ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2003, p. 2958–2961 Vol. 47, No. 9
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.9.2958–2961.2003

NOTES

Effects of Ser130Gly and Asp240Lys Substitutions in Extended-Spectrum β-Lactamase CTX-M-9

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Received 17 April 2003/Returned for modification 23 May 2003/Accepted 4 June 2003

In CTX-M-9 extended-spectrum β-lactamases (ESBLs), an S130G mutation induced a 40- to 650-fold increase in 50% inhibitory concentrations but decreased hydrolytic activity against cefotaxime. A D240K mutation did not modify enzymatic efficiency against ceftazidime. Residue K240 could interact with Q270 and therefore not with ceftazidime, in contrast with what was observed with certain TEM/SHV-type ESBLs.

CTX-M extended-spectrum β-lactamases (ESBLs) are generally characterized by much greater hydrolytic activity against cefotaxime than against ceftazidime (2, 3). Three recently described enzymes, CTX-M-15 (1, 12), CTX-M-16 (6), and CTX-M-19 (19), have greater enzymatic efficiency against ceftazidime. CTX-M-15 and CTX-M-16 harbor an Asp→Gly substitution in position 240. Position 240 is known to play a major role in the activity against ceftazidime of certain TEM/SHV-type ESBLs (10, 13). Nevertheless, positively charged residues (Lys and Arg) are always observed in these ESBLs. Currently, CTX-M enzymes are susceptible to inhibitors of β-lactamases, whereas TEM/SHV-type enzymes have acquired a high level of resistance to inhibitors, notably through an S130G substitution (4, 16, 18, 20), which is directly implicated in the inhibition process (7, 8).

By analogy with SHV/TEM-type enzymes, we investigated in the present study whether CTX-M-9 enzyme could improve its activity against ceftazidime or acquire resistance to inhibitors by D240K and S130G substitutions, respectively.

The CTX-M-9-encoding plasmid pClRio-7 (6) was used to introduce the mutations into $bla_{\text{CTX-M-9}}$ by site-directed mutagenesis (14) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). An S130G mutant was constructed by using the primers M9S130G-A (5′-GCCGCGTTGCAGTACGGCGACAATACCGCCATG-3′) and M9S130G-B (5′-ATGGCGGTATTGTCGCCTACTGCAACGCGGC3′), whereas a D240K mutant was constructed by using the primers M9D240K-A (5′-GATAAGACCGGCAGCGGCAAGTACGGCACCACCAATG-3′) and M9D240K-B (5′-CATTGTGGTGCCGTACTTGCCGCTGCCGTCTTATC-3′) (the mutated codons are underlined). All the mutant genes were sequenced on both strands by the dideoxy chain termination procedure of Sanger et al. (21) as previously reported (5). The MICs of β-lactams alone or in combination with β-lactamase inhibitors were determined by a dilution method in agar as previously reported (6). Table 1 lists the MICs of β-lactams for *Escherichia coli* DH5α strains producing CTX-M-9 and the S130G and D240K mutants.

High levels of resistance were observed for amoxicillin, ticarcillin, and cephalothin (MICs, 64 to >2,048 μg/ml) for the three β-lactamases. Combination with clavulanate did not restore the activity of amoxicillin and ticarcillin for S130G mutant-producing *E. coli*, whereas activity was restored with the strain producing the D240K mutant and CTX-M-9 (MICs, 256 to 512 μg/ml versus 8 to 32 μg/ml). The MICs of cefotaxime (0.25 versus 8 μg/ml), ceftazidime (0.12 versus 1 μg/ml), and aztreonam (0.12 versus 2 to 4 μg/ml) were lower for S130G mutant-producing *E. coli* than for D240K mutant-producing and CTX-M-9-producing *E. coli*. Overall, the S130G mutant induced a resistance phenotype similar to that conferred by inhibitor-resistant or overexpressed TEM-type penicillinase (8), whereas the resistance phenotype conferred by the D240K mutant was similar to that of parental enzyme CTX-M-9.

The two mutant enzymes were extracted from recombinant *E. coli* DH5α by sonication and purified by ion exchange and

TABLE 1. β-Lactam MICs for *E. coli* DH5α producing CTX-M-9, the S130G mutant, and the D240K mutant

Substrate(s)	MIC (μg/ml) for *E. coli* DH5α producing:			
	S130G mutant	D240K mutant	CTX-M-9	No enzyme
Amoxicillin	>2,048	>2,048	>2,048	2
Amoxicillin + CLA[a]	256	8	8	2
Ticarcillin	>2,048	>2,048	>2,048	2
Ticarcillin + CLA[a]	512	32	16	2
Piperacillin	≤64	≤64	256	2
Piperacillin + TZB[b]	4	2	2	2
Cephalothin	64	256	512	4
Cefotaxime	0.25	8	8	0.06
Ceftazidime	0.12	1	1	0.12
Aztreonam	0.12	2	4	0.06

[a] Clavulanate at a fixed concentration of 2 μg/ml.
[b] Tazobactam at a fixed concentration of 4 μg/ml.

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TABLE 2. Inhibition profiles of S130G, D240K, and CTX-M-9 enzymes

Agent	IC_{50} (μM)		
	S130G mutant	D240K mutant	CTX-M-9 (6)
Clavulanate	18 ± 1	0.025 ± 0.002	0.036 ± 0.001
Tazobactam	4.5 ± 0.2	0.006 ± 0.001	0.007 ± 0.001
Sulbactam	135 ± 8	3.0 ± 0.5	3.5 ± 0.2

gel filtration as previously described (6). The kinetic constants of purified enzymes (purity rates ≥ 96%) were obtained by a computerized microacidimetric method as previously described (15). The concentrations of inhibitors required to inhibit enzyme activity by 50% (IC_{50}s) were determined as previously reported (5).

Inhibition profiles of S130G, D240K, and CTX-M-9 enzymes are shown in Table 2. Those of the latter two were similar, whereas IC_{50}s of the S130G mutant were 40-fold (sulbactam) to 650-fold (tazobactam) higher than those of CTX-M-9.

The kinetic constants of the two mutants and of the parental enzyme CTX-M-9 are listed in Table 3. Compared to CTX-M-9, the S130G mutant showed similar K_m values but greatly decreased hydrolytic activity (k_{cat}), particularly on cephalosporins (cephalothin, 3 versus 3,000 s^{-1}; cefotaxime, 0.5 versus 450 s^{-1}). S130G substitution therefore induced resistance to β-lactam inhibitors but altered the extended-spectrum activity, as previously observed in the SHV/TEM-type complex mutant CMT-3 (18, 20, 23).

k_{cat} values for the D240K mutant were weakly increased for penicillin G, cefotaxime, and ceftazidime and similar to those of CTX-M-9 for amoxicillin, ticarcillin, piperacillin and aztreonam. K_m values were weakly increased for all β-lactams. The enzymatic efficiency (k_{cat}/K_m) of the D240K mutant was only weakly reduced for cefotaxime (1.5 versus 4 s^{-1} μM^{-1}) and not significantly increased for ceftazidime (0.005 versus 0.003 s^{-1} μM^{-1}) in contrast with that observed with CTX-M-16 (a

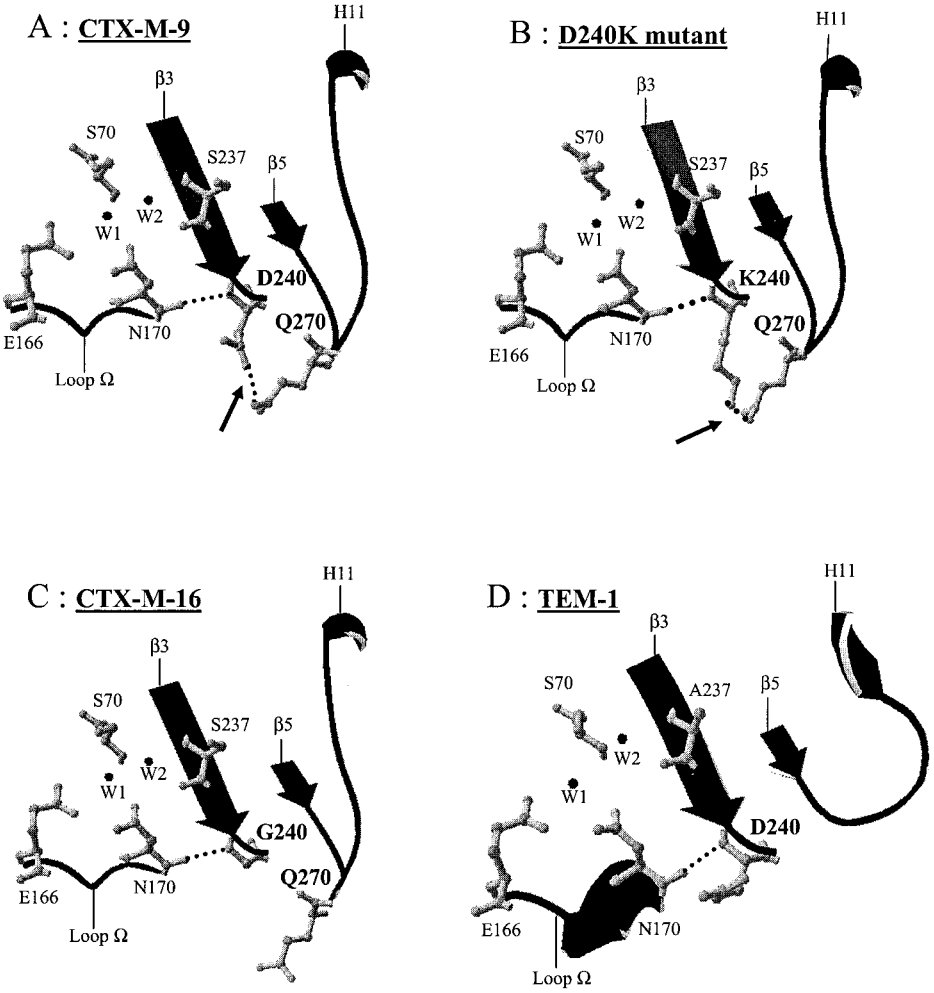

FIG. 1. Structures of CTX-M-9 (A), the D240K mutant (B), and CTX-M-16 (C) obtained by molecular modeling and the crystallographic structure of TEM-1 (D) (17). Two black dots indicate the positions of water molecules: W1, the catalytic water molecule maintained by the lateral chains of residues 166, 170, and 70, and W2, the water molecule of oxyanionic hole formed by the main chains of residues 70 and 237. The hydrogen bonds between residues 170, 240, and 270 are indicated by dashed lines.

TABLE 3. Kinetic constants of S130G, D240K, and CTX-M-9 enzymes

Agent	S130G mutant			D240K mutant			CTX-M-9 (6)		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Penicillin G	12 ± 0.2	15 ± 0.3	0.8	430 ± 5	136 ± 0.5	3	295 ± 3	25 ± 0.5	12
Amoxicillin	6 ± 0.1	29 ± 0.5	0.2	130 ± 2	62 ± 0.2	2	90 ± 1.5	20 ± 2.0	4.5
Ticarcillin	12 ± 0.2	60 ± 2	0.2	70 ± 2	12 ± 0.5	5.8	60 ± 2	35 ± 3.0	1.7
Piperacillin	12 ± 0.4	13 ± 1	0.9	160 ± 6	46 ± 3	3.5	110 ± 5	20 ± 1.0	5.5
Cephalothin	3 ± 0.1	147 ± 10	0.02	2,700 ± 15	325 ± 10	8.3	3,000 ± 135	150 ± 8.0	20
Cefotaxime	0.5 ± 0.05	154 ± 12	0.003	600 ± 11	370 ± 18	1.5	450 ± 22	120 ± 12	4
Ceftazidime	<0.01	ND[a]	ND	5 ± 0.2	765 ± 21	0.005	2 ± 0.3	600 ± 95	0.003
Aztreonam	<0.01	ND	ND	10 ± 0.2	436 ± 20	0.023	10 ± 2	220 ± 30	0.045

[a] ND, not determinable.

D240G mutant of CTX-M-9) (6) and K240-harboring SHV/TEM-type ESBLs (10, 13).

To investigate the role of residue 240, the structures of CTX-M-9, CTX-M-16, and the D240K mutant were modeled on the basis of the crystallographic structure of the CTX-M enzyme Toho-1 (11). The mutations were introduced as part of an automated procedure using Hyperchem v6.3 software (Hypercube Inc., Gainesville, Fla.). The residues, the catalytic water molecule, and the water molecules of the Toho-1 crystal were minimized using the Amber96 parameters (9) and a distance-dependent dielectric constant by conjugate gradient until the root mean square gradient was <0.1 kcal/mol. The major differences observed between the models obtained and TEM-1 crystallographic structure (17) are shown in Fig. 1.

In the CTX-M enzymes, the loop connecting the β5 strand and the H11 helix exhibited two additional residues and was oriented towards the C-terminal extremity of the β3 strand, unlike that in the TEM-type enzyme (Fig. 1D). In the CTX-M-9 and D240K models, residue Asn-270 established a hydrogen bond with D240 and K240 side chains, respectively (Fig. 1A and B), while this hydrogen bond is absent in the CTX-M-16 model and SHV/TEM-type enzymes (10, 13). In the crystal structure of Toho-1 acyl enzymes (22), the interaction between residues 270 and 240 is mediated by a water molecule. However, the calculated energy from a structure harboring a binding mediated by a water molecule was higher than that obtained from the model harboring a direct interaction of residues 270 and 240. This interaction may be involved in the positioning of β3-strand residues during the catalytic process and could therefore explain the similarities of kinetic constants between CTX-M-9 and D240K, as well as the differences observed with those of CTX-M-16 (6). Molecular modeling suggests that K240 of SHV/TEM-type ESBLs increases enzymatic efficiency against ceftazidime by establishing a hydrogen bond with ceftazidime (10, 13). In CTX-M, this interaction could be altered by the interaction between residues 240 and 270, the latter of which orients the K240 side chain away from the active site of the enzyme.

In conclusion, CTX-M-9 behaved like SHV/TEM-type β-lactamases with regard to an S130G substitution but not the substitution in position 240, probably because of a slight structural difference in the loop connecting the β5 strand and the H11 helix.

We thank Rolande Perroux, Marlene Jan, and Dominique Rubio for technical assistance.

This work was supported in part by a grant from the Ministère de l'Education Nationale de la Recherche et de la Technologie.

REFERENCES

1. **Baraniak, A., J. Fiett, W. Hryniewicz, P. Nordmann, and M. Gniadkowski.** 2002. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum β-lactamase (ESBL) in Poland. J. Antimicrob. Chemother. **50:**393–396.
2. **Barthélémy, M., J. Péduzzi, H. Bernard, C. Tancrède, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122:**15–22.
3. **Bauernfeind, A., J. M. Casellas, M. Goldberg, M. Holley, R. Jungwirth, P. Mangold, T. Rohnisch, S. Schweighart, and R. Wilhelm.** 1992. A new plasmidic cefotaximase from patients infected with *Salmonella typhimurium*. Infection **20:**158–163.
4. **Bermudes, H., F. Jude, E. B. Chaïbi, C. Arpin, C. Bebear, R. Labia, and C. Quentin.** 1999. Molecular characterization of TEM-59 (IRT-17), a novel inhibitor-resistant TEM-derived β-lactamase in a clinical isolate of *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **43:**1657–1661.
5. **Bonnet, R., J. L. M. Sampaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot.** 2000. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob. Agents Chemother. **44:**1936–1942.
6. **Bonnet, R., C. Dutour, J.-M. Sampaio, C. Chanal, D. Sirot, R. Labia, C. De Champs, and J. Sirot.** 2001. Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240→Gly. Antimicrob. Agents Chemother. **45:**2269–2275.
7. **Brown, R., R. Aplin, and C. Schofield.** 1996. Inhibition of TEM-2 beta-lactamase from *Escherichia coli* by clavulanic acid: observation of intermediates by electrospray ionization mass spectrometry. Biochemistry **35:**12421–12432.
8. **Chaïbi, E. B., D. Sirot, G. Paul, and R. Labia.** 1999. Inhibitor-resistant TEM β-lactamases: phenotypic, genetic and biochemical characteristics. J. Antimicrob. Chemother. **43:**447–458.
9. **Cornell, W., P. Cieplak, C. Bayly, I. Gould, K. Merz, D. Ferguson, D. Spellmeyer, T. Fox, J. Caldwell, and P. Kollman.** 1995. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. J. Am. Chem. Soc. **117:**5179–5197.
10. **Huletsky, A., J. Knox, and R. Levesque.** 1993. Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and three-dimensional modeling. J. Biol. Chem. **268:**3690–3697.
11. **Ibuka, A., A. Taguchi, M. Ishiguro, S. Fushinobu, Y. Ishii, S. Kamitori, K. Okuyama, K. Yamaguchi, M. Konno, and H. Matsuzawa.** 1999. Crystal structure of the E166A mutant of extended-spectrum β-lactamase Toho-1 at 1.8 A resolution. J. Mol. Biol. **285:**2079–2087.
12. **Karim, A., L. Poirel, S. Nagarajan, and P. Nordmann.** 2001. Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. FEMS Microbiol. Lett. **201:**237–241.
13. **Knox. J. R.** 1995. Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
14. **Künkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic and selection. Proc. Natl. Acad. Sci. USA **82:**488.
15. **Labia, R., J. Andrillon, and F. Le Goffic.** 1973. Computerized microacidimetric determination of β-lactamase Michaelis-Menten constants. FEBS Lett. **33:**42–44.
16. **Leflon-Guibout, V., V. Speldooren, B. Heym, and M.-H. Nicolas-Chanoine.** 2000. Epidemiological survey of amoxicillin-clavulanate resistance and corresponding molecular mechanisms in *Escherichia coli* isolates in France: new

genetic features of *bla*$_{TEM}$ genes. Antimicrob. Agents Chemother. **44:**2709–2714.
17. **Minasov, G., X. Wang, and B. Shoichet.** 2002. An ultrahigh resolution structure of TEM-1 beta-lactamase suggests a role for Glu166 as the general base in acylation. J. Am. Chem. Soc. **124:**5333–5340.
18. **Neuwirth, C., S. Madec, E. Siebor, A. Pechinot, J.-M. Duez, M. Pruneaux, M. Fouchereau-Peron, A. Kazmierczak, and R. Labia.** 2001. TEM-89 β-lactamase produced by a *Proteus mirabilis* clinical isolate: new complex mutant (CMT 3) with mutations in both TEM-59 (IRT-17) and TEM-3. Antimicrob. Agents Chemother. **45:**3591–3594.
19. **Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen, and P. Nordmann.** 2001. CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45:**3355–3361.
20. **Prinarakis, E. E., V. Miriagou, E. Tzelepi, M. Gazouli, and L. S. Tzouvelekis.** 1997. Emergence of an inhibitor-resistant b-lactamase (SVH-10) derived from SHV-5 variant. Antimicrob. Agents Chemother. **41:**838–840.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
22. **Shimamura, T., A. Ibuka, S. Fushinobu, T. Wakagi, M. Ishiguro, Y. Ishii, and H. Matsuzawa.** 2002. Acyl-intermediate structures of the extended-spectrum class A beta-lactamase, Toho-1, in complex with cefotaxime, cephalothin and benzylpenicillin. J. Biol. Chem. **277:**46601–46608.
23. **Sirot, D., R. Labia, P. Pouedras, C. Chanal-Claris, C. Cerceau, and J. Sirot.** 1998. Inhibitor-resistant oxy-2-derived β-lactamase produced by *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **42:**2184–2187.