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XENOLOGY OF BETA-LACTAMASES: ASSOCIATION OF ITS GENETIC SOURCES AND PUTATIVE PLEIOTROPISM

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Dissertação orientada por Dr. Ricardo Dias (BioFIG-FCUL)

e Prof. Dr. Francisco Dionísio (DBV-FCUL)

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This dissertation was fully performed at Center for Biodiversity, Functional and Integrative Genomics (BioFIG-FCUL) under the direct supervision of Dr. Ricardo Dias in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

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RESUMO

Em bactérias Gram-negativas, a fase final da síntese do peptidoglicano ocorre no lado periplasmático da membrana celular, envolvendo reacções de carboxipeptidação e transpeptidação mediadas por DD-peptidases membranares. Estas enzimas pertencem a uma família de proteínas conhecidas colectivamente como *Penicillin-Binding Proteins* (PBPs). As PBPs constituem o alvo preferencial dos antibióticos β-lactâmicos. A molécula do β-lactâmico actua como análogo do substrato enzimático, formando um complexo covalente acil-enzima muito estável, uma reacção que é irreversível e que resulta na inactivação da enzima. Consequentemente, dá-se o bloqueio da biossíntese da parede celular, o que pode resultar em lesões, perda de permeabilidade selectiva, e mesmo na lise celular.

As bactérias têm a capacidade de detectar interferências no metabolismo da sua parece celular e traduzir o stress resultante em sinais que induzem respostas defensivas. No decurso da sua evolução, as bactérias desenvolveram várias estratégias para lidar com os efeitos prejudiciais dos antibióticos, como os β-lactâmicos. Em Gram-negativas de relevância clínica, o mecanismo mais importante e frequente de resistência aos β-lactâmicos consiste na produção de β-lactamases. Estas enzimas hidrolisam a ligação amida do anel β-lactâmico, destruindo o local de ligação às PBPs bacterianas e inactivando assim o seu efeito antimicrobiano. Algumas β-lactamases requerem a ligação a iões zinco (Zn^{2+}) para romper o anel β-lactâmico, mas a maioria das enzimas são serina-hidrolases. Semelhantemente às PBPs, as serina-enzimas têm um motivo conservado Ser-x-x-Lys, constituindo a serina o resíduo do sítio activo. Apesar de partilharem uma estrutura semelhante ao nível do sítio activo, a ligação da PBP ao substrato β-lactâmico (via resíduo de serina) inactiva a enzima, ao passo que o mesmo substrato é hidrolisado pela β-lactamase, sendo a enzima liberta.

As serina-β-lactamases cromossomais podem ter evoluído a partir das PBPs, com quem partilham algumas homologia de sequência, ou apresentarem uma evolução paralela associada a pleiotropia funcional. Neste caso, estas enzimas teriam sido seleccionadas pela função secundária, associada ao consumo de antimicrobianos. Esta hipótese é apoiada pelo facto dos genes que codificam as β-lactamases serem geneticamente conservados, e a sua expressão se encontrar associada a mecanismos fisiológicos responsáveis pelo arranjo da parede celular. A evolução destes genes pode ter sido assim possível devido a pressões selectivas exercidas por organismos produtores de β-lactâmicos presentes no solo, ou pela importância pleiotrópica da sua função primária na fisiologia microbiana. Contudo, o uso clínico actual dos β-lactâmicos ser o principal factor selectivo a influenciar o isolamento de β-lactamases em organismos patogénicos.

Actualmente, as β-lactamases são classificadas de acordo com a sua estrutura primária (classificação de Ambler) ou com a sua função (classificação de Bush-Jacoby-Medeiros). A classificação de Ambler divide estas enzimas em quatro classes (A – D), com base em motivos de aminoácidos conservados. As enzimas das classes A, C e D são serina-β-lactamases, enquanto que as enzimas da classe B são conhecidas como Metalo-β-lactamases (MBLs). O esquema de classificação de Bush-Jacoby-Medeiros, por sua vez, baseia-se na similaridade funcional (perfil de substrato e inibição) das enzimas incluídas em cada classe do esquema de classificação de Ambler. Este sistema compreende quatro grupos funcionais principais (1 – 4), com vários subgrupos dentro do grupo 2 (a – f).

As enzimas do grupo 1 pertencem à classe C e hidrolisam preferencialmente cefalosporinas, não sendo normalmente inibidas pelo ácido clavulânico ou por tazobactam. Em bactérias Gram-negativas, as cefalosporinases cromossomais mais relevantes constituem o grupo de enzimas conhecidas colectivamente como β-lactamases AmpC (AmpCs). A informação existente sugere uma ligação estreita entre a indução destas enzimas e a reciclagem do peptidoglicano. Neste caso, a hidrólise de β-lactâmicos teria sido uma resposta evolutiva à pressão de selecção efectuada pelas cefalosporinas, de modo a proteger as bactérias da sua acção antimicrobiana. De facto, as AmpC cromossomais são expressas frequentemente como enzimas induzíveis, em resposta à exposição a β-lactâmicos. Estas enzimas não contribuem significativamente para a resistência clínica a estes antibióticos, uma vez que são normalmente expressas a baixos níveis, mas podem causar complicações terapêuticas severas caso os seus genes sejam translocados para plasmídeos. Recentemente, foram identificadas cefalosporinases de espectro hidrolítico alargado, com susceptibilidade reduzida a todo o tipo de cefalosporinas, incluindo as de quarta-geração (ex. cefepima e cefepiroma). Estas AmpCs de amplo espectro (ESACs) encontram-se relacionadas estruturalmente com as AmpC do grupo 1, como resultado de substituições, inserções e delecções de aminoácidos, e encontram-se reunidas no grupo 1e. A disseminação deste tipo de enzimas pode comprometer a utilidade clínica da maioria dos β-lactâmicos.

O grupo 2 inclui β-lactamases pertencentes às classes A e D. Estas enzimas têm mais afinidade para o ácido clavulânico do que para o tazobactam. Destacam-se as β-lactamases de espectro alargado (ESBLs) do subgrupo 2be, as quais derivam das enzimas do subgrupo 2b por apenas algumas substituições de aminoácidos. As ESBLs pertencem à classe A e, à semelhança de algumas enzimas do grupo 1, são capazes de hidrolisar cefalosporinas de quarta-geração. Contudo, as ESBL são susceptíveis ao ácido clavulânico e ao tazobactam, contrariamente às cefalosporinases do grupo 1. Nesta dissertação, o termo “ESBL” foi extendido às β-lactamases da classe D que se encontram reunidas no subgrupo 2de. Estas enzimas derivam das oxacilinases (OXAs) do subgrupo 2d e conseguem hidrolisar cefalosporinas de quarta-geração, retendo igualmente a capacidade de hidrolisar cloxacilina (ou oxacilina).

As MBLs da classe B encontram-se reunidas no grupo 3, sendo inibidas distintivamente pelo EDTA, mas não pelo ácido clavulânico ou pelo tazobactam. À excepção dos monobactamos (ex. aztreonam), que são hidrolisados pelas serina-enzimas, as metalo-enzimas conseguem hidrolisar todos as classes de β-lactâmicos. As MBLs encontram-se actualmente subdivididas em três subclasses estruturais (B1, B2 e B3), as quais se encontram alinhadas com dois subgrupos funcionais (3a e 3b), com base em similaridades funcionais. As enzimas do subgrupo 3a (subclasses B1 e B3) requerem a ligação de dois iões Zn^{2+} ao seu sítio activo para o desempenho da sua actividade hidrolítica de amplo espectro. Pelo contrário, as enzimas do subgrupo 3b (subclasse B2) são inibidas se ocorrer a ligação a um segundo ião Zn^{2+}. Estas metalo-enzimas possuem um espectro hidrolítico reduzido, actuando preferencialmente sobre carbapenemos.

As primeiras MBLs descritas foram identificadas em bactérias Gram-negativas de origem ambiental, sendo na sua maioria enzimas cromossomais e induzíveis, associadas provavelmente a uma outra função metabólica ainda desconhecida. Estas enzimas apresentam distribuição ubiquitária e são

reconhecidas actualmente como o reservatório mais importante destes genes de resistência. No entanto, as bactérias que as produzem são geralmente patogénios oportunistas, não associados frequentemente a infecções nosocomiais graves. De facto, são as metalo-enzimas encontradas em elementos transmissíveis, particularmente em integrões ou plasmídeos, que se encontram disseminadas globalmente em bactérias patogénicas de relevância clínica. Assim sendo, é de extrema importância científica e de Saúde Pública a real compreensão do modelo de evolução destas enzimas, como também a identificação dos reservatórios genéticos e avaliação do seu potencial na emergência de novas enzimas hidrolíticas de β-lactâmicos.

O presente estudo teve por objectivo clarificar as relações evolutivas entre as diferentes classes de β-lactamases, e também procurar compreender como elas se tornaram nas enzimas hidrolíticas de β-lactâmicos dos dias de hoje. Para tal, recorreu-se à análise filogenética e funcional de sequências nucleotídicas e aminoacídicas destas enzimas. Inicialmente, foram alinhadas 61 sequências aminoacídicas de β-lactamases de origem cromossomal, as quais foram posteriormente sujeitas a análise filogenética, recorrendo-se ao método da máxima parcimónia (MP). O critério de selecção destas sequências consistiu em reunir enzimas representativas de cada classe molecular de Ambler e grupo funcional de Bush-Jacoby-Medeiros descritos actualmente, de acordo com os respectivos esquemas de classificação. A partir da árvore filogenética inferida, foi possível organizar as sequências de β-lactamases em 8 grupos (ou *clusters*) (α – θ), os quais revelaram ser consistentes com os agrupamentos de classificação molecular e funcional em vigor para estas enzimas. Enzimas representativas de cada *cluster* foram então utilizadas para pesquisar proteínas ortólogas nas bases de dados públicas. A filogenia destas sequências foi reconstruída, igualmente, através da abordagem MP. A análise filogenética revelou que a maior parte dos ortólogos putativos surgiu recentemente, juntamente com as β-lactamases com as quais aparentam estar mais relacionados, visto haver pouca divergência em relação ao ancestral comum a partir do qual ocorreu o evento de especiação. Existem também ramos na árvore mais longos, particularmente no grupo das OXAs, os quais representam um grau de divergência mais elevado, resultante de um maior número de substituições de aminoácidos. Estes resultados sugerem que as oxacilinases podem ter surgido na fase inicial da evolução das β-lactamases.

Uma vez que a similaridade por homologia nem sempre é sinónimo de funções idênticas, a inferência de homologia pode não ser suficiente para efectuar previsões funcionais rigorosas. Assim sendo, recorreu-se às ferramentas *InterProScan* e *CDD* para a anotação funcional das sequências representativas de cada *cluster* e dos seus ortólogos putativos. Esta análise funcional revelou que todos os ortólogos putativos partilham alguns domínios conservados com a mesma superfamília de β-lactamases com quem aparentam estar relacionados. Estes domínios funcionais encontram-se relacionados com actividade hidrolítica de β-lactâmicos, o que sugere estarmos perante ortologia genuína. As metalo-enzimas da classe B e os seus ortólogos partilham um domínio funcional específico com outras enzimas da mesma superfamília, as glutationa tiolesterases. Estas enzimas hidrolisam S-D-lactoilglutationa em glutationa e D-ácido láctico, requerendo a ligação a dois iões Zn^{2+} como cofactor. Estes resultados sugerem a existência de uma possível pleiotropia funcional para as metalo-enzimas.

Por fim, foram estimados os rácios K_a/K_s para os genes codificantes das 61 β-lactamases cromossomais, visto que a identificação de selecção natural positiva poderia evidenciar alterações adaptativas na função. A filogenia destas sequências foi reconstruída, recorrendo-se ao método da máxima verosimilhança (ML). Um total de 8 genes revelou estar sob selecção positiva (K_a/K_s >1), o que pode indicar que as proteínas codificadas podem ter alterado a sua função, possivelmente devido à pressão selectiva dos β-lactâmicos. De facto, os genes que codificam as metalo-enzimas CAU-1 e Mbl1b de *C. crescentus* parecem enquadrar-se neste cenário, tendo sido sugerido que estas enzimas representam hidrolases ancestrais que foram seleccionadas positivamente em resposta à presença de β-lactâmicos produzidos por fungos no solo. Os resultados obtidos fortalecem esta hipótese, bem como a de pleiotropia funcional nas MBLs, evidenciada pelas previsões funcionais. O facto de se terem detectado genes que codificam para enzimas da família OXA sob selecção positiva, suporta a hipótese destas enzimas poderem ter tido uma função importante na *fitness* bacteriana, como referido anteriormente. Os genes que codificam as cefalosporinases de *P. aeruginosa*, PDC-1 e PDC-2, parecem partilhar um ancestral comum com as AmpCs. O facto destes genes aparentarem estar sob forte selecção positiva pode significar que a mutação que conferiu a vantagem selectiva na presença do β-lactâmico e, assim, a alteração na função, pode ter-se fixado no seu DNA. Observou-se um elevado grau de conservação no grupo das AmpCs, sugerido pelos baixos rácios K_a/K_s apresentados, o qual pode indicar que estes genes são essenciais para as bactérias e, como tal, evoluem mais lentamente do que os genes não-essenciais. De facto, foi sugerida a existência de uma relação estreita entre a indução das AmpCs e a reciclagem do peptidoglicano, antes da pressão selectiva imposta pelas cefalosporinas, o que pode significar que estas enzimas têm um papel crucial na fisiologia bacteriana.

Apesar de por vezes óbvia, nem sempre é possível demonstrar a existência de pleiotropia funcional. Não obstante, esta propriedade parece desempenhar um papel fundamental na evolução dos genes das β-lactamases. De facto, pensa-se que o efeito pleiotrópico pode aumentar quando as bactérias se deparam com novos desafios selectivos, como é o caso dos antibióticos β-lactâmicos. Dependendo de como o novo caracter fenotípico (função) afecta a *fitness* bacteriana, os genes tornam-se pleiotrópicos ou especializados numa única função. Os resultados apresentados neste estudo contribuíram para o reconhecimento de um certo grau de pleiotropia funcional nas β-lactamases, e para uma maior compreensão de como estas proteínas podem ter evoluído e se tornado em enzimas específicas, responsáveis pela hidrólise de β-lactâmicos. Estudos adicionais combinando análise da sintenia de genes, alinhamentos estruturais, filogenias de genes individuais, interacções proteína-proteína e análises de recombinação e selecção, poderão ajudar a identificar e clarificar os processos evolutivos destas enzimas.

ABSTRACT

In clinically important Gram-negative bacteria, the predominant mechanism for β-lactam resistance is the synthesis of β-lactamases. These enzymes hydrolyze the amide bond in the β-lactam ring of these antibiotics, inactivating their effect. A few β-lactamases are zinc-dependent hydrolases, or Metallo-β-lactamases, requiring at least one zinc ion for disrupting the β-lactam ring. However, most enzymes are serine hydrolases, operating via a serine-ester mechanism. The chromosomal serine β-lactamases may have evolved from the Penicillin-Binding Proteins, with whom they share sequence homologies, or present parallel evolution associated with functional pleiotropy, resulting from the selective pressures performed by β-lactam-producing organisms in the soil. However, the current clinical misuse of β-lactams seems to be the most important factor for the dissemination of β-lactam resistance among pathogenic bacteria.

The present study aimed to clarify the evolutionary relationships between the β-lactamases, and to understand how they became the specific β-lactam-hydrolyzing enzymes nowadays. Phylogenetic and functional analysis from nucleotide and amino acid sequences was used. Phylogenies were reconstructed using Maximum Parsimony. Analysis revealed that most putative orthologs have arisen recently along with some already described β-lactamases, although a higher degree of divergence was evidenced for the oxacillinases, suggesting they may have evolved at the early stages of β-lactamase evolution. Functional predictions revealed that all putative orthologs share conserved domains with the β-lactamase superfamily with whom they seem more closely related. Putative pleiotropism was also evidenced for the metalloenzymes. Finally, K_a/K_s ratios were estimated. Eight β-lactamase genes were found to be under positive selection, suggesting possible adaptive changes in function. Overall, this study has contributed to the acknowledgement of some level of functional pleiotropy within the β-lactamases. Further studies combining analysis of gene synteny, structural alignments, phylogenies of individual genes, protein-protein interactions, and analyses of recombination and selection would help identify and clarify the evolutionary processes of such enzymes.

Keywords: β-lactamase, orthologs, functional pleiotropy, phylogenetic analysis, functional predictions

LIST OF ABBREVIATIONS

In this dissertation, acronyms are expanded on first usage and whenever deemed necessary to improve clarity.

AmpC: Ampicillin Class C
BBLI: β-lactam / β-lactamase Inhibitor
BLAST: Basic Local Alignment Tool
CA: Clavulanic Acid
CDD: Conserved Domain Database
CHDL: Carbapenem-hydrolyzing class D β-lactamase
D-Ala: D-alanine
DNA: Deoxyribonucleic Acid
EDTA: Ethylenediaminetetraacetic Acid
HGT: Horizontal Gene Transfer
IS: Insertion Sequence
LS: Least Squares Methods
MDR: Multidrug-resistant
ME: Minimum Evolution
MEGA: Molecular Evolutionary Genetics Analysis
MGE: Mobile Genetic Element
ML: Maximum Likelihood
MP: Maximum Parsimony
MRSA: Methicillin-Resistant *Staphylococcus aureus*
MSA: Multiple Sequence Alignment
NaCl: Sodium Chloride
NAG: *N*-acetylglucosamine
NAM: *N*-acetylmuramic Acid
NCBI: National Center for Biotechnology Information
NJ: Neighbor-Joining
ORF: Open Reading Frame
PBP: Penicillin-Binding Protein
PSSM: Position-Specific Score Matrix
RBH: Reciprocal Best Hits
TZB: Tazobactam
UPGMA: Unweighted Pair-Group Method Using Arithmetic Averages

β-lactamase Abbreviations

ACT: AmpC-type
ACC: Ambler class C
ADC: *Acinetobacter* derived cephalosporinase
ARI: *Acinetobacter* resistant to imipenem
Bc-II: From *Bacillus cereus* type II
BEL: Belgium ESBL
BES: Brazil ESBL
BIL: Named after the patient (Bilal) from whom it was first isolated
BLA: From *Bacillus anthracis*
BRO: From (*Branhamella*) *Moraxella catarrhalis*
CARB: Active on carbenicillin
CAU: From *Caulobacter crescentus*
CcrA: Cefoxitin and carbapenem resistant
CepA: Cephalosporinase from *Bacteroides fragilis*, class A
CME: From *Chryseobacterium meningosepticum*
CMT: Complex mutant derived from TEM-1
CMY: Active on cephamycins
CphA: Carbapenem-hydrolyzing and first (A) from *Aeromonas hydrophila*
CTX-M: Active on cefotaxime, first isolated at Munich, Germany

DHA: Discovered at Dhahran Hospital in Saudi Arabia

ESAC: Extended-spectrum AmpC

ESBL: Extended-spectrum β-lactamase

FEC: Faecal *Escherichia coli*

FEZ: *Legionella* (*Fluoribacter*) *gormanii* endogenous zinc β-lactamase

FOX: Active on cefoxitin

GC1: From *Enterobacter cloacae* strain GC1

GES: Guiana ESBL

GIM: German imipenemase

GOB: From *Chryseobacterium meningosepticum*, class B

HugA: Hopital Universitaire de Genève, class A

IMI: Imipenem-hydrolyzing β-lactamase

IMP: Active on imipenem

IND: From *Chryseobacterium* (*Flavobacterium*) *indologenes*

IRT: Inhibitory resistant TEM

JOHN: From *Flavobacterium johnsoniae*

KHM: Kyorin Health Science MBL, from Kyorin University Hospital in Tokyo, Japan

KPC: *Klebsiella pneumoniae* carbapenemase

L1 / 2: Labile enzyme(s) from *Stenotrophomonas maltophilia*

LAT: Active on latamofex

LEN-1: From *K. pneumoniae* strain LEN-1

MBL: Metallo-β-lactamase

MIR: Discovered at Miriam Hospital in Providence, Rhode Island, USA

MOX: Active on moxalactam

MUS: From *Myroides odoratimimus*

NMC: Not metalloenzyme carbapenemase

NDM: New-Delhi MBL

NPS: From a national *Pseudomonas* survey

OXA: Active on oxacillin

P99: From *Enterobacter cloacae* strain P99

PaO1: From *Pseudomonas aeruginosa* strain PaO1

PC1: From *Staphylococcus aureus* strain PC1

PDC: *Pseudomonas*-derived cephalosporinase

PER: *Pseudomonas* extended resistant

PSE: *Pseudomonas*-specific enzyme

RAHN: From *Rahnella aquatilis*

RTG: Enzyme with RTG (arginine, threonine, glycine) triad in conserved box VII

Sfh: *Serratia fonticola* carbapenem hydrolase

SFO: From *Serratia fonticola*

SHV: Sulfhydryl reagent variable

SIM: Seoul imipenemase

SLM01: From *Morganella morganii* strain SLM01

SME: *Serratia marcescens* enzyme

SPM: São Paulo MBL

SRT: From *Serratia* resistant to β-lactam T-5575

TEM: Named after patient (Temoniera) from whom it was first isolated

THIN-B: From *Janthinobacterium lividum*, class B

TLA: Named after the Tlahuicas Indian tribe

TLE: TEM-like enzyme

TUS: From *Myroides odoratus*

VEB: Vietnam ESBL

VIM: Verona integron-encoded MBL, first isolated at Verona, Italy

INTRODUCTION

Peptidoglycan is a complex made of glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked by short stem peptides attached to the NAM (160, 209, 211). It constitutes a mesh-like layer outside the plasma membrane of bacteria and it is an essential component of the cell wall (100), preventing the cell from osmotic lysis and determinating its shape (100, 160, 211). In Gram-negative bacteria, the final stages of peptidoglycan biosynthesis take place at the periplasmic side of the membrane (209), involving distinct carboxypeptidation and transpeptidation reactions catalyzed by membrane-bound DD-peptidases (Figure 1a). These enzymes belong to a group of proteins known collectively as Penicillin-Binding Proteins (PBPs) (39, 104, 160, 192, 211). The term PBPs is a misnomer, since they are produced to complete the cross-linking of the peptide chains that confers rigidity to the peptidoglycan and viability to the bacterial cell, not to bind to penicillins (22). Inhibition of PBPs has harmful consequences in the cell wall structure, such as elongation, lesions, loss of selective permeability, and eventual cell death and lysis (77, 222).

PBPs are the primary targets of β-lactam antibiotics (39, 100, 209), a broad class of antibacterial agents which typically contain a β-lactam ring in their molecular structure and that include all penicillins, cephems (cephalosporins and cephamycins), monobactams and carbapenems (Holten and Onusko, 2000). The PBPs interact with their peptide substrates and β-lactam antibiotics through a similar mechanism (160), by forming a covalent acyl-enzyme complex with the antibiotic molecule via the serine residue at the enzyme's active-site (39, 209, 211). This is an irreversible reaction which inactivates the enzyme (160), blocking carboxypeptidation and transpeptidation reactions, and consequently impairing the later stages of cell wall biosynthesis (209, 222, 224) (Figure 1b).

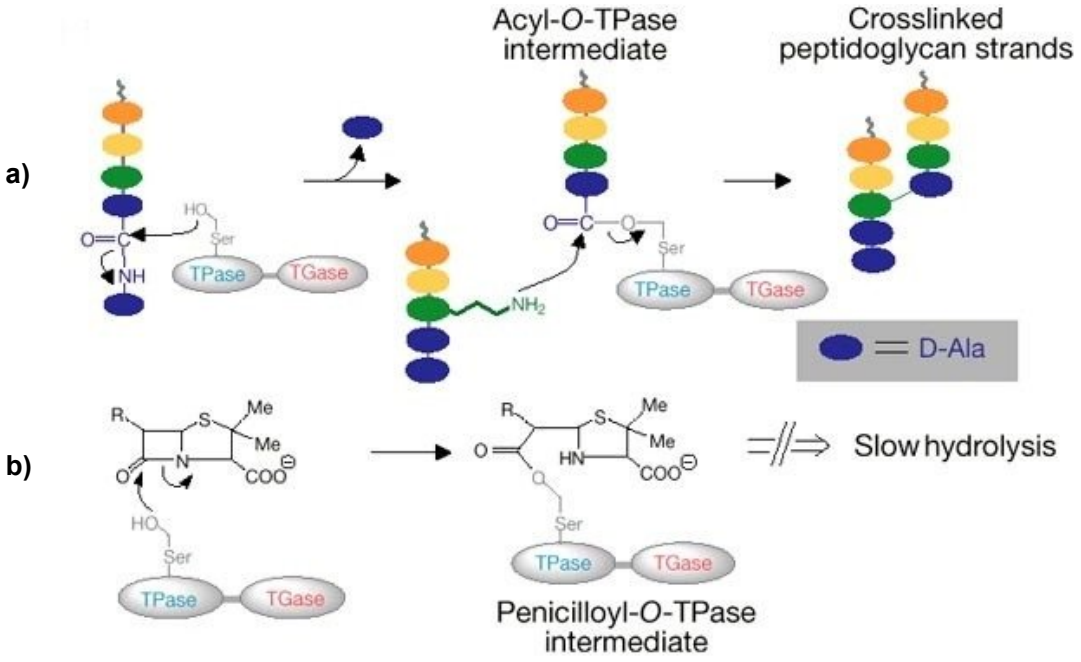

Fig. 1: Schematic representation of the biosynthesis of the cell wall (adapted from 236):

a) In the final step, peptide chains from adjacent glycan strains are cross-linked to each other by a peptide bond exchange (transpeptidation) between the free amine of the amino acid in the third position of the pentapeptide and the D-alanine (D-Ala) at the fourth position of the other peptide chain, releasing the terminal D-alanine of the precursor. Transpeptidation and carboxypeptidation reactions are mediated by DD-peptidases (PBPs).

b) β-lactams (e.g. penicillin) bind to the substrate-anchoring site of the PBP, acting as a substrate analog of D-alanyl-D-alanine, which is target of transpeptidation. The β-lactam-PBP intermediate is very stable, and it inactivates the enzyme. This interference with the normal transpeptidation reactions in the cell wall results in cellular lysis.

Perturbations in cell wall metabolism are sensed by bacteria and the resulting stress is translated into signals that induce defensive responses (100). In the course of evolution, bacteria have developed several strategies to deal with the severe effects caused by antibiotics such as β-lactams (218). Mechanisms frequently found in Gram-positive bacteria involve alteration of PBPs, which results in low affinity to β-lactams (191, 223), or acquisition of new PBPs (211), both responsible for β-lactam resistance. Alterations in PBPs of *Streptococcus pneumoniae* (219) and *Listeria monocytogenes* (182) confer high-level penicillin and imipenem resistance, respectively. High-level of resistance to methicillin and all other β-lactams in Gram-positive *Staphylococcus aureus* (MRSA) is due to acquisition of the *mecA* gene, which encodes the new PBP 2a with reduced affinity for β-lactams (127).

In clinically important Gram-negative bacteria, the predominant mechanism for resistance to β-lactam antibiotics is the synthesis of β-lactamases (12, 24, 34, 37, 38, 84, 86, 100, 123, 130, 132, 141, 205, 218), enzymes that catalyze the hydrolysis of the amide bond in the β-lactam ring of these antibacterial agents, inactivating their antimicrobial effect (72, 84, 86, 104, 123, 141). A few β-lactamases require a zinc ion to disrupt the β-lactam ring, but most enzymes are serine hydrolases (102, 130, 168, 190). The serine β-lactamases operate via a serine-ester mechanism (38, 130); following noncovalent binding of the enzyme to the antibiotic, the β-lactam ring is attacked by the free hydroxyl group on the side chain of the active-site serine residue, yielding a covalent acyl-enzyme ester intermediate. This intermediate is then deacylated from the serine by hydrolysis and the active enzyme is freed, as well as the hydrolyzed, inactive, drug (39, 130). Although the PBPs are also able to react with β-lactam molecules to give serine esters, these do not hydrolyze readily, unlike the similar esters produced by the β-lactamases (39, 77). Hydrolytic deacylation of the serine β-lactamases is possible due the presence of a glutamate residue located on a small peptide strand, the Ω-loop, which is a conserved structural motif that transverses the active-site of the enzyme (25, 39).

The serine β-lactamases are evolutionary related (39, 132, 224) and belong to a superfamily of penicillin-recognizing enzymes that also includes the DD-peptidases, and a variety of other PBPs (86, 104, 192); not only they bind to similar ligands, but also operate by a common acyl-enzyme mechanism (39, 77, 104, 160, 192). In addition, all these enzymes contain a conserved Ser-x-x-Lys motif, where the serine is the active-site residue (19, 104, 160, 192). Although the active-sites of the PBPs and β-lactamases are similar, the covalent binding of the PBPs to its β-lactam substrate

inactivates the enzyme, while the same substrate is hydrolyzed by the β-lactamases (90, 160). Sequence homologies suggests that the chromosomal β-lactamases may have evolved from the PBPs (30, 84, 108, 244), with whom they share several highly conserved amino acid sequences (141), or present a parallel evolution associated to functional pleiotropy. The class A and C serine enzymes apparently arose from a common PBP ancestor, an actinomycete DD-carboxypeptidase involved in bacterial wall peptidoglycan metabolism (90, 108, 141). The evolution of β-lactamase genes was probably a result of the selective pressure carried out by β-lactam-producing soil organisms in the natural environment (30, 84, 108, 130; 141). However, the current clinical misuse and overprescription of β-lactam antibiotics seems to be the most important factor for the selection and dissemination of β-lactam resistance in pathogenic bacteria (10, 30, 51, 84, 86, 141, 176, 190).

Classification Schemes

β-lactamases are currently classified according to their primary structure (namely, Ambler classification) (8) or their function (namely, Bush-Jacoby-Medeiros classification) (34, 35, 36, 37, 38). The Ambler classification scheme (8) divides β-lactamases into four major molecular classes, designated A to D, based on conserved and distinguishing amino acid motifs (8, 24, 33, 34, 35, 36, 37, 38, 84, 130, 168, 132, 176, 183, 193). Classes A, C and D are serine β-lactamases (38, 86, 132,176, 190), enzymes that hydrolyze their substrates through the serine-ester mechanism mentioned earlier. Class B enzymes, known as Metallo-β-lactamases (MBLs), utilize at least one active-zinc ion linked to a histidine or cysteine residue to react with the carbonyl group of the amide bond of most carbapenems, cephalosporins and penicillins, facilitating its hydrolysis (37, 38, 39, 84, 193, 239). Contrarily to the serine enzymes, MBLs lack the ability to hydrolyze monobactams and are not susceptible to clavulanic acid or tazobactam (38, 39, 132, 193, 239).

A functional classification is more important in Microbiology than classification based on amino acid homology, because it helps to correlate the properties of a specific enzyme with the observed resistance profile for a particular clinical isolate (38, 39, 130, 176). The Bush-Jacoby-Medeiros classification scheme (37), updated in 2010 (38), groups β-lactamases within each class of the Ambler system according to functional similarities, namely relative hydrolysis rates of a variety of substrates (substrate specificity) and sensitivity to various enzyme inhibitors (inhibitor susceptibility). This system comprises four main functional groups (1 – 4), with multiple subgroups under group 2 (a – f) (34, 37, 38, 84, 141, 176, 193).

Table 1 aligns functional groupings with molecular assignments as closely as possible, based on the available public information. A summary of functional characteristics, such as ability to hydrolyze specific β-lactam classes and susceptibility to the β-lactamase inhibitors clavulanic acid (CA) and tazobactam (TZB), is also included. A description of each of the functional groups and molecular classes follows. For the purpose of this dissertation, the term “class” will be employed to describe the molecular classes of the Ambler classification scheme. Likewise, the terms “group” and “subgroup” will refer to the functional clusterings within the Bush-Jacoby-Medeiros classification scheme.

Table 1: Classification schemes for bacterial β-lactamases (adapted from 38).

Functional group (Bush-Jacoby-Medeiros classification)	Molecular class (Ambler classification)	Defining characteristic(s)	Representative enzyme(s)
1	C	Preferential hydrolysis of cephalosporins and cephamycins Not inhibited by CA and TZB	*Escherichia coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1, DHA-1
1e	C	Hydrolysis of penicillins, cephamycins, extended-spectrum cephalosporins and monobactams Not inhibited by CA and TZB	GC1, CMY-37, SRT-1
2a	A	Preferential hydrolysis of benzylpenicillin Efficient hydrolysis of cephalosporins Inhibited by CA and TZB	PC1 and other staphylococcal penicillinases
2b	A	Efficient hydrolysis of penicillins and early cephalosporins Inhibited by CA and TZB	TEM-1, TEM-2, SHV-1
2be	A	Hydrolysis of penicillins, extended-spectrum cephalosporins and monobactams Inhibited by CA and TZB	ESBLs: TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1, SFO-1
2br	A	Efficient hydrolysis of penicillins and early cephalosporins Not well inhibited by CA	IRTs: TEM-30, SHV-10, SHV-49
2ber	A	Hydrolysis of penicillins, extended-spectrum cephalosporins and monobactams Less efficiently inhibited by CA and TZB	CMTs: TEM-50, TEM.68, TEM-121
2c	A	Efficient hydrolysis of carbenicillin Inhibited by CA	PSE-1, CARB-3
2ce	A	Increased hydrolysis of carbenicillin, cefepime and cefpirome Inhibited by CA	RTG-4
2d	D	Efficient hydrolysis of cloxacillin or oxacillin Not always inhibited by CA	OXA-1, OXA-10
2de	D	Hydrolysis of cloxacillin or oxacillin and extended-spectrum cephalosporins Not always inhibited by CA	ESBLs: OXA-11, OXA-15, OXA-18
2df	D	Hydrolysis of cloxacillin or oxacillin and carbapenems Not always inhibited by CA	OXA-23, OXA-48
2e	A	Efficient hydrolysis of cephalosporins Inhibited by CA and TZB	CepA
2f	A	Hydrolysis of carbapenems, cephalosporins, penicillins and cephamycins Poorly inhibited by CA, low inhibition by TZB	KPC-2, GES-2, SME-1, IMI-1, NMC-A, OXA-40
3a	B1	Hydrolysis of all β-lactams except monobactams Inhibited by EDTA and metal ion chelators Not inhibited by CA and TZB	IMP-1, NDM-1, VIM-1, CcrA, Bc-II, IND-1
	B3		L1, CAU-1, GOB-1, FEZ-1
3b	B2	Preferential hydrolysis of carbapenems Inhibited by EDTA and metal ion chelators Not inhibited by CA and TZB	CphA, Sfh-1

Group 1 / Class C β-lactamases

Group 1 β-lactamases belong to class C and are generally encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms (38, 235). These enzymes hydrolyze preferentially cephalosporins and are usually not inhibited by clavulanic acid or tazobactam, having also high affinity

for aztreonam (34, 35, 37, 38, 39). Representative enzymes include CMY-1 (14), CMY-2 (13), FOX-1 (122), MIR-1 (174), MOX-1 (95) and LAT-1 (233), all from *Klebsiella pneumoniae,* P99 from *Enterobacter cloacae* (75), PDC-1 from *Pseudomonas aeruginosa* 202) and BIL-1 from *Escherichia coli* (72). In Gram-negative bacteria, one of the most important chromosomal cephalosporinases is the group of enzymes collectively known as AmpC β-lactamases (19, 123). Data suggests that the AmpC enzymes have evolved to deal with cephalosporins rather than for some other cellular function (33, 101), although there is evidence suggesting a close relationship between the induction of these enzymes and peptidoglycan recycling (33, 90, 100, 101, 123, 130, 252). This may imply an alternative metabolic function of the class C β-lactamases at the early stages of its evolution, possibly some physiological role in cell wall metabolism (130, 141). In this context, the hydrolysis of β-lactams should be considered an evolutionary response to the challenge by cephalosporins, in order to defend bacteria against these antimicrobial agents. The β-lactam substrate would have provided the selective pressure that drove the evolution of the class C enzymes (33).

Chromosome-mediated AmpC β-lactamases are often expressed as inducible enzymes in response to β-lactam exposure (19, 38, 100, 101, 123, 235). In Gram-positive bacteria, the presence of a β-lactam molecule at the external face of the cytoplasmic membrane is sensed by the cell, which transmits this signal to the intracellular regulatory components that control β-lactamase expression (19). In Gram-negative bacteria, the induction system is more complex, involving four regulatory genes, *ampR*, *ampD*, *ampG* and *ampE*, which constitute the *amp* operon, together with the structural gene, *ampC* (19, 100, 101, 123, 252). When the β-lactam inducer is present, it interacts with the PBPs of the bacterial cell, preventing the cross-linking step of cell wall biosynthesis (19, 223). This interaction causes peptidoglycan breakdown products (muropeptides) to accumulate in the periplasm (19, 252), which are captured and transported by the transmembrane permease AmpG to the cytoplasm, serving as the induction signal to activate the transcriptional regulator, AmpR. When on its active form, AmpR acts as an activator for *ampC* transcription, stimulating β-lactamase synthesis (19, 100, 101, 123, 141, 252). AmpD is a cytosolic amidase that down-regulates β-lactamase expression, maintaining AmpR in its repressor form (100, 101, 141, 252), possibly assisted by the cytoplasmic membrane protein, AmpE (19, 123, 252).

Most members of the *Enterobacteriaceae* (e.g. *Citrobacter freundii, E. cloacae*, *Serratia marcescens*, *Morganella morganii*, *Proteus mirabilis*, *Yersinia enterocolitica*) and *P. aeruginosa* possess a naturally-occurring, chromosome-mediated β-lactamase (11, 15, 29, 30, 38, 64, 84, 119, 123, 202, 203, 235, 245, 252). Such chromosomal *ampC* genes are usually expressed at low level, regulated by induction of β-lactams (19, 38, 100, 101, 123, 134) or by mutation of regulatory genes (123, 134). In other organisms, one or more components of the induction system are missing (38); *E. coli*, *Acinetobacter baumannii*, *Shigella dysentery* and *Shigella flexneri* lacks an *ampR* gene, so that their AmpC enzymes are noninducible (101, 123, 252). Chromosome-mediated AmpC β-lactamases can also be expressed at continuously high level (123); spontaneous mutations, deletions or insertions in regulatory genes, most frequently in *ampD* (134), results in constitutive hyperproduction of the enzyme and in increased resistance to third-generation cephalosporins (12, 64, 134, 202, 203). Such derepressed phenotypes can also be achieved by less common mutations in *ampR* locus (12, 101), which results in the

production of more active transcriptional regulator AmpR (12, 100). In addition, mutations in *ampC* gene can expand the substrate specificity of the AmpC β-lactamases, contributing as well to naturally-occurring resistance to extended-spectrum cephalosporins (12, 134). Another form of chromosomal β-lactamase expression is at continuously low level (123), due to least common mutations in *ampG* (100, 101). Inactivation of AmpG also impairs the peptidoglycan recycling process, because in addition to modified β-lactamase regulation, such mutants have lost the ability to recycle muropeptides (100).

Most class C β-lactamases are produced in Gram-negative bacteria as chromosomal enzymes (30, 168). Such enzymes may not contribute to clinical β-lactam resistance, since they are often expressed at low levels, but may cause severe therapeutic problems if their genes are translocated onto plasmids (119, 245). The ability of plasmids harbouring resistance genes to spread between bacterial cells by conjugation (167, 169) greatly enhances the nosocomial dissemination of resistant strains, which represents a serious challenge to the treatment of infections (39, 123, 217).

All plasmid-mediated β-lactamases, including the AmpC enzymes, are likely to have chromosomal origins (101, 123, 130, 134). Plasmid-encoded AmpC enzymes have been described worldwide since 1989 (38, 101, 123, 235), mainly in bacteria without or with incomplete chromosomal *ampC* gene, such as *Klebsiella* spp., *E. coli*, *P. mirabilis*, *Salmonella* spp. and some *Shigella* spp., but also in bacteria with complete chromosomal *ampC*, such as *E. cloacae*, *Acinetobacter* spp. and *P. aeruginosa* (123). Generally, plasmid-encoded *ampC* genes lack regulatory gene *ampR*, and expression seems to be at continuously high level and noninducible (123). The exceptions are the DHA-1 enzyme from *Salmonella enteritidis* (11), as well as DHA-2 (71) and ACT-1 (29) enzymes from *K. pneumoniae*, which are inducibly expressed because their *ampC* genes are linked to *ampR* genes (101, 123). In addition to resistance to cephamycins, a combination of high level production of the ACT-1 β-lactamase with the loss of a major outer membrane porin can confer imipenem resistance in *K. pneumoniae* (29, 176, 241). This development is of particular concern for the Public Health, since that carbapenems, such as imipenem and meropenem, have been the drugs of last-resort for Gram-negative bacteria (123).

Most AmpC-type β-lactamases, with the exception of plasmid-borne ACC-1 from *K. pneumoniae* (15), confer resistance to cephamycins (cefoxitin and cefotetan) and to a lesser extent, to oxyimino-β-lactams (or oxyimino-cephalosporins), such as cefotaxime, ceftazidime, cefuroxime and ceftriaxone, and to the monobactam aztreonam (119, 134, 202, 203). AmpC-overproducing strains, additionally, have demonstrated increased resistance to extended-spectrum cephalosporins, particularly oxyimino-cephalosporins (134). Recently, cephalosporinases with broadened substrate activity have been reported (101, 134, 202, 203); these enzymes confer reduced susceptibility to all cephalosporins, including fourth-generation cephalosporins cefepime and cefpirome (128), having been termed Extended-Spectrum AmpC (ESAC) β-lactamases (12, 38, 39, 101, 134, 202, 203). ESACs are structurally related to the cephalosporinases from group 1, as a result of amino acid substitutions, insertions or deletions (38, 101, 134), and were assigned into group 1e (38). They are very likely chromosomally-encoded enzymes (134), and include enzymes such as SRT-1 from *S. marcescens* (140), GC1 from *E. cloacae* (168), PDC-2 from *P. aeruginosa* (202) and ADC-33 from *A. baumannii* (203). Plasmid-mediated ESACs, such as CMY-10 from *Enterobacter aerogenes* (119), CMY-19 from

K. pneumoniae (235) and CMY-37 from *C. freundii* (3), have also been described. Along with other resistance mechanisms, such as AmpC overproduction, increased drug efflux and decrease or loss of outer membrane permeability, ESACs were described as also contributing to carbapenem resistance in *P. aeruginosa* isolates (101, 202). Spread of such an extended-substrate activity phenotype may potentially compromise the clinical utility of most β-lactams (134).

Group 2 / Class A β-lactamases

Group 2 enzymes, which include classes A and D, represent the largest group of β-lactamases (37, 38). Contrarily to group 1 cephalosporinases, group 2 enzymes tend to have more affinity to clavulanic acid than for aztreonam (34, 35). Subgroup 2a penicillinases are a small cluster of β-lactamases that belong to class A and are predominant in Gram-positive bacteria (34, 35, 38). These enzymes have a relatively narrow-spectrum hydrolytic activity, acting preferentially in benzylpenicillin and other penicillin derivates, over cephalosporins, carbapenems or monobactams (38). Cloxacillin is not an effective enzyme inhibitor (34, 35), as are clavulanic acid and tazobactam (37, 38, 39). Subgroup 2a penicillinases are mainly chromosomal (37, 38), such as BLA-1 from *Bacillus anthracis* (46) and LEN-1 from *K. pneumoniae* (9), but there are some plasmid-encoded enzymes (38), such as PC1 from *S. aureus* (132, 251) and NPS-1 from *P. aeruginosa* (129, 173).

Subgroup 2b β-lactamases are broad-spectrum enzymes belonging to class A (34, 35, 37, 38, 39, 130). They readily hydrolyze penicillins and first-generation cephalosporins, such as cephalothin, cefazolin and cephaloridine (30, 38, 39, 84), presenting low hydrolysis rates for extended-spectrum cephalosporins, aztreonam and imipenem (34, 35, 130, 176). These enzymes are strongly inhibited by clavulanic acid and tazobactam (34, 35, 37, 38, 39). The most common and widely distributed enzymes TEM-1, TEM-2 and SHV-1 are included in this subgroup (34, 35, 37, 38, 39). TEM-1 is the most frequently found β-lactamase in Gram-negative bacteria (30, 130, 132, 176), mainly in *E. coli*, *K. pneumoniae* and *P. mirabilis* (84). This enzyme is transposon- (81, 175) and plasmid-mediated (7, 30, 84, 116), which explains its widespread dissemination among bacteria (30). For instance, transposons carrying TEM-1 genes have been responsible for the plasmid-mediated ampicillin and penicillin resistance reported in *Haemophilus influenzae* (30, 62, 132) and *Neisseria gonorrhoeae* (30, 63, 132), respectively. The SHV-1 β-lactamase is the most prevalent chromosomal β-lactamase in *K. pneumoniae* (30, 84, 130, 176, 234), although is plasmid-mediated in other species (130), such as *E. coli* (30, 84).

Substitutions in one or more amino acids in classic subgroup 2b β-lactamases resulted in enzymes with an extended-spectrum phenotype (30, 130, 176, 234). These enzymes, known as Extended-Spectrum β-lactamases (ESBLs), are capable of hydrolyzing latest-generation oxyimino-cephalosporins, as well as the monobactam aztreonam (24, 30, 37, 38, 39, 130, 176, 177), at rates usually >10% that for benzylpenicillin (34, 35, 37, 38, 176), and were assigned into group 2be (24, 30, 37, 38, 39, 176). Being mostly mutants of subgroup 2b TEM-1, TEM-2 and SHV-1 (30, 38, 130, 177), subgroup 2be ESBLs are class A enzymes that retain hydrolytic activity against penicillins and early cephalosporins (30, 37, 38, 39, 176), while remaining susceptible to clavulanic acid and tazobactam (24, 30, 34, 35, 37, 38, 39, 130, 176). This is the main feature differentiating ESBLs and group 1

AmpC-type enzymes, which are also capable to hydrolyze oxyimino-cephalosporins, but are not susceptible to such β-lactamase inhibitors (176).

Additionally to the TEM- and SHV-type ESBLs, CTX-M-type enzymes have been described (24, 30, 38, 84, 176). These plasmid-mediated enzymes have apparently originated from chromosomal β-lactamase genes of environmental *Kluyvera* spp. (24, 30, 38, 106, 176, 201, 244) that have been captured by mobile genetic elements (MGEs) (177), such as insertion sequence IS*Ecp1* (106, 177, 218). CTX-M β-lactamases are actually the most prominent class A / subgroup 2be ESBLs (24, 86, 176, 177, 199, 218), given its high dissemination among bacteria and the number of outbreaks reported worldwide (24, 30, 84, 106, 176, 177). They hydrolyze preferentially cefotaxime over ceftazidime (24, 30, 38, 84, 106, 176, 201), being so known as cefotaximases (24, 201). Another distinctive property of these enzymes is that unlike TEM or SHV ESBLs, they are better inhibited by tazobactam than by clavulanic acid or sulbactam (24, 30, 38, 84, 176). CTX-M-15 is the most widely distributed CTX-M ESBL, which was first reported in *E. coli* isolates from India (47, 84, 106, 177, 217). Multidrug-resistant (MDR) CTX-M-15-producing *E. coli*, particularly strain ST131 O25:H4, is now emerging globally in community and hospital settings, constituting an important public health threat (39, 47, 177). Other examples of class A / 2be ESBLs, not closely related to TEM, SHV or CTX-M, include plasmid-mediated and integron-associated enzymes (24, 30, 38, 39, 84, 176), such as VEB-1 (156, 183), GES-1 (184), TLA-1 (214), SFO-1 (138), BES-1 (23), as well as the chromosomally-encoded enzymes PER-1 (164), PER-6 (80), RAHN-1 (18) and BEL-1 (189).

The “ESBL” terminology was originally associated with class A / subgroup 2be β-lactamases, mainly TEM and SHV derivatives, capable of hydrolyzing oxyimino-cephalosporins and susceptible to β-lactamase inhibitors, such as clavulanic acid (24, 30, 37, 38, 84, 176). This term was expanded to include not only the CTX-M and VEB enzymes, but also unusual class C / group 1e AmpC cephalosporinases with increased activity against cefepime, and a subset of class D / subgroup 2de OXA enzymes (described later), both with extended-spectrum phenotypes, but not meeting the definition of subgroup 2be, since they are not inhibited by clavulanic acid (38, 176). For the purpose of this dissertation, the term “ESBL” will refer to class A / subgroup 2be and class D / subgroup 2de enzymes, as previously suggested (176).

Clinical introduction of β-lactam / β-lactamase inhibitor (BBLI) combinations could result in the emergence of subgroup 2b mutants with acquired resistance to clavulanic acid and sulbactam, while retaining the broad-spectrum of activity of their parent enzymes (30, 38, 130, 132), but that remain susceptible to tazobactam (30). Such variants belong to subgroup 2br (37, 38) and were originally given the designation Inhibitory-Resistant TEM (IRT) β-lactamases, given that the first mutants were TEM-1 and TEM-2 derivatives (30, 130), such as TEM-30 (IRT-2) from *E. coli* (40). However, there are also inhibitory-resistant variants of SHV-1 (30), such as SHV-49 from *K. pneumoniae* (57). IRT β-lactamases represent an adaptive resistance mechanism specifically developed by bacteria to overcome the activity of β-lactamase inhibitors (161). The point mutations that lead to the inhibitory-resistant phenotype take place at a few specific amino acid residues (30, 109, 132) and are distinct from those that lead to the ESBL phenotype (30). However, concurrence of amino acid substitutions in bla_{TEM} genes of the ESBL and IRT variants could result in enzymes that combine resistance to

clavulanic acid with an extended-spectrum phenotype. They are known as Complex Mutant TEM (CMT) β-lactamases of subgroup 2ber (30, 38, 39, 41, 199), and include enzymes such as TEM-50 (CMT-1) (217) and TEM-158 (CMT-9) (200) from *E. coli* (217), TEM-68 (CMT-2) from *K. pneumoniae* (67) and TEM-121 (CMT-4) from *E. aerogenes* (187).

The remaining class A β-lactamases are comprised within subgroups 2c, 2ce, 2e and 2f (38). Subgroup 2c enzymes preferentially hydrolyze carbenicillin and ticarcillin (penicillins), at a rate >60% that for benzylpenicillin, with cloxacillin or oxacillin being hydrolyzed at a rate <50% that for benzylpenicillin (36, 37, 38). They are well inhibited by clavulanic acid and tazobactam (38), and include the chromosomal enzyme from *Moraxella catarrhalis* BRO-1 (26) and the plasmid-mediated enzymes from *P. aeruginosa*, PSE-1 (CARB-2) (98) and CARB-3 (115). Recently, a carbenicillinase with extended activity against the latest-generation cephalosporins cefepime and cefpirome, RTG-4 (CARB-10), was described in *A. baumannii*. This enzyme was assigned into subgroup 2ce (38).

Subgroup 2e β-lactamases are cephalosporinases, which can lead to be confused with group 1 AmpC enzymes. However, contrarily to group 1 cephalosporinases, subgroup 2e enzymes are inhibited by clavulanic acid and have low affinity for aztreonam (34, 36, 37, 38, 39, 176). Representative enzymes include chromosomal CepA from *Bacteroides fragilis* (204), L2 from *Stenotrophomonas maltophilia* (237), CME-1 from *Chryseobacterium meningosepticum* (205), and HugA from *Proteus penneri* (125), as well as plasmid-mediated FEC-1 from *E. coli* (138). Class A β-lactamases are still represented by the serine carbapenemases from subgroup 2f, carbapenem-hydrolyzing enzymes that are less inhibited by clavulanic acid than by tazobactam (37, 38, 39, 176, 193). Bacteria expressing these enzymes, such as *K. pneumoniae*, *E. cloacae* and *S. marcescens*, have reduced susceptibility to imipenem (193). Chromosomal enzymes, such as SME-1 (154), IMI-1 (196), NMC-A (153) and OXA-40 (91) have been described for this subgroup (37, 38, 166, 193), but the plasmid-encoded enzymes, particularly the KPC and GES families, are those that raises more clinical concerns (38, 39, 193). The KPC carbapenemases have been more often associated with nosocomial infections by MDR Gram-negative pathogens, and continue to spread worldwide (39, 193).

Group 2 / Class D β-lactamases

Similarly to class A and C β-lactamases, class D β-lactamases possess an active-site serine (4, 86, 176, 193), representing the most diverse enzymes among the four molecular classes (190). Subgroup 2d β-lactamases belong to class D and includes enzymes that preferentially hydrolyze oxacillin or cloxacillin (4, 30, 36, 37, 38, 39, 84, 86, 176, 190), at a rate >50% that for benzylpenicillin (37, 38, 176), but that may also efficiently hydrolyze carbenicillin (38). These oxacillinases, or OXA-type β-lactamases (OXAs), are poorly inhibited by clavulanic acid (4, 30, 37, 38, 39, 84, 190), but can be inhibited by sodium chloride (NaCl) (36, 38, 190). Many bacteria encode OXA enzymes in their chromosome, but most oxacillinase genes are part of gene cassettes within class 1 integrons (4, 10, 190). These integrons are frequently associated with plasmids or transposons (197), which facilitates the spread of OXA genes among bacteria (4, 193). Chromosomal enzymes include OXA-12 from *Aeromonas jandaei* (formerly *Aeromonas sobria*) (190, 195), OXA-13 (149) and OXA-20 (155) from *P. aeruginosa*, OXA-22 from *Ralstonia pickettii* (165), OXA-29 from *Legionella gormanii* (73), OXA-48

from *Burkholderia pseudomallei* (163) and OXA-61 from *Campylobacter jejuni* (4). Plasmid-mediated oxacillinases of clinical importance include OXA-1 from *E. coli* (172), OXA-2 from *S. typhimurium* (147) and OXA-10 (PSE-2) from *P. aeruginosa* (97, 197). OXA-type enzymes now comprise the second large family of β-lactamases, preceded by TEM and followed by SHV (38, 39).

Several subgroup 2d / class D β-lactamases have expanded its hydrolytic spectrum towards oxyimino-cephalosporins, while retaining the ability to hydrolyze oxacillin or cloxacillin. These enzymes are regarded as ESBLs (30, 38, 39, 84, 176, 190) and belong to subgroup 2de (38, 39). OXA-type 2de ESBLs are mostly OXA-10 derivatives by few amino acid substitutions and are predominant in *P. aeruginosa* (30, 38, 176, 190). They include chromosomally-encoded enzymes, such as OXA-18 (180), but also plasmid-mediated enzymes, such as OXA-11 (85) and OXA-15 (52).

Some OXA-type β-lactamases may also hydrolyze carbapenems (38, 190), but they are not able to combine such carbapenem-hydrolyzing activities with traditional ESBL hydrolytic profiles (190, 193), at least at a significant level (193). Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are allocated in subgroup 2df (38), being more frequent in *A. baumannii* (38, 190, 193). With exception of the clinically important enzymes from *A. baumannii*, OXA-23 (ARI-1) (56) and OXA-58 (188), which have been shown to be plasmid-encoded, the CHDLs described so far in this opportunistic pathogen are very likely chromosomally-located (38, 190, 193). Both these acquired enzymes, OXA-23 and OXA-58, have demonstrated to contribute significantly to carbapenem resistance (190, 193). Plasmid-mediated OXA-48 from *K. pneumoniae* (50, 185) is another clinically relevant CHDL (38, 39), which has demonstrated to be the enzyme with the highest known catalytic efficiency for imipenem (185, 190). Naturally-occurring CHDLs include enzymes such as OXA-24 (28), OXA-25 (2), OXA-26 (2), OXA-27 (2), OXA-51 (OXA-Ab1) (31) and OXA-69 (32), all from *A. baumannii*, OXA-54 from *Shewanella oneidensis* (186), OXA-62 from *Pandoraea pnomenus*a (212) and OXA-134 from *Acinetobacter lwoffii* (68).

Group 3 / Class B Metallo-β-lactamases

Group 3 MBLs belong to class B (34, 36, 37, 38, 39). These metalloenzymes are characterized by its ability to hydrolyze carbapenems, but most can also hydrolyze cephalosporins and penicillins (37, 38, 39, 132, 193, 239). They require a bivalent metal ion (Zn^{2+}) at the active-site for optimal enzymatic activity (34, 36, 76, 193, 239), and are distinguishably inhibited by EDTA and metal ion chelators, but not clavulanic acid or tazobactam (34, 36, 37, 38, 39, 132, 166, 176, 193, 239). MBLs are currently subdivided, based either on their structure (subclasses B1, B2, B3) (38, 39, 76, 193, 239) or function (subgroups 3a and 3b) (38, 39). The three structural subclasses are aligned within the subgroups, according to functional similarities (38). Subgroup 3a comprises subclasses B1 and B3 (38, 39), both requiring two bound zinc ions at the active-site for their similar broad-spectrum hydrolytic activity. In contrast, subclass B2 enzymes are inhibited if a second zinc ion is bound and have a narrow-spectrum of activity, preferentially hydrolyzing carbapenems (38, 39, 76, 193, 239). Subclass B2 is associated with subgroup 3b (38, 39).

Some bacteria from environmental niches carry ubiquitously MBLs, mostly chromosomal and inducible (39, 193, 239), which are probably associated with another cellular function that is yet to be entirely

elucidated (239). These enzymes are now recognized as the most important reservoir for these β-lactamase genes (157). Therefore, it is important to clarify the evolutionary relationships between the described β-lactamases, to understand how they evolved and became the specific β-lactam-hydrolyzing enzymes nowadays, and to identify the genetic sources associated with the emergence of putative new β-lactam-hydrolyzing enzymes. Indeed, the first MBLs identified and described were environmental, chromosomal enzymes produced by Gram-negative bacteria (38, 39, 166, 193, 239), such as CphA from *Aeromonas hydrophila* (subclass B2) (38, 137), L1 from *S. maltophilia* (subclass B3) (49) and CcrA (CifA) from *B. fragilis* (subclass B1) (194), and also by some Gram-positive bacteria (39, 193, 239), such as Bc-II from *Bacillus cereus* (subclass B1) (99, 114, 126). The latter was the first MBL for which an amino acid sequence was determined (99), having been described as a lipoprotein-like, membrane-bound enzyme (26, 99). Membrane-bound hydrophobic forms of Gram-positive β-lactamases were also described for *Bacillus licheniformis* and *S. aureus* and have been proposed to be the precursors of the periplasmatic enzymes (26, 162). Chromosomal metalloenzymes also include Sfh-1 from *Serratia fonticola* (subclass B2) (208), GOB-1 from *Chryseobacterium meningosepticum* (subclass B3) (16), IND-1 from *Chryseobacterium indologenes* (subclass B1) (17), FEZ-1 from *L. gormanii* (subclass B3) (27), CAU-1 from *Caulobacter crescentus* (subclass B3) (55), THIN-B from *Janthinobacterium lividum* (subclass B3) (206), JOHN-1 from *Flavobacterium johnsoniae* (subclass B1) (157), TUS-1 and MUS-1 from *Myroides* spp. (subclass B1) (133). The chromosomally-encoded MBLs were usually found in bacteria that also expressed at least one serine β-lactamase (39, 193, 239). For instance, *S. maltophilia* can hydrolyze third-generation cephalosporins (and carbapenems) due to coregulated overexpression of the L1 MBL and its chromosomal class A enzyme, L2 (239). With exception of the L1 MBL from S. *maltophilia*, bacteria producing chromosomal metalloenzymes have not been frequently associated with severe nosocomial infections, as they are generally opportunistic pathogens (193, 239), and also because chromosomal MBL genes are not easily transferred (193).

Contrarily to the chromosomal MBLs, whose presence depends directly on the prevalence of the producing species (193), the metalloenzymes encoded by transferable genes have disseminated globally (39) and are now widely spread among clinical pathogenic bacteria (193, 239). The most common transferable MBLs include the IMP, VIM and GIM families (38, 39, 193, 239). All these metalloenzymes are allocated in subgroup 3a (38, 39) and are found as gene cassettes in a variety of integron structures (193, 239), mostly from class 1 (239). Clinical important transferable MBLs include integron-borne enzymes, such as IMP-1 (117, 242), VIM-1 (118) and GIM-1 (44), and also plasmid-mediated SPM-1 (152, 232), all originally found in clinical isolates of *P. aeruginosa* (193, 239). Other relevant acquired MBLs includes the SIM-1 (120) enzyme, located within a class 1 integron from *A. baumannii* (120, 193), and also the plasmid-mediated KHM-1 enzyme from *C. freundii* (213).

Recently, a novel MBL was identified in a clinical isolate of *K. pneumoniae* (39, 145, 250). This metalloenzyme, NDM-1, was found to be carried on a large 180-kb transmissible genetic element encoding other resistance determinants, including the broad-spectrum β-lactamase CMY-4, genes inactivating erythromycin, ciprofloxacin, rifampicin and chloramphenicol, and genes encoding an efflux pump. This genetic element was able to spread rapidly among other enterobacterial strains and also

nonfermentative bacteria, conferring resistance to all antibiotics, except colistin (39, 145, 250). NDM-1 was originally described as being integron-borne (250), but is now carried by a variety of plasmids, which were responsible for its widespread dissemination worldwide (39). An *E. coli* isolate in Germany was reported as having the bla_{NDM-1} gene probably integrated into the chromosome, which could be related with the genetic structures surrounding that gene. In addition to the NDM-1 MBL, the same isolate coproduced the CTX-M-15 ESBL, as well as the enzymes TEM-1, OXA-1 and OXA-2 (39, 179).

Genetic Environment

The species definition for prokaryotes has advanced over the last years, following the genomic era (144). The current paradigm is that bacterial species are more accurately described by their pan-genome (142, 144), which is the sum of two components: a core genome consisting of genes ubiquitously found in all strains, and an accessory genome containing partially shared and strain-specific genes (20, 113, 142, 144, 215). The core cluster genes are highly conserved within the species and tend to remain stable throughout evolution, and horizontal gene transfer (HGT) is less likely to be detected (20, 53, 113). These genes typically encode proteins that are essential for the survival of the organism (215), which are mainly associated with housekeeping functions (e.g. transcriptional and translational apparatuses), the cell envelope, regulatory roles and transport of nucleotides and amino acid (144). A considerable fraction of the core genome consists in hypothetical proteins or open reading frames (ORFs) with no known function (53, 54, 215), but whose presence (i.e. conservation) suggests that they may have an important function (53). The components of the core genome can be particularly useful for phylogenetic inference, i.e. for clarifying the evolutionary history of clonal lineages through time (144).

Unlike the core genome, the accessory (or adaptive) genome consists of genes that vary among strains within a species (20, 113, 142, 144, 215). These genes typically encode proteins that are responsible for adaptation to specific niches, hosts or environments, reflecting specific phenotypes that are advantageous under certain selective conditions (113, 144, 215). The bacterial pan-genome evolves mainly through HGT of elements of the accessory genome, along with mutations, deletions and rearrangements within the core genome (113). Since gene acquisition through HGT often results in biological fitness cost, the acquired traits must confer an advantage sufficient to overcome not only inactivation by mutation, but also elimination by segregation (113, 169). The accessory gene pool may include genes encoding virulence factors that promote bacterial persistence in several host species, as well as genes encoding resistance to multiple classes of antibiotics, which are closely associated with MGEs (113, 144, 215). The most common MGEs for HGT between bacteria are plasmids, genomic islands and bacteriophages, which are relatively complex, usually encoding regulatory and structural mechanisms for replication and transfer. MGEs carrying accessory genes can become chromosomally-integrated; they can carry smaller and simpler insertion sequences (IS), transposons and integrons, which can facilitate genome rearrangements, gene duplications and deletions, and capture of new genes (113, 144, 169, 215).

Although bacteria have intrinsic resistance mechanisms encoded within their core genome, such as multidrug efflux pumps and naturally-occurring β-lactamases, is the dissemination of antibiotic

resistance by horizontally-transmitted elements of the accessory genome which causes the greatest threat (29, 39, 113, 176). The MGEs on which such elements are carried can disseminate among both clinical and community isolates, creating the opportunity for the rapid emergence of MDR bacterial clones. Strains harbouring these MGEs are reservoirs for antibiotic resistance genes, and their further spread could probably limit future therapeutic options (39, 113).

Functional Pleiotropy

In contrast to long-held concepts whereby single genes encoded single functions, most genes are now recognized to have multiple qualitatively distinct functions, a phenomenon known as pleiotropy (or pleiotropism). Pleiotropy occurs when a single locus influences multiple (and apparently unrelated) phenotypic traits (93, 255). It is not yet clear whether selection pressures have substantially shaped the distribution of pleiotropic effects among genes, and what mechanisms account for the observed variability in pleiotropy. Nevertheless, pleiotropy is thought to increase as organisms face new selective challenges (e.g. antibiotics), with the existing genes providing the raw material from which evolution molds additional functions (78). A single mutation in a pleiotropic gene can simultaneously affect some or all traits, which may have opposite effects on fitness. Such antagonistic pleiotropic effects mean that some mutations may benefit the organism at least in one phenotypic trait, but at least one is detrimental to its fitness (93, 221, 225). Depending on how the functionality of the different traits affects fitness, the genes become pleiotropic or specialized on a single function. However, specializing on the trait that is currently more important to fitness detracts from allocation to the second function (78). Adaptive mutations, such as increased resistance to a particular antibiotic, may benefit the organism in the presence of the antimicrobial agent, but it may be deleterious in its absence, or reduce the affinity of the organism for its natural substrates. However, such pleiotropic fitness costs can be minimized by cost-compensatory mutations (79, 89, 170, 199), or by acquisition of other resistance determinants (199). Recent work has demonstrated antagonist pleiotropy in CTX-M mutants with an inhibitory resistant (IR) phenotype, selected under exposure to increasing concentrations of BBLI combinations. Three IR-CTX-M variants were detected, having mutations associated with different resistance patterns. The most frequent mutation, Ser130Gly, conferred a high level of resistance to BBLIs, while providing an antagonistic pleiotropic effect by simultaneously decreasing cephalosporin resistance. However, a double mutation on synthetically constructed CTX-M-10 enzyme, Ser130Gly / Lys169Ser, acted as a compensatory mutation, having partially restored the resistance against cephalosporins (199).

No natural CTX-M β-lactamase has been found to confer resistance to BBLIs (41, 199), although the Ser130Gly mutation has been previously described in non-CTX-M clinical isolates, such as TEM-76 (121, 199). Additionally, CMT variants, which retain activity on both BBLIs and cephalosporins, have also been described in clinical isolates (30, 38, 39, 41, 199). However, the evolution towards a complex mutant phenotype within the CTX-M cluster is unlikely, since pleiotropy is expected to generate fitness cost, i.e. increased fitness following exposure to extended-spectrum cephalosporins will certainly reduced fitness when in contact with the β-lactam inhibitor (199). Nevertheless, double resistances might emerge as a consequence of the selection of compensatory mutations in the

bla$_{CTX-M}$ gene or by coincidental acquisition via HGT of CTX-M enzymes and other β-lactamases providing inhibitor resistance (199). An example is the BBLI resistance in CTX-M-15 producers, which can be associated with the coproduction of OXA-1 β-lactamase (47, 199) or porin loss (199, 241).

As mentioned earlier, sequence homologies have lead to the assumption that the chromosomal β-lactamases may have evolved from the PBPs. Whether β-lactamase evolution is a product of physic structural changes or functional pleiotropism, it is widely accepted that it involves specific amino acid replacements (i.e. mutations). Since pleiotropy appears to be a general property of missense mutations, it should play a major role in β-lactamase evolution (243), although it is not always easy to differentiate close physical linkage of two distinct genes and genuine pleiotropy (225).

Phylogenetic Inference

Phylogenetic analysis of DNA or protein sequences has become an important tool for studying and reconstructing the evolutionary history of organisms. It not only allows one to clarify the phylogenetic relationships between species, but may also provide a way to identify the specific proteins or genes that can best explain the differences species or groups of species, according to the derived phylogenetic tree (54).

The recent advances in genome sequencing technology, following the genomic era, have generated a vast amount of genetic data, however with little or no determined functional information (60, 61, 86). Phylogenetic methods have provided the practical tools to predict gene function based on gene sequence (60, 86). Most of these functional prediction methods are based on the identification and analysis of sequence similarities that are thought to be due to homology, i.e. inherited from a common ancestor (60, 240). The inference of homology is crucial in identifying conserved functions and pathways, as functionally related proteins often share sequence similarity as conserved sequence motifs (1). However, the identification of homologies is not always sufficient to make specific functional predictions, since sequence similarity due to homology does not ensure identical functions (60) (e.g. PBPs and the β-lactamases). Gene duplication, for example, can result in homologs with divergent functions; such genes are called paralogs. If the homologous genes diverged from each other after speciation events, they are called orthologs (60, 82, 240). These genes tend to have similar function and they are of great utility in comparative analysis and in species phylogeny (74, 82). Additionally, homologous genes that have diverged from each other after being acquired through HGT (e.g. resistance genes in bacteria) are called xenologs (60, 82, 111, 240). Dividing genes according the different types of homology can improve the accuracy of functional predictions of uncharacterized genes (60). However, is not always easy to distinguish between orthologs and paralogs, particularly when there are many copies of duplicate genes in the genome (74).

Phylogenetic analysis involves primarily the identification of homologous sequences (nucleotide or amino acid) to the sequence of interest, through database searches (60, 240). The Basic Local Alignment Search Tool (BLAST) is particularly useful in finding regions of local similarity between sequences. This simple and robust algorithm compares nucleotide and amino acid sequences to the sequences in the public databases, and estimates the statistical significance of the matches found (5). The query sequences are then subjected to multiple sequence alignment (MSA), whose analysis is

particularly useful to identify some biologically relevant similarities, such as patterns or motifs preserved by evolution that play an important role in the structure and function of a group of related proteins (127). When coupled with experimental data and results of database searches, these motifs constitute a very powerful means of characterizing sequences of unknown function. A number of tools are available for protein sequence classification and comparison. The InterPro database (150) is a repository of collected and annotated protein signatures for identifying protein families and domains. Query protein sequences can be searched using the InterProScan software to identify signatures from the InterPro member databases (PROSITE, PRINTS, ProDom, SMART, TIGRFAMs) (151). The ability to compile results from different protein signature databases makes InterProScan the software of choice for protein function annotation (107).

Progressive alignment constitutes one of the simplest and most effective ways of multiply aligning a set of sequences, combining speed and simplicity with reasonable sensitivity (43, 48). ClustalW (231) and MUSCLE (58) are two popular programs for MSA assembly using this progressive approach. Progressive sequence alignment algorithms start by performing all possible pairwise alignments, starting with the most similar (closely related) pair and progressing to the most distantly related. All pairs of sequences are aligned separately in order to calculate a distance matrix, from which a guide tree representing the relationships between the sequences is generated. This guide tree is produced by a clustering method (e.g. Neighbour-Joining; UPGMA) and gives the order by which the sequences are progressively added to the growing MSA, until a global alignment is achieved (43, 58, 231).

MSAs generate a concise and information-rich summary of sequence data, which is very important for the construction of the phylogenetic tree. The information in the calculated tree can then be used to infer likely function of the sequence of interest (60, 158). The inference of phylogenies may be affected by the statistical methods applied for data analysis. Three methods – distance, maximum parsimony and maximum likehood – are commonly used for reconstructing phylogenetic trees (60, 148, 158). These methods differ essentially by how they handle the molecular data and by the approach taken when building trees (148). Distance methods comprise a large family of methods that determines the estimated evolutionary distance between each pair of sequences and then uses the calculated pairwise distances (dissimilarities) matrix to generate a tree that predicts the observed set of distances as accurately as possible (60, 158). The unweighted pair-group method using arithmetic averages (UPGMA) (220) is the simplest within this category. This clustering algorithm assumes a constant rate of evolution, and therefore it is generally not considered suitable for phylogenetic inference. For instance, if the evolutionary rate is not the same on different branches, two distant sequences may be joined simply because they are similar in not having changed. Nevertheless, UPGMA may prove useful as a quick guide to identify sequence similarities (148, 158). Least squares (LS) methods (45, 69) outcome this limitation by allowing different rates of nucleotide substitution for different branches of the inferred tree. This group of methods is characterized by being capable of measuring the discrepancy between the observed and predicted distances (158). Similarly to UPGMA, Neighbour-Joining (NJ) (210) is also a clustering method. This method is based on the principle of minimum evolution (ME), by which the best tree is the one with the smallest sum of branch length estimates (*S*). Following this criterion, the NJ algorithm computes the *S* values for different tree

topologies (i.e. branching patterns), comparing them and finally returning the tree with the smallest S value (158, 207). Although the NJ method is fast, simple and particularly useful when handling large databases (148), it has the disadvantage of returning only the single best solution out of several possible trees, instead of generating several trees with possible correct topologies (158).

Character-based methods, such as maximum parsimony and maximum likehood, are regarded as being more powerful than distance methods, because they use raw information rather than sequence information (158). Indeed, using the original sequence data can potentially minimize the loss of some information (148). Maximum parsimony (MP) (70) is a family of methods that seeks to identify the phylogenetic tree requiring the fewest number of nucleotide or amino acid substitutions over evolutionary time to explain a given topology (60, 148, 158). The most parsimonious tree is the one that minimizes the total tree length, i.e. the sum of the number of changes in each position. However, this does not mean that it always is the correct one (158). Although MP is quite efficient in obtaining the correct tree topology, it may also result in nonconforming trees if the rate of nucleotide substitution is fairly constant (227). Additionally, sequence similarity on long branches could mean independent nucleotide substitutions, rather than a close relationship between the sequences. Nevertheless, MP is considered a reliable method for analysis of closely related sequences (158).

The maximum likelihood (ML) approach (65) is particularly useful when there is greater variability among sequences (148), although because it uses all sequence information and requires search of all possible combinations of tree topology and branch length, it is extremely slow and computationally expensive (65, 158). ML methods have the advantage of including probability evolutionary models of nucleotide or amino acid substitution to explain the variation between the sequences (60, 65, 148, 158). For a given set of sequence data and based on the selected evolutionary model, the ML algorithm chooses parameter values of the model that produce a distribution that maximizes the probability of observing that data (i.e. the likelihood). This procedure is repeated for all possible tree topologies, and the topology that shows the highest likelihood is chosen as the final tree (65, 158).

Bootstrap confidence tests (59, 66) are the most widely used methods for estimating the reliability of an inferred phylogenetic tree. The original dataset is randomly sampled with replacement in order to generate pseudo-replicate datasets of the same size. Bootstrap trees are computed for each of these replicates. The frequency with which a given branch is found is recorded as the bootstrap proportion, and it can be used as a measure of the reliability of individual branches in the original tree. Essentially, bootstrapping is used to examine how often a particular cluster in a given tree appears when the nucleotides or aminoacids are re-sampled (158). Despite their simplicity, bootstrap tests can be very time-consuming. This is not a problem for fast analysis such as NJ, but can be prohibitive when using ML methods.

OBJECTIVES

The present study will aim to clarify the evolutionary relationships between the β-lactamases, and to understand how they became the specific β-lactam-hydrolyzing enzymes nowadays.

Amino acid sequences from already described β-lactamases will be aligned and subjected to phylogenetic analysis, followed by the search for putative orthologs. Functional predictions based on the detection of putative conserved domains will be performed for all sequences. Finally, to infer the magnitude of selective pressure acting on protein-coding genes, K_a/K_s ratios for β-lactamase nucleotide sequences are to be estimated. Positive selection is an indicator that encoded proteins may have undergone an adaptive changed function.

This study will be important to clarify if the β-lactam-hydrolyzing activity represents functional pleiotropy associated with the challenge by β-lactam antibiotics, or if is truly the core function of the β-lactamases. Understanding the main function of the β-lactamases, and possible alternative metabolic functions, could help identify the genetic sources associated with the emergence of putative new β-lactam-hydrolyzing enzymes.

METHODS

Data

Nucleotide sequences of 61 chromosomally-encoded β-lactamase genes, available at the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/), were used in this study. The criterion for the selection of these sequences was to gather representative enzymes from each molecular class and functional group, according to the classification schemes currently in use. These sequences are listed in Table 2.

Table 2: Sequences used to construct the phylogeny of the β-lactamases.

Enzyme name	Organism	Functional group (Bush-Jacoby-Medeiros classification)	Molecular class (Ambler classification)	GenBank accession number
AmpC-K12	*Escherichia coli*	1	C	J01611
AmpC-P99	*Enterobacter cloacae*	1	C	X07274
AmpC-SLM01	*Morganella morganii*	1	C	Y10283
AmpC-SR50	*Serratia marcescens*	1	C	X52964
PDC-1	*Pseudomonas aeruginosa*	1	C	FJ666065
AmpC-CHE	*Enterobacter cloacae*	1e	C	AJ278994
AmpC-Ear2	*Enterobacter aerogenes*	1e	C	AJ544162
CMY-37	*Citrobacter freundii*	1e	C	AB280919
GC1	*Enterobacter cloacae*	1e	C	D44479
PDC-2	*Pseudomonas aeruginosa*	1e	C	FJ666064
SRT-1	*Serratia marcescens*	1e	C	AB008454
BLA1	*Bacillus anthracis*	2a	A	AY453161
LEN-1	*Klebsiella pneumoniae*	2a	A	X04515
SHV-1	*Klebsiella pneumoniae*	2b	A	HM751102
SHV-11	*Klebsiella pneumoniae*	2b	A	AY528717
BEL-1	*Pseudomonas aeruginosa*	2be	A	GU250441
CTX-M-3	*Kluyvera ascorbata*	2be	A	AJ632119
PER-1	*Pseudomonas aeruginosa*	2be	A	AJ621265
PER-6	*Aeromonas allosaccharophila*	2be	A	GQ396303
RAHN-1	*Rahnella aquatilis*	2be	A	AF338038
BRO-1	*Moraxella catarrhalis*	2c	A	U49269
OXA-12	*Aeromonas sobria*	2d	D	U10251
OXA-13	*Pseudomonas aeruginosa*	2d	D	U59183
OXA-20	*Pseudomonas aeruginosa*	2d	D	EU503121
OXA-22	*Ralstonia pickettii*	2d	D	AF064820
OXA-29	*Legionella gormanii*	2d	D	AJ400619
OXA-42	*Burkholderia pseudomallei*	2d	D	AJ488302
OXA-61	*Campylobacter jejuni*	2d	D	AY587956
OXA-18	*Pseudomonas aeruginosa*	2de	D	U85514
OXA-24	*Acinetobacter baumannii*	2df	D	AJ239129
OXA-25	*Acinetobacter baumannii*	2df	D	AF201826
OXA-26	*Acinetobacter baumannii*	2df	D	AF201827
OXA-27	*Acinetobacter baumannii*	2df	D	AF201828
OXA-51	*Acinetobacter baumannii*	2df	D	AJ309734
OXA-54	*Shewanella oneidensis*	2df	D	AY500137
OXA-62	*Pandoraea pnomenusa*	2df	D	AY423074
OXA-69	*Acinetobacter baumannii*	2df	D	AY750911
OXA-134a	*Acinetobacter lwoffii*	2df	D	HQ122933
CepA	*Bacteroides fragilis*	2e	A	L13472
HugA	*Proteus penneri*	2e	A	AF324468
L2	*Stenotrophomonas maltophilia*	2e	A	EF126084
CME-1	*Chryseobacterium meningosepticum*	2e	A	AJ006275
IMI-1	*Enterobacter cloacae*	2f	A	U50278
NMC-A	*Enterobacter cloacae*	2f	A	Z21956
OXA-40	*Acinetobacter baumannii*	2f	A	AF509241

Table 2 (cont.):

Enzyme name	Organism	Functional group (Bush-Jacoby-Medeiros classification)	Molecular class (Ambler classification)	GenBank accession number
SME-1	*Serratia marcescens*	2f	A	Z28968
Bc-II	*Bacillus cereus*	3a	B1	M11189
CcrA	*Bacteroides fragilis*	3a	B1	M63556
IND-1	*Chryseobacterium indologenes*	3a	B1	AF099139
JOHN-1	*Flavobacterium johnsoniae*	3a	B1	AY028464
MUS-1	*Myroides odoratimimus*	3a	B1	AF441286
TUS-1	*Myroides odoratus*	3a	B1	AF441287
CAU-1	*Caulobacter crescentus*	3a	B3	AJ308331
GOB-1	*Chryseobacterium meningosepticum*	3a	B3	EF394442
FEZ-1	*Legionella gormanii*	3a	B3	Y17896
L1	*Stenotrophomonas maltophilia*	3a	B3	JF705928
Mbl1b	*Caulobacter crescentus*	3a	B3	AJ315850
THIN-B	*Janthinobacterium lividum*	3a	B3	AJ250876
CphA	*Aeromonas hydrophila*	3b	B2	AY261379
ImiS	*Aeromonas veronii*	3b	B2	Y10415
Sfh-1	*Serratia fonticola*	3b	B2	AF197943

Alignments

All sequence alignments and later phylogenetic reconstructions were carried out with Molecular Evolutionary Genetics Analysis (MEGA) v5.05 (229). This software is available free of charge at http://www.megasoftware.net/.

The amino acid sequences derived from the genes in Table 2 were aligned by the MUSCLE program (58) embedded within MEGA, using default parameters. This clustering algorithm returns faster and more accurate results than ClustalW, particularly when using amino acid sequences (158). Because the sequences were too dissimilar in length, poorly aligned regions from the constructed MSA were automatically removed with trimAl v1.3 (42). This program reads all columns in an alignment and computes a consistency score (S_c) from which the alignment with best consistency is chosen, and then trimmed to remove columns that are less conserved. Trimming allows that all sequences have the same length, spanning the entire alignment, which increases the quality of subsequent phylogenetic analysis. The trimming tool was ran with the option “automated1”, which implements an heuristic method that improves running time and decides the most appropriate mode for the automated selection of parameters, depending on the alignment characteristics. The online version of trimAl is available at http://phylemon2.bioinfo.cipf.es/utilities.html.

Phylogenetic Analysis

Phylogenies were reconstructed by maximum parsimony (MP) (70), a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given topology. The topology with the minimum tree length, i.e., that requires the smallest number of substitutions, is chosen to be the best tree (the most parsimonious tree) (60, 148, 158). The reliability of the inferred trees was estimated by the bootstrap test (59, 66), which was replicated 100 times for MP. A 70% bootstrap value cut-off was used, so that the derived condensed tree emphasizes reliable branching patterns.

Defining Orthologs

Representative enzymes from each identified cluster were scanned for putative orthologs using BLAST on Orthologous Groups (BLASTO) (253), a modified BLAST tool for searching orthologous group data. BLASTO performs standard protein-protein BLAST search (BLASTP) against the NCBI Clusters of Orthologous Groups (COG) database for unicellular organisms (230). The COG collection is built upon all-against-all protein sequence comparison in complete genomes using Gapped BLAST (6), which allows the identification of orthologs as Reciprocal Best Hits (RBH). If the protein products of at least three genes residing in distinct genomes are symmetrical best hits in each reciprocal all-against-all BLAST search (more than to any other proteins from the same genomes), they probably belong to an orthologous family (146, 230). These triangles are merged into "COGs", and then case-by-case analysis of each COG is performed, aiming to eliminate false positives (124, 230).

To prevent hidden parologies, we imposed an Expect value (*E*-value) cut-off of 1×10^{-5} on each BLASTO search. In addition, all hits referred to as β-lactamases (redundancy) were excluded. Filtering out redundancy and putative parology as often as possible can improve functional predictions, since paralogous genes are much more likely to have diverged functionally than orthologous genes, which tend to conserve protein function (53, 253). BLASTO tool is accessible online at http://oxytricha.princeton.edu/BlastO/.

Functional Annotation

Representative enzymes of each cluster and the identified putative orthologs were annotated using InterProScan (151), a tool that combines different protein signature recognition methods from the InterPro consortium (150) member databases into one resource. InterProScan is available at (http://www.ebi.ac.uk/Tools/pfa/iprscan/). To enhance the accuracy of functional predictions, we used the NCBI Conserved Domain Database (CDD) (135), a resource that provides annotation of domain footprints and conserved functional sites on protein sequences. CDD consists in a collection of multiple sequence alignments representing protein domains conserved in molecular evolution. Domain alignments in CDD are available as Position-Specific Score Matrices (PSSMs) for database searching. A PSSM is an alignment scoring matrix that provides substitution scores for each position in a protein sequence; a consensus sequence for each conserved domain is calculated, reporting the most frequent residues in aligned columns. Pre-calculated PSSMs within the CDD can be queried with protein sequences via the CD-Search interface (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), which uses Reverse-Position-Specific BLAST (RPS-BLAST) (135) for the rapid identification of conserved domains.

Estimation of Positive Selection

Protein-encoding nucleotide sequences were obtained from the NCBI database server and aligned with ClustalW (231), using default parameters. Before the alignment, all sequences were put in frame, so that every sequence began with the start codon ATG and ended with a stop codon (TAA, TAG or TGA). The ratio of the number of nonsynonymous nucleotide substitutions (d_N) per nonsynonymous

site (K_a) to the number of synonymous nucleotide substitutions (d_S) per synonymous site (K_s) was estimated for the resulting MSA, using the free online server K_a/K_s calculation tool available at http://services.cbu.uib.no/tools/kaks. The K_a/K_s ratio is an important means of understanding mechanisms of molecular evolution (158), and may provide information about the degree of selective pressure acting on a protein-coding gene. A K_a/K_s ratio >1 is taken as evidence of positive or Darwinian selection, while a K_a/K_s ratio <1 is taken as evidence of purifying selection (247).

RESULTS AND DISCUSSION

The evolutionary relationships of DNA or protein sequences can be inferred from reconstructed phylogenetic trees. Phylogenetic inference can also be applied for predicting functions of uncharacterized genes. However, because similarity due to homology does not ensure identical functions (pleiotropic effect), the inference of homology, although crucial, is not always sufficient to make accurate functional predictions. Functionally related proteins often share sequence similarity as conserved sequence regions. In molecular studies, built MSAs are often used to identify such conserved motifs across a group of sequences hypothesized to be evolutionarily related. These conserved sequence motifs can be used in conjunction with structural and mechanistic information to identify putative functional domains associated to specific catalytic activities (e.g. β-lactam-hydrolyzing activity). Additionally, proximity of such motifs to the active-site indicates that they are either structurally or functionally important for the enzyme catalytic activity. Because these are frequently used terms in this dissertation, we felt important to differentiate domains from motifs. Protein domains are conserved parts of a given protein sequence and structure that can evolve, function, and exist independently of the rest of the polypeptide chain. Each domain forms a compact three-dimensional structure, and often can be independently stable and folded. In turn, motifs are simple combinations of secondary structure (supersecondary structures), formed by consecutive sequences of primary structure. However, there are many protein sequences of which the three-dimensional structures are not known for certain, although a consensus in primary structure implies a similarity in tertiary structure. In this cases the term “motif” strictly applies to primary structure rather than supersecondary or tertiary structure.

In this study, we began by reconstructing the evolutionary relationships among 61 sequences experimentally identified as β-lactam-hydrolyzing enzymes (Table 2), according to the criterion mentioned in the methods section. Considering that the early β-lactamases were probably chromosomal and inducible enzymes associated with important regulatory roles in the cell (e.g. AmpC in peptidoglycan recycling), this raises the hypothesis that their genes remain highly conserved within the core genome. Taking this into account, and because components of the core cluster can be particularly useful for phylogenetic inference (144), we chose to conduct this study only with chromosomally-encoded enzymes.

The evolutionary relationships between the β-lactamases were represented as phylogenetic trees, using the MP approach. MP methods have the advantage of being relatively free from various assumptions that are required for nucleotide (or amino acid) substitution in other tree-building

methods, such as ML. MP is strictly a method for choosing among topologies, whereas ML estimation incorporates an explicit model of sequence evolution; this model accounts for multiple changes at a single position, so ML simultaneously estimates both branch lengths and phylogenetic tree topologies. For this reason, ML gives more reliable branch length estimates than MP. However, since any mathematical model is only a rough approximation of reality, model-free approaches such as MP may give more reliable trees than other methods, particularly when the extent of sequence divergence is low (158). Furthermore, there is evidence supporting that gene sequences evolve heterogeneously over time and are not identically distributed, a scenario for which ML can become strongly biased and statistically inconsistent (110).

Constructing a consensus tree from the replicate bootstrap trees and use it as a new inferred tree has been previously suggested for MP methods (66). This is partially because MP methods often produce several equally parsimonious trees, which can lead to wrong assumptions in what is the correct (most parsimonious) tree (158). Since the consensus tree is an "average" tree of many bootstrap trees, one could consider that it may be more reliable than the original one. However, there is no data supporting this hypothesis. For these reasons, it was chosen not to present consensus tree(s) for MP.

The robustness of tree topologies was evaluated by bootstrap. The bootstrap proportion is not a measure of how accurate the inferred tree is, it only provides information about the stability of the tree topology (the branching order), and it helps assess whether the sequence data is suitable to validate that topology (92). It is known to be a conservative measure, so a bootstrap proportion of 90% gives more than 90% confidence in that branch. There is no consensus about the significance of bootstrap frequencies, although proportions equal or higher than 70-75% are accepted as reliable (92, 246). Furthermore, bootstrap proportions of $\geq$70% usually correspond to a probability of $\geq$95% that the corresponding branch is real (92). Because lower cut-offs include more topologies with lower support (88), all interior branches with no statistical significance lengths (bootstrap proportions $<$70%) were eliminated. A condensed tree of 61 chromosomal β-lactamase amino acid sequences (Table 2) is presented in Figure 2. This tree is unrooted, showing all possible relationships of the included sequences, but lacking any statement on relative times of divergence. The frequency with which a given branch is found is recorded as the bootstrap proportion, and it can be used as a measure of the reliability of individual branches in the original tree.

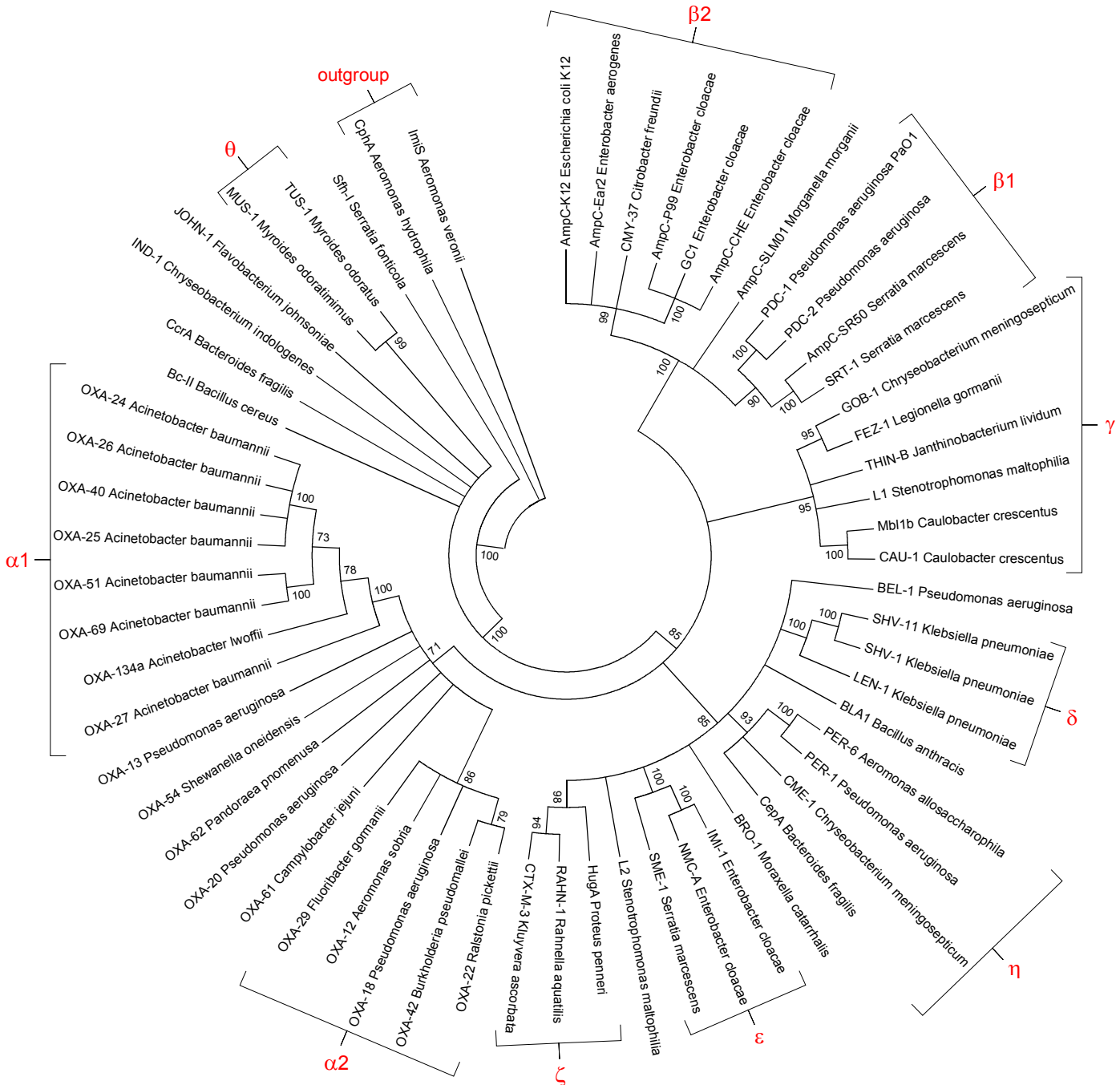

Fig. 2: Maximum parsimony (MP) analysis of β-lactamase sequences. The evolutionary history was inferred using the MP method (70). Tree #1 out of 8 most parsimonious trees is shown. The MP tree was obtained using the Close-Neighbour-Interchange algorithm (158) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 61 amino acid sequences, aligned with MUSCLE (58). All ambiguous positions were removed for each sequence pair. There were a total of 432 positions in the final dataset. Evolutionary analysis was conducted with MEGA v5.05 (229). Sequences are grouped within clusters (delimited by braces), which are identified with a specific letter from the Greek alphabet (in red). Enzyme name is followed by the name of the source organism. Numbers in figure represent the respective bootstrap proportions (for 100 replicates).

Based on visual inspection of the MP tree presented in Figure 2, β-lactamase sequences were organized into clusters. By "clustering", we mean organizing β-lactamase sequences into groups, in order to represent some measure of distance between them. These groupings were correlated with the molecular and functional classification currently in use for these enzymes. This information is summarized on Table 3.

Table 3: Clustering of the β-lactamases based on MP analysis (Fig. 2) and association with the current classification schemes.

Cluster	Sub-cluster	Enzyme Name	Functional group (Bush-Jacoby-Medeiros classification)	Molecular class (Ambler classification)
α	α1	OXA-27	2df	D
		OXA-134a	2df	D
		OXA-69	2df	D
		OXA-51	2df	D
		OXA-25	2df	D
		OXA-40	2f	A
		OXA-26	2df	D
		OXA-24	2df	D
	α2	OXA-22	2d	D
		OXA-42	2d	D
		OXA-18	2de	D
		OXA-12	2d	D
		OXA-29	2d	D
		OXA-12	2d	D
β	β1	PDC-1	1	C
		PDC-2	1e	C
		AmpC-SR50	1	C
		SRT-1	1e	C
	β2	AmpC-K12	1	C
		AmpC-Ear2	1e	C
		CMY-37	1e	C
		AmpC-P99	1	C
		GC1	1e	C
		Amp-CHE	1e	C
γ		GOB-1	3a	B3
		FEZ-1	3a	B3
		THIN-B	3a	B3
		L1	3a	B3
		Mbl1b	3a	B3
		CAU-1	3a	B3
δ		SHV-11	2b	A
		SHV-1	2b	A
		LEN-1	2a	A
ε		IMI-1	2f	A
		NMC-A	2f	A
		SME-1	2f	A
ζ		HugA	2e	A
		RAHN-1	2be	A
		CTX-M-3	2be	A
η		PER-6	2be	A
		PER-1	2be	A
		CME-1	2e	A
		CepA	2e	A
θ		MUS-1	3a	B1
		TUS-1	3a	B1

It is possible to define eight main clusters (α – θ) in the presented condensed tree (Fig.2), and such phylogenetic groupings are consistent with the current classification schemes for these β-lactamases. These enzymes present several degrees of amino acid identity between them, as it will be discussed, but their close structural relationship does not necessarily mean they are functionally related. Two enzymes may have low amino acid identity, but if they share conserved functional domains, a common fold, or similar active-site architecture, they probably have similar functions. Indeed, in most cases, enzymes that have converged on similar function have low sequence similarity, as well as a quite distinct protein folds. They would not have diverged from a common ancestor, but instead arisen independently and converged on the same active-site configuration as a result of natural selection for a particular biochemical function. Conversely, there are enzymes with very different biochemical functions, but which nevertheless have very similar three-dimensional structures and enough sequence identity to imply homology. Additionally, the different selection pressures acting on different regions within a protein-coding sequence can change amino acids. If such changes include functionally important residues (e.g., those in the active-site of an enzyme), this can ultimately change the enzyme function. Thus, determination of function from sequence and structure is complicated by the fact that enzymes of similar structure may not have the same function, even when they are evolutionary related (178).

Cluster α comprises enzymes currently classified as oxacillinases. With the exception of OXA-40, a class A / subgroup 2f carbapenem-hydrolyzing oxacillinase from *A. baumannii* with reduced susceptibility to imipenem, all enzymes in sub-cluster α1 belong to class D / subgroup 2df. The fact all these enzymes are encoded in the genome of the same organism, and that OXA-40 differs from OXA-24, -25 and -26 by only one or two amino acid changes (91), may explain the inclusion of OXA-40 in this sub-cluster. With the exception of OXA-18 from *P. aeruginosa*, a unique class D enzyme capable of hydrolyzing latest-generation oxyimino-cephalosporins and which is poorly inhibited by clavulanic acid, sub-cluster α2 includes oxacillinases from class D / subgroup 2d. This ESBL from subgroup 2de has 42% of identity with OXA-12 (180), which may justify their grouping in sub-cluster α2, along with other known class D enzymes.

Cluster β comprises enzymes which have been described as class C cephalosporinases. Two sub-clusters - β1 and β2 - were defined, both including enzymes from group 1 and from the structurally related group 1e (ESACs). All the enzymes in cluster β appear to be very closely related, particularly the pairs PDC-1 and PDC-2 from *P. aeruginosa*, AmpC-SR50 and SRT-1 from *S. marcescens*, and GC1 and Amp-CHE from *E. cloacae*. This suggests that the genes encoding such enzymes remained conserved within the core genome of the producing strains, which could imply the association with an important function rather than the hydrolysis of β-lactams, as previously suggested (33, 90, 100, 101, 123, 130, 141, 252). One could affirm that the hypothesis of an alternative metabolic function has been supported by the obtained results. To infer functional conservation among sequences, one approach would consist in exploring the existence of syntenic genes (genes that are sequential) within genomes, which have a very high probability of functional relatedness. By definition, synteny is the condition of two or more gene loci being on the same chromosome whether or not there is demonstrable linkage between them. During evolution, genome rearrangement increases with

increasing sequence divergence. Such rearrangements may physically separate two loci apart, which results in the loss of synteny between them, and consequently diminishing the probability of functional conservation. In contrast, exceptional conservation of synteny can reflect important functional relationships between genes (249). Another approach to further investigate protein function would be to characterize protein-protein interactions. These interactions occur when two or more proteins bind together, often to carry out their biological function. They are very important, particularly transient interactions, which are expected to control the majority of cellular processes. One biological effect of such interactions is the change in the specificity of a protein for its substrate, yielding a new function that neither protein can exhibit alone. Protein interaction methods (e.g. Co-Immunoprecipitation, Label Transfer Protein Interaction Analysis, Far-Western Blot Analysis) may therefore uncover unique, unforeseen functional roles for well-known proteins. Furthermore, previously unknown proteins may be discovered by their association with one or more known proteins. Knowing where, how and under which conditions proteins interact consists on the first step to understand the functional implications of these interactions (181).

Cluster δ groups three known class A penicillinases from *K. pneumoniae*. SHV-11 is a point mutation derivate of SHV-1 by four silent mutations (57), which explains their close relationship. Contrarily to the SHV-type enzymes, which belong to subgroup 2b, LEN-1 is a subgroup 2a penicillinase. This enzyme presents a homology of 90% to the amino acid sequence of SHV-1 (96), an observation which is in agreement with the phylogeny shown in Figure 2. All enzymes in cluster ε are class A serine carbapenemases from subgroup 2f. IMI-1 and NMC-A are encoded in the genome of the same organism, *E. cloacae*, with both DNA and encoded protein sequences sharing more than 95% of identity (196). This explains their closest relationship than with SME-1 from *S. marcescens*, although the later enzyme has shown amino acid identity of 70% with NMC-A (154). Although functionally related with the enzymes in this cluster, OXA-40 was grouped with other OXA-type enzymes in sub-cluster α1, such as OXA-24, -25 and -26, with whom it shares minor amino acid changes, as mentioned earlier.

Clusters ζ and η comprise enzymes currently described as class A β-lactamases, from different organisms. CTX-M-3 from *Kluyvera ascorbata* and RAHN-1 from *Rahnella aquatilis* appear as being closely related in the presented phylogenetic tree, and are grouped together in cluster ζ. This result can be explained by the fact that RAHN-1 shares, respectively, 73% and 71% amino acid identity with the plasmid-encoded enzymes CTX-M-1 and CTX-M-2 (18), which are probable descendants of CTX-M-3 (201). Indeed, the chromosomal cefotaximases of some *Kluyvera* spp., particularly CTX-M-3, are apparently the source of CTX-M-derived plasmid-borne enzymes (201). This is the case for the widespread dispersed CTX-M-15 β-lactamases, the clinically most important (39) and most prevalent ESBLs in global surveillance (177), which differs from CTX-M-3 by a single amino acid substitution (106). HugA was grouped in cluster ζ along with the CTX-M-3 and RAHN-1 ESBLs, since these enzymes share a high degree of homology, to which is associated a common hydrolytic profile. Indeed, HugA is able to efficiently hydrolyze ceftriaxone and cefuroxime (125). Although having the ability to hydrolyze oxyimino-cephalosporins and being inhibited by clavulanic acid (38), subgroup 2e enzymes such as HugA lack good penicillin-hydrolyzing activity, and are not regarded as ESBLs

(176). Indeed, the number of 2e enzymes is now stable and it is not expected to increase in the future, probably because many of these enzymes are now identified as ESBLs (38).

Cluster η includes enzymes presently described as subgroup 2be ESBLs, namely PER-1 from *P. aeruginosa* and PER-6 from *Aeromonas allosaccharophila*, and also CME-1 from C*. meningosepticum* and CepA from *B. fragilis*, both 2e enzymes. The PER-6 gene, bla_{PER-6}, shares 79% nucleotide identity with the bla_{PER-1} gene (80), which may explain their close relationship at amino acid sequence level, and consequently their similar extended-spectrum profile. CME-1 belongs to a distinct molecular subfamily among the class A β-lactamases found in Gram-negative bacteria, whose primary structure is rather divergent from those enzymes. One of its closest neighbours is the PER-1 ESBL, with whom it shares 39% amino acid identity (205). However, the overall functional behaviour of CME-1 resembles those of the *Bacteroides* cephalosporinases, such as CepA, with whom it shares 36% amino acid identity (205). The enzymes from *Bacteroides* species also form a distinct homology group which is significantly different from other class A β-lactamases (204). According to the obtained results and the available information regarding CME-1 and CepA, one may hypothesize that these enzymes have probably diverged rather early during the evolutionary history of the class A β-lactamases, although nowadays they are functionally related.

Clusters γ and θ comprise enzymes known as MBLs from subgroup 3a. Cluster γ includes enzymes from structural subclass B3, which require two bound zinc ions at the active-site for their broad-spectrum hydrolytic activity, similarly to the enzymes from subclass B1, grouped in cluster θ. FEZ-1 from *L. gormanii* is a recent member of the highly divergent subclass B3 lineage, being the MBL that shares the closest structural similarity (29.7% of identical residues) with the L1 metalloenzyme from *S. maltophilia*. Indeed, all the residues involved in coordination of the zinc ions in L1 were found to be conserved also in FEZ-1, suggesting an identical geometry of zinc coordination in the active-site for both enzymes (27). However, FEZ-1 exhibits distinctive substrate specificity from L1, with preference for cephalosporins and higher catalytic efficiency for meropenem than for imipenem, a hydrolytic profile shared by the GOB-1 metalloenzyme from *L. gormanii* (16, 27). MBLs from subclass B3 can be either monomeric or multimeric, a phenomenon which has not been observed for the metalloenzymes of other subclasses (143). Their similar substrate profile and common monomeric nature (16, 27, 143) may explain why FEZ-1 and GOB-1 appear as being more closely related than with the L1 multimeric MBL. According to the presented phylogenetic tree, the environmental metalloenzyme from *J. lividum*, THIN-B, appears to be more closely related to L1 than with FEZ-1 and GOB-1. Indeed, THIN-B has 35.6% identical residues to L1, and only 27.8% and 24.2% of identity to GOB-1 and FEZ-1, respectively (206). Despite being less common among pathogenic bacteria, environmental MBLs such as THIN-B are potentially very dangerous as resistance effectors due to their efficient hydrolysis of carbapenems, which are stable to hydrolysis by most β-lactamases and often represent the last-resort agents for MDR pathogens, and also because of their lack of susceptibility to the serine enzymes inhibitors (e.g. clavulanic acid) (123).

Cluster γ also includes the Mbl1b and CAU-1 metalloenzymes from C. *crescentus*, a widespread Gram-negative bacterium found in soil and aquatic habitats. Mbl1b has 32% amino acid identity with the L1 MBL (216). Sequence comparisons showed that CAU-1 has 35.1% identical residues with the

THIN-B metalloenzyme, being more divergent from L1 (32.9% identity), FEZ-1 (32.3%), and GOB-1 (30.7%) (55). The fact that both Mbl1b and CAU-1 share similar hydrolytic profiles and are produced by the same organism, may justify their closest phylogenetic relationship relatively to the other MBLs in cluster γ. In fact, it has been suggested that these proteins represent ancestral hydrolases that had been selected to counteract the effect of naturally-occurring fungal β-lactams in the natural environment of *C. crescentus* (216).

Members of the lineage of enzymes currently classified as subclass B1 MBLs are included in cluster θ, namely the enzymes of *Myroides* spp., TUS-1 and MUS-1. These β-lactamases are very closely related, sharing 73% of amino acid identity, but being distantly related to other metalloenzymes. A comparison of the amino acid sequences of TUS-1 and MUS-1 with those of other class B MBLs revealed a 42% amino acid identity with IND-1, the most closely related metalloenzyme (133). The phylogenies of IND-1 and other subclass B1 MBLs, such as Bc-II from *B. cereus*, CcrA from *B. fragilis* and JOHN-1 from *F. Johnsoniae*, have no bootstrap support under the imposed cut-off. However, JOHN-1 is known to have 41% amino acid identity with IND-1, and both share similar hydrolytic profiles (157). Likewise, CcrA shares 33.5% amino acid identity with the Bc-II metalloenzyme (194). The MBLs from subclass B2, CphA and ImiS, constitute the outgroup which can represent members that diverged rather early during the evolutionary history of the metalloenzymes lineage. Such outgroup is necessary to root the phylogenetic tree, allowing to determine the direction of evolution and providing some means of identifying ancestry (158). These enzymes belong to subgroup 3b, a smaller group of MBLs that preferentially hydrolyzes carbapenems when only one of the zinc binding sites is occupied. Contrarily to subgroup 3a MBLs, the presence of a second zinc ion is inhibitory to their enzymatic activity. CphA from *A. hydrophila* has 98% amino acid identity with ImiS from *A. veronii* (238). The results of these comparisons are consistent with the positioning of both enzymes as members of structural subclass B2. These metalloenzymes have a narrow-spectrum of activity compared to subgroup 3a MBLs. They have assumed clinical significance due to their carbapenemase activity, and constitute an important reservoir for such resistance determinants (157). The disposal of the B2 MBLs in the presented phylogenetic tree (Fig. 2) suggests these enzymes are very distant phylogenetically, having probably different origins.

More detailed analysis was restricted to representative sequences of the clusters listed on Table 3, randomly chosen. These sequences, along with their putative orthologs, are presented in Table 4.

Table 4: β-lactamases and their putative orthologs, identified by standard protein-protein BLAST search (BLASTO) against the NCBI Clusters of Orthologous Groups (COG) database for unicellular organisms. The *E*-value[a] and maximum identity[b] parameters for each putative ortholog are shown.

Cluster	Enzyme name	Organism	Putative ortholog(s)	Organism	*E*-value	Maximum identity	GenPept accession number
α1	OXA-24 OXA-69	*Acinetobacter baumannii*	hypothetical protein ABTJ_p0086	*Acinetobacter baumannii*	$2e^{-13}$	60%	YP_006291759
α2	OXA-18	*Pseudomonas aeruginosa*	hypothetical protein mll0916	*Mesorhizobium loti*	$3e^{-89}$	54%	NP_102617
	OXA-22	*Ralstonia pickettii*	hypothetical protein HMPREF1013_03386	*Bacillus* sp. 2_A_57_CT2	$8e^{-46}$	41%	ZP_08006772
β1	PDC-2	*Pseudomonas aeruginosa*	hypothetical protein ESA_01875	*Cronobacter sakazakii*	$1e^{-160}$	62%	YP_001437965
	AmpC-SR50	*Serratia marcescens*	hypothetical protein PROVALCAL_02707	*Providencia alcalifaciens*	$6e^{-137}$	51%	ZP_03319760
β2	CMY-37 AmpC-P99	*Citrobacter freundii* *Enterobacter cloacae*	hypothetical protein BN629460	*Saccharothrix espanaensis*	$7e^{-13}$	31%	YP_007037129
γ	L1	*Stenotrophomonas maltophilia*	hypothetical protein A176_2298	*Chondromyces apiculatus*	$9e^{-147}$	71%	ZP_11025925
	Mbl1b	*Caulobacter crescentus*	Zn-dependent hydrolase, glyoxylase	*Caulobacter* sp. AP07	$8e^{-136}$	66%	ZP_10754164
δ	SHV-1	*Klebsiella pneumoniae*	hypothetical protein CLONEX_01829	*Clostridium nexile*	$1e^{-132}$	65%	ZP_03289627
ε	NMC-A	*Enterobacter cloacae*	putative carbapenemase	*Klebsiella pneumoniae*	$1e^{-98}$	55%	AFV48348
ζ	RAHN-1	*Rahnella aquatilis*	hypothetical protein pC15-1a_016	*Escherichia coli*	$4e^{-150}$	72%	NP_957562
			hypothetical protein PROPEN_03714	*Proteus penneri*	$5e^{-120}$	66%	ZP_03805320
η	PER-1 CME-1	*Aeromonas allosaccharophila* *Chryseobacterium meningosepticum*	hypothetical protein HMPREF9455_03253	*Dysgonomonas gadei*	$4e^{-70}$	43%	ZP_08475087
θ	TUS-1	*Myroides odoratus*	hypothetical protein HMPREF9714_03545	*Myroides odoratimimus*	$1e^{-131}$	73%	ZP_17120145

[a] Measures the significance of BLAST hits, sequences that produce alignments to the query sequence. Each alignment has a bit score (*S*), which is a measure of similarity between the hit and the query. The *E*-value describes the number of hits one can "expect" to see by chance when searching a database of particular size. It decreases exponentially as the *S* of the match increases. Biologically significant hits will tend to have *E*-values much less than 1, meaning that there is less chance that the similarity between the hit and the query are due to mere coincidence.

[b] Corresponds to the percent similarity between the query and subject sequences over the length of the coverage area.

Orthologs are genes which have evolved from a common ancestor gene, and diverged due to speciation (60, 82, 240). These genes are likely to conserve function or activity, although orthology does not guarantee common function (74, 82), particularly when there is extensive sequence divergence due to high nonsynonymous over synonymous nucleotide substitution ratios, which is apparently caused by Darwinian selection (158, 247). For comparative analyses, such as phylogenetic analysis, it is important to identify orthologs that most likely retain similar function. Because sequence similarity can be related to functional similarity, errors in defining orthologs can greatly affect subsequent functional predictions. For defining orthologs, we queried the COG database with the amino acid sequences listed in Table 4. The COG collection uses the Reciprocal Best Hits (RBH) analysis as part of its ortholog prediction process. One problem with the RBH approach is that it will incorrectly predict a paralog as an ortholog when incomplete genome sequences or gene loss is involved (74). Due to this limitation, it is important to recognize that many of the current ortholog databases will likely contain false positives, making it difficult to obtain a dataset containing only true orthologs.

All putative orthologs are listed in Table 4. The representative sequences from each cluster and their putative orthologs were aligned, and the resultant MSA was trimmed, according to the procedures described in the methods section. A MP tree of 16 β-lactamase amino acid sequences and 14 putative orthologs is shown in Figure 3. For a question of fitting the image into the page, only the sequence name of each putative ortholog is visible.

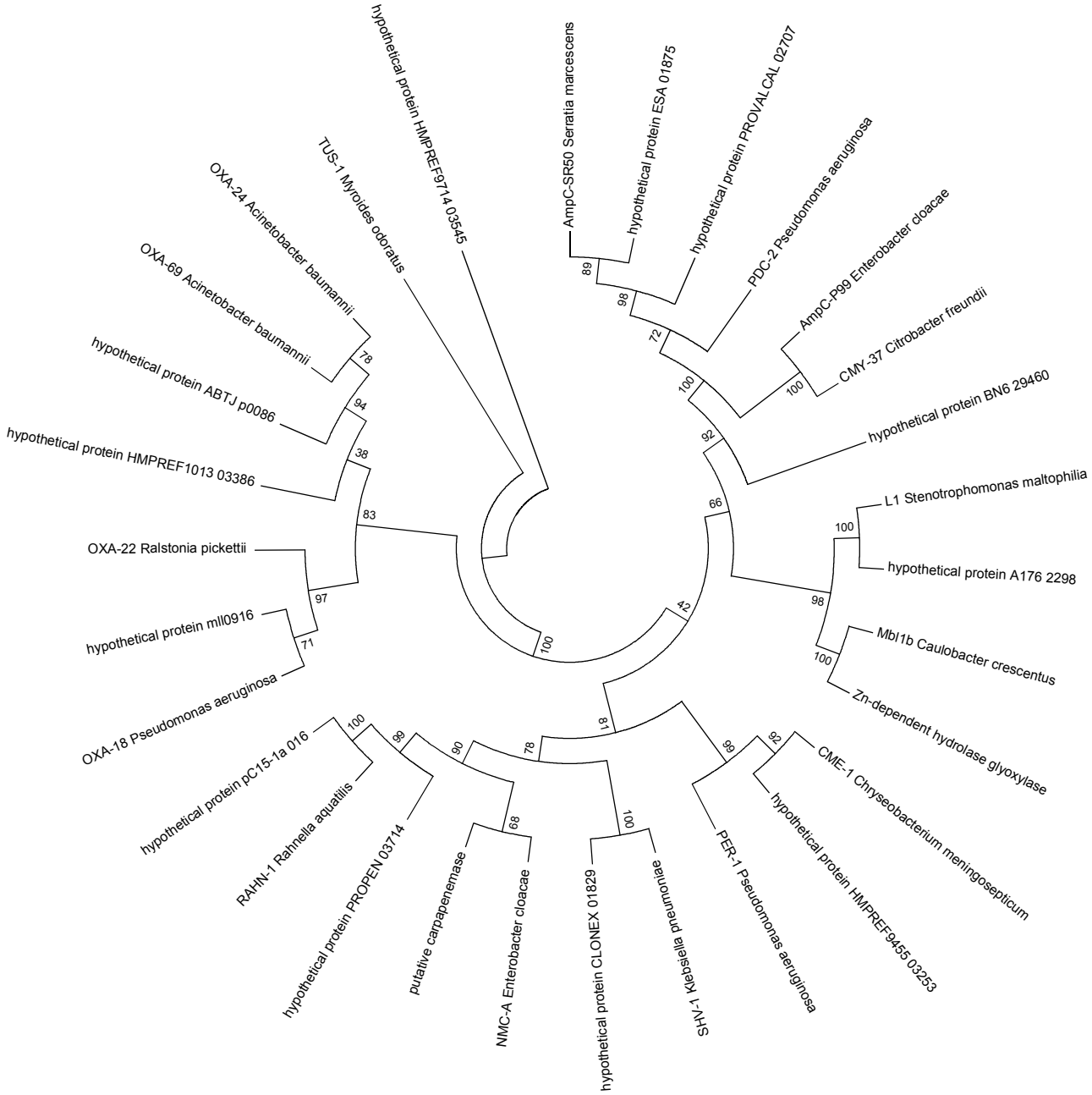

Fig. 3: Maximum parsimony (MP) analysis of β-lactamase sequences and their putative orthologs. The evolutionary history was inferred using the MP method (70). Tree #1 out of 2 most parsimonious trees is shown. The MP tree was obtained using the Close-Neighbour-Interchange algorithm (158) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 37 amino acid sequences, aligned with MUSCLE (58). All ambiguous positions were removed for each sequence pair. There were a total of 358 positions in the final dataset. Evolutionary analysis was conducted with MEGA v5.05 (229). Numbers in figure represent the respective bootstrap proportions (for 100 bootstrap replicates).

The MP tree in Figure 3 suggests a close phylogenetic relationship between most putative orthologs and some already described β-lactamases, some of them presenting little divergence from their common ancestors. This is the case for the following related groups: [AmpC-SR50; hypothetical protein ESA 01875], [L1; hypothetical protein A176_2298], [Mbl1b; Zn-dependent hydrolase, glyoxylase], [CME-1; hypothetical protein HMPREF9455_03253], [SHV-1; hypothetical protein CLONEX 01829], [NMC-A; putative carbapenemase], [RAHN-1; hypothetical protein pC15-1a_016], and [OXA-18; hypothetical protein mll0916]. The hypothetical protein HMPREF9714_03545, the putative ortholog for the TUS-1 metalloenzyme, constitutes the outgroup which roots the phylogenetic tree, representing the last common ancestor shared by all sequences. In turn, the hypothetical proteins PROVALCAL_02707 from *Providencia alcalifaciens*, PROPEN_03714 from *P. penneri*, and ABTJ_p0086 from *A. baumannii* appear to share a common ancestor with some known β-lactamases, respectively AmpC-SR50 from *S. marcescens*, RAHN-1 from *R. aquatilis*, and the OXA-type enzymes from *A. baumannii*, OXA-24 and OXA-69. The phylogeny of the OXA β-lactamases suggests these enzymes may have diverged earlier and thus have more information about evolution to give. There is growing evidence that such ancient evolutionary events have contributed considerably to the antibiotic resistance problem we face nowadays. Indeed, the OXA β-lactamase genes are very old, predating the divergence of the Cyanobacteria about 2.5 billion years ago (10), long before the current antibiotic Era. Thus, it seems likely that these genes have played an important role in bacterial fitness, tough the precise nature of this role is uncertain.

While orthology is one piece of evidence used to reconstruct functional evolution, no assumptions were made a priori about the relationship between orthology and functional conservation, although orthologs are likely to have the same function. Function is typically inferred by homology, on the assumption that it did not change if the common ancestral protein is recent enough. However, there are problems: what is the threshold for determining not only homology, but functional identity? Even if two proteins have 95% of identity in their amino acid sequences, if the remaining 5% happens to include active-site residues, these proteins may do completely different things. Thus, in order to avoid misannotations, it is important that such amino acid residues are included in analysis, and also be careful not to annotate proteins with functions that are more specific than the available evidence supports. The functional annotation of the β-lactamases and their putative orthologs was carried out using InterProScan (151) and searches within the NCBI Conserved Domains Database (CDD) (135) (see methods). The results are shown in Table 5.

Table 5: Functional annotation of the β-lactamases and their putative orthologs, using InterProScan[c] and searches within the NCBI CDD[d].

Sequence name	Organism	InterPro match (InterProScan)	List of domain hits (CDD)
OXA-24	*Acinetobacter baumannii*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
OXA-69	*Acinetobacter baumannii*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
hypothetical protein ABTJ_p0086	*Acinetobacter baumannii*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
OXA-18	*Pseudomonas aeruginosa*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]

Table 5 (cont.):

Sequence name	Organism	InterPro match (InterProScan)	List of domain hits (CDD)
OXA-22	*Ralstonia pickettii*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
hypothetical protein mll0916	*Mesorhizobium loti*	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
hypothetical protein HMPREF1013_03386	*Bacillus* sp. 2_A_57_CT2	Superfamily: Beta-lactamase / transpeptidase-like	COG2602: Beta-lactamase class D [defence mechanisms]
PDC-2	*Pseudomonas aeruginosa*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
hypothetical protein ESA_01875	*Cronobacter sakazakii*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
AmpC-SR50	*Serratia marcescens*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
hypothetical protein PROVALCAL_02707	*Providencia alcalifaciens*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
CMY-37	*Citrobacter freundii*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
AmpC-P99	*Enterobacter cloacae*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms] [PRK11289], Beta-lactamase / D-alanine carboxypeptidase
hypothetical protein BN629460	*Saccharothrix espanaensis*	Superfamily: Beta-lactamase / transpeptidase-like	COG1680: AmpC multi-domain, Beta-lactamase class C and other PBPs [defence mechanisms]
L1	*Stenotrophomonas maltophilia*	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases
hypothetical protein A176_2298	*Chondromyces apiculatus*	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases
Mbl1b	*Caulobacter crescentus*	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases

Table 5 (cont.):

Sequence name	Organism	InterPro match (InterProScan)	List of domain hits (CDD)
Zn-dependent hydrolase, glyoxylase	*Caulobacter* sp. AP07	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases
SHV-1	*Klebsiella pneumoniae*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
hypothetical protein CLONEX_01829	*Clostridium nexile*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
NMC-A	*Enterobacter cloacae*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
putative carbapenemase	*Klebsiella pneumoniae*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
RAHN-1	*Rahnella aquatilis*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
hypothetical protein pC15-1a_016	*Escherichia coli*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
hypothetical protein PROPEN_03714	*Proteus penneri*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
PER-1	*Aeromonas allosaccharophila*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
CME-1	*Chryseobacterium meningosepticum*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
hypothetical protein HMPREF9455_03253	*Dysgonomonas gadei*	Superfamily: Beta-lactamase / transpeptidase-like	pfam13354: Beta-lactamase enzyme family PRK15442: Beta-lactamase TEM; Provisional
TUS-1	*Myroides odoratus*	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases
hypothetical protein HMPREF9714_03545	*Myroides odoratimimus*	Superfamily: Metallo-hydrolase / oxidoreductase	smart00849: Lactamase_B, Metallo-beta-lactamase Superfamily COG0491: GloB multi-domain, Zn-dependent hydrolases, including glyoxylases

[c] "InterPro match" gathers a compilation of identified protein signatures through InterProScan searches within the InterPro consortium member databases.

[d] Searches within the NCBI CDD allowed elaborating a "List of domain hits", where the conserved domains identified on each query sequence are listed.

The results shown on Table 5 indicate that there is functional conservation between the β-lactamases sequences and the identified orthologs. Indeed, all putative orthologs share some conserved domains that are found in the same superfamily of β-lactamases with whom they seem more closely related (Figure 3). These domains have been described as being associated with β-lactam-hydrolyzing activity. These functional predictions are consistent with the phylogeny shown in Figure 3, and lead to the assumption that we are in the presence of genuine orthology. Two major superfamily clusters were recognized: the β-lactamase/transpeptidase-like and the metallo-hydrolase/oxidoreductase. A large number of PBPs, which includes the DD-peptidases, are represented in this first group of sequences. These enzymes synthesize cross-linked peptidoglycan from lipid intermediates, and contain a penicillin-sensitive transpeptidase carboxy-terminal domain. The active-site serine is conserved in all members of this family. Serine-utilizing hydrolases are classed A, C or D, according to sequence, substrate specificity, and kinetic behaviour. Although clearly related, however, the sequences of these three classes vary considerably outside the active-site. The serine class D enzyme OXA-69 was also annotated by InterProScan as a prokaryotic membrane lipoprotein (not shown on Table 5). Bacterial lipoproteins are an important set of membrane proteins with many different functions, from cellular physiology to cell division and virulence (112). Transmembrane topology prediction methods, such as Phobius (105), are important for structure determination of this class of proteins, and to understand how the topology of a membrane evolves from the amino acid sequence to the fully folded tertiary (three-dimensional) structure, which is directly related with protein function and thus more evolutionarily conserved than primary structure (21). Indeed, because protein structure is more conserved than protein sequence, the evolutionary relationships between proteins cannot be easily detected by standard alignment techniques. Structural alignments have been used to infer such relationships, being particularly useful when studying proteins that share very little common sequence. The basic principle of such alignment techniques consists on trying to establish homology between two or more protein structures, based on their shape and three-dimensional conformation. Although structural alignments can provide important insights about protein function and help classify protein families into functional superfamilies, their outputs regarding shared evolutionary ancestry can be confounded with the effects of convergent evolution, by which unrelated amino acid sequences converge on a common tertiary structure (139).

The class B MBLs and their orthologs share the protein domain *smart00849* (the metallo-β-lactamase protein fold), which is found in the metallo-hydrolase/oxidoreductase superfamily. Apart from the β-lactamases, a number of other proteins, which includes the glutathione thiolesterases (glyoxalase II family), contain this conserved domain (159). These enzymes catalyze the hydrolysis of intermediate substrate S-D-lactoyl-glutathione to reduced glutathione and D-lactic acid, requiring the binding of two zinc ions per molecule as cofactor, and a competence protein (ComE) that is essential for natural transformation in *Neisseria gonorrhoeae* (87). Glutathione is one of the most abundant thiols present in cyanobacteria and proteobacteria (also in few strains of Gram-positive bacteria), and in all mitochondria or chloroplast-bearing eukaryotes. In bacteria, in addition to its key role in maintaining the proper oxidation state of protein thiols, glutathione (in its reduced state) also serves a key function in protecting the cell from the action of low pH, chlorine compounds, and oxidative and osmotic

stresses. Additionally, glutathione has emerged as a posttranslational regulator of protein function under conditions of oxidative stress, by the direct modification of proteins via glutathionylation (136). Regarding ComE, this competence protein could be a transporter involved in DNA uptake, and it has been proposed that it binds DNA within the periplasm after specific DNA uptake has occurred. In *N. gonorrhoeae*, DNA transformation and the process of type-4 pili biogenesis are functionally linked and play a vital role in the lifestyle of this strictly human pathogen. The components of the pilus and its assembly machinery are either directly or indirectly involved in the transport of DNA across the outer membrane, although other factors unrelated to pilus biogenesis appear to facilitate further DNA transfer across the peptidoglycan layer and the inner membrane, before the transforming DNA is rescued in the recipient bacterial chromosome in a RecA-dependent manner (87).x

The MBLs and their orthologs also share the *GloB* multi-domain [COG0491], which is associated with zinc-dependent hydrolase activity. The catalytic diversity (and hence adaptability) of the zinc-dependent hydrolases explains how certain members of this superfamily might have easily evolved into metallo-β-lactamases in response to environmental and therapeutical antibiotics (159). Although the presented functional predictions suggest putative pleiotropism for the class B metalloenzymes, no other function rather than β-lactam-hydrolyzing activity has been clearly suggested for the remaining enzymes. Thus, identifying positive natural selection can be particularly interesting, because it provides evidence for adaptive changes in function. The nucleotide sequences on Table 2 were aligned and a ML tree was computed for the resulting MSA, which is shown in Figure 4.

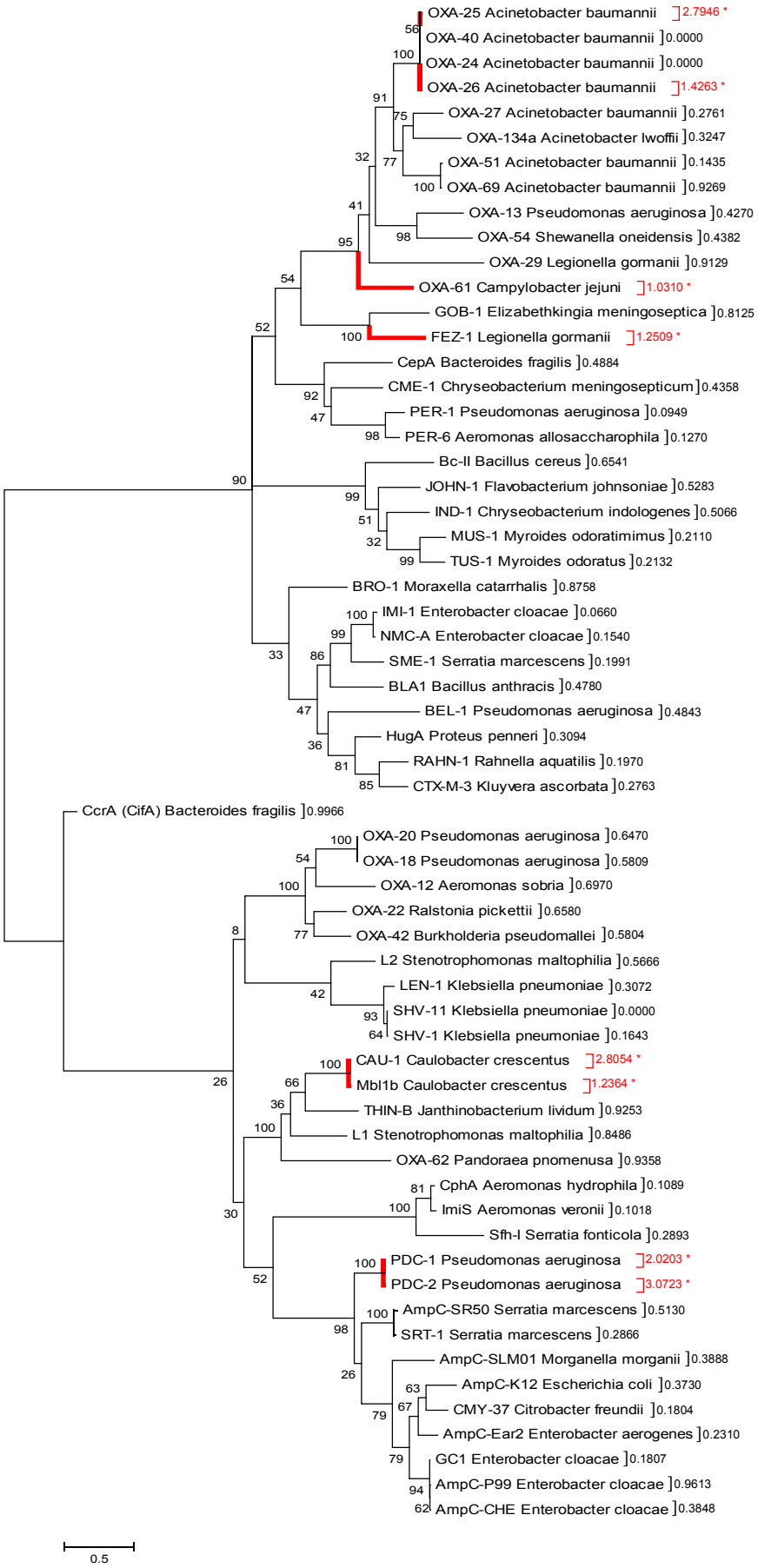

Fig. 4: Maximum likelihood (ML) analysis of β-lactamase nucleotide sequences and evidence for positive selection. The evolutionary history was inferred using the ML method based on the Tamura-Nei model (228). The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 61 nucleotide sequences, aligned with ClustalW (231). All ambiguous positions were removed for each sequence pair. There were a total of 995 positions in the final dataset. Evolutionary analyses were conducted in MEGA v5.05 (229). Enzyme name is followed by the name of the source organism. Numbers in figure represent the respective bootstrap proportions (for 100 replicates). K_a/K_s ratios are shown for each sequence. The eight branches for which K_a/K_s ratios were >1.0 are indicated in heavy red lines and an asterisk next to the respective K_a/K_s ratio.

The maximum likelihood (ML) approach (65) has been previously suggested for estimating K_a/K_s ratios (248), having the advantage of including probability evolutionary models for of nucleotide substitution to explain the variation between the sequences (60, 65, 148, 158). The K_a/K_s ratio is an indicator of the selective pressures on genes, and it is used to infer the direction and magnitude of Darwinian (natural) selection acting on protein-coding genes. It can identify pairwise combinations of genes or branches of nucleotide phylogenetic trees, where encoded proteins may have changed function. If an amino acid change is neutral, it will be fixed at the same rate as a synonymous (silent) mutation, thus $K_a/K_s = 1$. However, a combination of positive and purifying selection at different sites within a gene or at different times along its evolution may annul them, yielding an average value that maybe lower, equal or higher than one. If the amino acid change is deleterious, purifying selection will reduce its fixation rate, and there will be a tendency to conserve protein sequence (and function), thus $K_a/K_s <1$. Only when the amino acid change offers a selective advantage, is it fixed at a higher rate than a synonymous mutation, with $K_a/K_s >1$. Therefore, a K_a/K_s ratio significantly higher than one is convincing evidence for positive selective pressure. Although the K_a/K_s ratio is a good indicator of selective pressure at the sequence level, it only estimates evolutionary changes within protein-coding regions, not detecting changes in the regulatory regions of genes (247, 248).

According to Figure 4, a total of eight genes were found to be under positive selection (K_a/K_s ratio >1). Three of them belong to the OXA family, which supports the hypothesis that the encoded OXA enzymes may have had an important function in bacterial fitness, rather than β-lactam hydrolysis, as mentioned earlier. However, the K_a/Ks ratio for the gene coding OXA-61 was very close to one (1.0310), which could indicate a chimera of positive and stabilizing selection at different points within the gene, in the course of its evolution. Nevertheless, the K_a/K_s ratios within such genes are indicative of high selection pressures, suggesting that the encoded sequences are important to function. The genes coding for the CAU-1, Mbl1b and FEZ-1 metalloenzymes, and for the class C cephalosporinases from *P. aeruginosa*, PDC-1 and PDC-2, were also found to be under positive selection. The results for the two MBLs of *C. crescentus* are in agreement with the hypothesis that these proteins represent ancestral hydrolases that were positively selected in response to the presence of fungal β-lactam antibiotics in the soil, as previously suggested (216). Indeed, finding three MBL genes under positive selection strengthens the hypothesis of putative pleiotropy inferred from the functional predictions, and of the probable association of the metalloenzymes with another function yet to be elucidated, as previously suggested (239). Regarding PDC-1 and PDC-2, their genes appear in the presented phylogenetic tree (Figure 4) as sharing a common ancestor with the AmpC enzymes. The fact that the genes from the cephalosporinases of *P. aeruginosa* are under strong positive selection could mean that the mutation(s) that conferred the selective advantage in the presence of the β-lactam antibiotic, and thus change in function, was fixed in their DNA sequence. A great degree of conservation is observed within the AmpC gene cluster, which is suggested by their respective low K_a/K_s ratios. Such conservation would be the result of stabilizing (purifying) selection, which reduces the fixation rate of the mutation(s), preserving protein sequence, and thus function. The degree of conservation evidenced by the AmpC genes could indicate that these genes are essential to bacteria. Indeed, a close relationship between the induction of the AmpC enzymes and peptidoglycan recycling

has been previously suggested (33, 90, 100, 101, 123, 130, 252), which may imply a crucial role in cell wall metabolism for the class C β-lactamases (130, 141), before the challenge by cephalosporins. It has been proposed that such essential genes should evolve more slowly than nonessential genes. This is because purifying selection against amino acid replacements acting on essential genes is expected to be more stringent than for nonessential genes (103). This reflects into lower average values for K_a and K_s, and also K_a/K_s ratios <1, which has been observed for this particular gene cluster.

CONCLUDING REMARKS

Functional features of orthologous proteins, such as pleiotropy, may play a major role in molecular evolution. One hypothesis for the evolution of the β-lactamases is that the selection pressure caused by β-lactams has favoured the hydrolysis activity on such antibiotics, in detriment of an alternative metabolic function. Nonsynonymous nucleotide substitutions would have result in amino acid changes that altered their substrate specificity, and Darwinian selection may have led to their retention, rather than elimination by purifying selection. In fact, the current misuse and overprescription of β-lactam antibiotics seems to be the most important factor for the selection and dissemination of such phenotypic trait (β-lactam resistance) in pathogenic bacteria (10, 30, 51, 84, 86, 141, 176, 190). Although sometimes obvious, pleiotropy is not always easy to demonstrate. In this study, we could not clearly distinguish between the possibility that the early β-lactamase genes encoded functional enzymes that offered protection against naturally-occurring antibiotics, and the possibility that those genes encoded proteins with entirely different metabolic roles. However, putative pleiotropism has been evidenced, particularly for the class B metalloenzymes. Overall, the results presented here have contributed to the acknowledgement of some level of functional pleiotropy within the β-lactamases, and to a wider understanding of how these proteins might have evolved and become specific β-lactam-hydrolyzing enzymes. Further studies combining analysis of gene synteny, structural alignments, phylogenies of individual genes, protein-protein interactions, and analyses of recombination and selection would help identify and clarify the evolutionary processes of such enzymes.

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