CONVERGENT SELECTION OF CEFTAZIDIME RESISTANCE MUTATIONS AT POSITION 167 OF CTX-M-3 β-LACTAMASE IN HYPERMUTABLE *E. COLI* STRAINS

P1238

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INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) of the CTX-M family typically confer higher levels of resistance to cefotaxime (CTX) than to ceftazidime (CAZ) in bacterial strains-producers. Nevertheless, several CTX-M variants have been described (e.g. CTX-M-19, -23 and -35) that contain mutations at position 167 and confer predominant resistance to CAZ [5, 7]. It has been also demonstrated by Welsh et al. using in vitro mutagenesis of CTX-M-2 that the Pro→Ser mutation at position 167 is particularly important for resistance to CAZ [8]. Recently, we identified another CTX-M β-lactamase, CTX-M-42, differing from CTX-M-3 by a single Pro_{167}→Thr amino-acid substitution, and demonstrated that the above mentioned mutation was acquired in vivo in a hypermutable *E. coli* strain [6].

The role of hypermutability in accelerated development of CAZ resistance has been previously studied in experiments on TEM β-lactamase-producing strains [1, 2]. In this study, we used an in vitro selection with CAZ to simulate the evolution of CTX-M-3 in the mutator *E. coli* hosts and to characterize mutations increasing CAZ resistance.

MATERIALS AND METHODS

Hypermutable (mutator) strains. The selection experiments were conducted with both the clinical *E. coli* mutator strain Irk1224 producing the CTX-M-3 [6] and the laboratory mutator strain GM2995 (*mutD5*). The former strain was identified as a possible progenitor of the CTX-M-42-producing strain [6]. The latter strain lacks the proofreading activity of DNA polymerase III (as a result of mutation in the *dnaQ* gene) and thus confers up to 100000-fold proportional increase in the rates of all common point mutations [3].

Amplification and cloning of $bla_{CTX-M-3}$ gene. A DNA fragment containing the entire $bla_{CTX-M-3}$ gene and the upstream part of IS*Ecp1* element including the putative promoter region was amplified from Irk1224 and cloned in the pCC1 vector as previously described [6]. The resulting recombinant plasmid was introduced into the GM2995 by transformation.

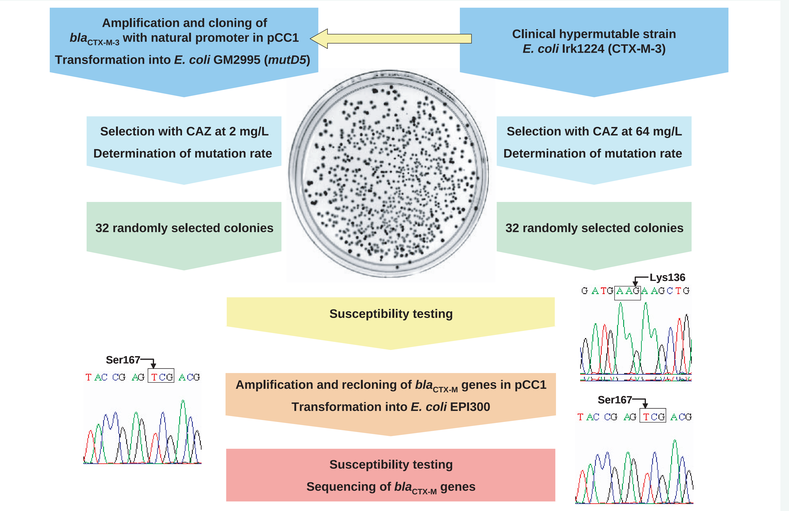
Mutagenesis and selection. Both the Irk1224 and GM2995 carrying the blaCTX-M-3 were subcultured in MH-broth for 14-16 h at 37°C and plated on agar containing CAZ at concentrations of 2x the MICs (64 mg/L for the Irk1224 and 2mg/L for the GM2995). The mutation rates were then determined as described elsewhere [4]. Thirty two colonies of each strain grown on selective plates were randomly selected for determination of susceptibilities to ampicillin (AMP), CAZ and CTX and characterization of mutations in the bla_{CTX-M} genes.

Characterization of mutations in bla_{CTX-M} genes. In order to avoid accumulation of excessive mutations and to distinguish the effects of mutations related or unrelated to the β-lactamase on resistance, the bla_{CTX-M} genes were reamplified from all selected clones and recloned in the pCC1 vector in *E. coli* EPI300 (Epicentre, Madison, WI). The bla_{CTX-M} genes and their promoters were then sequenced from plasmids using an ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Stafford, TX).

Susceptibility testing. The MICs of AMP, CAZ and CTX were determined for the selected mutator clones and *E. coli* EPI300 strains carrying the bla_{CTX-M} genes from these clones using an agar dilution method according to the CLSI (2005) guidelines.

RESULTS AND DISCUSSION

The general scheme of the mutagenesis and selection experiments conducted with the CTX-M-3-producing hypermutable strains is shown in the figure.



The total rates of mutations increasing CAZ resistance above 2x the MICs were 2E-8 and 2E-6 for the Irk1224 and GM2995, respectively.

Both strains yielded several types of mutants. As shown in Table 1, the majority of CAZ-resistant mutants (Mut. Type 1) had MICs of CTX equal or higher than those of CAZ. The bla_{CTX-M} genes from these mutants contained no changes relative to $bla_{CTX-M-3}$ and conferred the same levels of resistance to β-lactams when cloned and expressed in *E. coli* EPI300. Thus, the increased resistance of the 1st type mutants to CAZ was not related to the β-lactamase and could have resulted from alterations in outer membrane permeability. The selection of such mutants was previously reported by Elington et al. in experiments with TEM-type ESBL [2].

Another group of mutants was characterized by MICs of CAZ higher than those of CTX. Out of this group, 1 and 8 clones derived, respectively, from Irk1224 and GM2995 contained a single Pro167 → Ser substitution in the CTX-M. This mutation is found in the naturally occurring enzymes of the CTX-M-2 and CTX-M-9 clusters (CTX-M-35 and CTX-M-19) and, as shown by Welsh et al., can be readily selected with CAZ following in vitro mutagenesis of CTX-M-2 [8]. In addition, a Pro→ Thr substitution in the same position is observed in CTX-M-42 [6] and CTX-M-23 [7] of the CTX-M-1 cluster.

One mutant of Irk1224 carried a previously unknown substitution, Asn136 → Lys. Similar to Ser167, this mutation increased the MIC of CAZ but reduced that of CTX and, especially, of AMP, which may explain the absence of this substitution in the CTX-Ms from clinical isolates.

None of the selected mutants contained mutations in the promoter region of bla_{CTX-M} gene.

Table 1. Susceptibilities of mutator strains, their derivatives, and recombinant clones producing the WT and mutant CTX-M β-lactamases.

Strains and selected mutants	β-lactamase (mutation)	n*	MICs, mg/L					
			Mutator			*E.coli* EPI300**		
			AMP	CAZ	CTX	AMP	CAZ	CTX
Irk1224	CTX-M-3 (WT)	30	≥256	32	≥256	≥256	1	8
Irk1224 Mut.Type 1	CTX-M-3 (WT)	1	≥256	≥256	≥256	≥256	1	8
Irk1224 Mut.Type 2	CTX-M-3 (Ser167)	1	≥256	≥256	64	128	16	1
Irk1224 Mut.Type 3	CTX-M-3 (Lys136)		≥256	≥256	64	32	8	1
GM2995	CTX-M-3 (WT)		≥256	1	4	>256	1	8
GM2995 Mut.Type 1	CTX-M-3 (WT)	24	≥256	32	≥256	≥256	1	8
GM2995 Mut.Type 2	CTX-M-3 (Ser167)	8	128	32	1	128	16	1

* Number of selected mutants of each type.
** Susceptibilities of *E. coli* EPI300 strains carrying the blaCTX-M genes from respective mutants.

CONCLUSION

In this study we were able to reproduce the natural evolution of CTX-M β-lactamases by selecting CAZ resistance mutations at position 167 of CTX-M-3 in the hypermutable hosts.

We have also demonstrated the potentially important role of mutator strains in the acquisition of CAZ resistance.

REFERENCES
1. Ellington M.J., et al. Poster 2391. 44th ICAAC, 2004, Washington, DC.
2. Ellington M.J., et al. Poster 474. 15th ECCMID, 2005, Copenhagen, Denmark.
3. Fowler R.G., et al. Mol Gen Genet. 1974;133(3):179-91.
4. Galan J.C., et al. J Clin Microbiol. 2004;42(9): 4310-12
5. Kimura S., et al. Antimicrob Agents Chemother. 2003;48(5):1454-60
6. Stepanova M., et al. Poster C1-60/103. 45th ICAAC, 2005, Washington, DC.
7. Sturenburg E., et al. JAntimicrobial Chemotherapy . 2004;54: 406-9.
8. Welsh K.J., et al. Antimicrob Agents Chemother. 2005;49(3):1242-44