Single Amino Acid Replacements at Positions Altered in Naturally Occurring Extended-Spectrum TEM β-Lactamases

JESUS BLAZQUEZ, MARIA-ISABEL MOROSINI, MARIA-CRISTINA NEGRI, MARINA GONZALEZ-LEIZA, AND FERNANDO BAQUERO*

Servicio de Microbiología, Hospital Ramón y Cajal, Madrid, Spain

Received 16 February 1994/Returned for modification 26 April 1994/Accepted 17 October 1994

By directed mutagenesis, we constructed a set of seven TEM-1 derivatives containing single replacements in each one of the amino acids substituted in naturally occurring extended-spectrum TEM β-lactamases. The exact contribution of each mutation to the resistance phenotype was determined. In addition, mutant enzyme production and stabilities were studied. Five of seven mutations determined to some extent variations in cephalosporin and/or monobactam activity. Dramatic changes in the hydrolysis of ceftazidime and aztreonam occurred when a serine was at position 164. Changes at positions 104, 238, and 240 showed more leaky variation in activity towards cephalosporins and aztreonam. Replacements at positions 237 and 265 caused no variation in susceptibility to cephalosporins. Interestingly, the change from Glu to Lys at position 39 found in TEM-2, classically considered a neutral change, slightly but consistently increased the MIC of ceftazidime and aztreonam. The in vitro construction of mutations appearing in naturally occurring TEM-β-lactamases, studied in the same genetic context, may help to understand the evolution of extended-spectrum β-lactamases.

The production of TEM-type β-lactamases is the most prevalent mode of resistance to β-lactam antibiotics. TEM-1 β-lactamase is considered a broad-spectrum enzyme because it hydrolyzes both penicillins and cephalosporins (4). TEM-1, however, cannot efficiently inactivate new extended-spectrum cephalosporins, such as cefotaxime and ceftazidime, and monobactams, such as aztreonam. Molecular variants of TEM-1, termed extended-spectrum β-lactamases, emerged and disseminated probably as a consequence of the introduction of these extended-spectrum β-lactam antibiotics.

After years of intensive study of molecular variations involved in substrate profile alterations of TEM enzymes, it is now known that clinically resistant variants are the result of amino acid substitutions in one of several well-defined positions or of combinations of two to five of these substitutions (for a review, see reference 11). Figure 1 shows the modified positions in these resistant variants and the amino acids by which they are substituted in naturally occurring TEM-type β-lactamases. For each position, the replacement is with a particular amino acid, the only exception being Arg-164, which can be replaced by either Ser or His. Several authors constructed TEM-1 derivatives containing some of the above-described single mutations by either oligonucleotide-directed mutagenesis (10, 30) or subcloning of fragments containing those mutations (28). Biochemical and microbiological studies have been performed with some of these mutants. Nevertheless, none of these publications includes a comparative study of the variations in β-lactamase phenotype and stability resulting from all seven single mutations corresponding to the seven positions modified in naturally occurring β-lactamases. In addition, the microbiological activities were, in each one of these studies, measured in a different genetic context (promoter, plasmid, and strain), which precluded reliable phenotypic comparisons among the three studies.

In this work, we describe the construction and characterization of seven different mutants with changes in each one of the seven positions altered in clinical β-lactamases. In addition, we introduced these mutant derivatives into a strain lacking the outer membrane protein OmpF, as the interplay of TEM type β-lactamases and porin mutations leads to higher resistance levels (20, 24). It is expected that our study may serve to obtain new insights for understanding the selective process and the resulting evolution of extended-spectrum β-lactamases. To our knowledge, this is the first study including mutant TEM-1 derivatives with single mutations in all seven positions modified in naturally occurring extended-spectrum TEM-type β-lactamases.

MATERIALS AND METHODS

Escherichia coli strains and plasmids. The bacterial strains used for determination of MICs were RY1000 (pRJ159 ΔlacU169 Rpd, Δrha7 thi-1 recA56) and MH621 (pΔD159 ΔlacU169 Rpd, Δrha7 thi-1 recA56 ΔompF-lacZ210::Tn10 [Hyb]) (9). Strains for oligonucleotide-directed mutagenesis were C2326 [Δl Lac-1 thi-1 recA1 pCh105 (F', Cm')] (13) and TG1 (Δlac-pro) supE thi hsdS2 F' traD36 proA B' lacI52 lacZM15 [17]. The hybrid phage M13mp18Ap was constructed by cloning a BamHI-BamHI fragment from plasmid pK294Ap (8) containing the blaTEM-1 gene from transposon Tn3, encoding the TEM-1 β-lactamase, into the BamHI site of phage M13mp19. An EcoRI-SalI fragment from the hybrid phage M13mp18Ap was cloned in plasmid pBG19 (31) digested with the same restriction enzymes. This new hybrid plasmid was named pBGTEM-1. The blaTEM-1 gene from this plasmid was sequenced in its entirety to verify that it was the previously described gene coding for TEM-1. The mutant derivatives, constructed by directed mutagenesis, were made by substituting the adequate mutated fragment from M13mp18Ap, after mutagenesis and sequencing, for that of pBGTEM-1. Plasmids containing the mutant derivatives were named by adding the number of the β-lactamase (if previously described) or the amino acid change, as corresponded in each case, to the prefix pBGTEM-1.
Site-directed mutagenesis and selection of mutants. For all substitutions except Gly-240→Ser (TEM-19), site-directed mutagenesis was performed on M13mp18 or pAT264 (see text) containing the desired 3′-lactamase, showing the residues modified in extended-spectrum TEM enzymes. Arrows indicate the amino acids by which they are substituted in naturally occurring enzymes. At the bottom, a diagram of the blaTEM-1 gene, including internal relevant restriction sites, is shown. At the extremes, sites from both the vector (pBGSI9′) and cloned fragment, from pKT2561Ap (8), are boxed. B, BamHI; E, EcoRI; H, HindIII; He, HindE; P, PstI; S, SalI; Sm, SmaI. The circle labelled P indicates the position of the bla natural promoter from Tn3. Numbering is according to Ambler (1). Nt and Ct, N terminus and C terminus, respectively.

Isoelectric focusing. Analytical isoelectric focusing was performed in precast polyacrylamide gels (pH range, 3 to 9 and/or 4 to 6.5) by using a Pharmacia (Uppsala, Sweden) PhastSystem apparatus according to the instructions of the manufacturer. β-Lactamase activity was revealed by hydrolysis of the chromophore β-lactam nitrocefin (Oxoid).

Preparation of crude enzyme extracts. Strain RYC1000 containing plasmid pBGTEM-1 or one of its seven mutant derivatives was grown in Luria-Bertani medium until the culture reached an optical density at 600 nm of 1.00. The cells were harvested by centrifugation and washed twice with 0.1 M potassium phosphate buffer (pH 7.5).

For preparation of cell extracts, cells were ruptured by ultrasonic treatment at 4°C. Cell debris was removed by centrifugation. The supernatant was used for kinetic tests. As a negative control, extracts from strain RYC1000 containing vector pBGSI9′ were used for the tests.

Kinetics. β-Lactamase activities were determined by measuring the decrease in absorbance of cephaloridine at 275 nm. Kinetic studies were performed at a constant temperature of 25°C in a UVikon-940 spectrophotometer. One unit of β-lactamase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate in 1 min at 25°C in 0.1 M potassium phosphate buffer (pH 7.5). For specific enzyme activities, protein concentrations were determined as described before (15). Kinetic parameters were obtained by linear plots of the initial steady-state velocities at different substrate concentrations (5).

RESULTS

Microbiological activities. Tables 2 and 3 show the microbiological activities of some β-lactam antibiotics with strains RYC1000 and MH621 containing the wild-type (TEM-1) and mutant TEM derivatives.

(i) Gln-39 to Lys (TEM-2). The Gln-39 to Lys mutation did not change the susceptibility pattern observed for the wild-type enzyme with amoxicillin, amoxicillin plus clavulanic acid, cefotaxime, aztreonam, and meropenem. Susceptibility to cephaloridine and ceftazidime was slightly (twofold) but consistently decreased in more than five independent experiments and in both the agar dilution and E test.

(ii) Glu-104 to Lys (TEM-17). Susceptibility to amoxicillin, amoxicillin plus clavulanic acid, and meropenem was not affected by this mutation. Susceptibility to ceftazidime and aztreonam was significantly decreased (four- to eightfold).

(iii) Arg-164 to Ser (TEM-12). A decrease in susceptibility to ceftazidime and aztreonam, particularly dramatic in the case of ceftazidime (from 0.5 to 32 μg/ml in the OmpF− strain), was detected. The amoxicillin MIC remained ≥2,048 μg/ml, and the meropenem MIC was not affected.
(iv) Ala-237 to Thr. No significant variation in susceptibility was found for most of the β-lactams tested with this mutation. A slight decrease in the MIC of amoxicillin (in both OmpF<sup>b</sup> and OmpF<sup>m</sup> contexts) was consistently detected in several independent experiments.

(v) Glu-238 to Ser (TEM-19). The presence of this mutation originated a significant decrease in the MIC of amoxicillin plus clavulanate. On the other hand, an increase in the MIC of cefotaxime (only twofold in the OmpF<sup>b</sup> but eightfold in the OmpF<sup>m</sup> context) was regularly observed.

(vi) Glu-240 to Lys. The Glu-240 to Lys change originated a significant increase in resistance to cefotaxime (from 0.5 to 4 µg/ml in the OmpF<sup>b</sup> strain) and aztreonam (from 0.25 to 1 µg/ml). No changes in the activity of the other β-lactams were detected.

(vii) Thr-265 to Met. The Thr-265 to Met mutation did not produce major variations in susceptibility to the β-lactam antibiotics tested in either the OmpF<sup>b</sup> or OmpF<sup>m</sup> context. With cephaloridine, a twofold increase was observed in the OmpF<sup>m</sup> strain (Table 2).

**β-Lactamase activities.** As shown in Table 4, the mutations Lys-39, Lys-104, Thr-237, and Met-265 did not alter the affinity to cephaloridine. However, mutations Ser-164 and Ser-238 significantly increased (about five times) the affinity of β-lactamase and, to a minor extent, so did Lys-240. The maximal relative rates of hydrolysis for cephaloridine decreased in most tested mutations, particularly Ser-164 and Ser-238 (100 to 200 times). V<sub>max</sub> was not altered in the Met-265 mutant and increased slightly for the Lys-39 mutation. The highest relative hydrolytic efficiency (V<sub>max</sub>/K<sub>m</sub>) was found for the lys-39 (1.5, compared with 1 for the wild-type TEM-1 enzyme). The same catalytic efficiencies as TEM-1 were found for the mutants Lys-240, Thr-237, and Met-265. Ser-164 and Ser-238 showed the lowest efficiencies for cephaloridine (20 to 30 times weaker than TEM-1).

**DISCUSSION**

Some of the single mutants studied in this work have been previously obtained and characterized. Nevertheless, some of those mutants were constructed in different plasmids or genetic contexts. Indeed, the bla genes used for some of those constructions were not the original bla<sub>TEM-1</sub> genes but derivatives with theoretically “neutral” mutations (11, 30). The aim of this work was to construct a collection of TEM-1 derivatives in the same genetic context (plasmid, promoter, or strain) and define the change in the enzyme properties (kinetics, amount, and stability) and in the phenotype conferred by each one of these mutants.

The Lys-39 mutation (characterizing the TEM-2 enzyme) is frequent among extended-spectrum β-lactamases, being present in TEM-3, TEM-7, TEM-8, TEM-11, TEM-13, TEM-14, TEM-16, TEM-18, and TEM-24 (11, 19). Such frequency is higher than expected if the low frequency of TEM-2 among the...
natural *E. coli* isolates is considered (11). Our results suggest that the Lys-39 mutation by itself may provide the host strain with better survival at low concentrations of some cephalosporins or monobactams, thus increasing the possibilities for acquisition of new mutations. This effect could be increased by the higher promoter strength of the natural bla<sub>TEM-2</sub> gene from Tn<sub>1</sub> (7).

The change Glu-104→Lys was identified previously in TEM-3, TEM-4, TEM-6, TEM-8, TEM-9, TEM-14, TEM-15, TEM-16, TEM-17, TEM-18, TEM-24, and TEM-26 (6, 11, 19), and the change Glu-240→Lys was identified in TEM-5, TEM-10, and TEM-24 (6, 11). These changes at positions 104 and 240 consistently decreased susceptibility to ceftazidime and aztreonam. It has been suggested that enzymes with lysines at positions 104 and 240 could establish electrostatic interactions with the oxime acid group of ceftazidime and aztreonam (30).

The change Arg-164→Ser has been found previously in TEM-5, TEM-7, TEM-8, TEM-9, TEM-10, TEM-12, TEM-24, and TEM-26 (6, 11, 19). Dramatic changes in the hydrolysis of ceftazidime and aztreonam occurred when we introduced the mutation Ser-164. The results with these changes are very striking if we consider that, in the structure of TEM-1, the side chain of Arg-164 is not in a position to interact directly with the substrate. Nevertheless, the Arg to Ser change could alter the conformation or orientation of an omega loop, affecting the substrate profile of the enzyme (12, 22).

In general, our results with the mutations Lys-104, Ser-164, and Lys-240 are in accordance with those reported previously (28, 30). Soweik et al. (30) found a catalytic efficiency for cephaloridine in the Ser-164 mutant that was higher than ours, which can be related to the differences in the studied wild-type bla<sub>TEM-1</sub> gene and/or in the enzyme preparation. These three mutations, particularly Lys-104 and Ser-164, are in fact more frequently found among the more active extended-spectrum β-lactamases. In our work, the strains harboring such mutations required higher MICs of ceftazidime and aztreonam. The Ala-237→Thr change, found in TEM-5 and TEM-24 (6, 29), is apparently neutral in relation to the tested antibiotics except for amoxicillin, which had a decreased MIC. This decrease of activity for penicillins has been shown previously by Healey et al. (10).

The change Gly-238→Ser, found previously in TEM-3, TEM-4, TEM-8, TEM-14, TEM-15, and TEM-19 (11), produces a decrease in cefotaxime susceptibility. These results are consistent with those of Sougakoff et al. (28). Susceptibility to amoxicillin plus clavulanate is increased. Enzymatic activity for cephaloridine decreased with this mutation, although affinity to this substrate was increased sevenfold.

Thr-265→Met was found in TEM-4, TEM-9, TEM-13, and TEM-14 (11); nevertheless, this change had never been obtained previously as a single mutation. It was suggested by others that this mutation, far away from the active-site pocket, could be a functionally silent substitution (16), and our data give support to this view.

Modifications in the amino acid sequence of a β-lactamase may modify the stability of the protein, influencing the turnover of the enzyme and therefore the β-lactam/β-lactamase interplay and consequently the antibiotic activity. Our pulse and chase data show that none of the seven single mutations studied increases the amount or the stability of the TEM protein at the times tested, but we cannot exclude that differences could be detected at shorter time points.

The five TEM-1 natural mutations determining different rates of decrease in susceptibility to β-lactam antibiotics are not the only ones that could have been selected in nature. Many other TEM mutations have been constructed that are able to increase resistance to extended-spectrum cephalosporins (21–23). It remains a pending fact that these mutations have not yet been recovered either in clinical isolates or in stepwise in vitro selection tests.

The understanding of the evolution of extended-spectrum β-lactamases should be based on the data obtained by the in vitro reproduction of their evolutionary molecular intermediates. Since TEM-1 seemed to be the more widespread enzyme among plasmid-encoded β-lactamases, single point mutations leading to small decreases in the antimicrobial activity of the newest cephalosporins and monobactams could have been selected under the pressure of the low antibiotic concentrations obtained during clinical therapy.

**ACKNOWLEDGMENTS**

We thank P. Courvalin for the generous gift of plasmid pAT264, J. C. Pérez Díaz for helpful discussions, and L. de Rafael for English correction.

This research was supported by a grant from Zeneca Pharmaceutica-

**REFERENCES**


