1 # Evolution of β-lactamases and enzyme promiscuity

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15 **ABSTRACT**

16 β-lactamases represent one of the most prevalent resistance mechanisms against β-lactam antibiotics.
17 Beyond their clinical importance, they have also become key models in enzymology and evolutionary
18 biochemistry. A global understanding of their evolution, and sequence and functional diversity can therefore
19 aid a wide set of different disciplines. Interestingly, β-lactamases have evolved multiple times from distinct
20 evolutionary origins, with ancestries that reach back billions of years. It is therefore no surprise that these
21 enzymes exhibit diverse structural features and enzymatic mechanisms. In this review, we provide a bird's
22 eye view on the evolution of β-lactamases within the two enzyme superfamilies—*i.e.*, the penicillin-binding
23 protein-like and metallo-β-lactamase superfamily—through phylogenetics. We further discuss potential
24 evolutionary origins of each β-lactamase class by highlighting signs of evolutionary connections in protein
25 functions between β-lactamases and other enzymes, especially cases of enzyme promiscuity.

26

27 **ABBREVIATIONS**

28 **CEAS1/2:** carboxyethy arginine synthase; **CPS/CarA:** carbapenam synthetase; **CarB-E:** carbapenem
29 biosynthesis enzymes; **NRPS:** non-ribosomal protein synthetase; **ACVS:** L-δ-(α-aminoadipoy1)- L-
30 cysteinyl-D-valine synthetase; **SulI/M:** sulfazecin biosynthesis enzymes; **NocA/B:** nocardicin A
31 biosynthesis enzymes; **β-LS:** β-lactam synthetase; **IPNS:** isopenicillin N synthase; **DAOCS/DACS:**
32 deacetoxycephalosporin/deacetylcephalosporin C synthases; **PB:** penicillin-binding; **PBP:** penicillin-
33 binding protein; **HMW:** high molecular weight; **LMW:** low molecular weight; **SBL:** serine β-lactamase; **MBL:**
34 metallo-β-lactamase; **DnhA/B:** dinitroanisole O-demethylases; **ChD:** chlorothalonil dehalogenase.

35

36 **INTRODUCTION**

37 β-lactams are the most prevalently used class of antibiotics, and a staple therapeutic in combating bacterial
38 infections (Abbott *et al.*, 2013, Bush *et al.*, 2016). Since the initial discovery of penicillin in 1928, several
39 classes of β-lactams have been uncovered and further developed into semi-synthetic therapeutics. The
40 wide-spread use of these compounds in both clinical and industrial settings has inevitably selected for the
41 evolution and dissemination of bacterial defence mechanisms against these drugs (Bush, 2018, Rice,

42 2012). One major resistance mechanism is conferred by the expression of enzymes known as β-
43 lactamases, which hydrolyze β-lactams to render these compounds inactive (Bush, 2018).

44 The first plasmid-mediated β-lactamase, TEM-1, was discovered in the 1960's (Bradford, 2001).
45 Since then, many distinct and highly diverged β-lactamase classes (Ambler class A, B, C and D) harbouring
46 various enzyme families (*e.g.*, SHV-, KPC-, VIM-, NDM-, OXA-types) have been discovered in clinical
47 pathogens (Rice, 2012). The high sequence and functional diversity allow the entire family of β-lactamases
48 to degrade virtually all classes of β-lactams (Bonomo, 2017, Tooke *et al.*, 2019). The underlying molecular
49 and structural mechanisms for β-lactam degradation have been extensively studied, aiding the
50 development of newer, more effective β-lactam antibiotics and β-lactamase inhibitors (Bush, 2018). Besides
51 their clinical implications, these enzymes have become indispensable model systems across various
52 disciplines such as enzymology and evolutionary biology (Bonomo, 2017, Tooke, et al., 2019). The
53 knowledge gained from many proof-of-principle studies of evolutionary dynamics have provided crucial
54 foundations for developing effective protein engineering and laboratory evolution strategies (Salverda *et*
55 *al.*, 2010, Socha *et al.*, 2013, Yang *et al.*, 2020).

56 Our current understanding of β-lactamase evolution is almost entirely centred on clinically isolated
57 β-lactamases and their variants. However, this may only be the tip of the iceberg as an even greater plethora
58 of β-lactamase diversity exists in microbes living in the environment (Berglund *et al.*, 2017, Bush, 2018),
59 and leaves many important questions about these enzyme families unaddressed. How did these β-
60 lactamases evolve? Who are the ancestors? What is the molecular basis that promotes their evolution?
61 Furthermore, human production and application of β-lactam antibiotics may have completely changed the
62 evolutionary dynamics of β-lactamases in the environment. Are new classes of β-lactamases emerging due
63 to our antibiotic consumption? Not only are such questions interesting to explore for the sake of fundamental
64 science, answering these questions will also aid our ability to limit the growth of β-lactam resistances and
65 reveal key concepts useful in designing and engineering novel enzymes in the laboratory. In this review,
66 we provide a birds-eye view on the evolution and diversity of β-lactamases, by presenting phylogenetic
67 trees of the two protein superfamilies harbouring β-lactamases from different classes. We then discuss the
68 evolutionary origins of these classes by highlighting recently disclosed promiscuous β-lactamase activity of
69 non-β-lactamase enzymes within each superfamily. Since the evolution of new enzymatic functions is more

70 likely to recruit and optimize promiscuous enzymes than to generate a function *de novo,* enzymatic
71 promiscuity can be regarded as possible evolutionary connections between related enzymes of a
72 superfamily (Baier *et al.*, 2016, Keshri *et al.*, 2020, Khersonsky *et al.*, 2010, Zou *et al.*, 2015).
73
74 **Penicillin binding proteins, and β-lactams**
75 In order to discuss the origins of β-lactamases, we first briefly describe how β-lactam antibiotics act in the
76 bacterial cell on their inhibition targets, the penicillin-binding proteins (PBPs), and how these antibiotics are
77 produced in nature (**Figure 1a**).
78
79 ***Penicillin binding proteins and inhibition***
80 PBPs are a family of diverse enzymes responsible for the synthesis, regulation and maintenance of
81 peptidoglycan in bacterial cell walls (Sauvage *et al.*, 2008). These enzymes can be broadly classified into
82 high molecular weight (HMW) and low molecular weight (LMW) PBPs. HMW-PBPs are multi-domain
83 proteins that incorporate and polymerize peptidoglycan groups into the cell wall, and are functionally
84 subdivided into class A (transpeptidase and transglycosylase) and class B (transpeptidase only) (Sauvage,
85 et al., 2008). LMW-PBPs (class C PBPs, subclassified into type-4, -5, -7 and -AmpH) are mainly involved
86 in the peptidoglycan maturation and recycling, possessing DD-carboxypeptidase and/or DD-endopeptidase
87 activity (Sauvage, et al., 2008). PBPs generally share a catalytic penicillin-binding (PB) domain with a
88 nucleophilic serine in the active site to catalyze their reactions. Due to the structural similarity between β-
89 lactams and D-Ala-D-Ala (**Figure 1a**), the natural substrate of the PBPs, the serine residue attacks the β-
90 lactam ring to form an irreversible, inhibitory acyl-enzyme complex (Sauvage, et al., 2008). Different classes
91 of β-lactams differ in their specificity towards different HMW- and LMW-PBPs (Sauvage, et al., 2008).
92
93 ***β-lactam biosynthesis pathways***
94 To date, there are at least five distinct classes of natural β-lactam compounds whose independent
95 biosynthetic pathways are known (**Figure 1a**): 1) penicillins and cephalosporins, *2*) monocyclic β-lactams
96 (*e.g.*, nocardicin), *3*) monobactams (*e.g.*, sulfazecin), 4) carbapenems and 5) clavams (Bush, et al., 2016,
97 Hamed *et al.*, 2013, Townsend, 2016), all of which can inhibit PBPs (Bush, 2018, Bush, et al., 2016).

98 Intriguingly, the key enzymes in these biosynthetic pathways that catalyze the β-lactam ring formation have
99 evolved independently several times in history (Townsend, 2016). For example, penicillins and
100 cephalosporins share the same pathway, and their structural backbones are synthesized by a non-
101 ribosomal peptide synthetase (NRPS) called L-δ-(α-aminoadipoy1)-L-cysteinyl-D-valine
102 synthetases (ACVS), followed by β-lactam ring formation through oxidoreduction by isopenicillin N synthase
103 (IPNS). The initial compound of this reaction is a penicillin and that can be further converted to a
104 cephalosporin through the deacetoxycephalosporin/deacetylcephalosporin C synthases (DAOCS/DACS)
105 (Hamed, et al., 2013, Townsend, 2016). Monobactams and monocyclic β-lactams backbones are also
106 synthesized by separate, distantly related NRPS, where the β-lactam ring for each class is formed by
107 different enzymatic modules within the NRPS complex (Long *et al.*, 2018, Oliver *et al.*, 2018, Townsend,
108 2016). The NRPS for these three classes of β-lactams are likely to have evolved independently *via*
109 convergent evolution (Townsend, 2016). For carbapenems and clavams, the β-lactam ring formation is
110 catalysed by carbapenem synthetase (CPS) and β-lactam synthetase (β-LS), respectively, by ATP
111 activation followed by intramolecular acylation (Townsend, 2016). CPS and β-LS are homologous enzymes
112 that evolved independently from the asparagine synthetases, which are involved in ammonia and amino
113 acid metabolisms (Townsend, 2016).
114 It is unclear when these β-lactam biosynthetic pathways evolved, but it is likely that they have co-
115 evolved with β-lactamases for up to a few billion years (Hall *et al.*, 2004). These β-lactam synthetic
116 pathways are found in distinct bacterial (*e.g., Streptomyces*, *Flavobacterium*, *Burkholderia*) and fungal (*e.g.*,
117 *Aspergillus*, *Acremonium*) species and they have only been transferred to a limited set of organisms (Tahlan
118 *et al.*, 2013). The original function of β-lactams in nature, and the selection pressure to evolve their synthetic
119 pathways are also unknown. In general, these compounds are naturally produced at low concentrations in
120 the environment and it is unclear if they primarily functioned as antibiotics. It has also been proposed that
121 β-lactams play a role in cell-to-cell communication as sub-lethal concentrations of these compounds can
122 induce diverse cellular responses in bacteria (Andersson *et al.*, 2014). Nonetheless, multiple convergent
123 evolution of the biosynthetic pathways suggests that β-lactams, with the capability for PBP inhibition, have
124 played key functional roles among the bacterial community.
125

126 **β-lactam resistance and β-lactamase evolution**

127 Several different mechanisms have evolved among bacteria in response to β-lactam compounds: 128 *i*) mutations leading to β-lactam-insensitive PBPs, *ii*) changes in cell-wall permeability through the 129 expression of active efflux pumps or porins, and *iii*) the production of β-lactamases. While mechanism *i*) is 130 largely observed among Gram-positive bacteria, mechanism *iii*) is most often reported for Gram-negative 131 bacteria (Bush, 2018, Worthington *et al.*, 2013).

132 To date, more than 50,000 β-lactamase and close homologous gene sequences have been 133 reported in the NCBI database. These enzymes are classified into two main groups, the serine β-134 lactamases (SBLs) and the metallo-β-lactamases (MBLs), based on their structural fold and catalytic 135 mechanism. SBLs belong to the PBP-like superfamily (**Figure 1b**, Pfam ID: CL0013), which consists of 136 HMW-PBPs, LMW-PBPs and other metabolic enzymes including esterases (Abe *et al.*, 2002, Ohki *et al.*, 137 2009). SBLs are further subclassified into Ambler class A, C, and D, with highly diverse substrate 138 specificities. Interestingly, some SBLs are inhibited by certain clavam compounds, and this inhibitory 139 character has fostered to the development of clinical SBL inhibitors such as clavulanic acid (Bush, 2018, 140 Bush, et al., 2016). MBLs, also classified as Ambler class B β-lactamases, are members of the MBL 141 superfamily (Pfam ID: CL0381), which encompass a wide variety of metal-dependent hydrolytic enzymes 142 (**Figure 1b**), such as phosphodiesterases, glyoxalases, sulfatases, lactonases and phosphotriesterases 143 (Baier *et al.*, 2014, Bebrone, 2007). Most MBLs exhibit broad substrate specificity, and act on virtually all 144 β-lactams except monobactams. Akin to β-lactam biosynthesis pathways, the different ancestral origins of 145 SBLs and MBLs suggest that β-lactamases arose from multiple, independent convergent evolutionary 146 events.

147 Each class of β-lactamase has spread to diverse bacterial species, and orthologous genes exhibit 148 enormous sequence diversity with sequence identities as low as 20% between members of each 149 class (Hall, et al., 2004). Based on those observations, Hall and Barlow estimated that class A β-lactamases 150 evolved more than two billion years ago, which greatly predates the clinical introduction of β-lactam agents 151 (Bush, 2018, Hall, et al., 2004). Indeed, functional β-lactamase genes and conjugative plasmids have been 152 identified in diverse environments, undisturbed by human influence (Bush, 2018, Perry *et al.*, 2016). Thus, 153 a few billion years of evolution, especially co-evolution with the biosynthetic pathways, has likely caused

154 the generation of immense diversity within both β-lactamase sequences and functions. More recent
155 selective pressures imposed by the extensive use of β-lactams have probably accelerated the mobilization
156 and spread of these enzymes (Medeiros, 1997). Indeed, even low β-lactam concentrations have been
157 shown to accelerate the evolution of β-lactamases and demonstrated strong selective advantages in cells
158 expressing these enzymes (Andersson, et al., 2014, Fröhlich *et al.*, 2020). Since the discoveries of the first
159 β-lactamases (TEM-type and SHV-type) in the 1960's, more than 100 different types of these enzymes—
160 partially originating from environmental bacteria—have been identified in pathogens (Alcock *et al.*, 2020).
161 Moreover, β-lactamases transferred to pathogens have continuously evolved to expand their specificity and
162 efficiency (Bush, 2018).

163

164 **Convergent evolution of β-lactamases within the PBP-like superfamily**

165 The PBP-like superfamily is a large protein superfamily with over 450,000 sequences in the UniProt
166 database (Pfam ID: CL0013). A large fraction of these sequences is HMW- and LMW-PBPs with
167 transpeptidase, transglycosylase, DD-carboxypeptidase and DD-endopeptidase activities (Sauvage, et al.,
168 2008). However, some sequences in the superfamily perform other enzymatic functions including esterase,
169 acyltransferase, methyl-butanoyltransferase, 6-aminohexanoate-dimer hydrolase and β-lactamase
170 activities (**Figure 2a**) (Abe, et al., 2002, Grijseels *et al.*, 2018, Ohki, et al., 2009). Moreover, many
171 sequences within this superfamily remain uncharacterized, and there may exist many more unidentified
172 enzymatic functions. The PB domain is shared by members of the PBP-like superfamily and exhibits a
173 common αβα structural fold along with conserved SXXK and semi-conserved SXN and KTG(T/S) active
174 site motifs (**Figure 1a** and **2b**).

175 SBLs, or class A, C and D β-lactamases, represent ~10% (about 40,000 sequences) of the PBP-
176 like superfamily. Compared to HMW- and LMW-PBPs, these enzymes retain the same β-lactam acylation
177 mechanism, using the asparagine of the SXN motif and the hydroxyl groups in the KTG(T/S) motifs to
178 stabilize/orient the substrate while the serine in the SXXK motif nucleophilically attacks the β-lactam ring
179 (Sauvage, et al., 2008). However, unlike the PBPs, the SBLs have acquired the ability to hydrolyze the
180 acyl-enzyme intermediate, where a distinct molecular mechanism has been evolved within each of the three
181 SBL classes to facilitate the β-lactam product release (Tooke, et al., 2019). Within the phylogenetic tree,

182 these classes are positioned in distinctly separate branches, suggesting that they evolved independently
183 from different ancestors (**Figure 2a**).
184
185 ***Evolution of class A SBLs***
186 Class A SBLs (*e.g.*, TEM-, SHV-, KPC-types) are well studied enzymes as they were the first β-lactamases
187 discovered in the 1960s (Liakopoulos *et al.*, 2016, Salverda, et al., 2010). This class also represents the
188 largest sequence cluster among all other classes with over 20,000 sequences. The evolutionary origin of
189 class A SBLs has been proposed to be the type-5 LMW-PBPs which is supported by their ~ 30% sequence
190 identity. Sequence and structural comparisons between TEM-1 and cyanobacterial PBP-A (LMW-PBP)
191 suggested that the evolution of class A SBLs required large structural rearrangements including insertions
192 and deletions which resulted in enzymes generally shorter (<350 residues) than LMW-PBPs (>400
193 residues). One structural feature that is perhaps crucial to this evolutionary transition is the extension and
194 sequence change of the active site Ω-loop between PBPs and SBLs. The X-loop likely re-shaped the active
195 site and enabled SBLs to hydrolyze the acyl-enzyme complex by utilizing a deacylation water activated by
196 E166, a conserved residue within this loop (**Figure 2c**) (Matagne *et al.*, 1998).
197 The functional connection between SBL and type-5 LMW-PBP enzymes is further strengthened as
198 promiscuous β-lactamase activities have been reported in PBP-4, PBP-A, and PBP-5 (Moon *et al.*, 2018,
199 Smith *et al.*, 2013, Urbach *et al.*, 2008, van der Linden *et al.*, 1994). The deletion of PBP-4 from
200 *Enterococcus faecalis* increased the strains susceptibility against the cephalosporin ceftriaxone by more
201 than >200 fold (Moon, et al., 2018). Conversely, overexpression of PBP-4 increased resistance of *E.*
202 *faecalis* towards other β-lactams such as penicillin and imipenem by 4-fold suggesting promiscuous β-
203 lactamase activities of PBP-4 (Moon, et al., 2018). Moreover, substituting the SBLs' conserved E166 into
204 the Ω-loop of PBP-A from *Thermosynechococcus elongatus* can increase the penicillin deacylation rate by
205 90-fold (Urbach, et al., 2008). Other site-directed mutagenesis studies showed that T217A/S in *Escherichia*
206 *coli* PBP-5 increased the penicillin deacylation rate by up to 3-fold (van der Linden, et al., 1994). In addition,
207 reverse evolution of TEM-1 toward carboxypeptidase activity has been explored (Chang *et al.*, 1990).
208 Inserting a 28 amino acid long segment of *E. coli* PBP-5 into TEM-1 led to detectable carboxypeptidase

209 activity (100-fold lower than the activity of PBP-5), and simultaneously reduced activity against
210 benzylpenicillin by 500,000-fold (Chang, et al., 1990).

211 While these observations imply a plausible evolutionary connection between the class A SBLs and
212 type-5 LMW-PBPs, the class A SBLs may not have evolved directly from the PBPs but rather from other
213 enzymes within the superfamily that diverged from the PBPs. Indeed, there are sequence clusters more
214 closely related to class A SBLs (Figure 2a), with ~40% sequence identity and similar lengths to class A
215 SBLs (290-350 residues). To our knowledge, however, no structural or biochemical data for any of these
216 sequences is currently available, and it is possible that these uncharacterized sequences may perform
217 different functions from either SBLs or PBPs.

218

219 ***Evolution of class C SBLs***

220 Class C SBLs contain ~9,000 sequences and are typically 370-400 residues in length. Based on our
221 phylogenetic tree (**Figure 2a**), the class C SBLs seem to have originated from the AmpH-type LMW-PBPs
222 as PBPs are one of the closest characterized clades with a sequence identity of ~30%. Unlike other SBLs,
223 class C enzymes have experienced only moderate structural re-arrangement evolving from AmpH-type
224 LMW-PBPs (~400 residues), which is reflected in their similar sequence lengths. The deacylation
225 mechanism within the class C is also distinct from other SBLs, where K67 is thought to polarize the side
226 chain of Y150, which then activates a water molecule to carry out the deacylation step (**Figure 2c**) (Tripathi
227 *et al.*, 2016). K67 and Y150 are also conserved within AmpH-type LMW-PBPs and esterases. Thus, the
228 evolution of class C SBLs seems to have occurred with only minor structural re-arrangements. Based on
229 our sequence alignment, we speculate that deletion of the active site `R2´loop in AmpH-type LMW-PBPs
230 may have promoted this process, but generally we believe that substitutions around the active site may
231 have been responsible for this evolutionary transition.

232 Interestingly, the neighbouring sequence cluster has also evolved to several other enzymatic
233 functions including esterase, acyltransferase, methyl-butanoyltransferase and 6-aminohexanoate-dimer
234 hydrolase, suggesting highly divergent evolution has occurred within these branches (**Figure 2a**). This
235 functional diversity suggests that β-lactamase activity could have been a promiscuous activity that was later
236 refined and selected for. Indeed, several enzymes close to this cluster have shown to exhibit promiscuous

237 β-lactamase activity including AmpH from *E. coli*, and various esterases such as EstU1 (**Table 1**). One
238 particular study aimed to understand the promiscuous β-lactamase activity of EstU1 by comparing its
239 structure to the chromosomally encoded β-lactamase AmpC of *E. coli* (Cha *et al.*, 2013). The structure of
240 EstU1 was solved with cephalothin covalently bound to its active site serine, revealing that the relevant and
241 conserved catalytic residues of class C SBLs are present (including K67 and Y150), but that the overall
242 active site of EstU1 appears suboptimal for β-lactam hydrolysis (Cha, et al., 2013).
243
244 ***Evolution of class D SBLs***
245 The class D SBLs (230-320 residues) contain over 16,000 sequences, and are highly distant from all other
246 sequence clusters in the phylogeny (**Figure 2a**). We found the closest related sequences, and thus their
247 putative ancestor to be HMW-PBPs (500-850 residues). HMW-PBPs are anchored in the periplasmic
248 membrane by their transmembrane domain. For transferases such as HMW-PBPs, there is generally no
249 water involved in the catalytic reaction and thus HMW-PBPs exhibit highly limited ability to catalyse the
250 deacylation of β-lactams (Goffin *et al.*, 1998). Consequently, the evolution of class D SBL may have
251 required substantial structural rearrangements, including the loss of the transmembrane domain that could
252 be reflected in the significant reduction in their length. Interestingly, in class D SBLs, the deacylation water
253 of the product is activated by a carbamylated K73 which is stabilised by W157 (**Figure 2c**) (Lohans *et al.*,
254 2017). Such organization is not observed in the HMW-PBPs, therefore it must have been acquired during
255 the evolution of class D SBLs. While little is known about promiscuous β-lactamase activity of HMW-PBPs,
256 the HMW-PBP2x of *Streptococcus pneumoniae* has been shown to possess low deacylation capacity
257 towards penicillin (Chesnel *et al.*, 2002). Site-directed mutagenesis on F450, which is structurally close to
258 E166 in class A SBLs, demonstrated that F450H and F450D increased the deacylation rate by 10- and 45-
259 fold, respectively (Chesnel, et al., 2002). Directed evolution on PBP2x showed that a combination of G336A,
260 F450L and N452H drastically increased this rate by 10^5-fold, elevating the bacterial cefotaxime resistance
261 by 10-fold (Peimbert *et al.*, 2003). It is hypothesised that N452H could act as a potential proton acceptor
262 and may activate a water molecule, thus increasing the β-lactam hydrolysis rate (Peimbert, et al., 2003).
263 Class D SBL can be subclassified into the D1 and D2 SBLs (**Figure 2a**). The D1 cluster, such as
264 OXA-1-like variants, appears to have evolved first from HMW-PBPs, then diverged into the D2 SBLs (*e.g.*,

265 OXA-48-like variants) and the BlaR-like transcription factors. The PB domain of BlaR senses the presence
266 of β-lactams in the environment by attacking the β-lactam and forming the covalent acyl-enzyme complex
267 to allosterically alter the conformation of its DNA-binding domain, which then regulates the expression of
268 class A SBLs and PBPs (Belluzo *et al.*, 2019, Duval *et al.*, 2003). Consequently, BlaR has almost
269 completely lost its ability to release the β-lactam product (Belluzo, et al., 2019, Duval, et al., 2003).
270

271 **Convergent evolution within the MBL superfamily**

272 MBLs, or class B β-lactamases, are members of the MBL superfamily, which is host to a diverse set of
273 proteins with over 480,000 sequences in the UniProt database (Pfam: CL0381) (**Figure 3a**). Members of
274 the MBL superfamily perform a wide range of functions including a breadth of hydrolysis reactions
275 (esterase, lactonase, lactamase, phosphoric esterase, sulfate esterase, dehalogenase, *etc.*), oxido-
276 reduction, sulfur dioxygenation, and DNA repair and uptake (Baier, et al., 2014, Bebrone, 2007, Dominski,
277 2007). The primary functional domain in the MBL superfamily exhibits a characteristic αββα fold, which is
278 generally about 200-300 residues; the size of each protein can deviate substantially depending on the
279 enzyme subfamily, and some are fused to other domains (*e.g.*, alkylsulfatase). Most members exhibit two
280 conserved active site metal binding motifs involving H116/H118/H196 and D120/H121/H263 as well as a
281 D221 that coordinates both metals (all numbering according to class B scheme (Galleni *et al.*, 2001))
282 (**Figure 3b-c**) (Bebrone, 2007). The class B β-lactamases (subclass B1/B2/B3) make up only 1.5% of the
283 entire MBL superfamily with ~6300 sequences. Phylogenetics suggests that there were two convergent
284 evolutionary events, with one resulting in the B1 and B2 subclasses and the other resulting in the B3
285 subclass. Both events involve changes in the metal binding motif which could shift metal geometries (Cheng
286 *et al.*, 2018, Park *et al.*, 2020b), as well as changes to the overall shape of the active site that affect substrate
287 specificity (Chen *et al.*, 2020, Sun *et al.*, 2018).
288

289 ***Evolution of class B1 and B2 MBLs***

290 B1 and B2 MBLs are much closer to each other than other MBL subfamilies and are thought to be
291 evolutionary related, though their sequence identities are typically only 20%. There are currently ~2800 B1
292 and ~140 B2 sequences available. A key mechanism underlying the evolution of B1/B2 subclasses seem

293 to be changes in the metal binding motif (**Figure 3c**). In the B2 subclass, an H116N substitution in the metal
294 binding motif leads to a distinctive mono-metal active site. Most B1 enzymes retain binding of both metals,
295 but a subset exhibits a mono-metal active site by an H116G substitution (Berglund *et al.*, 2021).
296 Furthermore, in both B1 and B2 MBLs, position 121 is no longer metal binding and D221 is replaced with a
297 cysteine that only coordinates a single metal. Examples of functional mono- and di-metal active sites are
298 found across the MBL superfamily, and certain enzymes can function in both forms (Catlin *et al.*, 2020,
299 Cheng *et al.*, 2018, Kim *et al.*, 2020, Lisa *et al.*, 2017, Tu *et al.*, 2017). In addition, depending on a mono-
300 or di-metal active sites state, these enzymes exhibit distinct chemical mechanisms (Lisa, et al., 2017). The
301 active site of B1 enzymes exhibits an extended 'L3' loop (residues 56 to 66) (Fröhlich *et al.*, 2020) relative
302 to other MBLs that is involved in substrate recognition/specificity (Chen, et al., 2020, Sun, et al., 2018). In
303 contrast, the B2 active site is enclosed with an extended 'L8' loop (residues 150 to 165).
304 With regards to specific ancestors, the function of an immediate neighbouring cluster to B1/B2
305 MBLs is unknown (Baier, et al., 2014). The closest characterized sequences to the B1/B2 enzymes are the
306 dinitroanisole O-demethylases (dnhA and dnhB) and the chlorothalonil dehalogenase (Chd) (**Figure 3a**)
307 (Baier, et al., 2014). The Chd structure exhibits remarkable similarity to the B2 enzyme cphA, including the
308 L8 loop and a single metal binding site due to the H116S substitution (Catlin, et al., 2020). We therefore
309 speculate that B2 enzymes first evolved from sequences close to Chd, which may have different functions,
310 and then further diverged into the B1 class by reacquiring the second metal binding site.
311
312 ***Evolution of class B3 MBLs***
313 B3 MBLs (~3400 sequences) represent another independent evolutionary event, as they are extremely
314 distant from the B1 and B2 MBLs. B3 enzymes also have modified active site motifs, where D221 is
315 replaced by a S221 that no longer coordinates the metal directly (**Figure 3c**). This change in metal binding
316 has been shown to change the metal binding geometry (Park, et al., 2020b). The overall shape of the B3
317 active site is unique in that the 'L10' loop (residues 220 to 237) extends directly upward from position 221.
318 The B3 enzymes also have an extended L8 loop like the B2 enzymes. Taken together, the class B β-
319 lactamases have convergently evolved toward the same functionality through different mechanistic
320 solutions, which include both changes to the metal binding sites and active site loops.

321 Phylogenetically, the B3 MBLs do not seem to have any closely related, characterized neighbours,
322 but the glyoxylases and lactonases appear to be some of the closer families (Baier, et al., 2014) (**Figure 3a**).
323 Although the MIM-1 and MIM-2 enzymes exhibit strong lactonase activity within the B3 subfamily, their
324 evolution is clearly quite distant from the lactonases (Miraula *et al.*, 2016). Further characterization of
325 intermediate sequences between the B3 enzymes, glyoxylases and lactonases are needed to narrow down
326 their evolutionary origins.

327

328 ***VarG represents a new class of β-lactamases within the MBL superfamily***

329 Intriguingly, there appears to be a new subclass of β-lactamases within the MBL superfamily, following the
330 recent discovery of VarG, an enzyme found within a drug resistance regulon of *Vibrio Cholerae* (Lin *et al.*,
331 2017). VarG is highly specific to carbapenems and less effective against other β-lactam classes, which is
332 similar to B2, but different from B1/B3 MBLs. Although there is no structure of VarG available, the sequence
333 suggests that it retains the conserved MBL superfamily metal-binding motif, unlike class B MBLs
334 (**Figure 3b**). Phylogenetics shows VarG forms a close-by but distinct cluster from the B1/B2 subclasses
335 (Berglund, et al., 2021), and thus it may share a common evolutionary ancestor with the B1/B2 enzymes
336 (closely related to dnhA and dnhB). (**Figure 3a**). Interestingly, the VarG-like cluster contains sequences
337 from organisms that produce antibacterial metabolites, such as *Pseudoalteromonas* (UniProt ID:
338 A0A1C0TWG5), *Amycolatopsis* (A0A3N2GPA3) and *Brevibacillus* (A0A0F7EFG4) indicating similar
339 sequences may have evolved in the context of antibiotic pressure. Moreover, VarG itself represents a very
340 small set of related sequences, mostly identified among *Vibrio* species, and thus, this enzyme may
341 represent a relatively recent evolution of β-lactamases within the superfamily as it has not disseminated to
342 the same degree as other class B enzymes.

343

344 ***Widespread promiscuity in the MBL superfamily enhances evolution for β-lactamases***

345 As we discussed above, the origins of class B MBLs are largely unknown. However, many subfamilies
346 spanning the entire phylogeny of the MBL superfamily show promiscuous activity against β-lactams
347 (**Figure 3a** and **Table 2**), suggesting that the superfamily has substantial foundations for the evolution of
348 β-lactamases. Notably, the presence of promiscuous activities is not limited to bacterial species

349 experiencing pressure from β-lactam antibiotics, and has been detected in a number of eukaryotic, archaeal
350 and marine metagenomic enzymes (Baier, et al., 2014, Diene *et al.*, 2020, Diene *et al.*, 2019, Lee *et al.*,
351 2019, Limphong *et al.*, 2009). Moreover, functional transitions to and from β-lactamase activity have been
352 experimentally demonstrated. The laboratory evolution of glyoxalase II demonstrated that a functional
353 transition to β-lactamase activity is possible, where the resulting glyoxalase variant (evMBL8) only retains
354 59% sequence identity and confers 100-fold higher resistance to cefotaxime in *E. coli* (Park *et al.*, 2006). A
355 modified evMBL8 β-lactamase (evMBL9) was improved by 1.5 to 10-fold for a broad range of β-lactam
356 substrates with further evolution (Sun *et al.*, 2013). Class B1 enzymes have also been converted into
357 efficient phosphonatases where the overall hydrolysis rate could be improved by 35 to 3600-fold through
358 laboratory evolution (Baier *et al.*, 2019). Thus, the evolution of class B β-lactamases has been supported
359 by the wealth of promiscuous enzymes within the superfamily, and three different events led to distinct
360 molecular solutions.

361

362 **CONCLUSION**

363 Multiple convergent evolution of β-lactamases illuminates how versatile enzyme can foster the same target
364 function with different molecular solutions. The enzyme superfamilies we have discussed provide diverse
365 yet approachable models for the evolution of antibiotic resistance. Further efforts to divulge the evolutionary
366 origins and evolutionary pathways will be required to gain deeper insights into the evolutionary dynamics
367 and structure-activity relationships of these enzymes. Such knowledge can then be further applied to better
368 understand the reaction mechanisms and functional transitions that may be useful in enzyme engineering.
369 Notably, the co-evolutionary dynamics between β-lactam biosynthetic pathways and β-lactamases remain
370 to be addressed, and are crucial in illuminating the global view of antibiotic resistance evolution. In addition,
371 furthering our understanding of the broad genetic diversity and interplay between β-lactam biosynthesizers
372 and degraders can enhance our ability to discover and design more effective antibiotic agents and
373 inhibitors.

374

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380

381 **FIGURE LEGENDS**

382 **Figure 1. Evolution and ecological role of β-lactamases.** (**a**) The upper half shows the schematic
383 synthesis pathways of five different β-lactams classes and their structures. Enzymes that synthesize the β-
384 lactam backbone are in the upper row and enzymes forming the β-lactam ring have shaded backgrounds.
385 In the middle, the inhibitory effects of β-lactams are highlighted in green lines, and the hydrolysis of β-
386 lactams by β-lactamases are highlighted in yellow lines. In the inset to the right, the structural analogy
387 between D-Ala-D-Ala, the natural PBP substrate, and the β-lactams is illustrated. This schematic also
388 depicts key interactions formed between the substrate and the PBP/SBL active site through the three
389 generally conserved and semiconserved active site motifs SXXK, SXN and KTG(T/S). Activation of the
390 nucleophilic serine and subsequent attack on the peptide backbone is shown in arrows. The bottom shows
391 the evolutionary connection of the SBL and MBL enzymes to their originating superfamilies. (**b**) Schematic
392 of the sequence-function network within the enzyme superfamilies that led to the evolution of the SBLs and
393 MBLs. Nodes depict clusters of similar sequences, with notable representatives labelled by name. Small,
394 unlabelled nodes indicate hypothesized or uncharacterized sequence intermediates. Edges between
395 sequence clusters indicate relatedness by sequence similarity. Major functions in the family are colored,
396 where ‘other functions’ refer to enzymatic functions other than β-lactamase or PBP activity.
397

398 **Figure 2. Phylogeny and structures of the PBL-like superfamily.** (**a**) Phylogenetic tree of all SwissProt
399 and select TrEMBL sequence in the PBL-like superfamily (black) clustered at 40% identity (387 sequences
400 final) along with specific sequences of interest (colored). (**b**) Multiple sequence alignment of representative
401 SBLs from class A, C, D and other PBP-like superfamily members, highlighting the functional and
402 conserved/ semiconserved amino acids according to their side-chain properties: basic (blue), polar (green)
403 and non-polar (orange). (**c**) Crystal structures of the active site loops from class A (KPC-2, turquoise), C

404 (AmpC, blue) and D (OXA-48, green) SBLs. An inset depicts the activation mechanism of the deacylation
405 water below each SBL class, with active site residues key to deacylation labelled and depicted in stick
406 representation; the 'R' group represents the remainder of the hydrolysed β-lactam ring acylated to S70.
407
408 **Figure 3. Phylogeny and structures of the MBL superfamily.** (**a**) Phylogenetic tree of all SwissProt
409 sequences in the MBL superfamily (black) clustered at 40% identity (103 sequences final), along with
410 specific sequences of interest (colored or starred). (**b**) Multiple sequence alignment of representative
411 class B β-lactamases and MBL superfamily members, highlighting the representative metal-binding sites
412 for each subclass in colors corresponding to the side-chain property: basic (blue), acidic (red) and polar
413 (green). (**c**) Overall structure and active-site arrangements of representatives from the class B β-
414 lactamases (class B1: VIM-2, B2: CphA, B3: FEZ-1), and MBL superfamily members of eukaryotic (Glx2-
415 5), archaeal (Igni18) and metagenomic (PNGM-1) origin. Active site loops (L3/L8/L10) in the class B
416 enzymes that are distinct from other MBL superfamily members are highlighted by black outlines.
417

Table 1: Enzymes with promiscuous β-lactamase activity within the PBP-like superfamily

Protein name	Native function	Host organisms	Gene accession [a]	PDB ID [b]	Promiscuous β-lactamase activity against: [c]				Ref.
					Chromogenic [d]	Penicillins	Cephalosporins	Carbapenems	
EstC	Carboxylesterase	Metagenome	ACH88047.1	-	**Yes**	No	No	-	Rashamuse *et al.* (2009)
Est22	Carboxylesterase	Metagenome	AGT17593.1	-	**Yes**	-	**Yes**	-	Mokoena *et al.* (2013)
EstSTR1	Carboxylesterase	Soil metagenome	AJE68931.1	-	-	-	**Yes**	-	Jeon *et al.* (2016)
EstU1	Carboxylesterase	Metagenome	AFU54388.1	4ivk	**Yes**	-	**Yes**	-	Cha, et al. (2013) Jeon *et al.* (2011)
EstM-N1	Carboxylesterase	Soil metagenome	AEA07653.1	-	**Yes**	-	-	-	Yu *et al.* (2011)
EstM-N2	Carboxylesterase	Soil metagenome	AEA07655.1	-	**Yes**	-	-	-	Yu, et al. (2011)
PsEstA	Carboxylesterase	*Paenibacillus sp.*	AHL66978.1	-	**Yes**	-	**Yes**	-	Kwon *et al.* (2019)
EstCS3	Carboxylesterase	Unculturable organism	ARE60547.1	-	-	**Yes**	**Yes**	-	Park *et al.* (2020a)
PBS-2	Carboxylesterase	*Paenibacillus sp.*	AHL66978.1	-	-	**Yes**	**Yes**	-	Kim *et al.* (2014)
ORF006	Carboxylesterase	Unculturable organism	AAY90129.1	-	No	No	**Yes**	No	Elend *et al.* (2006)
CcEstA	Carboxylesterase	*Caulobacter crescentus CB15*	YP_002515630	5gkv	**Yes**	-	-	-	Ryu *et al.* (2016)
Tp47	Unknown	*Treponema pallidum*	WP_013945211.1	1o75	-	**Yes**	-	-	Cha *et al.* (2004)
MSMEG_2433	Carboxylesterase	*Mycobacterium smegmati*	ABK71878.1	-	**Yes**	**Yes**	**Yes**	-	Bansal *et al.* (2015)
PBP-5	Carboxylesterase	*Escherichia coli/ Pseudomonas*	NP_415165 WP_048264223	1nzo	-	**Yes**	**Yes**	**Yes**	Nicholas *et al.* (1988), Smith, et al. (2013), van der Linden, et al. (1994)
PBP-A	Carboxylesterase	*Thermosynechococcus elongatus*	P72161	4k91	-	**Yes**	-	-	Urbach *et al.* (2009)
AmpH	Endopeptidase/ carboxypeptidase	*Escherichia coli*	NP_414910	-	**Yes**	-	-	-	Gonzalez-Leiza *et al.* (2011)
PBP2x	Transpeptidase	*Streptococcus pneumoniae*	AFC91898.1	1pmd	-	**Yes**	-	-	Chesnel, et al. (2002)

1. All IDs are NCBI accessions.
2. PDB ID = Protein Data Bank identification number
3. Empty entries (-) means the activity was not tested.
4. Chromogenic substrates include Nitrocefin or Centa.

Table 2: Enzymes with promiscuous β-lactamase activity within the MBL superfamily

Protein name	Native function	Host organism	Gene accession [a]	PDB ID [b]	Promiscuous β-lactamase activity against: [c]				Ref.
					Chromogenic [d]	Penicillins	Cephalosporins	Carbapenems	
MBLAC2	B-cell exome biogenesis	*Homo sapiens*	Q68D91	-	**Yes**	**Yes**	No	No	Diene, et al. (2019)
SNM1A	DNA cross-link repair proteins	*Homo sapiens*	Q6PJP8	4b87	**Yes**	**Yes**	No	No	Diene, et al. (2019)
SNM1B	DNA cross-link repair proteins	*Homo sapiens*	Q9H816	5aho	**Yes**	**Yes**	No	No	Diene, et al. (2019)
MetbaB	Ribonuclease	*Methanosarcina barkeri*	WP_048116343	-	**Yes**	**Yes**	-	**Yes**	Diene, et al. (2020)
Glx2-1	Glyoxalase II	*Arabidopsis thaliana*	O24495	-	**Yes**	-	**Yes**	**Yes**	Limphong, et al. (2009)
Igni18	Unknown	*Ignicoccus hospitalis*	A8ABX8	6hrg	-	**Yes**	**Yes**	**Yes**	Perez-Garcia *et al.* (2021)
Mblp01	Phytase	*Soil metagenome*	A0A411PXB6	-	-	**Yes**	**Yes**	No	Villamizar *et al.* (2019)
Mblp02	Phytase	*Soil metagenome*	A0A411PXC0	-	-	**Yes**	**Yes**	**Yes**	Villamizar, et al. (2019)
PNGM-1	Ribonuclease	*Marine metagenome*	A0A2U8UYM6	6j4n	-	-	**Yes**	**Yes**	Lee, et al. (2019)
Chd	Chlorothalonil dehalogenase	*Pseudomonas aeruginosa*	C9EBR5	6uxu	**Yes**	-	-	-	Baier, et al. (2014)
Gly3	Sulfur dioxygenase	*Arabidopsis thaliana*	Q9C8L4	2gcu	**Yes**	-	-	-	Baier, et al. (2014)
Glx2-5	Glyoxalase II	*Arabidopsis thaliana*	Q9SID3	1xm8	**Yes**	-	-	-	Baier, et al. (2014)
ZiPD	Ribonuclease	*Escherichia coli*	P0A8V0	2cbn	**Yes**	-	-	-	Baier, et al. (2014)
Tm0207	Unknown	*Thermotoga maritima*	Q9WY50	1vjn	**Yes**	-	-	-	Baier, et al. (2014)
Tm1679	Unknown	*Thermotoga maritima*	Q9X207	3h3e	**Yes**	-	-	-	Baier, et al. (2014)
4-Pyridoxolactonase	4-Pyridoxolactonase	*Mesorhizobium loti*	Q988B9	3aj3	**Yes**	-	-	-	Baier, et al. (2014)
PqqB	PQQ biosynthesis	*Pseudomonas putida*	Q88QV5	1xto	**Yes**	-	-	-	Baier, et al. (2014)
TupBlac	Ribonuclease	*Tupanvirus deep ocean*	A0A6N1NX79	-	**Yes**	**Yes**			Colson et al. (2020)

1. All gene accessions are for UniProt except for WP_048116343 (NCBI).
2. PDB ID = Protein Data Bank identification number
3. Empty entries (-) means the activity was not tested.
4. Chromogenic substrates include Nitrocefin or Centa.

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