

Phylogenetic Analysis Shows That the OXA β -Lactamase Genes Have Been on Plasmids for Millions of Years

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Abstract. The OXA genes encode a class of β -lactamases that confer resistance to a wide range of β -lactam antibiotics. To determine whether the diversity of the OXA β -lactamases is the result of recent or ancient events, and to determine whether mobilization of the OXA genes from chromosomes to plasmids occurred recently or long ago, we have constructed a Bayesian phylogeny of the OXA β -lactamase genes. Analysis of that phylogeny shows that much of the diversity is the result of ancient events and that the OXA genes were mobilized from chromosomes to plasmids on at least two independent occasions that occurred millions of years ago. That observation contradicts the commonly held impression that mobilization of antibiotic resistance genes is strictly the result of modern use of antibiotics.

Key words: OXA — β -lactamase — Positive selection — Gene mobilization

Introduction

The rapid evolution of antibiotic resistant microbes has allowed us the unique opportunity to observe evolutionary processes over the course of a few decades instead of the several millennia that are generally required for these processes to occur. Because antibiotics have been in use only for the past 60 years,

we have detailed information about the genetic changes that have occurred to produce resistant microbes as well as the selective pressures that have driven those changes. In many cases the antibiotics that selected specific adaptations are known. The TEM β -lactamases are a group of resistance genes for which we have sufficient data to correlate selective pressures with specific mutations. Microbial resistance to β -lactam antibiotics, including penicillin, ampicillin, cephalosporins, and their modern synthetic derivatives, is primarily the result of the synthesis of β -lactamases, enzymes that degrade β -lactam antibiotics. The TEM family of β -lactamases is plasmid-borne and all are descended from the TEM-1 allele (Barlow and Hall 2002b). TEM-1 is primarily a penicillinase that is relatively inactive toward third- and fourth-generation cephalosporins. Many of its descendants, the so-called “extended-spectrum” TEM β -lactamases exhibit greatly increased activity toward the modern β -lactam antibiotics. Since the first appearance of the extended-spectrum TEM β -lactamases in the 1980s, the protein substitutions arising in the new TEM alleles have been determined (<http://www.lahey.org/studies/webt.htm>). The phenotypic effects of those substitutions have been determined *in vivo* by measuring the minimum inhibitory concentration (MIC) for a variety of β -lactam substrates and *in vitro* by kinetic measurements (Franceschini et al. 1998; Neuwirth et al. 2000; Perilli et al. 2000). The structural consequences of many of those amino acid substitutions have been determined by crystal structure analysis (Knox 1995; Matagne et al. 1998, Orenca et al.

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2001). The overall picture painted by these analyses has been that within 3 years, the TEM β -lactamases can evolve the ability to confer resistance to most β -lactam antibiotics that are in widespread clinical use. (Medeiros 1997). Another family, the OXA β -lactamases, has also evolved the ability to hydrolyze an extended range of substrates.

Not all β -lactamase genes are located on plasmids. Members of both the AmpC family and the OXA family are found both on chromosomes and on plasmids. Plasmid-borne resistance genes can spread rapidly by horizontal transfer. Phylogenetic analysis has shown that members of the AmpC family have been mobilized from chromosomes to plasmids at least six times (Barlow and Hall 2002a). The AmpC β -lactamase allele from the chromosome of *Citrobacter freundii* has been mobilized to a plasmid, and while we cannot be certain of the exact time that this gene was mobilized, its debut in clinical isolates during the early 1990s and the relatively few differences in the plasmid-borne and chromosomal *ampC* sequences suggest that the gene was mobilized quite recently. The *ampC* genes from several other species have also been recently mobilized (Barlow and Hall 2002a).

The repeated demonstrations of the evolution of increased substrate range and the mobilization of resistance genes in response to the current use of antibiotics have served as a caution that the usefulness of an antibiotic exists for a limited amount of time. However, it is not the case that antibiotic resistance genes have evolved and been mobilized exclusively as a response to the modern clinical use of antibiotics. There is growing evidence that resistance genes were capable of conferring resistance long before the discovery of modern antibiotics. For example, two antibiotic-sensitive strains of *Citrobacter freundii* that were collected in the preantibiotic era carry *ampC* genes whose products are fully as active toward as wide a range of substrates as are the AmpC β -lactamases that are found on plasmids. The only requirement for those preantibiotic-era *ampC* genes to become resistance determinants is that they must be expressed at a higher level. The enzymes produced by these genes were highly efficient at hydrolyzing β -lactam antibiotics long before antibiotics ever came into clinical use (Barlow and Hall 2002a).

The TEM β -lactamases paint a clear picture in which the extant diversity is the consequence of recent evolution in response to the clinical use of antibiotics. In contrast, the AmpC β -lactamases paint a different picture in which most of the diversity is the result of ancient evolution but in which mobilization of those genes from chromosomes to plasmids occurred recently (Barlow and Hall 2002a). It is already well understood that the OXA family of β -lactamases is very diverse (Sanschagrin et al. 1995) and that, like the AmpC family, the OXA genes are found both on

chromosomes and on plasmids. To determine whether that diversity is the result of recent or ancient events, and to determine whether mobilization of the OXA genes from chromosomes to plasmids occurred recently or long ago, we have constructed a Bayesian phylogeny of the genes encoding the OXA β -lactamases and the OXA homologues that exist in the chromosomes of several bacteria species. Comparison of that phylogeny with a phylogeny of small ribosomal RNAs has allowed us also to estimate the times at which those mobilizations occurred. Estimates of the ratio of replacement-to-silent nucleotide substitutions along each of the branches of that phylogeny have allowed us to conclude that these genes have been under strong positive selection several times in the very distant past. These results add to the growing evidence that ancient evolutionary events have contributed considerably to the antibiotic resistance problem we currently face.

Methods

The Sequences

Table 1 lists the sequences of the OXA β -lactamases and their homologues that were used to construct the phylogeny shown in Figs. 1 and 2. All of the OXA sequences that are so identified in GenBank are included. OXA homologues were identified by a TBLASTN search of the 144 complete and incomplete microbial genomes that were available in December 2001 using the OXA-1 amino acid sequence as a search probe. Only sequences with an *E* value $<10^{-13}$ were included.

Alignment

Before alignment several amino acid sequences were truncated by deleting the N-terminal residues as shown in Table 2.

The protein sequences derived from the genes in Table 1 were aligned with ClustalX 1.8 (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). CodonAlign for Macintosh and for PC (Windows) computers, and source code that can be compiled for other platforms, is available at no charge at <http://www.rochester.edu/college/BIO/labs/Hall-Lab/index.html>.

Phylogenetic Reconstruction

Phylogenies were constructed by the Bayesian method (Mau and Newton 1997; Mau et al. 1999; Rannala and Yang 1996) as implemented by the program MrBayes (Huelsenbeck 2000). MrBayes is available at no charge from <http://brahms.biology.rochester.edu/software.html>. The evolutionary model was the general time-reversible model (Tavaré 1986). Among-site variation in evolutionary rate was estimated separately for the first, second, and third

Table 1. Sequences used to construct the phylogeny

Name	Accession No. or reference	Plasmid or chromosomal location	Organism	Phylogenetic group
OXA-1	AJ238349	Plasmid		
OXA-2	M25261	Plasmid	<i>Salmonella typhimurium</i>	
OXA-3	L07945	Plasmid		
OXA-5	X58272	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-10	U37105	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-15	U63835	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-17	AF060206	Chromosome	<i>Pseudomonas aeruginosa</i>	Proteobacteria, γ group
OXA-18	U85514	Chromosome	<i>Pseudomonas aeruginosa</i>	Proteobacteria, γ group
OXA-19	AF043381	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-22	AF064820	Chromosome	<i>Ralstonia pickettii</i>	Proteobacteria, β group
OXA-24	Bou et al. (2000)	Chromosome	<i>Acinetobacter baumannii</i>	Proteobacteria, γ group
OXA-25	AF201826	Chromosome	<i>Acinetobacter baumannii</i>	Proteobacteria, γ group
OXA-26	AF201827	Chromosome	<i>Acinetobacter baumannii</i>	Proteobacteria, γ group
OXA-27	AF201828	Chromosome	<i>Acinetobacter baumannii</i>	Proteobacteria, γ group
OXA-28	AF231133	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-29	AJ400619	Chromosome	<i>Legionella gormanii</i>	Proteobacteria, α group
OXA-30	AF255921	Chromosome	<i>Shigella flexneri</i>	Proteobacteria, γ group
OXA-31	AF294653	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-32	AF315351	Plasmid	<i>Pseudomonas aeruginosa</i>	
OXA-34	AF350424		<i>Pseudomonas aeruginosa</i>	
OXA-35	AF315786	Plasmid	<i>Pseudomonas aeruginosa</i>	
	SANGER 28450	Chromosome	<i>Burkholderia pseudomallei</i>	Proteobacteria, β group
Similar to β -lactamase	Z99105	Chromosome	<i>Bacillus subtilis</i>	Gram positive
	TIGR 1282	Chromosome	<i>Staphylococcus epidermis</i>	Gram positive
Methicillin resistance gene	AP003358	Chromosome	<i>Staphylococcus aureus</i> Mu50	Gram positive
	SANGER 1496	Chromosome	<i>Clostridium difficile</i>	Gram positive
	DOE 985	Chromosome	<i>Cytophaga hutchisonii</i>	Flexibacteria–Cytophaga–Bacteroides group
	TIGR 24	Chromosome	<i>Shewanella putrefasciens</i>	Proteobacteria, γ group
	DOE 63737	Chromosome	<i>Nostoc punctiforme</i>	Cyanobacteria
	DOE 156889	Chromosome	<i>Magnetococcus</i> MC-1	Proteobacteria, magneotactic group
Putative periplasmic β -lactamase	CJ11168X1	Chromosome	<i>Campylobacter jejuni</i>	Proteobacteria, ϵ group
Probable β -lactamase	AE004964	Chromosome	<i>Pseudomonas aeruginosa</i>	Proteobacteria, γ group
Class D β -lactamase	AE008024	Chromosome	<i>Agrobacter tumefaciens</i>	Proteobacteria, α group
	CUCGC446	Chromosome	<i>Legionella pneumophila</i>	Proteobacteria, α group

positions of sites within codons. Four chains, with a “temperature” of 0.2 for the heated chains, were run for 1,000,100 generations, sampling trees every 100 generations. The log likelihood of the trees had converged on a constant value by generation 10,000, i.e., after saving 100 trees. The consensus tree, with branch lengths, was calculated from the final 9601 trees visited, well after convergence had occurred.

One of the advantages of Bayesian inference of phylogeny is that the results are easy to interpret. For example, the sum of the posterior probabilities of all trees is 1. Moreover, the posterior probability of any single clade is simply the sum of the posterior probabilities of all trees that contain that clade. The consensus trees calculated by MrBayes do not include the posterior probabilities of the clades, thus each entire set of trees was imported into PAUP* (Swofford 2000) and the same trees used by MrBayes to calculate a consensus were used to calculate a 50% majority rule consensus in PAUP* (Swofford 2000). The resulting tree shows the posterior probabilities of the clades, i.e., the percentage of time that those taxa are included in the clade.

The consensus trees calculated by MrBayes were imported into PAUP* for the purposes of displaying and printing the tree.

Estimation of Positive Selection

Estimation of the nonsynonymous (d_N) and synonymous (d_S) substitution rates is an important means of understanding mechanisms of molecular evolution. A d_N/d_S ratio > 1 is taken as evidence of positive selection, while a d_N/d_S ratio < 1 is taken as evidence of purifying selection (Yang 1998). The Codeml program of the PAML package (Yang 1998) was used to estimate d_N/d_S ratios in the phylogeny shown in Fig. 1.

Results

Phylogeny of OXA Genes and Their Homologues

A Bayesian phylogeny of the OXA β -lactamases and their homologues, in which most of the clades are well supported, is shown in Fig. 1. OXA genes are found both on plasmids and in chromosomes. The chrom-

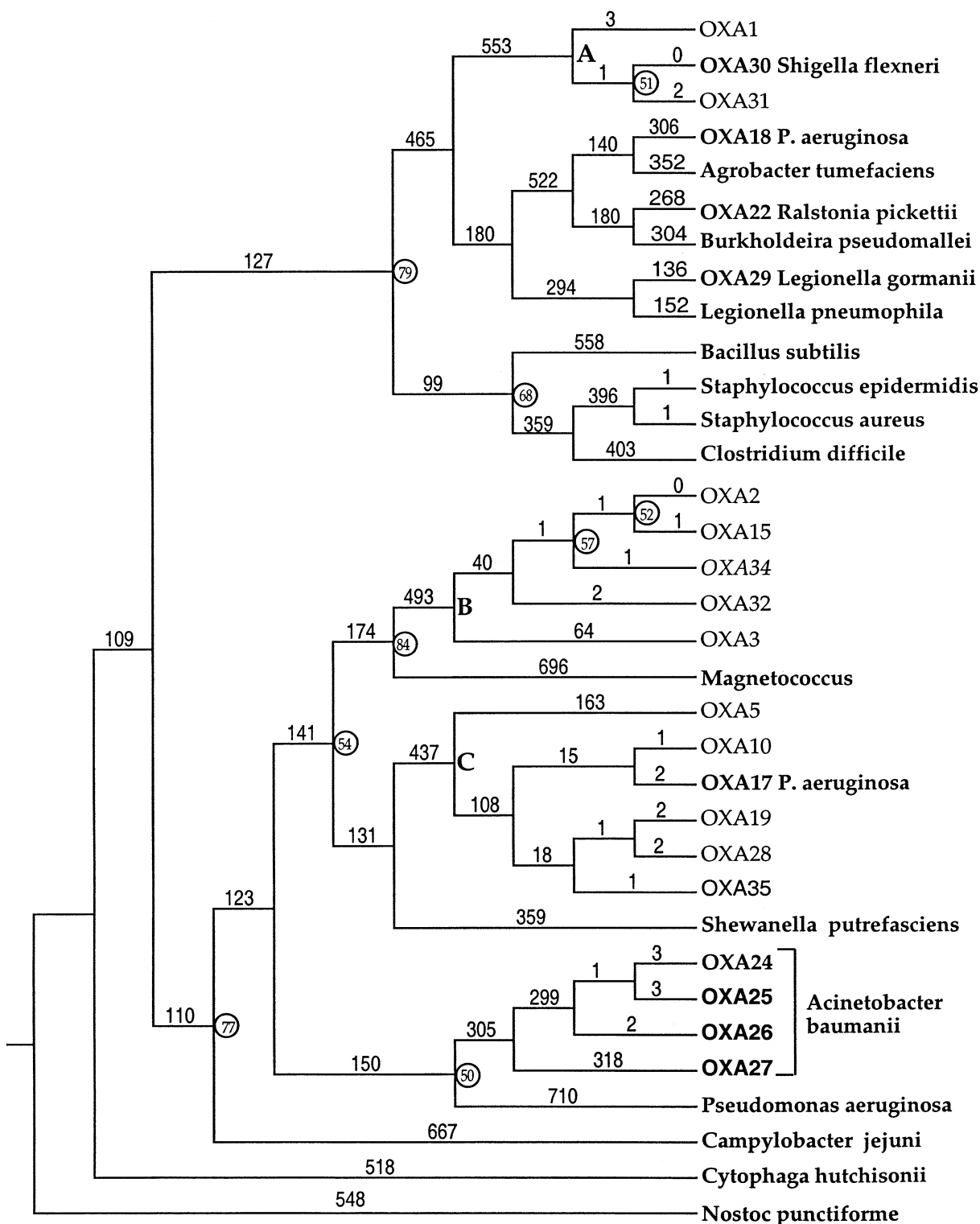


Fig. 1. Bayesian phylogeny of OXA genes and their homologues. Branch lengths, indicated above each branch, are in nucleotide substitutions. Posterior probabilities of clades were >85% except where indicated by a percentage value within a circle at a node. OXA genes were mobilized from chromosomes to plasmids along

the branches leading to nodes A, B, and C. **Boldface** indicates taxa in which the OXA genes or homologues are chromosomal; *regular typeface* indicates plasmid-borne alleles. OXA-34 is *italicized* to indicate that we do not know whether it is chromosomal or plasmid-borne.

osomal OXA genes are found exclusively in the Gram-negative Proteobacteria group, and most are in the γ subdivision. Most of the OXA homologues are also in

the proteobacteria, but two are in other Gram-negative groups and four homologues are in Gram-positive bacteria. The phylogeny of the OXA homologues

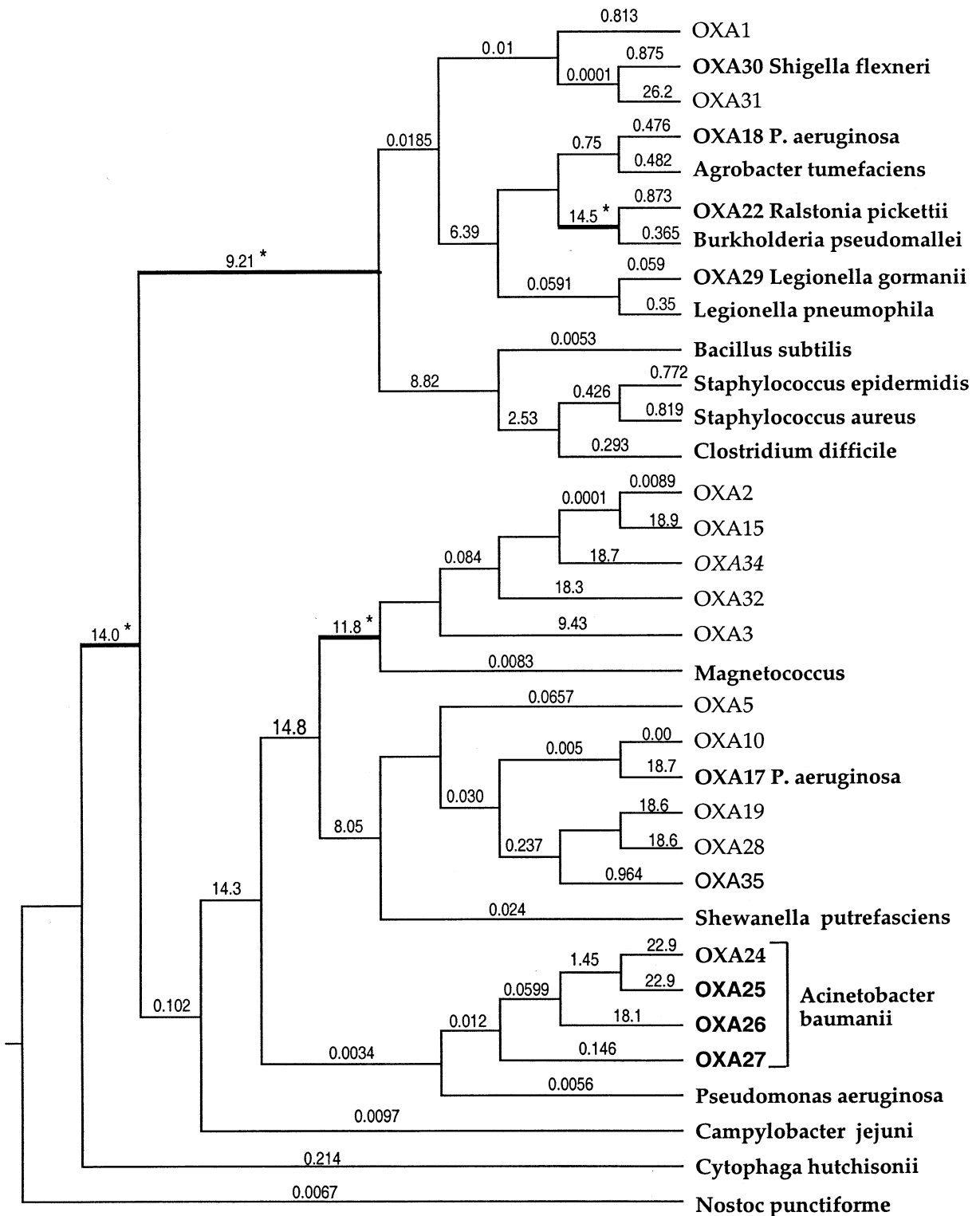


Fig. 2. Evidence for positive selection along the OXA tree. d_N/d_S ratios are indicated above branches. The four branches for which d_N/d_S ratios were significantly >1.0 are indicated by *heavy lines* and an *asterisk* next to the d_N/d_S ratio. *Boldface* indicates taxa in

which the OXA genes or homologues are chromosomal; *regular typeface* indicates plasmid-borne alleles. OXA-34 is *italicized* to indicate that we do not know whether it is chromosomal or plasmid-borne.

would make it appear that the Gram-positive bacteria are a clade within the Proteobacteria, a placement that is in strong disagreement with standard bacteria systematics. The phylogeny thus suggests that an an-

cestral OXA homologue was horizontally transferred into the Gram-positive bacteria.

Other horizontal transfer events include the recent transfers of a plasmid-borne allele into *Shigella flex-*

Table 2. Genes truncated for the purpose of alignment

Sequence	N-terminal amino acids deleted
<i>Burkholderia pseudomallei</i>	130
<i>Staphylococcus epidermidis</i>	286
<i>Staphylococcus aureus</i> Mu50	286
<i>Clostridium difficile</i>	272
<i>Magnetococcus</i> MC-1	249

neri, a plasmid-borne OXA allele into *Pseudomonas aeruginosa* (OXA-17), and a transfer from the α subgroup of proteobacteria into *Pseudomonas aeruginosa* (OXA-18).

The phylogeny shows that the OXA genes have been mobilized to plasmids at least three times along the branches leading to the nodes labeled A, B, and C. Several of the plasmid-borne alleles (OXA-1 and -31; OXA-2, -15, -34, and -32; OXA-10, -19, -28, and -35) have arisen recently as is evidenced by little divergence from their common ancestors. OXA-3 and OXA-5, however, are at the tips of quite long branches, suggesting that they have been plasmid-borne for a long time.

We have estimated the times at which OXA genes were mobilized to plasmids (nodes B and C) and the time at which OXA was transferred from the Proteobacteria to the Gram-positive group by calibrating the OXA tree with universal trees that have been estimated based on large combined protein sequence data sets (Brown et al. 2001; Feng et al. 1997). Feng et al. (1997) estimated the divergence of the Gram-positive Eubacteria at about 2.2 billion years ago. We have taken several distances (Table 3) from the tree in Fig. 2 of Brown et al. (2001) and estimated divergence times on the assumption that Gram positives diverged from the Proteobacteria about 2.2 billion years ago. Comparison of the OXA tree (Fig. 1) with those divergence times yields an estimate of an average of 1.26 ± 0.27 substitutions per Myr. Using that calibration we estimate that the mobilization at Node B occurred about 42 ± 9 Myr and that at node C about 116 ± 25 Myr ago.

Brown et al. (2001) put the divergence of *B. subtilis* from *S. aureus* at about 320 Myr and the divergence of the *B. subtilis*/*S. aureus* group from the *Streptococcus* group at about 575 Myr. Based on the OXA tree we would estimate the divergence of *B. subtilis* from *S. aureus* at about 520 Myr. Because OXA homologues are not present in the completely sequenced genomes of *Streptococcus pyogenes* or *Streptococcus pneumoniae*, horizontal transfer must have taken place between 320 and 575 Myr according to the Brown et al. tree and sometime prior to 520 Myr according to the OXA tree.

Positive Selection

Codeml model 1 (Yang 1998), which estimates the d_N/d_S ratio along each branch independently, was used to detect selection along the branches of the tree shown in Fig. 1. The log likelihood of that model was $-19,637.26$. Those ratios are shown in Fig. 2. That tree was also used to estimate the d_N/d_S ratio under the one-ratio model, model 0 of Codeml, in which all branches are assumed to have the same d_N/d_S ratio. The log likelihood of model 0 was $-19,781.38$. The likelihood ratio test (Yang 1998), in which twice the difference in log likelihoods ($2\Delta\ln$) is compared with the χ^2 distribution, was used to test whether the free-ratio model fits the data significantly better than the homogeneous-ratio model. The free-ratio model involves 63 parameters, 1 for each branch, while the single-ratio model involves 1 parameter, thus there are 62 degrees of freedom for the χ^2 distribution. $2\Delta\ln$ is 288.24 and the difference is significant, at $p \ll 0.001$.

To assess the significance of the branches that appear to be under positive selection (d_N/d_S ratio, >1.0), the d_N/d_S ratio was estimated for those branches using two two-ratio models. Model 2a estimates one d_N/d_S ratio for the branch of interest and another d_N/d_S ratio, the “background ratio,” for all other branches. Model 2b assumes that the d_N/d_S ratio for the branch of interest is 1.0 and estimates the background d_N/d_S ratio for all other branches. The likelihood ratio test was used to estimate whether the d_N/d_S ratio of the branch of interest was significantly greater than 1.0 by comparison of $2\Delta\ln$ with the χ^2 distribution for one degree of freedom. By that criterion four branches were under significant positive selection ($p < 0.05$). Those branches are indicated by heavy lines in Fig. 2 and the d_N/d_S ratios along those branches are marked with asterisks.

Discussion

The finding that there have been four branches, three of them early in the evolution of the OXA family, that were under significant positive selection indicates that the OXA genes have played important roles in the fitness of some bacteria. This analysis does not permit us to say what that role was. We cannot distinguish between the possibility that early OXA genes encoded functional β -lactamases that offered protection against naturally occurring antibiotics and the possibility that those genes encoded proteins with entirely different metabolic roles.

The OXA β -lactamase genes are very old, pre-dating the divergence of the cyanobacteria about 2 billion years ago. Most of the diversity of the OXA genes is the result of ancient events. During much of their history the OXA genes have been mobile, being

Table 3. Calibration of divergence times

Divergence of groups	Distance on Brown et al. tree (AA)	Estimated time (Myr) ^a	Nucleotide substitutions on OXA tree	Estimated nucleotide substitutions on OXA tree per Myr
Gram + and γ proteobacteria	2433	2200		
β and γ groups of proteobacteria	1100	995	1480	1.49
α and γ groups of proteobacteria	1166	1054	1501	1.42
α and β groups of proteobacteria	800	723	469	0.65
ϵ and γ groups of proteobacteria	1800	1628	1089	0.67
<i>S. aureus</i> and <i>B. subtilis</i>	350	316	658	2.08
<i>S. aureus</i> / <i>B. subtilis</i> group and <i>Streptococcus pyogenes/pneumoniae</i> group	633	572	—	—
Mean				1.268
SE				0.27

^a Divergence times were estimated based on the assumption that the Gram positives diverged from the proteobacteria about 2.2 billion years ago (Feng et al. 1997).

transferred between lineages and being mobilized to plasmids on several occasions during the last 500 million years. One such mobilization (node A in Fig. 1) is recent, the other two (nodes B and C in Fig. 1) occurred millions of 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. While we think that we have used the best estimates available, our conclusions about the age of OXA genes and the times at which chromosomal OXA genes were mobilized to plasmids are only as good as those estimates. However, even if the divergence times that we used to calibrate our estimates were off by an order of magnitude, we cannot escape the conclusion that the OXA genes are ancient and that they were mobilized millions of years ago, long before the current era of antibiotic use.

Screening of the Murray collection of *Enterobacteriaceae* that were isolated between 1954 and 1971 showed that about 20% of isolates carried transmissible plasmids but that none of the isolates were resistant to commonly used antibiotics (Datta and Hughes 1983). That study is often cited as evidence that mobilization of antibiotic resistance genes to plasmids has been driven by the clinical use and misuse of antibiotics (Davies 1997). Although the recent mobilizations of the TEM and AmpC β -lactamase genes, and the rapid spread of those plasmids, make it clear that antibiotic use contributes powerfully to the mobilization and spread of resistance genes, the finding that OXA β -lactamase genes have

been on plasmids for millions of years makes it clear that the presence of antibiotic resistance genes on plasmids is not solely the result of the clinical use and misuse of antibiotics.

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