ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2000, p. 2182–2184 Vol. 44, No. 8
0066-4804/00/$04.00+0


# Selection of Naturally Occurring Extended-Spectrum TEM β-Lactamase Variants by Fluctuating β-Lactam Pressure

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Received 22 October 1999/Returned for modification 27 February 2000/Accepted 21 April 2000

**Despite the large number of in vitro mutations that increase resistance to extended-spectrum cephalosporins in TEM-type β-lactamases, only a small number occur in naturally occurring enzymes. In nature, and particularly in the hospital, bacteria that contain β-lactamases encounter simultaneous or consecutive selective pressure with different β-lactam molecules. All variants obtained by submitting an *Escherichia coli* strain that contains a $bla_{TEM-1}$ gene to fluctuating challenge with both ceftazidime and amoxicillin contained only mutations previously detected in naturally occurring β-lactamases. Nevertheless, some variants obtained by ceftazidime challenge alone contained mutations never detected in naturally occurring TEM β-lactamases, suggesting that extended-spectrum TEM variants in hospital isolates result from fluctuating selective pressure with several β-lactams rather than selection with a single antibiotic.**

TEM-type β-lactamases are the main mechanism of β-lactam resistance in enteric gram-negative microorganisms. The evolution and spread of β-lactamases in these bacteria seem to be the consequence of the evolution and consumption of β-lactam antibiotics (14). Because the use of the new β-lactam agents was not followed by a substantial drop in the use of the old ones, the result was a net diversification of the selective network. This situation created a complicated adaptive problem for enteric bacteria. In fact, a very efficient mutation of a β-lactamase that leads to an improved rate of hydrolysis of a new type of β-lactam may result in a lack of efficient hydrolysis of older (but always present) antibiotic substrates (4).

By using directed mutagenesis procedures, several groups of investigators have produced a large number of artificial amino acid changes that are able to extend the substrate spectrum of TEM-1 β-lactamases (7, 16, 17, 18, 19, 22). Interestingly, only some of them were found in naturally occurring extended-spectrum TEM β-lactamases (5, 6). The reason for such discrepancy may be that only those mutations or combinations of mutations able to adapt to highly fluctuating environments, such as those produced as a result of hospital-based chemotherapy, can be selected and fixed in naturally occurring bacterial populations. If this were the case, β-lactamase variants selected in vitro by use of a single β-lactam drug must be different from those selected by alternate use of two different β-lactam antibiotics such as an extended-spectrum cephalosporin and a penicillin. To explore this possibility, 18 flasks with 5 ml of Mueller-Hinton medium plus 0.06 μg of ceftazidime per ml were inoculated with 50 μl of an overnight culture of *Escherichia coli* K-12 strain RYC1000 (*araD139 ΔlacU169 rpsL Δrib7 thiA gyrA recA56*) that contained plasmid pBGTEM-1 (3). The flasks were incubated overnight at 37°C. Two series of nine flasks each were made: the first was submitted to successive daily serial passages in medium with increasing doubling concentrations of ceftazidime until growth was obtained at 32 μg/ml. The challenge with ceftazidime-amoxicillin in the second series was identical to that with ceftazidime alone, except that between each ceftazidime passage an overnight challenge in medium with a fixed concentration (16 μg/ml) of amoxicillin was performed. Plasmid DNA was extracted from each of the 18 bacterial populations that grew in the last flask (32 μg of ceftazidime per ml) for each evolution experiment. DNA was introduced by transformation into new RYC1000 competent cells (20). Transformants were selected on agar plates that contained kanamycin (40 μg/ml). Selection with β-lactam antibiotics was avoided in order to overcome the possible selection of new mutations. Ten transformants from each flask were purified, and their resistances to ceftazidime and amoxicillin were tested by streaking them, in parallel with the nonchallenged control, against ceftazidime and amoxicillin disks located on the center of a petri dish. Those clones that grew closer to the ceftazidime disk than the control were selected for further study. Clones that showed similar ceftazidime resistance phenotypes but that showed differences in their resistance to amoxicillin were considered different and were selected.

Only one ceftazidime-resistant phenotype per flask was detected among the 10 transformants analyzed from each of the nine flasks submitted to fluctuating selection. From the nine flasks submitted to continuous selection with ceftazidime alone, two flasks (flasks 1 and 3) harbored isolates with two different resistance phenotypes. The 20 resistant variants (9 from the fluctuating selection experiment and 11 from the continuous selection one) were purified and submitted to phenotypic (MIC) (15) and genotypic characterization. The ceftazidime MIC increased for all 20 clones (Table 1). Plasmid DNA extracted from each 1 of the 20 selected mutant clones was purified, and the nucleotide sequences of the whole $bla_{TEM}$ genes were obtained (21). Several $bla_{TEM}$-specific primers were used to sequence the whole $bla_{TEM}$ gene, including the promoter region. Table 1 shows the nucleotide changes and the corresponding deduced amino acid changes found in each of the mutant genes. With continuous antibiotic challenge (ceftazidime), five different types of variants were obtained: L169R, D179Y, D179G, R164S, and R164H (for two, one, one, six, and one isolates, respectively). Three of these changes (L169R, D179Y, and D179G) have never been detected among naturally occurring TEM enzymes from bacteria isolated in the hospital environment. Only two different mutations

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TABLE 1. Nucleotide changes, corresponding deduced amino acid changes, number of isolates selected under continuous or fluctuating challenge conditions, and MICs of ceftazidime and amoxicillin for *E. coli* K-12 strain RYC1000 harboring plasmid pBGS19 containing $bla_{TEM}$ genes (wild-type and mutant derivatives)

| TEM-1 mutation | Codon change | No. of isolates selected by: | | MIC (μg/ml)[a] | |
|---|---|---|---|---|---|
| | | Continuous selection | Fluctuating selection | Ceftazidime | Amoxicillin |
| None | | | | 0.25 | >1,024 |
| L169R | CTG→CGG | 2 | 0 | 4 | 8 |
| D179Y | GAC→TAC | 1 | 0 | 4 | 32 |
| D179G | GAC→GGC | 1 | 0 | 8 | 64 |
| R164S | CGT→AGT | 6 | 5 | 8 | >1,024 |
| R164H | CGT→CAT | 1 | 4 | 8 | >1,024 |

[a] All variants that contained the same mutation expressed the same level of resistance.

were obtained with fluctuating β-lactam selection pressure: R164S and R164H (for five and four isolates, respectively). These two mutations have previously been detected in TEM β-lactamases from naturally occurring strains.

The results presented above led us to suspect that the three changes not previously found in naturally occurring enzymes confer a phenotype deleterious for amoxicillin-hydrolyzing activity. Table 1 shows that, as expected, the three variants (L169R, D179Y, and D179G) showed decreased resistance to amoxicillin (lower MIC) with respect to the resistance for the strains that express TEM-1, R164S, or R164H. Nevertheless, the amoxicillin MIC was greater than 16 μg/ml (the concentration of amoxicillin used in the fluctuating selection experiments) for the D179Y and D179G variants. Thus, this amoxicillin concentration should apparently not be able to eliminate these two variants. This apparent paradox can be explained by the fact that our experiments were done in liquid medium, in which all possible beneficial variants must compete with one another. Thus, by this phenomenon of clonal interference, only the fittest variant is fixed in a population.

To date, about 45 naturally occurring extended-spectrum TEM variants have been described. All of these variants are the result of substitutions in 1 of 16 determined positions or combinations of positions for some of them. These amino acids (present in the mature protein), numbered as described by Ambler (1), and those that replace them are Q39K, A42V, L51P, G92D, E104K, S130G, H153R, R164S or R164H, M182T, G218E, A237T or A237G, G238S, E240K, T265M, and S268G (as determined from the Lahey website [http://www.lahey.org/studies/temtable.htm]). The changes at positions 39, 104, 164, 238, 240, and 265 occur in 40, 47, 47, 33, 24, and 20% of the extended-spectrum enzymes, respectively, as calculated from data from the Lahey website updated on 1 September 1999. All the extended-spectrum β-lactamases contain at least one of these changes. The high prevalence of these mutations suggests a direct role in the enlargement of the spectrum of activity. The other 10 changes are much less represented among the enzymes, and they may act as compensating or modulating mutations (4, 10, 11) or may have been fixed by random genetic drift.

Despite the limited number of strains examined, the simple experimental approach used in this work may help provide an understanding of the evolution of a single gene in natural fluctuating environments. The resulting evolution of variant enzymes so that they can perform novel catalytic functions may be described in terms of changes in a protein's space (12, 13). These changes have been described on some occasions as "protein differentiation" (9). Indeed, the capability of each enzyme to catalyze specific reactions under continuous or fluctuating selection conditions can be defined as the fitness of that protein under such environmental scenarios. The concept of evolution in a catalytic task space (12) may be applied here, as in our fluctuating selection environment the enzyme should deal with more than one β-lactam substrate, thus forcing protein evolution to carry out new but related reactions. Our experimental fluctuating selection conditions do not cover all possible selective β-lactam combinations that can be expected in the natural hospital environment. Nevertheless, the type of variants obtained by our selective procedure corresponds in an adequate way to the repertoire of enzymes prevalent in the clinical setting. We cannot exclude the possibility that in nature a certain number of nonprevalent enzyme variants with lower levels of catalytic fitness may be present in particular locations and may be protected by a structured environment (where competence is diminished) or by compensatory or modulating mutations (4, 10, 11). It may be possible that most of the β-lactamase mutations previously considered neutral or nearly neutral are, in fact, "active" changes able to modulate the enzymatic activity against different substrates or to compensate for some enzymatic deficiencies. Some examples have been described. The Q39K change was previously referred to as not completely neutral (3), and furthermore, molecular modeling provided a possible structural basis for such behavior (8). Also, the theoretically neutral M182T change found in the inhibitor-resistant TEM IRT-3 (2) and in some extended-spectrum TEM β-lactamases was shown to be a compensatory mutation (10, 11). Finally, the A237T mutation seems to equilibrate the substrate preference of the enzyme (4).

The results presented here strongly suggest that naturally occurring extended-spectrum TEM variants are the result of fluctuating selection pressure with several β-lactams more than they are the result of selection with an individual type of antibiotic.

We thank L. de Rafael for correction of the English.

This work was supported in part by a grant from Eli Lilly (Spain). Standard antibiotic powders were kindly provided by SmithKline Beecham Laboratories (amoxicillin) and Glaxo-Wellcome Laboratories (ceftazidime).

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