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The Metallo-β-Lactamases Fall into Two Distinct Phylogenetic Groups

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Abstract. The Ambler Class B metallo-β-lactamases fall into two distinct phylogenetic groups based on the observation that there is no significant sequence homology between the sequences of members of different groups. Structural alignments confirm that those groups are no more closely related to each other than are the three classes of serine β-lactamases, Classes A, C, and D. We present phylogenies of these two groups and suggest a new classification scheme for the β-lactamases.

Key words: Metallo-β-lactamases — Ambler Class B — Serine β-lactamases

Introduction

Production of β-lactamases is the most common mechanism of bacterial resistance to β-lactam antibiotics. β-Lactamases belong to two major groups, both of which catalyze the hydrolysis of the amide bond within the β-lactam ring and thereby inactivate the antibiotic, but the groups are structurally and mechanistically unrelated. The first group, the serine β-lactamases, uses an active site serine for hydrolysis, and the second group, the metallo-β-lactamases, uses a metal ion, usually zinc. The metallo-β-lactamases are considered to be potentially particularly dangerous because they are active toward the carbapenem β-lactam antibiotics, which are often the "last-resort" drugs for multidrug-resistant pathogens. Ambler originally recognized these two groups in 1980 when he classified the serine β-lactamases as Class A and the metallo-β-lactamases as Class B, although he recognized at that time that there would probably be a need for additional classes as new β-lactamases were found (Ambler 1980). The following year the Class C β-lactamases, primarily cephalosporinases and related to the AmpC β-lactamase of *E. coli*, were recognized (Jaurin and Grundstrom 1981). In 1987 the discovery of the OXA-1 β-lactamase established yet another class, Class D (Ouellette et al. 1987). Both Classes C and D are serine β-lactamases, but they are distinguished from each other and from Class A because there is no detectable sequence homology among the three classes.

An early review of the metallo-β-lactamases (Rasmussen and Bush 1997) suggested that the Class B enzymes should be divided into three subclasses, B1, B2, and B3. At that time the only member of subclass B3 was the L1 enzyme. That scheme has been generally accepted and incorporated into a general numbering scheme for the amino acid sequences of Class B enzymes (Galleni et al. 2001). In reporting the discovery of one member of subclass B3, THIN-B, Rossolini et al. presented an unrooted phylogenetic tree that included members of all three subgroups (Rossolini et al. 2001). Here we show that the "B3" subclass of Class B is as distinct from the "B1 + B2" subclass as the three classes of serine β-lactamases, A, C, and D, are from each other.

During the process of constructing a phylogeny of the metallo-β-lactamases we noticed that the amino

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Table 1. Accession numbers of genes and protein structures

DNA sequences			
Gene Name	Organism	Location	Accession No.
Subclass B1 + B2			
bla2	*Bacillus anthracis*	Chromosome	AF367984
BlaB-1	*Chryseobacterium meningosepticum*	Chromosome	AF189298
BlaB-2	*Chryseobacterium meningosepticum*	Chromosome	AF189300
BlaB-3	*Chryseobacterium meningosepticum*	Chromosome	AF189301
BlaB-5	*Chryseobacterium meningosepticum*	Chromosome	AF189303
BlaB-6	*Chryseobacterium meningosepticum*	Chromosome	AF189302
BlaB-7	*Chryseobacterium meningosepticum*	Chromosome	AF189304
BlaB-8	*Chryseobacterium meningosepticum*	Chromosome	AF189305
blm	*Bacillus cereus*	Chromosome	M11189
CcrA	*Bacteroides fragilis*	Chromosome	M63556
cfiA	*Bacteroides fragilis*	Chromosome	M34831
CGB-1	*Chryseobacterium gleum*	Chromosome	AF339734
CphA	*Aeromonas hydrophila*	Chromosome	X57102
CphA2	*Aeromonas hydrophila*	Chromosome	U60294
ImiS	*Aeromonas veronii*	Chromosome	Y10415
IMP-1	*Serratia marcescens*	Plasmid	AF416297
IMP-2	*Acinetobacter baumannii*	Chromosome	ABA243491
IMP-4	*Acinetobacter baumannii*	Chromosome	AF244145
IMP-5	*Acinetobacter baumannii*	Unknown	AF290912
IMP-6	*Serratia marcescens*	Plasmid	AB040994
IMP-8	*Klebsiella pneumoniae*	Plasmid	AF322577
IMP-9	*Shigella flexneri*	Plasmid	AY033653
IMP-10	*Pseudomonas aeruginosa*	Plasmid	AB074434
IMP-11	*Pseudomonas aeruginosa*	Plasmid	AB074437
IND-1	*Chryseobacterium indologenes*	Chromosome	AF099139
IND-2	*Chryseobacterium indologenes*	Chromosome	AF219129
IND-2a	*Chryseobacterium indologenes*	Chromosome	AF219130
IND-3	*Chryseobacterium indologenes*	Chromosome	AF219131
IND-4	*Chryseobacterium indologenes*	Chromosome	AF219135
JOHN-1	*Flavobacterium johnsoniae*	Chromosome	AY028464
shfI	*Serratia fonticola*	Chromosome	AF197943
VIM-1	*Achromobacter xylosoxidans* subsp. *denitrificans*	Plasmid	AJ278514
VIM-2	*Pseudomonas aeruginosa*	Chromosome	AF191564
VIM-3	*Pseudomonas aeruginosa*	Chromosome	AF300454
Subclass B3			
CAU-1	*Caulobacter crescentus*	Chromosome	AJ308331
FEZ-1	*Fluoribacter gormanii*	Chromosome	Y17896
GOB-1	*Chryseobacterium meningosepticum*	Chromosome	AF090141
L1	*Stenotrophomonas maltophilia*	Chromosome	X75074
L1c	*Stenotrophomonas maltophilia*	Chromosome	AJ251814
L1d	*Stenotrophomonas maltophilia*	Chromosome	AJ251815
L1e	*Stenotrophomonas maltophilia*	Chromosome	AJ272109
Mbl1	*Caulobacter crescentus*	Chromosome	AJ315850
Mbl511	*Stenotrophomonas maltophilia*	Chromosome	AJ289086
THIN-B	*Janthinobacterium lividum*	Chromosome	AJ250876

Protein structures			
PDB No.	Function	Subclass	Name
2BMI	Metallo-β-lactamase	B1	CcrA
1DDK	Metallo-β-lactamase	B1	IMP-1
1BVT	Metallo-β-lactamase	B1	blm
1SML	Metallo-β-lactamase	B3	L1
1BLS	Serine -β-lactamase	C	AmpC, *Enterobacter cloacae*
1FR6	Serine -β-lactamase	C	AmpC, *Citrobacter freundii*
1K4F	Serine -β-lactamase	D	OXA-10
1H8Z	Serine -β-lactamase	D	OXA-13
1M40	Serine -β-lactamase	A	TEM-1
1HZO	Serine -β-lactamase	A	BlaB, *Proteus vulgaris*

Table 2. Comparisons[a] of subclass B1 + B2 with subclass B3 metallo-β-lactamase protein sequences

Subclass B1 + B2	VIM-2	IMP-9	cfiA	BlaB3	Blab8	IND-2	JOHN1	CphA	shfl
blm	4×10^{-37}	1×10^{-28}	5×10^{-33}	1×10^{-38}	1×10^{-38}	3×10^{-33}	5×10^{-35}	5×10^{-14}	2×10^{-13}
VIM-2	N/A	5×10^{-24}	1×10^{-30}	3×10^{-23}	1×10^{-23}	9×10^{-20}	5×10^{-18}	3×10^{-12}	2×10^{-13}
IMP-9		N/A	5×10^{-40}	7×10^{-31}	8×10^{-30}	9×10^{-24}	6×10^{-27}	2×10^{-11}	6×10^{-06}
cfiA			N/A	1×10^{-28}	2×10^{-29}	2×10^{-26}	2×10^{-33}	2×10^{-13}	2×10^{-12}
BlaB3				N/A	1×10^{-128}	9×10^{-56}	2×10^{-67}	3×10^{-15}	1×10^{-12}
Blab8					N/A	3×10^{-54}	2×10^{-67}	6×10^{-15}	3×10^{-70}
IND-2						N/A	5×10^{-53}	1×10^{-13}	4×10^{-15}
JOHN1							N/A	3×10^{-18}	3×10^{-15}
CphA								N/A	7×10^{-78}
Subclass B3	**mbl-1**	**FEZ1**	**GOB1**	**L1**	**L1c**	**L1d**	**L1e**	**mbl511**	**THINB**
CAU-1	1×10^{-156}	3×10^{-35}	3×10^{-31}	6×10^{-34}	9×10^{-35}	4×10^{-35}	1×10^{-32}	1×10^{-23}	8×10^{-39}
mbl-1	N/A	4×10^{-35}	3×10^{-31}	2×10^{-33}	2×10^{-34}	1×10^{-34}	3×10^{-32}	3×10^{-23}	1×10^{-37}
FEZ1		N/A	4×10^{-45}	1×10^{-31}	3×10^{-31}	1×10^{-32}	1×10^{-30}	9×10^{-21}	1×10^{-30}
GOB1			N/A	1×10^{-21}	3×10^{-21}	8×10^{-23}	6×10^{-23}	1×10^{-17}	5×10^{-22}
L1				N/A	1×10^{-137}	1×10^{-141}	1×10^{-124}	9×10^{-96}	5×10^{-38}
L1c					N/A	1×10^{-140}	1×10^{-127}	1×10^{-101}	8×10^{-38}
L1d						N/A	1×10^{-132}	2×10^{-98}	4×10^{-40}
L1e							N/A	4×10^{-84}	2×10^{-38}
mbl511								N/A	8×10^{-30}

[a] Values are E-scores from pairwise BLAST (BLAST 2) alignments.

acid sequences of 10 of those enzymes (subclass B3 in Table 1) bore no recognizable homology to those of the remaining 34 enzymes. Table 1 lists the enzymes and their GenBank accession numbers. Because a phylogenetic tree, or a dendrogram, depicts the descent of a set of genes from a common ancestor, it is a fundamental principle that only genes that are truly homologous, i.e., descended from a common ancestor, can be on the same tree. Sequence-based phylogenetic reconstruction programs all depend on comparison of homologous sites (bases or amino acids) that are obtained by aligning the sequences so as to maximize the number of identical or similar residues in a column of homologous sites. Although ultimately all sequences may indeed be descended from a common ancestral sequence, during the process of evolution some sequences become so diverse that all traces of their relationship become lost and their sequence relatedness approaches that of any two randomly chosen sequences. Alignment of such unrelated sequences is meaningless, as are the phylogenetic trees that include such unrelated sequences. Alignment programs, such as ClustalX (Thompson et al. 1997), will align any set of sequences, whether or not they are related; phylogeny programs will construct phylogenies based on those spurious alignments, and it is not uncommon to see such meaningless trees in the literature (Bush et al. 1995; Rossolini et al. 2001).

We used the BLAST 2 Sequences tool (Tatusova and Madden 1999) that is implemented at the NCBI BLAST site (http://www.ncbi.nlm.nih.gov/blast/index.html) to assess the homology of the 10 suspect sequences to each other and to 10 of the remaining 34 metallo-β-lactamases. BLAST 2 attempts to align two sequences, and if it is able to find a significant alignment, reports both a bit score and an "E" value. The "E" value can be thought of as the probability of an equally good alignment to a random protein of similar length. Although that probability is actually $1 - e^{-\text{E-score}}$, at values < 0.05 the E-score and the probability of a random match are virtually indistinguishable. Table 2 shows that within the group of 10 suspect sequences (subclass B3) the E-scores are all $< 10^{-21}$, and that within the other group of 10 (subclasses B1 + B2) the E-scores are all $< 10^{-5}$. In pairwise comparisons between the two groups there are no significant alignments. Either BLAST was unable to create an alignment or the E-score was > 1.0, i.e., the probability of an equally good alignment to a random protein of similar length was > 0.63. The results mean that the 10 suspect metallo-β-lactamases have no detectable sequence homology to the remaining 34 metallo-β-lactamases and should therefore not be included in a single sequence alignment and should not be included on the same sequence-based phylogenetic tree.

Among the three classes of serine-β-lactamases there does exist homology that is detectable at the three-dimensional structure level. It is well recognized that there is likewise structural homology among all of the metallo-β-lactamases (Galleni et al. 2001), including subclass B3. To determine whether the difference in structural homology between subclasses B1 + B2 and subclass B3 is similar to the differences among Classes A, C and D, we used the VAST application at NCBI (http://www.ncbi.nlm.nih.gov/Structure/RESEARCH/iucrabs.html) to compare the structures of the three subclass B1 metallo-β-lactamases and the single subclass B3 metallo-β-lac-

Table 3. Comparisons[a] of structures by VAST structural alignments

Structure {class} (length)	1DDK	1BVT	1SML		
1BM1 {B1} (232)	214 (0.97)	219 (0.96)	194 (0.84)		
1DDK {B1} (220)		206 (0.94)	190 (0.86)		
1BVT {B1} (227)			184 (0.82)		
1SML {B3} (269)					
	1FR6	1K4F	1H8Z	1M40	1HZO
1BLS {C} (361)	356 (0.99)	208 (0.84)	191 (0.77)	195 (0.74)	199 (0.73)
1FR6 {C} (361)		206 (0.83)	196 (0.79)	196 (0.75)	197 (0.72)
1K4F {D} (247)			233 (0.94)	224 (0.91)	211 (0.85)
1H8Z {D} (247)				209 (0.85)	196 (0.79)
1M40 {A} (263)					258 (0.98)
1HZO {A} (271)					

[a] Values are the number of aligned amino acids (fraction of aligned amino acids).

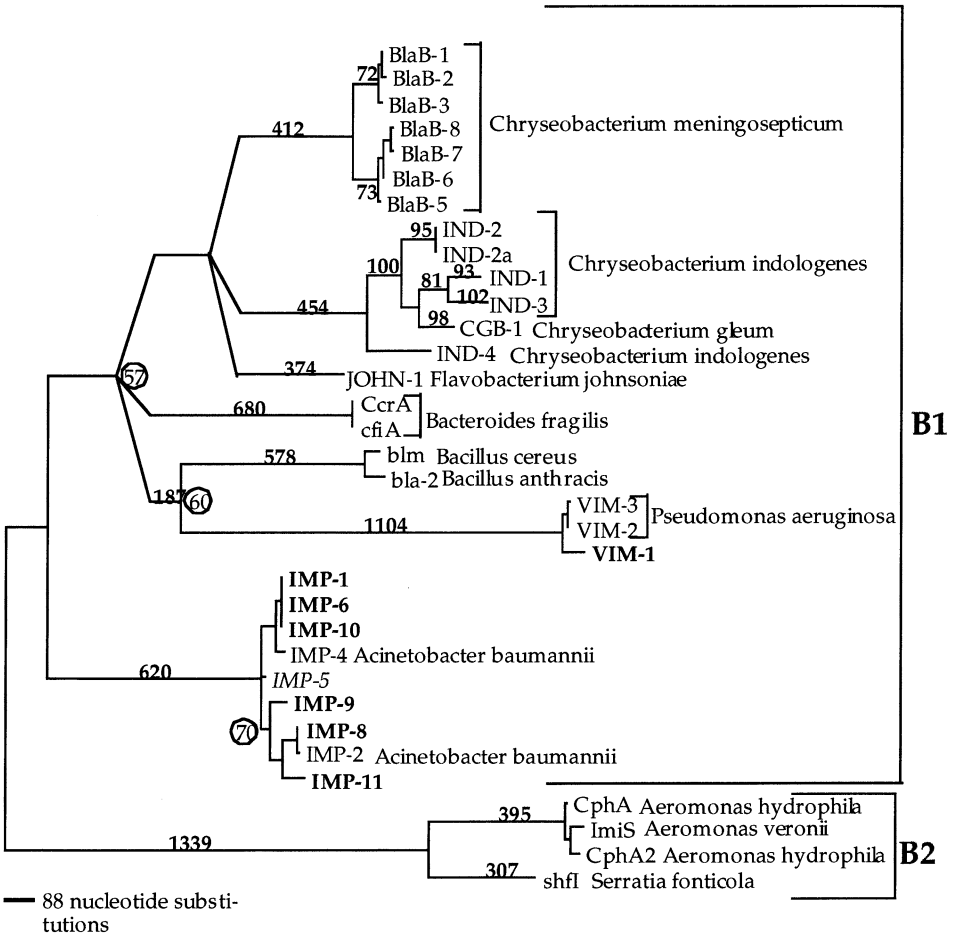

Fig. 1. Phylogeny of the subclass B1 + B2 metallo-β-lactamases. Protein sequences were aligned with ClustalX (Thompson et al. 1997) as previously described (Barlow and Hall 2002). The corresponding DNA coding sequences were gapped according to the protein alignment using CodonAlign (Hall 2001), and a Bayesian phylogeny was constructed using MrBayes (Huelsenbeck and Ronquist 2001) as previously described (Barlow and Hall 2002) from the consensus of 9200 trees. Branch lengths, in nucleotide substitutions, are indicated above the branches except for very short branches, where lengths can be estimated from the scale near the bottom of the figure. Except as indicated by numbers in *circles*, the confidence in clades, i.e., the percentage of the trees in which those taxa are included in that clade, is >80%. *Boldface* indicates plasmid-borne alleles.

tamase that are in the structure database. The VAST program creates a structural alignment (Madej et al. 1995) of two protein structures and reports the number of aligned amino acids. Note that the identity or similarity of those aligned amino acids is not considered in generating the structural alignment.

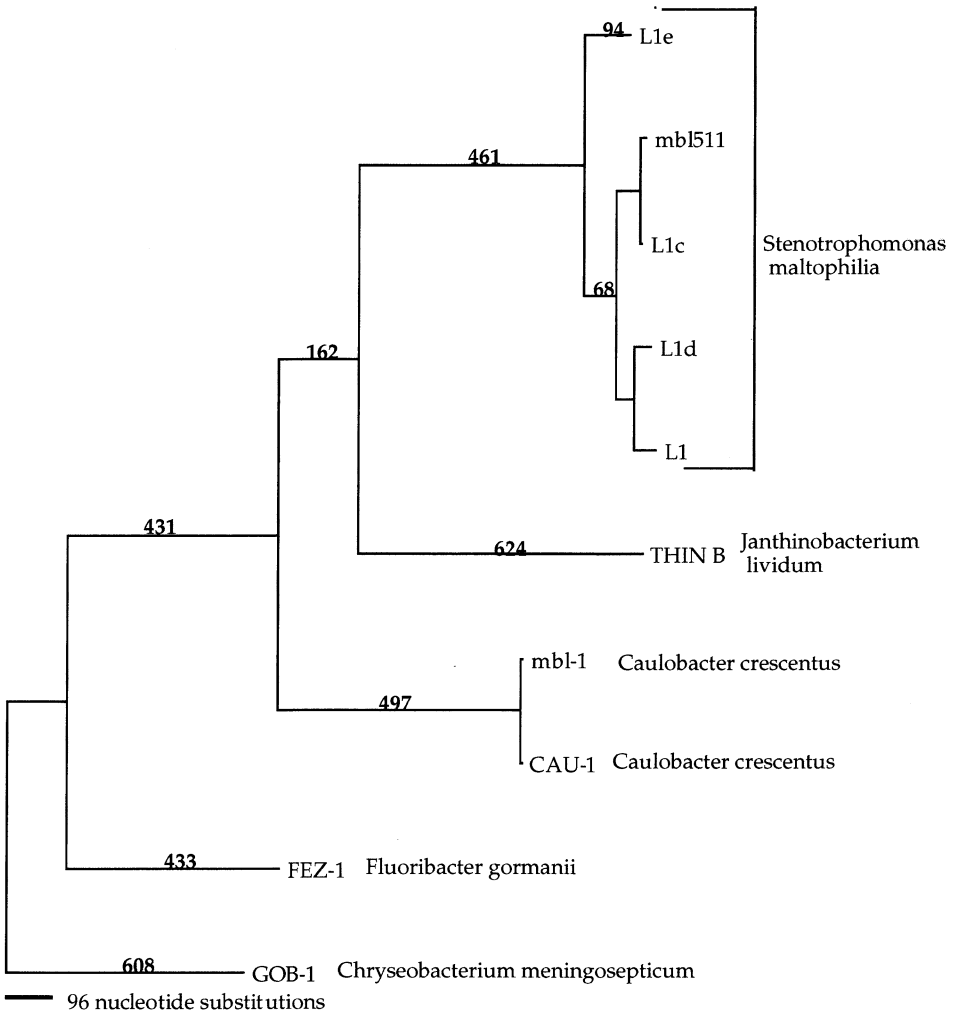

Fig. 2. Phylogeny of the subclass B3 metallo-β-lactamases. Phylogenies were determined as for Fig. 1. The confidence in each clade is > 95%.

Table 3 reports the result of those comparisons as the number of aligned amino acids and the fraction of aligned amino acids for each pairwise comparison. The fraction of aligned amino acids is calculated as the number of aligned amino acids divided by the length of the shorter sequence.

The structures of three subclass B1 and one subclass B3 enzyme are in the Protein Structure Database (PDB). Within subclass B1 0.96 ± 0.01 (mean ± SE) of the residues are structurally aligned, whereas between subclass B1 enzymes and the subclass B3 enzyme 0.84 ± 0.01 of the residues align. To see how those results compare with similar results for the three classes of serine-β-lactamases we selected two enzymes from each class (Classes A, C, and D) for which there are structures in the PDB. The pairwise comparisons within classes gave 0.97 ± 0.02 aligned residues. Comparison of Class A vs Class C gave 0.74 ± 0.01, between Class A and Class D 0.85 ± 0.02, and between Class C and Class D 0.81 ± 0.02 aligned residues. The structural differences between subclass B1 and subclass B3 are therefore comparable to the differences among the three serine β-lactamase classes.

We have constructed Bayesian phylogenies (Figs. 1 and 2) of the two groups of metallo-β-lactamases.

The two major clades that correspond to the subclasses B1 and B2 (Galleni et al. 2001; Rasmussen and Bush 1997) are fully supported. Subclass B2 consists entirely of chromosomally located genes of Gram-negative bacteria, while subclass B1 includes two chromosomally located genes of Gram-positive organisms (*Bacillus cereus* and *Bacillus anthracis*), suggesting the likelihood of horizontal transfer into those Gram-positive chromosomes. Subclass B1 consists of two clades, the smaller of which consists of IMP alleles that are primarily found on plasmids of Gram-negative organisms. The two chromosomal alleles may well be the result of horizontal transfers from plasmids into *A. baumanii* chromosomes.

The subclass B3 metallo-β-lactamase genes (Fig. 2) are all in Gram-negative organisms, and most are located within chromosomes. The L1 alleles of *Stenotrophomonas maltophilia* have been reported as being on plasmids (Avison et al. 2001), but the authors of that report note that the "plasmid" is a 200-kb DNA element that they refer to as "plasmid-like"

and for which there is no evidence of mobilization. With respect to their ability to be trasferred from cell to cell the L1 alleles should probably be considered to behave like chromosomal alleles.

VAST reports that subclasses B1 and B3 are structural neighbors. They are therefore clearly descended from a common ancestor, as are Classes A, C, and D of the serine-β-lactamases. Although descended from a common ancestor, they have diverged so much that all traces of that history have disappeared from their sequences. For that reason members of subclasses B1 + B2 and subclass B3 cannot be placed onto the same sequence-based phylogenetic tree. Note that VAST reports no structural relationship between the metallo- and the serine-β-lactamases.

Alignments that include both subclass B1/B2 and subclass B3 sequences have frequently been reported (Bellais et al. 2000; Boschi et al. 2000; Poirel et al. 2000; Rossolini et al. 1996; Wang et al. 1999). Because the presence of unrelated sequences can interfere with the proper alignment of related sequences, those alignments should not be trusted, nor should trees (Bellais et al. 2000; Poirel et al. 2000; Rossolini et al. 1996) that are based upon those alignments.

As currently employed, the Ambler nomenclature is hardly ideal, as it assigns the same class rank to the three major lineages of serine-β-lactamases as it does to the entire family of metallo-β-lactamases. The current scheme promotes spurious alignments and phylogenetic trees of the metallo-β-lactamases. We urge the β-lactamase community to consider creating a new classification scheme for β-lactamases. We suggest dividing the β-lactamases into two groups, the serine-β-lactamases and the metallo-β-lactamases. Within the serine-β-lactamases would be three subclasses corresponding to the present Classes A, C, and D. Within the metallo-β-lactamases would be two subclasses corresponding to the present Class B subclass B1 + B2 and subclass B3. The proposed scheme would recognize that members of the two major classes are unrelated to each other at any level, and that within those classes members are related to each other at the structural level, but that there is no detectable relationships between members of different subclasses at the sequence level.

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