ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1998, p. 1042–1044 Vol. 42, No. 5
0066-4804/98/$04.00+0

A237T as a Modulating Mutation in Naturally Occurring Extended-Spectrum TEM-Type β-Lactamases

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Received 25 August 1997/Returned for modification 17 December 1997/Accepted 3 March 1998

A TEM-1 β-lactamase derivative containing the single amino acid substitution A237T slightly increased (from 24 to 32 μg/ml) the cephalothin MIC for *Escherichia coli* RYC1000 but did not influence the activities of cefotaxime, ceftazidime, and aztreonam (MICs of 0.03, 0.12, and 0.06 μg/ml, respectively). Despite its apparent neutrality, addition of the A237T mutation to the pair of mutations characterizing TEM-10 (R164S and E240K) had a strong effect on substrate preference. Ceftazidime and aztreonam MICs decreased from 128 and 16 μg/ml to 16 and 2 μg/ml, respectively. In contrast, the cefotaxime MIC increased from 0.5 to 4 μg/ml. The acquisition of apparently neutral or even deleterious mutations results in a very effective mechanism of resistance to different β-lactams that may be simultaneously or subsequently present in the environment. We propose here that the mutation in position 237 is an example of a modulating mutation and that consideration of this type of mutation may be important for understanding the evolution of β-lactamases.

The evolution and spread of β-lactamases in enteric bacteria seem to be the mirror consequences of the evolution and consumption of β-lactam antibiotics. New variant TEM-β-lactamase molecules have evolved under the pressure of new expanded-spectrum cephalosporins and monobactams. Nevertheless, the use of the new β-lactam agents has not been followed by a substantial drop in the use of the old ones, and the result is a net diversification of the selective network. This situation may create a complicated adaptive problem for enteric bacteria. In fact, an efficient mutation of a β-lactamase leading to an improved hydrolysis rate of a new type of β-lactam may lack efficiency against the old antibiotic substrates. Although an ever-increasing number of amino acid substitutions are being described, most naturally occurring extended-spectrum TEM derivatives are the result of amino acid replacements in one of seven positions. These amino acid replacements, numbered according to Ambler (1), are as follows: Q39K, E104K, R164S or R164H, A237T, G238S, E240K, and T265M (10). Combinations of these amino acid substitutions have also been reported (4, 5, 7, 10). The A237T substitution was early found to be responsible for a decrease in the TEM enzymes' preference for penams (benzyl-penicillin and ampicillin) versus cephems (cephalosporin and cephalothin [CE]) (9). A deeper analysis of a broader range of substrates shows that this cephem versus penem preference is not generally applicable to every member of these groups of β-lactams. In fact, no differences were found when the cephem versus penam preferences of TEM-1 and a TEM derivative containing the A237T substitution were compared for the cephems cephaloridine (CER), cefotaxime (CTX), and ceftazidime (CAZ) (3). On the other hand, this apparent neutrality of A237T may be questioned by comparing the susceptibilities of *Escherichia coli* derivatives, obtained by directed mutagenesis, containing TEM-10 (with R164S and E240K) with those containing TEM-5 (harboring R164S and E240K plus A237T). The presence of A237T in this case was associated with significant alterations in the susceptibilities to CE, CTX, CAZ, and aztreonam (ATM). In this paper, the potential relevance of this substitution to the activities and evolution of the TEM enzymes was studied. For such a purpose, all possible combinations of the R164S, E240K, and A237T mutations were constructed and the resulting substrate specificities were evaluated.

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MATERIALS AND METHODS

***E. coli* K-12 strains and plasmids.** The bacterial strains used in this work were *E. coli* RYC1000 (*araD139 ΔlacU169 rpsL Δrib7 thiA gyrA recA56*) and MC4100 [F⁻, *araD139* Δ(*argF-lacU169*) *rpsL flbB5301 fruA25 relA1 rbsR*] (6). An MC4100 nalidixic acid-resistant derivative (MC4100-Nx) was obtained by inoculating the wild-type strain onto a plate containing this antibiotic at a concentration of 40 μg/ml. Plasmid pBGTEM-1 was constructed by cloning an *Eco*RI-*Sal*I fragment from the hybrid phage M13mpΩAp (3) into the plasmid pBGS19⁻ (18), which had been digested with the same restriction enzymes. Plasmids containing the mutant derivatives were named by adding the number of the β-lactamase (if described) or the amino acid change (depending on the case) to the prefix pBGTEM.

Antibiotic susceptibility testing. Agar dilution assays were performed and interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (12). Standard antibiotic powders were kindly provided by pharmaceutical companies as follows: amoxicillin (AM), SmithKline Beecham Laboratories; CER and CE, Eli Lilly and Co.; CAZ, Glaxo-Wellcome; CTX, Hoechst Roussel Pharmaceuticals; ATM, Bristol-Myers Squibb.

Recombinant DNA techniques. Standard recombinant techniques were performed as previously described (14). Nucleotide sequencing was carried out by the dideoxynucleotide chain termination method (15) with Sequenase (U.S. Biochemicals) and bla_{TEM-1} specific primers.

Construction of mutants. Construction of the three single mutants, by site-directed mutagenesis on M13mpΩAp (11), was performed as previously described (3).

(i) TEM-10. The gene encoding TEM-10 with the R164S and E240K amino acid substitutions was constructed by replacing the *Pst*I fragment of pBGTEM-K240 with its homolog from pBGTEM-12. The resulting hybrid plasmid was designated pBGTEM-10, and the pI of the TEM-10 enzyme was 5.6.

(ii) R164S-A237T. The gene encoding a TEM variant with the R164S and A237T changes was constructed by replacing the *Pst*I fragment of pBGTEM-T237 with that from pBGTEM-12. The pI of the TEM-S164-T237 enzyme was 5.2.

(iii) A237T-E240K. The gene encoding a TEM variant with the A237T and E240K changes was constructed by replacing the *Pst*I fragment of pBGTEM-5 (see below) with that from pBGTEM-1. The pI of the TEM-T237-K240 enzyme was 5.9.

(iv) TEM-5. The TEM-5 derivative contains the changes R164S, A237T, and E240K. The gene encoding TEM-5 was constructed by replacing the *Sca*I-*Eco*RI fragment of pBGTEM-12 (containing the 5′ region of the *bla* gene) with that of plasmid pAT268 (16), which contains the two additional mutations A237T and E240K. The pI of the TEM-5 enzyme was 5.6.

In all cases the gene for each mutant derivative was completely sequenced (both strands) to verify that it had only the desired mutation(s).

TABLE 1. Microbiological activities of β-lactam antibiotics with strain RYC1000 producing β-lactamases

β-lactamase (TEM type)	MIC (μg/ml)					
	AM	CER	CTX	CAZ	ATM	CE
No TEM[a]	≤32	4	0.03	≤0.06	≤0.015	2
TEM-1	>2,048	32	0.03	0.12	0.06	32
S164 (TEM-12)	>2,048	16	0.12	4	0.25	8
T237	2,048	32	0.03	0.12	0.06	32
K240	>2,048	64	0.03	0.5	0.25	32
S164-T237	1,024	16	0.25	1	0.12	32
S164-K240 (TEM-10)	>2,048	16	0.5	128	16	16
T237-K240	2,048	64	0.06	0.25	0.06	128
S164-T237-K240 (TEM-5)	1,024	32	4	16	2	128

[a] Strain RYC1000 containing plasmid pBGS19⁻.

Competition experiments. A nalidixic acid-resistant mutant of *E. coli* MC4100 (MC4100-Nx) was obtained. To ensure the neutrality of the nalidixic acid resistance marker, mixed cultures were prepared with identical inocula of each of the two isogenic strains containing the wild-type TEM-1 enzyme and the A237T derivative, i.e., MC4100 (pBGTEM-1) plus MC4100-Nx (pBGTEM-T237) and MC4100-Nx (pBGTEM-1) plus MC4100-Nx (pBGTEM-T237). These mixtures were submitted to selective pressure for 4 h in tubes with different concentrations of either CAZ or CTX (0.008, 0.016, 0.032, 0.064, 0.12, 0.25, 0.5, and 1 μg/ml). Cultures without antibiotic were used as controls. After challenge, aliquots were treated with β-lactamase (*Enterobacter cloacae* type IV; Sigma Co., St. Louis, Mo.) for 20 min to prevent carry-over and were then inoculated onto drug-free broth. After overnight incubation, dilutions were plated onto agar plates containing kanamycin at a concentration of 40 μg/ml. The final proportion of each one of these strains was studied by streaking 100 colonies onto agar plates containing nalidixic acid (40 μg/ml).

Isoelectric focusing. Analytical isoelectric focusing was performed in precast polyacrylamide gels (pH 4.0 to 6.5) by using a PhastSystem apparatus (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. β-lactamase activity was identified by the hydrolysis of the chromophore β-lactam nitrocefin (Oxoid, Basingstoke, United Kingdom).

RESULTS

Phenotypic characterization of strain harboring the mutant TEM-T237. The MICs of several β-lactam antibiotics against the RYC1000 strain containing either the wild-type β-lactamase TEM-1 or its variant TEM-T237 were determined by the agar dilution method as previously described (12). As shown in Table 1, with the exception of AM, no difference was found in the activity of any of the antibiotics against the strains containing either of the enzymes. Although this double-dilution method of MIC determination is the one most commonly used in clinical microbiology laboratories, very small differences in susceptibility can be overlooked. We therefore determined the MICs of CE, CTX, CAZ, and ATM by using Epsilon test strips, an agar diffusion-based procedure which enables discrimination of smaller differences than is possible with the classical MIC determination methods (2). Again, MICs for the two strains showed no differences for any of the β-lactams tested, except for CE. The strain containing TEM-T237 consistently showed a slightly increased CE MIC compared with that containing TEM-1 (32 versus 24 μg/ml). This result in part confirmed the data previously reported for this mutation (9). Such a small difference in MICs may be overlooked by conventional MIC testing based on doubling dilutions. When the mutations R164S and E240K were also present, the A237T mutation significantly increased the MIC of CTX (from 0.5 to 4 μg/ml), while decreasing to the same degree the activity against CAZ (from 128 to 16 μg/ml) (Table 1).

To better document the putative neutrality of the change A237T for CAZ and CTX in the absence of other mutations, we used the method of competitive selection in mixed strains MC4100 and MC4100-Nx, harboring either TEM-1 or the A237T variant derivative. Irrespective of the host strain used, the proportion of the A237T variant derivative was not significantly altered with respect to that for the control strain after challenge with different CAZ or CTX concentrations, thus showing that the A237T change has a neutral phenotype in the absence of other mutations.

Characterization of TEM variants constructed by directed mutagenenesis. To determine the exact contribution of the A237T mutation to the TEM activity in the presence of the R164S and/or E240K mutation, we constructed by directed mutagenesis all possible combined derivatives and studied the corresponding phenotypes.

Table 1 shows the susceptibilities of each constructed mutant in the strain RYC1000 to AM, CER, CE, CTX, CAZ, and ATM as determined by agar dilution assay.

(i) Single mutations. As previously described (3, 16, 17), the change R164S was responsible for a dramatic increase in the CAZ MIC, with slight increases in the ATM and CTX MICs. An increase in susceptibility to CE was also observed. E240K, in accordance with previous communications (3, 13), slightly increased the CAZ and ATM MICs, with a very modest increase in the CER MIC. As was mentioned above, the change A237T did not produce changes in resistance to the tested β-lactams, except for a decrease in the AM MIC and a slightly decreased susceptibility to CE.

(ii) Double mutations. The double combination causing the greatest effect was found in TEM-10, which contained the changes R164S and E240K. This combination produced very dramatic increases in the MICs of CAZ and ATM, a smaller but significant decrease in CTX susceptibility, and slight decreases in the MICs of CER and CE. The addition of the A237T mutation to the R164S or the E240K mutation modestly decreased susceptibility to CTX and CE and decreased the MICs of CAZ and ATM.

(iii) Triple mutation (TEM-5). The presence of the A237T mutation in addition to the other two mutations (R164S and E240K) changed the pattern of susceptibility to the tested β-lactams with respect to that of TEM-10. TEM-10 conferred high CE, CAZ, and ATM MICs (16, 128, and 16 μg/ml, respectively) and low-level resistance to CTX (MIC, 0.5 μg/ml). The addition of mutation A237T (resulting in TEM-5) increased the MICs of CTX and CE to 4 and 128 μg/ml, respectively, but decreased the MICs of CAZ and ATM to 16 and 2 μg/ml, respectively.

Preliminary kinetic results with CAZ and CTX, with crude cell extracts obtained from strains containing TEM-10 and TEM-5, are in agreement with the results of these susceptibility studies (data not shown).

DISCUSSION

In the TEM enzymes, A237 is a substrate-binding residue that acts by forming a hydrogen bond between the substrate and the protein backbone (19). Binding of specific β-lactam antibiotics will be influenced differently by the A237T mutation because these antibiotics are differently constrained in the binding site. The rotation of the side chain of the threonine residue provides another hydrogen bond, which may facilitate the interaction of CTX with the binding site. This type of situation has also been observed in a class A β-lactamase of *Proteus vulgaris*, where the reverse type of substitution, S237A, produces a decrease in activity on oxyimino-cephalosporins (20). It was previously reported that the A237T replacement increases the cephem versus penam preference of TEM enzyme (9). Our results confirm this result for CER and AM but not for CTX. In the present study, the simultaneous presence

of the R164S and E240K mutations, in addition to that of the A237T mutation, was required to significantly increase the CTX hydrolysis rate.

In order to adapt to environmental fluctuations in antibiotic challenges the plasticity of the TEM binding site allows the substrate binding to be fine tuned by small changes. The possibility that the activity against various β-lactams could be modulated could provide a selective advantage to bacterial cells harboring extended-spectrum TEM-type β-lactamases. By means of cross-infection in closed habitats (typically in intensive care units) the same bacterial organism may settle in different patients who were being treated with different β-lactams. Because of sequential bacterial infections in immunosuppressed patients, treatment with different β-lactams, perhaps changing from CTX or CE to CAZ or ATM, may create a fluctuating selective environment.

The data presented here are consistent with the concept of mutation saturation in enzymatic evolution proposed by Hartl et al. (8). A neutral or nearly neutral mutation in a defined environment can be selected, and fixed, in a different environment in which this mutation confers a selective advantage. The case of the A237T change contained in TEM derivatives extends the hypothesis beyond two defined environments (for which the mutation is neutral or favorable) to an undefined number of fluctuating ones. TEM-10, the naturally occurring derivative containing the mutations R164S and E240K, confers a very high level of resistance to CAZ (128 μg/ml), a medium level of resistance to CE (16 μg/ml), and a low level of resistance to CTX (0.5 μg/ml). The presence of the mutation A237T in TEM-5, another naturally occurring derivative, in addition to the other two mutations optimizes a relatively high level of resistance to CAZ, ATM, CE, and CTX. In this work we show that neutral, nearly neutral or even deleterious (for a given environment) mutations can be fixed in fluctuating environments, even though these mutations may decrease bacterial fitness in some of these environments. New mutations appearing in TEM-type β-lactamases must increase the host bacterial cells' ability to survive in the strong counterselective forces of a rapidly varying environment. Mutations such as A237T may buffer the difficulties of maintaining the bacterial fitness of cells containing a wild-type A237 in the presence of fluctuating challenges of CAZ, ATM, CE, and CTX and perhaps of other β-lactam antibiotics not tested in this work.

The results shown here strongly suggest that mutations resulting in very weak phenotypic changes (or none for some substrates) in the absence of other specific changes may play an important role in the evolution and selection of derivative enzymes, with increased activities against various substrates in highly fluctuating environments. The existence of the A237T mutation, and others previously defined as neutral or nearly neutral, in TEM β-lactamases could be explained by their contribution in modulating the substrate preference of the enzyme under yet-undefined variably selective challenges.

ACKNOWLEDGMENTS

We thank J. C. Pérez-Díaz for generous technical support in his laboratory, A. Valencia and L. S. Pulido for helpful and thoughtful discussions on β-lactamase structure, and L. de Rafael and T. Coque for English corrections.

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