An Analysis of Why Highly Similar Enzymes Evolve Differently

Fahd K. Majiduddin* and Timothy Palzkill*,†,[1]

**Verna and Marrs McLean Department of Biochemistry and Molecular Biology and the †Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030*

Manuscript received September 26, 2002
Accepted October 31, 2002

ABSTRACT

The TEM-1 and SHV-1 β-lactamases are important contributors to resistance to β-lactam antibiotics in gram-negative bacteria. These enzymes share 68% amino acid sequence identity and their atomic structures are nearly superimposable. Extended-spectrum cephalosporins were introduced to avoid the action of these β-lactamases. The widespread use of antibiotics has led to the evolution of variant TEM and SHV enzymes that can hydrolyze extended-spectrum antibiotics. Despite being highly similar in structure, the TEM and SHV enzymes have evolved differently in response to the selective pressure of antibiotic therapy. Examples of this are at residues Arg164 and Asp179. Among TEM variants, substitutions are found only at position 164, while among SHV variants, substitutions are found only at position 179. To explain this observation, the effects of substitutions at position 164 in both TEM-1 and SHV-1 on antibiotic resistance and on enzyme catalytic efficiency were examined. Competition experiments were performed between mutants to understand why certain substitutions preferentially evolve in response to the selective pressure of antibiotic therapy. The data presented here indicate that substitutions at position Asp179 in SHV-1 and Arg164 in TEM-1 are more beneficial to bacteria because they provide increased fitness relative to either wild type or other mutants.

PENICILLIN and other β-lactam antibiotics have been available since the 1940s to treat bacterial infections and are among the most often used antimicrobial agents (Ghuysen 1991; Navarre and Schneewind 1999). The extensive use of these antibiotics has unfortunately led to the emergence of resistant strains of bacteria (Abraham and Chain 1940). Among these strains, the production of β-lactamase enzymes is the most common mechanism of resistance (Abraham and Chain 1940; Frere 1995). β-Lactamases catalyze the hydrolysis of the amide bond present in the β-lactam ring to create an ineffective antimicrobial agent. The enzymes can be divided into four classes (A, B, C, and D) based on primary sequence homology (Ambler 1980). The class A, C, and D enzymes utilize an active site serine to hydrolyze β-lactam antibiotics (Matagne *et al.* 1998). In contrast, class B enzymes, also known as the metallo-β-lactamases, utilize zinc ions to catalyze the hydrolysis of β-lactams (Livermore and Woodford 2000). The rapid spread of β-lactam resistance, both within and between species, is facilitated by the transmission of β-lactamase-encoding genes via mobile genetic elements such as transposons and plasmids (Ghuysen 1991; Massova and Mobashery 1998).

The TEM-1 and SHV-1 β-lactamases are plasmid-encoded class A enzymes that are an important source of β-lactam resistance (Ghuysen 1991; Massova and Mobashery 1998). The TEM-1 and SHV-1 enzymes are 68% identical in amino acid sequence and their three-dimensional structures are virtually superimposable. For example, there is only a 1.4-Å rms deviation of the overall enzyme structures and only a 0.23-Å rms deviation of the catalytically important residues (Jelsch *et al.* 1993; Kuzin *et al.* 1999). The TEM-1 and SHV-1 enzymes also share similar substrate profiles in that both readily hydrolyze the older β-lactam antibiotics such as penicillin, ampicillin, and cephalosporin C (Heritage *et al.* 1999).

Avoiding the action of β-lactamases is an important means of maintaining the efficacy of β-lactam antibiotics. One approach has been the use of β-lactamase inhibitors, such as clavulanic acid or sulbactam, in conjunction with an older antibiotic such as amoxacillin (Rolinson 1991). Another means of circumventing antibiotic resistance has been the use of extended-spectrum β-lactam antibiotics such as ceftazidime (CAZ) and cefotaxime (CTX), which are poorly hydrolyzed by the TEM-1 and SHV-1 β-lactamases (Maveyraud *et al.* 1996). Because of their efficacy, these drugs have been widely used, which, in turn, has placed strong selective pressure on bacteria to evolve resistance to these agents (Palzkill 1998; Goussard and Courvalin 1999; Diekema *et al.* 2000; Lesch *et al.* 2001). This has resulted in the emergence of variant β-lactamases with amino acid substitutions at critical residues that either facilitate the hydrolysis of extended-spectrum β-lactam antibiotics or avoid the action of the inhibitors (Raquet *et al.* 1994; Arlet *et al.* 1997; Bonomo *et al.* 1997; Rasheed *et al.* 1997; Heritage *et al.* 1999; Yang *et al.* 1999; Kuro-

[1]*Corresponding author:* Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: timothyp@bcm.tmc.edu

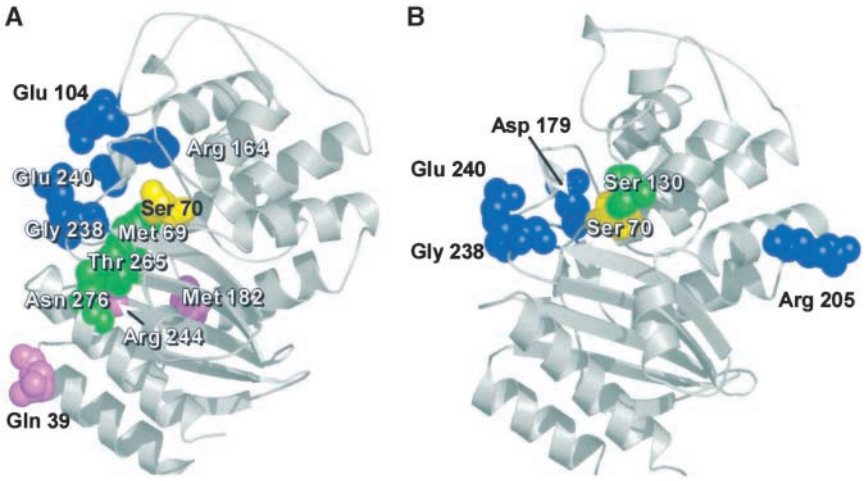

FIGURE 1.—Structural representation of TEM-1 and SHV-1 β-lactamases. Substitutions occurring at amino acid positions that alter the substrate specificity or inhibition profile are highlighted in TEM-1 (A) and SHV-1 (B). Substitutions resulting in resistance to extended-spectrum cephalosporins are in blue, inhibitor resistance is in green, and substitutions found in both extended-spectrum β-lactamases and inhibitor-resistant isolates are in purple.

KAWA *et al.* 2000; RANDEGGER *et al.* 2000). Nearly 100 variants of TEM-1 and >30 variants of SHV-1 β-lactamase have emerged among resistant clinical isolates in the past several years (Figure 1). The list of β-lactamase variants continues to grow each year, which is indicative of the ongoing evolution of these enzymes.

Many of the variant TEM-1 and SHV-1 enzymes possess identical substitutions at positions Glu104, Gly238, or Glu240 (SOWEK *et al.* 1991; RAQUET *et al.* 1994; RASHEED *et al.* 1997; PALZKILL 1998; RANDEGGER *et al.* 2000). For example, TEM-19 and SHV-2 both contain serine substitutions at position Gly238, while TEM-93 and SHV-5 both possess serine substitutions at position Gly238 as well as a lysine substitution at position Glu240. However, despite the many similarities between TEM and SHV, some positions in the enzymes exhibit different patterns of substitution in response to selective pressure. This is illustrated by the different β-lactamase variants that appear in resistant clinical isolates with substitutions at positions 164 and 179. For example, residue Arg164 in TEM-1 is substituted either by serine (TEM-12) or by histidine (TEM-29; RAQUET *et al.* 1994) among resistant isolates while substitutions at Arg164 have not been identified among SHV variants. Similarly, substitutions at residue Asp179 are found among SHV variants but have never been observed among TEM variants from resistant isolates (Figure 2). It is possible that this observation is an artifact of sampling and that, as more variants are sequenced, the Arg164 and Asp179 substitutions will be discovered. However, despite constant surveillance for resistant strains at the national level, no variants with substitutions at position 179 in TEM-1 or at position 164 in SHV-1 have been identified (MARANO *et al.* 2000). Therefore, while the TEM-179 and SHV-164 mutants may exist undetected in natural populations, it is clear that they are present in greatly reduced frequencies relative to the TEM-164 and SHV-179 variants.

Residues Arg164 and Asp179 are part of the ω-loop, a signature structural motif of class A β-lactamases that contributes to the substrate specificity profile of these enzymes (PETROSINO and PALZKILL 1996; MATAGNE *et al.* 1998). The ω-loop consists of 19–20 amino acids and is located between positions 160 and 180 in class A enzymes (PETROSINO and PALZKILL 1996). For TEM-1 β-lactamase, amino acid substitutions in the ω-loop structure alter the substrate specificity of the enzyme. Disrupting the ω-loop structure may allow for bulkier substrates, such as ceftazidime, to enter the active site and to be hydrolyzed (PALZKILL *et al.* 1994; PETROSINO and PALZKILL 1996). Interestingly, the structural environment around positions Arg164 and Asp179 in TEM-1 and SHV-1 is identical (KUZIN *et al.* 1999). Therefore, substitutions at these positions would be expected to exert a similar impact on the structure and function of the enzymes.

An interesting question is why two enzymes with such high degrees of similarity at both the sequence and structural levels differ in the spectrum of amino acid substitutions acquired in response to an identical selective pressure. To address this question, position Arg164 was systematically substituted in both the TEM-1 and SHV-1 enzymes with the remaining 19 amino acids, and the effects of these substitutions on the cefotaxime and ceftazidime resistance profiles of *Escherichia coli* strains containing the various mutants were determined. In addition, competition experiments were performed to assess the relative fitness of the various mutants. The results of these experiments indicate that the determining factor for whether a substitution becomes prevalent in a population is the fitness that substitution provides relative to the fitness provided by other substitutions and not relative to the fitness provided by the wild-type enzyme. Thus, simply providing for fitness higher than that of wild type is not sufficient to ensure the success of a mutant in a population. Rather, the mutant must compete effectively with all other possible mutants. Examining the differences between TEM-1 and SHV-1 variants will facilitate our understanding of the evolutionary

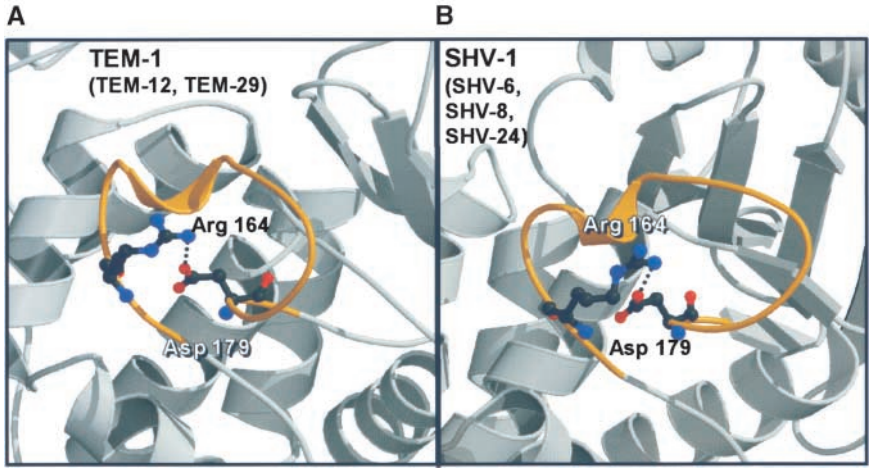

FIGURE 2.—Positions Arg164 and Asp179 in the TEM-1 and SHV-1 β-lactamases. The two highlighted positions, Arg164 and Asp179, are equivalent in both environment and side chain conformation in TEM-1 (A) and SHV-1 (B). The salt bridge formed between Arg164 and Asp179 is also highlighted. The ω-loop structure is indicated in orange. Substitutions occurring at these positions result in an extended-spectrum β-lactamase phenotype. The clinical isolates associated with the substitutions are indicated in parentheses.

response to the selective pressure of β-lactam antibiotic therapy and may allow for the deployment of alternative antimicrobial treatment regiments.

MATERIALS AND METHODS

Site-directed mutagenesis: Site-directed mutagenesis was carried out using a two-step polymerase chain reaction (PCR) procedure using Klentaq polymerase (Clontech, Palo Alto, CA; Ho *et al.* 1992; PETROSINO *et al.* 1999). Primers were designed such that each of the 19 remaining amino acids could be introduced at the codon for Arg164. In the first round of PCR two sets of primers, which included the outside primers and the mutagenic primers for position Arg164, were utilized to amplify the bla_{TEM} and bla_{SHV} genes. The PCR products generated from this first step were then mixed together and amplified using only the outside primers to generate the full-sized *bla* genes with the site-directed mutant at position 164. The outside primers contained *Sac*I and *Xba*I restriction enzyme sites for cloning into the pTP123 vector. All primers were obtained from Integrated DNA Technologies (Skokie, IL).

Bacterial strains and cloning: All of the TEM-1 and SHV-1 variants were cloned as *Sac*I/*Xba*I fragments into *Sac*I/*Xba*I-digested pTP123, which contains a Cam^r gene for chloramphenicol resistance and is derived from the pBC SK± vector (PETROSINO *et al.* 1999). All TEM- and SHV-containing vectors were electroporated into the *E. coli* XL-1 blue [*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, (F′::Tn*10*(Tet^r), *proAB*, δlacI^q (*laqZ*)*M15*)] strain (BULLOCK *et al.* 1987). Vectors encoding TEM-1, TEM Arg164Ser, TEM Asp179Gly, SHV-1, SHV Asp179Gly, and SHV Arg164Ser were electroporated into both *E. coli B* Ara^+ and Ara^- strains, which are genotypically equivalent except for the mutation affecting the utilization of L(+)-arabinose. This difference in arabinose utilization acts as a marker for the competition experiments (described below). Both the *Sac*I and *Xba*I enzymes were obtained from New England Biolabs (Beverley, MA).

DNA sequencing: Correct clones were identified by DNA sequencing either by manually using the Amersham Life Science Sequenase PCR product sequencing kit or by automated sequencing using either the Beckman-Coulter CEQ2000 capillary sequencer or the Applied Biosystems Instruments 377 automated sequencer. All protocols were followed according to the manufacturer's specifications.

MIC determination: Minimum inhibitory concentrations (MIC) were determined using the E-test strip method (VAIANI *et al.* 2000). For these experiments, *E. coli* XL-1 blue cells harboring a variant TEM or SHV gene were grown to an OD_{600} of 0.2 and 100 μl of the culture was spread on agar plates. The E-test strip was then placed on the agar plate and incubated overnight at 37°. The MIC was determined on the basis of the intersection of bacterial growth with the E-test strip.

Enzyme purification: The SHV-1, SHV Arg164Ser, SHV Asp179Gly, and TEM Asp179Gly enzymes were purified. For this purpose, 10 ml overnight cultures were diluted into 1 liter of Luria-Bertani (LB) medium supplemented with 12.5 μg/ml chloramphenicol and grown at 37° until an OD_{600} of 0.4 was reached. The cultures were then induced with 1 mM isopropyl thiogalactoside to express the β-lactamases and grown an additional 12–16 hr. Cells were harvested by centrifugation at 3000 × *g* for 20 min at 4° in a Sorvall RC50 centrifuge and then frozen at −80°. Cells were treated with 20% glucose, 1 mM EDTA, and 20 mM Tris-HCl pH 8.0 to release the periplasmic contents. The insoluble fraction was removed by centrifugation in a Sorvall RC50 centrifuge at 20,000 × *g* for 15 min at 4° and the soluble fraction was concentrated and dialyzed into 50 mM HEPES buffer, pH 8.0, to 1 mg/ml protein using an Amicon Centriprep-10 column (Millipore, Bedford, MA). Protein concentrations were determined using the Bradford method (Bio-Rad Bradford reagent kit). The concentrated soluble fraction (1 ml) was separated on a Sephadex G-75 gel filtration column (Tosoh Biosep, Montgomeryville, PA) and fractions containing active β-lactamase were identified using nitrocefin (Becton Dickinson, Sparks, MD). The active fractions were then concentrated using the Amicon Centriprep-10 column and protein concentrations were determined using the Bradford assay. Protein purity was assessed via SDS-PAGE.

Enzyme kinetic analysis: Kinetic parameters were determined for SHV-1 β-lactamase, SHV Arg164Asp, SHV Arg164Ser, SHV Asp179Gly, and TEM Asp179Gly enzymes to characterize the contributions of these amino acids toward the hydrolysis of ceftazidime. Kinetic parameters were also determined for ampicillin (Sigma, St. Louis), ceftazidime (Eli Lilly), cefotaxime (Sigma), and cephaloridine (Sigma). Substrate concentrations ranged from 1 to 700 μM, depending on the substrate. The change in absorbance of substrate upon hydrolysis was measured using a Beckman DU 640 spectrophotometer at the desired wavelength for each substrate. The changes in absorbance values were used to determine initial velocities. K_m and k_{cat} values were calculated using the enzyme kinetics program (Trinity Software, Plymouth, NH).

Competition experiments: *E. coli B* Ara^+ and Ara^- strains

containing the TEM-1, TEM Arg164Ser, TEM Asp179Gly, SHV-1, SHV Asp179Gly, and SHV Arg164Ser enzymes were grown to stationary phase overnight at 37°. A total of 12 competition experiments were performed as follows: TEM-1 (Ara^+) *vs.* TEM Arg164Ser (Ara^-), TEM-1 (Ara^-) *vs.* TEM Arg164Ser (Ara^+), TEM-1 (Ara^+) *vs.* TEM Asp179Gly (Ara^-), TEM-1 (Ara^-) *vs.* TEM Asp179Gly (Ara^+), TEM Arg164Ser (Ara^+) *vs.* TEM Asp179Gly (Ara^-), TEM Arg164Ser (Ara^-) *vs.* TEM Asp179Gly (Ara^+), SHV-1 (Ara^+) *vs.* SHV Asp179Gly (Ara^-), SHV-1 (Ara^-) *vs.* SHV Asp179Gly (Ara^+), SHV-1 (Ara^+) *vs.* SHV Arg164Ser (Ara^-), SHV-1 (Ara^-) *vs.* SHV Arg164Ser (Ara^+), SHV Arg164Ser (Ara^+) *vs.* SHV Asp179Gly (Ara^-), and SHV Arg164Ser (Ara^-) *vs.* SHV Asp179Gly (Ara^+). Each variant was mixed in equal volume with the competing variant. Each mixture was then diluted 1:100 into 10 ml of LB media supplemented with chloramphenicol and ceftazidime. A range of 0–0.5 μg/ml ceftazidime was used for the competition experiments. The competition was carried out over 16 hr at 37°. After the competition, each culture was diluted to 10^3 cells/ml and 100 μl was spread on tetrazolium-arabinose indicator agar plates. The colonies were allowed to grow overnight at 37°. Red and white colonies were counted and used to determine the percentage of each variant in the total cell population.

RESULTS

Minimum inhibitory concentrations for ceftazidime and cefotaxime of all 19 variants of TEM-1 and SHV-1: To determine whether SHV-1 β-lactamase is able to tolerate mutations at position Arg164 and to compare SHV Arg164 mutants against TEM Arg164 mutants, TEM-1 and SHV-1 variants were constructed by replacing the wild-type arginine at position 164 with the remaining 19 naturally occurring amino acids using PCR-based mutagenesis (Ho *et al.* 1992; Petrosino *et al.* 1999). The MIC values for ceftazidime and cefotaxime of all 19 TEM-1 and SHV-1 variants at position Arg164 were determined using the E-test strip method (Vaiani *et al.* 2000). It was found that all substitutions at position 164 in the SHV-1 enzyme, with the exception of tryptophan, resulted in increased ceftazidime resistance for the *E. coli* strain containing the mutants relative to the strain expressing the wild-type enzyme. Interestingly, all of the single-base-pair substitutions that can occur from the arginine codon would result in amino acid substitutions that improve ceftazidime hydrolysis for the SHV enzyme. However, natural isolates harboring these SHV mutants have not been found, even though surveillance for resistance is ongoing and the list of SHV mutants is growing. Similarly, it has been shown in previous studies that substitutions at position Asp179 in TEM-1 result in increased ceftazidime resistance, yet no clinical isolates have been identified with substitutions at position Asp179 (Vakulenko *et al.* 1995).

TEM-1 Arg164 mutants were constructed in the exact manner as the SHV Arg164 mutants to directly compare the MIC results for both sets of variants. MIC determinations yielded results similar to those published by Vakulenko *et al.* (1999) for substitutions at Arg164 in the TEM-1 enzyme. All substitutions except isoleucine, leucine, and proline provided a MIC equal to or higher than the wild-type value for ceftazidime (Figure 3; Table 1). Four of the six possible substitutions resulting from single-base-pair changes of the arginine codon result in enzymes that confer higher MICs for ceftazidime. Three of these substitutions, Arg164Ser, Arg164His, and Arg164Cys, occur in the clinical setting (Weber *et al.* 1990; Arlet *et al.* 1995; Perilli *et al.* 2002). The fourth possible substitution, glycine, has yet to be identified among resistant clinical isolates.

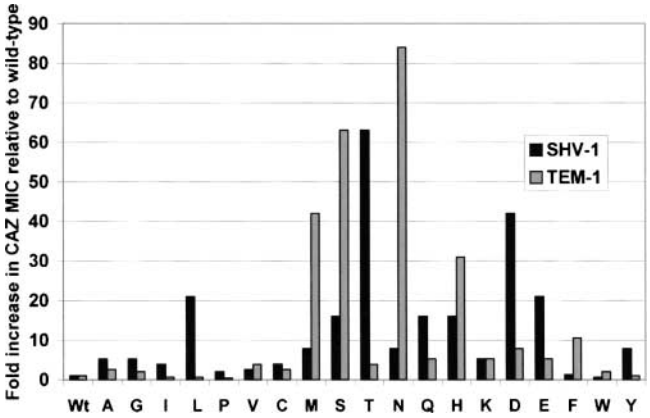

Figure 3.—Relative ceftazidime MIC values for each substitution compared to parental enzyme. The fold increase in ceftazidime MIC relative to wild-type TEM-1 or SHV-1 is shown.

The SHV-1 and TEM-1 position 164 mutants were also examined for the effect of the substitutions on resistance to another third-generation cephalosporin, cefotaxime. None of the SHV Arg164 variants, except the SHV Arg164Phe variant, exhibited a significant increase in cefotaxime resistance relative to wild type (Table 1). Only three substitutions in TEM-1, Arg164Asn, Arg164Met, and Arg164Gly, resulted in a significant increase in the cefotaxime MIC relative to wild type. Only glycine can be attained from a single-base-pair change from the wild-type arginine codon and thus far no TEM Arg164Gly variants have been identified. It is believed that substitutions at Gly238, another position that is identical in both enzymes, play an important role in altering substrate specificity for cefotaxime hydrolysis (Cantu *et al.* 1996, 1997; Cantu and Palzkill 1998).

Kinetic analysis of SHV and TEM variants: On the basis of the MIC data, SHV-1 variants with substitutions at Arg164 exhibit increased resistance to ceftazidime and therefore should exist among resistant natural isolates. To demonstrate that these substitutions directly alter enzyme catalysis, kinetic parameters were determined for the SHV Arg164Ser, SHV Asp179Gly, and TEM Asp179Gly enzymes. The kinetic parameters of these mutants and the TEM Arg164Ser mutant (Bradford *et al.* 1994) were compared to the wild-type TEM-1 and SHV-1 enzymes to assess the difference in catalytic efficiency.

Kinetic parameters were determined for cephalori-

TABLE 1

MIC for ceftazidime of the TEM-1 and SHV-1 β-lactamase variants

SHV MICs			TEM MICs	
CAZ (μg/ml)	CTX (μg/ml)	Mutation	CAZ (μg/ml)	CTX (μg/ml)
1.0–1.5	0.016	R164A	0.50–0.75	0.047–0.064
1.0–1.5	0.032–0.047	R164G	0.38–0.5	0.75–1.0
0.75–1.0	0.047	R164I	0.125–0.19	0.047–0.064
4.0–6.0	0.064	R164L	0.125	0.016–0.032
0.38–0.5	0.023–0.032	R164P	0.094–0.125	0.032–0.047
0.5–0.75	0.075–1.0	R164V	0.75	0.032–0.047
0.75–1.0	0.032	R164C	0.5	0.032
1.5	0.064–0.094	R164M	8.0–12.0	0.75–1.0
3.0–4.0	0.047	R164S	12.0	0.094
12.0–16.0	0.064	R164T	0.75	0.032–0.047
1.5–2.0	0.032	R164N	16.0–24.0	0.25–0.38
3.0	0.064	R164Q	1.0	0.047
3.0	0.094	R164H	6.0	0.064–0.094
1.0–1.5	0.032–0.064	R164K	1.0	0.032–0.047
0.19	0.047–0.064	R164R (wt)	0.19	0.023–0.032
8.0	0.064–0.094	R164D	1.5–2.0	0.032–0.047
4.0	0.094	R164E	1.0	0.047
0.25–0.38	0.19	R164F	2.0	0.064–0.094
0.125	0.032	R164W	0.38	0.032
1.5	0.023	R164Y	0.19	0.047
4.0	0.094	D179G	4.0	0.047

dine, cefotaxime, ampicillin, and ceftazidime for each mutant and for wild-type SHV-1. The K_m, k_{cat}, and k_{cat}/K_m values are listed in Tables 2 and 3. As predicted from the MIC data, the SHV Arg164Ser enzyme catalyzes the hydrolysis of ceftazidime more efficiently than wild-type SHV-1. A substitution from arginine to serine at position 164 in the SHV-1 enzyme results in a 2.5-fold increase in catalytic efficiency for ceftazidime hydrolysis relative to the wild-type SHV-1 enzyme. However, the SHV Asp179Gly substitution results in an even larger (5.5-fold) increase in ceftazidime hydrolysis relative to wild type. The relatively small difference in catalytic efficiency between the SHV-1 and SHV Arg164Ser enzymes may explain why the SHV Arg164Ser variant does not exist among natural isolates. This lower catalytic efficiency may not be sufficient for the bacteria containing the SHV Arg164Ser variant to compete with bacteria expressing the SHV Asp179 variants. In contrast, the

TABLE 2

K_m and k_{cat} values for SHV-1, SHV Arg164Asp, SHV Arg164Ser, TEM1, and TEM Arg164Ser

	SHV-1	SHV R164S	SHV D179G	TEM-1[a]	TEM R164S[b]	TEM D179G
K_m (μM)						
AMP	54 ± 14.6	5.4 ± 0.5	26.6 ± 5.9	50 ± 2	NA	8 ± 2
Ceph	242.12 ± 17.4	178.5 ± 55	NM[d]	697 ± 12	100	NM[d]
CTX	240.5 ± 24	175.6 ± 68	527.8 ± 57	1100	230	1783 ± 422
CAZ	NM[c]	NM[c]	168.2 ± 18	NM[c]	270×10^3	NM[c]
k_{cat} (s^{-1})						
AMP	1773 ± 126	227.8 ± 8.6	2 ± 0.1	1428 ± 24	NA	8.24 ± 0.3
Ceph	331.9 ± 14.9	4.6 ± 0.64	NM[d]	947 ± 9	46	NM[d]
CTX	58.7 ± 3	0.68 ± 0.1	2.6 ± 0.15	1.8	2.3	2.6 ± 0.15
CAZ	NM[c]	NM[c]	0.63 ± 0.04	NM[c]	1.4	NM[c]

NA, not available.

[a] Cantu *et al.* (1997).

[b] Bradford *et al.* (1994).

[c] NM, not measured. Only catalytic efficiency was measured by assuming $K_m \gg [S]$ and therefore $V = (k_{cat}/K_m) \times [E][S]$.

[d] Cephaloridine was no longer commercially available.

TABLE 3

Catalytic efficiency values for mutant and wild-type SHV and TEM β-lactamases

	SHV-1	SHV R164S	SHV D179G	TEM-1[a]	TEM R164S[b]	TEM D179G
k_{cat}/K_m (M^{-1} s^{-1})						
AMP	34.5×10^6	42.3×10^6	$0.08\ 0.01 \times 10^6$	29×10^6	NA	1.1×10^6
Ceph	1.4×10^6	0.027×10^6	NM	1.36×10^6	0.46×10^6	NM
CTX	0.25×10^6	0.0042×10^6	0.005×10^6	0.0016×10^6	0.010×10^6	0.005×10^6
CAZ	684	1703	3733	55.1	5200	740
Relative k_{cat}/K_m						
AMP	1	1.23	0.002	0.84	NA	0.030
Ceph	1	0.019	NM	0.97	0.33	NM
CTX	1	0.017	0.020	0.0064	0.04	0.020
CAZ	1	2.5	5.5	0.081 (1)	7.6 (94)	1.14 (14)

NA, not available; NM, not measured.
[a] Cantu *et al.* (1997).
[b] Bradford *et al.* (1994).

TEM Arg164Ser enzyme exhibits a 94-fold increase in catalytic efficiency for ceftazidime hydrolysis when compared to wild-type TEM-1 (Table 3) and a 7-fold increase in efficiency relative to the TEM Asp179Gly enzyme (Table 3). These large differences in catalytic efficiency may explain why TEM Arg164 variants are found among clinical isolates while TEM Asp179 variants are not.

The presence or absence of a variant enzyme in a natural population depends on the ability of the enzyme to provide greater fitness to the bacteria harboring it. Furthermore, the improved fitness must not only be greater than the fitness provided by wild-type enzyme but must also be higher than the fitness provided by other variants. By this hypothesis, the Arg164 mutants in TEM-1 β-lactamase exist because they are able to outcompete both wild-type TEM-1 and TEM Asp179 mutants (Vakulenko *et al.* 1999). Similarly, SHV Asp179 mutants predominate among natural isolates because in the presence of ceftazidime they provide greater fitness than do either wild-type SHV-1 or any of the SHV Arg164 mutants. To test this hypothesis, competition experiments were conducted to compare the relative fitness of bacteria harboring these enzymes in the presence of ceftazidime.

Competition experiments between TEM mutants and SHV mutants: Competition experiments were performed between two *E. coli* strains that were either able (Ara^+) or unable (Ara^-) to utilize arabinose and that harbored different β-lactamase genes. The competition between these β-lactamase-containing strains was for growth in the presence of increasing concentrations of ceftazidime. Equal amounts of each strain, based on optical density, were mixed together and then diluted 1:100 into Luria-Bertani media supplemented with increasing concentrations of ceftazidime. After growth to stationary phase in the competition media, ~100 cells were plated onto agar plates supplemented with arabinose and tetrazolium red. On these agar plates, the Ara^+ strain forms white colonies, while colonies from the Ara^- strain appear red. The number of red and white colonies therefore indicates the ratio of β-lactamase-containing strains, which, in turn, is an indication of the relative fitness of each strain. The relative fitness provided by six β-lactamases, SHV-1, TEM-1, SHV Arg-164Ser, SHV Asp179Gly, TEM Arg164Ser, and TEM Asp179Gly, was tested in two sets of competition experiments. One set included competition between bacteria containing the wild-type SHV-1 or TEM-1 enzyme *vs.* bacteria possessing a substituted enzyme. In the second set of competition experiments, bacteria expressing the β-lactamase variant enzymes were competed against each other.

In the first set of experiments, bacteria harboring TEM-1 β-lactamase were competed against bacteria harboring either TEM Arg164Ser or TEM Asp179Gly. From the MIC data and kinetic analysis of these enzymes, it was hypothesized that both mutant enzymes would exhibit increased fitness relative to the strain containing the wild-type TEM-1 enzyme when these bacteria were grown in increasing concentrations of ceftazidime. This was confirmed in that bacteria harboring either the TEM Arg164Ser enzyme or the TEM Asp179Gly enzyme were able to outcompete bacteria harboring the wild-type TEM-1 enzyme (Figures 4A and 5A). This explains why TEM Arg164Ser mutants exist in the clinical setting, but does not indicate why TEM Asp179Gly mutants are absent among clinical isolates.

To answer why TEM Asp179Gly enzymes do not exist in the clinical setting, competition experiments were performed in increasing concentrations of ceftazidime between bacteria harboring the TEM Asp179Gly enzyme and bacteria expressing the TEM Arg164Ser enzyme. On the basis of the higher catalytic efficiency of TEM Arg164Ser for ceftazidime, it was hypothesized that the TEM Arg164Ser would provide greater fitness to bacteria under conditions of increasing ceftazidime concen-

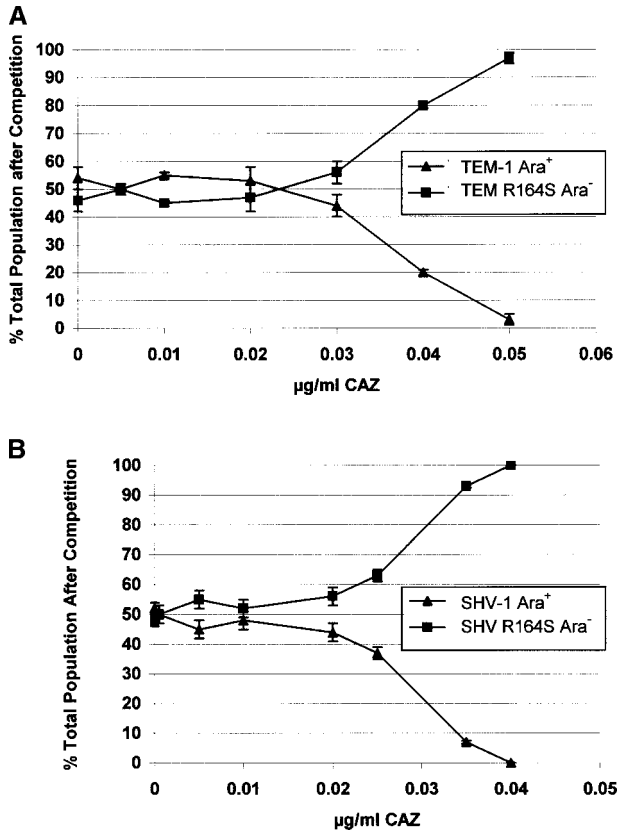

FIGURE 4.—Competition experiments between Arg164 variants and parental enzymes. Each point is an average from three to five competition experiments. All competition experiments were carried out in reciprocal strains. (A) Competition experiment between TEM-1 (triangle) and TEM Arg164Ser (square). (B) Competition experiment between SHV-1 (triangle) and SHV Arg164Ser (square).

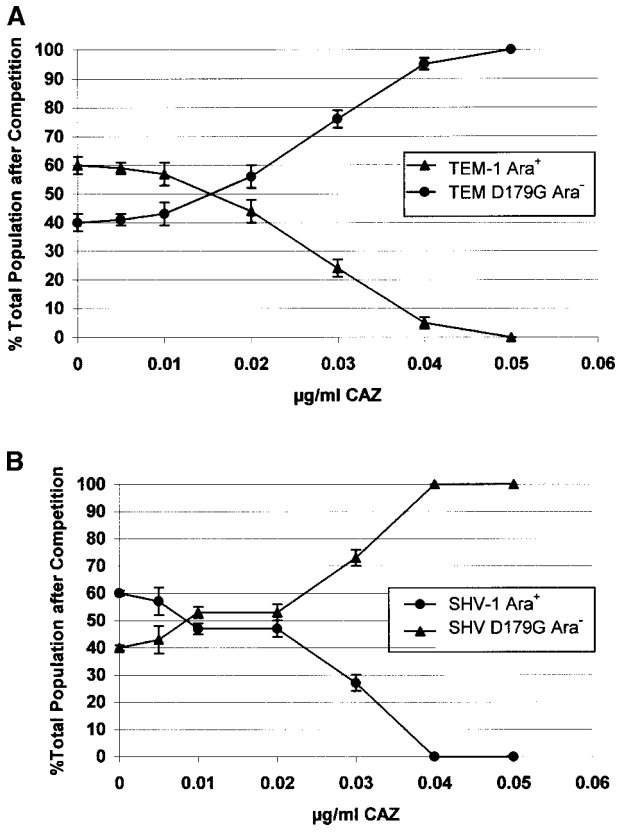

FIGURE 5.—Competition experiments between Asp179 variants and parental enzymes. (A) Competition experiment between TEM-1 (triangle) and TEM Asp179Gly (circle). (B) Competition between SHV-1 (triangle) and SHV Arg179Gly (circle).

tration. TEM Arg164Ser-expressing bacteria do outcompete bacteria harboring TEM Asp179Gly enzymes when ceftazidime concentrations are increased in the competition media (Figure 6A). This result suggests that bacteria containing the TEM Asp179Gly enzyme, despite being more fit than bacteria containing wild-type TEM-1, are not present at detectable frequencies in natural populations because they do not compete effectively with bacteria expressing TEM Arg164Ser β-lactamase.

Competition experiments were also performed between strains containing the wild-type SHV-1 enzyme *vs.* those containing either SHV Arg164Ser or SHV Asp179Gly variants. As shown in Figures 4B and 5B, both the SHV Arg164Ser and SHV Asp179Gly enzymes provide bacteria with improved fitness relative to bacteria containing the wild-type SHV-1 enzyme. Analogous to the situation with the TEM enzymes, this explains the presence of SHV Asp179Gly in natural populations but does not explain the absence of the SHV Arg164Ser mutant.

To address the absence of SHV Arg164Ser, bacteria expressing the SHV Arg164Ser and SHV Asp179Gly enzymes were competed against one another to determine which mutant provides greater fitness under competitive conditions. On the basis of the catalytic efficiencies of the SHV Arg164Ser and SHV Asp179Gly enzymes, it was expected that bacteria harboring the SHV Asp179Gly enzyme would outcompete bacteria containing the SHV Arg164Ser enzyme because the SHV Asp179Gly enzyme hydrolyzes ceftazidime more efficiently. This was found to be the case as expression of the SHV Asp179Gly enzyme did provide its host with increased capacity to survive under the ceftazidime selection conditions relative to bacteria expressing the SHV Arg164Ser enzyme (Figure 6B). Thus, the situation is the opposite of that encountered with TEM in that, in the case of SHV, bacteria containing the Arg164Ser mutant cannot compete effectively with bacteria harboring the Asp179Gly enzyme and therefore the Arg164Ser mutant does not accumulate to any appreciable frequency in natural populations.

DISCUSSION

Closely similar enzymes would be expected to respond to amino acid substitutions and thus to evolve in a comparable fashion. For example, in the case of the TEM-1

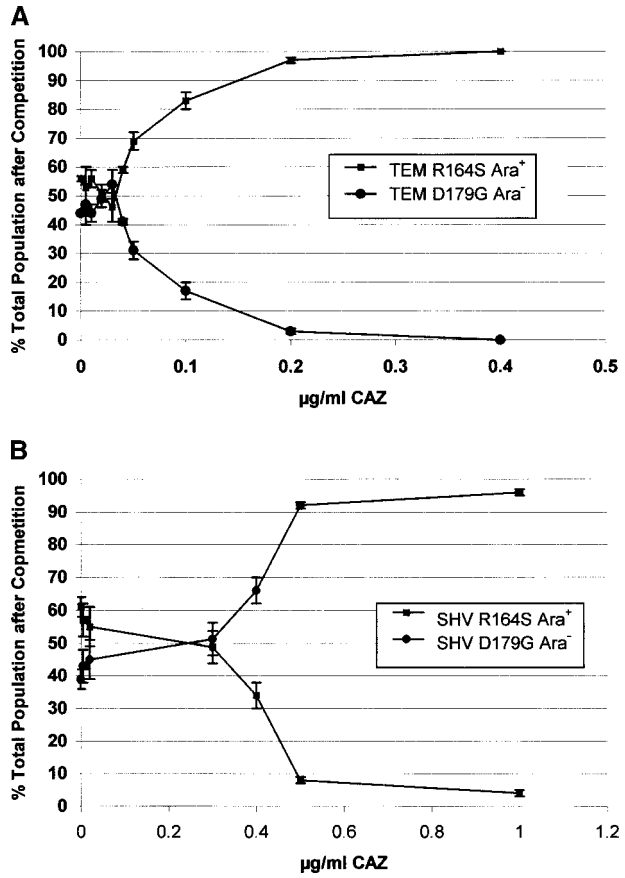

FIGURE 6.—Competition experiments between Ser164 variants and Asp179 variants. (A) Competition experiment between TEM Arg164Ser (square) and TEM Asp179Gly (circle). (B) Competition between SHV Arg164Ser (square) and SHV Arg179Gly (circle).

and SHV-1 β-lactamases, substitutions found in one enzyme among clinical isolates resistant to extended-spectrum β-lactam antibiotics would be expected to be found in the other enzyme. However, this is not the case for the TEM-1 and SHV-1 enzymes at positions Arg164 and Asp179. It is known from this work as well as from the results of others that substitution of Asp179 in TEM-1 and Arg164 in SHV-1 does yield enzymes that provide for higher levels of ceftazidime resistance than the wild-type counterparts do and yet TEM-179 and SHV-164 mutants have not been identified in natural populations. For example, VAKULENKO *et al.* (1999) demonstrated for the TEM-1 enzyme that substitutions at either residue Arg164 or Asp179 result in an extended-spectrum β-lactamase phenotype. In addition, HUJER *et al.* (2001) carried out studies in which the SHV Arg164Ser and Arg164His substitutions were compared to SHV Asp179Asn extended-spectrum β-lactamase. On the basis of the results, it was concluded that an SHV extended-spectrum β-lactamase with substitutions at position Arg164 would be found among clinical isolates of infectious bacteria.

In the competition experiments reported here it was observed that *E. coli* cells carrying plasmids encoding the TEM Arg164Ser variant possess increased fitness in the presence of ceftazidime relative to those encoding the wild-type TEM-1 enzyme. Similarly, strains containing the SHV Asp179Gly mutant display increased fitness relative to those containing the wild-type SHV-1 enzyme. These results were expected on the basis of the known evolution of these variants. The competition experiments also indicated that the SHV Asp179Gly variant is more fit than the SHV Arg164Ser variant. In contrast, for TEM-1, the Arg164Ser mutant is more fit than the Asp179Gly mutant. The fact that this result mirrors the distribution of mutants found in the natural setting suggests that the most important factor for determining whether a mutant evolves is not the competition between the mutant and wild-type enzyme, but rather the competition between the mutant and all other mutants present in the population. The fact that only the most fit mutants, *i.e.*, TEM Arg164Ser and SHV Asp179Gly, predominate in natural populations, despite the fact that many other substitutions at these positions provide increased fitness relative to wild type, suggests that the competition occurring among mutants in nature is very rigorous. The exact stringency of the selection is unclear since it is difficult to estimate precisely the concentration of ceftazidime experienced by bacteria in the natural setting. An estimated peak concentration of 35 μg/ml ceftazidime can be made, however, on the basis of an intravenous dose of 35 mg ceftazidime per kilogram body weight (VINKS *et al.* 1996). This concentration is consistent with a very strong selective pressure on the mutants since it is about twofold higher than the MIC of the most resistant Arg164 mutants.

Although the competition experiments account for the observed distribution of mutants in natural populations, they do not explain the physical basis for the differences seen upon substituting positions Arg164 and Asp179 in TEM-1 and SHV-1. It is known from the X-ray structures of the TEM-1 and SHV-1 enzymes that a salt bridge is formed between position Arg164 and Asp179 and that this salt bridge provides structural stability to the ω-loop (JELSCH *et al.* 1993; KUZIN *et al.* 1999). Also it is believed that when the conformation of the ω-loop is disrupted, the substrate profile of the β-lactamase is altered (PETROSINO and PALZKILL 1996). The extended-spectrum β-lactamase phenotype is an outcome of substitutions at position Arg164 in TEM-1 and of substitutions at position Asp179 in SHV-1 that disrupt the salt bridge and therefore the substrate profile of the enzymes. However, it is not obvious from examination of the TEM-1 and SHV-1 structures why substitutions at position 164 in the TEM enzyme result in larger increases in catalytic efficiency than do substitutions at position 179 and vice versa in the SHV enzyme. The conformations of the 164 and 179 side chains are the same in the two enzymes (JELSCH *et al.* 1993; KUZIN *et al.* 1999). This finding suggests that a subtle structural

difference between the TEM and SHV enzymes results in a significantly different response to amino acid substitutions and in a correspondingly different evolutionary response to the selective pressure of antibiotic therapy.

A possible explanation for the difference in mutational response of TEM-1 and SHV-1 is provided by residue Met182. It is known that the Met182Thr substitution, which is commonly found among extended-spectrum TEM-1 enzymes, suppresses certain deleterious substitutions by altering enzyme folding and stability (Huang and Palzkill 1997; Sideraki *et al.* 2001). In our study, substituting Arg164 in TEM-1 with cysteine does not have as profound an increase in MIC for ceftazidime when compared to the serine or histidine substitutions (Table 1). However, two recently identified variants from clinical isolates, TEM-87 and TEM-91, contain an Arg164Cys substitution (Perilli *et al.* 2002). Interestingly, these extended-spectrum β-lactamases also contain the Met182Thr substitution (Figure 1). Two other TEM-1 variants, TEM-43 and TEM-63, also have Arg164 substitutions in conjunction with threonine substitution at position 182. Thus, the Met182Thr substitution may exert a positive effect on TEM-1 variants with substitutions at residue Arg164. It will be of interest to determine if the Met182Thr substitution also influences the phenotype of substitutions at position 179. If the Met182Thr substitution has a positive effect on TEM-1 variants, would it also have the same effect on SHV-1 variants? In this regard, it is important to note that the wild-type SHV-1 enzyme already possesses threonine at position 182. Thus, it is possible that the difference between TEM-1 and SHV-1 at position 182 influences the differential response of these enzymes to the selective pressure of antibiotic therapy.

We thank Hariharan Jayaram for assistance with Figures 1 and 2 and Carla Perez for assistance with DNA sequencing. We also thank Richard E. Lenski for generously providing the *E. coli* Ara^+ and Ara^- strains. This work was supported by National Institutes of Health grant no. AI32956 to T.P.

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Communicating editor: H. Ochman