

Evolution of the serine β -lactamases: past, present and future

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Abstract

We present a protein structure-based phylogeny of Classes A, C and D of the serine β -lactamases, and a new, detailed, sequence-based phylogeny of the Class A β -lactamases. In addition, we discuss the historical evolution of Classes C and D. The evolutionary histories of all three classes indicate that the serine β -lactamases are ancient enzymes, originating over two billion years ago, and that some have been on plasmids for millions of years. We also discuss the recent, antibiotic-era, evolution of the serine β -lactamases in response to the clinical use of β -lactam antibiotics. We also discuss a method that is being used to predict the future evolution of β -lactamases in response to selection with new drugs.

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1. Introduction

In 2002, the global market for antibiotics was estimated at US\$ 25 billion (Coates et al., 2002), of which about 50% was β -lactam antibiotics. The cost of resistance to β -lactam antibiotics is estimated to be between US\$ 10 and 17 billion annually (Palumbi, 2001). Because β -lactam antibiotics have been heavily used since the 1940s, many strains of bacteria resistant to β -lactam antibiotics have emerged in clinical care settings. As the frequency of microbial resistance to β -lactams—as well as other antimicrobials—continues to increase, the usefulness of the antibiotics currently available diminishes and the willingness of pharmaceutical companies to invest in the discovery and development of new antimicrobials wanes.

Resistance to β -lactam antibiotics is an especially severe threat because β -lactams kill a broad spectrum of bacteria and have very low toxicity to humans (Livermore, 1996). The primary mechanism of β -lactam resistance is the enzymatic cleavage of the β -lactam ring by β -lactamases. Because of the importance of β -lactam antibiotics, β -lactamases have been extensively studied (Medeiros, 1997). Representatives of every class of serine β -lactamases

have been crystallized, their spread and the mutations that occur within them have been well documented as have the phenotypes they confer (Medeiros, 1997). Yet, until recently, there has been little formal analysis addressing the evolutionary patterns and histories of the serine β -lactamases. Recent advances in genome sequencing technology have provided us with a lot of data about microbial genomes, and recent advances in phylogenetic methods have given us practical tools for analyzing the genetic data (Huelsenbeck and Ronquist, 2001). The availability of sequence data and improved phylogenetic methods have provided greater insight into the origins of resistance genes. Newly developed methods for in vitro evolution of resistance genes have provided a way of experimentally determining realistic evolutionary pathways that may be taken in nature (Barlow and Hall, 2002a, 2003a,b). By combining detailed analysis of the ancient evolution of serine β -lactamases and analysis of the evolutionary possibilities that exist for β -lactamases with the well-recorded emergence, spread, and mutations of serine β -lactamases that have caused them to become the substantial threat that they are, we have been able to significantly increase our understanding about the evolutionary forces and dynamics that have shaped and will continue to shape β -lactamases. While much work is still required in this area, this review summarizes many of the recent advancements in understanding the evolution of the β -lactamases.

Resistance to antibiotics, including β -lactams, typically appears within 2–3 years of introduction of that antibiotic

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into clinical use. A collection of over 400 enterobacterial strains isolated between 1954 and 1971 included no antibiotic resistant strains (Datta and Hughes, 1983), yet today over 20% are resistant to ampicillin or amoxicillin (London et al., 1993; Bruinsma et al., 2003). Antibiotic resistance arises quickly and spreads rapidly, especially when resistance genes are horizontally transferred via plasmids and integrons among individuals, among species, and even among bacterial kingdoms. If we are to understand the processes that led, and continue to lead, to the spread of antibiotic resistance, and especially if we hope to predict how resistance to new antibiotics will arise and spread, it is essential to understand the evolution of resistance genes. An understanding of past, pre-antibiotic era evolution helps us to understand where resistance genes came from and why they appear so quickly after the introduction of a new antibiotic into clinical use. An understanding of present evolution provides insight into why some resistance genes seem to evolve phenotypically in response to modified drugs, while others appear to diversify genetically without much change in phenotype. One of the most practical applications of evolution is the prediction of the future evolution of resistance genes in response to the introduction of new antibiotics. If we can accurately anticipate how resistance genes will evolve, we may be able to design optimal strategies for the use of antimicrobials that will maximize the useful life of the drugs.

2. Structural phylogeny of the serine β -lactamase family

Serine β -lactamases use an active-site serine to hydrolyze the four-membered β -lactam ring that is common to β -lactam antibiotics. The serine β -lactamases have been divided into three classes, A, C, and D, based on sequence similarity (Ambler, 1980; Jaurin and Grundstrom, 1981; Ouellette et al., 1987) and all are related to the DD peptidases (Medeiros, 1997). It is important to draw a clear distinction between similarity and homology. Similarity between two proteins simply means the tendency to have similar or identical amino acids at equivalent positions in the proteins. Homology means that the two proteins are descended from a common ancestor, and sequence homology means that similar amino acids at equivalent positions are similar *because* they are descended from a common ancestor. Similarity does not necessarily mean homology. Unrelated proteins that evolved independently may have similar sequences because there are a limited number of molecular solutions to the problem of achieving a particular function. Conversely, homologous proteins, proteins that share a common ancestor, may have diversified so much that all traces of homology at the sequence level have been lost. That is the case for the serine β -lactamases: it has long been recognized that there is sequence similarity within the classes, but not between members of different classes (Medeiros, 1997). At the same time, it has long been recognized that the structures of the proteins of different classes

are sufficiently similar that it is almost certain that the three classes are homologous (Medeiros, 1997).

Phylogenetic trees, sometimes called dendrograms, are used to represent the historical relationships among sequences. Because there is no detectable sequence homology between classes of the serine β -lactamases it is not possible to construct valid phylogenetic trees based upon sequence alignments that include members of different classes of the serine β -lactamase family. The dendrogram (phylogenetic tree) of β -lactamases that accompanies the Bush–Jacoby–Medeiros functional classification paper (Bush et al., 1995) is an excellent example of a tree that is based on an alignment that includes unrelated sequences. While this tree summarizes the fact that there are distinct groups of β -lactamases, it is misleading in that it implies that the relationships among those groups can be detected by sequence alignment. Such a tree shows little more than a table in which related genes are sorted out by family or class. Hall and Barlow (2003) recently constructed a phylogenetic tree of the serine β -lactamases by applying methods developed for sequence-based alignments of proteins to alignments based on the protein structures. Unlike sequence-based alignments that identify homologous amino acids based on sequence similarity, structure-based alignments identify homologous amino acids based on their positions within the protein structure. Fig. 1 shows a structure-based phylogeny of the serine β -lactamases and their relatives the DD-peptidases and the EstB esterase. That phylogeny reveals only the topology of the tree, i.e. the order of descent, but does not provide any information with respect to the lengths of the branches; i.e. information about how long ago the classes diverged from each other. The absence of branch length information results from the necessity of eliminating from the alignment regions that do not occupy similar positions in the structures.

The main conclusion from the structure-based phylogeny is that the divergence of the Class C serine β -lactamases

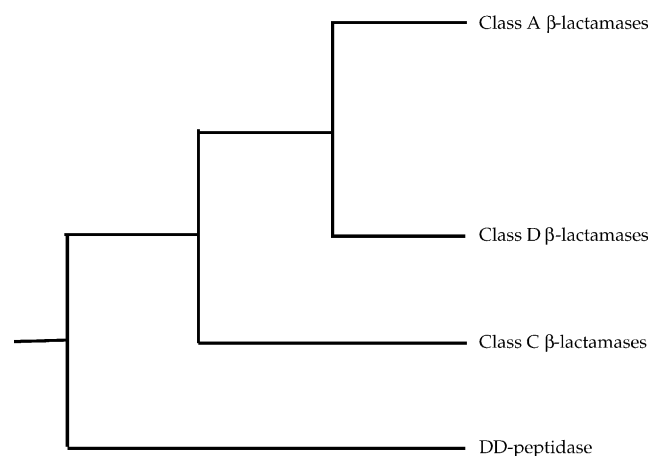


Fig. 1. Structure-based phylogeny of the serine β -lactamases. Branching order only is indicated on this figure; branch lengths are not proportional to distances. Modified from Hall and Barlow (2003).

predates the divergence of Classes A and D from a common ancestor. This does not necessarily imply that the Class C β -lactamases evolved the ability to hydrolyze β -lactams before the ancestor of Classes A and D. This means that the ancestor of Classes A and D was pushed down two independent evolutionary pathways, perhaps by selection from different selective pressures. Class C enzymes, on the other hand, have been following the same evolutionary pathway, or have been changing at a slower rate, and so they have not split into distinct groups as distantly related as Class A and Class D are to each other.

While it is impossible to identify the evolutionary forces that caused the divergence of Class C, and Classes A and D from each other, it is interesting to note some similarities between the phylogeny of the serine β -lactamases and the phenotypes of the β -lactamases.

For example, many Class A enzymes have been characterized primarily as penicillinases and are very good at hydrolyzing penicillin and ampicillin. While they have been able to evolve the ability to hydrolyze most cephalosporins, many Class A enzymes often specialize at hydrolyzing a specific subset of cephalosporins.

Likewise, Class D enzymes have primarily been classified as penicillinases although they specialize in the hydrolysis of oxacillin, cloxacillin, methicillin, and carbenicillin (Naas and Nordmann, 1999). Some OXAs are better at hydrolyzing those β -lactams than benzyl penicillin. Many OXAs have evolved the ability to confer resistance to cephalosporins, and like the Class A enzymes, they often specialize in the hydrolysis of a specific subset of the cephalosporins (Naas and Nordmann, 1999).

In contrast, the Class C enzymes originally emerged with the ability to hydrolyze a very broad spectrum of cephalosporins, and they have not evolved very much in response to the modern clinical usage of β -lactam antibiotics (Medeiros, 1997; Barlow and Hall, 2002b).

Evolution is inevitably linked with classification, indeed for many years evolutionary biology was virtually synonymous with classification of organisms into families, classes, genera and species. Phylogenies are, in a sense, a way to organize those classifications so as to illuminate genetic relationships and lines of descent. It is only fairly recently that phylogenetics has been applied to molecules. Another way to classify molecules is by function, as exemplified by the Bush–Jacoby–Medeiros scheme for classifying the β -lactamases (Bush et al., 1995; Medeiros, 1997). If important functional changes have been historically unique, i.e. if each arose once in response to a specific selective pressure, then functional classification should be highly congruent with phylogenetic classification. If functional changes have arisen multiple times, perhaps in response to similar selective pressures, then we may well find members of the same functional group that are only distantly related.

In an evolutionary biologist's perfect world, a molecular phylogeny of resistance genes would be completely congruent with a phylogeny of the organisms that host those genes.

Horizontal gene transfer, however, makes the real world far from perfect. Plasmids move genes promiscuously among species, genera, and even bacterial kingdoms. While we usually think of genes as moving from the chromosomes within which they evolved onto plasmids, it is not uncommon to find that genes have moved from plasmids into the chromosomes of new hosts with which they had no historical relationship.

3. Evolution of the Class A serine β -lactamases

The Class A β -lactamases were the first β -lactamases to be identified in penicillin-resistant isolates of bacteria and they continue to be the most common β -lactamases today. Penicillin-resistant *Staphylococcus aureus* producing a Class A β -lactamase started appearing within a few years of the clinical introduction of penicillin in the 1940s (Medeiros, 1997). In 1963, the plasmid-borne Class A resistance gene, TEM-1, was discovered. TEM-1's relative, SHV-1, was discovered in 1974. TEM and SHV are both distributed across the globe and TEM-1 has spread through many different species of bacteria. For years, TEM-1 and its descendants have been some of the most heavily researched antibiotic resistance genes. This has been, in part, because they are one of the most commonly encountered groups of resistance genes, but also results from the extraordinary phenotypic plasticity that has been demonstrated by the TEMs (Medeiros, 1997). In the 1980s, as a response to the extremely high frequencies with which penicillin resistant bacteria were encountered, cephalosporins and monobactams came into heavy use. While those antibiotics are structurally related to penicillins, in that they all contain a β -lactam ring, they are also different enough that the penicillinases were unable to hydrolyze them efficiently. The TEM β -lactamases have demonstrated the ability to evolve greatly increased activity towards the cephalosporins and the monobactams, so much so that the TEM enzymes seriously threaten the utility of those antibiotics. The TEM enzymes have also demonstrated the ability to evolve resistance to molecules that have been specifically designed as class A β -lactamase inhibitors.

Because TEM β -lactamases have contributed so heavily to β -lactam resistance, and because they have demonstrated so much phenotypic plasticity, it has come as a bit of a surprise that a new group of Class A β -lactamases, the CTX-Ms, is rapidly increasing in frequency. While their increasing frequency could be the result of their increased efficiency for hydrolyzing cefepime when compared to the TEMs, the cause of their increase is not completely understood. It has previously been demonstrated that the TEM-29 enzyme is one amino acid substitution away from conferring significantly increased levels of cefepime resistance (Barlow and Hall, 2003a). It therefore seems that there is likely another, yet undetermined cause for the increasing frequency of CTX-M alleles.

3.1. Past evolution of the Class A β -lactamases

Fig. 2 shows a Bayesian phylogeny of the Class A β -lactamases. Gram-positive diverged from gram-negative bacteria at Node A. It is important to note that, in the interest of readability, the figure is a representative, rather than a complete, representation of Class A sequences. In particular, the sequences that are descended from TEM-1 (over 100 different alleles) and SHV-2a (over 50 alleles) are not included in Fig. 2. A detailed phylogeny of the TEM β -lactamases is given in (Barlow and Hall, 2002a), and a detailed phylogeny of the SHV β -lactamases is shown in Fig. 3. Similarly, because a detailed phylogeny of the CTX-M-related sequences will be presented elsewhere (Barlow et al., in preparation) only a few representatives of the major CTX-M groups are shown. Table 1 provides the GenBank accession numbers of the sequences shown in Figs. 2 and 3. The Class A phylogeny (Fig. 2) was rooted with the group that includes CGA1, CME2, and CFXA2 because those genes are in the chromosomes of species within the *Cytophaga–Flexibacter–Bacteroides* (CFB) group, the group that is closest to the root of the Eubacterial tree among the groups represented in this phylogeny (Brown et al., 2001). In general, the positions of chromosomal sequences on the tree correspond well to positions of the host organisms on Eubacterial trees, but there is evidence of horizontal transfer events such as the presence of PER1 in *Pseudomonas areuginosa* near the root of the tree, and the presence of a cluster of sequences from gram-positive bacteria, all descended from Node B, that is nestled well within the gram-negative proteobacteria group. Within that group there was a subsequent horizontal transfer back into the gram-negative *Haemophilus influenzae*.

The CFB group of the Eubacteria arose well before the divergence of gram-positive from gram-negative bacteria (Node A) (Brown, Douady, Italia, Marshall and Stanhope, 2001), an event that occurred about 2.2 billion years ago (Feng et al., 1997). Using that node and divergence time we estimate that there were an average of about 0.75 nucleotide substitutions per million years (Myr) across the Class A phylogeny. That is about half the substitution rate that we previously estimated for the OXA β -lactamases (Barlow and Hall, 2002c). Because Class A β -lactamases are present in the chromosomes of organisms in the CFB group and in both gram-positive and gram-negative chromosomes, we estimate that the Class A β -lactamases arose around 2.4 billion years ago. Similarly, we estimate that the horizontal transfer into the gram-positive bacteria at Node B occurred about 800 million years ago. We also estimate that the TEM and SHV enzymes diverged around 400 million years ago. Any conclusions about the timing of events during the evolution of bacteria should be taken with a certain amount of caution because the values for bacterial divergence times are all fairly speculative. Lacking a fossil record, many of the estimates are based on assumptions about amino acid substitution rates by comparison with those rates in vertebrates. To

the extent that those assumptions are violated the estimated divergence times may be in significant error.

Given the wide distribution and ancient origin of Class A β -lactamases, we should not be surprised by the rapid appearance of strains that express those genes following the introduction of β -lactam antibiotics into medical and agricultural use.

3.2. Present evolution of the Class A β -lactamases

In the context of this article “present evolution” is taken as synonymous with evolution since the beginning of the antibiotic era, about 1950.

Three groups of Class A β -lactamases stand out in terms of rapidly expanding numbers during the antibiotic era: the TEM, the SHV and the CTX-M enzymes. Based on the phylogeny in Fig. 2, the TEM and SHV enzymes diverged from each other 300–400 million years ago, well before mammals appeared on the earth. The TEMs are all descended from TEM1 (Barlow and Hall, 2002a), and most are either extended-spectrum or inhibitor resistant β -lactamases. The TEM descendants probably evolved within the antibiotic era. The SHV enzymes likewise descended from an as-yet-unidentified common ancestor, and the short branch lengths within the SHV phylogeny indicate that they probably arose within the antibiotic era (Barlow and Hall, 2002a). Those descendants include both penicillinases and extended spectrum β -lactamases. Fig. 3 shows a Bayesian tree of the SHV β -lactamases and their close relatives, LEN-1 and LEN-2. The tree was rooted by using TEM-1 as an out-group. The ancestral sequences at Node 1 and Node 2 were estimated using the program MrBayes 2.1 (Huelsenbeck and Ronquist, 2001), and the estimated amino acid sequence at Node 1 is identical to that of SHV-36, while the amino acid sequence of Node 2 is estimated to be identical to that of SHV-2a. The Bush–Jacoby–Medeiros scheme classifies β -lactamases functionally according to relative rates of hydrolyzing a variety of substrates and sensitivity to various β -lactam inhibitors (Bush et al., 1995). These results suggest that the ancestral SHV probably had the extended spectrum phenotype (Bush–Jacoby–Medeiros group 2be), and that the broad spectrum (Bush–Jacoby–Medeiros group 2b) phenotype enzymes such as OHIO1, OHIO2, and SHV1 were derived from that extended spectrum SHV ancestor.

Thus, it appears that unlike the TEM enzymes, that experienced great phenotypic evolution, the SHV enzymes diversified but experienced little phenotypic evolution.

The first CTX-M enzyme was isolated in 1986, and over the next 9 years only an additional four members of that family were isolated (Bonnet, 2004) (see Bonnet, 2004 for a recent review of the CTX-M enzymes). Since that time there has been a virtual explosion in the discovery of CTX-M family β -lactamases and at this time at least 49 different members of the family have been identified. In contrast to the TEM and SHV enzymes, whose rapid appearance reflected

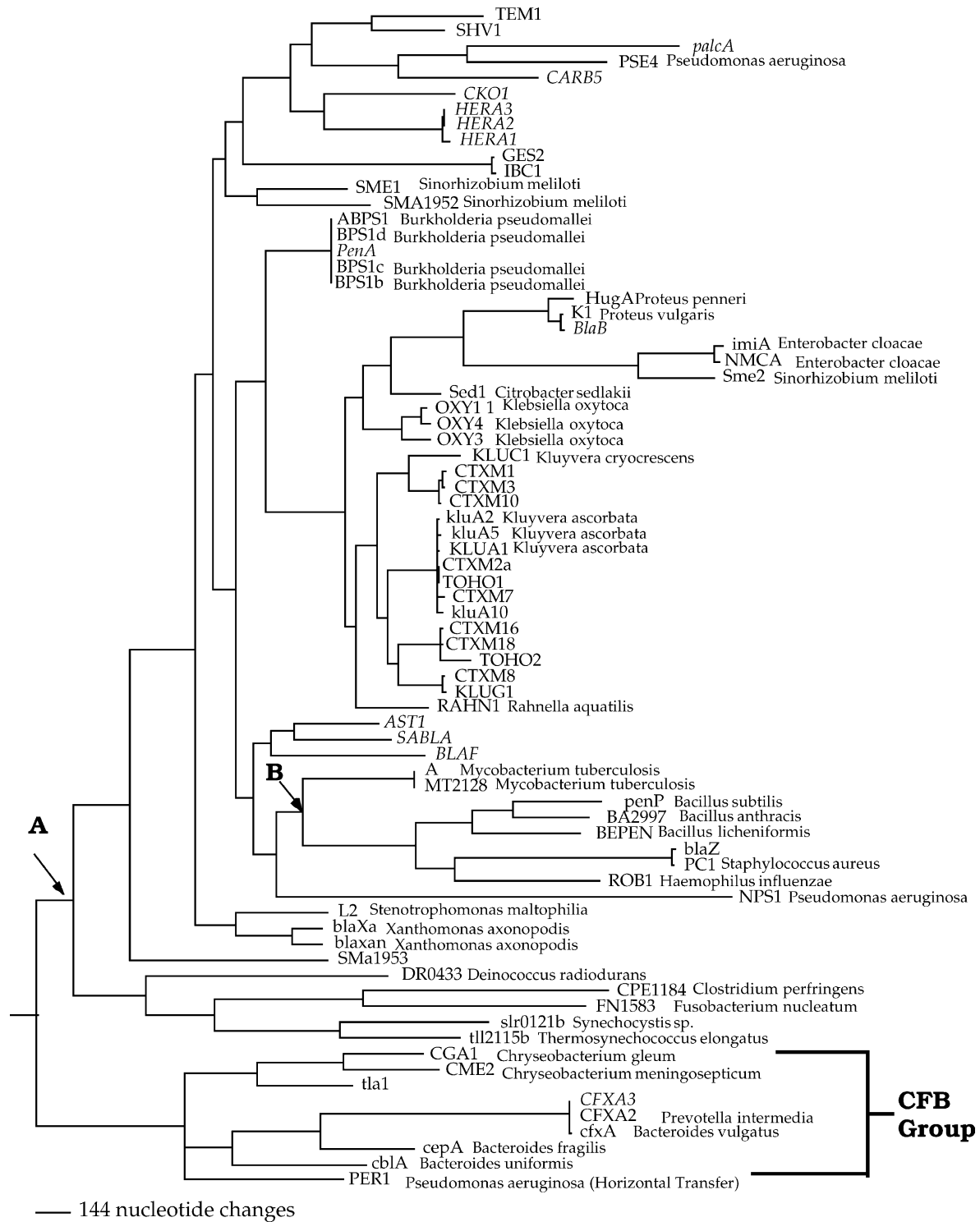


Fig. 2. Bayesian phylogeny of the Class A β -lactamases. The protein sequences derived from the genes listed in Table 1 were aligned using Clustal X v1.81 (Thompson et al., 1997) using the Gonet 250 similarity matrix with a gap opening penalty of 35 and a gap extension penalty of 0.75 for the pairwise alignment stage, and a gap opening penalty of 15 and a gap extension penalty of 0.3 for the multiple alignment stage. The corresponding DNA coding sequences (see Table 1 for accession numbers) were aligned by introducing triplet gaps between codons corresponding to gaps in the aligned protein sequences by using the program CodonAlign (Hall, 2001). The phylogeny was constructed by the Bayesian method (Rannala and Yang, 1996; Mau and Newton, 1997; Mau et al., 1999) as implemented by the program MrBayes (Huelsenbeck and Ronquist, 2001). Branch lengths are proportional to distances as measured in nucleotide substitutions per site. For simplicity the distance scale has been converted to nucleotides. The tree was rooted using the CFB group as an outgroup. Node A indicates the divergence of Gram positive from Gram negative bacteria, and Node B indicates a horizontal transfer from the gram-negative Proteobacteria into gram-positive bacteria.

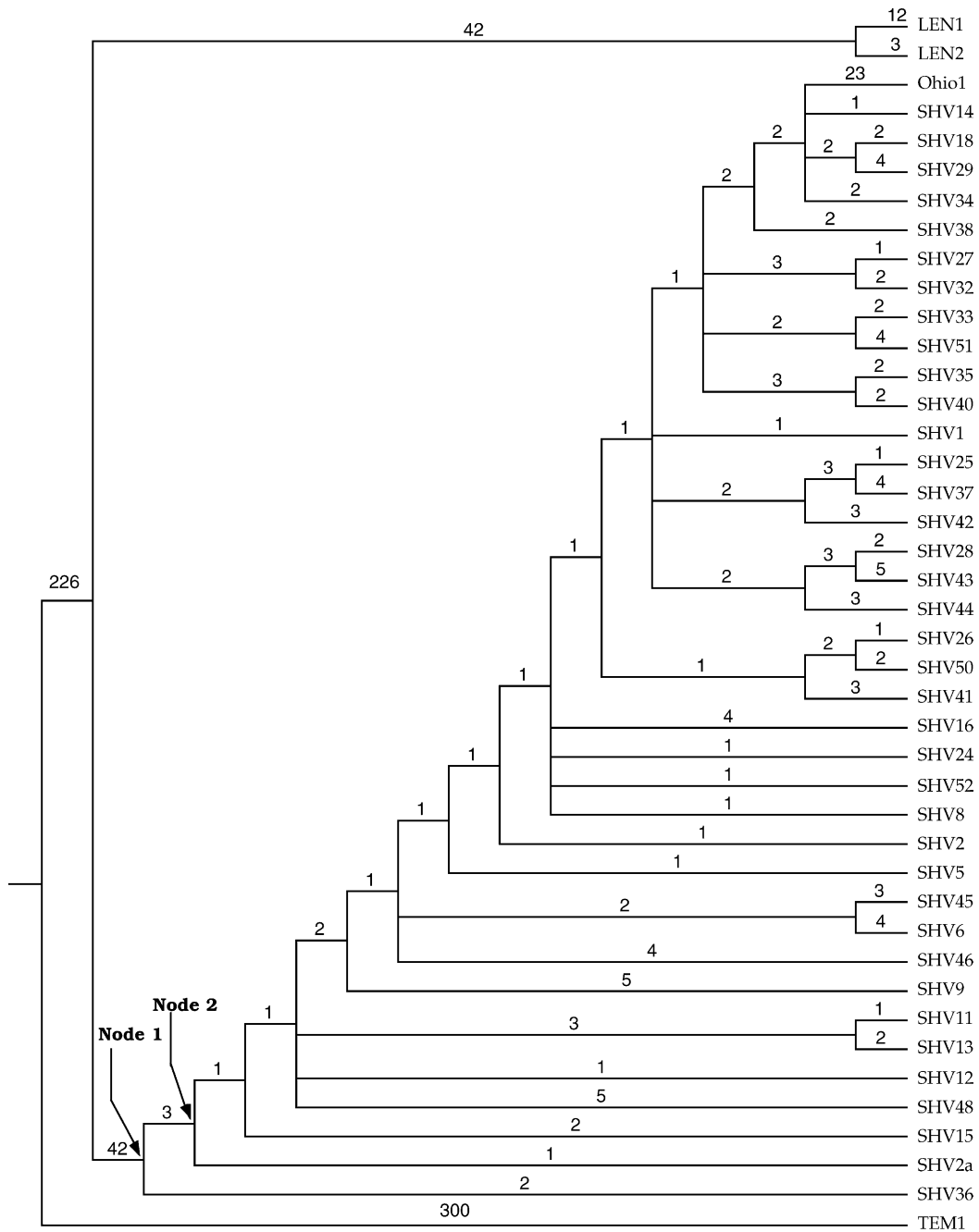


Fig. 3. Bayesian phylogeny of the SHV β -lactamases. The phylogeny was constructed as for Fig. 2. Branch lengths in nucleotide substitutions are indicated above each branch. The branches are not drawn proportionally to those lengths. Nodes 1 and 2 are discussed in the text.

rapid evolution during the antibiotic era, the CTX-M family appears to have diverged from a common ancestor 200–300 million years ago. Despite their explosive appearance, at least in terms of our awareness, little phenotypic evolution has accompanied the diversification of the CTX-M family.

3.3. Future evolution of the Class A β -lactamases

If we knew how antibiotic resistance genes were going to evolve in the near future in response to the continued use

of current drugs and in response to the introduction of new drugs we should be able to design optimal drug use strategies, and pharmaceutical companies could predict the useful lifetimes of new drugs even before introducing them into clinical use. Furthermore, being able to predict the emergence of resistance to an antibiotic can also help pharmaceutical companies determine which antibiotics will have the longest useful period in the clinical setting and can act as a guide for which drugs are most worth sending through very costly clinical trials. At this time our only practical response

Table 1
Class A and SHV accession numbers

Taxon name	Organism	Gene location	Accession number
Class A β -lactamases			
A	<i>Mycobacterium tuberculosis</i>	Chromosome	U67924
ABPS1	<i>Burkholderia pseudomallei</i>	Chromosome	AF326770
AST1	<i>Nocardia asteroides</i>	Chromosome	AF279904
BA2997	<i>Bacillus anthracis</i> str. A2012	Chromosome	NC_003995
BEPEN	<i>Bacillus licheniformis</i>	Chromosome	V00093
BlaB	<i>Proteus vulgaris</i>	Chromosome	D37831
BLAF	<i>Mycobacterium fortuitum</i>	Unknown	L25634
blaXa	<i>Xanthomonas axonopodis</i> pv. citri str. 306	Chromosome	NC_003919
blaxan	<i>Xanthomonas campestris</i> pv. Campestris str. ATCC 33913	Chromosome	NC_003902
blaZ	<i>Staphylococcus aureus</i> ssp. Aureus N315	Plasmid	NC_003140
BPS1b	<i>Burkholderia pseudomallei</i>	Chromosome	AF441237
BPS1c	<i>Burkholderia pseudomallei</i>	Chromosome	AF441238
BPS1d	<i>Burkholderia pseudomallei</i>	Chromosome	AF441239
CARB5	<i>Acinetobacter calcoaceticus</i> ssp. Anitratus	Chromosome	AF135373
cbIA	<i>Bacteroides uniformis</i>	Chromosome	L08472
cepA	<i>Bacteroides fragilis</i>	Chromosome	U05888
cfxA	<i>Bacteroides vulgatus</i>		U38243
CFXA2	<i>Prevotella intermedia</i>	Chromosome	AF118110
CFXA3	<i>Capnocytophaga ochracea</i>	Unknown	AF472622
CGA1	<i>Chryseobacterium gleum</i>	Chromosome	AF339733
CKO1	<i>Citrobacter koseri</i>	Unknown	AF477396
CME2	<i>Chryseobacterium meningosepticum</i>	Chromosome	AF033200
CPE1184	<i>Clostridium perfringens</i>	Chromosome	NC_003366
CTXM1	<i>Escherichia coli</i>	Plasmid	AJ416340
CTXM10	<i>Escherichia coli</i>	Plasmid	AF255298
CTXM16	<i>Klebsiella pneumoniae</i>	Plasmid	AY033516
CTXM18	<i>Klebsiella pneumoniae</i>	Plasmid	AF325133
CTXM2a	<i>Proteus mirabilis</i>	Plasmid	AJ416343
CTXM3	<i>Citrobacter freundii</i>	Unknown	Y10278
CTXM7	<i>Salmonella typhimurium</i>	Plasmid	AJ005045
CTXM8	<i>Citrobacter amalonaticus</i>	Plasmid	AF189721
DR0433	<i>Deinococcus radiodurans</i>	Chromosome	NC_001263
FN1583	<i>Fusobacterium nucleatum</i> ssp. Nucleatum ATCC 25586	Chromosome	NC_003454
GES2	<i>Pseudomonas aeruginosa</i>	Plasmid	AF326355
HERA1	<i>Escherichia hermannii</i>	Unknown	AF311385
HERA2	<i>Escherichia hermannii</i>	Unknown	AF398334
HERA3	<i>Escherichia hermannii</i>	Chromosome	AF398335
HugA	<i>Proteus penneri</i>	Chromosome	AF324468
IBC1	<i>Enterobacter cloacae</i>	Plasmid	AF208529
imiA	<i>Enterobacter cloacae</i>	Chromosome	U50278
K1	<i>Proteus vulgaris</i>	Chromosome	D29982
KLUA1	<i>Kluyvera ascorbata</i>	Chromosome	AJ272538
kluA10	<i>Kluyvera ascorbata</i>	Chromosome	AJ427467
kluA2	<i>Kluyvera ascorbata</i>	Unknown	AJ251722
kluA5	<i>Kluyvera ascorbata</i>	Chromosome	AJ427463
KLUC1	<i>Kluyvera cryocrescens</i>	Chromosome	AY026417
KLUG1	<i>Kluyvera georgiana</i>	Chromosome	AF501233
L2	<i>Stenotrophomonas maltophilia</i>	Chromosome	AF299368
MT2128	<i>Mycobacterium tuberculosis</i> CDC1551	Chromosome	NC_002755
NMCA	<i>Enterobacter cloacae</i>	Chromosome	Z21956
NPS1	<i>Pseudomonas aeruginosa</i>	Chromosome	AY027589
OXY1.1	<i>Klebsiella oxytoca</i>	Chromosome	Z30177
OXY3	<i>Klebsiella oxytoca</i>	Chromosome	AF491278
OXY4	<i>Klebsiella oxytoca</i>	Chromosome	AY077481
palcA	<i>Providencia alcalifaciens</i>	Unknown	AJ438771
PC1	<i>Staphylococcus aureus</i>	Chromosome	M25252
PenA	<i>Burkholderia mallei</i>	Chromosome	AY032868
penP	<i>Bacillus subtilis</i>	Chromosome	NC_000964
PER1	<i>Pseudomonas aeruginosa</i>	Chromosome	Z21957
PSE4	<i>Pseudomonas aeruginosa</i>	Chromosome	J05162
RAHN1	<i>Rahnella aquatilis</i>	Chromosome	AF338038
ROB1	<i>Haemophilus influenzae</i>	Chromosome	AF022114

Table 1 (Continued)

Taxon name	Organism	Gene location	Accession number
SABLA	<i>Streptomyces albus</i>	Chromosome	M28303
Sed1	<i>Citrobacter sedlakii</i>	Chromosome	AF321608
SHV1	<i>Klebsiella pneumoniae</i>	Plasmid	X98098
slr0121b	<i>Synechocystis</i> sp. PCC 6803	Chromosome	NC_000911
SMA1952	<i>Sinorhizobium meliloti</i>	Plasmid	NC_003037
SMA1953	<i>Sinorhizobium meliloti</i>	Plasmid	NC_003037
SME1	<i>Sinorhizobium meliloti</i>	Chromosome	NC_003047
Sme2	<i>Serratia marcescens</i>	Chromosome	AF275256
TEM1		Plasmid	AF309824
tlal	<i>Escherichia coli</i>	Plasmid	AF148067
tl2115b	<i>Thermosynechococcus elongatus</i> BP-1	Chromosome	NC_004113
TOHO1	<i>Escherichia coli</i>	Plasmid	D37830
TOHO2	<i>Escherichia coli</i>	Plasmid	D89862
SHV β -lactamases			
LEN1	<i>Klebsiella pneumoniae</i>	Chromosome	X04515
LEN2	<i>Klebsiella pneumoniae</i>	Chromosome	AY037780
Ohio1	<i>Enterobacter cloacae</i>	Plasmid	M33655
SHV1	<i>Klebsiella pneumoniae</i>	Plasmid	X98098
SHV11	<i>Shigella dysenteriae</i>	Plasmid	Y18299
SHV12	<i>Klebsiella pneumoniae</i>	Plasmid	AY008838
SHV13	<i>Klebsiella pneumoniae</i>	Plasmid	AF164577
SHV14	<i>Klebsiella pneumoniae</i>	Unknown	AF226622
SHV15	<i>Escherichia coli</i>	Plasmid	AJ011428
SHV16	<i>Klebsiella pneumoniae</i>	Chromosome	AF072684
SHV18	<i>Klebsiella pneumoniae</i>	Plasmid	AF132290
SHV2	<i>Klebsiella pneumoniae</i> ssp. Ozaenae	Unknown	X53433
SHV24	<i>Escherichia coli</i>	Plasmid	AB023477
SHV25	<i>Klebsiella pneumoniae</i>	Unknown	AF208796
SHV26	<i>Klebsiella pneumoniae</i>	Unknown	AF227204
SHV27	<i>Klebsiella pneumoniae</i>	Unknown	AF293345
SHV28	<i>Klebsiella pneumoniae</i>	Plasmid	AF299299
SHV29	<i>Klebsiella pneumoniae</i>	Unknown	AF301532
SHV2a		Plasmid	X53817
SHV32	<i>Klebsiella pneumoniae</i>	Chromosome	AY037778
SHV33	<i>Klebsiella pneumoniae</i>	Chromosome	AY037779
SHV34	<i>Escherichia coli</i>	Plasmid	AY036620
SHV35	<i>Klebsiella pneumoniae</i>	Plasmid	AY070258
SHV36	<i>Klebsiella pneumoniae</i>	Plasmid	AF467947.1
SHV37	<i>Klebsiella pneumoniae</i>	Unknown	AF467948
SHV38	<i>Klebsiella pneumoniae</i>	Chromosome	AY079099
SHV40		Unknown	AF535128.1
SHV41	<i>Klebsiella pneumoniae</i>	Unknown	AF535129
SHV42	<i>Klebsiella pneumoniae</i>	Unknown	AF535130
SHV43	<i>Klebsiella pneumoniae</i>	Unknown	AY065991
SHV44	<i>Klebsiella pneumoniae</i>	Chromosome	AY259119
SHV45	<i>Klebsiella pneumoniae</i>	Unknown	AF547625
SHV46	<i>Klebsiella oxytoca</i>	Plasmid	AY210887
SHV48	<i>Acinetobacter baumannii</i>	Plasmid	AY259164
SHV5	<i>Klebsiella pneumoniae</i>	Plasmid	X55640
SHV50	<i>Klebsiella pneumoniae</i>	Chromosome	AY288915
SHV51	<i>Klebsiella pneumoniae</i>	Chromosome	AY289548
SHV52	<i>Escherichia coli</i>	Plasmid	AY223863
SHV6	<i>Escherichia coli</i>	Plasmid	U20270
SHV8	<i>Escherichia coli</i>	Chromosome	U92041
SHV9	<i>Klebsiella pneumoniae</i>	Plasmid	S82452

to the introduction of a new drug is to limit it to “last resort” use when other drugs have failed. At a time when most pharmaceutical companies are abandoning antibiotic discovery research, limiting the use of new drugs provides an additional strong disincentive to continue antibiotic discovery efforts.

We (Barlow and Hall, 2002a) recently developed an in vitro evolution method that uses error-prone PCR to experimentally predict how a given resistance gene will evolve in response to selection with a new or existing drug. Briefly, the method uses an error-prone polymerase to introduce

mutations into the gene of interest at the rate of one to two mutations per molecule. The mutated gene copies are cloned into a suitable vector to produce a library of 10^6 to 10^7 mutant alleles in a suitable host, usually *Escherichia coli*. The library is expanded and populations that include ~ 10 copies of each mutant allele are subjected to selection for increased resistance to the drug(s) of interest by growing the populations in medium containing increasing concentrations of the drug. The highest concentration of drug that permits growth typically results in a population that includes only one to three different mutant alleles. That population is used as the starting point for another round of PCR mutagenesis and selection, and those cycles of mutation and selection continue until no further increase in resistance is obtained. Clones from the final round of selection are characterized and the mutant allele from each distinct different phenotype is sequenced. A sufficient number of parallel experiments are done that at each round the probability that any possible single or double amino acid replacement allele has been missed by chance is $<10^{-4}$. Finally, the “best” allele that conferred the highest level of resistance is tested by site-directed mutagenesis to determine whether there is an order in which the mutations could arise such that the allele could actually arise in nature by each mutation improving the resistance level.

To determine whether the Barlow–Hall method accurately predicts natural in vivo evolution we began with the TEM-1 allele and selected for the extended-spectrum phenotype using the drugs that are believed to have selected that phenotype for the TEMs in nature. It turns out that despite the large number of extended-spectrum TEMs that have been identified, only nine different amino-acid substitutions have independently arisen multiple times. Because those substitutions have been independently selected multiple times in nature, those are the substitutions that are most likely to have been responsible for the evolution of new phenotypes. In our in vitro experiments, we recovered seven of those same substitutions repeatedly, indicating that the Barlow–Hall method accurately mimics natural evolution.

We then applied that method to determine whether TEM-1 could evolve to confer a clinical level of resistance to the recently introduced β -lactam cefepime (Barlow and Hall, 2003a). No naturally-occurring TEM alleles confer clinical resistance to cefepime (MIC = 32 $\mu\text{g/ml}$) and the MIC of cefepime for the TEM-1 allele is only 0.5 $\mu\text{g/ml}$. Seven of the eight parallel experiments yielded a TEM allele that conferred an MIC of at least 32 $\mu\text{g/ml}$; i.e. clinically resistant as defined by the NCCLS breakpoints (National Committee for Clinical Laboratory Standards, 1999), and of those one allele conferred an MIC of 256 $\mu\text{g/ml}$. Those alleles had from two to six amino acid substitutions, but all had a substitution at position 164 (R164H in six of eight cases), and six alleles included an I173V substitution. Site-directed mutagenesis showed that the most resistant allele can arise by an R164H substitution that increases the MIC from 0.5 to 2 $\mu\text{g/ml}$, fol-

lowed by an I173V substitution that increases the MIC to 32 $\mu\text{g/ml}$ followed by an R178S substitution that increases the MIC to 256 $\mu\text{g/ml}$; i.e. there is a natural evolutionary pathway to the most resistant allele. Those results permitted us to predict that with the continued clinical use of cefepime the TEM β -lactamases will evolve to confer cefepime resistance, moreover the cefepime resistant alleles are likely to include R164H and I173V substitutions. Indeed, TEM-29, which differs from TEM-1 only by an R164H substitution, requires only an I173V substitution to confer cefepime resistance (Barlow and Hall, 2003a).

4. Evolution of the Class C β -lactamases

By the mid-1980s, it was known that the Class C β -lactamases were widely distributed among the chromosomes of the Enterobacteriaceae (Jaurin and Grundstrom, 1981; Lindberg and Normark, 1986), but it was not until the end of that decade, in 1989, that the first plasmid-borne Class C β -lactamase was reported (Bauernfeind et al., 1989). Because of their chromosomal locations, Class C β -lactamases were not initially subject to the rapid dissemination that was associated with the plasmid-borne TEM alleles. By now nearly 100 different Class C β -lactamases, both chromosomal and plasmid borne have been reported.

4.1. Past evolution of the Class C β -lactamases

A little less than 2 years ago we presented a Bayesian phylogeny of the Class C β -lactamases that were known at the time (Barlow and Hall, 2002b). At that time, it was not realized that the divergence of the Class C β -lactamases pre-dated the divergence of the Class A and Class D enzymes (see Fig. 1). It was thought that their distribution was limited to the Proteobacteria, a group that arose about 1.8 billion years ago (Brown et al., 2001). At this time at least one Class C β -lactamase has been identified in the chromosome of the gram-positive bacterium *Mycobacterium smegmatis* (GenBank accession AY442183). Given that the Class C β -lactamases diverged before the widely distributed Class A β -lactamases and Class D β -lactamases, and that the present-day distribution of Class C β -lactamases is limited almost exclusively to the gram-negative Proteobacteria, it seems likely that the Class C β -lactamases were eliminated from the chromosomes of gram-positive bacteria shortly after the gram-positive–gram-negative divergence.

4.2. Present evolution of the Class C β -lactamases

In 2002 (Barlow and Hall, 2002b), over 20 different plasmid-borne Class C alleles had been detected in clinical isolates over the previous 11 years and the rate at which new plasmid-borne Class C alleles were being detected

was accelerating. We were interested in learning whether these plasmid-borne alleles were evolving phenotypically in a manner similar to that of the TEM β -lactamases, and we were interested in how often independent chromosomal genes had been mobilized to plasmids.

Analysis of the phylogenetic tree showed that Class C β -lactamase genes had been independently mobilized to plasmids at least six times. Most of the CMY alleles and all of the LAT alleles were descended from a single plasmid-borne allele, CMY-2, that was clearly descended from a *Citrobacter freundii* chromosomal allele. The descendent alleles differed from CMY-2 by one to five nucleotide substitutions, and included one chromosomal allele that had been transferred into the chromosome of *Proteus mirabilis*. Other plasmid-borne groups of alleles are descended from chromosomal alleles of *Morganella moranii*, *Enterobacter cloacae*, *Hafnei alvei*, *Aeromonas sobria*, and *Aeromonadacea* species. In all cases, the short branch lengths within the groups of plasmid-borne alleles indicated that the mobilization events were recent.

To assess the phenotypic evolution of the recently-arisen plasmid-borne alleles we cloned CMY-2 and five of its descendant alleles into a common expression vector and transformed them into a common host. To provide a baseline for comparison we also cloned two chromosomal Class C alleles from *C. freundii* strains that had been isolated in the 1920s, well before the beginning of the antibiotic era. Both of those strains were sensitive to all β -lactam antibiotics that we considered. We determined the MICs of 16 β -lactam antibiotics for each of the cloned Class C alleles. Because those alleles were expressed in the same genetic background, from the same vector, and under the inducible expression of the same promoter we could be confident that any differences in MIC reflected differences in the in vivo activities of the encoded β -lactamases.

Over the 16 drugs tested there was very little difference in the levels of resistance conferred by CMY-2 and its five descendant alleles, indicating that essentially no phenotypic evolution accompanied the diversification of those alleles. More surprisingly, there was no difference between the resistance conferred by those six originally plasmid-borne alleles that had come from β -lactam-resistant clinical isolates and that conferred by the two chromosomal alleles that had come from pre-antibiotic era β -lactam-sensitive strains. It is clear that cryptic, or unexpressed, antibiotic resistance genes can provide a reservoir from which resistance can emerge by activation of those genes and subsequent mobilization to plasmids.

4.3. Future resistance of Class C β -lactamases

Cefepime is a relatively new β -lactam antibiotic, having received FDA approval only in 1996. The Class C CMY-2 gene and its descendants do not confer resistance to cefepime, however they do confer resistance to an older closely-related drug, ceftazidime (Barlow and Hall, 2002b).

Given its activity toward ceftazidime, we were curious whether CMY-2 had the evolutionary potential to confer resistance to cefepime. We used the Barlow–Hall in vitro evolution method described above to assess that potential (Barlow and Hall, 2003b).

CMY-2 confers a cefepime MIC of 2 $\mu\text{g/ml}$, whereas the MIC for clinical resistance is 32 $\mu\text{g/ml}$ (National Committee for Clinical Laboratory Standards, 1999). In seven independently evolved lines derived from CMY-2 six reached an MIC of 32 $\mu\text{g/ml}$ and one an MIC of 64 $\mu\text{g/ml}$. The resistant evolved alleles encoded enzymes that differed from CMY-2 by two to five amino acid substitutions. The allele that conferred the highest level of resistance was that with five mutations. As was the case for in vitro evolution of TEM-1, we showed by site-directed mutagenesis that there is an order in which those substitutions occur such that each increases the resistance to cefepime. We therefore concluded that an allele that confers an MIC of at least 64 $\mu\text{g/ml}$ can arise in nature.

A significant difference between these results and those obtained when evolving the TEM-1 allele is that there were no substitutions that were common to all, or even to a majority, of the evolvents. All alleles, however, did contain at least one substitution in the region between amino acids 291 and 298, indicating the importance of that region to the hydrolysis of cefepime.

Surprisingly, the activity of the parent allele toward the closely related drug ceftazidime had little predictive value for the ultimate activity toward cefepime of the evolvents. TEM-1 confers an MIC of 1 $\mu\text{g/ml}$ for ceftazidime, while CMY-2 confers an MIC of 256 $\mu\text{g/ml}$ for ceftazidime; yet a TEM-1 evolvent was able to achieve an MIC of 256 $\mu\text{g/ml}$ for cefepime, while the best CMY-2 evolvent achieved an MIC of only 64 $\mu\text{g/ml}$ for ceftazidime. This should serve as a caution that activity toward structurally related drugs is not a good predictor of future activity toward a drug of interest.

5. Evolution of the Class D β -lactamases

The Class D β -lactamases are a diverse group of enzymes whose genes are located both on plasmids and in the chromosomes of a wide range of gram-negative bacteria (Sanschagrin et al., 1995; Barlow and Hall, 2002c). Because the enzymes appear to specialize in hydrolyzing oxacillin many of the alleles have been designated OXA. Nearly 60 different OXA alleles have been isolated (<http://www.lahey.org/studies/webt.htm>).

About 2 years ago, we constructed a Bayesian phylogeny of the OXA β -lactamases and some of their homologs (Barlow and Hall, 2002c). We found that, as remains true today, the distribution of the Class D OXA alleles was limited to gram-negative bacteria, but that some homologs of the OXA genes were found in the chromosomes of gram-positive bacteria. Because those gram-positive alleles

cluster well within the gram-negative proteobacteria, the phylogeny strongly suggests that an ancestral OXA homolog was horizontally transferred into the gram-positive bacteria. Additional horizontal transfer events within the gram-negative bacteria were also apparent.

The phylogeny showed that the OXA genes have been mobilized to plasmids at least three different times, and we were able to estimate the times at which those mobilization events occurred. By using several distances from the tree in Fig. 2 of Brown et al. (2001) and using the date of 2.2 billion years ago for the divergence of gram-positive and gram-negative bacteria (Feng et al., 1997) we were able to calibrate the branch lengths on the OXA tree to about 1.26 ± 0.27 nucleotide substitutions per million years. Using that calibration, one mobilization of Class D genes to plasmids occurred about 116 ± 25 million years ago and another 42 ± 9 million years ago. The third mobilization was too recent to estimate accurately. We also estimated that the horizontal transfer event into the gram-positive lineage occurred between 575 and 520 million years ago.

Those findings are important because they show that Class D β -lactamase genes have been on plasmids and have been moving between bacterial kingdoms for millions of years. In turn, that means that mobilization to plasmids and horizontal transfer is not a phenomenon that has been driven exclusively by the modern use of antibiotics in medicine and agriculture.

6. Conclusions

The past evolution of each of the three classes of the serine β -lactamases indicates that the Class A β -lactamases have been around for a very long time and that they have been diversifying, moving onto plasmids, on those plasmids moving among species and even bacterial kingdoms, and moving from plasmids into chromosomes for much of their evolutionary history. The well-known study of the pre-antibiotic era Murray collection (Datta and Hughes, 1983) that found no resistance among those strains created a false picture that allowed us to attribute the existence of antibiotic resistance genes entirely to the modern use of antibiotics. The Datta study looked only at resistance phenotypes, but the existence of fully-functional but phenotypically silent (cryptic) Class C β -lactamase genes in pre-antibiotic era *C. freundii* (Barlow and Hall, 2002b) makes it clear that considering resistance phenotypes alone can be misleading. Other cryptic antibiotic resistance genes have been found in *Salmonella* (Magnet et al., 1999; Salipante and Hall, 2003) and in *Bacillus subtilis* (Salipante et al., 2004), and many of the “homologs” of antibiotic resistance genes that are found during the sequencing of bacterial genomes may well be similar cryptic genes. The phylogenies of all three classes of the serine β -lactamases suggest that there are many antibiotic resistance genes lurking within bacterial genomes, and it is not unreasonable to

expect that those genomes include resistance genes that are unrelated to any of the currently known resistance genes. If the products of some of those genes have specificities for drugs that are yet to be discovered, we should expect them to emerge when those new drugs are put into clinical use.

The Class A CTX-M genes may well provide an example of a group that is emerging only now in response to the introduction of cefepime into clinical use. Although the first CTX-M was detected 18 years ago, the explosive growth in the number reported over the last 2–3 years suggests a population explosion in response to a recent strong selective pressure.

The dramatic phenotypic evolution of the TEM β -lactamases to generate not only enormous diversity, but both extended-spectrum and inhibitor-resistant enzymes derived from TEM-1 is clearly the exception, rather than the rule. Most of the genes that have recently been mobilized to plasmids and subsequently diversified have exhibited little phenotypic evolution. When it was assumed that the extended-spectrum SHV enzymes evolved from SHV-1, which is primarily a penicillinase, the SHV enzymes were taken as another example of rapid phenotypic evolution. Phylogenetic analysis, on the other hand, suggests that the ancestral SHV was already extended-spectrum, and that enzymes such as SHV-1, OHIO-1, etc. evolved to become better penicillinases at the expense of being able to hydrolyze cephalosporins as effectively.

Because the TEMs are the exception, rather than the rule, does that mean that we should ignore the TEM phenomenon as we consider the potential of other resistance genes for phenotypic evolution? Perhaps not. The failure of most other genes to exhibit significant phenotypic evolution may simply reflect the fact that they were *already* capable of hydrolyzing most modern β -lactams effectively; i.e. they did not have much of anywhere to go in phenotypic space. On the other hand, our experience with in vitro evolution of TEM-1 and CMY-2 suggests that TEM-1 is considerably more plastic than CMY-2. If the structure of the TEM enzymes makes them significantly more plastic than most β -lactamases, then TEM may indeed be an exception, in which case we should revise our expectations of general rapid phenotypic evolution when a new drug is introduced.

Our in vitro evolution studies showed that both the Class A TEM and the Class C CMY enzymes have the potential to evolve to confer clinical resistance to cefepime, but with the introduction of cefepime into clinical use neither has yet done so in nature. Why not? Possibly because the CTX-M enzymes, that already hydrolyze cefepime effectively, were able to emerge and to occupy the cefepime-hydrolysis niche more easily than either TEM or CMY could evolve a cefepime-hydrolysis phenotype.

The Class D enzymes are already very effective at hydrolyzing most β -lactam antibiotics, so it is not surprising that they have exhibited little phenotypic evolution. Recently

a few of the OXA enzymes have been shown to have activity toward carbapenems such as imipenem (Bou et al., 2000). In general, it has been thought that only the metallo- β -lactamases are capable of increasing resistance to carbapenems. It might be worthwhile applying the Barlow–Hall in vitro evolution approach to the carbapenem-hydrolyzing OXA alleles to determine whether those genes have the potential to increase that activity to a level that would confer clinical resistance to carbapenems.

No evolutionary paper would be complete without an element of pure speculation. Let us speculate for a moment about the reasons that β -lactamases should be so widely distributed throughout the genomes of the Eubacteria. We suggest that during the early evolutionary history of the Eubacteria the biosphere was not nearly as rich in organic molecules as it is today, and that synthesis of antibiotics, including β -lactams, may have been a common means of sequestering organic resources from competitors, perhaps by killing and eating those competitors. If that were the case β -lactamases would have evolved both as protection from self-generated β -lactams and from the β -lactams of competitors. As the biosphere became organically richer the importance of that competitive mechanism may have diminished, leading to losses of β -lactam-synthesizing genes. As fewer competitors synthesized β -lactams, β -lactamase genes might have been lost. Although today we see relatively few β -lactam-synthesizing organisms compared with the number that make β -lactamases, there is some evidence that at one time β -lactam-synthesis was widely distributed. Although there is a wide variety of β -lactam synthesizing pathways, depending upon the final product, all of those pathways share the gene ACV synthetase that completes the first step of β -lactam synthesis. The ACV synthetase gene is very widely distributed throughout the Eubacterial kingdom, being present in alpha, beta and gamma subdivisions of the Proteobacteria, in the Firmicutes (gram-positive bacteria), the Actinobacteria (high G + C, gram-positive bacteria), and the Cyanobacteria. That wide distribution is consistent with the idea that at one time most bacteria produced antibiotics, and that β -lactamases evolved very early as a means of protection against those molecules.

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