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## Class C $\beta$ -Lactamases: Molecular Characteristics

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1 **Class C  $\beta$ -Lactamases: Molecular Characteristics**

2

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11 Running Head: Class C  $\beta$ -Lactamases: Molecular Characteristics

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52 **SUMMARY**

53 Class C  $\beta$ -lactamases or cephalosporinases can be classified into two functional groups (1,  
54 1e) with considerable molecular variability ( $\leq 20\%$  sequence identity). These enzymes are  
55 mostly encoded by chromosomal and inducible genes and are widespread among bacteria,  
56 including *Proteobacteria* in particular. Molecular identification is based principally on  
57 three catalytic motifs (<sup>64</sup>SXSK, <sup>150</sup>YXN, <sup>315</sup>KTG), but more than 70 conserved amino-acid  
58 residues ( $\geq 90\%$ ) have been identified, many close to these catalytic motifs. Nevertheless,  
59 the identification of a tiny, phylogenetically distant cluster (including enzymes from the  
60 genera *Legionella*, *Bradyrhizobium*, and *Parachlamydia*) has raised questions about the  
61 possible existence of a C2 subclass of  $\beta$ -lactamases, previously identified as serine  
62 hydrolases. In a context of the clinical emergence of extended-spectrum AmpC  $\beta$ -  
63 lactamases (ESACs), the genetic modifications observed *in vivo* and *in vitro* (point  
64 mutations, insertions or deletions) during the evolution of these enzymes have mostly  
65 involved the  $\Omega$ - and H-10/R2-loops, which vary considerably between genera, and, in some  
66 cases, the conserved triplet <sup>150</sup>YXN. Furthermore, the conserved deletion of several amino-  
67 acid residues in opportunistic pathogenic species of *Acinetobacter*, such as *A. baumannii*,  
68 *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* (deletion of residues 304-306), and in *Hafnia*  
69 *alvei* and *H. paralvei* (deletion of residues 289-290), provides support for the notion of  
70 natural ESACs. The emergence of higher levels of resistance to  $\beta$ -lactams, including  
71 carbapenems, and to inhibitors such as avibactam is a reality, as the enzymes responsible  
72 are subject to complex regulation encompassing several other genes (*ampR*, *ampD*, *ampG*,  
73 etc.). Combinations of resistance mechanisms may therefore be at work, including

74 overproduction or change in permeability, with the loss of porins and/or activation of efflux  
75 systems.

76

## 77 **KEYWORDS**

78 AmpC  $\beta$ -lactamases, cephalosporinases, ESAC, extended-spectrum, phylogeny, primary  
79 structure

80

## 81 **INTRODUCTION**

82  $\beta$ -lactamases remain an important natural or acquired mechanism of resistance to  $\beta$ -lactam  
83 antibiotics. New enzymes of this type, belonging to the molecular classes defined by  
84 Ambler and then completed by several authors (1-3), have been regularly discovered since  
85 the 1980s. The class C  $\beta$ -lactamases (BLCs), also known as AmpC or cephalosporinases,  
86 have a long history marked by the gradual loss of efficacy for the treatment of many  
87 bacterial infections, due initially to their large inactivation spectrum, including penicillins,  
88 the first cephalosporins (e.g. cephalothin), and cephamycins (e.g. ceftazidime), together with  
89 the general absence of an inhibitory effect of clavulanic acid, sulbactam and tazobactam  
90 (4-6). The next problem encountered was the emergence of constitutive or overproduced  
91 mutants, eventually overcome by the development of oxyiminocephalosporins, such as  
92 cefotaxime and ceftazidime (7). However, plasmid-borne cephalosporinases were  
93 subsequently discovered, particularly in species without chromosomal *ampC* genes (e.g.  
94 *Klebsiella pneumoniae*, *Salmonella enterica*, and *Proteus mirabilis*) and in *Escherichia*  
95 *coli*, which possesses an intrinsic *ampC* gene usually not expressed. These enzymes are  
96 derived from chromosomally encoded enzymes specific to other species, such as

97 *Enterobacter cloacae* and *Citrobacter freundii*, and their discovery raised new fears,  
98 allayed by the discovery of cefepime and ceftazidime (4,8). However, a new step in  
99 resistance development was then detected, with the discovery of extended-spectrum  $\beta$ -  
100 lactamases AmpC (ESAC), mutants or variants with an extended inactivation spectrum for  
101 oxyiminocephalosporins (ceftazidime, cefotaxime, cefepime and ceftazidime) due to the  
102 mutation of certain sites, in the R2-loop (9,10), for example, by substitution, deletion or  
103 insertion. Carbapenem resistance mediated by a combination of mechanisms, including a  
104 constitutive species-specific cephalosporinase and porin loss, has emerged more recently  
105 (11,12). Finally, the recent development of novel enzyme inhibitors, such as avibactam,  
106 has elicited considerable medical interest due to its ability to inhibit serine  $\beta$ -lactamases  
107 (classes A, C and D). However, this has already led to the selection of clinical mutants  
108 resistant to ceftazidime-avibactam combinations, most of them bearing deletions of various  
109 sizes in the  $\Omega$ -loop region of AmpC (13,14), similarly to  $\beta$ -lactamases from other classes  
110 (e.g. KPC-3).

111 Various methods for detecting resistant clinical isolates have been proposed, particularly  
112 for resistance to oxyiminocephalosporins and carbapenems. These methods include  
113 phenotypic tests, enzymatic methods based on hydrolysis, immunochromatographic  
114 assays, and molecular tests designed to test for the presence of particular genes, encoding  
115 class C  $\beta$ -lactamases, for example (15). Whole-genome sequencing (WGS) is a very useful  
116 approach for the precise identification of mechanisms of resistance to  $\beta$ -lactams, and has  
117 provided a large body of sequence data. Nevertheless, genotype-to-phenotype  
118 extrapolations are not straightforward, due to the variability of gene expression and  
119 polymorphisms linked to silent mutations at diverse sites, depending on the  $\beta$ -lactamase

120 considered (16). Improvements in our knowledge should make it possible to improve  
121 analyses of the resistance mechanisms detected, particularly against  $\beta$ -lactams, in the  
122 future. These mechanisms are numerous and differ considerably between species. The  
123 current classification of  $\beta$ -lactamases into four molecular classes is based on motifs  
124 involved in binding and hydrolysis, such as <sup>70</sup>SXXK, <sup>130</sup>SDN and <sup>234</sup>KTG for class A (17),  
125 and on diverse residues involved in determining affinity, either increasing the inactivation  
126 spectrum (e.g. ESBLs) or decreasing it (e.g. IRT) (18-20). A more detailed comparative  
127 analysis of the primary structure of a large number of proteins would facilitate the  
128 classification of enzymes into groups of clusters displaying common structural features, as  
129 for the enzymes of class A (21). BLCs seem to have a lower level of structural diversity,  
130 but the abundance of data now available in databases [e.g. Beta-Lactamase DataBase  
131 (BLDB, <http://bldb.eu/>) (22), Bacterial Antimicrobial Resistance Reference Gene Database  
132 (<https://www.ncbi.nlm.nih.gov/bioproject/313047>) (23), CARD  
133 (<https://card.mcmaster.ca/>) (24)] suggests that a more detailed analytical approach, based  
134 on several thousand sequences, would now be justified.

135 When we began this analysis in 2019, several numbering schemes had been proposed for  
136 class C  $\beta$ -lactamases, which were unified in a standardized structure-based numbering  
137 scheme published in 2020 (25). Accurate comparisons of protein sequences have improved  
138 structural classification, by providing a clearer identification of polymorphisms by species,  
139 a more precise identification of the bacterium updated in line with the continual changes  
140 in taxonomy, a better understanding of the residues or zones involved in the possible  
141 extension of the inactivation spectrum by species or bacterial group, and with respect to  
142 both substrates and enzyme inhibitors, such as avibactam. The ACC-type  $\beta$ -lactamases



143 appeared to be plasmid-encoded enzymes originating from *Hafnia alvei* with an unusual  
144 susceptibility pattern characterized by resistance to expanded-spectrum cephalosporins  
145 such as ceftazidime, cefotaxime, and, sometimes, cefpirome, and by susceptibility to  
146 cefepime and inhibition by cefoxitin (see the section on “natural ESACs” for more details).

147

#### 148 **PHYLOGENETIC COMPARISON**

149 The serine  $\beta$ -lactamases have been divided into three classes (A, C and D) based on  
150 sequence similarity (1,2,26). A protein structure-based phylogeny clearly distinguished  
151 between these classes (27-29). The amino acid-based phylogenetic tree based on 3943 class  
152 C sequences reveals large differences between the major groups (*Acinetobacter*,  
153 *Aeromonadales*, *Burkholderiales*, *Enterobacterales*, *Pseudomonadales*, *Rhizobiales*), each  
154 of which displays at least 24 % amino-acid sequence identity (Fig. 1).

155

156 Figure 1 here

157

158 Highly conserved and major clusters, including many variants displaying >75% sequence  
159 identity with each other, were found for the following genera: *Escherichia/Shigella*,  
160 *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Morganella*, *Serratia*. Other clusters  
161 (*Acinetobacter*, *Aeromonas*, *Burkholderia*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Yersinia*,  
162 etc.) are less conserved, with a percent amino-acid identity between sequences of less than  
163 50%. These findings highlight the need for more accurate taxonomic approaches in the  
164 future. Sequences from the *Proteobacteria* were particularly prevalent, but BLCs were  
165 widely distributed between bacterial groups, with the exception of Gram-positive bacteria,

166 in which they were rare (Table 1) (28,29). Finally, a separate cluster including several  
167 genera (e.g. *Bradyrhizobium*, *Legionella*, *Parachlamydia*) was identified and found to  
168 contain generic serine hydrolases and some carboxylesterases VIII with weak  $\beta$ -lactamase  
169 activity (Fig. 1).

170

171 Table 1 here

172

173 Table 2 here

174

## 175 **PRIMARY STRUCTURE/SEQUENCE ANALYSIS**

### 176 **Highly conserved motifs and residues**

177 A standardized amino-acid numbering scheme was recently developed, through  
178 collaboration, for class C  $\beta$ -lactamases (25). This numbering scheme has greatly facilitated  
179 molecular comparisons between class C enzymes. Indeed, “SANC” (structural alignment-  
180 based numbering of class C  $\beta$ -lactamases) preserves the usual numbering of the major  
181 catalytic residues (<sup>64</sup>S, <sup>67</sup>K, <sup>150</sup>Y, and <sup>315</sup>K). Three highly conserved motifs are currently  
182 used to characterize this molecular class (<sup>64</sup>SXSK, <sup>150</sup>YXN and <sup>315</sup>KTG) (17,28), but other  
183 conserved residues have been characterized and can be used for the more accurate  
184 identification of AmpC enzymes (Tables 2 and 3). They include 37 residues strictly  
185 conserved (100%) and 33 residues highly conserved (90-97%) in a dataset of 32  
186 representative class C  $\beta$ -lactamases examined in a previous study (25). Proline (P) and  
187 aromatic (F, W, Y) residues are overrepresented at these positions compared to class A  $\beta$ -  
188 lactamases. Indeed, prolines are present at positions 18, 26, 94, 118, 122, 140, 192, 213,

189 277, 330, 345 for class C  $\beta$ -lactamases, but only at positions 107, 183 and 226 for class A  
190  $\beta$ -lactamases. Similarly, strictly conserved aromatic residues are present at positions 60,  
191 138, 150, 199, 203, 221, 271, 322, 325, and 328 for class C  $\beta$ -lactamases, but only at  
192 positions 66, 210 and 229 for class A  $\beta$ -lactamases. If aromatic amino acids (F, Y and W)  
193 are considered together, as a single category, additional conserved positions emerge for  
194 BLCs: 43, 69, 134, 135, 170, 188, 233, 260, 276 and 344 (Table 3).

195

196 Table 3 here

197

198 In a representative set of 50 typical AmpC (see Table 2), the total number of amino acids  
199 in these enzymes ranged from 358 (*P. fluorescens* TAE4) to 397 (PDC-1) with a mean  
200 value of  $383 \pm 5.2$ , and the mean of molecular mass was estimated at  $41.64 \pm 0.58$  kDa.  
201 The number of amino acids may be much higher for enzymes with fused domains (29,64).  
202 AmpC enzymes typically have alkaline isoelectric points (pI), with a mean pI of  $8.48 \pm$   
203  $1.22$  for this representative set. Nevertheless, low predicted pI values were obtained for  
204 some enzymes (4.37 for CHR-1, 5.27 for BlaE, 4.59 for RHO-1 and 5.59 for IDC-1) (4,58).  
205

## 206 **Enterobacterales**

207 The Enterobacterales (EB) is one of the major groups emerging from the phylogram in Fig.  
208 1. This group was clearly separated into two major clusters based on the analysis of 413  
209 protein sequences: EB1, including *Buttiauxella*, *Cedecea*, *Citrobacter*, *Edwardsiella*,  
210 *Erwinia*, *Escherichia*, *Enterobacter*, *Klebsiella aerogenes*, *Lelliottia*, *Morganella*,  
211 *Pluralibacter*, *Serratia fonticola*, *Yersinia*, and *Xenorhabdus*, and EB2, including

212 *Budvicia*, *Cronobacter*, *Erwinia*, *Hafnia*, *Pantoea*, *Photorhabdus*, *Pragia*, *Providencia*,  
213 *Regiella*, *Rouxiella*, *Serratia*, and *Siccibacter* (84). Most species generally cluster together  
214 in the same genus or cluster, but, surprisingly, several species (e.g. *Erwinia teleogrylli*, *S.*  
215 *fonticola* and *Y. ruckeri*) were located at some distance from their main clusters. The  
216 following conserved residues distinguished between the EB1 and EB2 groups: D47K,  
217 V65L, G71A, V72T, G74A, W101F, Y112H, Q120F, S154G, F158L, W201Q, A255T,  
218 R258G, G270M, and H314N. A deletion in position 116 and an insertion in position 204a  
219 were also identified in EB2 (25,85). These groups displayed a high degree of diversity (e.g.  
220 55-100% sequence identity for the whole EB1 group). Consensus sequences (CS) were  
221 therefore determined for the main species.

222 For *Escherichia* species (*E. coli/Shigella*, *E. albertii*, and *E. fergusonii*), sequence identity  
223 ranged from 88 to 100% and 11 consensus sequences were obtained from 367 protein  
224 sequences. The *E. coli sensu stricto* population is now considered to have a strong  
225 phylogenetic structure, with the identification of at least 12 phylogroups (A, B1, B2, C, D,  
226 E, F, G, H, clade 1, clade III and clade IV) (86).

227 Several conserved residues were found to be specific for phylogroups A, A1 and B1 (<sup>235</sup>R,  
228 <sup>238</sup>M, <sup>239</sup>N, <sup>241</sup>R, <sup>245</sup>N), whereas others were specific for phylogroups B2 and D (<sup>235</sup>Q, <sup>238</sup>L,  
229 <sup>239</sup>K, <sup>241</sup>L, <sup>245</sup>N/T) (16). All four clusters (A-D) carry RMNRE residues in the 235-245  
230 region (85), and this key feature was observed in clinical isolates able to generate extended-  
231 spectrum AmpC β-lactamases (ESACs), as described above. The <sup>287</sup>SGN triplet is present  
232 in the sequence of such enzymes (87). Phylogroup B2 contains the specific conserved  
233 residues <sup>175</sup>K, <sup>193</sup>S, <sup>282</sup>I, <sup>288</sup>D, <sup>296</sup>R and <sup>300</sup>P, and is separated from phylogroup D by the  
234 residues <sup>185</sup>T and <sup>244</sup>T, in particular.

235 Finally, 78 protein sequences from uncultured bacteria obtained by metagenomic analyses  
236 on human fecal and environmental samples, originally found in the NCBI database as class  
237 C CMY-LAT-MOX-ACT-MIR-FOX  $\beta$ -lactamases, belong to this major cluster. They  
238 have been assigned to *E. coli*, *Shigella*, *E. fergusonii*, or *E. albertii*, and are characterized  
239 by a typical triplet (<sup>6</sup>HSE) (88-90).

240 One particular feature of interest is that all the protein sequences obtained from the genus  
241 *Hafnia* (*H. alvei*, *H. paralvei*) carry a two-residue deletion (residues 289-290) in H-10 and  
242 the R2-loop. The plasmid-encoded enzymes ACC-1 and ACC-4 are more than 99.4%  
243 identical to the AmpC of *H. paralvei*, a newly identified bacterial species (91,92).

244

#### 245 **Aeromonas**

246 The second major group identified included 318 protein sequences from *Aeromonas*, a  
247 genus for which many new species have been discovered over the last two decades. This  
248 is also the group in which the first inducible cephalosporinases (AsbA1, CAV-1, CepH,  
249 CepS) were detected, in *A. hydrophila* (43), *A. jandei* (93), *A. sobria* (94) and *A. caviae*  
250 (41). The group is highly diverse, with a sequence identity ranging from 50 to 100%.  
251 Thirty-six species from this group have been identified, including about twenty species  
252 pathogenic to humans, such as *A. caviae*, *A. dhakensis*, *A. veronii*, and *A. hydrophila* (95).  
253 Phylogenetic analysis identified two CS for *A. caviae*, *A. dhakensis*, *A. hydrophila*, and *A.*  
254 *salmonicida*, with sequence identities of 80 to 100%, suggesting that new species may be  
255 discovered in the future (96,97). Some of these species displayed a particular primary  
256 structure modification: the deletion of two or three residues (positions 301 to 303) in the  
257 R2-loop, as in *A. caviae* and in *A. dhakensis* (25).

258

259 **Acinetobacter**

260 Genus *Acinetobacter* has a long history of taxonomic changes, but is dominated by *A.*  
261 *baumannii*, the principal genomic species, which plays a major role in nosocomial  
262 infections, particularly in intensive care units (98,99). This major group is heterogeneous,  
263 with 500 protein sequences and 26 species, and with sequence identities ranging from 42  
264 to 100 % (100). The denomination ADC for “*Acinetobacter*-derived cephalosporinase”,  
265 followed by a number to distinguish between individual enzymes, was proposed because  
266 of the large numbers of *A. baumannii* variants or members of the Abc complex (101)  
267 (<http://bldb.eu/BLDB.php?prot=C#ADC>). In a previous analysis of 103 genomes, the  
268 *bla*<sub>ADC</sub>-like gene was found to be present in 13 validly named species, eight genomic  
269 species and six taxa (102). Three major clusters were identified: one cluster including the  
270 *A. baumannii*-*A. calcoaceticus* or Abc complex and a number of other species: *A. lactucae*  
271 (formerly known as *A. dijkshoorniae*), *A. nosocomialis*, *A. oleivorans*, *A. pittii*, and *A.*  
272 *seifertii*, a second cluster containing nine species: *A. apis*, *A. albensis*, *A. bereziniae*, *A.*  
273 *bohemicus*, *A. celticus*, *A. guillouiae*, *A. johnsonii*, *A. kyonggiensis*, and *A. rudis*, and a  
274 third cluster including a number of other species: *A. baylyi*, *A. beijerinckii*, *A. gyllenbergii*,  
275 *A. haemolyticus*, *A. junii*, *A. proteolyticus*, *A. parvus*, *A. soli*, *A. ursingii*, *A. tjernbergiae*,  
276 and *A. venetianus* (100). A large number of protein sequences were initially misidentified,  
277 for two reasons. The first was the substantial taxonomic modifications that had taken place  
278 and the second was the preferential use of standard biochemical methods and automated  
279 systems or devices widely used in clinical bacteriology laboratories, such as API 20NE,  
280 VITEK 2, Phoenix, Biolog, and MicroScan WalkAway, potentially leading to incorrect

281 identification. MALDI-TOF can facilitate correct identification of the members of the Abc  
282 complex to species level, provided that an accurate database is used (15,99). Accurate  
283 identification of the clinically important members of the Abc complex (*A. baumannii*, *A.*  
284 *pittii*, *A. nosocomialis*, *A. seifertii* and *A. dijkshoorniae*) and *A. calcoaceticus*, which is  
285 considered pathogenic, is possible only by molecular methods such as DNA-DNA  
286 hybridization (gold standard method) and DNA sequence-based analysis on various types  
287 of DNA sequences (16S rRNA, *rpoB*, 16S-23S intergenic spacer) (15). The 16S rRNA  
288 sequencing method is highly reliable at genus level, but discriminates poorly between  
289 species. By contrast, *rpoB* genes are highly variable and considered appropriate for  
290 *Acinetobacter* species identification. The multiplex PCR based on species-specific genes,  
291 such as *gyrB*, is simple, rapid (results obtained within 2 hours), and reproducible, but  
292 limited to major species: *A. baumannii*, *A. nosocomialis*, *A. pittii* and *A. calcoaceticus*.

293 One structural feature distinguishing these clusters was a conserved deletion of three  
294 residues in the R2-loop (residues 304-306) for the first two clusters (103,104). The third  
295 cluster, including *A. baylyi* producing ADC-8 with a low susceptibility pattern, particularly  
296 for cephalosporins, did not display this deletion (104,105). ADC-1 and ADC-68, naturally  
297 produced by *A. baumannii*, carry this deletion of three residues in the R2-loop (residues  
298 304-306) and were considered to be extended-spectrum AmpC (ESACs). Such enzymes  
299 typically hydrolyze penicillins and narrow- and expanded-spectrum cephalosporins and  
300 aztreonam, but not cefepime. Acquired resistance to cefepime or cefpirome has been  
301 reported to be associated with amino-acid substitutions — R148Q (close to YXN), V211A  
302 (within the  $\Omega$ -loop), and N287S (in helix H-10) — also conferring higher levels of  
303 resistance (4 to 64 times higher) to ceftazidime and cefotaxime (106,107). Ceftazidime

304 resistance resulted from overproduction of the ADC  $\beta$ -lactamase and the provision of  
305 strong promoter sequences by the insertion sequence IS*Aba1* (106,108).

306 In conclusion, the complexity of this bacterial group suggests that other species are likely  
307 to be identified, particularly given the very small size of some of the clusters identified.

308

### 309 **Pseudomonas**

310 A phylogenetic analysis of 582 class C protein sequences from *Pseudomonas* yielded a  
311 broad distribution, with at least 36 clusters. Sequence identity varied considerably, from  
312 42 to 100 %, between the 33 species producing class C  $\beta$ -lactamases from this group.  
313 Heterogeneity levels were particularly high for some species, such as *P. putida*, for which  
314 at least 11 clusters were observed, and for *P. fluorescens*, suggesting misidentification, as  
315 reported by subsequent comparative studies of genomes (109-114). Indeed, the  
316 *Pseudomonas* group is among the most diverse in terms of the species it contains, but, with  
317 the exception of *P. aeruginosa*, for which 430 sequences have been analyzed, its taxonomy  
318 is still under revision.

319 *P. aeruginosa* is one of the principal pathogens isolated from immunosuppressed patients  
320 and cases of hospital-acquired infections associated with multiresistance (115). It also  
321 causes chronic lung infections in patients with cystic fibrosis (116). The effective treatment  
322 of *P. aeruginosa* infections is challenging, because several mechanisms of resistance to  
323 antibiotics, including  $\beta$ -lactamases and efflux pump overexpression, have evolved in this  
324 species (117). An inducible chromosomal AmpC-type enzyme was identified, with a wild-  
325 type inactivation spectrum including various  $\beta$ -lactams, such as aminopenicillins, the  
326 oldest cephalosporins, and cephamycins. Following its overproduction due to mutations



327 altering the peptidoglycan recycling process, this cephalosporinase is a major source of  
328 resistance to ticarcillin, piperacillin, ceftazidime, and aztreonam (118,119). However,  
329 additional missense mutations have extended its inactivation spectrum to cefepime and  
330 cefpirome. Many variants, also observed as natural polymorphisms, have been named  
331 according to a nomenclature specifically developed for *P. aeruginosa*, as PDCs  
332 (*Pseudomonas*-derived cephalosporinases) (118,119). A phylogenetic analysis of 430  
333 sequences displaying 87 to 100 % identity yielded two clusters (C1, C2). The main cluster  
334 (C1), containing 416 protein sequences, had a consensus sequence with several variants  
335 (120). The second cluster (C2) was very small, comprising only 14 sequences. The  
336 polymorphism observed in cluster C1 had already been described in 44 antibiotic-  
337 susceptible strains with amino-acid substitutions at positions 53, 71, 79, 149, 178, and 365  
338 (119). However, other variants were observed, in particular in positions 121, 153, 174, 178,  
339 213, 216, 219, and 293, which served as mutation hotspots, thus extending the substrate  
340 specificity of PDC  $\beta$ -lactamases (see the section on ESACs).

341

#### 342 **Plasmid-encoded enzymes**

343 Chromosomal class C  $\beta$ -lactamases predominate, but plasmid-mediated enzymes are  
344 nevertheless important, and the protein sequences of 256 such enzymes were examined.  
345 Since their emergence in 1989, several types of plasmid-encoded AmpCs and their variants  
346 have been identified: ACC, ACT, CFE, CMY-2/BIL/LAT, DHA, FOX, MIR and  
347 MOX/CMY-1 (4,8,22). CMY-2 is the most common plasmid-encoded enzyme family,  
348 detected worldwide in isolates that do not naturally produce a cephalosporinase (*P.*  
349 *mirabilis*, *K. pneumoniae* and *S. enterica*, in particular), and also in *E. coli*, which has a

350 weak expression of the natural cephalosporinase. This family displays little variability,  
351 with only about 8% of amino acids varying in a comparison of 92 sequences, including  
352 BIL-1 and LAT-1. Other families varied in terms of the number of protein sequences and  
353 percent identity: ACC (2 and 99 %), ACT (10 and 86 %), CFE (2 and 92 %), DHA (15 and  
354 97 %), FOX (13 and 94 %), MIR (5 and 99 %), MOX/CMY-1 (10 and 75 %). Since the  
355 1990s, many modifications have been made to bacterial taxonomy, with the definition of  
356 new bacterial species, particularly in the genera *Citrobacter*, *Enterobacter*, and  
357 *Aeromonas*. Finally, phylogenetic analysis confirmed several changes in the identification  
358 of the progenitors for chromosomal and plasmid-encoded cephalosporinases (25,121-124)  
359 (Table 4). The rare class C  $\beta$ -lactamases SLC-1 and PAC-1, encoded by a gene in a  
360 chromosome-inserted Tn1721-like transposon, probably originate from an  
361 enterobacterium, but the origins of other enzymes (integron-encoded IDC-types, Alaska  
362 soil metagenomes LRA10-1, LRA13-1, LRA18-1) remain unknown (58,64,74,75).

363

364 Table 4 here

365

### 366 **Serine hydrolase, a class C $\beta$ -lactamase or a carboxylesterase VIII ?**

367 BLCs are usually genus-specific and chromosomally encoded, found mostly in Gram-  
368 negative bacteria, and more specifically in  $\alpha$ -,  $\beta$ - and  $\gamma$ -*Proteobacteria*. However, several  
369 clusters (e.g. *Legionella*, *Bradyrhizobium*, *Parachlamydia*) are clearly divergent, with low  
370 levels of sequence identity (between 10 and 20 %) (28) (Fig. 1). This early divergence is  
371 related to a limited number of highly conserved motifs or residues, most reported in Table  
372 3: <sup>43</sup>Y/G, <sup>54</sup>V/T, <sup>58</sup>T, <sup>60</sup>F/E, <sup>63</sup>A/G, <sup>64</sup>SXXK, <sup>94</sup>P, <sup>96</sup>L, <sup>109</sup>L, <sup>111</sup>T, <sup>138</sup>W, <sup>140</sup>P, <sup>145</sup>G, <sup>148</sup>R/Y,

373 <sup>150</sup>YXN/H, <sup>187</sup>T, <sup>202</sup>G/Y, <sup>229</sup>D, <sup>271</sup>W, <sup>315</sup>KXG, <sup>330</sup>P, <sup>335</sup>G, <sup>337</sup>V, <sup>339</sup>L, <sup>341</sup>N, <sup>357</sup>L and <sup>360</sup>L.  
374 Several bacteria from *Bacteroidetes* and *Firmicutes* (e.g. *Pelosinus fermentans*,  
375 *Sediminibacterium salmoneum*) also carry enzymes with some of these highly conserved  
376 residues and motifs: <sup>27</sup>G, <sup>44</sup>G, <sup>58</sup>T, <sup>60</sup>F, <sup>61</sup>E/F, <sup>63</sup>G, <sup>64</sup>SXXK, <sup>67</sup>K, <sup>94</sup>P, <sup>115</sup>G, <sup>144</sup>G, <sup>150</sup>YXN,  
377 <sup>200</sup>A, <sup>202</sup>G, <sup>220</sup>A/S, <sup>222</sup>G/S, <sup>229</sup>D, <sup>315</sup>K/HT/SG, <sup>319</sup>T, <sup>321</sup>GF, <sup>337</sup>V and <sup>341</sup>N. These enzymes  
378 are generally identified as serine hydrolases in databases, but with no additional  
379 bacteriological and enzymatic details that could be used to determine whether or not they  
380 should be considered as class C  $\beta$ -lactamases.

381 One family of enzymes (microbial carboxylesterases VIII, EC 3.1.1.1) mostly identified in  
382 metagenomic libraries from various environmental samples, including EstC, EstM-N1/N2,  
383 Est22, EstU1 and EstSRT1, appears to be phylogenetically and structurally related to BLCs  
384 (Fig. 1). These enzymes generally hydrolyze nitrocefin (EstC), cephalothin (Est22),  
385 cephaloridine, cefazolin (EstU1), and oxyiminocephalosporins (EstSRT1) (131-137). The  
386 EstB and Est22 enzymes selectively deacetylate cephalosporin-based substrates leaving the  
387 amide bond of the  $\beta$ -lactam ring intact (136,137), while for EstU1 it was not clear from the  
388 HPLC data if the observed pattern against cephalosporin substrates was due to  
389 deacetylation or amide bond hydrolysis of the  $\beta$ -lactam ring (131). The catalytic  
390 efficiencies of EstSRT1 for cephalothin, cefotaxime, and cefepime are similar to that of  
391 EstU1 for cefazolin (131). These enzymes bear the catalytic nucleophilic motif <sup>100</sup>S, <sup>103</sup>K  
392 and <sup>218</sup>Y (<sup>64</sup>S, <sup>67</sup>K and <sup>150</sup>Y according to SANC numbering), as well as other residues highly  
393 conserved in BLCs (Table 3). They have an overall structure consisting of a mixed  $\alpha/\beta$   
394 domain and a small helical domain, similar to that of class C  $\beta$ -lactamases (134,138-140).

395 The classification of these carboxylesterases as BLCs does not appear to be appropriate,  
396 given the total absence of  $\beta$ -lactamase activity for some of these enzymes, their low levels  
397 of *in vitro* antibacterial activity against  $\beta$ -lactams, including cephalosporins, their primary  
398 structure and their low levels of sequence identity to genuine BLCs. Nevertheless, these  
399 findings suggest that the promiscuous  $\beta$ -lactamase activity of some of these family VIII  
400 esterases may have evolved from BLCs or *vice versa*, and that some may have closer  
401 evolutionary relationships to BLCs (133).

402

### 403 **SECONDARY AND TERTIARY STRUCTURES**

404 The first crystallographic structure of an AmpC was reported in 1994, for the covalent  
405 complex between a phosphonate transition-state analog and the *E. hormaechei* P99  
406 (formerly known as *E. cloacae* P99) cephalosporinase, which belongs to the ACT family  
407 (141). Many structures from different families of class C  $\beta$ -lactamases have since been  
408 published, including ACC (142,143), ACT (141,144-156), ADC (103,157-162), CMH  
409 (163), CMY (154,164-168), EC (165,169-206), FOX (207-209), MOX (210,211), PDC  
410 (212-221) and TRU (222) enzymes, together with the halophilic  $\beta$ -lactamase (HaBLA)  
411 from *Chromohalobacter* sp. 560 (47) and the class C  $\beta$ -lactamase from a psychrophilic  
412 organism, *Pseudomonas fluorescens* (223). More than 200 structures of class C  $\beta$ -  
413 lactamases have been described, and an updated list of these structures can be found in the  
414 Beta-Lactamase DataBase (BLDB, <http://bldb.eu/S-BLDB.php>) (22).

415 The typical three-dimensional structure of class C  $\beta$ -lactamase contains two mixed  $\alpha/\beta$   
416 domains: one composed of nine antiparallel  $\beta$ -sheets and three  $\alpha$ -helices (H1, H10 and  
417 H11) on one side, and the other, composed of three small antiparallel  $\beta$ -sheets and eight  $\alpha$ -

418 helices (H2 to H9) on the other side (Fig. 2). Two additional structural elements important  
419 for the interaction with substrates,  $\Omega$ -loop and R2-loop, are located between H6 and H8  
420 (residues 189-225), and close to H10 (residues 288-309), respectively (see ref. (25) for a  
421 detailed analysis of residues involved in these regions). Structurally, class C  $\beta$ -lactamases  
422 belong to the *Beta-lactamase* PFAM family (PF00144) and share the same overall fold  
423 with class A and class D  $\beta$ -lactamases, penicillin-binding proteins (PBPs) and family VIII  
424 carboxylesterases.

425

426 Figure 2 here

427

## 428 **REGULATION AND EXPRESSION OF CLASS C $\beta$ -LACTAMASES**

429 In some Enterobacteriales (other than *E. coli*) and *P. aeruginosa*, *ampC* gene expression is  
430 weak and inducible. This induction involves several proteins (AmpR, AmpD and AmpG)  
431 and two mucopeptides (4,224-227). Nevertheless, the molecular mechanisms mediating  
432 AmpC overproduction in *P. aeruginosa* appear to be more complex than initially thought,  
433 as other proteins, such as AmpDh2, AmpDh3, and DacB (PBP4), have since been identified  
434 as involved in these mechanisms (228-230).

### 435 **Genetic context and regulation**

436 The *ampR* and *ampC* genes are linked. AmpR is encoded by a gene located immediately  
437 upstream from *ampC* and acts as a transcriptional activator, binding to the intercistronic  
438 region upstream from the *ampC* gene promoter. The *ampD* gene encodes an *N*-acetyl-  
439 anhydromuramyl-L-alanine amidase involved in recycling the products of peptidoglycan  
440 catabolism, including, in particular, the 1,6-anhydro-*N*-acetylmuramyl-tripeptide

441 (anhNAM-tripeptide). AmpD releases the tripeptide, which is then recycled for synthesis  
442 of the UDP-NAM pentapeptide (uridine 5'-pyrophosphoryl-*N*-acetylmuramic acid-  
443 pentapeptide), for integration into the neo-peptidoglycan. There is therefore a permanent  
444 balance between these two components in the cytoplasm.

445 Under normal conditions, AmpR associates with the UDM-NAM pentapeptide, and the  
446 resulting complex binds to the intercistronic region, thereby preventing transcription from  
447 both the *ampR* and *ampC* promoters. However, the presence of excess anhNAM-tripeptide  
448 alters this binding and promotes an increase in *ampC* transcription. Two clinical situations  
449 can result in an excessive increase in anhNAM-tripeptide levels: substantial degradation of  
450 the peptidoglycan due to the presence of  $\beta$ -lactams in the periplasmic space, and changes  
451 in the AmpD amidase preventing the recycling of this component.

452 In *E. cloacae* complex and *C. freundii*, the main cause of *ampC* overexpression is  
453 represented by amino-acid substitutions in AmpD, resulting in the constitutive production  
454 of large amounts of AmpC and increasing resistance to cefotaxime and ceftazidime.  
455 Several resistant clinical isolates or *in vitro* mutants displaying AmpC overproduction have  
456 been identified in the Enterobacterales, including *C. freundii* and *E. cloacae* with various  
457 amino-acid substitutions (e.g. W7G, H34A, S37R, Y63F, R80H, G82C, A94P/V, Y102,  
458 E116A, L117R, R108 D121G, D127G, N150I, H154N, A158D, K162H/Q, D164E/A,  
459 W171, A172L) capable of triggering constitutive  $\beta$ -lactamase production (231-235). In  
460 some cases, the *ampD* gene is truncated by a premature stop codon or an IS1 insertion, or  
461 an IS4321 element may be inserted into the promoter (231,236,237).

462 In *P. aeruginosa*, acquired resistance to amino- and ureido-penicillins, cephamycins, and,  
463 to a lesser extent, to oxyiminocephalosporins (ceftazidime, cefotaxime, ceftriaxone) and

464 aztreonam, is very often related to overproduction of the PDC  $\beta$ -lactamase, which is also  
465 linked to virulence (238). Several variants with *ampD* mutations displaying resistance *in*  
466 *vivo* and *in vitro* have been reported (239-242). As in the Enterobacterales, the principal  
467 genetic mechanisms involved missense mutations creating amino-acid substitutions  
468 (A84G, D61Y, G148A, A136V, G148A, S175L) or insertion sequences (IS1669).

469 The LysR-type transcriptional regulator AmpR was initially identified as involved in  
470 regulating the inducible class C  $\beta$ -lactamases produced by various Gram-negative rods,  
471 such as *C. freundii*, *E. cloacae*, *S. marcescens* and *P. aeruginosa*. However, recent results  
472 indicate that in *P. aeruginosa* AmpR regulates the expression of many genes involved in  
473 other pathways, such as quinolone resistance, quorum sensing and associated virulence  
474 phenotypes (243). Phylogenetic analyses revealed the presence of AmpR homologs in  
475 many  $\alpha$ -,  $\beta$ -, and  $\gamma$ -*Proteobacteria*, and several highly conserved residues were found in *C.*  
476 *freundii*, *E. cloacae*, and *P. aeruginosa* (243,244). AmpR loss leads to susceptibility to  $\beta$ -  
477 lactams, whereas constitutively high levels of *ampC* expression were reported for clinical  
478 isolates and *in vitro* strains with mutations resulting in AmpR amino-acid substitutions: *C.*  
479 *freundii* (S35F, G102D/E, D135A, Y264N) (4,245,246), *E. cloacae* (T84I, R86C,  
480 D135N/V, E274K) (231,247), and *P. aeruginosa* (A12R, D135N, G154R, G237A)  
481 (239,248-250). Transposon mutagenesis of *P. aeruginosa* strain PAO-1 and  
482 complementation experiments have generated two mutants with stronger  $\beta$ -lactam  
483 resistance due to transposition into two new genes (*mpl*, *nuoN*) (251).

484 AmpG, an intrinsic membrane protein, displays permease activity mediating the transport  
485 of muropeptides from the periplasm to the cytoplasm, such transport being essential for the  
486 induction of class C  $\beta$ -lactamases (252-254). Deletion of the gene encoding this protein in

487 *P. aeruginosa* results in a lower level of bacterial resistance to ampicillin. AmpG proteins  
488 are widespread and highly variable in Gram-negative bacteria (e.g. Enterobacterales, *A.*  
489 *baumannii*, *P. aeruginosa*). Genetic experiments showed that *ampG* genes from *E. coli* and  
490 *A. baumannii* can complement AmpG function in *P. aeruginosa* (253). In this species, the  
491 site-directed mutagenesis of some highly conserved AmpG residues (G29A, G29V,  
492 A129V, A197S and A197D mutants) results in a loss of resistance to ampicillin; *ampG*  
493 mRNA levels were found to be normal for two other mutants (A129T and A129D), but the  
494 proteins encoded had much lower levels of activity (253).

#### 495 **AmpC overproduction in *E. coli***

496 In *E. coli*, in the absence of the *ampR* gene, the expression of *ampC* is usually weak, under  
497 the control of a naturally non-efficient promoter (4). In this species, acquired resistance to  
498 old or narrow-spectrum cephalosporins, cephamycins (e.g. cefoxitin), or even  
499 oxyiminocephalosporins, is related to *ampC* overexpression due to the selection of a  
500 stronger promoter by mutation, or, less often, by insertion (255,256). The most important  
501 factors responsible for strengthening the *ampC* promoter are mutations creating a  
502 consensus -35 box (TTGACA) by T/A transversion at position -32 or C/T transition at  
503 position -42, and base-pair insertions increasing the distance between the -35 and -10 boxes  
504 to 17 or 18 bp (4,256,257). Mutations have also been identified within the attenuator region  
505 and the -10 box, but such mutations have little effect on *ampC* expression. Similar promoter  
506 mutations have been reported in strains from livestock, such as pigs or calves (258,259).

#### 507 **AmpC overproduction in *A. baumannii***

508 Overexpression of the chromosomal *ampC* gene in *A. baumannii* was observed following  
509 the acquisition of an IS element, mostly IS*Aba1*, resulting in a strong promoter and



510 resistance to expanded-spectrum cephalosporins (cefotaxime, ceftazidime) and aztreonam  
511 (260).

### 512 **Transferable $\beta$ -lactamases**

513 Most plasmid-encoded class C  $\beta$ -lactamases mobilized by transposons and insertion  
514 sequence elements, with the exception of the ACT-1, CFE-1, and DHA-types, are in a  
515 “derepressed” state (45,58,261,262). The genes encoding the ACC-1 and CMY-2 types are  
516 transcribed under the control of a strong promoter in the mobile element (*ISEcp1* or *IS26*)  
517 (263,264). The high level of *bla<sub>MIR-1</sub>* gene expression is due to the presence of a more  
518 efficient hybrid promoter upstream from the natural promoter (265). All these genes are  
519 often associated with integrons inserted in close proximity and forming composite  
520 structures with other transposable elements, including a gene cassette in a class 1 integrons  
521 characterized by rapid spread (58). Gene cassettes are under the control of a constitutive  
522 promoter specific for the gene cassette array. Their expression level is determined not only  
523 by promoter strength, but also by distance from the promoter (266). The removal of *ampC*  
524 gene cassettes from the integron may constitute a less costly control mechanism than the  
525 continuous overproduction of many plasmid-borne class C enzymes (267). However, three  
526 plasmid-encoded enzyme types with an *ampR* gene (ACT-1, CFE-1, DHA-1) and a genetic  
527 organization identical to those of chromosomal enzymes rarely cause resistance to  
528 oxyiminocephalosporins despite constitutively high levels of AmpC activity due to an  
529 amino-acid substitution (D135A) (243,246).

530 In conclusion, given the complexity of the regulation of *ampC* expression, the variability  
531 of these genes in databases and the small number of clinical examples of acquired  
532 resistance with the identification of hot spots for at least *ampR*, *ampD* and *ampG*, it is not

533 currently appropriate to analyze such mutants by genomic procedures, particularly in the  
534 absence of the initial susceptible clinical isolate. Classical methodologies are preferable for  
535 the detection of AmpC  $\beta$ -lactamase overproduction. These methods include phenotypic  
536 approaches assessing synergy between a cephamycin or an oxyiminocephalosporin and an  
537 inhibitor, such as cloxacillin or boronic acid (4). Nevertheless, the determination of  $\beta$ -  
538 lactamase activity with or without imipenem or ceftaxime induction, together with  
539 determinations of the mRNA levels for these genes by real-time RT-PCR, is probably much  
540 more accurate (119,253,268-270).

541

#### 542 **ACQUIRED EXTENDED-SPECTRUM CEPHALOSPORINASES (ESACs)**

543 In Gram-negative bacteria, such as the Enterobacterales, various genetic events may  
544 increase the level of resistance, particularly to oxyiminocephalosporins, such as  
545 ceftazidime, cefotaxime, and, to a lesser extent, cefepime, and ceftipime. The evolution of  
546 such resistance led to a new denomination: ESAC, for extended spectrum AmpC  $\beta$ -  
547 lactamases (271). The first ESAC was identified in an *E. cloacae* isolate (GC1) in Japan in  
548 1992, based on the duplication of three amino acids at positions 208-210 ( $\Omega$ -loop) and  
549 constitutive production (10).

550 In *E. coli*, AmpC is constitutively produced in small amounts in the absence of the *ampR*  
551 gene, under the control of a weak promoter (4). Within this species, acquired resistance to  
552 old or narrow-spectrum cephalosporins (e.g. ceftaxime) is related to the overproduction of  
553 AmpC due to the selection of a strong promoter generated by mutation, or, less often, by  
554 insertion (255,256). A second step in the development of resistance involved the  
555 acquisition of plasmid-encoded AmpCs with a higher level of resistance to ceftazidime and

556 aztreonam, for example (8). Finally, an even higher level of resistance (e.g. to cefepime or  
557 cefpirome) was developed following the emergence of an ESAC at low prevalence ( $\leq 1\%$ )  
558 in human clinical isolates and even isolates from cattle (16,272-274).

559 The genetic determinism of ESACs, leading to an increase in the catalytic efficiency of  
560 these enzymes against extended-spectrum cephalosporins, is based principally on missense  
561 mutations resulting in amino-acid substitutions, with structural modifications to the R1 ( $\Omega$ -  
562 loop between residues 189 and 225) or R2 (H10 helix between residues 280 and 292 and/or  
563 R2-loop between residues 286 and 310) binding sites. Insertions or duplications in H10 or  
564 the R2 loop were also observed (Table 5).

565

566 Table 5 here

567

568 Most ESACs belong to phylogenetic group A, but others belong to phylogenetic group B1  
569 and are characterized by the presence of the following conserved residues: <sup>235</sup>R, <sup>238</sup>M, <sup>239</sup>N,  
570 <sup>241</sup>R, <sup>245</sup>D, <sup>281</sup>S, <sup>288</sup>G and <sup>296</sup>H (16,87,273,276). Nevertheless, two isolates with different  
571 residues in these positions have been reported (281). A single *E. coli* strain (HKY28)  
572 isolated from urine in Japan was found to produce an ESAC with a tripeptide deletion  
573 (<sup>286</sup>GS<sup>289</sup>D) altering binding to extended-spectrum cephalosporins, but also to sulbactam  
574 and tazobactam (277) (Table 5). Reversion of the deletion through a nine-base insertion  
575 restored the typical inhibitor-resistant phenotype of class C enzymes and decreased the  
576 level of resistance to cefepime and cefpirome. Finally, other positions affecting the ESAC  
577 phenotype were identified by the selection of mutants *in vitro* or by site-directed

578 mutagenesis, resulting in a greater structural flexibility or affinity for extended-spectrum  
579 cephalosporins (195,320).

580 Many BLCs (e.g. those of *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Morganella*, and  
581 *Serratia*) are inducible, opening up possibilities for higher levels of production, resulting  
582 in stronger resistance to aminopenicillins, old cephalosporins and ceftazidime or  
583 cefotaxime, and even to cefepime if the mutated enzyme is overproduced (321).

584 Additionally, a significant proportion of strains has constitutive overproduction of class C  
585 enzymes, often after selection with  $\beta$ -lactam antibiotics. However, the risk of transitions  
586 between susceptibility and intermediate resistance (S/I) and between susceptibility and  
587 resistance (S/R) to cefepime is species-dependent. It is particularly high for the *E. cloacae*  
588 complex (66.3%), non-negligible for *H. alvei* (36.4%), moderate for *Citrobacter* and  
589 *Proteus* (18.1-21.9%) and *S. marcescens* (12.3%), low for *K. aerogenes* (1.1%) and  
590 inexistent for *M. morgani* (0%) (322). As summarized in Table 5, expansion of the  
591 inactivation spectrum results from missense mutations at several mutation hotspots (around  
592 triplet <sup>150</sup>YAN,  $\Omega$ -loop, H10-helix and R2-loop), from duplications or deletions of 2-4  
593 amino acids in the H10 helix (position 293), or insertions of amino acids into the  $\Omega$ -loop.

594 In some cases, two such events may be combined (Table 5). Various examples of increased  
595 catalytic efficiency ( $k_{cat}/K_m$ ) of these enzymes, particularly against extended-spectrum  
596 cephalosporins (ceftazidime, cefotaxime, cefepime), are illustrated in Table 6.

597

598 Table 6 here

599

600 In *P. aeruginosa*, acquired resistance to amino- and ureidopenicillins, cephamycins, and,  
601 at low levels, to oxyiminocephalosporins (ceftazidime, cefotaxime, ceftriaxone) and  
602 monobactams (aztreonam), is frequently related to overproduction of the AmpC  $\beta$ -  
603 lactamase (14,118,238,240,319). ESACs emerged in *P. aeruginosa* several years after  
604 Enterobacterales, and such enzymes were identified mainly from clinical sources, but also  
605 from *in vitro* studies. They differ from the wild-type AmpC of *P. aeruginosa* by various  
606 amino-acid substitutions, deletions or insertions in four regions in the vicinity of the active  
607 site: the  $\Omega$ -loop, the H10-helix, the H2-helix, and the C-terminal end of the protein  
608 (119,318,319) (Table 5).

609 Genus *Aeromonas* may have been the origin of several chromosomal (e.g. MOX-3, MOX-  
610 10) or plasmid-encoded  $\beta$ -lactamases (e.g. MOX-1, MOX-2, CMY-1, CMY-8, CMY-19)  
611 identified on several occasions from clinical isolates, mostly in Enterobacterales (e.g. *K.*  
612 *pneumoniae* and *E. coli*). The first plasmid-encoded enzyme, MOX-1, was identified in a  
613 *K. pneumoniae* isolate with a high level of resistance to various broad-spectrum  $\beta$ -lactams,  
614 including moxalactam, flomoxef, ceftizoxime, cefotaxime, and ceftazidime (323).  
615 According to its kinetic parameters, cephalothin was its ideal substrate, and it had good  
616 activity against benzylpenicillin, but poor activity against cloxacillin and piperacillin.  
617 Moxalactam and cefoxitin were also hydrolyzed, but ceftazidime and cefepime were poor  
618 substrates, with very high  $K_m$  values (Table 6). Finally, aztreonam was found to inhibit  
619 MOX-1 (69). The X-ray crystallographic structure of MOX-1 suggested that residues 303-  
620 306 show a significant structural flexibility, possibly underlying the unique substrate  
621 profile of this enzyme, which can hydrolyze penicillins, cephalothin, expanded-spectrum  
622 cephalosporins, cefepime and moxalactam (210). The position of H10-helix in both MOX-

623 1 and CMY-10 is shifted further away from the catalytic serine residue compared with the  
624 AmpCs from *E. coli*, *E. hormaechei* P99 (formerly known as *E. cloacae* P99) and *E.*  
625 *cloacae* GC1, whereas that of the AmpC from *P. aeruginosa* occupies an intermediate  
626 position. Such structural features may underlie the extended substrate profile of MOX-1  
627 and CMY-10, which are also considered to be ESBLs, but have never been called ESACs  
628 (164,210). Finally, the susceptibility profile of these enzymes is characterized by a high  
629 level of hydrolysis for cephamycins, oxacephems (e.g. cefoxitin) and moxalactam,  
630 increased resistance to cefotaxime compared with ceftazidime and aztreonam, and  
631 resistance to cefpirome and/or cefepime (50,129,293,313,316,324,325). The deletion of  
632 three residues in the R2-loop (positions 301-303) appears to be responsible for the  
633 expanded spectrum activity of CMY-10, and further mutation around this deletion in the  
634 P99 enzyme extended its substrate spectrum by widening the R2 region (Table 5) (164).  
635 Globally, all chromosomal (e.g. MOX-3, MOX-10, MOX-11) or plasmid-encoded (e.g.  
636 MOX-1, MOX-2, MOX-14, CMY-1, CMY-8, CMY-8b, CMY-9, CMY-10, CMY-11,  
637 CMY-19)  $\beta$ -lactamases from this family feature the sequence  
638 <sup>289</sup>AKVILEANPTAA $\Delta\Delta\Delta$ PRESG<sup>309</sup>S in the conserved R2 region (25). An increase in  
639 resistance to cefepime was also achieved by an additional amino-acid substitution (I292S)  
640 in the H10-helix region, as observed in CMY-11 and CMY-19 relative to CMY-9 (313)  
641 (Table 5).  
642 The other enzymes from *Aeromonas* include a cluster of plasmid-encoded FOX-type  
643 enzymes without the amino-acid deletion at positions 301-303. These enzymes have a  
644 susceptibility profile characterized by a higher degree of resistance to cefoxitin (the origin  
645 of the family name, FOX) and to ceftazidime compared with cefotaxime (280,328,333-

646 335). No ESACs have been identified among FOX variants (22). A tripeptide deletion  
647 (<sup>286</sup>GN<sup>288</sup>S) in the R2-loop of *E. coli* HKY28 led to an extended hydrolysis spectrum, but  
648 the same deletion by site-directed mutagenesis in FOX-4 did not increase catalytic  
649 efficiency for ceftazidime, cefotaxime, or cefepime, despite large differences in  $K_m$  and  $k_{cat}$   
650 values (Table 6). A decrease in the MIC for cefoxitin was obtained in both *E. coli* HKY28  
651 and FOX-4  $\Delta$ 286-288, together with a slight increase in susceptibility to clavulanate,  
652 sulbactam and tazobactam (329).

653 Structural studies have confirmed the importance of the  $\Omega$ - and R2-loops for modulating  
654 the catalytic activity of class C  $\beta$ -lactamases. In CMY-10, a three-amino acid deletion in  
655 the R2-loop appears to underlie the extended spectrum activity (164), whereas a two-amino  
656 acid deletion in the R2-loop of CMH-family AmpC enzymes from *E. cloacae* Ent385 leads  
657 to reduced susceptibility to ceftazidime-avibactam and cefiderocol (163). Similarly, the  
658 Y221H mutation in CMY-136 induces an important change in the confirmation of the  $\Omega$ -  
659 loop, widening the active site cavity and conferring resistance to many  $\beta$ -lactams and  
660 combinations, including ceftolozane/tazobactam (167).

661

## 662 **NATURAL ESACs**

663 The term “naturally occurring ESAC” has been proposed for the enzymes in *E. coli* isolates  
664 with increased hydrolysis of oxyiminocephalosporins, including cefepime and cefpirome  
665 (MICs greater than or equal to 16  $\mu$ g/ml and 0.5  $\mu$ g/ml for ceftazidime and cefepime,  
666 respectively, without a positive synergy test with clavulanic acid), and resistance to  
667 amoxicillin and to amoxicillin/clavulanic acid (16). As for class A ESBLs, which are  
668 sometimes encoded by chromosomal genes (e.g. OXY-types for *K. oxytoca*) (336), both

669 chromosomal and natural ESACs have been identified in *A. baumannii* (103), in which  
670 these  $\beta$ -lactamases are the principal source of resistance (99). The genes encoding ADC-  
671 type  $\beta$ -lactamases are non-inducible and generally expressed at low levels, resulting in  
672 resistance to penicillins and narrow-spectrum cephalosporins (33,101). However,  
673 overexpression may be observed following the acquisition of an IS element, principally  
674 IS*Aba1*, providing a strong promoter, and this may lead to resistance to expanded-spectrum  
675 cephalosporins (cefotaxime, ceftazidime) and aztreonam (260). All ADC-type  $\beta$ -  
676 lactamases (about 450 protein sequences examined) are characterized by a deletion of three  
677 residues (positions 304-306) in the R2-loop that enhances their catalytic efficiency against  
678 these clinically important drugs compared with enzymes without this deletion  
679 (103,104,162). Clinical variants with higher levels of resistance (4 to 64-fold increase) have  
680 been detected, with V211A ( $\Omega$ -loop) or N287S (H10-helix) substitutions (106) (Table 5).  
681 Moreover, acquired resistance to cefepime or ceftazidime and increased hydrolysis  
682 efficiency (ESAC) were induced by the P210R substitution and duplication of A215 in the  
683  $\Omega$ -loop (ADC-33) or by the substitution R148Q in the P2-loop (ADC-56) (107,317). ADC-  
684 68 has seven amino-acid substitutions compared with ADC-1, one of which (<sup>321</sup>G) is  
685 located in the C-loop and other two (<sup>192</sup>A and <sup>217</sup>D) in the  $\Omega$ -loop. The overall structures of  
686 ADC-68 and ADC-1 are conserved, but there are marked structural differences in the  $\Omega$ -  
687 loop and the C-loop. In particular, residues <sup>217</sup>D and <sup>321</sup>G in ADC-68 make a major  
688 contribution to the structural differences between these two ADC-type  $\beta$ -lactamases (162).  
689 There is a two-amino acid deletion (residues 301-302) in the R2-loop of AmpC from two  
690 species, *A. dhakensis* and *A. caviae* (25), but an equivalent deletion in AQU-1 does not  
691 seem to affect the usual  $\beta$ -lactam resistance phenotype. However, cefotaxime monotherapy



692 should be used with caution for severe *A. dhakensis* infections, because such treatment  
693 could lead to the selection of variants with constitutively high levels of  $\beta$ -lactamase  
694 production. Nevertheless, cefepime susceptibility was conserved in all cases studied (34).  
695 A specific structural alteration to the R2-loop (deletion of two amino acids in positions  
696 289-290) was observed in all *Hafnia* protein sequences, and this feature is unique among  
697 Enterobacterales (25,85,142) (Table 5). Furthermore, ACC-1 confers an unusual and  
698 unique pattern of susceptibility to  $\beta$ -lactams, with higher MICs for ceftazidime and  
699 cefotaxime than for ceftazidime (4,280). In addition to the two-amino acid deletion in  
700 positions 289-290 mentioned above, ACC-1 features two other remarkable structural  
701 alterations, one in the  $\Omega$ -loop (<sup>213</sup>ME instead of <sup>213</sup>PG) and another along the active-site  
702 rim (<sup>120</sup>F instead of <sup>120</sup>Q) (25,142). Moreover, ACC-2, a cephalosporinase encoded by an  
703 inducible chromosomal gene, displays marked hydrolysis activity against ceftazidime in  
704 particular (309), in opposition to the widely accepted view that ceftazidime is resistant to  
705 hydrolysis by class C enzymes, even when they are overproduced (337). ACC-2 is also  
706 strongly inhibited by ceftazidime (309).

707 In conclusion, these natural ESACs constitute a threat because of their resistance to  
708 cefepime and/or ceftazidime, or even to carbapenems if they are overproduced, combined  
709 with porin loss and/or efflux pump activation.

710

## 711 **CARBAPENEM RESISTANCE**

712 Carbapenem resistance has become a serious threat to public health (338). It has spread  
713 worldwide in Gram-negative bacteria, and is continuing to increase due to the production  
714 of plasmid-encoded carbapenemases by Enterobacterales and by nonfermenting organisms

715 (mainly *A. baumannii* and *P. aeruginosa*) (339,340). The main mechanism underlying  
716 carbapenem resistance is the production of highly transmissible carbapenemases, such as  
717 KPC-types (class A), IMP-, NDM- or VIM-types (class B) and OXA-types (class D).  
718 Carbapenems, which diffuse efficiently across the native outer membrane of Gram-  
719 negative bacteria, are poor substrates for class C  $\beta$ -lactamases (4). However, they may act  
720 as inhibitors in some cases, due to the high affinity of AmpCs for these antibiotics,  
721 suggesting that resistance may be acquired by a mechanism known and sometimes  
722 described as “trapping” (341), in which the cephalosporinase has a good affinity for the  
723 substrate combined with a very slow hydrolysis (342-345).

724 Nevertheless, several clinical failures and *in vitro* studies have been published concerning  
725 chromosomally encoded and transmissible class C  $\beta$ -lactamases with weak carbapenem  
726 hydrolysis activity, suggesting poor carbapenemase activity (287,339,346). Such resistance  
727 was always obtained from the combination of a high production of  $\beta$ -lactamase (a  
728 hyperproducing mutant of a chromosomally-encoded enzyme or a plasmid-mediated  
729 enzyme) with another mutation-driven mechanism, such as porin loss (OmpC, OmpF,  
730 OmpG, OmpK35, OmpK36) or efflux overexpression, which was essential for carbapenem  
731 resistance, preferentially against ertapenem, then imipenem and finally meropenem  
732 (280,347) (Table 7). The prevalence of such non-enzymatic mechanisms is low and differs  
733 between bacteria, with relatively few occurrences among Enterobacterales (mostly in *K.*  
734 *pneumoniae*, but also in *E. coli*, *P. mirabilis*, and *S. enterica*).

735 Within the genus *Enterobacter*, carbapenem resistance is primarily due to the  
736 hyperproduction of chromosomal *ampC* associated with defective penetration of the  
737 carbapenemases in the bacterium (348). However, *E. kobei* from the *E. cloacae* complex

738 group naturally produces an ACT-28-type AmpC  $\beta$ -lactamase that hydrolyzes imipenem  
739 more efficiently (349) and several plasmid-encoded AmpCs show intrinsically low  
740 carbapenemase activity (CMY-10, and to a lesser extent, CMY-2 and ACT-1) (280,346).  
741 Although in *A. baumannii* carbapenem resistance is mainly related to class B (VIM-, IMP-  
742 and NDM-type) and class D (OXA-type) carbapenemases, the combination of  
743 overproduced class C  $\beta$ -lactamase and porin loss was also proposed for this species, which  
744 produces a natural ESAC with a deletion in the R2-loop and a low binding affinity for  
745 imipenem (103,162,350-352). No carbapenemase activity was detected in crude extracts,  
746 for example, so it was assumed that porin loss (CarO, Omp22-33, Omp33-36, Omp37,  
747 Omp43, Omp44, and Omp47) was the most likely mechanism (339,350,353-355).

748 Carbapenems (e.g. imipenem and meropenem) are important drugs to treat *P. aeruginosa*  
749 infections. Carbapenem resistance is mediated by various mechanisms, including the  
750 production of a carbapenemase (mostly class B enzymes), the overproduction of efflux  
751 pumps (mostly MexA-MexB), the overproduction of class C enzymes (e.g. PDC-type  $\beta$ -  
752 lactamases) and decreases in porin expression (mostly OprD), with combinations of these  
753 mechanisms observed in many cases (119,268,356,357). In *P. aeruginosa*, transmissible  
754 class B carbapenemases or MBLs are considered to be the most clinically relevant source  
755 of resistance. Class D (oxacillinases) enzymes are also frequently encountered, and  
756 sometimes even class C  $\beta$ -lactamases, which are not real carbapenemases and have a low  
757 carbapenem hydrolysis capacity. The combination of PDC overproduction with a decrease  
758 in outer membrane permeability (OprD) and/or the overexpression of efflux systems  
759 (MexA-MexB-OprM) has been shown to lead to carbapenem resistance (119,268,356,358-  
760 360) (Table 7). Various studies on clinical isolates have highlighted the difficulty of clearly

761 defining the contribution of each mechanism, as resistance is generally a multifactorial  
762 phenomenon. However, most imipenem- and meropenem-resistant isolates display high  
763 levels of PDC-type enzymes and increased efflux pump expression (268).

764

765 Table 7 here

766

## 767 **RESISTANCE TO INHIBITORS**

### 768 **Early inhibitors**

769 The first inhibitors to be developed (e.g. clavulanate, sulbactam, tazobactam) were inactive  
770 against a majority of class C  $\beta$ -lactamases, but some older combinations have been  
771 revamped (e.g. ceftolozane-tazobactam) because ceftolozane has a reduced affinity for  
772 PDC enzymes and is not, therefore, hydrolyzed (371). Ceftolozane also has a high potency  
773 against *P. aeruginosa* when used alone. Resistance may be acquired during treatment and  
774 is mediated principally by transmissible  $\beta$ -lactamases (e.g. ESBLs and serine  
775 carbapenemases, such as KPCs). Several missense mutations or deletions in PDC genes  
776 have also been reported in *P. aeruginosa* isolates displaying overexpression of these genes  
777 and in studies performed *in vitro*, but the underlying mechanisms of resistance (such as  
778 combination with porin deficiency and overexpression of various efflux pumps) remain  
779 poorly defined (119,318,319,366,372-376).

### 780 **Avibactam**

781 Novel non- $\beta$ -lactam (diazabicyclooctane or DBO)  $\beta$ -lactamase inhibitors, such as  
782 avibactam, with a high affinity for a wide range of Ambler class A (e.g. ESBLs and KPC-  
783 types), class C and some D  $\beta$ -lactamases (e.g. OXA-48) have been developed and showed

784 encouraging results in clinical trials (371,377). Avibactam is currently available in  
785 combination with ceftazidime, and is being used to combat multidrug-resistant Gram-  
786 negative bacterial infections, which are on the rise worldwide, including carbapenem-  
787 resistant Enterobacterales in particular. These inhibitors are not active against class B  
788 metallo- $\beta$ -lactamases (e.g. NDM, VIM) and resistance to the ceftazidime-avibactam  
789 combination or combinations of avibactam with other antibiotics have been reported in  
790 patients treated since 2015 and in screens *in vitro* (377-383). This type of resistance has  
791 yet to be fully elucidated, but several possible underlying mechanisms have been reported.  
792 The most frequently encountered resistance mechanism is enzymatic, due to mutations  
793 identified mostly in transmissible class A (e.g. KPC and ESBLs) and some class D (e.g.  
794 OXA-48)  $\beta$ -lactamases. Other combined mechanisms have been reported, including  
795 membrane impermeability and efflux (366,384), and there have been several reports of  
796 PBP3 mutations leading to ceftazidime-avibactam resistance (385-387). Finally, the  
797 overexpression of chromosomally- or plasmid-encoded AmpCs, with or without mutations  
798 and even in combination with porin deficiency appears to make only a small contribution  
799 to resistance (13,14,214). Avibactam inhibits the *E. cloacae* CHE ESAC less efficiently  
800 than other *E. cloacae* AmpC proteins, due to a subtle rearrangement of the binding site  
801 (13). *In vitro* studies with *P. aeruginosa* showed that high-level resistance to combinations  
802 of avibactam with ceftazidime or aztreonam occurs with low frequency. In all cases, the  
803 mutant displayed alterations to the chromosomal *ampC* gene, mostly deletions of various  
804 sizes (5 to 19 residues) in the  $\Omega$ -loop region of AmpC (14). Eight highly conserved residues  
805 made key contributions to binding interactions with avibactam (<sup>64</sup>S, <sup>67</sup>K, <sup>120</sup>Q, <sup>150</sup>Y, <sup>152</sup>N,  
806 <sup>315</sup>K, <sup>316</sup>T and <sup>346</sup>N). The PAC-1 enzyme, recently identified in four *P. aeruginosa* isolates

807 and encoded by a gene on a chromosome-inserted Tn1721-like transposon, mediates very  
808 high levels of resistance to  $\beta$ -lactams, such as ceftazidime, cefepime, and ceftolozane alone  
809 or in combination with avibactam or tazobactam (74). Resistance to the ceftazidime-  
810 avibactam combination has been attributed to a deletion of two amino acids in the R2 loop  
811 of the AmpC  $\beta$ -lactamase produced independently by *Enterobacter*, which simultaneously  
812 causes resistance to cefepime and carbapenems and reduced susceptibility to cefiderocol,  
813 a novel siderophore cephalosporin (299).

814 Six carbapenem-resistant clinical isolates of *K. pneumoniae* in China were recently shown  
815 to produce KPC-2, a class A carbapenemase, and CMY-172, a new CMY-2-like class C  $\beta$ -  
816 lactamase (291). CMY-172 mediates high levels of resistance to  $\beta$ -lactams, including  
817 ceftazidime, cefotaxime, and cefepime, and to the ceftazidime-avibactam combination.  
818 This resistance results from a major modification (<sup>290</sup>KVA deletion) to the R2-loop (ESAC)  
819 and an amino-acid substitution, N346I (Table 5). The variant retains all the highly  
820 conserved residues for binding to avibactam (<sup>64</sup>S, <sup>67</sup>K, <sup>120</sup>Q, <sup>150</sup>Y, <sup>152</sup>N, <sup>315</sup>K, <sup>316</sup>T) except  
821 for <sup>346</sup>N (216). In addition, the residue in position 346, which is well conserved among  
822 AmpC-type enzymes, modulates the hydrolysis spectrum of cephalosporinases (327).

823 More generally, *in vitro* resistance to ceftazidime-avibactam and aztreonam-avibactam was  
824 examined among Enterobacterales, showing that resistance associated with changes to  $\beta$ -  
825 lactamases was seen only for mutants of AmpC. The mutants R148H, G156R/D, N346Y  
826 or small deletions at positions 289-294 were obtained with ceftazidime-avibactam, whereas  
827 with aztreonam-avibactam the Y150C or N346Y substitutions were observed (388).

828 **Relebactam**

829 The newer imipenem-relebactam combination recently approved by the FDA has  
830 antimicrobial activity against Enterobacterales and *P. aeruginosa* strains producing class  
831 A and C  $\beta$ -lactamases, through the strong inhibition of KPC and AmpC enzymes (371,389).  
832 Isolates resistant to this combination, due to porin loss, have been reported in  
833 *Enterobacteriaceae* (390,391). There are *in vivo* and *in vitro* examples of resistance to this  
834 combination in *P. aeruginosa* mediated by class C enzymes, but resistance was again  
835 multifactorial, dependent on OprD loss, cephalosporinase overproduction, and mutations  
836 of the genes encoding the MexAB-OprM efflux system pump and the peptidoglycan  
837 recycling machinery (371,392,393).

#### 838 **Vaborbactam**

839 Meropenem-vaborbactam is a fixed-dose combination of a carbapenem antibiotic and a  
840 novel boronic acid-based  $\beta$ -lactamase inhibitor. It has *in vitro* activity against  
841 Enterobacterales (e.g. *K. pneumoniae*) producing class A (e.g. ESBL, KPC), class C  
842 (mostly plasmid-encoded enzymes), and also some class D enzymes (157,159,175,394).  
843 The impact of vaborbactam on class C enzymes appears to be limited, but resistance may  
844 nevertheless result from combinations of mechanisms, including the overexpression of  
845 ESAC mutants after cefepime treatment, associated with OmpK35 and OmpK36 porin  
846 deficiencies due to the insertion of an IS903-like element, as recently reported in a clinical  
847 isolate of *E. hormaechei* (299). This isolate was multidrug-resistant, with resistance even  
848 against ceftazidime-avibactam, meropenem-vaborbactam and imipenem-relebactam  
849 combinations. This resistance should serve as an important warning that cephalosporins  
850 and carbapenems should be used in a judicious manner, whether used alone or in  
851 combination with these novel inhibitors.

852

## 853 **NEW CEPHALOSPORINS**

### 854 **Cefiderocol**

855 Cefiderocol is an advanced injectable siderophore cephalosporin active against MDR and  
856 XDR Gram-negative rods, including producers of various  $\beta$ -lactamases, including  
857 members of Enterobacterales, *P. aeruginosa*, *A. baumannii*, *Stenotrophomonas maltophilia*,  
858 *B. cepacia* and *B. pseudomallei* (395-398). Through its catechol siderophore moiety,  
859 cefiderocol exploits iron-transport systems *via* a Trojan-horse strategy, navigating the  
860 bacterial periplasm and evading various  $\beta$ -lactamases, including ESBLs, KPC (class A),  
861 NDM, IMP, VIM (class B) and various class C and D enzymes (OXA-23, OXA-24, OXA-  
862 48 and OXA-51), as well as other mechanisms of resistance (399-401). Resistance to  
863 cefiderocol may be due to a two-amino acid deletion in the R2 loop of the AmpC of *E.*  
864 *cloacae* complex (163,299), but can also be acquired through the loss of energy-  
865 transducing proteins or catecholate receptors, such as PirA, PiuA, PiuD and TonB in  
866 Enterobacterales, *P. aeruginosa* and *A. baumannii* (400,402-404).

867

## 868 **CONCLUSIONS**

869 BLCs have a highly variable primary structure, with minimum sequence identity levels as  
870 low as 20% (28). However, the three principal catalytic motifs that are characteristic for  
871 this molecular class (<sup>64</sup>SXSK, <sup>150</sup>YXN and <sup>315</sup>KTG) are highly conserved, together with at  
872 least 70 other residues displaying >90% conservation. Some of these other residues,  
873 including <sup>60</sup>F, <sup>61</sup>E, <sup>63</sup>G, <sup>145</sup>G, <sup>148</sup>R, <sup>318</sup>T, <sup>321</sup>G, <sup>322</sup>F, <sup>325</sup>Y and <sup>328</sup>F, are located around the  
874 conserved catalytic motifs. Generally, with the exception of a few sequences corresponding



875 to serine hydrolases often identified by BLAST analysis, structural classification should be  
876 based on the notion of “genus”. Genera contain highly variable numbers of species, and  
877 future taxonomic reorganizations are possible, as already observed for the genus *Erwinia*,  
878 for example, with the discovery of *E. teleogrylli*, or for the genus *Pseudomonas*, with  
879 definition of the species *P. putida* and *P. fluorescens*.

880 The considerable increase in the number of protein sequences present in databases for  
881 several species has made it possible to confirm the existence of a certain degree of  
882 polymorphism, the large number of clusters observed (more than 10 for *E. coli/Shigella*)  
883 making it possible to define consensus sequences for each cluster or phylogroup (16). By  
884 contrast, enzymes of the DHA- and FOX-types, encoded by plasmid-borne genes, and the  
885 species from which they originate, *M. morgani* and *A. allosaccharophila*, respectively,  
886 display little polymorphism.

887 With the development of high-throughput sequencing, this notion of *ampC* gene  
888 polymorphism complicates the detection of variants responsible for a significant increase  
889 in the MICs of  $\beta$ -lactams, particularly for oxyiminocephalosporins (e.g. cefotaxime,  
890 ceftazidime, cefepime, and cefpirome), monobactams (e.g. aztreonam), and carbapenems.  
891 However, most infections are identified in hospital inpatients, from whom several isolates  
892 of the same species can be obtained, therefore facilitating the detection of such variants.  
893 Nevertheless, in the absence of an isolate obtained at the start of treatment, certain variants,  
894 such as insertions, duplications or deletions, appear to be easy to detect, whereas others,  
895 such as the substitution of a single residue in a hotspot, may be much harder to identify.  
896 The diverse examples of ESAC-producing strains observed in clinical practice demonstrate  
897 that genetic modifications similar to those described above essentially affect three regions

898 (around the <sup>150</sup>YXN motif, the Ω-loop and the H10-helix/R2-loop). Surprisingly, these  
899 regions vary to different extents in different species, with a limited number of conserved  
900 residues. The notion of a “consensus sequence” should therefore favor the possible  
901 detection of ESACs in the framework of increasingly frequent genotype/phenotype  
902 analyses (Fig. 3). Such diversity at the heart of species, particularly for the H10-helix/R2-  
903 loop region, could account for the considerable variation of MICs between substrates, or  
904 the diversity of the β-lactam resistance and kinetic constants reported. Wild-type bacterial  
905 species have variable MICs for β-lactams (<https://mic.eucast.org/>), suggesting that  
906 combinations of other, non-enzymatic resistance mechanisms may be at work.

907

908 Figure 3 here

909

910 It should not be forgotten that the chromosomal *ampC* gene is subject to regulation, and  
911 thus is dependent on other genes, particularly as it is inducible in most of the bacterial  
912 species producing the corresponding enzyme. Diverse genetic events can, therefore, occur  
913 in the intercistronic space, generating a strong promoter, or in other genes, such as *ampR*,  
914 *ampD*, and *ampG* genes, to generate mutants displaying derepression or overexpression.  
915 The analysis would not really be complete without an examination of several systems  
916 involved in the transfer of β-lactams across the cell wall, such as porins (e.g. OmpC, OmpF,  
917 OmpK15, OmpK36), and efflux systems (e.g. AcrAB-TolC, MexAB-OprM) (387,405-  
918 409).

919 For medical biologists, the identification of a β-lactamase from one of the four known  
920 molecular classes is based on the presence of at least three motifs (17). The accumulation

921 of a large number of protein sequences in current databases, with the development of WGS  
922 in particular, has made it possible to compare almost 4000 protein sequences from class C  
923  $\beta$ -lactamases, facilitating classification within clusters or taxa. This class is as diverse as  
924 class A, with about 20% identity between sequences, but its genetic footprint may be  
925 enriched in fewer than 80 highly conserved residues, justifying the exclusion of certain  
926 carboxylesterases with  $\beta$ -lactamase activity. In addition, the existence of major taxa in  
927 bacterial genera, such as *Citrobacter*, *Enterobacter*, *Acinetobacter* and *Aeromonas*, and of  
928 recent and more long-standing taxonomic reorganizations provides an indispensable aid to  
929 diagnosis at species level, particularly in light of the potential failings of the phenotypic  
930 methods currently used in laboratories. This type of analysis, based on primary structure,  
931 makes it possible to propose one or several consensus sequences for a species, potentially  
932 improving the characterization of possible polymorphisms of the  $\beta$ -lactamase. In the  
933 absence of the susceptible strain of the bacterium at the start of infection, prior knowledge  
934 of the diverse genetic modifications likely to lead to an extended spectrum of inactivation,  
935 which vary between bacterial species, should make it possible to detect these modifications  
936 (substitutions, duplications, insertions, deletions). The recent adaptation of a numbering  
937 system for this molecular class of enzymes has led to comparisons of protein sequences  
938 and emergence of the notion of a “natural ESAC”. Finally, medical biologists should not  
939 ignore possible interactions with other genes likely to lead to hyperproduction, which are  
940 particularly important for this molecular class.

941

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948

949

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- 2317

2318 **AUTHOR BIOS**

2319 **Alain Philippon** is a veterinarian (Ecole Nationale Vétérinaire d'Alfort, 1963) graduated  
2320 in biochemistry (University of Orsay 1965) and bacteriology-immunology (Institut  
2321 Pasteur, 1966) who prepared his Ph.D. thesis at the Commissariat à l'Energie Atomique  
2322 (CEA). Initially trained on *Brucella* and experimental bovine brucellosis at Nouzilly  
2323 (Institut National de la Recherche Agronomique/INRA) between 1966 and 1970, he started  
2324 to analyze bacterial resistance to antibiotics, mostly to  $\beta$ -lactams (susceptibility patterns,  
2325 ESBL, and plasmid-encoded AmpC.....) at CHU Cochin, Paris, France, and in  
2326 collaboration with François Le Goffic and Roger Labia (ENS Ulm) in 1972. As Professor  
2327 Emeritus of Microbiology at the University of Paris Descartes and previous Head of the  
2328 Bacteriology Laboratory at Hopital Cochin, he published more than 200 scientific papers  
2329 and mentored 20 doctoral and postdoctoral researchers. He was also codirector of a course  
2330 on medical bacteriology at Institut Pasteur of Paris for a decade.



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2332

2333 **Guillaume Arlet** is a doctor (René Descartes University, Paris, 1979), graduate in Medical  
2334 Biology in 1984, in Biochemistry and Molecular Biology in 1986 (Denis Diderot  
2335 University, Paris) and Bacteriology (Institut Pasteur, 1986). He did his Ph.D (University  
2336 Paris-Sud, 1989-1992) under the supervision of Alain Philippon. Associate Professor at

2337 Saint-Louis Hospital and Denis Diderot University (1991-1999), he became Professor at  
2338 Pierre & Marie Curie University (now Sorbonne University), Head of the medical  
2339 bacteriology department, first at Tenon Hospital (1999-2012) and then within the Eastern  
2340 Parisian Hospitals Group (2012-2018). He headed the teaching department of clinical  
2341 Bacteriology (2007-2018). He joined in 2014 the Center for Immunology and Infectious  
2342 Diseases (INSERM U1135) at Sorbonne Université. He supervised ten Ph.D students and  
2343 published more than 210 scientific papers mainly on  $\beta$ -lactamases (ESBL, AmpC  $\beta$ -  
2344 lactamases, carbapenemases), their genetic supports, and in relation to virulence of their  
2345 bacterial hosts. He is currently Professor Emeritus.



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2347

2348 **Roger Labia** graduated from the world-renowned Ecole Polytechnique in Paris, France  
2349 (1962 to 1964), with specialization in mathematics, physics, and chemistry. In relation with  
2350 his high and early interest in chemical and biological problems, he joined scientific  
2351 research, first at the Pasteur Institute (starting in 1965). In 1969, he received a Ph.D.,  
2352 studying the organic synthesis of natural compounds. Subsequently, he spent one  
2353 postdoctoral year at Ottawa University, Canada (1969 to 1970), where he began studying

2354 biochemistry and bacteriology. Back in France, he started a research program on  
2355 antibiotics, including their mode of action and mechanisms of resistance. This allowed him  
2356 to develop multiple collaborations with chemists and bacteriologists from France and other  
2357 countries. He has published more than 320 scientific papers, mostly in high-impact  
2358 international journals. He has been involved in teaching and directed about 50 theses.



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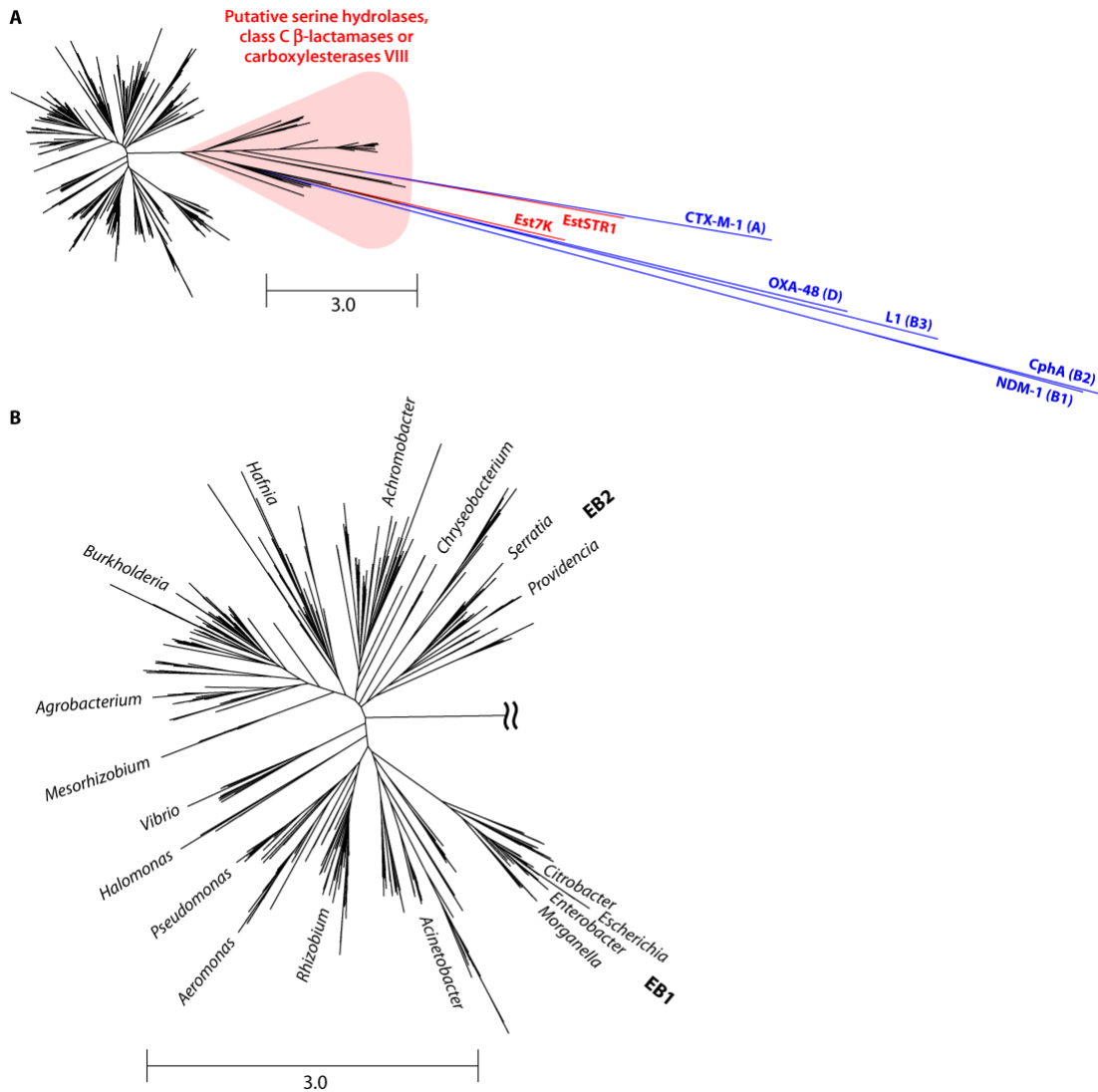
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2361 **Bogdan I. Iorga** received in 2001 a Ph.D in molecular chemistry from the Ecole  
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2363 Modeling and Structural Crystallography team at the Institut de Chimie des Substances  
2364 Naturelles (CNRS, Université Paris-Saclay, Gif-sur-Yvette, France). He is author of 1  
2365 book, 6 book chapters, 4 patents and more than 100 publications. He is the developer of  
2366 the Beta-Lactamase DataBase (BLDB, <http://bldb.eu>) and is involved in several French  
2367 and European projects related to the antimicrobial resistance. His main research interests  
2368 include the design of biologically active compounds, the study of structure-function  
2369 relationships in different classes of  $\beta$ -lactamases and the development of innovative  
2370 protocols in molecular modeling. His recent work focuses on the development of tools for  
2371 *in silico* prediction of antibiotic susceptibility from genomic data using machine learning  
2372 and deep learning approaches.



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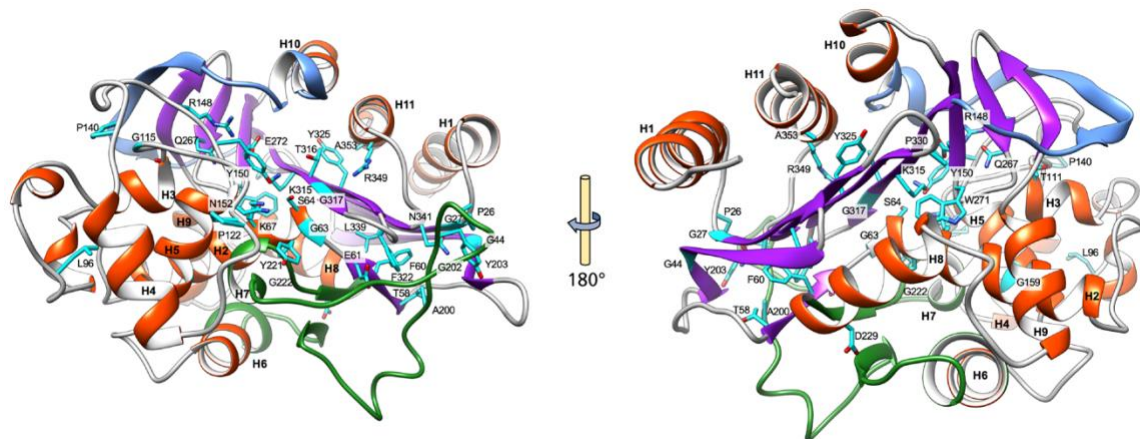
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2376 **FIG 1 (a)** Phylogram for representative and putative class C  $\beta$ -lactamases, compared with  
 2377  $\beta$ -lactamases from classes A, B and D. **(b)** Focused view on the phylogram of class C  $\beta$ -  
 2378 lactamases. The protein sequences of representative enzymes are listed in (85,97,104,114).  
 2379 The sequences were filtered using CD-HIT (<https://github.com/weizhongli/cdhit>) at 90 %  
 2380 sequence identity, then aligned with Clustal Omega (410). The tree was constructed using  
 2381 RAxML (411) and the phylogram generated using FigTree (version 1.4.3). The tree was  
 2382 unrooted.

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2384

2385 **FIG 2** Representative three-dimensional structure for class C  $\beta$ -lactamases. The structure  
 2386 of *E. hormaechei* P99 (formerly known as *E. cloacae* P99) (PDB code 1BLS) (141) is  
 2387 colored in orange ( $\alpha$ -helixes) and purple ( $\beta$ -strands). The  $\Omega$ - and R2-loops are colored in  
 2388 green and blue, respectively. The most conserved residues (see Table 3) are represented as  
 2389 sticks and colored in cyan. The numbering of residues follows the SANC nomenclature  
 2390 (25).

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	Motif SXSK		Motif YXN			Omega-loop					
	60	70	140	150	160	190	200	204a	210	220	230
<i>E. coli</i>	TLFELGSVSKTFTGVLG		PAWAPGTQRLYANSSIGLFGA			WINVPPAEKKNYAWGYR	EGKAVHVS	PGALDAE	TYG	V	GKSTIEDM
<i>C. freundii</i>	TLFELGSVSKTFNGVLG		PQWTPGAKRLYANSSIGLFGA			WITVPSQSEQKNYAWGYR	EGKPVHVS	SPGLDAE	AYG	V	GKSSVIDM
<i>C. portucalensis</i>	TLFELGSVSKTFNGVLG		PQWAPGAKRLYANSSIGLFGA			WITVQNEQKDYAWGYR	EGKAVHVS	SPGLDAE	AYG	V	GKSSVIDM
<i>E. asburiae</i>	TLFELGSISKTFFTGVLG		PQWKPQTTRLYANASIGLFGA			WINVPKAEAEHYAWGYR	DGKAVHVS	SPGMLDAE	AYG	V	GKTNVQDM
<i>E. hormaechei</i>	TLFELGSISKTFFTGVLG		PQWKPQTTRLYANASIGLFGA			WINVPKAEAEHYAWGYR	DGKAVRVSP	GMLDAQ	AYG	V	GKTNVQDM
<i>E. kobei</i>	TLFELGSISKTFFTGVLG		PQWKPQTTRLYANASIGLFGA			WINVPKAEAEHYAWGYR	DGKAVHVS	SPGMLDAE	AYG	V	GKTNVQDM
<i>E. roggenkampii</i>	TLFELGSISKTFFTGVLG		PQWKPQTTRLYANASIGLFGA			WINVPKAEAEHYAWGYR	EGKAVHVS	SPGMLDAE	AYG	V	GKTNVQDM
<i>M. morgani</i>	TLFELGSVSKTFNGVLG		PSWKPGDMRLYANSSIGLFGA			FITVPEQAQSYAYGYK	NKKPVRVSP	GLDAE	SYG	V	GKSASKDM
<i>K. aerogenes</i>	TLFELGSVSKTFNGVLG		PQWAPGSKRLYANASIGLFGA			WINVPPAQSKDYAWGYR	DGKAVHVS	SPGLDAE	AYG	V	GKSSIEDM
<i>S. marcescens</i>	TLFEVGSLSKTFATLA		PKHPAGSYRVVSNLIGLGLGM			YVQVFAAQMANYAQGY	SKDDK	FVRVNP	GLDAE	SYG	IKSNARDL
<i>H. paralvei</i>	TLFEVGSLSKTFATLA		PADAAGTHRIVYVSNIGTLLGM			YLVKVPADQMENYAWG	YNKDEP	VHVNM	EILGNE	AYG	IKTSSDDL
<i>A. allosaccharophila</i>	TLFEIGSVSKTLTATLG		PVYPAGTHRQVSNPSIGLFGH			YIQVPEAMANYAYGY	SKEDK	PIRVTP	GLDAE	AYG	IKTSSADL
<i>A. caviae</i>	TLFEIGSVSKTLTATLG		PAYSFGSHRQVANPSIGLFGY			YLVNVEQAMGHYAYG	YKEDK	PIRVTP	GLDAE	AYG	IKTSSADL
<i>A. baumannii</i>	TLFEIGSVSKLFTATA		PKNPIGEYRQVSNPSIGLFGK			YVNVKTKMQNYPGYN	QENQ	PIRVNP	GLDAP	AYG	VKSTLPDM
<i>P. aeruginosa</i>	TLFEIGSVSKTFATLA		PTYAPGSQRLVSNPSIGLFGY			HLDVPEAALAQYAYG	YKEDDR	PLRVG	PLDAE	YG	VKSTSAADL

	H10/R2-loop			Motif KTG		
	290	300	310	315	330	340
<i>E. coli</i>	NGSGNKIALAAHPVKAITPPTPAVRA			HKTGATGGFGSYVAFIPEKELGIVMLAN		
<i>C. freundii</i>	NGSDSKVALAALPAVEVNPAPAVKA			HKTGSTGGFGSYVAFVPEKNLGIIVMLAN		
<i>C. portucalensis</i>	NGSDSKVALAALPAVEVNPAPAVKA			HKTGSTGGFGSYVAFVPEKNLGIIVMLAN		
<i>E. asburiae</i>	EGSDNKVALAPLPAREVNPAPPNAS			HKTGSTGGFGSYVAFIPEKQLGIIVMLAN		
<i>E. hormaechei</i>	EGSDSKVALAPLPVAEIVNPAPPVKA			HKTGSTGGFGSYVAFIPEKQLGIIVMLAN		
<i>E. kobei</i>	EGSDNKVALAPLPVAEIVNPAPPVKA			HKTGSTGGFGSYVAFIPEKELGIVMLAN		
<i>E. roggenkampii</i>	GGSDNKVALAPLPVAEIVNPAPPVKA			HKTGSTGGFGSYVAFIPEKQLGIIVMLAN		
<i>M. morgani</i>	NGVTNEVALQPHVPTD-NQVQPYNRA			HKTGATTFGAYVAFIPEKQVAIVILAN		
<i>K. aerogenes</i>	NGSDNKVALAATPVTAIVNPAPPVKA			HKTGSTGGFGSYVAFIPQDGLGIIVMLAN		
<i>S. marcescens</i>	EGNNAGMTMNGTPATAITPPQPELRA			NKTGSTGGFSTYAVFIPAKNIAVIVMLAN		
<i>H. paralvei</i>	TGND--MAMTKSVATPIVPLPPQEN			NKTGSTNGFGAYIAFVPAKMKGIIVMLAN		
<i>A. allosaccharophila</i>	AGNSPAVSLQANPVTRFAVPKAMGEQ			NKTGSTGGFGAYVAFVPAARGIAIVMLAN		
<i>A. caviae</i>	AGNSPAMIYNANPAAAPAAATGHPVL			NKTGSTNGFGAYVAFVPAKGIIVMLAN		
<i>A. baumannii</i>	DSNSEQIVMKPNKVTAIISK--EPSV			HKTGSTNGFGTYVVFIPKENIGLVMLTN		
<i>P. aeruginosa</i>	AGNSTPMALQPHRIARLPAPQALEGQ			NKTGSTNGFGAYVAFVPRDLGLVILAN		

2395

2396 **FIG 3** Consensus partial protein sequences of species or their progenitors susceptible to  
 2397 expand their spectrum of inactivation. For *E. coli*, the consensus sequence was calculated  
 2398 from protein sequences of clusters A, B, C and D (84,96,100,113). Residue boxed in gray  
 2399 indicates 100 % conserved. Underlined positions indicate at least two different residues  
 2400 (polymorphism).

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2404 **Table 1.** Overview of bacteria producing class C  $\beta$ -lactamases

Class	Order	Genus	
<i>Alphaproteobacteria</i>	Rhizobiales	<i>Agrobacterium</i> , <i>Bosea</i> , <i>Bradyrhizobium</i> , <i>Inorhizobium</i> , <i>Mesorhizobium</i> , <i>Methylobacterium</i> , <i>Microvirga</i> , <i>Ochrobactrum</i> , <i>Phyllobacterium</i> , <i>Pseudorhodoplanes</i> , <i>Rhizobium</i>	
	Rhodobacterales	<i>Rhodobacter</i> , <i>Ruegeria</i> , <i>Silicibacter</i> , <i>Sulfitobacter</i>	
	Rhodospirillales	<i>Dongia</i>	
<i>Betaproteobacteria</i>	Burkholderiales	<i>Achromobacter</i> , <i>Bordetella</i> , <i>Burkholderia</i> , <i>Caballeronia</i> , <i>Collimonas</i> , <i>Cupriavidus</i> , <i>Herbaspirillum</i> , <i>Janthinobacterium</i> , <i>Massilia</i> , <i>Noviherbaspirillum</i> , <i>Pandoraea</i> , <i>Paraburkholderia</i>	
	Neisseriales	<i>Chromobacterium</i> , <i>Laribacter</i> , <i>Snodgrassella</i>	
	Rhodocyclales	<i>Thauera</i>	
<i>Gammaproteobacteria</i>	Aeromonadales	<i>Aeromonas</i>	
	Alteromonadales	<i>Shewanella</i>	
	Cellvibrionales	<i>Microbulbifer</i>	
	Enterobacterales	<i>Budvicia</i> , <i>Buttiauxella</i> , <i>Cedecea</i> , <i>Citrobacter</i> , <i>Cronobacter</i> , <i>Edwardsiella</i> , <i>Enterobacter</i> , <i>Erwinia</i> , <i>Escherichia</i> : <i>Shigella</i> , <i>Ewingella</i> , <i>Hafnia</i> , <i>Klebsiella</i> , <i>Lelliottia</i> , <i>Morganella</i> , <i>Pantoea</i> , <i>Photorhabdus</i> , <i>Pluralibacter</i> , <i>Pragia</i> , <i>Providencia</i> , <i>Regiella</i> , <i>Rouxiella</i> , <i>Serratia</i> , <i>Siccibacter</i> , <i>Xenorhabdus</i> , <i>Yersinia</i>	
		Legionellales	<i>Legionella</i>
		Oceanospirillales	<i>Aidingimonas</i> , <i>Chromohalobacter</i> , <i>Halomonas</i> , <i>Salinicola</i>
		Pseudomonadales	<i>Acinetobacter</i> , <i>Pseudomonas</i> , <i>Psychrobacter</i>
		Vibrionales	<i>Vibrio</i>
		Xanthomonadales	<i>Dyella</i> , <i>Lysobacter</i> , <i>Xanthomonas</i>
	<i>Deltaproteobacteria</i>	Myxococcales	<i>Myxococcus</i>
<i>Terrabacteria</i>	Actinobacteria	<i>Mycobacterium</i>	
	Negativicutes	<i>Pelosinus</i>	
FCB group	Chitinophagales	<i>Sediminibacterium</i>	
	Cytophagales	<i>Dyadobacter</i> , <i>Emticicia</i> , <i>Siphonobacter</i>	

	Flavobacteriales	<i>Chryseobacterium</i>
	Sphingobacteriales	<i>Sphingobacterium</i>
PVC group	Chlamydiales	<i>Chlamydia</i>
	Parachlamydiales	<i>Parachlamydia</i>
Unclassified		<i>Dependentiae</i>

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2407 **Table 2.** Representative class C  $\beta$ -lactamases

<b>Bla</b>	<b>Origin of name</b>	<b>Accession number</b>	<b>Genomic localisation<sup>a</sup></b>	<b>Organism</b>	<b>Number of residues</b>	<b>References</b>
ACC-1	<u>A</u> mbler <u>C</u> lass <u>C</u> -1	AJ133121	P	<i>Klebsiella pneumoniae</i>	386	(30)
ACT-1	<u>A</u> mpC <u>T</u> ype	U58495	P	<i>Klebsiella pneumoniae</i>	381	(31)
ABA-1 (ADC-2)	<u>A</u> cinetobacter <u>b</u> aumannii	AY177427	IS	<i>Oligella urethralis</i>	383	(32)
ABAC-1 (ADC-3)	<u>A</u> cinetobacter <u>b</u> aumannii	AY178995	Chr	<i>Acinetobacter baumannii</i>	383	(32)
ADC-1	<u>A</u> cinetobacter-derived cephalosporinase	AJ009979	Chr	<i>Acinetobacter baumannii</i>	383	(33)
AQU-1	<u>A</u> eromonas <u>a</u> quariorum	AB765393	Chr	<i>Aeromonas dhakensis</i>	380	(34)
AsbA1	<u>A</u> eromonas <u>s</u> obria	U10250	In	<i>Aeromonas jandaei</i>	381	(35)
<u>BIL</u> -1 (CMY-2-like)	Name of patient ( <u>B</u> ilal)	X74512	P	<i>Escherichia coli</i>	383	(36,37)
BlaE	Gene name	AY442183	Chr	<i>Mycobacterium smegmatis</i>	380	(38,39)
BUT-1	<u>B</u> uttiauxella sp.	AJ415568	Chr	<i>Buttiauxella sp.</i>	383	(40)
CAV-1	<u>A</u> eromonas <u>c</u> aviae	AF462690	Chr	<i>Aeromonas caviae</i>	382	(41)
CDA-1	<u>C</u> edecea <u>d</u> avisae	KJ650399	Chr	<i>Cedecea davisae</i>	382	(42)
CepH	<u>C</u> ephalosporinase <u>h</u> ydrophila	AJ276030	Chr	<i>Aeromonas hydrophila</i>	382	(43)
CepS	<u>C</u> ephalosporinase <u>s</u> obria	X80277	Chr	<i>Aeromonas sobria</i>	382	(44)
CFE-1	<u>C</u> itrobacter <u>f</u> reundii	AB107899.	P	<i>Escherichia coli</i>	381	(45)
CHR-1	<u>C</u> hromohalobacter sp.	AB070219	Chr	<i>Chromohalobacter sp.</i>	396	(46,47)
CMA-1	<u>C</u> ronobacter <u>m</u> alonaticus	KF640251	Chr	<i>Cronobacter malonaticus</i>	375	(48)
CMH-1	<u>C</u> hi <u>M</u> ei <u>H</u> ospital	JQ673557	P	<i>Enterobacter cloacae</i>	381	(49)
CMY-1	Active on <u>c</u> eph <u>a</u> mycins	X92508	P	<i>Klebsiella pneumoniae</i>	382	(50)

CMY-2	Active on <u>cephamycins</u>	X91840	P	<i>Klebsiella pneumoniae</i>	381	(51)
CSA-1	<i>Cronobacter sakazakii</i>	KF623543	Chr	<i>Cronobacter sakazakii</i>	375	(48)
DHA-1	<u>Dhahran</u> (Saudi Arabia)	Y16410	P	<i>Salmonella enteritidis</i>	379	(52,53)
Ear-1	<i>Enterobacter aerogenes</i>	AJ544162	Chr	<i>Enterobacter aerogenes</i>	381	(54)
EDC-1	<i>Edwardsiella</i> AmpC	EF467366	Chr	<i>Edwardsiella tarda</i>	386	–
<u>ENT-1</u>	<i>Buttiauxella</i> <i>agrestis</i> CF01 <u>Ent1</u>	AJ489827	Chr	<i>Buttiauxella agrestis</i>	390	(55)
ERH-1	<i>Erwinia rhapontici</i>	AY288518	Chr	<i>Erwinia rhapontici</i>	379	(56)
FOX-1	Active on <u>cefoxitin</u>	X77455	P	<i>Klebsiella pneumoniae</i>	382	(57)
IDC-1	<u>Integron</u> <u>derived</u> cephalosporinase	MN985649	In	sediment metagenome	395	(58)
<u>K12</u> (EC-1)	<i>Escherichia coli</i> <u>K12</u>	J01611	Chr	<i>Escherichia coli</i>	377	(59,60)
LAT-1	Active on <u>latamoxef</u>	X78117	P	<i>Klebsiella pneumoniae</i>	381	(61,62)
LHK-1	<i>Laribacter hongkongii</i>	AY632070	Chr	<i>Laribacter hongkongii</i>	388	(63)
LRA10-1	$\beta$ -lactam <u>resistance</u> from <u>Alaska</u>	EU408357	?	uncultured bacteria (soil)	375	(64)
LRA13-1 <sup>b</sup>	$\beta$ -lactam <u>resistance</u> from <u>Alaska</u>	EU408352	?	uncultured bacteria (soil)	609*	(64)
LRA18-1	$\beta$ -lactam <u>resistance</u> from <u>Alaska</u>	EU408355	?	uncultured bacteria (soil)	386	(64)
LYL-1	<i>Lysobacter lactamgenus</i>	X56660	Chr	<i>Lysobacter lactamgenus</i>	385	(65)
MIR-1	<u>Miriam</u> hospital	M37839	P	<i>Klebsiella pneumoniae</i>	381	(66,67)
MOX-1	Active on <u>moxalactam</u>	D13304	P	<i>Klebsiella pneumoniae</i>	382	(68,69)
OCH-1	<i>Ochrobactrum anthropi</i>	AJ401618	Chr	<i>Ochrobactrum anthropi</i>	390	(70)
<u>P99</u> (ACT-89)	<i>Enterobacter hormaechei</i> <u>P99</u>	X07274	Chr	<i>Enterobacter hormaechei</i>	397	(71)
PAO-1 (PDC-1)	<i>Pseudomonas aeruginosa</i> ( <i>Pseudomonas</i> - <u>derived</u> cephalosporinase)	AY083595	Chr	<i>Pseudomonas aeruginosa</i>	397	(72,73)
PAC-1	<i>Pseudomonas aeruginosa</i> Class <u>C</u>	KY285014	Tn	<i>Pseudomonas aeruginosa</i>	381	(74)

SLC-1	<i>Serratia liquefaciens</i> Class C	DQ022079	–	Uncultured bacteria (soil)	379	(75)
PSI-1	<i>Psychrobacter immobilis</i>	X83586	Chr	<i>Psychrobacter immobilis</i>	401	(76,77)
RHO-1	<i>Rhodobacter sphaeroides</i>	CP000144	Chr	<i>Rhodobacter sphaeroides</i>	380	(78)
SR50 (SRT-1-like)	<i>Serratia marcescens</i> SR50, <i>Serratia resistant</i>	X52964	Chr	<i>Serratia marcescens</i>	376	(79)
SST-1	Susceptible strain	AB008455	Chr	<i>Serratia marcescens</i>	378	(80)
TRU-1	Formerly <i>Aeromonas tructi</i>	EU046614	Chr	<i>Aeromonas enteropelogenes</i>	382	(81)
YEC-1	<i>Yersinia enterocolitica</i> cephalosporinase	X63149	Chr	<i>Yersinia enterocolitica</i>	388	(82)
YRC-1	<i>Yersinia ruckeri</i> cephalosporinase	DQ185144	Chr	<i>Yersinia ruckeri</i>	383	(83)

2408 <sup>a</sup> Chr, chromosome; In, integron; P, plasmid; Tn, transposon; IS, insertion sequence.

2409 <sup>b</sup> fusion between two  $\beta$ -lactamases (class C and class D)

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2412 **Table 3.** Conserved residues in class C  $\beta$ -lactamases

Position <sup>a</sup>	Residue (> 90 % conserved)	Secondary structure <sup>b</sup>	% conserved <sup>b</sup>
18	P	H1	94
26	P	S	97
27	G	E	100
29	A	E	97
36	G	T	97
43	F/Y/W	E	100
44	G	E	100
54	V		91
58	T		100
60	F	E	100
61	E	E	100
63	G	G	100
<b>64</b>	<b>S</b>	G	100
<b>66</b>	<b>S</b>	H2	100
67	K	H2	100
71	G/A	H2	100
73	L	H2	94
77	A	H2	91
94	P	G	94
96	L	G	100
109	L	H3	97
110	A/G	H3	100
111	T	T	100
113	T/S		100
115	G	S	100
116	G		96
118	P		94
119	L	S	97
122	P		100
123	D/E	T	100
134	F/Y/W	H4	100
135	Y/F	H4	97
138	W		97
140	P		100
145	G	T	97
148	R	E	100
<b>150</b>	<b>Y</b>		100
<b>152</b>	<b>N</b>	H5	100
155	I	H5	91
156	G	H5	97

159	G	H5	100
170	F/Y	H6	100
187	T/S	Ω	100
188	Y/W/F	Ω	100
191	V	Ω	97
192	P	Ω	97
199	Y	Ω	97
200	A	Ω	100
202	G	Ω	100
203	Y	Ω	100
210	R/H	Ω	100
211	V	Ω	91
213	P	Ω	94
214	G	Ω	94
221	Y	Ω H7	100
222	G	Ω	100
224	K	Ω	94
229	D	H8	100
260	Y/W/F	E	97
267	Q	E	100
269	L	S	97
271	W		100
272	E	E	100
277	P	S	97
286	G	H10 R2	97
<b>315</b>	<b>K</b>	E	100
<b>316</b>	<b>T</b>	E	100
<b>317</b>	<b>G</b>	E	100
319	T		97
321	G	S	97
322	F		100
325	Y	E	100
328	F	E	97
330	P	E	100
335	G/A	E	100
337	V	E	91
339	L	E	100
340	A	E	97
341	N	S	100
345	P		97
349	R	H11	100
353	A	H11	100

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2414 <sup>a</sup> According to the “SANC” class C  $\beta$ -lactamases numbering scheme described in (25).  
2415 Residues in boldface type are involved in the catalytic mechanism and/or in substrate  
2416 binding.

2417 <sup>b</sup> From the alignment of 32 representative class C  $\beta$ -lactamases examined in (25). H:  
2418  $\alpha$ -Helix; S/T: Bend or Turn; E/B:  $\beta$ -Strand or  $\beta$ -Bridge; G:  $3_{10}$ -Helix;  $\Omega$ :  $\Omega$ -loop; R2: R2-  
2419 loop.

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2423 **Table 4.** Class C  $\beta$ -lactamases and species-specific progenitors

Enzyme/Strain <sup>a</sup>	First identification	Updated identification	% <sup>b</sup>	References
ACC-1* <sup>c</sup>	<i>Hafnia alvei</i>	<i>Hafnia paralvei</i>	99.7	(30), this review
ACT-1* <sup>c</sup>	<i>Enterobacter cloacae</i>	<i>Enterobacter asburiae</i>	98.4	(31), this review
Aer-1	<i>Enterobacter aerogenes</i>	<i>Klebsiella aerogenes</i>	98.9	(54,125)
AQU-1	<i>Aeromonas aquariorum</i>	<i>Aeromonas dhakensis</i>	99.2	(34,126)
AsbA1	<i>Aeromonas sobria</i>	<i>Aeromonas jandaei</i>	94.5	(35), this review
BIL-1	<i>Citrobacter freundii</i>	<i>Citrobacter portucalensis</i>	96.8	(37), this review
BUT-1	<i>Buttiauxella</i> sp.	<i>Scandinavium goteborgense</i>	99.0	(40,127)
BUT-2	<i>Buttiauxella agrestis</i>	<i>Scandinavium goteborgense</i>	99.5	(55,127)
CAV-1	<i>Aeromonas caviae</i>	<i>Aeromonas allosaccharophila</i>	97.1	(41,121)
CFE-1*	<i>Citrobacter freundii</i>	<i>Citrobacter europaeus</i>	99.2	(45), this review
CFE-2*	<i>Citrobacter freundii</i>	<i>Citrobacter werkmanii</i>	95.0	(128), this review
CMY-1* <sup>c</sup>	<i>Aeromonas hydrophila</i>	<i>Aeromonas sanarellii</i>	95.3	(50), this review
CMY-2*	<i>Citrobacter freundii</i>	<i>Citrobacter portucalensis</i>	98.4	(51), this review
EDC-1	<i>Edwardsiella tarda</i>	<i>Edwardsiella piscicida</i>	99.1	–, this review
FOX-1*	<i>Aeromonas caviae</i>	<i>Aeromonas allosaccharophila</i>	94-98	(57,121)
LAT-1*	<i>Citrobacter freundii</i>	<i>Citrobacter portucalensis</i>	97.4	(62), this review
MIR-1* <sup>c</sup>	<i>Enterobacter cloacae</i>	<i>Enterobacter roggkampii</i>	99.7	(25,71)
MOX-1*	<i>Aeromonas hydrophila</i>	<i>Aeromonas sanarellii</i>	94.5	(69,121)
MOX-2*	<i>Aeromonas</i> sp.	<i>Aeromonas caviae</i>	98.9	(121,129)
MOX-9*	<i>Aeromonas caviae</i>	<i>Aeromonas media</i>	98.0	(121,130)
<u>P99</u> (ACT-89)	<i>Enterobacter cloacae</i>	<i>Enterobacter hormaechei</i>	97.9	(25,71), this review
TRU-1	<i>Aeromonas tructi</i>	<i>Aeromonas enteropelogenes</i>	97.1	(81), this review

2424 <sup>a</sup> Plasmid-encoded enzymes are labelled by an asterisk2425 <sup>b</sup> Percentage of identity with consensus sequence2426 <sup>c</sup> Other plasmid-encoded types: ACC-4 (99.5 % for *H. paralvei*), ACT-3, ACT-6, ACT-8,2427 and ACT-10 ( $\geq$ 97.6 % for *E. asburiae*); CMY-17, CMY-55, CMY-132, and CMY-161 ( $\geq$

2428 98.1 % for *A. sanarellii*); MIR-4 (99.4 % for *E. roggenkampii*). Their respective  
2429 phylogenies can be found in (84,96,100,113) and (123,124).

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2434 **Table 5.** Localization of ESAC-associated mutations in chromosomal or plasmid-encoded class C  $\beta$ -lactamases, the exact position of

2435 each mutation and the specific regions associated with each cluster

<b>Bla or strain <sup>a</sup></b>	<b>Location</b>	<b>First numbering</b>	<b>Updated numbering <sup>b</sup></b>	<b>References</b>
<b><i>E. coli</i></b>				
<u>MEV</u>	H10-helix	S282 duplication	idem	(275)
<u>ECB33</u>	H10-helix	I283 duplication	I284	(276)
<u>HKY28</u>	H10-helix	286GSD deletion	idem	(277)
<u>EC16</u>	H10-helix	S287C	idem	(16)
<u>EC13</u>	H10-helix	S287N	idem	(16,87)
<u>8009162</u>	H10-helix, R2-loop	A292V	idem	(273)
<u>EC80</u>	H10-helix, R2-loop	L293P	idem	(278)
<u>BER</u>	R2-loop	293AA insertion	idem	(279)
<u>7014517</u>	R2-loop	295ALA insertion	idem	(273)
<u>EC15</u>	R2-loop	H296P	idem	(16,280)
<u>EC14</u>	R2-loop	V298L	idem	(16,280)
<u>KL</u>	H11-helix	V350F	idem	(281)
<b><i>Citrobacter</i></b>				
<u>CHA</u>	close to YSN	R148H	idem + Q196H	(87,282)
<u>CMY-107</u>	$\Omega$ -loop	Y199C	idem	(283)
<u>CMY-27</u>	$\Omega$ -loop	W221C	W201C	(284)
<u>CMY-30</u>	$\Omega$ -loop	V231G	V211G	(285)
<u>CMY-42</u>	$\Omega$ -loop	V231S	V211S	(286,287)
<u>CMY-95</u>	$\Omega$ -loop	V211A	idem	(278)
<u>CMY-32</u>	$\Omega$ -loop	G214E	idem	(288)
<u>CMY-54</u>	$\Omega$ -loop	217EL insertion	216aEL insertion	(289)
<u>GN346</u>	$\Omega$ -loop	E219K	idem	(290)
<u>CMY-136</u>	$\Omega$ -loop	Y221H	idem	(167)

CMY-172	R2-loop	290KVA N346I	deletion+	idem	(291)
CMY-2	R2-loop	A292P or L293P		idem	(292)
CMY-33	R2-loop	293LA deletion		idem	(293,294)
CMY-44	R2-loop	293LAAL deletion		idem	(293)
CMY-69	R2-loop	A295P		A294P	(295)
CMY-99	R2-loop	P306T		idem	(296)
CMY-37	R2-loop	L316I		L296I	(297)
<b><i>Enterobacter</i></b>					
<u>LN04004SS1</u>	Ω-loop	213K-226G deletion		idem	(298)
<u>GC1</u>	Ω-loop	208AVR duplication		idem	(10,151)
<u>CHE</u>	R2-loop	289SKVALA294 deletion		idem	(9)
<u>Ent630</u>	R2-loop	292-293 AL deletion		idem	(299)
<u>P99 (ACT-89)</u>	R2-loop	L293P		idem	(300)
<u>MHN</u>	R2-loop	V298E		idem	(301)
<u>LN04004SS1</u>	H11-helix	N366H		N346H	(298)
<b><i>K. aerogenes</i></b>					
<u>EA6/13/17/20</u>	H3-helix	Q90H, W101C, L107Y		idem	(302)
<u>Ea595</u>	H10-helix, R2-loop	V291G		idem	(303)
<u>Ear2</u>	R2-loop	L293P		idem	(54)
<b><i>S. marcescens</i></b>					
<u>520R</u>	H2-helix	T64I		T70I	(304)
<u>SRT-1</u>	Ω-loop	E213K		E219K	(80)
<u>ES46, ES71</u>	Ω-loop	E235K		E219K	(305)
<u>SMSA</u>	Ω-loop	S220Y		idem	(306)
<u>HD</u>	R2-loop	287MNGT deletion		293MNGT deletion	(307)
<b><i>Hafnia</i></b>					
ACC-4*	Ω-loop	V211G deletion	+ 289-290	idem	(308), this review
ACC-1*	R2-loop	289-290 deletion		idem	(25,309), this review

ACC-2	R2-loop	289-290 deletion <sup>c</sup>	idem	(309), this review
<b><i>Aeromonas</i></b>				
CMY-9	R2-loop	E85D + 299-301 deletion	E61D + 301-303 deletion	(310-312)
CMY-19	R2-loop	I292S + 299-301 deletion	I292S + 301-303 deletion	(311-313)
CMY-1	R2-loop	299-301 deletion	301-303 deletion <sup>d</sup>	(164,313,314)
MOX-1	R2-loop	303-305 deletion	301-303 deletion <sup>d</sup>	(210)
MOX-2	R2-loop	303-305 deletion	301-303 deletion <sup>e</sup>	(129), this review
MOX-13	R2-loop	303-305 deletion + N346I	301-303 deletion <sup>e</sup>	(315), this review
CMY-10	R2-loop, H11-helix	E85D + 299-301 deletion	E61D + 301-303 deletion	(164,311,314,316)
<b><i>A. baumannii</i></b>				
ADC-56	H5-helix	R148Q	idem	(107)
ADC-53	Ω-loop	V208A	V211A	(106)
ADC-33	Ω-loop	P210R + 215A duplication	P213R + 218aA duplication	(317)
ADC-51	R2-loop	N283S	N287S	(106)
ADC-1	R2-loop	304-306 deletion	idem deletion <sup>f</sup>	(103)
ADC-68	Ω-loop	G220D + R320G	G217D + R321G	(162)
<b><i>P. aeruginosa</i></b>				
PDC-222	H2-helix	T96I	T70I	(318)
PDC-78	H2-H3-loop, Ω-loop	R100H + G216R	R100H + R215G	(119)
PDC-82	H3-H4-loop	F121L + M175L	F121L + M174L	(119,319)
PDC-73	H5-helix	P154L	P153L	(119)
PDC-82	H6-helix	M175L	M174L	(119)
PDC-50	Ω-loop	V213A	V211A	(119)
PDC-74	Ω-loop	G216R	G215R	(119)
PDC-86	Ω-loop, H7-helix	E221K	E219K	(119)
PDC-80	Ω-loop, H7-helix	E221G	E219G	(119)

PDC-85	$\Omega$ -loop, H7-helix	Y223H	Y221H	(119)
PDC-223	$\Omega$ -loop	229G-247E deletion	202G-219E deletion	(318)
PDC-221	$\Omega$ -loop	E247K	E219K	(119)
PDC-88	H10-helix, R2-loop	290TP deletion	289TP deletion	(119)
PDC-89	H10-helix, R2-loop	290TPM deletion	289TPM deletion	(119)
PDC-91	H10-helix, R2-loop	290TPMA deletion	289TPMA deletion	(119)
PDC-44	R2-loop	L294P	L293P	(119)
PDC-92	R2-loop	294LQ deletion	293LQ deletion	(119)
PDC-76	H11-helix	N347I	N346I	(119)

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2437 <sup>a</sup> The strain names are underlined

2438 <sup>b</sup> According the Standard Numbering Scheme (25)

2439 <sup>c</sup> All AmpC sequences of *H. paralvei* and *H. alvei* examined have this deletion

2440 <sup>d</sup> This cluster includes CMY-1, MOX-1, CMY-8, CMY-10, CMY-11, CMY-19, and MOX-14

2441 <sup>e</sup> These enzymes were located in different clusters (122)

2442 <sup>f</sup> This deletion was observed for all *A. baumannii* sequences, and particularly for ADC-7 (101).

2443 \* Plasmid-encoded

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2446 **Table 6.** ESAC and effects on kinetic constants for groups of enzymes with different phenotypes<sup>a</sup>

Bla or strain <sup>b</sup>	Mutation	Ceftazidime (CAZ)			Cefotaxime (CTX)			Cefepime (FEP)			References
		$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{cat}/K_m$ (μM · s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{cat}/K_m$ (μM · s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{cat}/K_m$ (μM · s <sup>-1</sup> )	
CMY-2	–	0.004	0.5	0.008 <sup>c</sup>	0.007	0.001	7	–	–	–	(282)
CMY-2	R148H	0.67	0.6	1.12 <sup>c</sup>	NM	0.003	NM	–	–	–	(282)
CMY-2	–	0.01	0.02	0.5	<0.01	0.005	<2	–	–	–	(283) (326)
CMY-107	–	0.14	0.15	0.9	0.8	0.075	10.7	–	–	–	(283)
CMY-30	–	0.4	0.14	2.9	1.7	0.3	5.7	–	–	–	(326)
CMY-42	–	0.5	0.3	1.7	0.2	0.08	2.5 <sup>c</sup>	–	–	–	(286)
CMY-2	–	0.005	0.15	0.033	0.007	0.001	7	0.37	412	9 · 10 <sup>-4</sup>	(167,282,327)
CMY-136	–	6.26	2360	0.003	4.71	20	0.24	1.79	3588	5 · 10 <sup>-4</sup>	(167)
CMY-2	–	–	–	–	NM	1.8	NM	NM	108.1	NM	(288)
CMY-32	–	–	–	–	0.9	4.05	0.22	NM	988.9	NM	(288)
ACC-2	–	0.03	5.2	0.006 <sup>c</sup>	0.02	19	0.001	3.6	147	0.024	(309)
ACC-4	–	1.5	15	0.1	2.7	9.4	0.29	0.14	73	0.002	(308)
Ser <sup>s</sup>	–	<0.5	50 <sup>d</sup>	<0.01	2	4	0.5	<0.5	100 <sup>d</sup>	<0.005 <sup>c</sup>	(306)

Ser <sup>R</sup>	S220Y	520	570	0.9	800	980	0.8	330	1000	0.33	(306)
FOX-3	–	0.273	1.18	0.231	0.081	0.076	1.06	ND	ND	$3.32 \cdot 10^{-3}$	(328)
FOX-8	–	$2.6 \cdot 10^{-3}$	0.382	$6.8 \cdot 10^{-3}$	$18.2 \cdot 10^{-3}$	$10.95 \cdot 10^{-3}$	1.66 <sup>c</sup>	ND	ND	$1.36 \cdot 10^{-3}$	(328)
FOX-4	–	1.33	13	10	1.33	0.23	5.69	11.01	1071	0.01	(329)
FOX-4	306GNSΔ	0.84	6.44	0.13	0.24	0.087	2.75	2.5	103	0.02	(329)
CMY-9	–	1.8	560	$3.2 \cdot 10^{-3}$	0.27	0.28	0.96	NM	950	ND	(313)
CMY-19	–	0.085	3.7	0.023	0.33	31	0.011	1.8	630	$2.9 \cdot 10^{-3}$	(313)
CMY-8	–	0.091	48	$1.9 \cdot 10^{-3}$	0.36	2.3	0.16	–	–	–	(310)
CMY-9	–	0.53	120	$4.4 \cdot 10^{-3}$	0.48	3.4	0.14	–	–	–	(310)
MOX-1	–	ND	311	ND	–	–	–	ND	211	ND	(69)
CMY-1	–	–	–	–	0.01	0.015	0.67	–	–	–	(330)
CMY-10	–	5	33.9	0.15	–	–	–	–	–	–	(164)
ACT-89 (P99)	–	$6.1 \cdot 10^{-3}$	18.4	$3.2 \cdot 10^{-4}$	–	–	–	–	–	–	(164)
ACT-89 (P99)	–	<1	20 <sup>d</sup>	ND	0.5	0.5 <sup>d</sup>	1	1	15 <sup>d</sup>	0.067	(9)
<u>CHE</u>	289-294Δ	<1	1 <sup>d</sup>	ND	0.5	0.05 <sup>d</sup>	10	2	3 <sup>d</sup>	0.67	(9)



ACT-89 (P99)	–	0.065	28	$2.3 \cdot 10^{-3}$	–	–	–	–	–	–	(331)
ACT-89 (P99)	L293C	0.041	7	$5.9 \cdot 10^{-3}$	–	–	–	–	–	–	(331)
ACT-89 (P99)	–	0.013	15	$8.7 \cdot 10^{-4}$	–	–	–	0.5	100	$4.7 \cdot 10^{-3}$	(300)
ACT-89 (P99)	L293P	0.10	10	0.01	–	–	–	3.1	24	0.13	(300)
Ear	–	ND	16 <sup>d</sup>	ND	0.15	>500	ND	0.4	126	0.003	(54)
Ear2	–	ND	9.8 <sup>d</sup>	ND	0.15	10 <sup>d</sup>	0.015	0.4	9.1	0.044	(54)
ADC-1	–	0.7	16.0	0.044	0.16	0.5	0.32	–	–	–	(103) (162)
ADC-68	–	1.66	147.7	0.01	18.5	117.5	0.16	–	–	–	(162)
ADC-11	–	0.01	10	0.001	0.2	2.5 <sup>d</sup>	0.1	1	1800	$5.5 \cdot 10^{-4}$	(317)
ADC-33	–	4	30	0.13	1	0.5 <sup>d</sup>	2	10	1300	$7.7 \cdot 10^{-3}$	(317)
ADC-1	–	1.255	265	$4.7 \cdot 10^{-3}$	–	–	–	–	–	–	(260)
ADC-5	–	0.011	232	$4.7 \cdot 10^{-5}$	–	–	–	–	–	–	(260)
ADC-5	P167S	$2.5 \cdot 10^{-3}$	120	$2.1 \cdot 10^{-5}$	–	–	–	–	–	–	(260)

ADC-5	<u>P167S/D242G/</u> <u>Q163K/G342R</u>	1.235	90	0.014	–	–	–	–	–	–	(260)
ADC-30	–	0.05	1.39	0.04	0.18	0.51	0.32	–	–	–	(107)
ADC-56	–	0.1	1.42	0.07	0.27	1	0.27	0.2	17.17	0.011	(107)
PDC-1	–	0.004	20	$2 \cdot 10^{-4}$	0.02	6	$3.3 \cdot 10^{-3}$	0.08	800	$1 \cdot 10^{-4}$	(118)
PDC-2	–	0.01	20	$5 \cdot 10^{-4}$	0.15	5	0.03	2	850	$2.4 \cdot 10^{-3}$	(118)
PDC-3	–	0.02	35	$5.7 \cdot 10^{-4}$	0.15	8	0.019	2	1300	$1.5 \cdot 10^{-3}$	(118)
PDC-5	–	0.015	30	$5 \cdot 10^{-4}$	0.1	5	0.02	2.5	1700	$1.5 \cdot 10^{-3}$	(118)
PDC-5	–	0.01	7.3	$1.3 \cdot 10^{-3}$	0.07	0.14	0.5	>0.15	>250	$6 \cdot 10^{-4}$	(332)
PDC-5	<u>N346Y</u>	0.06	19	$3.2 \cdot 10^{-3}$	0.2	1.2	0.17	>0.17	>300	$5.5 \cdot 10^{-4}$	(332)

2447 <sup>a</sup> NM,  $k_{cat}$  not measurable; ND, not determined

2448 <sup>b</sup> Strain names are underlined

2449 <sup>c</sup> Value computed from  $k_{cat}$  and  $K_m$ , which is different from the value reported in the original paper

2450 <sup>d</sup>  $K_i$  values ( $\mu$ M) were determined instead of  $K_m$  values, using cefalothin as a reporter substrate

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2454 **Table 7.** Chromosomal or plasmid-encoded class C  $\beta$ -lactamases: mechanisms of acquired resistance to carbapenems

Bla or <u>strain</u>	Species	Location <sup>a</sup>	Mechanisms	Fold-increase MICs <sup>b</sup>					Reference
				CAZ	FEP	IMP	ERT	MER	
<u>MEV</u>	<i>E. coli</i>	Chr	ESAC + OmpC decrease + OmpF loss	>128	>16	8	>16	8	(275)
ACC-1	<i>E. coli</i>	P	+ OmpC/OmpF loss	>64	64	4	>128	4	(280)
ACT-1	<i>E. coli</i>	P	+ OmpC/OmpF loss	>256	>16	128	>512	32	(280)
CMY-2	<i>E. coli</i>	P	+ OmpC/OmpF loss	>256	>16	256	>512	256	(280)
DHA-1	<i>E. coli</i>	P	+ OmpC/OmpF loss	>512	64	16	256	8	(280)
FOX-1	<i>E. coli</i>	P	+ OmpC/OmpF loss	>512	>128	4	>256	16	(280)
CMY-2	<i>E. coli</i>	P	Overproduction + OmpC/OmpF loss	>256	>16	>64	>256	>32	(361)
CMY-2	<i>E. coli</i>	P	Overproduction + OmpC insertion IS1			>64		>256	(287)
CMY-2	<i>E. coli</i>	P	Overproduction + OmpC/OmpF loss	>256	>128	>256		>512	(344)
<u>EC14</u>	<i>E. coli</i>	Chr	ESAC + OmpC/OmpF loss	>256	>128	16	>32	1	(362)
CMY-4	<i>S. enterica</i>	P	+ OmpF loss	>512	>64	>16			(363)
ACT-1	<i>K. pneumoniae</i>	P	+ Omp42-kDa loss	>128	8-32	8- >16			(31)
ACT-1	<i>K. pneumoniae</i>	P	+ OmpK35/36 insertion + PhoE decrease	64- 256		128		256	(364)
DHA-1	<i>K. pneumoniae</i>	P	+ OmpK36 loss		>128	>32		>32	(365)
DHA-1	<i>K. pneumoniae</i>	P	+ OmpK35/36 loss + AcrAB/OqxAB			32	>32		(366)
ACC-1	<i>K. pneumoniae</i>	P	+ OmpK35/36 loss	>128	16	8	32	16	(347)
FOX-1	<i>K. pneumoniae</i>	P	+ OmpK35/36 loss	>512	32	64	128	64	(347)
MOX-1	<i>K. pneumoniae</i>	P	+ OmpK35/36 loss	64	8	32	32	32	(347)

<u>EA-Z</u>	<i>K. aerogenes</i>	Chr	+ Omp40-kDa loss				16		(367)
<u>E15</u>	<i>K. aerogenes</i>	Chr	+ OmpK35/36 loss	>256			>16	>16	(368)
<u>E11</u>	<i>E. cloacae</i>	Chr	Overproduction + OmpK35/36 loss	>256			>16	>16	(368)
<u>144</u>	<i>E. cloacae</i>	Chr	Overproduction + porin loss	64			16	>128	(369)
<u>213</u>	<i>P. rettgeri</i>	Chr	Overproduction + porin loss	4			8	>128	(369)
<u>ACT-28</u>	<i>E. kobei</i>	Chr	Overproduction + OmpC- like protein	>256	>64		>128	>512	(349)
<u>A-1</u>	<i>A. baumannii</i>	Chr	Overproduction + Omp33/36 kDa decrease	32			>64		(350)
<u>A(4) or B(4)</u>	<i>A. baumannii</i>	Chr	Overproduction + Omp37/44/47 kDa decrease	>32	>32		8-16	>16	(370)
<u>Paeβ-04</u>	<i>P. aeruginosa</i>	Chr	Overproduction ESAC + <i>mexB</i> increase	32	16		16	16	(360)
<u>3-D8</u>	<i>P. aeruginosa</i>	Chr	Overproduction + <i>mexB</i> increase + OprD loss + <i>dacB</i>	128	64		32	>256	(358)
<u>AM339</u>	<i>P. aeruginosa</i>	Chr	Overproduction + <i>mexA</i> and <i>mexC</i> and <i>mexX</i> increase + OprD loss	>32	>128		>16	>16	(268)

2455 <sup>a</sup> Chr, chromosome; P, plasmid.

2456 <sup>b</sup> CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; ERT, ertapenem; MER, meropenem

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