

# Predicting Evolutionary Potential: *In Vitro* Evolution Accurately Reproduces Natural Evolution of the TEM $\beta$ -Lactamase

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## ABSTRACT

To evaluate the validity of our *in vitro* evolution method as a model for natural evolutionary processes, the TEM-1  $\beta$ -lactamase gene was evolved *in vitro* and was selected for increased resistance to cefotaxime, cefuroxime, ceftazadime, and aztreonam, *i.e.*, the “extended-spectrum” phenotype. The amino acid substitutions recovered in 10 independent *in vitro* evolvants were compared with the amino acid substitutions in the naturally occurring extended-spectrum TEM alleles. Of the nine substitutions that have arisen multiple times in naturally occurring extended-spectrum TEM alleles, seven were recovered multiple times *in vitro*. We take this result as evidence that our *in vitro* evolution technique accurately mimics natural evolution and can therefore be used to predict the results of natural evolutionary processes. Additionally, our results predict that a phenotype not yet observed among TEM  $\beta$ -lactamases in nature—resistance to cefepime—is likely to arise in nature.

THERE is a long history of using microbial systems as models to study the evolution of novel enzyme functions (MORTLOCK 1984). Those studies have depended on rare spontaneous mutations as the source of variation and have therefore been limited to situations in which a single mutation can yield a novel phenotype at a level that is easily detected by selection in the laboratory. *In vitro* evolution offers an alternative in which random mutations can be introduced at rates that are orders of magnitude greater than those at which living organisms could survive and thus permits screening a much higher fraction of mutation space than can be achieved *in vivo*. It is possible, *in vitro*, to introduce mutations into a single target gene at rates that ensure that most copies carry multiple mutations. Because of this, *in vitro* evolution has become a powerful and widely used tool for engineering proteins to increase activities and/or to extend substrate ranges (CRAMERI *et al.* 1996; MOORE and ARNOLD 1996; ARNOLD and MOORE 1997; MINSHULL and STEMMER 1999; SCHMIDT-DANNERT and ARNOLD 1999; COCO *et al.* 2001; LASSNER and BEDBROOK 2001).

There is also growing evidence that *in vitro* evolution mimics natural evolutionary processes and can therefore be used as a predictor of natural evolutionary processes (VAKULENKO *et al.* 1998; ZACCOLO and GHERARDI 1999; LONG-MCGIE *et al.* 2000; ORENCIA *et al.* 2001).

To assess the validity of a given *in vitro* evolution method as a predictor of natural evolution we need to compare the outcomes of the two processes. Ideally, we would begin

with a natural ancestral gene, evolve that gene *in vitro* under selective conditions similar to those encountered by natural descendants of that gene, and compare the profile of resulting amino acid substitutions obtained in the laboratory with those that occurred in nature. Very few examples in nature will allow us to obtain an ancestral gene, to know the conditions under which it evolved, and to obtain the descendants that evolved under those conditions. Fortunately, the recent rapid evolution of antibiotic resistance has provided us with exactly such a system.

The evolution of antibiotic resistance has had an enormous impact on modern medicine, industry, and science. Drug resistance has threatened public health and has caused doctors and hospitals to revise their strategies for treating infections and illness (COURVALIN and TRIEU-CUOT 2001). The evolution of resistance has also provided an invaluable opportunity to study natural evolution in a short time frame.

Because of their efficiency, specificity, and general absence of toxicity,  $\beta$ -lactam antibiotics, including penicillin, ampicillin, and the cephalosporins and monobactams and their derivatives, account for ~50% of global antibiotic consumption (LIVERMORE 1996; MATAGNE *et al.* 1998). Resistance to  $\beta$ -lactams is the result of expressing  $\beta$ -lactamases, enzymes that degrade and inactivate  $\beta$ -lactams. The most common  $\beta$ -lactamases are the TEM  $\beta$ -lactamases that are encoded by the TEM-1 gene and its descendants. TEM-1 is globally distributed (MEDEIROS 1997) and exists at high frequencies in antibiotic-resistant bacteria (CHANAL *et al.* 2000; YAN *et al.* 2000).

The evolution of the TEM  $\beta$ -lactamases has occurred largely in response to selection imposed by the clinical use of  $\beta$ -lactam antibiotics. At this time, >90 different al-

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leles descended from TEM-1 have been isolated in clinics and hospitals. TEM alleles are distinguished strictly by differences in amino acid, not nucleotide, sequences. For a complete list of the currently identified TEM alleles see <http://www.lahey.org/studies/webt.htm>. The TEM-1  $\beta$ -lactamase hydrolyzes only penicillins and very early cephalosporins effectively, but hydrolyzes more modern cephalosporins and monobactams ineffectively. Within 3 years of the clinical debuts of most new  $\beta$ -lactam antibiotics, descendants of TEM-1 have typically evolved the ability to hydrolyze the new drugs effectively (MEDEIROS 1997). At this time there are only a handful of  $\beta$ -lactam antibiotics that the TEMs cannot yet hydrolyze. Among the best-studied TEM  $\beta$ -lactamases are the so-called "extended-spectrum" TEMs that have evolved the ability to hydrolyze modern  $\beta$ -lactams such as cefotaxime, aztreonam, cefuroxime, and ceftazidime. Because both the nucleotide sequences and the phenotypes of these descendants of TEM-1 are known, and because we are confident that those phenotypes evolved in response to clinical use of those drugs (MEDEIROS 1997), we can directly compare the evolved "extended-spectrum" descendants of TEM-1 with *in vitro* evolved descendants that have been selected for resistance to the same drugs.

Through the directed evolution of the TEM-1  $\beta$ -lactamase, several researchers have been able to recover substitutions that have arisen in nature. STEMMER (1994) used gene shuffling to introduce many mutations into TEM-1, selected mutants that conferred resistance to cefotaxime, and, after several rounds of shuffling and selection, recovered an allele that conferred a level of resistance that was 64-fold greater than that of any TEM alleles that had yet been characterized. VAKULENKO *et al.* (1998) mutagenized TEM-1 using Taq DNA polymerase under error-prone conditions, selected for resistance to the  $\beta$ -lactamase inhibitor clavulanic acid, and successfully isolated several mutations that conferred the inhibitor-resistant phenotype. ZACCOLO and GHERARDI (1999) developed a novel mutagenesis method on the basis of the use of nucleoside analogs to introduce 8–28 mutations per copy into the TEM-1 gene and also recovered mutants with very high levels of resistance to cefotaxime. Some of those mutants, obtained with a single round of mutagenesis and selection, were as resistant to cefotaxime as the mutant obtained by Stemmer after three rounds of selection and mutagenesis.

Each of these studies recovered amino acid substitutions that are present in naturally occurring extended-spectrum or inhibitor-resistant TEM  $\beta$ -lactamases, but only two explicitly addressed the issue of using directed evolution to predict the natural evolution of TEM  $\beta$ -lactamases (VAKULENKO *et al.* 1998). They found that they recovered all of the amino acid substitutions that had been implicated in inhibitor resistance in naturally occurring TEM  $\beta$ -lactamases, but found no additional single-amino-acid replacements that conferred the in-

hibitor-resistant phenotype. Because they could be confident that they had screened all possible single-amino acid replacements they were able to conclude that no new single-replacement inhibitor-resistant TEM  $\beta$ -lactamases are likely to appear in nature, but they also cautioned that they could not rule out the possibility that new multiple-substitution inhibitor-resistant TEM  $\beta$ -lactamases would appear.

ORENCIA *et al.* (2001) have argued that experimental evolution can be used as a valid predictor of natural evolution and indeed claimed that experimental evolution has already been used to make predictions about how evolution will occur in nature. They claim that the M182T substitution observed in that same study predicted the appearance of M182T in TEM-20 in 1995. In fact, M182T appeared in nature in an allele now designated TEM-32 in 1993 (BLAZQUEZ *et al.* 1993), a year prior to the Stemmer article. Because three of the substitutions recovered in Stemmer's ST-2 allele (STEMMER 1994) later appeared together in the naturally occurring TEM52 allele, ORENCIA *et al.* (2001) also claim that through the creation of his mutant allele, Stemmer predicted the emergence of TEM52. However, all of the substitutions in TEM-52 had been reported by 1993, at least a year before Stemmer published his article. The fact that these substitutions later appeared in the same allele is not surprising because many naturally occurring TEMs consist of unique combinations of the same substitutions. Although it is indeed the case that the three substitutions that appeared in 1998 in TEM-52 were present in Stemmer's ST-2 allele (STEMMER 1994), there were three additional substitutions in the ST-2 allele. It is thus difficult to consider that particular combination a "prediction." To our knowledge, no one has yet predicted a substitution that will confer antibiotic resistance before that substitution has arisen in nature.

We are not convinced that it is possible to generalize the validity of *in vitro* evolution as a predictor of natural evolution over all *in vitro* evolution methods. For instance, the mutagenic spectra of the various methods differ significantly from each other and from spontaneous mutagenic spectra that have been measured (see Table 1) and we consider it unlikely that such dissimilar mutagenic spectra would yield equally valid predictions of natural evolution. It is our purpose here to test the specific *in vitro* evolution method that we use with respect to its validity as a predictor of natural evolution. If the method validly predicts natural evolution then it can be used to predict the natural evolution of novel substrate specificities for the TEM  $\beta$ -lactamases, to predict the evolution of other  $\beta$ -lactamases, to predict the evolution of other antibiotic resistance genes, and to predict the evolution of other functions in microorganisms.

The several studies that have used *in vitro* evolution to improve the activity of TEM  $\beta$ -lactamases have all selected for improved activity toward a single drug, and in most cases the goal has been to maximize the resis-

tance conferred by the mutant allele. While the results of each of those studies may well represent a technical *tour de force*, we do not think that they represent a very realistic approach to predicting natural evolution. For instance, selection for improved resistance to a single drug fails to take into account the fact that in nature extant alleles are likely to encounter a variety of drugs. Indeed, no systematic approach for using *in vitro* evolution to predict how evolution will occur in nature has yet been described.

For evolution to act the same way *in vitro* that it does *in vivo*, three features of natural evolution should be preserved in an artificial evolutionary system. First, the target gene should be randomly mutated and the mutagenic spectrum of the enzyme used to mutate it should be similar to the mutagenic spectrum of the organism from which the gene was taken. Second, the selective pressure used for the *in vitro* evolution of a gene should be similar to the selective pressure in nature. Third, selection for advantageous phenotypes already conferred by the gene should be maintained during the *in vitro* evolution of that gene.

In this article we expand upon the preliminary work that others have done to show that our method of *in vitro* evolution mimics natural evolutionary processes, and we develop a systematic approach for using *in vitro* evolution as a general predictor of natural evolution and assess the validity of this approach in more general terms than have previously been addressed.

## MATERIALS AND METHODS

**Bacterial strains and plasmids:** *Escherichia coli* strain DH5 $\alpha$ E [F<sup>-</sup>  $\phi$ 80d*lacZ* $\Delta$ M15  $\Delta$ (*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r<sup>-</sup>m<sup>+</sup>) *deoR* thi-1 *phoA supE44*  $\lambda$ <sup>-</sup> *gyrA96 relA1 gal*-; GIBCO BRL, Gaithersburg, MD] was used as the host for all plasmids.

Plasmid pACSE3 was used as the vector for cloning and expressing TEM alleles. pACSE3 was created in three steps. In step 1 the *Msd* fragment (base pairs 2938–3980) of pACYC184 (CHANG and COHEN 1978) was replaced with base pairs 6–622 of pSE380 (Invitrogen, San Diego) to yield the plasmid pACSE. In step 2 the *lacI<sup>q</sup>* gene (base pairs 3269–167) of pSE380 was PCR amplified using primers with *SpeI* and *XbaI* sites and ligated into the *SpeI* site of pACSE to create pACSE2. In step 3 site-directed mutagenesis was used to (a) replace the *NcoI* restriction site (base pairs 533–539) with a *BspHI* restriction site and (b) destroy a *BspHI* site in the tetracycline resistance gene. Finally, a *BspHI* fragment (base pairs 534–648 of pACSE2) was deleted to create pACSE3. Plasmid pACSE3 carries the p15A origin of replication (5–10 copies/cell), a gene for tetracycline resistance, the *lacI<sup>q</sup>* gene that encodes the Lac repressor, and a regulatory region that includes the lac-repressor-regulated pTac promoter, a strong ribosome-binding site and initiation codon, and a multiple cloning region, all derived from pSE380. The chloramphenicol resistance gene of pACYC184 is partially deleted.

Plasmids pACTEM1 and pACTEM3 are pACSE3, into which the TEM-1 and TEM-3 genes, respectively, have been cloned as described below.

**Media:** L-broth is 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 g/liter glucose. L-tetracycline medium is L-broth

containing 15 mg/liter tetracycline. Mueller-Hinton broth (Difco), the standard medium for assessing antibiotic resistance, was prepared according to the manufacturer's instructions. SOC medium is 20 g tryptone and 5 g/liter yeast extract with 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, and 20 mM glucose. Solid media contained 15 g/liter agar.

**Recombinant DNA methodology:** Plasmids were prepared from overnight cultures grown in L-tetracycline broth. Plasmids were purified using the QIAGEN (Chatsworth, CA) QuickSpin kit according to manufacturer's instructions.

TEM-1, TEM-3, and the mutated TEM alleles were amplified with the FailSafe (Epicentre, Madison, WI) polymerase system using FailSafe Buffer A and the following cycling program: denaturation at 94° for 30 sec, annealing at 65° for 30 sec and extension at 72° for 90 sec repeated for 25 cycles, followed by 1 cycle of 72° for 5 min. TEM-1 was amplified from pBR322 (BOLIVAR *et al.* 1977) and TEM-3 from *Klebsiella pneumoniae* that harbors a naturally occurring TEM-3 plasmid. Both genes were amplified using primers P1 (GGGGGGT**TCATGAGTATT** CAACATTTCCGTGTCG), which has a *BspHI* site (boldface type), and P2 (CCGAGCTCTTGGTCTGACAGTTACCAATGC), which has a *Sad* site.

The resulting amplicons were purified with QIAGEN PCR purification columns according to the manufacturer's instructions and digested with the restriction endonucleases *BspHI* (New England Biolabs) and *Sad* (New England Biolabs, Beverly, MA). The plasmid pACSE3 was digested with the restriction endonucleases *BspHI* and *Sad* and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). Digested amplicons and vector were purified with QIAGEN PCR purification columns, combined, ligated with T4 DNA Ligase (GIBCO BRL), and transformed into strain DH5 $\alpha$ E by electroporation.

**Mutagenesis:** Mutant TEM alleles were created by introducing random mutations into TEM-1 with the Genemorph PCR mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used for the PCR amplification were P3 (TCATCCGGCTCGTATAATGTGTGGA) and P4 (ACTCTCTTCCGGGCGCTATCAT). Those primers flank the multiple cloning site of pACSE3 and are used to amplify inserts cloned into this vector.

The mutation frequency is determined by the number of amplification cycles using the Mutazyme enzyme in the Genemorph kit. The number of cycles was chosen to introduce, on average, two mutations into the TEM coding sequence.

The resulting mutated amplicons were further amplified with the FailSafe system as described above, digested with *BspHI* and *Sad* endonucleases, ligated into plasmid pACSE3, and electroporated into strain DH5 $\alpha$ E. After allowing 90 min for expression in SOC medium at 37°, an aliquot of the transformed culture was suitably diluted and spread onto L-tetracycline plates to estimate the total number of transformants, *i.e.*, the total library size. The remainder of the culture was diluted into L-tetracycline broth and allowed to grow overnight to expand the library. An aliquot of the expanded library was stored frozen in 9% dimethylsulfoxide at -80°.

The following day nine colonies were randomly chosen from an L-tetracycline plate, boiled to make a crude DNA preparation, and used as template in a PCR reaction with primers P3 and P4. The resulting amplicons were subjected to agarose gel electrophoresis, and the effective library size was estimated from the fraction of colonies containing insert-bearing plasmids.

**Expression of TEM alleles:** Cloned TEM alleles from pACSE3-based plasmids are expressed from the pTac promoter that is tightly regulated by the lac repressor that is encoded by the *lacI* gene on plasmid pACSE3. Expression of the TEM alleles was induced by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). To determine the concentration of IPTG required to induce

the expression of TEM at appropriate levels, the minimum inhibitory concentrations (MICs) of piperacillin, cephalothin, and cefuroxime for a strain of DH5 $\alpha$ -E carrying a naturally occurring plasmid bearing TEM-3 were determined. The concentration of IPTG required to give an equivalent MIC for those antibiotics for the DH5 $\alpha$ E-carrying plasmid pACTEM3 was determined to be 50  $\mu$ M IPTG.

**Antibiotics and determination of MICs:** The following  $\beta$ -lactam antibiotics were used in this study: ampicillin (Sigma, St. Louis), piperacillin (Sigma), piperacillin + tazobactam (Lederle), temocillin (SmithKline Beecham), ticarcillin (SmithKline Beecham), ticarcillin + clavulanate (SmithKline Beecham), cephalothin (Sigma), cefuroxime (Sigma), ceftriaxone (Roche), ceftazidime (Glaxo Wellcome), cefotaxime (Sigma), cefepime (Bristol-Myers Squibb), and aztreonam (Bristol-Myers Squibb). Stock solutions of antibiotics were prepared in 0.1 M NaPO<sub>4</sub> buffer, pH 7.0, and stored at  $-80^{\circ}$  in single-use aliquots.

MICs were determined from 0.5-ml cultures at a titre of  $10^5$  cells/ml in Mueller-Hinton broth (Difco) containing 50  $\mu$ M IPTG. Twofold serial dilutions of the antibiotics were added to each culture and the cultures were grown overnight at  $37^{\circ}$ . The MIC is defined as the lowest concentration of antibiotic that completely prevented visible growth of the culture.

**Disk diffusion test for antibiotic resistance:** Mueller-Hinton agar containing 50  $\mu$ M IPTG was spread with  $\sim 10^8$  cells from standing overnight cultures in L-tetracycline broth of the strains being tested. The plates were then stamped with a set of antibiotic discs (BBL) containing cephalothin (30  $\mu$ g), piperacillin (100  $\mu$ g), ceftazidime (30  $\mu$ g), cefotaxime (30  $\mu$ g), cefuroxime (30  $\mu$ g), and aztreonam (30  $\mu$ g). The resistance phenotype is indicated by the zone of inhibition around each disk after incubation of the plate overnight at  $37^{\circ}$ .

**Selection for extended-spectrum TEM alleles:** Fifty-milliliter cultures in Mueller-Hinton broth containing 50  $\mu$ M IPTG, each containing a number of cells equivalent to 10 times the total library size, were adjusted to cefotaxime concentrations, in twofold increments, ranging from 0.125  $\mu$ g/ml (the TEM-1 MIC) to 32  $\mu$ g/ml (the cefotaxime MIC for TEM-3 in our hands). Cultures were incubated for 36 hr at  $37^{\circ}$ , a time sufficient for a single cell to grow to culture saturation. The inoculum of 10 times the total library size ensured that the probability that any member of the library was absent from a culture was  $<10^{-4}$ . The culture that grew at the highest concentration of cefotaxime was selected and grown in a similar dilution series of cultures in cefuroxime-containing medium, followed by similar selections in ceftazidime and aztreonam at concentrations from the TEM-1 MIC up to twice the concentration that is defined by the NCCLS as indicating clinical resistance (NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS 1999). To ensure that resistance to ampicillin, piperacillin, and cephalothin had been retained, the culture was sequentially grown in the presence of each drug at twice the clinical MIC concentration.

A population was considered to be fully resistant to a drug only if it grew at the highest concentration used for selection. At the end of the first round of selection no population was fully resistant to any of the four "extended-spectrum" drugs. Plasmid was prepared from the final culture grown in cephalothin-containing medium and subjected to mutagenesis and cloning as described above. A second, identical, round of selection was carried out, after which the population was fully resistant to all four extended-spectrum drugs.

To assess the number of unique alleles, plasmid was purified from the final second-round culture, electroporated into DH5 $\alpha$ E, and 15 individual transformants were selected on L-tetracycline agar. Phenotypic differences were assessed by disk diffusion tests. In eight of the nine evolved lines the phenotypes of the 15 individuals were indistinguishable, sug-

gesting that a single mutant clone dominated the culture, a phenomenon known as clonal displacement. One individual was retained, phenotypically characterized on several drugs (Table 2), and sequenced (Table 3).

Eight similar experiments were conducted, differing only in the order with which the mutagenized libraries were challenged by the extended-spectrum drugs. Experiments 1, 2, and 3 were as described above. For experiments 4 and 5, the order of selection was cefuroxime, ceftazidime, aztreonam, cefotaxime; for experiments 6 and 7, the order was ceftazidime, aztreonam, cefotaxime, cefuroxime; and for experiments 8 and 9, the order was aztreonam, cefotaxime, cefuroxime, ceftazidime.

**Sequencing:** For TEM-1, TEM-3, and each TEM allele that was selected, the sequence was verified or determined from an amplicon of the TEM insert. Amplification reactions were carried out using Taq PCR Master Mix (QIAGEN) with primers P3 and P4 under the conditions described above. The amplicons were purified with the QIAGEN PCR purification columns and sequenced on an ABI Prism 377 DNA sequencer with the ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) according to the manufacturer's protocol.

**Phylogenetic analysis:** Sequences were obtained from GenBank. A complete list of accession numbers and references is available at [http://www.rochester.edu/College/BIO/labs/HallLab/TEM\\_Phylo.html](http://www.rochester.edu/College/BIO/labs/HallLab/TEM_Phylo.html). Sequences were aligned using ClustalX (THOMPSON *et al.* 1997) using default gap penalties for nucleotide alignments.

Phylogenies were constructed by the Bayesian method (RANALALA and YANG 1996; MAU and NEWTON 1997; MAU *et al.* 1999) as implemented by the program MrBayes (HUELSENBECK 2000). MrBayes is available from <http://brahms.biology.rochester.edu/software.html>. The Bayesian method seeks the most likely trees given the data (the alignment) and the evolutionary model. The posterior probabilities of the phylogeny, branch lengths, and substitution parameters cannot be calculated directly; however, they can be approximated by a Markov chain Monte Carlo (MCMC) process by sampling trees from the posterior probability distribution. A variant of MCMC, called metropolis-coupled Markov chain Monte Carlo (MCMCMC), runs several chains, some number of which are heated. A heated Markov chain has the posterior probability of a tree raised to some power  $i$ . Heated Markov chains can more easily cross deep likelihood valleys. The effect of heating is to fill in valleys and lower peaks; hence, a heated Markov chain can better explore the parameter space. Using the MCMCMC algorithm, a swap of the states between two chains is attempted at each step. If the swap is accepted, then the states for the two chains are exchanged. If a swap occurs between a heated chain and the cold chain, the cold chain might cross a large valley that it would normally cross with only a very small probability. A more detailed discussion of the method and its implementation is provided in HALL (2001).

The evolutionary model was the general time reversible model (TAVARÉ 1986), and among-site variation in evolutionary rate was estimated separately for first, second, and third positions of sites within codons. Four chains, with a "temperature" of 0.2 for the heated chains, were run for 200,000 generations, sampling trees every 100 generations. The ln likelihood of the trees had converged on a constant value by generation 30,000, *i.e.*, after saving 300 trees. The consensus tree was calculated from the final 1700 trees visited, well after convergence had occurred.

One of the advantages of Bayesian inference of phylogeny is that the results are easy to interpret. For example, the sum of the posterior probabilities of all trees will be 1. Moreover, the posterior probability of any single clade is simply the sum of the posterior probabilities of all trees that contain that

TABLE 1  
Mutational spectra

Mutation	Spontaneous (%)			<i>Taq</i> <sup>c</sup> (%)	Nucleoside analogs (%) <sup>d</sup>	Mutazyme <sup>e</sup> (%)
	<i>lacI</i> <sup>a</sup>	<i>ebgR</i> <sup>b</sup>	<i>E. coli</i> average			
AT to GC	6	9	8	31	75	10
GC to AT	56	24	40	21	20	45
GC to TA	16	42	29	7	<1	20
GC to CG	4	4	4	0	0	9
AT to CG	9	11	10	7	5	4
AT to TA	9	11	10	34	0	11

Values shown are the percentages of base substitutions.

<sup>a</sup> HALLIDAY and GLICKMAN (1991).

<sup>b</sup> HALL (1999).

<sup>c</sup> SHAFIKHANI *et al.* (1997).

<sup>d</sup> ZACCOLO and GHERARDI (1999).

<sup>e</sup> GeneMorph PCR mutagenesis kit instruction manual (Stratagene).

clade. The consensus tree calculated by MrBayes does not include the posterior probabilities of the clades; thus the entire set of trees was imported into PAUP\* (SWOFFORD 2000) and the same trees used by MrBayes to calculate a consensus were used to calculate a 50% majority rule consensus in PAUP\* (SWOFFORD 2000). The resulting tree shows the posterior probabilities of the clades.

The consensus trees calculated by MrBayes were imported into PAUP\* for the purposes of displaying and printing the tree. The tree was rooted by using the SHV sequences as a monophyletic outgroup.

## RESULTS AND DISCUSSION

The target gene TEM-1 was mutated by random PCR mutagenesis with the GeneMorph PCR mutagenesis kit (Stratagene), because the spectrum of mutations introduced by the Mutazyme polymerase in that kit is similar to the average mutation spectrum of *E. coli* (Table 1) and because the frequency of mutations can be controlled. Conditions were chosen to introduce, on average, two mutations per copy into the TEM-coding sequence. That mutation rate ensured that all possible single-point mutations were sampled and that a fraction of all possible double- and triple-point mutations were sampled in each experiment. Mutations were introduced at that frequency because, in nature, mutations usually occur one at a time and very rarely in pairs. A low mutation rate is important because mutations that may be deleterious when introduced one at a time may confer a fitness advantage when introduced simultaneously. Such mutations would probably not go to fixation in nature because it is unlikely that they would arise simultaneously in the same genome. High mutation rates also cause more neutral and deleterious mutations to hitchhike their way to fixation.

Nine independent libraries of mutated TEM alleles were created as described in MATERIALS AND METHODS. The average library size was  $1.3 \times 10^6$  insert-bearing transformants per experiment.

Extended-spectra TEM alleles typically confer ele-

vated resistance to one or more of the following drugs: cefotaxime, cefuroxime, ceftazidime, or aztreonam. In addition, they confer resistance to ampicillin, piperacillin, and cephalothin.

The selective pressures that have directed the natural evolution of the TEM  $\beta$ -lactamase have not been high concentrations of a single antibiotic such as STEMMER (1994) employed in the directed evolution of TEM-1 for cefotaxime resistance. BLAZQUEZ *et al.* (2000) showed that there is probably pressure in nature for the maintenance of resistance to multiple antibiotics. During *in vivo* evolution experiments in which selective pressure was alternated between ceftazidime and amoxicillin, they recovered only mutations that had already been found in naturally occurring TEM alleles. However, when continuous selection for ceftazidime was applied, mutations that had not been observed in nature were recovered, and in all cases, those mutations caused a drop in the ampicillin resistance.

We selected for increased resistance by subjecting the population from each mutagenized library to selection for growth in the presence of cefotaxime, cefuroxime, ceftazidime, and aztreonam as described in MATERIALS AND METHODS. Those antibiotics were selected because they have been in clinical use for several years, TEM-1 does not confer resistance to them, and many of TEM-1's descendants do confer resistance to them. Drug concentrations ranged from the minimum concentration that inhibited cells expressing the TEM-1  $\beta$ -lactamase up to twice the concentration that the NCCLS (NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS 1999) defines as indicating clinical resistance. The exception was cefotaxime, for which the maximum concentration used was the MIC for strains expressing the TEM-3  $\beta$ -lactamase. Selection for new resistance phenotypes was followed by selection for the maintenance of resistance to ampicillin, piperacillin, and cephalothin, in that order. Those antibiotics were selected because TEM-1 confers resistance to them and because natural TEM alleles have

TABLE 2  
MICs of evolved TEM alleles

Drug <sup>a</sup>	Clinical MIC <sup>b</sup>	Isolate no.										
		1	2	3	4	5	6	7a	7b	8	9	
AMC	32	>4096	4096	4096	4096	4096	>4096	>4096	4096	>4096	4096	4096
PIP	128	2048	2048	2048	2048	2048	2048	4096	1024	2048	2048	2048
TZP	128	16	16	64	32	128	64	64	32	32	32	32
TIC	128	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	2048	2048	>4096
TIM	128	256	128	128	128	256	128	128	64	128	128	128
CEF	32	512	512	1024	512	512	64	256	64	64	64	256
CXM	32	16	16	1024	1024	1024	1024	128	64	32	32	512
(GRO)	64	0.063	0.25	64	64	64	64	32	16	32	32	64
(CTX)	64	0.125	512	512	256	512	256	32	16	32	32	32
(CAZ)	32	0.25	64	64	64	512	64	2048	512	2048	2048	2048
FEP	32	0.063	8	8	8	8	8	16	4	64	64	8
(ATM)	32	0.125	64	64	64	128	64	1024	128	1024	1024	512

The MICs of the antibiotic in which the lines were first selected appear italic.

<sup>a</sup> Abbreviations for  $\beta$ -lactams are the following: AMC, ampicillin; PIP, piperacillin; TZP, piperacillin and tazobactam (8:1); TIC, ticarcillin; TIM, ticarcillin and clavulanate; CEF, cephatothrin; CXM, cefuroxime; GRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazadime; FEP, cefepime; ATM, aztreonam. The "extended-spectrum" drugs used for selection are in parentheses.

<sup>b</sup> The indicated MICs are the NCCLS breakpoints for resistance (NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS 1999).

been subjected to them through extensive clinical use. The selection strategy is realistic because naturally occurring TEM alleles have evolved in response to different antibiotics (KITZIS *et al.* 1988; CHANAL *et al.* 1992).

None of the populations reached the desired level of resistance in one round of selection and were therefore subjected to a second round of mutagenesis and subjected to the same selection that had been applied during the initial round. After the second round of selection, all populations grew at the maximum concentration of each of the drugs used.

Following the second round of mutagenesis and selection, to determine the extent of phenotypic variation in each population, plasmid was purified from each population and transformed into DH5 $\alpha$ E to eliminate any phenotypic variation that resulted from selection on the host. Fifteen transformants from each population were chosen and phenotypes were identified by disk diffusion tests. For every population except line 7, only one phenotype was recovered, indicating that in most populations one allele conferred the greatest fitness advantage and the carrier of that allele eliminated all others in the population. One isolate from each population (two from line 7) was retained for phenotypic characterization and sequencing.

**Phenotypic evolution:** Table 2 shows that the order of selection is very important in determining the phenotype that is recovered. In each case resistance to a given drug was greatest when that drug was used first in the selection procedure. Clonal displacement, the rapid domination of the population by the fittest alleles, probably ensures that very few alleles remain after selection in the initial drug used. Clearly selection imposed by low-to-moderate concentrations of antibiotics selects for high levels of resistance. For example, populations 1–3, selected for growth in 32  $\mu$ g/ml cefotaxime, include alleles that confer resistance to cefotaxime at MICs of either 256 or 512  $\mu$ g/ml. In every population, resistance was selected at a much higher concentration than the concentration of the first antibiotic to which they were first exposed. This suggests that the greater the level of resistance, the greater the fitness advantage even at low concentrations of that antibiotic. Alleles that conferred even higher levels of resistance may well have been present immediately after selection in the first drug, but those that may have conferred lower resistance levels in subsequent drugs may well have been lost.

Table 2 also shows that there is indirect selection for resistance to antibiotics that were not used for selection. For example, selection in cefotaxime and cefuroxime caused ceftriaxone resistance to evolve. Selection in ceftazadime and aztreonam caused cefepime resistance to increase greatly.

**Sequence evolution:** Table 3 shows the mutations and resulting amino acid substitutions that were recovered during the nine *in vitro* evolution experiments, while Table 4 shows the amino acid substitutions that are

**TABLE 3**  
Mutations recovered from *in vitro* evolved TEM alleles

DNA site <sup>a</sup>	Mutation	Amino acid substitution <sup>b</sup>									
		1	2	3	4	5	6	7a	7b	8	9
11	A>G					Q6R					
45	G>A	s									
105	A>G							s	s		
199	C>G			A42G							
220	C>T			s							
304	G>A	(E104K)	(E104K)	(E104K)	(E104K)	(E104K)	(E104K)			(E104K)	
484	C>A							(R164S)	(R164S)	R164N <sup>c</sup>	(R164S)
485	G>A						(R164H)			R164N <sup>c</sup>	
496	G>A										E168K
498	G>A									s	
511	A>G						I173V	I173V		I173V	
512	T>G										I173T
539	T>C	(M182T)					(M182T)				
546	A>G										s
562	T>C		s								
571	C>T									s	
660	C>T		s								
697	T>A							s			
703	G>T								A237S		A237S
706	G>A	(G238S)	(G238S)	(G238S)	(G238S)	(G238S)	(G238S)				
709	G>A							(E240K)	(E240K)		(E240K)
748	G>C							D254H			
749	A>G					D254G					
756	G>A	s									
782	C>T		(T265M)			(T265M)					
784	A>G	T266A									
787	G>A						G267R				
825	C>A										s

Substitutions that have arisen multiple times in nature are in parentheses. s, a silent mutation.

<sup>a</sup> Position in the coding sequence.

<sup>b</sup> Substitutions are given as the TEM-1 amino acid according to the IUPAC single-letter code, the position in the protein numbered according to AMBLER *et al.* (1991), and the mutant amino acid. E104K means that the glutamate at position 104 of TEM-1 was replaced by lysine.

<sup>c</sup> The R164N mutation is the result of two base substitutions.

present in the naturally occurring extended-spectrum TEM alleles. The bottom row in Table 4 indicates the number of times that each amino acid substitution has arisen *independently*. Independent occurrence is based on analysis of the TEM phylogeny (Figure 1). For instance, although the Q39K substitution is present in nine naturally occurring TEM alleles, it arose only once in TEM-2. The E104K substitution, in contrast, has arisen independently seven times. We assume that substitutions that have arisen and been fixed more than once have been fixed by positive selection; *i.e.*, they contribute directly to a fitness advantage conferred by that allele. Substitutions that have arisen only once may be advantageous, neutral, or even slightly deleterious.

Nine mutations have arisen independently multiple times in nature: L12F, E104K, R164S, R164H, M182T, A237T, G238S, E240K, and T265M. Seven of those, E104K, R164S, R164H, M182T, G238S, E240K, and T265M, were recovered during our *in vitro* evolution experiments (Ta-

ble 4). Of those, all except R164S were recovered multiple times in independent experiments. The A237S substitution was also recovered twice. Although that substitution has not been recovered in natural TEM alleles, the A237T substitution has arisen independently at least twice in nature, and we note that serine and threonine differ by a single methyl group. The other mutation we recovered multiple times that has not been found in nature is I173V.

These results demonstrate that *in vitro* evolution with the Mutazyme polymerase recovers substantially the same mutations that are selected in nature. We take this as evidence that our method of *in vitro* evolution accurately mimics nature and can validly be used to predict natural evolution not only for antibiotic resistance genes but for other genes of interest.

Although this project was designed primarily to validate *in vitro* evolution as a good mimic of natural evolution, these results do allow us to begin making predictions

**TABLE 4**  
**Amino acid substitutions in naturally occurring extended-spectrum TEM alleles**

Enzyme	Amino acid position														
	21	39	42	51	92	104	153	164	182	237	238	240	244	265	268
TEM-1	L	Q	A	L	G	E	H	R	M	A	G	E	R	T	S
TEM-2 <sup>a</sup>		K													
TEM-3		K				K					S				
TEM-5								S		T		K			
TEM-6						K		H							
TEM-8		K				K		S			S				
TEM-9	F					K		S						M	
TEM-10								S				K			
TEM-11		K						H							
TEM-12								S							
TEM-15						K					S				
TEM-17						K									
TEM-20									T		S				
TEM-21		K				K	R				S				
TEM-22		K				K				G	S				
TEM-24		K				K		S		T		K			
TEM-25	F										S			M	
TEM-26						K		S							
TEM-27								H				K		M	
TEM-28								H				K			
TEM-29								H							
TEM-42		K	V								S	K		M	
TEM-43						K		H	T						
TEM-47											S	K		M	
TEM-48	F										S	K		M	
TEM-49	F										S	K		M	G
TEM-53	F										S				
TEM-54													L		
TEM-60		K		P		K		S							
TEM-66		K			D	K					S				
TEM-72									T		S	K			
No. of times the mutation has arisen	3	1	1	1	1	7	1	H5 S7	3	G1 T2	5	6	1	2	1

Data are from <http://www.lahey.org/studies/webt.htm>. The number of times each mutation has arisen independently is based on the phylogeny shown in Figure 1.

<sup>a</sup> TEM-2 is not an extended-spectrum allele but is shown because the Q39K substitution that is found in nine extended-spectrum alleles arose in TEM-2.

about the evolution of antibiotic resistance. For instance, natural TEM alleles that confer clinical resistance to cefepime have not yet been recovered. The highest level of resistance to cefepime that has been reported is a MIC of 8 µg/ml (PERILLI *et al.* 2000; REBUCK *et al.* 2000). Clones 6 and 8 exhibit cefepime resistance increased to the level of clinical resistance. Because the I171V substitution was recovered repeatedly in alleles with increased cefepime resistance, it seems likely that the I171V substitution is important for that phenotype.

We note that the clones most resistant to cefepime are the least resistant to cefuroxime. We speculate that cefepime resistance conferred by a TEM allele has not yet been observed in nature because of the high use of cefuroxime (<http://www.rxlist.com/top200a.htm>) and the relatively recent introduction of cefepime.

We have provided strong evidence that *in vitro* evolu-

tion reproduces natural evolution that has already occurred and that, when used properly, *in vitro* evolution can make strong predictions about how natural evolution will proceed in the future. Evolutionary biologists can now proceed to use *in vitro* evolution with confidence to predict the evolution of their favorite genes. There is also an immediate and practical application of *in vitro* evolution to antibiotic resistance: As new antibiotics are developed, *in vitro* evolution can be used to predict how known resistance genes are likely to evolve in response to use of the new antibiotic. In turn, the *in vitro* evolved genes that confer resistance to the novel antibiotic can be used as a test bed for the next generation of drug. *In vitro* evolution can also be used to search for resistance phenotypes that are mutually exclusive and, from this information, devise prescription strategies that inhibit the evolution of resistance.

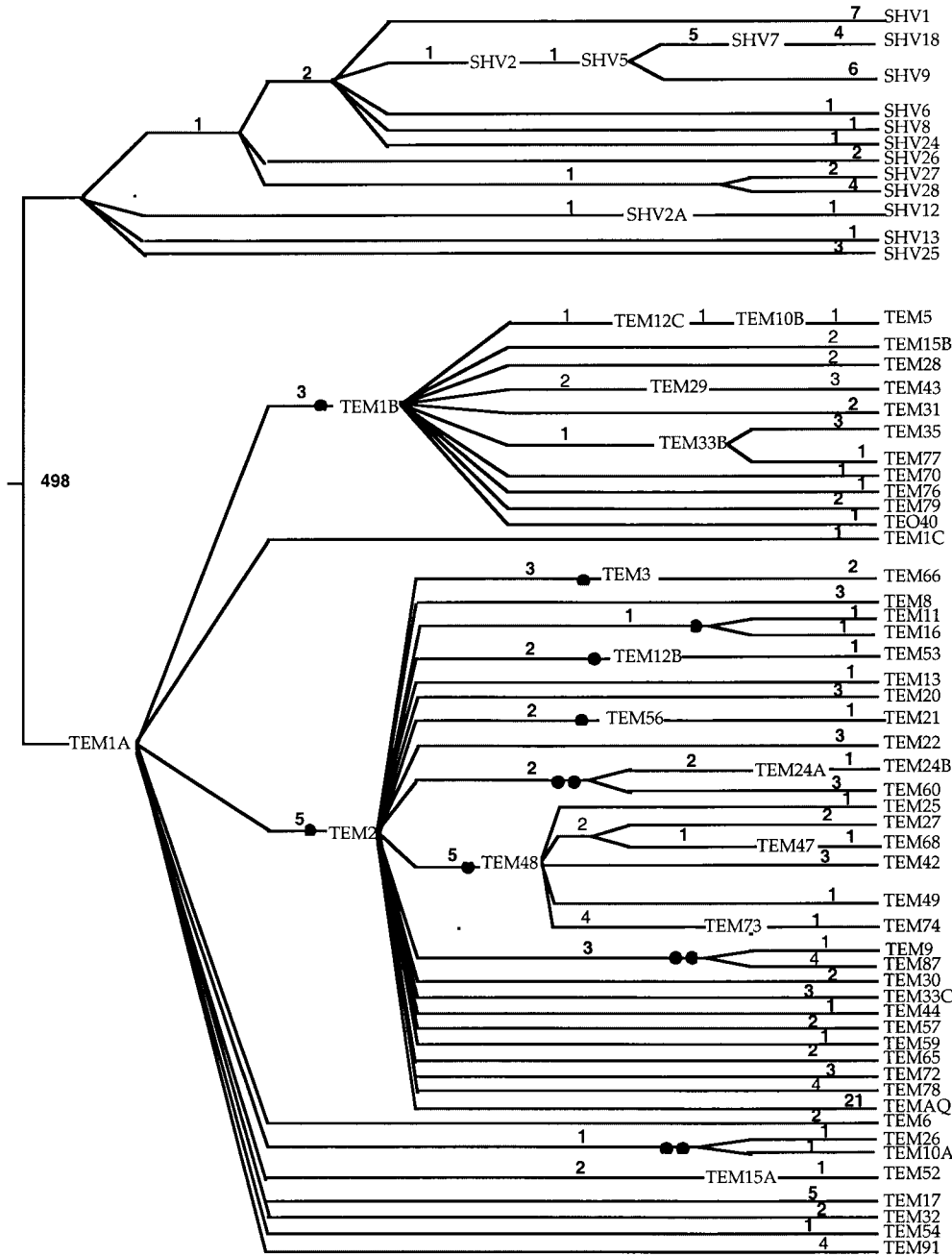


FIGURE 1.—Phylogeny of the TEM and SHV  $\beta$ -lactamases. Branch lengths, in nucleotide substitutions, are adjacent to the branches. Except as indicated all clades have posterior probabilities of >75%. Clades indicated by a single dot have probabilities of 60–75%, and those with two dots have probabilities of 50–59%. Because TEM  $\beta$ -lactamases are named strictly according to differences in protein sequence (<http://www.lahey.org/studies/webt.htm>) variants that differ only by silent mutations are assigned names such as TEM-1a, TEM-1b, and so on. A complete list of accession numbers and references is available at [http://www.rochester.edu/College/BIO/labs/HallLab/TEM\\_Phylo.html](http://www.rochester.edu/College/BIO/labs/HallLab/TEM_Phylo.html).

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