Experimental Prediction of the Evolution of Cefepime Resistance From the CMY-2 AmpC β-Lactamase

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ABSTRACT

Understanding of the evolutionary histories of many genes has not yet allowed us to predict the evolutionary potential of those genes. Intuition suggests that current biochemical activity of gene products should be a good predictor of the potential to evolve related activities; however, we have little evidence to support that intuition. Here we use our *in vitro* evolution method to evaluate biochemical activity as a predictor of future evolutionary potential. Neither the class C *Citrobacter freundii* CMY-2 AmpC β-lactamase nor the class A TEM-1 β-lactamase confer resistance to the β-lactam antibiotic cefepime, nor do any of the naturally occurring alleles descended from them. However, the CMY-2 AmpC enzyme and some alleles descended from TEM-1 confer high-level resistance to the structurally similar ceftazidime. On the basis of the comparison of TEM-1 and CMY-2, we asked whether biochemical activity is a good predictor of the evolutionary potential of an enzyme. If it is, then CMY-2 should be more able than the TEMs to evolve the ability to confer higher levels of cefepime resistance. Although we generated CMY-2 evolvants that conferred increased cefepime resistance, we did not recover any CMY-2 evolvants that conferred resistance levels as high as the best cefepime-resistant TEM alleles.

FOR more than 30 years microorganisms have been used as model systems to study the evolution of new functions (LeBlanc and Mortlock 1971; Mortlock 1984; Hall 1999a). Much of that work involved the isolation of mutants that grow in new environments or that metabolize new carbon sources to determine which mutations cause the new phenotypes (Mortlock *et al.* 1965; Stemmer 1994; Hall and Malik 1998). None of those studies has attempted to predict which genes will evolve or what the nature of the evolved products will be. In effect, experimental evolutionists did exactly what evolutionary biologists have always done: explain what has happened by characterizing the outcomes. Recently, attention has begun to shift in the direction of understanding the evolutionary potential that is inherent in current genomes (Hall 1995, 1999a,b; Hall and Malik 1998), and predicting the evolution of antibiotic resistance genes has served both as an excellent model and as a practical application for experimental evolution (Vakulenko *et al.* 1998; Barlow and Hall 2002b). While *in vitro* evolution has been used to make predictions about the evolutionary potential of some genes (Barlow and Hall 2003), it is still unknown whether information about the biochemical activity of a protein can serve as an accurate predictor of evolutionary potential (Hall 2001).

In several *in vivo* experimental evolution systems it was observed that a trace level of activity toward a novel substrate was a good predictor of an enzyme's ability to evolve biologically effective activity toward that substrate (Clarke 1984; Hall 1984). The *ebg* system of *Escherichia coli* provided a particularly good example of biochemisty as a predictor of evolutionary potential (Hall 1999a). Wild-type *ebg* enzyme has extremely low activity toward lactose, lactulose, and galactosyl arabinose, with k_{cat}/k_m values such that lactose > lactulose > galactosyl-arabinose, and has no detectable activity toward lactobionic acid. Single amino acid replacements give good activity toward either lactose or lactulose and increase activity toward galactosyl-arabinose, but not to a level permitting growth on that substrate. Neither of the single-replacement enzymes has detectable activity toward lactobionate, and it was not possible to isolate lactobionate-hydrolyzing mutants of the wild-type enzyme or of either of the single-mutant enzymes. The combination of the two substitutions increased activity toward galactosyl-arabinose sufficiently to permit growth on that substrate and resulted in very low, but detectable, activity toward lactobionate. As the result of a third mutation, the double mutant, with detectable lactobionate activity, was able to evolve sufficient activity to permit growth on lactobionate. This, and other studies, led to the paradigm that current enzymatic activity toward a poor substrate is a good predictor of the potential to evolve increased activity that is selectively advantageous. A reasonable extension of that paradigm is that the better the current activity toward a substrate, the easier it will be (the fewer mutations will be required) for that en-

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zyme to evolve a high level of activity toward that substrate; *i.e.*, the closer an enzyme is to a desired level of activity, the shorter the distance in phenotypic space that it must travel to reach its goal. That assumption is often the basis of choosing among candidate enzymes when deciding on the most likely candidates during directed evolution of enzymes for industrial purposes. The antibiotic resistance model provides an excellent means to address the accuracy and generality of that assumption.

Throughout the past 60 years detailed records of the occurrence and mechanisms of antibiotic resistance have been kept (Medeiros 1997). In many cases the specific mutations that cause a microbe to acquire a new resistance phenotype have been identified (Knox 1995). The TEM family of class A (Ambler 1980) β-lactamase resistance genes has been one of the best studied because it is globally distributed and, although it started out primarily conferring resistance to penicillins, it has evolved the ability to confer resistance to most of the β-lactam antibiotics. Currently, >90 TEM alleles differ in amino acid sequence, and many confer different resistance phenotypes (see http://www.lahey.org/studies/webt.htm and also http://www.rochester.edu/College/BIO/labs/HallLab/TreesMenu.html). The class C (Ambler 1980) *ampC* antibiotic resistance gene family, which is so distantly related to class A that homology is detectable only at the structural level, also confers resistance to the β-lactam antibiotics. Whereas the TEMs originally conferred resistance to penicillins and had to evolve the ability to confer resistance to cephalosporins, the *ampC*'s primarily confer resistance to cephalosporins.

ampC is located in the chromosomes of the Enterobacteriacae group, which includes *E. coli* and its close relatives. In the late 1980s an *ampC* gene was first detected on plasmids in resistant strains of bacteria (Bauernfeind *et al.* 1989), and in 1990 an *ampC* gene from *Citrobacter freundii* was first observed on a plasmid (Bauernfeind *et al.* 1990). That allele, CMY-2, has since become distributed throughout several species of bacteria worldwide and is able to confer resistance to some antibiotics that the TEM alleles cannot. Although a handful of alleles that are descended from CMY-2 and that differ in amino acid sequence have been isolated, they do not appear to have evolved any new phenotypes (Barlow and Hall 2002a). While it is possible that there has not been sufficient time and/or selective pressure for CMY-2 to give rise to dramatically different alleles, it is also possible that CMY-2 lacks the evolutionary plasticity that has been observed in the TEM β-lactamases.

Cefepime is a relatively new antibiotic that received FDA approval in 1996 and has since been used somewhat less than other β-lactam antibiotics. While different in terms of size, electrostatic charge, and sidechain stereochemistry, ceftazidime is the β-lactam antibiotic that is most similar to cefepime (Figure 1). Ceftazidime is readily hydrolyzed by the CMY-2 enzyme (Barlow and Hall 2002a) and by some of the evolved TEM β-lactamases (Medeiros 1997). The ancestral allele TEM-1, however, exhibits very little activity toward ceftazidime. Although CMY-2 and some of the TEMs confer resistance to ceftazidime, none of those alleles confer resistance to cefepime. We recently used *in vitro* evolution to show that, although it lacks significant activity toward ceftazidime, the TEM-1 β-lactamase does have the potential to evolve the ability to confer resistance to cefepime (Barlow and Hall 2003).

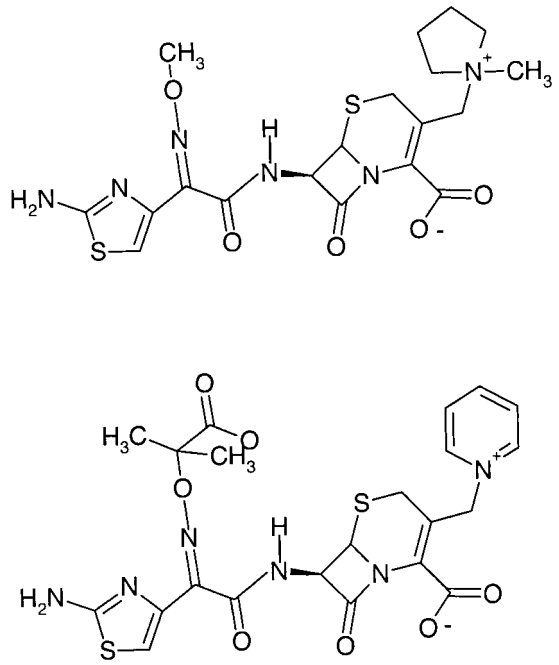

Figure 1.—Structures of cefepime (top) and ceftazidime (bottom).

Our *in vitro* evolution system is designed specifically to consider only mutations that occur in the coding sequences of the proteins under consideration. In nature both regulatory mutations, including promoter mutations, and mutations that affect other cellular properties such as permeability to the drug could affect the level of resistance and thus affect fitness. We have chosen to exclude those mutations from consideration to focus our attention on mutations that alter the properties of the protein. Mutations in the coding sequence can potentially affect the catalytic properties such as k_{cat} and k_m, and some mutations may alter the stability of the protein or the stability of the mRNA and thus change the steady-state level of the protein within the cell. The specific activity of the β-lactamase (activity per cell) will be a function of both the steady-state level of the protein and the catalytic properties of the protein, and it is that specific activity that is reflected in the minimum inhibitory concentration (MIC) of the drug that is the substrate of the β-lactamase.

If biochemical activity is a good predictor of evolutionary potential, then we would predict that, because it can

TABLE 1

MICs of CMY-2 evolvants (in micrograms per milliliter)

Drug	Clinical resistance MIC	pACSE2[a]	CMY-2	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6	Clone 7
Ampicillin	32	8	2048	1024	1024	1024	1024	512	2048	1024
Pipericillin	128	2	256	512	512	512	512	512	512	512
Cefuroxime	32	16	128	512	1024	512	512	256	512	256
Cefotaxime	64	0.5	64	128	128	128	128	128	128	128
Ceftazidime	32	1	256	2048	2048	2048	2048	1024	1024	2048
Aztreonam	32	1	64	128	128	128	256	64	256	128
Cefepime	32	0.03125	2	64	32	32	32	32	32	32

[a] MIC for the host strain carrying the vector only, *i.e.*, the background level of resistance.

already hydrolyze ceftazidime, CMY-2 will readily evolve the ability to confer high levels of resistance to cefepime and that it will become better at hydrolyzing cefepime than will the evolved TEM alleles. In this article, we have used the same *in vitro* evolution method that was used to create cefepime-resistant TEM alleles (Barlow and Hall 2003) to determine the potential of CMY-2 to evolve the ability to confer cefepime resistance.

MATERIALS AND METHODS

E. coli strain DH5αE (F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA*1 *recA*1 *hsdR*17(r− m+) *deoR* thi-1 *phoA supE*44 λ$^-$ *gyrA*96 *relA*1 gal−; GIBCO, Gaithersburg, MD) was used as the host for all plasmids.

Plasmid pACSE3, a low-copy-number vector derived from pACYC184 (Barlow and Hall 2002b), was used as the vector for cloning and expressing CMY-2 and its evolvants during random PCR mutagenesis and selection. Plasmid pACSE2 was used as the vector for the site-directed mutagenesis of CMY-2 and expression of CMY-2 and the alleles generated by site-directed mutagenesis. Because pACSE2 and pACSE3 differ only at two restriction sites and because those differences do not alter any of the functions of the vector, expression from those vectors is identical. Site-directed mutagenesis was performed according to the manufacturer's instructions using the Quick-Change kit from Stratagene (La Jolla, CA).

In vitro mutagenesis, cloning, sequencing, and determination of MICs of antibiotics and the disc diffusion test were performed as previously described (Barlow and Hall 2002b). Selection of evolved mutants was done as previously described (Barlow and Hall 2002b). Briefly, the CMY-2 gene was mutagenized using the error-prone polymerase Mutazyme (Stratagene) in a PCR reaction under conditions that generated an average of two mutations per molecule. Mutagenized amplicons were independently cloned into pACSE3 in seven separate experiments and transformed into *E. coli* strain DH5-αE, and the sizes of the resulting seven libraries were estimated by plating serial dilutions onto l-tetracycline medium. Each of the seven mutant libraries was amplified by growth in the presence of tetracycline to select for retention of the plasmid, and twofold serial dilutions of cefepime (64–0.5 μg/ml) were inoculated with a number of cells corresponding to at least 10 times the library size. Cells from the highest concentration of cefepime that permitted growth were used to inoculate a second cefepime dilution series; these cells were inoculated into ampicillin (64 μg/ml), and finally cells from the ampicillin culture were passaged once again through a dilution series of cefepime (64μg/ml –0.5μg/ml). Multiple passages through cefepime ensured that cefepime-resistant alleles dominated the culture. The single passage through ampicillin required that the mutant alleles also maintain the ability to confer resistance to ampicillin, a commonly used β-lactam. Because ampicillin remains a heavily used antibiotic, naturally evolving alleles are likely to encounter ampicillin selection frequently, and maintenance of the ampicillin resistance phenotype is likely to be important in nature.

Following selection, plasmid from each library was prepared from the highest concentration of cefepime at which growth occurred. Those plasmid preparations were then used as starting material for the next round of mutagenesis and selection. The process of mutation and selection was repeated until the culture was able to grow at 64 μg/ml cefepime or until there was no improvement in cefepime resistance relative to the previous round.

RESULTS AND DISCUSSION

Resistance to an antibiotic can be quantified by determining the MIC of the antibiotic on a bacterial strain. The MIC is the lowest concentration of the antibiotic that can completely block microbial growth. An MIC of 32 μg/ml is the breakpoint for clinical resistance to cefepime (National Committee for Clinical Laboratory Standards 2001), but the MIC of cefepime on *E. coli* expressing CMY-2 is 2 μg/ml (Table 1).

We previously reported that we are able to evolve alleles derived from TEM-1 that increase the MIC of cefepime from 0.5 μg/ml to 256 μg/ml through an *in vitro* evolution method that accurately mimics natural evolution (Barlow and Hall 2002b, 2003). We used the same method to evolve CMY-2 alleles that confer resistance to cefepime. We created seven independent libraries of mutant CMY-2 alleles and selected those alleles that confer the highest level of cefepime resistance. We repeated rounds of mutation and selection for each library until either we achieved an MIC of 64 μg/ml cefepime (twice the clinical resistance MIC) or there was no improvement in MIC relative to the previous round for that library. Six of the seven libraries showed no improvement after round 3, and the plasmid

TABLE 2

Nucleotide and amino acid substitutions of the CMY-2 evolvants

		Amino acid substitutions						
Nucleotide	Mutation	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6	Clone 7
30	G → A					S		
110	C → A						T17N	
168	A → G			S				
228	G → T				S			
228	G → A			S				
246	A → G						S	
393	C → T						S	
405	C → T		S					
415	C → T		S					
435	T → C				S			
480	G → T				S			
505	C → T	L149F						
511	G → T	A151S						
525	T → G	I175M						
537	C → T	S						
541	C → T		L161S[a]					
542	T → C		L161S[a]					
543	G → A	S						
580	A → C		M174L					
630	G → A				S			
720	C → A						S	
742	A → G			I228V				
768	C → G					S		
828	G → A						S	
880	C → T		S					
932	T → C			V291A				
932	T → G						V291G	
934	G → C		A292P	A292P	A292P			
937	T → C					L293P[a]		L293P[a]
938	T → C					L293P[a]		L293P[a]
941	C → A						A294E	
947	T → C	L296P						
953	C → T	A298V						
972	C → T				S			
1088	G → A				S323N			
1100	C → A							P347H
1107	T → C					S		
1108	G → A	V350I	V350I					

S, silent mutation.
[a] Amino acid substitutions that resulted from two mutations in the same codon.

prepared from round 3 was used for further characterization and sequence analysis of the CMY-2 evolvants. After four rounds of mutation and selection, library number 1 increased in cefepime resistance to an MIC of 64 μg/ml, at which point we discontinued mutation and selection on that library as well. We transformed the final plasmid preparations for each library into naïve DH5α-E and determined the resistance phenotypes for five transformants in each library by the disc diffusion method. Prior experience with evolution of the TEM-1 β-lactamase (Barlow and Hall 2003) indicated that during the selection process a single clone typically came to dominate the population (clonal displacement). In keeping with that experience we found only one resistant phenotype in the population derived from any given library. A single clone exhibiting increased resistance to cefepime was chosen as a representative of the final population selected from each library, and the clone was named with the corresponding library number. The MICs of several antibiotics for each clone were determined as were the sequences of the evolved CMY-2 alleles contained within each clone.

The MICs for the evolved alleles are shown in Table 1. The highest MIC of cefepime was 64 μg/ml for clone 1. For the other six clones the MIC was 32 μg/ml. That result was surprising because one *in vitro* evolved TEM

TABLE 3

MICs of allele 1 intermediates (in micrograms per milliliter)

Drug	Clinical resistance MIC	pACSE2	CMY-2	L296P	A298V	L296P A298V	L296P A298V L149F	L296P A298V L149F A151S	L296P A298V L149F A151S V350I
Ampicillin	32	8	2048	1024	2048	1024	1024	1024	1024
Pipericillin	128	2	256	128	512	256	256	512	512
Cefuroxime	32	16	128	256	512	512	512	512	512
Cefotaxime	64	0.5	64	64	128	128	128	128	128
Ceftazidime	32	1	256	1024	512	1024	1024	2048	2048
Aztreonam	32	1	64	64	128	128	128	128	128
Cefepime	32	0.03125	2	16	2	16	16	64	64

allele reached an MIC of 256 μg/ml and another reached an MIC of 128 μg/ml. Although the unevolved CMY-2 allele confers a resistance level that is fourfold greater than that conferred by TEM-1, TEM-1 was able to evolve the ability to confer high levels of resistance more readily than was CMY-2.

The mutations present in the seven representative clones are shown in Table 2. While two substitutions have been independently selected twice, and one has been independently selected three times, there does not appear to be any single substitution that is crucial for the increase in resistance to cefepime. All alleles, however, contain at least one substitution between amino acids 291 and 298, which demonstrates that mutations in that region are important for the evolution of cefepime resistance. That pattern sharply contrasts with the pattern that we obtained when we evolved TEM alleles that could confer cefepime resistance. All eight of the TEM alleles we recovered had an amino acid substitution at residue 164 and six of those alleles had a substitution at residue 173. The majority of the substitutions in the TEM alleles were in the ω-loop region; thus the patterns are similar in that in each case the majority of the mutations responsible for cefepime resistance are confined to a small region of the enzyme.

Although the CMY-2 evolvants did not reach a resistance level as high as that of the TEMs, they are capable of conferring clinical resistance to cefepime. Because natural mutations generally occur one at a time and because our *in vitro* mutagenesis technique simultaneously introduces multiple substitutions, it is possible to recover phenotypes from *in vitro* mutagenesis that would never arise in nature (Hall 2002). For example, if two substitutions are individually deleterious, but advantageous when both are present, they would probably not go to fixation in nature, but they might well be recovered through *in vitro* evolution procedures that introduce mutations at a high frequency. To verify that the mutations we recovered in the best allele, allele 1, can also be recovered from natural evolution, we determined if a pathway exists between CMY-2 and allele 1 in which the five substitutions found in allele 1 can be introduced one at a time such that each additional substitution confers an increase in cefepime resistance. We isolated an individual from each of the three rounds of mutagenesis and selection that preceded the recovery of clone 1. Because we had already shown that clonal displacement causes one resistance allele to dominate the population during selection (Barlow and Hall 2002b, 2003), we were reasonably confident that the allele collected from each intermediate round would be the ancestor of the allele selected in the next round. We found that after

TABLE 4

Zones of inhibition for allele 1 intermediates (diameter in millimeters)

	CMY-2	L296P	A298V	L296P A298V	L296P A298V L149F	L296P A298V L149FA151S	L296P A298V L149F A151S V350I
	25	16	24	14	13	11	11
	25	16	24	14	11	11	10
	25	16	23	14	12	11	10
	25	17	24	14	12.5	11	9
	25	16	24	14	13	11	9
Mean	25	16.2	23.8	14	12.3	11	9.8
Standard error	0	0.2	0.2	0	0.3742	0	0.3742

TABLE 5

Biochemical factors predicting evolutionary potential of TEM-1 and CMY-2

β-Lactam	Clinical resistance MIC	MIC (μg/ml)		
		pACSE2	TEM-1	CMY-2
Cefepime	32	0.03	0.5	2
Ceftazidime	32	1	1	256

the first round CMY-2 contained the substitutions L296P and A298V. The allele taken from the second round contained the substitution L149F in addition to the two substitutions that appeared in the first round. In the third round, the substitution A151S was added and the substitution V350I was added in the fourth and final round. Because three of the five mutations had already been added one at a time, verification of a natural pathway required only the separate introduction of the first two substitutions, L296P and A298V, through site-directed mutagenesis. Those two substitutions were individually created within CMY-2 by site-directed mutagenesis, and the phenotypes of those alleles and all of the intermediate alleles were determined.

The MICs for the alleles involved in the evolutionary pathway of allele 1 are shown in Table 3. Some of the alleles appeared to confer the same levels of cefepime resistance and we could not determine whether there was a natural evolutionary pathway to allele 1 from the MIC data. Because MIC data resolve only twofold differences in resistance level, we further characterized the cefepime resistance phenotypes by the disc diffusion test using discs containing 30 μg of cefepime. Smaller zones of inhibition indicate that the cells are able to grow at a higher concentration of cefepime and that the CMY-2 evolvant alleles in those cells confer increased levels of resistance. Five disc diffusion tests were done for each allele and the mean diameters of the zones of inhibition and the standard errors are shown in Table 4. Those data show that addition of each substitution slightly increases resistance to cefepime and that a pathway of single substitutions in which resistance is always increased does exist. This means that, in nature, it is possible for CMY-2 to evolve cefepime resistance that is at least as high as the resistance of clone 1.

In vitro evolution has demonstrated that both the TEM-1 and the *C. freundii* CMY-2 β-lactamases have the potential to evolve clinical levels of resistance to cefepime. It seems likely that plasmids carrying cefepime resistance genes, derived from one of these sources, will appear in nature in the near future. That conclusion is supported by the recent finding (Vakulenko *et al.* 2002) that another *ampC* β-lactamase, that of *Enterobacter cloacae*, can evolve a lower level of cefepime resistance (8 μg/ml) by PCR mutagenesis.

Two biochemical factors suggest that CMY-2 should have more potential than TEM-1 for evolving cefepime resistance. First, CMY-2 confers a level of cefepime resistance fourfold higher than TEM-1 confers (Table 5). Second, TEM-1 confers no detectable resistance to the structurally similar drug ceftazidime (Figure 1), while CMY-2 confers high-level resistance to ceftazidime (Table 5). In contrast to those expectations, comparison of the *in vitro* evolution of CMY-2 with that of TEM-1 shows that the biochemical activity of a protein is not always a good predictor of evolutionary potential. TEM-1 is more able than CMY-2 to give rise to alleles that confer significantly higher levels of resistance to cefepime.

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