Current Research in Translational Medicine 67 (2019) 115–122

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General review

Structure-based classification of class A beta-lactamases, an update

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ARTICLE INFO

Article history:
Received 28 February 2019
Accepted 21 May 2019
Available online 31 May 2019

Keywords:
Class A beta-lactamases
Subclass
Cluster
Primary structure
Classification

ABSTRACT

Beta-lactamase (EC 3.5.2.6) synthesis, particularly in Gram-negative bacilli, is a major mechanism of natural and acquired resistance to beta-lactams, sometimes accompanied by impermeability and/or active efflux. These enzymes have been classified into four molecular classes (A–D). The serine enzymes of class A, which may be encoded by the bacterial chromosome or transferable elements and are susceptible to clinically available inhibitors (clavulanic acid, sulbactam, tazobactam, avibactam), are prevalent considering other molecular classes (B,C,D). The continual rapid development of genomic approaches and tremendous progress in automatic sequencer technology have resulted in the accumulation of massive amounts of data. A structure-based classification of class A beta-lactamases based on specific conserved motifs involved in catalytic mechanisms and/or substrate binding (S^{70}XXK, S^{130}DN, K^{234}TG), together with E166 (Ambler numbering) and at least 24 other amino-acid residues or analogs such as G45, F66, V80, L81, L91, L101, P107, A134, L138, G143, G144, G156, L169, T181, T182, P183, was validated on 700 amino-acid sequences, including 132 representative types, but mostly probable enzyme sequences, many produced by environmental bacteria. Two subclasses (A1, A2), six major clusters or groups (e.g. natural limited-spectrum beta-lactamases (LSBL), wider spectrum beta-lactamases (WSBL), and various other clusters were identified on the basis of conserved ($\geq$ 90%) and specific motifs, and residues such as S^{70}TFKAL, S^{130}DNTAANL, R^{164}XEXXLN, V^{231}GDKTG for subclass A1, S^{70}VFKFH, S^{130}DNNACDI,E^{166}XXM, and V^{231}AHKTG for subclass A2, a probable disulfide bridge C77-C123 and G236, A237, G238, and R244 for the LSBL group. This great diversity of primary structures was used as the basis for a structure-based and phylogenetic classification.

1. Introduction

The serine beta-lactamases of molecular class A (CABL) are the most important enzymatic source of both natural and acquired resistance to beta-lactams, particularly in Gram-negative bacilli (e.g. *Enterobacteriaceae*,*Pseudomonas aeruginosa*, *Acinetobacter baumannii*), and but more rarely, in Gram-positive bacteria [1,2]. Time-consuming phenotypic and biochemical identification methods have identified several functional groups, which can now be further classified by molecular methods [3]. Thus, in medicine, more than 2000 class A enzymes have been identified. Most belong to particular functional types found in Gram-negative bacilli, such as TEM, CTX-M, KPC, or CARBA. These functional types are highly diverse, with many molecular variants demonstrating their ability to extend their inactivation spectrum to the latest-generation cephalosporins, even carbapenems and inhibitors through mutations [3–5]. There has been a continual, rapid development of genomic approaches and considerable progress in sequencing technology, with the recent advent of machines for whole-genome sequencing which has led to the generation of massive amounts of data. However, the accurate molecular definition of CABL remains poorly known, and several databases in 2018 still define "class A beta-lactamases" as proteins of more than 440 amino acids sharing only the following three motifs: SXXK, SDN, and KTG, and much more rarely, EXXLN [3]. Two studies comparing protein sequences recently reported considerable diversity among CABLs, with the identification of various groups and clusters, providing the basis for a more accurate molecular identification, including conserved and specific residues [6,7]. With the considerable increase in the number of sequences in databases, it is now possible to confirm the main molecular characteristics of CABLs in a large sample of

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http://dx.doi.org/10.1016/j.retram.2019.05.003
2452-3186/

representative enzyme amino-acid sequences (132) and to identify those probably or hypothetically highly predominant in genomes (568).

2. Phylogenetic studies

A number of new enzymes have been added to the list of 114 representative beta-lactamases studied to date: ARL-1, AXC-1, BKC-1, CARB-17, CBP-1, CRH-1, CRP-1, CRS-1, CzoA, GPC-1, HMS-1, LUS-1, OIH-1, PAD-1, RHOCA, RUB-1, SEL-1, VCC-1 (Supplementary Table S1). In addition to the 174 probable or hypothetical beta-lactamases already compared, another 394 protein sequences have now been identified as belonging to class A following searches of three databases on NCBI, Uniprot and BLDB sites [8]. Each sequence generally corresponds to a single species or family, because they differ from each other by one to four amino-acid substitutions, defining the CTX-, GES-, SHV-, and TEM-families, the criteria for molecular selection being as recently tested [6]. A phylogenetic study (Fig. 1) was performed following multiple sequence alignment (ClustalO), with the neighbor-joining method [9].This phylogenetic study allows to highlight 6 major clades or groups.

Considerable enzyme diversity was observed, with the identification of several super clades within group A (subclass A2), which forms a group separated from the other groups by a large phylogenetic distance (Fig. 1, Supplementary Fig. S1) [6,7,10,11]. Most of these enzymes are produced by bacteria of the CFB group (*Cytophaga-Flavobacterium-Bacteroides*) or from various orders and genera of Bacteroidetes: Bacteroidales, Chitinophagales, Cytophagales, Flavobacteriales and Sphingobacteriales. Some protein sequences were obtained from other phyla, such as the Fibrobacteres-Acidobacteria group; two orders and several genera from the phylum Cyanobacteria: Nostocales, and Spirochaetales. A few of the bacterial species were classified as *Proteobacteria.* The possibility of horizontal gene transfer from the CFB group to *Gammaproteobacteria*, such as enterobacteria, was previously suggested for the PER-1, VEB-1, TLA-1 beta-lactamases [10]. However, we were unable to identify the progenitors involved in such a transfer. Based on phylogeny data, the PER and VEB types appear to have different progenitors (Fig. 1). Subclass A2 is highly diverse, with a wide distribution of the representative enzymes, and various clusters were clearly identified. Most of the beta-lactamases concerned were naturally encoded by the chromosome (species-specific), and it was often possible to identify highly probably progenitors, as e.g. for the CfxA type (genus *Prevotella*); the CepA and CblA types (genus *Bacteroides*); the CME type (genus *Elizabethkingia*) . . . These enzymes were functionally classified as hydrolyzing predominantly cephalosporins, but also penicillins and aztreonam (functional group 2e) [1,3,12,13]. Significant synergy with an inhibitor (e. g. clavulanate, avibactam) was generally observed with such substrates [14]. Within this subclass, the various clusters displayed only low levels of identity, below 25% in some cases, clearly demonstrating their different polyphyletic origins. A comparison of representative beta-lactamases (CfxA, CepA, CblA-1) among anaerobic bacteria (*Bacteroides*, *Prevotella* etc.) revealed a similar low level of identity (e.g. 37% between CepA and CfxA, 42% between CfxA and CblA-1). Nevertheless, the single cluster containing CfxA-1 and 10 protein sequences from various species of *Prevotella* and *Bacteroides* demonstrated the existence of a transposon, such as Tn4555 [15,16].

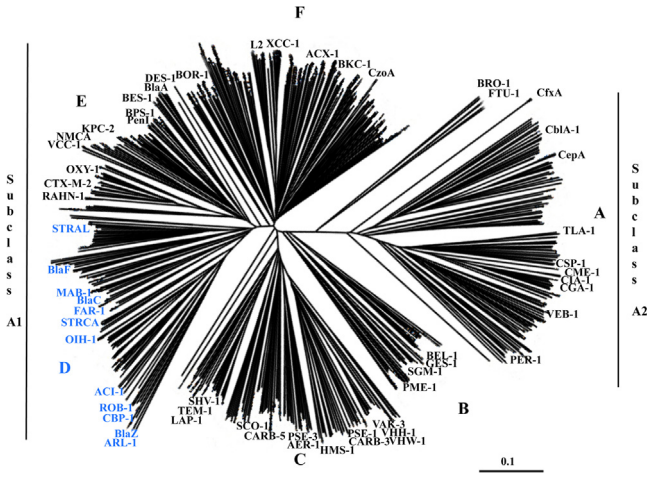

Fig. 1. Unrooted phylogram for 700 class A beta-lactamases. Several representative enzymes are shown (see also Supplementary Table 1). Putative enzymes are indicated by a UniProt or GenBank accession number (GI or WP), but most contain the following conserved motifs or residues common to class A beta-lactamases: S^{70}XXK, S^{130}DN, E^{166} and K^{234}TG. ClustalO was used to align the sequences and the tree was constructed by the neighbor-joining method [9,86,87]. The figure was created with Seaview [88].

The compositions of groups A, C, D, E and F are listed in supplementary FIG. S1-S5. The composition of **group B** is as follows: cluster 1 (PME-1; *Acidobacteria bacterium (A0A192WNJ8); Acidomonas methanolica (GI:*754682365*); Altererythrobacter epoxidivorans (A0A0M3T9N4); Altererythrobacter marensis* (A0A0G3XED2); *Erytrobacter longus* (GI:736965560); *Gluconobacter japonicus* (A0A177G0D0); *Hydrocarboniphaga daqingensis* (A0A1M5ND10); *Novosphingobium nitrogenifigens* (F1Z6G2); *Porphyrobacter cryptus* (GI:653237942); *Porphyrobacter neustonensis* (A0A192D3 × 4); *Sphingomonas sanguinis* (A0A147ILI6); *Neoasaia chiangmaiensis* (A0A1U9KS83); cluster 2: BEL-1; GES-1; IBC-1; SGM-1; *Blastomonas fulva* (A0A222 × 0U7); *Sphingobium chungbukense* (A0A0M3ARR2); *Sphingobium indicum* (A0A1L5BLN4); *Sphingomonas paucimobilis* (GI:612114377); *Sphingopyxis baekryungensis* (GI:550924335); *Sphingopyxis baekryungensis* (GI:550924335); *Sphingopyxis ummariensis* (A0A1Y6FTJ6); *Sphingopyxis witflariensis* (A0A246K5M3); cluster 3: *Acidocella facilis* (GI:651314737); *Acetobacter indonesiensis* (A0A0D6N9I5); *Acetobacter tropicalis* (A0A094ZTL7); *Celeribacter baekdonensis* (K2K1M7); *Bradyrhizobium* diazoefficiens (A0A1L5M1G8); *Bradyrhizobium lablabi* (WP_079588090.1); *Bradyrhizobium liaoningense* (A0A151FNV2); *Bradyrhizobium neotropicale* (A0A176Z6A1); *Bradyrhizobium yuanmingense* (A0A1C3URT4); *Gluconobacter frateurii* (A0A0K8N3A8); *Komagataeibacter hansenii* (D5QCA1); *Tardiphaga robiniae* (A0A163YVQ1); *Zymomonas mobilis* (A0A2R9NW34).

Groups B consists largely of enzymes from Gram-negative *Alphaproteobacteria* (Fig. 1). These bacteria may be symbiotic with plant roots (Rhizobiales), photosynthetic, mainly aquatic (Rhodospirillales), or phytopathogenic, and some are known to break down certain aromatic compounds (Sphingomonadales). BEL-1, GES-1, and PME-1 extended-spectrum and plasmid-encoded beta-lactamases have been identified in various *Gammaproteobacteria* species, including *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. Several molecular variants were identified [8]. Identity was highest between BEL-1 and GES-1 (about 52%).

Groups C and E include enzymes from Gram-negative bacilli, mostly from the *Gammaproteobacteria*, but with some from the other classes (*Alphaproteobacteria* and, more rarely, *Betaproteobacteria*), and many representative beta-lactamases, some specifically encoded by the chromosome and others by plasmids.

Group C was previously split into four clusters known as the LSBL (limited spectrum beta-lactamases)(LSBL 1 to 4), TEM/SHV and CARB clusters [6,7]. However, this major group of enzymes was found to be much more diverse, with a larger number of clusters containing mostly “probable” enzymes (Supplementary Fig. S2). This group is characterized by various representative beta-lactamases belonging to group 2b or 2c, according to the functional classification [12,13]. Most of these representative enzymes belong to the same clade as *K. pneumoniae* OKP-A, OKP-B, and SHV-1/OHIO-1 beta-lactamases and *K. variicola* LEN-1, and the ORN-1 (*Raoultella ornitholytica*), PLA-1 (*R. planticola*), and TER-1 (*R. terrigena*) enzymes of *Raoultella*. The transferable TEM-1 clusters with GIL-1 (*Citrobacter gillenii*) and RUB-1 (*Serratia rubideae*), together with several putative enzymes from enterobacterial species (e.g. *Hamiltonella defensa*, *Kluyvera intermedia*,

Pectobacterium carotovorum) confirming the probable origin of TEM-1 enzymes from Enterobacteriaceae. A new sequence from a *Kluyvera intermedia* isolate (FOSA7093) was recently obtained from a pancreatic specimen from a patient who had been hospitalized for a long period [17]. Other representative enzymes: RHOCA (*Rhodobacter capsulatus*), BlaP, RTG-2, SCO-1, HER-1 (*Escherichia hermannii*), and CKO-1/MAL-1)(*Citrobacter koseri*/*Levinea malonatica*) were distributed between the various clusters and displayed low levels of similarly, the lowest identity observed being 36%. The representative enzymes SCO-1, BlaP and RTG-2 were found in clusters of sequences from bacteria from terrestrial, aquatic or marine environments, and with those from the *Alphaproteobacteria* (Rhizobiales, Rhodobacterales, Rhodospirillales) and *Gammaproteobacteria* (Alteromonadales, Oceanospirillales) classes. A cluster of vibrios was identified, containing the representative enzymes VHH-1, VHW-1 and CARB-17 (*Vibrio parahaemolyticus*) and putative enzymes from several *Vibrio* species. The CARB-2 and CARB-3 enzymes were phylogenetically distant from this cluster of vibrios, with a percentage identity between VHH-1 and PSE-1 of only 47%. Note also the weak relationship between VAK-3 and CARB-17 (52%). HMS-1 beta-lactamase was found in a tiny cluster corresponding to Alteromonadales and Oceanospirillales, the most halotolerant or halophilic species, with a percent identity of 62 to 100% (*Colwellia chukchiensis*). This beta-lactamase was also recently identified, mostly in *Proteus mirabilis* isolates in China [18].

Gram-positive bacteria (group D) highlight the great diversity of these enzymes, with 20 clusters already reported (Fig. 1, Supplementary Fig. S3) [6,7]. A number of these species are known to be pathogenic. However, the possible role of a class A beta-lactamase in *C. botulinum,* with the inducible enzyme CBP-1 (group 2a) isolated in a child [19], is particularly noteworthy. *C. botulinum* isolates from infants or wound botulism are usually susceptible to several antibiotics, including penicillins. However, penicillin resistance has already been reported in *Clostridium butyricum* from a patient with neonatal necrotizing enterocolitis and up to 7.5% of strains are resistant [20]. This analysis expanded the role of new beta-lactamase inhibitors or combinations, such as meropenem-clavulanate, in mycobacteria such as *Mycobacterium tuberculosis* [21,22].

Finally, ACI-1 ESBL beta-lactamase has been identified in several species (e.g. *Acidaminococcus fermentans, Dialister succinatiphilus*, *Megasphaera elsdenii* . . .) of Negativicutes (Gram-negative Firmicutes). Most were obtained from the digestive microbiomes of humans and animals, probably the *aci1* gene was thought to be acquired via a transposon [23,24].

Within other Gram-negative bacteria, group E differed significantly from group B, with the initial identification of several clusters, such as ESBL1, ESBL2, CARBA and BURK [6]. The representative beta-lactamases were classified as functional group 2be or less frequently, 2f enzymes. This group could also be labeled WSBL (wider spectrum beta-lactamases because of their intrinsic capacity to hydrolyze oxyiminocephalosporins. Fig. 1 and Supplementary Fig. S4 confirm the existence of this group, with other clusters of probable enzymes. The ESBL1 cluster was clearly associated with various chromosomal enzymes specific to *Enterobacteriaceae* species, such as FONA, KLUA-1, OXY-1, and RAHN-1, some of which are the progenitors of plasmid type CTX-M beta-lactamases. Not far removed phylogenetically is the genus *Pantoea* cluster, including the species *P. vagans*, *P. stewartii*, *P. ananatis*, which beta-lactamase share little identity with other members of this cluster (between 57 and 63%).

The CARBA for carbapenemase cluster (functional group 2f) was enriched with a new representative enzyme, VCC-1, originally identified in a strain of *Vibrio cholerae* isolated from a batch of shrimps imported into Canada from India, but recently from coastal waters of Germany [25]. Interestingly two beta-lactamases produced by *Chromobacterium* species (*Betaproteobacteria*), CRH-1 (*C. haemolyticum*) and CRP-1 (*C. piscinae*), were recently identified as belonging to this cluster. They displayed a high level of identify to KPC-type enzymes, up to 76% in some cases [26].

Several putative enzymes were added to the old cluster of probable enzymes produced by several species of *Yersinia*, ERP-1 and BES-1. *Betaproteobacteria* includes groups of Gram-negative aerobic or facultative bacteria that often have versatile degradation capacities, including, in particular, the genus *Burkholderia,* which includes species pathogenic in humans and animals, such as *B. pseudomallei*, *B. mallei*, and the opportunistic pathogens, *B. thailandensis*, *B. cepacia*, *B. cenocepacia*, and *B. multivorans* [27,28]. Phylogenetic comparisons showed that these enzymes were closely related and clustered together. They displayed 64 to 99% amino acid sequence identity, the lowest level of identity being that between PenI and PenB (64%). Two clusters were distinguished: cluster 1 (*B. pseudomallei*, *B. mallei*, *B. thailandensis*, *B. glumae*, *B. oklahomensis*, *B. plantaris*, *B. singularis*), and cluster 2 (*B. cenocepacia*, *B. multivorans*, *B. vietnamensis*, *B. ambifaria*, *B. contaminans*, *B. dolosa*, *B. ubonensis*). The LUT-1 and MIN-1 sequences were obtained from various members of the *Betaproteobacteria* and shown to be phylogenetically different. The cluster including LUT-1 also included other probable enzymes produced by several other *Chromobacterium* species, including *Chromobacterium violaceum,* a model environmental opportunistic and resistant pathogen causing infections in humans [29–31].

Group F (Supplementary Fig. S5) includes various environmental bacterial species [7] mostly from the classes *Alphaproteobacteria* (50%), *Gammaproteobacteria* (26%), *Betaproteobacteria* (22%) and *Deltaproteobacteria* (2%). Note the great diversity of *Alphaproteobacteria*, with ten genera from Rhizobiales, nine from Rhodospirillales and two from Caulobacterales. Most of the species concerned are bacteria from the environment (soil, water), of the Gram-negative bacillus type. However, they currently have only very limited pathogenicity, at least in humans, requiring an immunocomprised background as an essential promoting factor [32–39]. Only one plasmid-encoded beta-lactamase, BKC-1, has been reported to be produced by only two strains of *K. pneumoniae* that also produce ESBLs, in Brazil [40]. Moreover, BKC-1 is a weak carbapenemase [5]. The tremendous species diversity is also illustrated by the existence of various clusters, such as that of BKC-1, with the genera *Shinorhizobium*/*Paramesorhizobium,* with percent identity values ranging from 64 to 84%. PAD-1 is a chromosomal class A weak carbapenemase with an amino-acid sequence very different from those of enzymes identified in a clinical context, such as BKC-1 (66%) and KPC-2 (47%) [5,41]. Supplementary Fig. S5 illustrates the existence of other clusters, such as the *Xanthomonas*/*Stenotrophomonas* cluster, with two chromosomal enzymes, XCC-1 and L2 as opportunistic pathogens in humans [42]. Two putative class A beta-lactamases were originally assigned to a cluster grouping together sequences from the genera *Comamonas* and *Delftia*, and CzoA was recently reported in *Comamonas testosteroni,* environmental bacterium shown to cause several types of infection [43,44]. Finally, these environmental bacteria of limited pathogenicity include opportunistic pathogens such as *Bordetella bronchiseptica* with BOR-1 (functional group 2b) [45,46]. AXC-1, a novel inducible beta-lactamase, was recently identified in a meropenem-resistant clinical isolate of *Achromobacter xylosoxidans* [47].

3. Specific molecular characteristics

3.1. A single molecular class

The mean length of the 700 protein sequences studied here was 298 ± 12 (standard deviation) amino acids (aa), for a molecular

mass of less than 35 Md. Lately, a tiny difference was reported between enzymes of subclasses A1 and A2, respectively 285 and 300 aa [11].

The following motifs and residues, or their analogs*, are highly conserved (≥ 99%): S70XXK, S130DN, E166, K234TG. However, rare exceptions have been noted, such as the presence of a C70 residue (*Legionella hackeliae*, *Tatlockia micdadei*) and a G70 residue (*Vibrio mytili*). The presence of a G70 is particularly astonishing, because the role of the serine is central for the acylation step of beta-lactams. The G130 residue is identified in three putative sequences (*Bordetella hinzii*, *Francisella tularensis*, *Francisella novicida*), contrasting with the representative beta-lactamases BRO-1, FTU-1 and FPH-1. Interestingly in several TEM- and SHV-type enzymes, S130 G confers resistance to inactivation by the enzyme inhibitors as clavulanic acid [48]. Finally, the S130DG triplet has already been reported in several species of *Mycobacterium* [6]. It has now also been identified in other phyla (e.g. *Acidobacteria*, *Actinobacteria*, Bacteroidetes). The E166 residue essential for enzymatic activity is absent from several putative sequences: *L. hackeliae*, *T. micdadei*, and *Rubidibacter lacunae*. The E166PEL motif is present in some *Legionella* species (e.g. *L. brunensis*, *L. lubrilucens*), but a different pattern (P166XEA) is observed in other species (e.g. *L. adelaidensis*, *L. feelei*). Several E166 substitutions in beta-lactamases have been investigated [49–51].

In conclusion, several motifs characterize class A as clearly illustrated by the past, but indidualization of subclasses was unknown [52,53]. Otherwise twenty-four additional residues or analogs* are found, making it possible to characterize this molecular class more clearly and to identify representative and probable beta-lactamases (Fig. 2) [4,6,54,55]. These residues* are as follows (700 sequences): G45 (99%), F66 (95%), V80 (96%), L81 (96%), L91 (91%), L101 (97%), P107 (97%), A134 (96%), L138 (98%), G143 (94%), G144 (94%), G156 (92%), L169 (96%), T181 (95%), T182 (97%), P183 (93%), L190 (92%), L199 (97%), L207 (95%), M211 (99%), L225 (91%), V231 (92%), G232 (96%), I261 (91%).

* Footnote: The following residues are considered equivalent: A and G; S and T; D and E; I, L, M and V; F, Y, and W; K, R and H; N and Q.

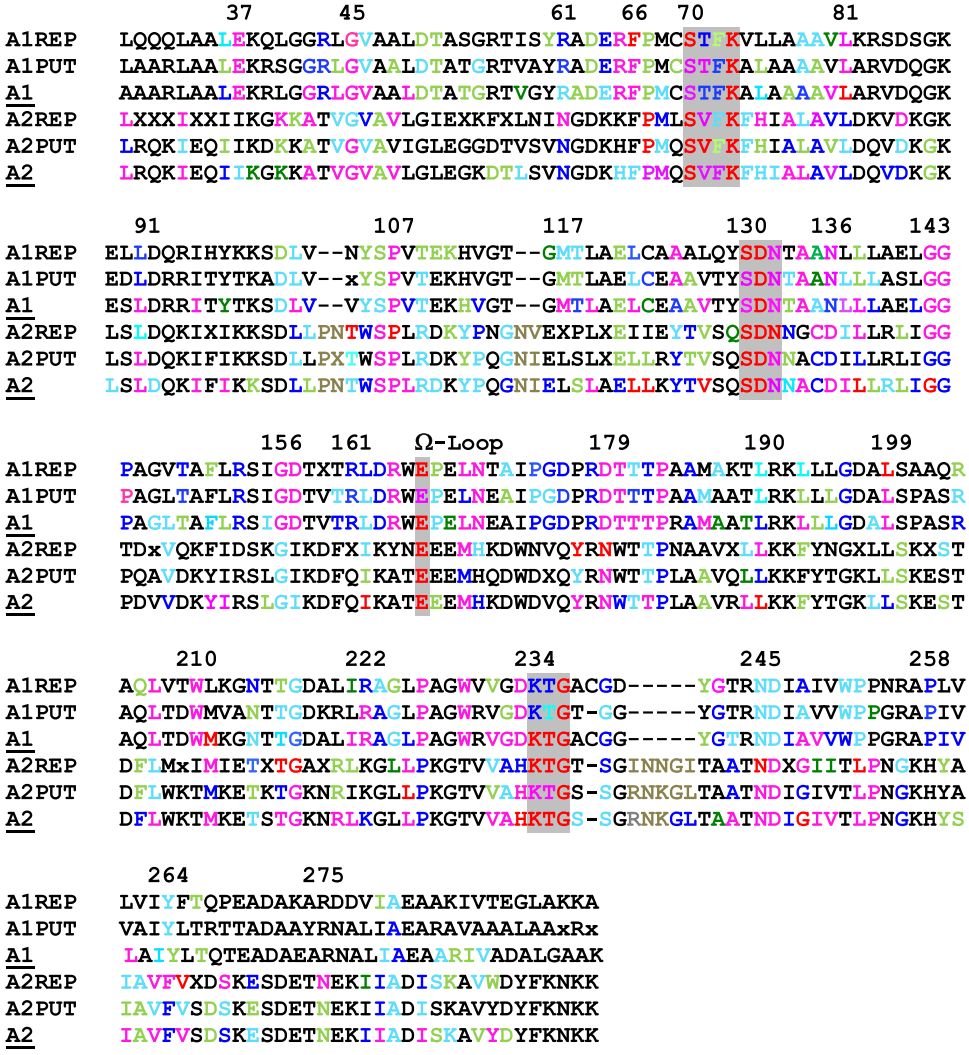

Fig. 2. ABL consensus sequences after multiple alignments of the amino-acid sequences of the subclass A1 (132 REP for representative, 447 PUT for putative) and subclass A2 (14 REP, 107 PUT) beta-lactamases. Underlined A1 and A2 correspond to residues or analogs, as follows (A = G; D = E; K = R=H; N = Q; T = S; I = L=M = V; F = Y=W). Signal peptides and N-terminal ends have been omitted because they display little sequence identity. Numbering is as described by Ambler et al. [54]. The residues involved in the catalytic mechanism and/or substrate binding are shaded. Additional residues typical of subclass A2 are colored in gray. Dashes indicate gaps within the alignment, and X indicates variable residues. A colorimetric scale was used to express the percentage of residues in each subclass deduced from Jalview [89], where red indicates a 100% conserved residue, purple indicates a 90 to 99% conserved residue, dark blue indicates an 80 to 89% conserved residue, light blue indicates a 70 to 79% conserved residue, and green indicates a 60 to 69% conserved residue (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.2. Identification of two subclasses

Subclass A1 was characterized by the other conserved residues R61, R65, T71, G78, A125, N136, D157, R164, N170, D179, T180, T181, T182, P183, W210, R222, W229, and D233 (Fig. 2). By contrast, the beta-lactamases of subclass A2 were characterized, in particular, by the other residues N61, V71, C135, D136, Y177, N179, H233, V263, F264, and V265.

Finally, four major motifs with residues involved in the catalytic mechanism and/or in substrate binding were defined to distinguish between the enzymes of subclass A1 (S70TFKAL, S130DNTAANL, R164XEXXLN, V132GDKTG), and subclass A2 (S70VFKFH, S130DNNACDI, E166XXM, and V232AHKTG).

3.3. Molecular characteristics of groups B, C, and E

These enzymes of group B mostly result in an ESBL phenotype. This group is characterized by the following conserved residues: C69, C238, R/K222, S/T237, R220 (for the *Sphingobium* family), and R244 (for the GES and BEL families). The conserved C69 and C238 residues may form a disulfide bridge, modifying the overall shape of the active site [5,56]. The disruption of this disulfide bridge has different effects on GES and CARBA enzymes. Some GES variants display phenotypic variability, potentially extending their spectrum of activity to carbapenemases through a single-amino-acid substitution, D170 N or D170S [5]. Surprisingly, all BEL-type enzymes have an N170 residue, but no activity towards carbapenems. A T237 residue is present in GES enzymes (except GES-12), whereas the enzymes of the BEL and SGM families have an S237 residue. The T237A substitution results in GES-11 hydrolyzing aztreonam and ceftazidime twice as efficiently as GES-12 [5]. The highest MICs obtained with GES enzymes, for cefotaxime and aztreonam, for example, were conferred by combinations of up to four substitutions. The G243 residue present in all the enzymes of the BEL cluster and most of those of the GES cluster is not conserved in other class A beta-lactamases. The G243S and G243A substitutions are associated with variable efficiencies of oxyimino-beta-lactam hydrolysis [5].

Group C consists of natural and originally limited-spectrum beta-lactamases (LSBLs), including various types of predominantly chromosomal enzymes, but also a number of plasmid-encoded or transposable element-encoded enzymes. With the addition of two representative enzymes (HMS-1, RHOCA), all representative enzymes were classified according to their penicillinase activity (functional group 2a, 2b or 2c) [12,13]. Only a few residues distinguish these enzymes from other groups of enzymes, such as those of group C: C77 (99%) and C123 (100%) with a probable disulfide bridge, D85 (84%), G236 (100%), A237 (93%), G238 (95%), and R244 (100%). N and D aminoacids are never represented at positions 245 and 246 in LSBL enzymes. As previously reported, when R is present at position 244, a different residue is found at position 220 or 276 of the corresponding enzyme [4]. Representative carbenicillin-hydrolyzing enzymes are unique among class A beta-lactamases in possessing an R residue at position 234 and a T or S residue at position 235 (e.g. CARB-types, MP-1, SCO-1, VAK-3, VHH-1) [57]. Curiously, HMS-1 has been classified as belonging to group 2b rather than 2c [12]. All these molecular characteristics were detected in 91 protein sequences of putative enzymes distributed between at least 15 clusters. Finally, in this group of enzymes, which cannot naturally inactivate oxyimino-beta-lactams and aztreonam and are not naturally resistant to inhibitors (e.g. clavulanate), well-defined substitutions were observed only in various TEM and SHV mutants [4,58–60]. The BLDB website lists 227 TEM variants and 203 SHV variants with amino-acid substitutions (March 2019) [5].

The representative enzymes of the group E were clearly identified as particularly reactive against cephalosporins, and aztreonam, but they react poorly with ceftazidime [5,61–63]. Finally, these enzymes, grouped together under the acronym WSBL, as opposed to LSBL, are also highly susceptible to beta-lactamase inhibitors. Some residues* are highly conserved in this group ($\geq$ 90%): C69, Q128, Y129, F160, T171 and T216. The T/S237 residue is also conserved (88%). Nevertheless, as for the ESBL1 cluster, various susceptibility patterns related to certain key residues were readily identified for species in which the beta-lactamase was chromosomally encoded. Some enzymes have an A residue in position 237, whereas most of the others have an S or T residue, which has been implicated in extension of the substrate specificities of TEM and SHV ESBLs to cefotaxime. The A237S substitution in the OXY-1 enzyme increases resistance to cefotaxime and cefepime [64]. CTX-M enzymes can also evolve through particular point mutations (P167S and D240 G substitutions) observed in clinical practice to inactivate ceftazidime more efficiently. The relative rate of oxyimino-beta-lactam hydrolysis is decreased by R276 substitution, suggesting that this residue is involved in extending the activity spectrum of the enzyme. Only three probable *Pantoea* enzymes were added to this cluster, and these enzymes are probably ESBLs, based on the presence of the S237 and R276 residues. The previous cluster, ESBL2, included only two other probable *Yersinia* enzymes (*Y. nurmii*, *Y. pekkanenii*), which also had T/S237 and R276 residues. The enzymes of this cluster mostly have C69 and C123 residues, by contrast to ESBL1 enzymes (which have only C69). Interestingly, the predicted susceptibility pattern for the ERP-1 ESBL enzyme in *Erwinia persicina* was unusual and placed in group 2be because no basic residues in positions 220, 244 and 276 [65].

The CARBA cluster mostly contains strong carbapenemases of functional group 2f, principally from *Enterobacteriaceae*, with the exception of VCC-1 (*Vibrio cholerae*) [5,66–69]. This cluster includes chromosomally encoded enzymes (BIC, IMI-1, NmcA, SFC, SME-types, and VCC-1), more troublesome plasmid-encoded types (KPC, and FRI) [5]. However, other criteria identified several other enzymes chromosomal (BKC-1, FPH-1, PenA, SHV-38) and plasmid-encoded (GES) enzymes as weak carbapenem-hydrolyzing enzymes. Chromosome-encoded enzymes, including VCC-1, mediate resistance to carbapenem uncoupled to resistance to extended-spectrum cephalosporins, whereas KPC-type enzymes mediate resistance to both carbapenems and extended-spectrum cephalosporins. Several conserved residues characterize this cluster: two C residues at positions 69 and 238, forming a disulfide bridge that modifies the shape of the active site by changing the distances between several active-site residues [70]. Comparing multiple sequence alignment with GES-types, a tiny difference appeared considering location of 238C for enzymes of CARBA cluster and 239C for GES-types [6]. Curiously, disruption of the disulfide bridge in the GES-5 enzyme by the C69 G substitution results in minor decreases of beta-lactam MICs, but causes arrest of bacterial growth of several CARBA enzymes [56]. With the exception of KPC enzymes, which selectively hydrolyze extended-spectrum cephalosporins, all other types of beta-lactamases have an additional residue (indel) at a position close to 140. Finally, all the enzyme types are characterized by the presence of a S/T237 residue. The S-to-A substitution at this position decreased *k*cat values for imipenem and reduced cephalothin hydrolysis rates by a factor of 5 [71]. However, no single residue has yet been identified as responsible for carbapenem resistance [5,56,72,73].

Several CABLs in the genus *Burkholderia* have been given diverse names (BPS-1, PenA, PenI) for the enzyme from the species *B. pseudomallei*, which was identified as an ESBL (group functional 2be) [74]. The MICs of cefotaxime and aztreonam are significantly higher than that of ceftazidime (CAZ). Two types of mutations have been implicated in CAZ resistance (CAZ-R) in clinical isolates (C69Y and P167S) and in clavulanate resistance (S72 F) [74–77].

Observations for the *B. pseudomallei* beta-lactamase have been confirmed by in vitro studies (PenA/PenL) [78–80]. Interestingly, a single amino-acid deletion (Q168del) may expand the in vitro spectrum of inactivation to ceftazidime. Additional single amino-acid deletions have recently been reported. The *Burkholderia cepacia* complex includes two opportunistic pathogens, *B. cepacia* and *B. multivorans*. PenA and PenB enzymes, originally described as penicillinases, were subsequently shown to be inhibitor-resistant weak carbapenemases [15,74]. PenA overproduction was observed in CAZ-R mutants, and the PenB enzyme was found to have an F72 residue, accounting for clavulanate resistance [80–82]. The role of PenA in the clinical resistance of *B. multivorans* to β-lactams remains unclear, but another mechanism of acquired resistance involves the loss of a penicillin-binding protein and a porin [74].

4. Conclusions

The molecular identification of 700 representative and mainly probable class A beta-lactamase protein sequences (81%) must first be based on the three motifs involved in substrate binding and/or hydrolysis (70SxxK, 130SDN and 234 K/R T/S G) and the residue 166E but also on about twenty conserved residues whose possible role remains unknown (e.g. G45, F66, V80, L81, L91, L101, P107, A134, L138, G143, G144, G156, L169, T181, T182, P183). Other residues or patterns, less conserved (between 80–89%) are more recently discovered such as the 105Y important for substrate recognition [83]. Finally, the number of amino acids varies little (298 ± 12). The diversity of this class is considerable, with only 16% identity between CfxA and CARB-2 (PSE-1) or PER-1 and 20–24% between PER-1 and several CTX-Ms. The phylogenic classification individualizes at least 6 major groups including the recently reported one justifying the proposal of a new A2 subclass by identifying different residues, especially at the active site (71 V, 75H, 133 N, 135CD, 169 M, 233 H), the other groups constituting the A1 subclass (71 T, 75 K, 133 T, 136 N L, 164R, 169 L N, 233D) [6,7,11]. The lack of sufficient crystallographic data for A2 subclass enzymes does not currently allow them to be assigned a peculiar role. Recently, the variable activity of avibactam, a new broad-spectrum inhibitor according to the residue at position 169 in the Ω-loop has been reported [83].

The phylogenic individualization of a group or a cluster comprising several representative enzymes makes it possible to envisage a filiation, more enzymatic for the probable ones. Thus, in the LSBL group comprising 125 protein sequences, the cluster (> 70% identity) comprising TEM-1, GIL-1 and RUB-1 encompasses several enterobacterial sequences (Supplementary Fig. S2). Of the *Kluyvera* species including *K. ascorbata*, *K. cryocrescens*, and *K. georgina* naturally producing beta-lactamases belonging to another major group (WSBL), bacterial identification appeared doubtful for *K. intermedia* and *K. intestini*. Indeed, it has just been corrected in *P. ursingii* and *P. diazotrophicus* [84]. In a second step, the analysis of the sequence (s) of probable beta-lactamases will also have to take into account the possible residues known to be involved in a modification of the spectrum (extension or limitation). The final analysis for bacterial species of this cluster indicates that the sequences encode enzymes classifiable in the broad-spectrum 2b beta-lactamases [12,13].

It will thus be possible in a more or less near future with artificial intelligence to analyze and predict quickly and automatically the type of beta-lactamase after analysis of the sequence especially as the analysis will have to take into account many mutants regularly reported in the scientific literature, but also other genes implicated for overproduction e.g. promoter, *amp*R, and even coding for porins. Nevertheless Karen Bush has rightly reaffirmed the need to characterize bacteriologically and enzymatically such highly probable beta-lactamases, especially in clusters without enzymes yet identified [85].

Author contributions

The literature review, data analysis and manuscript writing were performed by AP, and RL, while HJ and ER reviewed the manuscript and provided technical assistance.

Disclosure of interest

No funding.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.retram.2019.05.003.

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