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Predicting Evolution by In Vitro Evolution Requires Determining Evolutionary Pathways

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In an early example of DNA shuffling, Stemmer (W. P. C. Stemmer, Nature 370:389-390, 1994) demonstrated a dramatic improvement in the activity of the TEM-1 β-lactamase toward cefotaxime as the consequence of six amino acid substitutions. It has been pointed out (B. G. Hall, FEMS Microbiol. Lett. 178:1-6, 1999; M. C. Orencia, J. S. Yoon, J. E. Ness, W. P. Stemmer, and R. C. Stevens, Nat. Struct. Biol. 8:238-242, 2001) that the power of DNA shuffling might be applied to the problem of predicting evolution in nature from in vitro evolution in the laboratory. As a predictor of natural evolutionary processes, that power may be misleading because in nature mutations almost always arise one at a time, and each advantageous mutation must be fixed into the population by an evolutionary pathway that leads from the wild type to the fully evolved sequence. Site-directed mutagenesis was used to introduce each of Stemmer's six substitutions into TEM-1, the best single mutant was chosen, and each of the remaining five substitutions was introduced. Repeated rounds of site-directed mutagenesis and selection of the best mutant were used in an attempt to construct a pathway between the wild-type TEM-1 and Stemmer's mutant with six mutations. In the present study it is shown (i) that no such pathway exists between the wild-type TEM-1 and the supereffective cefotaxime-hydrolyzing mutant that was generated by six amino acid substitutions via DNA shuffling (Stemmer, Nature 370:389-390, 1994) but that a pathway to a fourfold more efficient enzyme resulting from four of the same substitutions does exist, and (ii) that the more efficient enzyme is likely to arise in nature as the result of a single mutation in the naturally occurring TEM-52 allele.

In vitro evolution by DNA shuffling and other methods that introduce multiple simultaneous mutations has become a powerful and widely used tool for the engineering of proteins to increase activities and/or to extend substrate ranges (1, 5, 6, 9–11, 16). One of the earliest papers on DNA shuffling used that technique to increase the level of resistance to cefotaxime conferred by the TEM-1 β-lactamase 32,000-fold (18). That increase was the result of six amino acid substitutions, three of which occur repeatedly in naturally occurring "extended-spectrum" alleles of the TEM β-lactamase, plus a promoter mutation that increased the level of expression about 2.5-fold. The measure of the in vivo effectiveness of an antibiotic resistance protein such as TEM is the MIC of the drug substrate; the higher the MIC is, the more effective the protein is. That effectiveness is determined by the catalytic efficiency of the protein, the in vivo substrate concentration, and factors such as stability and folding efficiency that determine the concentration of active protein in the cell. The protein with six substitutions reported by Stemmer (18) conferred a cefotaxime MIC of 640 μg/ml, considerably higher than the cefotaxime MICs conferred by the products of any naturally occurring TEM alleles.

Does Stemmer's result predict that TEM alleles that confer cefotaxime MICs of 640 μg/ml will arise in nature as cefotaxime use continues? Blazquez et al. (3) suggest that fluctuating selection as TEM alleles encounter other β-lactam antibiotics might prevent such supereffective cefotaxime alleles from arising. Fluctuating selection is a special case of the "cost" of mutations that may be advantageous under one selective condition, the presence of cefotaxime, but disadvantageous under another selective condition. A second possible barrier to a supereffective cefotaxime-hydrolyzing TEM β-lactamase might be the absence of an evolutionary pathway from existing alleles to the supereffective allele.

The power of DNA shuffling lies in its ability to speed up evolutionary processes by the simultaneous introduction of multiple mutations into existing sequences. That power may be misleading as a predictor of natural evolutionary processes because in nature mutations almost always arise one at a time and each mutation must be fixed into the population by selection. (For populations as large as microbial and plasmid populations, fixation of any particular neutral mutation that might potentially be advantageous in the presence of another otherwise neutral mutation can largely be ignored.) With respect to increased antibiotic resistance, this means that during selection for antibiotic resistance each mutation must confer a measurable increase in effectiveness in order to be fixed into the population. When the fully evolved allele requires six amino acid substitutions, one can easily imagine two possibilities: (i) there is some order in which those mutations could occur such that each mutation improves the effectiveness of the gene product, i.e., each mutation improves fitness, in which case there is a clear pathway to the fully evolved mutant with six mutations; and (ii) at some point in the pathway, for example, after three advantageous mutations have accumulated, none of the remaining three mutations improves the effectiveness of the enzyme. In that case the evolutionary pathway will terminate with the triple mutant. In the second case, although it is known that the mutant with six mutations can exist and that it

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TABLE 1. Drug sensitivities of mutant TEM-1 alleles

Amino acid substitution	MIC (μg/ml)				Improvement for cefotaxime[a]
	Ampicillin	Piperacillin	Cephalothin	Cefotaxime	
No plasmid	2	4	16	0.0625	
pBR322	4,096	4,096	128	0.0625	
TEM-1 (estimated)[b]				0.012	
A42G	4,096	4,096	256	0.0625	—[c]
G92S	4,096	4,096	256	0.0625	—
E104K	4,096	4,096	128	0.25	21
M182T	4,096	4,096	256	0.0625	—
G238S[d]	**1,024**	**128**	**64**	**0.5**	**42**
R241H	4,096	4,096	256	0.0625	—
A42G, E104K	8,192	4,096	256	0.25	21
G92S, E104K	8,192	4,096	256	0.0625	—
E104K, M182T	8,192	8,192	512	0.125	10
E104K, R241H	4,096	2,048	256	0.0625	—
A42G, G238S	2,048	1,024	256	4	333
G92S, G238S	2,048	256	64	0.5	42
E104K, G238S	**2,048**	**256**	**128**	**8**	**667**
M182T, G238S	2,048	512	128	2	167
G238S, R241H	2,048	256	64	0.5	42
G238S, E104K, A42G	**2,048**	**1,024**	**512**	**64**	**5,333**
G92S, E104K, G238S	2,048	256	128	8	667
E104K, M182T, G238S	4,096	1,024	256	32	2,667
E104K, G238S, R241H	2048	256	256	8	667
A42G, G92S, E104K, G238S	4,096	1,024	512	128	10,667
A42G, E104K, M182T, G238S	**4,096**	**4,096**	**2,048**	**1,024**	**85,333**
A42G, E104K, G238S, R241H	4,096	1,024	512	128	10,667
A42G, G92S, E104K, M182T, G238S	4,096	1,024	512	128	10,667
A42G, E104K, M182T, G238S, R241H	**4,096**	**1,024**	**512**	**256**	**21,333**
A42G, G92S, E104K, M182T, G238S, R241H	2,048	1,024	512	256	21,333

[a] The improvement for cefotaxime is relative to the estimated cefotaxime MIC for TEM-1.
[b] See text for estimation of the cefotaxime MIC for the wild-type TEM-1 gene.
[c] —, the cefotaxime MIC is indistinguishable from that for the host; therefore, relative resistance cannot be calculated.
[d] Data for mutations with the highest resistance to cefotaxime in their class (single mutation, double mutation, etc.) are highlighted in boldface.

is fitter than the triple mutant, there is no pathway that can lead to the mutant with six mutations.

Escherichia coli strain DH5αE [F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r-m^+) *deoR thi-1 phoA supE44* λ^- *gyrA96 relA1 gal*] (Gibco) was used as the host for all plasmids.

L broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 g of glucose per liter) was used as a general growth medium. Mueller-Hinton broth (Difco), the standard medium used for the assessment of antibiotic resistance, was prepared according to the instructions of the manufacturer.

Plasmids were prepared from overnight cultures grown in L broth containing 15 μg of tetracycline per ml. Plasmids were purified with the Qiagen QuickSpin kit according to the instructions of the manufacturer.

Stock solutions of antibiotics were prepared in 0.1 M $NaPO_4$ buffer (pH 7.0), filter sterilized, and stored at −80°C in single-use aliquots.

Amino acid substitution mutations were introduced into the TEM-1 β-lactamase gene of plasmid pBR322 by site-directed mutagenesis with the QuickChange PCR mutagenesis kit of Stratagene, Inc., according to the instructions of the manufacturer. *E. coli* strain DH5αE transformants were selected on L-broth plates containing 15 μg of tetracycline per ml, and the TEM genes of the resulting plasmids were sequenced to confirm the incorporation of the desired mutation and the absence of any other mutations.

The MIC of each antibiotic was determined by growing the plasmid-bearing strain overnight in L broth containing 15 μg of tetracycline per ml, diluting the culture to about 10^5 cells per ml in Mueller-Hinton broth (Difco), and adding to 500-μl aliquots of the diluted culture in 48-well microtiter plates two-fold serial dilutions of the drug being tested. The plates were incubated for 24 h, and the MIC was taken as the lowest concentration of drug that prevented growth that was detectable by eye as turbidity of the culture.

To determine whether there is a pathway from TEM-1 to the mutant with six mutations (mutant ST-3) reported by Stemmer (18), each of the six amino acid substitutions was introduced by site-directed mutagenesis into the TEM-1 allele borne on plasmid pBR322 (Table 1). The substitution that gave the greatest increase in resistance to cefotaxime was chosen, and each of the remaining five substitutions was introduced into that allele. Similarly, the best double mutant was chosen, and each of the remaining four mutations was introduced into that allele. That pattern was followed until, after six rounds of selection, the ST-3 allele of Stemmer (18) had been reconstructed. The rationale is that in nature population sizes are large enough that all possible alleles with single mutations are continuously being generated by spontaneous mutations. Given typical spontaneous mutation rates of 2×10^{-10} to 4×10^{-10} base substitutions per site per cell division, bacterial populations as small as 10^{10} cells will include at least one representative of each possible mutation. When challenged with a β-lactam antibiotic, if the

members of a population are free to compete with each other, the allele that confers the greatest resistance will replace the wild-type allele and subsequent mutations will arise in the new genetic background (2). Indeed, the selection imposed by 32 μg of cefotaxime per ml selects efficiently for high levels of resistance (MICs, 256 to 512 μg/ml) (2). Similar results were obtained with three other β-lactam antibiotics, the MICs of which for the mutants exceeded the concentrations used for selection by 32- to 64-fold (2). Although neutral and slightly deleterious alleles will also arise and may even persist for some time, their frequencies remain so low that it is very unlikely that a particularly advantageous combination will arise as the consequence of a modestly advantageous mutation occurring in the rare background of such a neutral or slightly deleterious allele.

The MICs of four β-lactam antibiotics, ampicillin, piperacillin, cephalothin, and cefotaxime, were determined for each mutant allele. Ampicillin is a widely used penicillin that was introduced into use in 1965, while piperacillin is a more modern penicillin that was introduced in 1981. Cephalothin is a cephalosporin that was introduced in 1964, while cefotaxime is an extended-spectrum cephalosporin that was introduced in 1981. The breakpoint for clinical resistance to ampicillin is an MIC of at least 32 μg/ml, the breakpoint for clinical resistance to piperacillin is at least 128 μg/ml, the breakpoint for clinical resistance to cephalothin is at least 32 μg/ml, and the breakpoint for clinical resistance to cefotaxime is at least 64 μg/ml (13). Note that more recent NCCLS guidelines define *E. coli* and *Klebsiella* isolates that produce any extended-spectrum β-lactamase as being "resistant," regardless of the MIC conferred by that enzyme (12). The host, *E. coli* strain DH5αE, is sensitive to all four antibiotics. The TEM-1 allele confers resistance to ampicillin, piperacillin, and cephalothin but does not detectably increase the level of resistance to cefotaxime (Table 1).

Because the TEM-1 allele does not detectably increase the level of resistance to cefotaxime relative to the level of resistance of the host, the effectiveness of TEM-1 with respect to the hydrolysis of cefotaxime cannot be determined directly from the MIC. The effective MIC can, however, be estimated from the literature. Stemmer (18) showed that the naturally occurring TEM-3 and TEM-15 alleles, both of which include the E104K and G238S substitutions, confer the same cefotaxime MIC (10 μg/ml). Franceschini et al. (7) measured the efficiency with which the TEM-1 and TEM-3 enzymes hydrolyze cefotaxime in vitro. The k_{cat}/K_m for TEM-1 was 0.0015 $\mu M^{-1}\ s^{-1}$ and that for TEM-3 was 1.0 $\mu M^{-1}\ s^{-1}$, indicating that TEM-1 hydrolyzes cefotaxime 0.15% as efficiently as TEM-3 does and, by extension, 0.15% as efficiently as the mutant with the E104K and G238S substitutions that is equivalent to TEM-15. Table 1 shows that in my hands the cefotaxime MIC for the mutant with the E014K and G2328S substitution is 8 μg/ml, giving an estimated MIC for the TEM-1 enzyme of 0.012 μg/ml. That estimate assumes that the Q39K substitution that is present in TEM-3 and absent in TEM-15 has no effect on the cefotaxime hydrolysis rate. On another substrate, cephaloridine, Q39K by itself increases k_{cat}/K_m by 50% relative to that for TEM-1 (4). A similar effect with respect to cefotaxime hydrolysis by TEM-3 would give an estimated cefotaxime MIC for TEM-1 of 0.018 μg/ml. In the absence of any direct evidence of such an effect, the improvement in the level of resistance of each mutant relative to that of TEM-1 is expressed relative to the estimated MIC of 0.012 μg/ml.

The single substitution that confers the greatest increase in the level of resistance to cefotaxime is G238S, with a 42-fold improvement. That allele, however, also reduces the level of resistance to ampicillin, piperacillin, and cephalothin dramatically. Fluctuating selection during which the G238S allele encounters one of those drugs might prevent fixation of G238S in nature. The second most effective allele was E104K, which gives a 21-fold improvement in the level of resistance without suffering any decrease in levels of resistance to the other drugs. Therefore, for round two, both E104K and G238S were chosen, and all other substitutions were introduced into each background.

The best double mutant was one with the combination of the E104K and G238S substitutions, a combination that resulted in a 667-fold improvement in resistance relative to that for TEM-1. Therefore, whichever of the two single substitutions was fixed into the population, the same double mutant would be fixed.

The best triple mutant was that with the A42G, E104K, and G238S substitutions, in which the level of resistance was improved 5,333-fold relative to that for TEM-1, while the best mutant with four mutations was the one with the A42G, E104K, M182T, and G238S substitutions, which was 85,333 times more effective than TEM-1. The M182T substitution is known to act by affecting the folding pathway for the TEM β-lactamases, thereby increasing the concentration of active enzyme (17). Although by itself the M182T substitution has no effect on the cefotaxime MIC, in combination with other substitutions it always increases the level of resistance (Table 1).

Introduction of either of the remaining substitutions, G92S or R241H, reduced the level of resistance to cefotaxime; therefore, neither mutant with five mutations would be fixed into the population by selection.

Finally, the mutant with six mutations was exactly as resistant to cefotaxime as the better of the two mutants with five mutations. In the pBR322 background, the mutant with six mutations is resistant to cefotaxime at 256 μg/ml (Table 1). Stemmer's mutant with six mutations carried an additional promoter mutation that increased the level of expression two- to threefold (18), and the cefotaxime MIC for the mutant was 640 μg/ml, exactly 2.5 times the level of resistance conferred by the same sequence in pBR322.

It was expected either that there would be a clear evolutionary pathway leading to the mutant with six mutations or that the pathway would terminate early, resulting in a failure to achieve the level of resistance conferred by the mutant with six mutations. A third, unanticipated result was obtained: the evolutionary pathway terminated with the mutant with four mutations, but that mutant conferred resistance four times better than the mutant with six mutations. In effect, the introduction of multiple simultaneous mutations by DNA shuffling resulted in leaping over the adaptive peak and landing at a lower adaptive level. This result emphasizes the importance of reconstructing evolutionary pathways when using any in vitro evolution method that introduces simultaneous multiple mutations to predict natural evolution.

This result predicts that a supereffective cefotaxime-hydrolyzing TEM β-lactamase will arise in nature in response to the continued clinical use of cefotaxime. The sequence of the β-lactamase with the A42G, E104K, M182T, and G238S substitutions, which confers resistance to cefotaxime fourfold more effectively than the best allele of Stemmer (18) does, is just a single mutation (C to G at base pair 119 of the TEM-52 coding sequence) away from the sequence of the naturally occurring TEM-52 β-lactamase (E104K, M182T, G238S) (15). The TEM-52 β-lactamase confers a cefotaxime MIC of 32 μg/ml (Table 1). Introduction of the A42G substitution not only increases the cefotaxime MIC 32-fold to 1,024 μg/ml, but it also increases the MICs of piperacillin and cephalothin fourfold each. Thus, fluctuating selection in the presence of those drugs will also favor the appearance in nature of a new TEM β-lactamase that hydrolyzes cefotaxime extremely efficiently. Because selection for high levels of resistance does not require exposure to high levels of cefotaxime (2), the appearance of a TEM β-lactamase with the A42G, E104K, M182T, and G238S substitutions is expected even if cefotaxime concentrations in the environment do not reach especially high levels.

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