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Independent Origins of Subgroup Bl + B2 and Subgroup B3 Metallo-β-Lactamases

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Abstract. The metallo-β-lactamases constitute Class B in the Ambler classification of β-lactamases and are divided into three subclasses: Bl, B2, and B3. Bayesian phylogenies of the Subclass B1 + B2 and Subclass B3 metallo-β-lactamases and their homologs show that the β-lactam-hydrolyzing function evolved independently within each group. In Subclass B1 + B2 that function evolved about 1 billion years ago, and in Subclass B3 it evolved before the divergence of the Gram-positive and Gram-negative eubacteria, about 2 billion years ago. These results lend additional support to the proposal that the metallo-β-lactamases should be divided into two distinct classes.

Key words: Metallo-β-lactamases — Subgroup B1 + B2 — Subgroup B3 — Ambler classification

Introduction

Because of their broad spectrum and minimal side effects, β-lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems, currently account for more than 50% of the antibiotics prescribed worldwide (Livermore 1996). Penicillin was one of the earliest antibiotics to come into common clinical use, and over the past 60 years a plethora of β-lactam-resistant microorganisms has emerged (Medeiros 1997). The most common means of β-lactam resistance is the synthesis of β-lactamases, enzymes that inactivate β-lactam antibiotics by hydrolyzing the β-lactam ring (Nikaido and Normark 1987). The most frequently encountered β-lactamases are the serine-β-lactamases, which use a catalytic serine as part of the β-lactam ring hydrolysis mechanism.

A completely unrelated and less frequently encountered group of enzymes, the metallo-β-lactamases, also act by cleaving the β-lactam ring of β-lactam antibiotics but do so by an entirely different mechanism that involves a catalytic metal Zn^{2+} ion (Bush 1998). Metallo-β-lactamases were first identified nearly 40 years ago (Sabath and Abraham 1966) and are now known to be present in at least 20 species of bacteria (Hall et al. 2003). Metallo-β-lactamases are considered to be a particular threat because (1) they are not inactivated by clinically useful β-lactamase inhibitors and (2) they exhibit activity toward carbapenems, a class of β-lactam antibiotics that is generally not hydrolyzed by the serine β-lactamases. Metallo-β-lactamases have classically been categorized as Ambler Class B (Ambler 1980) and subdivided into three subclasses, B1, B2, and B3 (Rasmussen and Bush 1997). Although there is structural homology between subclass B1 + B2 and subclass B3 metallo-β-lactamases (Galleni et al. 2001; Hall et al. 2003), there is no detectable sequence homology between members of subclass B1 + B2 and members of subclass B3 (Hall et al. 2003).

We have previously presented phylogenies of the experimentally determined subclass B1 + B2 and subclass B3 metallo-β-lactamases (Barlow and Hall 2003). Here we extend that study to include homologs

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Table 1. Sequences, organisms, and accession numbers

Sequence name	Organism	Abbreviated taxonomy	Accession No.
		Subclass B1 + B2	
actIORF5	*Streptomyces coelicolor* A3(2)	Bacteria; Firmicutes; Actinobacteria	X63449
BA1	*Bacillus anthracis* str. Ames	Bacteria; Firmicutes; *Bacillus*/*Clostridium* group	TIGR_198094
BF1	*Burkholderia fungorum*	Bacteria; Proteobacteria; β subdivision	DOE_134537
bla2	*Bacillus anthracis*	Bacteria; Firmicutes; *Bacillus*/*Clostridium* group	AF367984
BlaB1	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189298
BlaB2	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189300
BlaB3	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189301
BlaB5	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189303
BlaB6	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189302
BlaB7	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189304
BlaB8	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF189305
blm	*Bacillus cereus*	Bacteria; Firmicutes; *Bacillus*/*Clostridium* group	M11189
BM1	*Burkholderia mallei*	Bacteria; Proteobacteria; β subdivision	TIGR_13373
CcrA	*Bacteroides fragilis*	Bacteria; CFB group; Bacteroidetes	M63556
cfiA	*Bacteroides fragilis*	Bacteria; CFB group; Bacteroidetes	M34831
CGB1	*Chryseobacterium gleum*	Bacteria; CFB group; Flavobacteria	AF339734
CH3	*Colwellia* sp. 34H	Bacteria; Proteobacteria; γ subdivision	TIGR_167879
CphA	*Aeromonas hydrophila*	Bacteria; Proteobacteria; γ subdivision	X57102
CphA2	*Aeromonas hydrophila*	Bacteria; Proteobacteria; γ subdivision	U60294
Gp289	*Geobacter metallireducens*	Bacteria; Proteobacteria; Δ subdivision	NZ_AAAS01000001
ImiS	*Aeromonas veronii*	Bacteria; Proteobacteria; γ subdivision	Y10415
IMP1	*Serratia marcescens*	Bacteria; Proteobacteria; γ subdivision	AF416297
IMP10	*Pseudomonas aeruginosa*	Bacteria; Proteobacteria; γ subdivision	AB074434
IMP11	*Pseudomonas aeruginosa*	Bacteria; Proteobacteria; γ subdivision	AB074437
IMP2	*Acinetobacter baumannii*	Bacteria; Proteobacteria; γ subdivision	ABA243491
IMP4	*Acinetobacter baumannii*	Bacteria; Proteobacteria; γ subdivision	AF244145
IMP5	*Acinetobacter baumannii*	Bacteria; Proteobacteria; γ subdivision	AF290912
IMP6	*Serratia marcescens*	Bacteria; Proteobacteria; γ subdivision	AB040994
IMP8	*Klebsiella pneumoniae*	Bacteria; Proteobacteria; γ subdivision	AF322577
IMP9	*Shigella flexneri*	Bacteria; Proteobacteria; γ subdivision	AY033653
IND1	*Chryseobacterium indologenes*	Bacteria; CFB group; Flavobacteria	AF099139
IND2	*Chryseobacterium indologenes*	Bacteria; CFB group; Flavobacteria	AF219129
IND2a	*Chryseobacterium indologenes*	Bacteria; CFB group; Flavobacteria	AF219130
IND3	*Chryseobacterium indologenes*	Bacteria; CFB group; Flavobacteria	AF219131
IND4	*Chryseobacterium indologenes*	Bacteria; CFB group; Flavobacteria	AF219135
JOHN1	*Flavobacterium johnsoniae*	Bacteria; CFB group; Flavobacteria	AY028464
MC1	*Methylococcus capsulatus*	Bacteria; Proteobacteria; γ subdivision	TIGR_414
MM1	*Magnetococcus sp.* MC-1	Bacteria; Proteobacteria; magnetotactic cocci	DOE_156889
RM1	*Ralstonia metallidurans*	Bacteria; Proteobacteria; β subdivision	DOE_119219
RP1	*Rhodopseudomonas palustris*	Bacteria; Proteobacteria; α subdivision	DOE_1076
RS01746	*Ralstonia solanacearum*	Bacteria; Proteobacteria; β subdivision	AL646080
RS05663	*Ralstonia solanacearum*	Bacteria; Proteobacteria; β subdivision	AL646084
shfI	*Serratia fonticola*	Bacteria; Proteobacteria; γ subdivision	AF197943
SP1	*Silicibacter pomeroyi*	Bacteria; Proteobacteria; α subdivision	TIGR_178391
SP2	*Silicibacter pomeroyi*	Bacteria; Proteobacteria; α subdivision	TIGR_178391
SSO2519	*Sulfolobus solfataricus*	Archaea; Crenarchaeota; Thermoprotei	AE006849
TM0681	*Thermotoga maritima*	Bacteria; Thermotogae; Thermotogales	NC_000853
VIM1	*Achromobacter xylosoxidans* subsp. *denitrificans*	Bacteria; Proteobacteria; β subdivision	AJ278514
VIM2	*Pseudomonas aeruginosa*	Bacteria; Proteobacteria; γ subdivision	AF191564
VIM3	*Pseudomonas aeruginosa*	Bacteria; Proteobacteria; γ subdivision	AF300454
		Subclass B3	
AF1748	*Archaeoglobus fulgidus*	Archaea; Euryarchaeota	NC_000917
CAU1	*Caulobacter crescentus*	Bacteria; Proteobacteria; α subdivision	AJ308331
DR1430	*Deinococcus radiodurans*	Bacteria; *Thermus*/*Deinococcus* group	NC_001263
DR2557	*Deinococcus radiodurans*	Bacteria; *Thermus*/*Deinococcus* group	NC_001263
EC1	*Erwinia chrysanthemi*	Bacteria; Proteobacteria; γ subdivision	TIGR_198628
FEZ1	*Fluoribacter gormanii*	Bacteria; Proteobacteria; γ subdivision	Y17896
GOB1	*Chryseobacterium meningosepticum*	Bacteria; CFB group; Flavobacteria	AF090141
Gp2047	*Geobacter metallireducens*	Bacteria; Proteobacteria; Δ subdivision	NZ_AAAS01000009
L1	*Stenotrophomonas maltophilia*	Bacteria; Proteobacteria; γ subdivision	X75074
L1c	*Stenotrophomonas maltophilia*	Bacteria; Proteobacteria; γ subdivision	AJ251814

Table 1. Continued

Sequence name	Organism	Abbreviated taxonomy	Accession No.
L1d	*Stenotrophomonas maltophilia*	Bacteria; Proteobacteria; γ subdivision	AJ251815
L1e	*Stenotrophomonas maltophilia*	Bacteria; Proteobacteria; γ subdivision	AJ272109
mbl1	*Caulobacter crescentus*	Bacteria; Proteobacteria; α subdivision	AJ315850
mbl511	*Stenotrophomonas maltophilia*	Bacteria; Proteobacteria; γ subdivision	AJ289086
MJ0296	*Methanocaldococcus jannaschii*	Archaea; Euryarchaeota	NC_000909
MS1	*Mycobacterium smegmatis*	Bacteria; Firmicutes; Actinobacteria	TIGR_1772
MTH1267	*Methanothermobacter thermautotrophicus* str. Delta H.	Archaea; Euryarchaeota	AE000893
NA1	*Novosphingobium aromaticivorans*	Bacteria; Proteobacteria; α subdivision	NC_002719
PH1213	*Pyrococcus horikoshii*	Archaea; Euryarchaeota	NC_000961
SSO1157	*Sulfolobus solfataricus*	Archaea; Crenarchaeota	NC_002754
SS03132	*Sulfolobus solfataricus*	Archaea; Crenarchaeota	NC_002754
ST0874	*Sulfolobus tokodaii*	Archaea; Crenarchaeota	NC_003106
STM3737	*Salmonella enteridis* serovar *typhimurium* LT2	Bacteria; Proteobacteria; γ subdivision	NC_003197
THINB	*Janthinobacterium lividum*	Bacteria; Proteobacteria; β subdivision	AJ250876

of the experimentally determined metallo-β-lactamases, and we show that the β-lactam-hydrolyzing function has evolved twice, arising independently within the B1 + B2 and the B3 subgroups.

Materials and Methods

Identification of Metallo-β-Lactamase Homologs

Metallo-β-lactamase homologs were identified by a tblastn search (Altschul et al. 1990, 1997) of the NCBI Microbial Genomes database using the following experimentally determined Subclass B1 + B2 (Imp-I [gi15866617], BlaBl [gi9587056], CphA [gi38824], and VIM2 [gi7381449]) and Subclass B3 (CAU-I [gi21425614], FEZ1 [8980430], L1 [gi525299], and GOB1 [gi6164597]) protein sequences as queries. The query sequences were chosen to represent the major clades within their subgroups. Table 1 lists the accession numbers and organisms associated with those sequences. Sequences that aligned with a query over at least 69% of either sequence length and had an *E*-score $\leq 10^{-4}$ were considered to be candidates for homologous proteins. Most sequences aligned over >69% or <40% of the length of the query. A pairwise blast (Tatusova and Madden 1999) alignment was done between each candidate and the query sequence that had identified it. A candidate was confirmed as a homolog only if met the same criteria in the pairwise blast as it had in the original tblastn search. Table 1 lists all of the experimentally determined metallo-β-lactamases and homologs that were used in this study.

Phylogenetic Reconstructions

The Subclass B1 + B2 and Subclass B3 protein sequences derived from the genes in Table 1 were aligned separately 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/HallLab/index.html. Both the protein and the DNA alignments, in Nexus format, are available from B.G.H. on request to drbh@mail.Rochester.edu.

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 and Ronquist 2001). MrBayes is available at no charge from http://morphbank.ebc.uu.se/mrbayes3/. The evolutionary model was the general time reversible model (Tavaré 1986). Among-site variation in evolutionary rate was estimated separately for first, second, and third positions of sites within codons. Four chains, with a "temperature" of 0.2 for the heated chains, were run for 3,000,100 generations, sampling trees every 100 generations. The ln likelihood of the trees had converged on a constant value by generation 100,000, i.e., after saving 1000 trees. The consensus tree, with branch lengths, was calculated from the final 29,001 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.

Cloning Metallo-β-Lactamase Homolog Genes

All genes were amplified using the Failsafe PCR System (Epicentre Technologies). *Tm0681* was amplified from *Thermotoga maritima* genomic DNA (American Type Culture Collection, ATCC number 43589D) using primers F1 and R1 with Premix Buffer F. In order to increase the efficiency of protein synthesis, Primer Fl (5′-GGGGGGGTACCAGTATCCCCATAGAAAGGCCG**ATG**C 3′) incorporated a mismatched base to change the wild-type F-methionine start codon (**TTG**) to (**ATG**). Primer Fl also included a *Kpn*I site upstream of the start codon, and Primer Rl (5′-GGG GGGAGCTCATATCTATATTCGAACGATCACG-3′) included a *Sac*I site downstream of the stop codon. *SSO2519* was amplified

from *Sulfolobus solfataricus* genomic DNA (American Type Culture Collection, ATCC number 35092D) using primers F2 and R2 with Premix Buffer C. Primer F2 (5′-GGGGGCC**ATG**GA AGTTCAATATAAGTTCGAAA-3′) included a *Nco*I site that contains the start codon, and Primer R2 (5′-GGGGGGAG CTCGTCGCAACAACGTTAGAAACAATC-3′) included a *Sac*I site downstream of the stop codon. *NA1* was amplified from *Novosphingobium aromaticivorans* genomic DNA (American Type Culture Collection; ATCC number 700278D) using primers F3 and R3 with Premix Buffer G. Primer F3 (5′-GGGGGGGTACC TGCGAGTCCTATTAGTCGA**TAA**GACC-3′) included a *Kpn*I site upstream of the start codon. A **TAA** stop codon was included to terminate translation that initiates within the multiple cloning site of the plasmid. Primer R3 (5′- GGGGGGAGCTCCGAA CACGGTAACTTTCCAGCTCA-3′) included a *Sac*I site downstream of the stop codon. *STM3737* was amplified from genomic DNA of *Salmonella enterica* serovar typhimurium strain LT2 using primers F4 and R4 with Premix Buffer H. Primer F4 (5′-GGGGGGGTACCGGCGCTTCAATCAGGA**TAA**CATT-3′) included a *Kpn*I site upstream of the start codon. A **TAA** stop codon was included to terminate translation that initiates within the multiple cloning site of the plasmid. Primer R4 (5′-GGGGGG AGCTCGCCGCCGGTGAGTTATTTCAT-3′) included a *Sac*I site downstream of the stop codon.

Amplicons were purified with QIAquick PCR Purification Kit (Qiagen). The *SSO2519* amplicon was digested with restriction endonucleases *Nco*I and *Sac*I (New England Biolabs), whereas the *NA1*, *Tm0681*, and *STM3737* amplicons were digested with *Kpn*I and *Sac*I (New England Biolabs). Each was ligated into similarly digested plasmid pACSE2 (Barlow and Hall 2002a) and transformed into strain DH5α-E (F^- *ϕ80d1acZΔM15*Δ(lacZYA–argF) *U169* end*A1* rec*A1* hsdR*17*(*r*−*m*+) deoR *thi-1* phoA supE*44* λ^- gyrA*96* relA*1 gal*-) (GIBCO). The products of the ligations were designated pSSO2519, pNAl, pTm0681, and pSTM3737, respectively. The authenticity of the sequence of each cloned gene was confirmed by sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

The metallo-β-lactamase homolog genes carried on the plasmid constructs are expressed from the strong pTAC promoter under the control of the plasmid-encoded *lacI*^{q} gene by induction with IPTG (isopropyl-β-*D*-thiogalactopyranoside). Gene expression in all plasmids except pSSO2519 was achieved using IPTG at a concentration of 1 m*M*. Induction of plasmid pSSO2519 with 1 m*M* IPTG was found to prevent growth of the strain carrying it, therefore gene expression in that plasmid was induced with 125 μ*M* IPTG.

Antibiotics and Determination of Antibiotic Resistance

Ampicillin (Sigma), cefotaxime (Sigma), cefepime (Bristol–Myers Squibb), cefoxitin (Merck), cefuroxime (Sigma), ceftazidime (Glaxo Wellcome), aztreonam (Bristol-Myers Squibb), imipenem (Merck), meropenem (Zeneca), and piperacillin (Sigma) were used in this project. Stock solutions of antibiotics were prepared in 0.1 *M* $NaPO_4$ buffer, pH 7.0, filter sterilized, and stored at −80°C in single-use aliquots. All minimum inhibitory concentrations (MICs) were determined in Mueller Hinton (Difco) broth containing an appropriate concentration of IPTG (1 m*M* or 125 μ*M*) according to Barlow and Hall (2002a). Disk Diffusion tests employed disks obtained from BBL that contained aztreonam (30 μg), cefotaxime (30 μg), ceftazadime (30 μg), cefuroxime (30 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg) or pipericillin (100 μg). Muller–Hinton agar containing 1 m*M* or 125 μ*M* IPTG was spread with 2.7×10^6 cells of the strain to be tested, a single disk was placed in the center of the plate, and the plate was incubated overnight at 37°C. For each test five replicate plates were spread with DH5α-E carrying plasmid pACSE2 (control) and five plates were spread with DH5α-E carrying the gene of interest cloned into the same plasmid vector (experimental). The following day the diameters of the zones of growth inhibition were measured and the mean and standard error of zone diameters were calculated. The smaller the zone, the more resistant is the strain to the antibiotic.

Results and Discussion

Homologs of the Subclass B1 + B2 and Subclass B3 metallo-β-lactamases were identified as described in Materials and Methods. No homolog was identified by both a Subclass B1 + B2 and a Subclass B3 query sequence. However, the sequence SSO2519 from the archaebacterial organism *Sulfolobus solfataricus* is homologous to the Subclass B1 + B2 metallo-β-lactamase BlaBl ($E = 3 \times 10^{-6}$) and is also homologous to two of the Subclass B3 *homologs*, AF1748 ($E = 2 \times 10^{-8}$) and PH1213 ($E = 2 \times 10^{-6}$).

It is important to distinguish between the families of metallo-β-lactamase proteins that are identified on the basis of sequence homology and the subsets of those proteins that have biologically detectable β-lactam-hydrolyzing activity. We refer to those proteins for which β-lactam hydrolysis has been experimentally demonstrated as "true" metallo-β-lactamases, and to the remainder as "homologs."

Subclass B1 + B2 Phylogeny

DNA sequences of the true Subclass B1 + B2 metallo-β-lactamases and their homologs were aligned by Clustal 1.8 and CodonAlign as described in Materials and Methods.

Figure 1 shows the Bayesian phylogeny of the Subclass B1 + B2 metallo-β-lactamases and their homologs. With the single exception of the SSO2519 sequence of the archaebacterium *Sulfolobus solfataricus*, all of the Subclass B1 + B2 sequences are found in the Eubacteria. Sequence SSO2519 was therefore used as an outgroup to root the Subclass B1 + B2 tree. The true metallo-β-lactamases, shown in boldface in Figure 1, form a single clade arising from the node designated "A" in Fig. 1.

Although the homologs have not been reported to have metallo-β-lactamase activity, that does not necessarily mean that the activity is absent. We cloned the closest relative of the true Subclass B1 + B2 metallo-β-lactamases, sequence TM0681 from *Thermotoga maritima*, and the most distant relative, sequence SSO2519 from *Sulfolobus solfataricus*, into the expression vector pACSE2. To determine whether either protein had biologically significant metallo-β-lactamase activity, we expressed each in *E. coli* Kl2 strain DH5-αE and compared the minimum inhibitory concentrations of each of 10 β-lactam antibiotics (see Materials and Methods) in strains that expressed the homolog enzymes with a

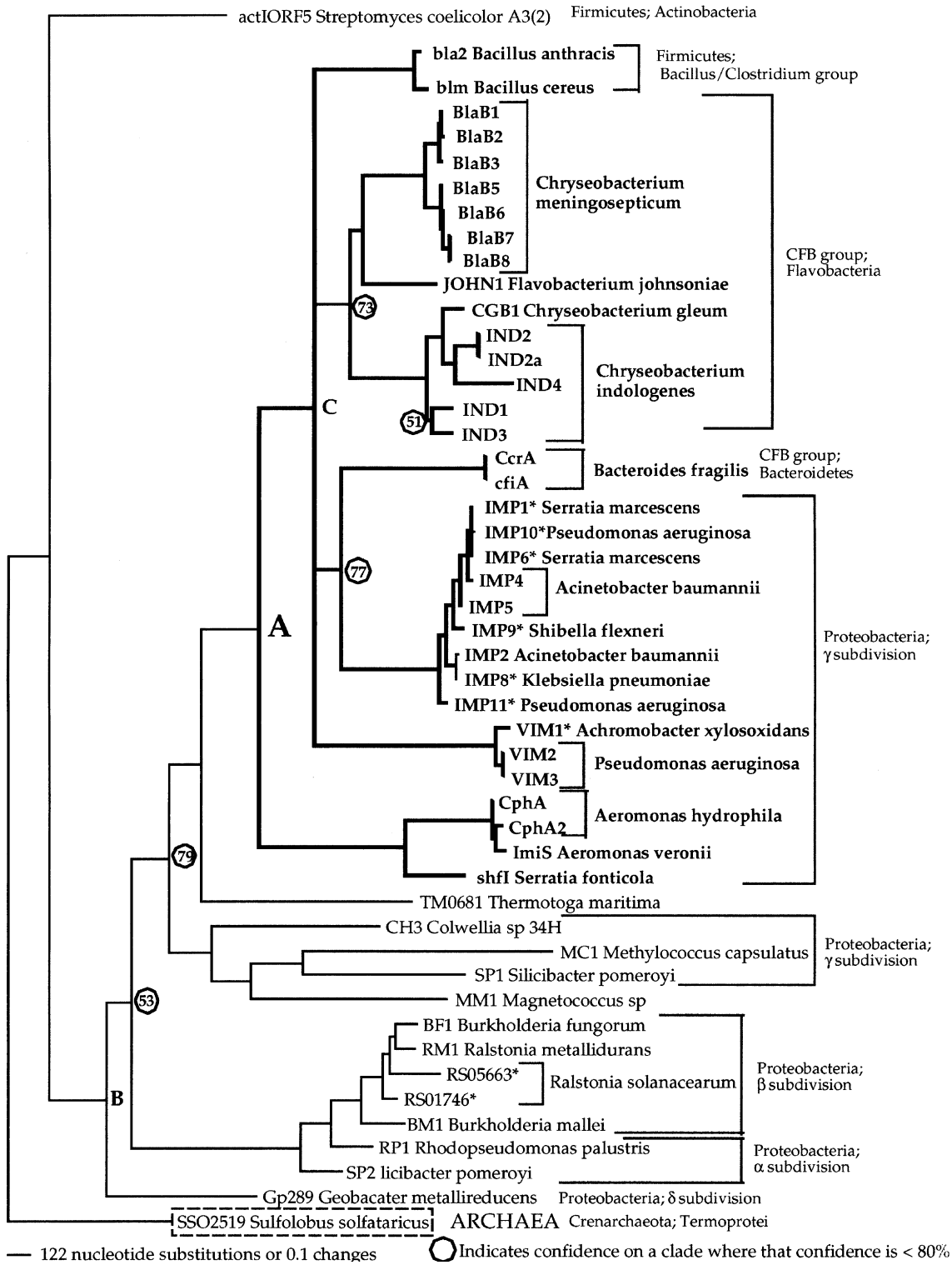

Fig. 1. Bayesian phylogeny of Subclass B1 + B2 metallo-β-lactamases and their homologs. Boldface indicates experimentally determined metallo-β-lactamases. Asterisks indicate plasmid-borne alleles. Lines are proportional to branch lengths. Posterior probabilities of clades were ≥80% except where indicated by a percentage value within a circle at a node. Except for the *Sulfobolus solfataricus* sequence that is enclosed within a dashed box, all sequences are from the Eubacteria. The true metallo-β-lactamases are descended from Node A. The branches of Clade A have been slightly thickened for clarity. The significance of Nodes labeled B and C is discussed in the text.

control strain that carried just the vector (Table 2). For neither enzyme did expression of the homolog increase the MIC. MIC determinations depend upon twofold increases in drug concentration, limiting their resolution to twofold differences. We previously found that some alleles that conferred identical MICs

Table 2. MICs for DHSα-E carrying cloned homolog genes

Drug	Clinical Resistance	MIC (μg /ml)[a]				
		pACSE2 (control)	pTM0681	pSS02519	pSTM3737	pNA1
Ampicillin	32	2	2	2	2	4
Aztreonam	32	0.0625	0.125	0.03125	0.0625	0.125
Cefotaxime	64	0.03125	0.0625	0.01563	0.03125	**0.125**
Ceftazidime	32	0.25	0.0625	0.125	0.125	0.25
Cefoxitin	32	4	4	2	4	8
Cefuroxime	32	2	4	4	4	**8**
Cefepime	32	0.01563	0.01563	0.01563	0.03125	0.03125
Imipenem	16	0.25	0.125	0.25	0.25	0.25
Meropenem	16	0.00391	0.00391	0.00391	0.00391	0.00781
Pipercillin	128	0.5	1	0.5	1	1

[a]Only MICs that are more than twice the MIC for the pACSE2 control are considered to be significant (boldface).

Table 3. Disk diffusion tests for antibiotic sensitivity

Drug	Sensitive[a]	Diameter of zone of inhibition, mm (mean ± SE of five replicate samples)							
		TM0681		SSO2519		STM3737		NA1	
		Cont[b]	Exp[c]	Cont	Exp	Cont	Exp	Cont	Exp
Ampicillin	17	22.2 ± 0.6	23.0 ± 0.3	21.0 ± 0.4	20.6 ± 0.4	21.6 ± 0.2	**18.4 ± 0.2**[d]	21.6 ± 0.2	**14.0 ± 0**
Aztreonam	22	31.6 ± 0.6	34.2 ± 1	38 ± 0.3	38.4 ± 0.2	36.2 ± 0.4	33 ± 1	36.2 ± 0.4	40.4 ± 0.4
Cefotaxime	23	36.6 ± 0.4	35.6 ± 0.2	37.8 ± 0.6	40.2 ± 0.7	36.6 ± 0.4	**35 ± 0.4**	36.6 ± 0.4	**34.8 ± 0.4**
Cefoxitin	18	24.6 ± 0.2	24.8 ± 0.2	25.2 ± 0.2	25.8 ± 0.2	24.6 ± 0.2	**23.0 ± 0**	24.6 ± 0.2	**19.4 ± 0.2**
Ceftazidime	18	35.8 ± 0.4	36 ± 0.3	29.8 ± 0.2	35.6 ± 0.4	35.8 ± 0.4	**29.6 ± 0.2**	35.8 ± 0.4	**33.4 ± 0.9**
Cefuroxime	18	27.4 ± 0.2	28 ± 0.3	22.6 ± 0.4	24 ± 0.3	27.4 ± 0.2	**25.4 ± 0.4**	27.4 ± 0.2	28.6 ± 0.4
Cefepime	18	34.6 ± 0.7	34.8 ± 0.7	38 ± 0.4	40.4 ± 0.8	34.6 ± 0.7	36 ± 0.4	34.6 ± 0.7	35.6 ± 0.4
Imipenem	16	30.8 ± 0.7	31.4 ± 0.4	32.4 ± 0.2	34 ± 0.3	30.8 ± 0.7	29.6 ± 0.2	30.8 ± 0.7	32.4 ± 0.4
Meropenem	16	17.8 ± 0.2	18.4 ± 0.2	19.4 ± 0.2	19 ± 0	17.8 ± 0.2	**15.2 ± 0.2**	17.8 ± 0.2	16.6 ± 0.5
Piperacillin	21	28 ± 0.3	31 ± 0.8	29.4 ± 0.2	31.6 ± 0.5	28 ± 0.3	31.8 ± 0.2	28 ± 0.3	27.8 ± 0.2

[a]A larger zone of inhibition indicates clinical sensitivity to the drug (National Committee for Clinical Laboratory Standards 1999).
[b]Control, strain DH5α-E carrying the vector pACSE2.
[c]Experimental, strain DH5α-E carrying the indicated gene cloned into pACSE2.
[d]**Boldface** indicates a zone that is significantly smaller than the control zone by unpaired *t*-test ($p < 0.05$).

could be resolved by the disk diffusion test for antibiotic resistance (Barlow and Hall 2003). Disk diffusion tests measure a zone of growth inhibition when a disk containing a standard amount of drug is placed on a plate that has been spread with about 10^6 cells of the strain to be tested. The smaller the zone of inhibition, the more resistant is the strain. Table 3 shows that neither of the Subclass B1 + B2 homologs significantly decreased the diameter of the zone of inhibition, indicating that neither homolog possesses biologically detectable metallo-β-lactamase activity. It is therefore reasonable to conclude that metallo-β-lactamase activity arose once at Node A.

The lowest-branching eubacterial lineage of the Subclass B1 + B2 phylogeny is actIORF5 from the Gram-positive organism *Streptomyces coelicolor*. The immediate descendants of the node labeled "B" are members of the Proteobacteria bacteria. The polytomy at the node labeled "C" includes branches that lead to (1) a clade of the Gram-positive *Bacillus/Clostridium* group, (2) a clade of the CFB group Flavobacteria, (3) a clade that includes both Proteobacteria of the γ subdivision and CFB group Bacteriodetes, and (4) another clade of Proteobacteria of the γ subdivision. While there are disagreements about the detailed branching order of the Universal Tree (Brown et al. 2001; Gupta 2001; Woese 1987), it is generally agreed that the Proteobacteria are the most recent group of the Eubacteria and that the CFB and *Bacillus/Clostridium* group arose before the Proteobacteria. Within the Proteobacteria, the γ subdivision is believed to be the most recent. Thermotoga is believed to be one of the most ancient of the Eubacteria. The most reasonable explanation for the topology of the Subclass B1 + B2 tree is that those sequences that are closely related to the true Subclass B1 + B2 metallo-β-lactamases arose in the Proteobacteria (node B), and that the true metallo-β-lactamases arose shortly after the γ subdivision diverged from the β subdivision of the Proteobacteria (node A). Shortly after that time it appears that there was a horizontal transfer into the *Bacillus/Clostridium*

group of the Firmicutes and another transfer to the CFB Flavobactaeria group. Slightly after that, there appears to have been another horizontal transfer to the CFB Bacteroidetes group. In addition, there appears to have been a horizontal transfer into *Themotoga maritima* just before the true metallo-β-lactamases evolved.

The γ subgroup of the Proteobacteria diverged from the β subgroup about 995 million years ago (Barlow and Hall 2002b). If, as the phylogeny suggests, the true Subclass B1 + B2 metallo-β-lactamases arose around the time that the γ diverged from the β subgroup of the Proteobacteria, it means that true metallo-β-lactamases are nearly 1 billion years old.

The majority of the Subclass B1 + B2 genes are located on the chromosomes of their hosts, but there are several plasmid-borne alleles, indicated by asterisks in Fig. 1. True Subclass B1 + B2 metallo-β-lactamases have been mobilized to plasmids twice, once for the IMP group and once for the VIM group; and a homolog has been mobilized once in *Ralstonia solanacearum*.

Subclass B3 Phylogeny

DNA sequences of the true Subclass B3 metallo-β-lactamases and their homologs were aligned by Clustal 1.8 and CodonAlign as described in Materials and Methods.

Figure 2 shows the Bayesian phylogeny of the Subclass B3 metallo-β-lactamases and their homologs. The Subclass B3 phylogeny was rooted with the archeal sequences AF1748, PH1213, and MTH1267 based upon a conceptual rooting with the SSO2519 sequence that is homologous to two of the Subclass B3 homologs, AF1748 and PH1213. The conceptual rooting was done by constructing a phylogeny of the Subclass B3 metallo-β-lactamases and their homologs plus SSO2519 and using SSO2519 as the outgroup. SSO2519 is not homologous to all of the taxa and its inclusion distorts the alignment, thus that is not a legitimate tree. In general, however, the topology of the illegitimate tree was similar to that of the legitimate tree that lacked SSO2519. In particular, with SSO2519 as the outgroup, AF1748, PH1213, and MTH1267 were the most basal taxa. That, together with the biological rationale that Archeae should be an outgroup to the Eubacteria, justifies the choice of outgroup. Indeed, even if all of the archaeal sequences are used as an outgroup the topology of the tree is unchanged from that shown.

The true Subclass B3 metallo-β-lactamases are all members of a clade that is descended from the node labeled A in Fig. 2. However, that clade also includes four sequences that are not known to be true metallo-β-lactamases (NA1, STM3737, MS1, and EC1). Two of those sequences, NA1 and STM3737, were cloned and expressed in *E. coli* to determine whether they encode proteins with biologically detectable metallo-β-lactamase activity. The STM3737 sequence encoded no significant metallo-β-lactamase activity as determined from the MICs of 10 β-lactam antibiotics, but the NA1 sequence encoded an activity that increased the MIC from 0.03125 to 0.125 μg/ml for cefotaxime and from 2 to 8μg/ml for cefuroxime (Table 2). The more sensitive disk diffusion tests (Table 3) show that STM3737 increases resistance to six drugs, and that NAl increases resistance to four drugs including cefoxitin. It appears likely that true metallo-β-lactamase activity arose once in the Subclass B3 lineage, at Node A, but that the function has been at least partially inactivated in several descendant lines.

Only one clade of Subclass B3 metallo-β-lactamases is found on plasmids, those found in *Stenotrophomonas maltophilia*. Those are indicated as being on plasmids because they have been reported to be located on a large, 200-kilobase, plasmid (Avison et al. 2001). Those authors, however, refer to the element as ''plasmid-like'' and point out that there is no evidence that the element can transfer from one cell to another. They suggest that it may be more accurate to consider the element as part of a fragmented chromosome.

While homologs of the Subclass B3 metallo-β-lactamases are present in both the Archaea and the Eubacteria, true metallo-β-lactamases evolved only in the Eubacteria. The presence of the true metallo-β-lactamase, GOBI, in *Chryseobacterium meningosepticum*, a member of the CFB group, suggests that the function may well have evolved before the divergence of the Gram-positive from the Gram-negative Eubacteria about 2.2 billion years ago (Feng et al. 1997).

The SSO2519 sequence of the archaebacterial organism *Sulfolobus solfataricus* is a legitimate homolog of the Subclass B1 + B2 metallo-β-lactamases and is also a homolog of the Subclass B3 Archaeal sequences AF1748 and PH1213. That is entirely consistent with the conclusion, based on protein structural considerations, that the Subclass B1 + B2 and Subclass B3 metallo-β-lactamases are derived from a common ancestor (Hall et al. 2003).

Conclusions

Taken together, the Subclass B1 + B2 and Subclass B3 phylogenies suggest that the metallo-β-lactamase family probably originated in the Archaea. The true β-lactamase function has clearly evolved twice, independently, in the Eubacteria. That finding supports and reinforces our earlier conclusion (Hall et al. 2003) that the Subclass B1 + B2 and the Subclass B3 metallo-β-lactamases should be considered separate

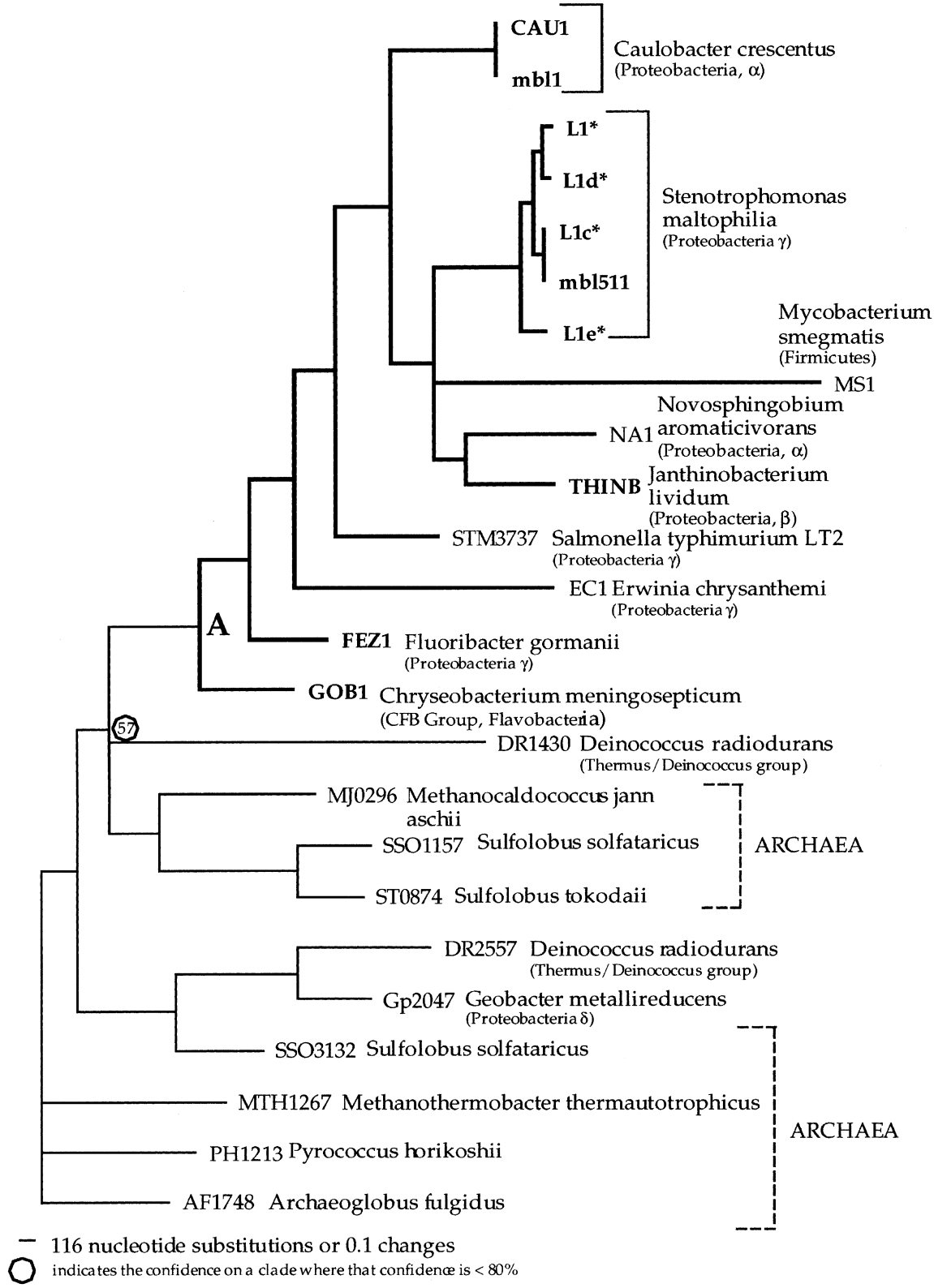

Fig. 2. Bayesian phylogeny of Subclass B3 metallo-β-lactamases and their homologs. **Boldface** indicates experimentally determined metallo-β-lactamases. Asterisks indicate plasmid-borne alleles (but see text). Lines are proportional to branch lengths. Posterior probabilities of clades were ≥80% except where indicated by a percentage value within a circle at a node. The true metallo-β-lactamases are descended from Node A. The branches of Clade A have been slightly thickened for clarity.

classes of the metallo-β-lactamases, just as Classes A, C, and D are separate classes of the serine β-lactamases.

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