazobactam. It also caused a three-fold reduction in the relative rate of hydrolysis of cefotaxime. These results indicate that Arg-276 in CTX-M-type β-lactamases may be implicated in hydrolysis of oxyimino-β-lactams; they do not, however, support the hypothesis that Arg-276 is the functional equivalent of Arg-244 found in other class A β-lactamases.

Keywords: CTX-M-type β-lactamase; Arginine 276; Cefotaxime resistance

1. Introduction

Plasmid-mediated β-lactamases related to the class A chromosomal β-lactamase of *Klebsiella oxytoca* have been recently found in enterobacterial isolates. This cluster of β-lactamases (referred here as 'CTX-M-type') includes CTX-M-1/MEN-1 and CTX-M-2 [1], CTX-M-3 [2], CTX-M-4 [3], CTX-M-5 [4], Toho-1 [5] and Toho-2 [6]. CTX-M-type enzymes confer resistance to penicillins, classical cephalosporins and various oxyimino-β-lactams such as cefotaxime and aztreonam but are virtually inactive against ceftazidime. They are also susceptible to inhibition by clavulanate and tazobactam. Class A β-lactamases possess Glu-166 (Ambler's numbering) and the motifs ^{70}SXXK^{73}, ^{130}SDN^{132} and ^{234}KTG^{236} [7]. Position 276 is occupied by an arginine in all CTX-M-type enzymes.

Arg-276 may be equivalent to Arg-244 found in TEM, SHV and other class A β-lactamases [5,6,8]. Arg-244 is critical for β-lactam hydrolysis and inhibition by mechanism-based inactivators (reviewed in [8] and [9]). In this study we examined the role of Arg-276 by comparing the properties of CTX-M-4 with those of the R276N mutant which was constructed by site-directed mutagenesis.

* Corresponding author. Tel.: +30 (1) 7771139; Fax: +30 (1) 7709180; E-mail: lstbact@hotmail.com

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2. Materials and methods

2.1. Bacterial strains and plasmids

The *Escherichia coli* strains JM109 and ES1301 *mutS* (Promega, Madison, WI, USA) were used for cloning and mutagenesis. The latter strain suppresses mismatch repair in vivo. The *E. coli* strain C600 was used for the preparation of β-lactamase extracts. The plasmid pMSL1, which encoded the CTX-M-4 β-lactamase, was a derivative of pBCSK(+) (Stratagene, La Jolla, CA, USA) [3]. The plasmid pMSL2 was a derivative of pAlter-Ex2 (Promega) containing an intragenic *Pst*I-*Bam*HI fragment (0.9 kbp) which included the codon 276 of the $bla_{\mathrm{CTX-M-4}}$ gene [3].

2.2. Site-directed mutagenesis

The pAlter-Ex2 mutagenesis kit (Promega) was used for site-specific mutagenesis. The mutagenic primer (5′-AAGGCGGAAAGCCGTAATGATGT-TCTGGCT-3′) was 30 nucleotides long and contained three mismatches in the triplet corresponding to codon 276 (AAT [Asn] instead of CGG [Arg]). The primer was prepared in an Applied Biosystems (Foster City, CA, USA) DNA synthesizer. Mutagenesis was performed in plasmid pMSL2. A *Pst*I-*Bam*HI fragment (0.9 kbp), including the mutagenized codon, was introduced into *Pst*I-*Bam*HI digested pMSL1 resulting in plasmid pRN276 that encoded the CTX-M-4(R276N) mutant β-lactamase. The DNA sequence was determined by the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH, USA). The mutant gene was entirely sequenced to confirm the desired exchanges in the nucleotide sequence.

2.3. β-Lactam susceptibility testing

Susceptibility to β-lactams of *E. coli* JM109 clones producing CTX-M-4 or CTX-M-4(R276N) β-lactamases were determined by a microdilution method with Mueller-Hinton broth (Unipath, Basingstoke, UK) [10]. The initial screening for clones producing mutant β-lactamases was performed by the disk diffusion method.

2.4. Preparation of β-lactamase extracts

For the preparation of β-lactamase extracts, *E. coli* C600 cells transformed with pMSL1 or pRN276 were grown overnight in Tryptone-Soya broth (Unipath). β-Lactamases were released by sonication of cell suspensions. The extracts were clarified by ultracentrifugation, desalted with Econo-Pac 10DG columns (Bio-Rad Laboratories, Richmond, CA, USA) and concentrated by ultrafiltration with Centriprep-10 filters (Amicon, Witten, Germany). The protein contents were determined with a protein assay kit (Bio-Rad). β-Lactamase activities were quantitated with nitrocefin (Unipath) and expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein. Isoelectric focusing (IEF) was performed in polyacrylamide gels containing ampholytes covering a pH range from 3.5 to 9.5 (Pharmacia-LKB, Uppsala, Sweden). β-Lactamase bands were visualized with nitrocefin (Unipath).

2.5. β-Lactamase substrate and inhibition profiles

Hydrolysis of nitrocefin, penicillin G, cephalothin, and cefotaxime was monitored spectrophotometrically at 37°C in phosphate buffer (50 mM, pH 7), on a temperature-controlled double-beam spectrophotometer with a 1.0-cm pathlength cuvette [11]. The wavelengths of maximal absorption differences and extinction coeffi-cients were as described previously [12]. The maximum rates of hydrolysis (relative $V_{\max}$) were expressed as relative to that of nitrocefin which was set at 100. Inhibition profiles of β-lactamases were determined with clavulanate and tazobactam as described previously [13]. The reporter substrate was nitrocefin (100 μM). Before the addition of nitrocefin, the inhibitor was preincubated with the enzyme for 5 min at 37°C. The amount of each enzyme preparation was normalized to give 100 μM nitrocefin hydrolyzed per min. The 50% inhibitory concentrations (IC_{50}s) were determined from inhibitor concentration versus percent inhibition plots.

3. Results and discussion

Susceptibility to β-lactams for *E. coli*

JM109(pMSL1) and *E. coli* JM109(pRN276) is shown in Table 1. The differences in the MICs of penicillins and penicillin-inhibitor combinations were not substantial. Both strains were highly resistant to amoxycillin and ticarcillin. The MIC values of amoxycillin, piperacillin, ceftazidime-clavulanate and ticarcillin-clavulanate combination were one dilution higher in the strain that expressed the R276N mutant β-lactamase. The latter differences were consistent and reproducible. The MICs of the penicillin-inhibitor combinations amoxycillin-clavulanate, ampicillin-sulbactam and piperacillin-tazobactam were similar for the two strains (8, 32 and 2 mg l^{-1}, respectively), as were the MICs to ceftazidime (2 mg l^{-1}) and cefepime (16 mg l^{-1}). Replacement of Arg-276 by Asn caused more pronounced changes in the resistance levels to some oxyimino-β-lactams conferred by CTX-M-4. The MICs of cefotaxime, ceftriaxone and aztreonam were lower by three, two and four dilutions, respectively, while those of ceftazidime and cefepime remained unaffected. The similar reduction in the MICs of penicillins in the presence of inhibitors for the two strains indicated that the mutant β-lactamase retained the wild-type levels of susceptibility to inhibitors. Accordingly, the introduced mutation caused a marginal increase in susceptibility to β-lactamase inhibitors. The IC_{50} of clavulanate, from 1.0 μM, increased to 1.6 μM, and that for tazobactam increased from 0.1 to 0.2 μM.

As measured by nitrocefin hydrolysis the CTX-M-4 and its Arg-276→Asn mutant were produced by *E. coli* JM109 in quantities equal to 100–110 units of activity, indicating that the levels of expression of the enzymes were similar. In IEF experiments, it was observed that the exchange of the positively charged arginine with the neutral asparagine lowered the isoelectric point (p*I*) of the enzyme from 8.4 to 8.0 (Fig. 1).

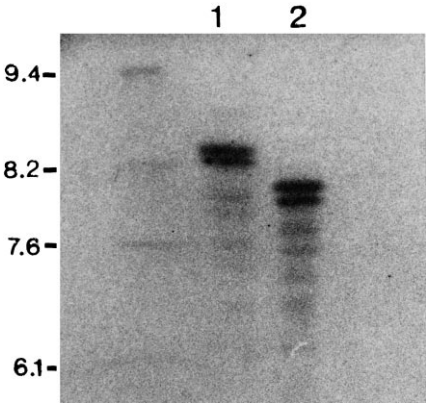

Fig. 1. Isoelectric focusing of CTX-M-4 and CTX-M-4(R276N) β-lactamases (lanes 1 and 2 respectively). Isoelectric points of standard β-lactamases are indicated in the left.

As shown in Table 2, the replacement of Arg-276 by Asn resulted in a three-fold reduction in the relative hydrolysis rate of cefotaxime. It also seemed that the introduced mutation caused a slight increase in the relative hydrolysis rate of penicillin G and cephalothin.

Molecular modeling studies suggested that the guanidium group of Arg-276 lies in a position equivalent to that of Arg-244 in TEM and SHV β-lactamases [8]. The results of the present study, however, do not indicate a similar function for these two res-

Table 1
MICs of β-lactams for *E. coli* JM109 clones producing CTX-M-4 wild-type and R276N mutant β-lactamases

Antibiotic	MIC (mg l^{-1}) for strain (enzyme):		
	E. coli JM109(pMSL1) (CTX-M-4)	*E. coli* JM109(pRN276) (CTX-M-4[R276N])	*E. coli* JM109
Amoxicillin	1024	2048	2
Ticarcillin	>2048	>2048	1
Ticarcillin-clavulanate[a]	16	32	1
Piperacillin	256	512	0.5
Ceftazidime-clavulanate[b]	0.25	0.5	0.18
Cefotaxime	512	64	0.06
Ceftriaxone	512	128	0.06
Aztreonam	32	2	0.06

[a]The inhibitor was at a fixed concentration of 2 mg l^{-1}.
[b]The inhibitor was at a fixed concentration of 4 mg l^{-1}.

idues. In CTX-M-4, replacement of Arg-276 by Asn, an amino acid with a shorter and uncharged side-chain, did not cause a significant reduction in susceptibility to mechanism-based inhibitors. In contrast, substitution of Ser, Cys, Thr or His for Arg-244 in TEM-1 β-lactamase rendered the enzyme highly resistant to clavulanate [14]. The guanidium group of Arg-244 activates a structurally conserved water molecule (W673) which is essential for inactivation by clavulanate [15]. The interaction of CTX-M-type enzymes with β-lactamase inhibitors may proceed differently and the activation of a water molecule located near the Arg-276 side chain may not be critical for inhibition, though these predictions will require further study.

The lower levels of resistance to oxyimino-β-lactams conferred by the CTX-M-4 (R276N) and the reduced relative hydrolysis rates of cefotaxime compared with the parental β-lactamase give support to the hypothesis that Arg-276 is important for the extension of the substrate specificity [5,6]. The attenuation of the catalytic efficiency observed in the mutant β-lactamase may be due to the inability of the side chain of Asn to attract the C_3 (C_4) β-lactam carboxylate and form with it a hydrogen bond as proposed for TEM-1 [16,17]. However, substitution of Asn for Arg-276 did not abolish the extended-spectrum capability of CTX-M-4. Again, it should be noted that replacement of the geometrically equivalent Arg-244 in TEM-1 [14], SHV-1 and SHV-5 [18] and OHIO-1 (R.A. Bonomo, personal communication) by a smaller and uncharged amino acid caused a much more drastic reduction of hydrolysis of penicillins and cephalosporins. We have also shown that Ser-237, which is present in all plasmid-mediated CTX-M-type β-lactamases, is involved in oxyimino-β-lactam hydrolysis in CTX-M-4 but is not solely responsible for the substrate specificity of the enzyme [3]. It is evident that the extended-spectrum properties of CTX-M-type β-lactamases should be considered as 'intrinsic' and not as the result of a few point mutations enabling some ancestral penicillinases to hydrolyze expanded-spectrum β-lactams. In conclusion Arg-276 in CTX-M-4 β-lactamase is implicated in the interaction with β-lactam substrates and β-lactamase inhibitors but its function may be different from that of Arg-244 found in other class A β-lactamases.

Table 2
Substrate profiles of CTX-M-4 β-lactamase and CTX-M-4(R276N) mutant enzyme

Substrate	CTX-M-4	CTX-M-4(R276N)
	Relative V_{max} ± S.D.	
Nitrocefin	100 ± 2	100 ± 2
Penicillin G	21.4 ± 4.2	36.2 ± 5.0
Cephalothin	12.9 ± 1.1	14.3 ± 1.8
Cefotaxime	9.6 ± 0.3	3.2 ± 0.4

Equal activities of enzymes (100 nmol of nitrocefin hydrolyzed per min) were used.

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