



Past and Present Perspectives on β -Lactamases

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ABSTRACT β -Lactamases, the major resistance determinant for β -lactam antibiotics in Gram-negative bacteria, are ancient enzymes whose origins can be traced back millions of years ago. These well-studied enzymes, currently numbering almost 2,800 unique proteins, initially emerged from environmental sources, most likely to protect a producing bacterium from attack by naturally occurring β -lactams. Their ancestors were presumably penicillin-binding proteins that share sequence homology with β -lactamases possessing an active-site serine. Metallo- β -lactamases also exist, with one or two catalytically functional zinc ions. Although penicillinases in Gram-positive bacteria were reported shortly after penicillin was introduced clinically, transmissible β -lactamases that could hydrolyze recently approved cephalosporins, monobactams, and carbapenems later became important in Gram-negative pathogens. Nomenclature is based on one of two major systems. Originally, functional classifications were used, based on substrate and inhibitor profiles. A later scheme classifies β -lactamases according to amino acid sequences, resulting in class A, B, C, and D enzymes. A more recent nomenclature combines the molecular and biochemical classifications into 17 functional groups that describe most β -lactamases. Some of the most problematic enzymes in the clinical community include extended-spectrum β -lactamases (ESBLs) and the serine and metallo-carbapenemases, all of which are at least partially addressed with new β -lactamase inhibitor combinations. New enzyme variants continue to be described, partly because of the ease of obtaining sequence data from whole-genome sequencing studies. Often, these new enzymes are devoid of any phenotypic descriptions, making it more difficult for clinicians and antibiotic researchers to address new challenges that may be posed by unusual β -lactamases.

KEYWORDS ESBL, MBL, β -lactam, β -lactamase, carbapenemase, cephalosporinase, penicillinase

One of the most studied enzyme families is the group of enzymes known as β -lactamases, with more than 28,900 citations in Medline (<https://www.ncbi.nlm.nih.gov/pubmed/>). These enzymes, whose most obvious role is to inactivate β -lactam antibiotics, have driven research in academic laboratories since the early 1940s (1, 2). Perhaps more importantly, they have been responsible for scores of pharmaceutical research programs that have attempted to find ways to protect effective antibiotics from destruction. β -Lactamases provide intellectual challenges for academic investigators with their deceptively simple mechanisms of action, as enzymes whose hydrolysis activity approaches that of a “fully efficient enzyme” with diffusion-limited reaction rates (3). Attempts to provide economically viable anti-infective agents that can circumvent the action of these enzymes have yielded substantial economic rewards to successful pharmaceutical companies. As a result, the safe and effective β -lactam antibiotics have become one of the most widely prescribed classes of antibacterial agents (4). Many review articles have been published about various aspects of these intriguing enzymes (5–11), but they have tended to focus on narrowly targeted sets of β -lactamases. In this minireview, a short history of the β -lactamases is presented, emphasizing key points that have driven both academic and pharmaceutical science

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TABLE 1 Table of Firsts: the dates, organisms, and locations of the first of a series of β -lactamase-producing isolates with long-term clinical significance

Original β -lactamase name (currently recognized name)	Yr of first verified isolation	Organism	Location	First description in literature	Reference(s)
Penicillinase (chromosomal AmpC)	1940	<i>Bacillus coli</i> (<i>Escherichia coli</i>)	England	1940	1
Penicillinase	1942	<i>Staphylococcus aureus</i>	England	1942	65
OXA	1962	<i>Salmonella enterica</i> serovar Typhimurium, <i>Escherichia coli</i> ^a	England	1965 1967	87, 215
TEM-1	1963	<i>Escherichia coli</i>	Greece	1965	85
SHV-1	1972	<i>Klebsiella pneumoniae</i>	Unknown	1972	216
Transferable ESBL (SHV-2)	Pre-1983	<i>K. pneumoniae</i>	Germany	1983	217
Serine (class A, group 2f) carbapenemase (SME-1)	1982 1985	<i>Serratia marcescens</i>	England (London) USA (Minnesota)	1990 1986	148, 150
Plasmid-encoded AmpC (MIR-1)	1988	<i>K. pneumoniae</i>	USA (Massachusetts)	1990	141
Plasmid-encoded MBL (IMP-1)	1988	<i>Pseudomonas aeruginosa</i>	Japan	1991	151
Inhibitor-resistant TEM (TEM-30)	1991	<i>E. coli</i>	France (Paris)	1994	118
KPC-type (KPC-2)	1996	<i>K. pneumoniae</i>	USA (North Carolina)	2000	158
NDM-1	2006	<i>K. pneumoniae</i>	India (New Delhi)	2009	175, 176

^aAnderson and Datta described a *Salmonella* Typhimurium isolate from 1962 that later was confirmed to produce the OXA-2 enzyme (215). Egawa et al. described an *E. coli* isolate in 1967 that produced the OXA-1 enzyme (87).

over the past 75 years. High points in their history will be discussed, based on the “Table of Firsts” shown as Table 1. This history is not meant to be a comprehensive review of all the current literature on β -lactamases but is meant to tell a story about where these enzymes came from, how they have driven antibiotic discovery programs, and what challenges they pose for today.

TOPLINE VIEW OF β -LACTAMASES

β -Lactamases are versatile enzymes with a limited range of molecular structures found in a diversity of bacterial sources. Their commonality is the ability to hydrolyze chemical compounds containing a β -lactam ring. As shown in Fig. 1, they have been classified biochemically into two broad divisions according to the mechanism by which they perform hydrolysis, either through the formation of an acyl enzyme with an active-site serine (Fig. 2) (12) or via a hydrolytic reaction facilitated by one or two essential zinc ions in the active sites of metallo- β -lactamases (MBLs) (13). After sequence analyses became available for key β -lactamases, four molecular classes, A, B, C, and D, were eventually assigned based on molecular size and homology between active-site amino acid motifs (14–16). However, biochemical differences between penicillinases and cephalosporinases had been recognized well before sequences were available (17), and known β -lactamases were distinguished based on functional capabilities related to substrate and inhibitor profiles (Table 2). Early classification schemes relied upon relative hydrolysis rates of penicillins and early cephalosporins, together with the enzymatic response to protein-modifying agents (18, 19). As additional substrates and inhibitors were introduced into clinical practice and gene sequencing became inexpensive and routine, both molecular and functional characteristics were combined into a more comprehensive classification scheme (20, 21). Today, at least 17 functional groups associated with the four molecular classes have been distinguished (20), with the major groups shown in Fig. 1. Enzymes are still differentiated with respect to the relative hydrolysis of the β -lactam substrates, penicillins, cephalosporins, carbapenems, and monobactams. Further differentiation is possible based on reactions with the class A β -lactamase inhibitor clavulanic acid (22), the broad-spectrum serine β -lactamase inhibitor avibactam (23), and the metal ion chelator EDTA to identify MBLs (24).

β -LACTAMASE ORIGINS

The antibacterial effect of a β -lactam results in inhibition of the growth of replicating bacteria by acylating an active-site serine in essential penicillin-binding proteins (PBPs) (25, 26). During the terminal stages of cell wall biosynthesis, these enzymes are thus prevented from effecting the cross-linking of peptide chains to form peptidoglycan,

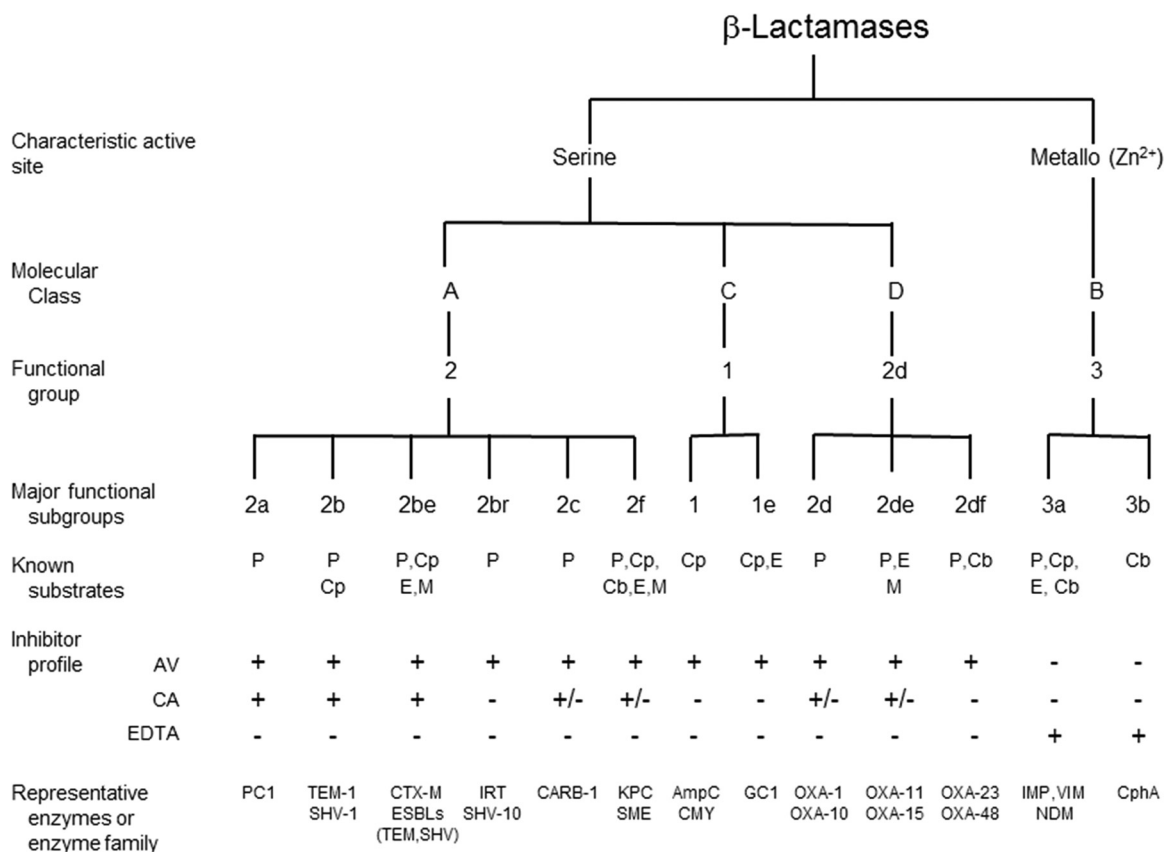


FIG 1 Molecular and functional relationships among β-lactamases (adapted from references 20 and 201 with permission). AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cp, cephalosporin; E, expanded-spectrum cephalosporin; M, monobactam; P, penicillin.

resulting in cell death. Tipper and Strominger proposed similarities between the structures of penicillin and D-Ala-D-Ala, the terminal amino acids of the nascent acetylmuramyl-pentapeptide fragment, to explain the antimicrobial activity of penicillin (27). Because the majority of β-lactamases contain an active-site serine that also can be acylated by β-lactam molecules, it is not unexpected to find mechanistic and structural similarities between the two sets of enzymes (28, 29). Fisher and Mobashery have cogently outlined a history of studies demonstrating the biochemical commonality between the two acyl enzymes (30). Molecular modeling of various serine β-lactamases and PBP structures has demonstrated three-dimensional similarities with conserved folding patterns and preservation of topology at the active site, in spite of low amino acid identities (28, 31).

The general reaction mechanism for both enzymes is shown in Fig. 2. It is commonly assumed that PBPs were the precursors of the β-lactamases, with the k_3 rate for deacylation increased dramatically for β-lactamases, compared to PBPs that exhibit a fast acylation step (k_2) compared to a slow deacylation step. Formation of the PBP-acyl

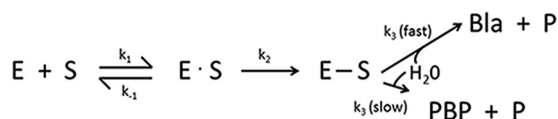


FIG 2 General reaction mechanism for binding of a β-lactam substrate (S) to a PBP (E) or a serine β-lactamase (E). Reversible formation of a Michaelis complex (E · S) which proceeds to a stable acyl enzyme (E—S) caused by reaction with the active-site serine. Hydrolysis occurs to form the microbologically inactive ring-opened β-lactam (P) and either enzymatically active PBP (slow hydrolysis of acyl enzyme) or β-lactamase (Bla, high hydrolysis rate).

TABLE 2 Characterization of β -lactamases based on information existing at the time of publication

Yr	Basis for characterization ^a	Conclusion(s)	Reference or source
1968	Substrate profile, reaction with antiserum	Three different groups of enzymes, typical cephalosporinases, cephalosporinase/penicillinase, and penicillinases (including those produced by R factors)	Sawai et al. (18)
1970	Substrate profile, inhibition by pCMB and cloxacillin, reaction with antiserum, electrical charge	Eight distinctive types of β -lactamases	Jack and Richmond (92)
1973	Substrate profiles, inhibition by cloxacillin and pCMB, electrophoretic mobility, molecular wt	Five classes of β -lactamases (I, II, III, IV, and V)	Richmond and Sykes (19)
1976	Substrate profiles, inhibition by cloxacillin and pCMB, isoelectric focusing, immunological relatedness, molecular wt; chromosomal or plasmid location considered	Five classes of β -lactamases (distinct from Richmond and Sykes)	Sykes and Matthew (218)
1980	Amino acid sequences from purified proteins	Molecular classes A and B defined based on four class A enzymes and one class B enzyme	Ambler (14)
1981	Amino acid sequence translated from <i>ampC</i> nucleotide sequence	Molecular class C defined	Jaurin and Grundström (15)
1988	Amino acid sequence translated from <i>bla_{PSE-2}</i> nucleotide sequence	Molecular class D defined	Huovinen et al. (16)
1988	Substrate profile, inhibition by clavulanic acid, aztreonam, and EDTA, molecular class designations for eight enzymes based on active site or full amino acid sequence data	Functional classification scheme with three groupings proposed for 28 enzymes	Bush (187)
1989	Substrate profile, inhibition by clavulanic acid, aztreonam, and EDTA, isoelectric point, molecular size, molecular class designations based on nucleotide sequences	Functional classification assigned to groups 1, 2 (six subgroups), 3, and 4 for 84 enzymes, aligned with molecular classes for 21 enzymes	Bush (219-221)
1995	Substrate profile, inhibition by clavulanic acid, sulbactam, tazobactam, aztreonam, cloxacillin, pCMB, and EDTA, molecular size, isoelectric point, molecular class designations based on nucleotide sequences	Functional classification for groups 1, 2 (eight subgroups), 3, and 4, for 217 enzymes, aligned with molecular classes for 118 enzymes	Bush et al. (21)
1996–2015	Amino acid sequences, initially for naturally occurring acquired TEM, SHV, and OXA ESBLs and IRTs; later expanded to all β -lactamase families	Curated numbering assignments for sequential β -lactamases in all major families	Lahey database (http://www.lahey.org/Studies/)
2010	Substrate profile, inhibition by clavulanic acid, tazobactam, or EDTA; molecular classification	Functional classification for groups 1 (2 subgroups), 2 (12 subgroups), 3 (2 subgroups), and 4	Bush and Jacoby (20)
2012	Genomic (DNA) data	Genotypic data for <i>Klebsiella</i> spp., including curation of nomenclature for OKP, LEN, and OXY β -lactamases	Institut Pasteur (http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html)
2013	Nucleotide or amino acid sequences for multiple resistance determinants for all antibiotic classes	Curated collection of characterized molecular sequences and phenotypes to predict functionality associated with antibiotic resistance genes	Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) (222, 223)
2015	DNA sequences in NCBI database that encode proteins conferring or contributing to resistance to β -lactam and other antibiotics	Annotated sequence records for representative DNA sequences in the NCBI database, including both naturally occurring and laboratory-derived β -lactamases	NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047)
2016	Commonalities of amino acid sequences of 285 class A β -lactamases	Separation of class A β -lactamases into two distinct subclasses, A1 and A2	Philippon et al. (73)
2017	Amino acid sequences, biochemical data (kinetics of hydrolysis), X-ray crystallographic structures	Well-annotated compilation of structure-function relationships of β -lactamases from all classes, including laboratory mutants	BLDB (http://blbdb.eu) (197)

^apCMB, *p*-chloromercuribenzoate.

enzyme complex is considered by many to be an irreversible reaction compared to the lifetime of a bacterial cell, with half-lives of acyl enzymes ranging from a relatively short 9 to 15 min for *Pseudomonas aeruginosa* PBP2 and PBP3 with amdinocillin or aztreonam (32, 33) to a half-life of more than 24 h for nitrocefin with PBP2a from *Staphylococcus aureus* (34). Some PBPs may have evolved to function as weak β -lactamases with a slow turnover of the β -lactam substrate (28); for example, cefotaxime deacylation rates are 70- to 80-fold higher for PBP2x variants than that for the wild-type enzyme in *Streptococcus pneumoniae* (35).

β -Lactamases are ancient enzymes that existed even in the absence of the pressure of therapeutic antibiotics. In 1979, Hamilton-Miller claimed that "penicillinase was born on December 28, 1940" (36), the date Abraham and Chain reported that a bacterial enzyme was capable of destroying penicillin (1). Little did he know that modern phylogenetic analyses have estimated the age of serine β -lactamases to be more than 2 billion years old (37), with plasmid-encoded β -lactamases appearing millions of years ago (38). Recent explorations of remote environmental niches have identified functional β -lactamase-like activity in locations, such as a region of the Lechuguilla Cave in New Mexico (USA) estimated to have been isolated for over 4 million years (39). Sampling of the cave resulted in the growth of 93 bacterial strains, with as many as 62% capable of hydrolyzing β -lactams. However, no sequences of known β -lactamase sequences were identified. The same group also examined metagenomic DNA from 30,000-year-old permafrost sediments east of Dawson City, Yukon (Canada) (40). In these samples, deduced amino acid sequences with 53% to 84% identities to the ubiquitous TEM β -lactamases clustered into the following two enzyme groups: (i) β -lactamases from various streptomycetes, and (ii) uncharacterized β -lactamase-like hydrolytic proteins. In a slightly younger sample of cold-seep sediments of Edison seamount (Papua, New Guinea) estimated to be 10,000 years old, 30 clones produced genes encoding β -lactamases primarily belonging to enzymes in the TEM family (41). Additional metagenomic analyses of ancient and naive environmental samples identified a novel metallo- β -lactamase (MBL), isolated from a 14th century bone sample, with an unusual substrate profile unlike that of other known MBLs; its sensitivity to clavulanic acid and to carbapenems suggested that it may have emerged to provide resistance to currently unknown β -lactam-containing molecules (42). In addition to these β -lactamase-type enzymes in historical samples, β -lactamase production has been identified in multiple remote pristine Antarctic soil samples (43), in ice cores in glaciers outside Antarctica (44), and in remote South American populations that have rarely, or never, been treated with commercial β -lactam antibiotics (45, 46). Curiously, an IMP MBL, a somewhat infrequent contributor to β -lactam resistance in contemporary clinical isolates (47), was found in approximately half of the glacial samples but not from Greenland or Antarctica (44). β -Lactamases have also been identified in fungi, where they are suspected of serving to inactivate plant or microbial naturally occurring β -lactam compounds (48). However, there are still organisms with no confirmed β -lactamases, notably *S. pneumoniae*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Mycoplasma* spp. and the *Chlamydiae*, possibly due to the production of homologous enzymes with the ability to inactivate β -lactams (49).

The history of β -lactamases can hardly be written without referring to the history of β -lactam-containing antibacterial agents, including the penicillins, cephalosporins, carbapenems, and monobactams, all of which contain a 4-membered 2-azetidinone ring (50). Although most of the β -lactam-containing agents in use today are synthetic or semisynthetic molecules, β -lactams as therapeutic agents originate from naturally occurring sources, identified initially because of their antibacterial activities in agar-based assays. Penicillin (penicillin G or benzylpenicillin) was first recognized because it prevented the growth of staphylococci on a petri dish contaminated with the common mold *Penicillium notatum* (51). Cephalosporin C, the starting point for the cephalosporins, was isolated from sewage sludge in Sardinia (52). Carbapenems were found in various β -lactam-producing actinomycetes from soil samples (53), and monobactams were isolated from bacteria in New Jersey soil and water samples (54). All these

iterations of unmodified β -lactams existed in natural environments and placed selective pressure on the neighboring bacteria to evade a milieu of deleterious agents. When β -lactam-producing organisms compete with nonproducing bacteria in the same environmental niche, survival strategies are quickly developed. Because bacteria, possibly the first living organisms, have existed for 2.5 to 4 billion years (55, 56), there has been a long incubation period for naturally occurring β -lactam biosynthesis to be perfected and for neighboring bacteria to learn how to survive in the presence of these molecules. Interestingly, mammalian enzymes, including serum proteins (57) and renal dehydropeptidase (dipeptidase) (58), are also capable of hydrolyzing β -lactams.

β -LACTAMASES IN GRAM-POSITIVE BACTERIA

Resistance to β -lactams can be mediated by multiple mechanisms. Because it is in the best interest of the bacteria to maintain active PBPs, the first line of defense would appear to be for PBPs to alter their affinities for β -lactams while maintaining their physiological function (28). This approach has been utilized most effectively by Gram-positive bacteria, where a primary resistance mechanism has been acquisition of PBPs with decreased affinity for common β -lactams (59). However, it took over 20 years of penicillin usage before clinical isolates of penicillin-resistant *S. pneumoniae* (60, 61) and penicillin/methicillin-resistant *S. aureus* (MRSA) were reported as a result of production of low-affinity PBP variants (62–64). Instead, penicillinases, or β -lactamases that hydrolyze penicillin, emerged as the initial resistance mechanism in *S. aureus* to challenge the therapeutic use of this agent (65). The drug was used more extensively and promiscuously to treat nosocomial streptococcal infections previously associated with high mortality (66), but, as collateral damage, staphylococci initially fully susceptible to penicillin rapidly developed resistance as a result of penicillinase production. In a single English hospital, the percentage of penicillinase-producing staphylococci increased from 14% in 1946 to 59% in 1948 (67) and up to 80% in 1953 (66). More recently, a 2013 study of U.S. clinical isolates showed that 86.5% of all *S. aureus* isolates (75.6% of methicillin-susceptible isolates) contained the penicillinase gene *blaZ* (68), indicating that a subpopulation of methicillin-susceptible staphylococci has survived in the absence of either a low-affinity PBP or the production of penicillinase. A transferable penicillinase was also reported in a few clinical isolates of *Enterococcus faecalis* in the 1980s (69), with identical or closely homologous amino acid sequences as staphylococcal penicillinases (70). A genomic analysis in 2005 demonstrated at least two divergent origins (71). However, penicillinase-producing enterococci have not been observed for the past decade and appear to have disappeared from these pathogens (72). Another analysis of recent genomic data noted similarities among a set of 88 class A β -lactamases from Gram-positive bacteria and two enzymes from Gram-negative bacteria, the ROB-1 penicillinase from *Haemophilus influenzae*, and the ACI-1 enzyme from *Acidaminococcus fermentans* (73), possibly due to “trans-Gram transfer” of β -lactamase genes between Gram-positive and Gram-negative bacteria.

In addition to the observed penicillinase production in Gram-positive clinical isolates, enzymes with β -lactam-hydrolyzing properties from numerous environmental bacilli were used by theoretical enzymologists as prototypical biochemical examples of well-behaved enzymes (74, 75). Early studies with β -lactamases from *Bacillus cereus* (76), *Bacillus anthracis* (77), and *Bacillus licheniformis* (78) provided tools on which future biochemical studies of β -lactamases were based. Assay development, enzyme purification, induction properties, and identification of a metal-dependent β -lactamase from these bacilli were activities critical to the establishment of β -lactamases as important enzymological entities (75, 79, 80). It is notable that the classical structural classification of β -lactamases initially proposed by Ambler in 1980 was based on five known amino acid sequences, four of which were from enzymes originating in Gram-positive bacteria. These included three class A penicillinases, PC1 from *S. aureus*, penicillinase from *B. licheniformis* 749C, and the *B. cereus* 569/H β -lactamase I. The only class B metallo- β -lactamases (MBL) that had been sequenced and studied in any detail at the time was the zinc-containing *B. cereus* 569/H β -lactamase II (14). The first report of β -lactamases

other than class A and B enzymes in Gram-positive bacilli described a set of oxacillin-hydrolyzing (class D) penicillinases from a set of environmental strains in 2016 (81).

β -LACTAMASES IN GRAM-NEGATIVE BACTERIA

1940 to 1985 and the emergence of modern β -lactamases. In Gram-negative bacteria, β -lactamases have played a critical clinical role and have served as the primary resistance mechanism for the β -lactam antibiotics. The first enzyme with β -lactamase activity reported in the literature in 1940 was from *Bacillus coli* (1), now assumed to be the class C, AmpC chromosomal cephalosporinase from *Escherichia coli* (Table 1). As β -lactam resistance began to be more frequently recognized in Gram-negative pathogens, it was shown that many enteric bacteria and *P. aeruginosa* produced species-specific inducible chromosomal β -lactamases (82, 83). However, it is the mobile β -lactamases in Gram-negative bacteria that have created a more insidious threat to the β -lactams. Although plasmid-encoded penicillinases were first reported in staphylococci (84), the genes encoding these enzymes were not readily transferred to other species, other than for the few enterococcal strains that appeared in the 1980s carrying a staphylococcal penicillinase gene (69). In contrast, transferable genetic elements encoding a wide variety of β -lactamases became the most prevalent mechanism leading to the emergence of β -lactam resistance among Gram-negative bacteria, with few species barriers existing for their transmission. When β -lactamases on "R-factors" were first described in 1965 (85), only a limited number of these enzymes were identified, related either to the transferable penicillinase on R_{TEM} (86), now known to be TEM-1, or to a transferable enzyme that hydrolyzed cloxacillin (87), an enzyme in the OXA family of β -lactamases. These mobile *bla* genes were soon disseminated among most enteric bacteria (88). We now understand that β -lactamase-encoding genes can be acquired horizontally by different means but mainly by plasmid acquisition. Gene mobilization mechanisms may include genetic elements, such as transposons, gene cassettes, integrons, and insertion sequences (89–91).

Sawai et al. in 1968 (18), Jack and Richmond in 1970 (92), and Richmond and Sykes in 1973 (19) attempted to group the known β -lactamases from Gram-negative rods in a meaningful way based on biochemical properties and functionality. Over time, these approaches have seen considerable refinement. As shown in Table 2, a limited number of different β -lactamases served as the starting point for a logical classification scheme based on biochemical characteristics.

The consensus was that the β -lactamases known by the mid-1970s constituted the range of β -lactam-hydrolyzing enzymes in relevant Gram-negative bacteria. The introduction of isoelectric focusing (IEF) (82, 93) aided by the ease of β -lactamase detection using nitrocefin as a colorimetric activity indicator (94) allowed investigators to analyze bacterial extracts for the presence of β -lactamase activity with minimal effort. Until the mid-1980s, it was possible to make a reasonable estimate of the number of β -lactamases produced per strain and the putative identity of an enzyme, based on isoelectric points from IEF gels. Biochemical properties were determined using purified enzymes; published molecular sizes, many of which were incorrect, were based on data obtained from gel exclusion chromatography, and amino acid composition was deduced from peptide analyses of purified proteins in a process that took months to obtain a single enzyme sequence. This was the environment in which the first definitions of molecular classes A, B, and C emerged (14, 15). Eventually, class D was added, based on nucleotide sequencing of the *bla*_{PSE-2} gene (16), and it represents the most diverse of the molecular classes.

During the late 1970s and early 1980s, a number of surveillance studies were conducted to assess β -lactamase production in Gram-negative bacteria. In a set of five studies published between 1979 and 1985, the distribution of β -lactamases from almost 1,800 ampicillin-resistant enteric bacteria were evaluated, based primarily on IEF profiles (95–99). The TEM-1 and TEM-2 penicillinases were the most common enzymes, with an average of 63% (range, 42 to 85%) of the isolates producing one of these enzymes. In these isolates, an enzyme designated SHV-1 was produced by an average

of 9.9% of the isolates (range, 1.7 to 24.0%), primarily as a chromosomal enzyme in *Klebsiella pneumoniae*, with OXA enzymes produced on average at 7.8% (range, 0.4 to 15.4%). As was common in the early years of β -lactam resistance due to transferable β -lactamases, few isolates produced more than one plasmid-encoded enzyme, with multiple transferable β -lactamases generally reported in no more than 3% of the isolates. An exception was a set of *Klebsiella* isolates from a 1983 Spanish study in which 30% of the strains produced at least two plasmidic enzymes, primarily combinations of SHV-1 with TEM-1 or TEM-2 (97). This compilation set the stage for the future evolution of β -lactamases.

The facile transmission of the R-factor encoded "RTEM" (TEM-1) into a multiplicity of Gram-negative pathogens is associated with triggering one of the most productive periods of antibiotic development, especially for the β -lactams. In 1974, reports of ampicillin resistance in meningitis patients infected with *Haemophilus influenzae* led to the discovery that these strains produced an acquired TEM-type β -lactamase (100). At that time, ampicillin was the preferred treatment of sepsis or pediatric meningitis caused by *H. influenzae* type *b*, and the new TEM-producing strains were no longer responsive to therapeutic levels of ampicillin (101). This observation was followed quite soon by the alarming finding that the TEM β -lactamase was freely being transferred into *Neisseria gonorrhoeae* by conjugal mating when a 24.5-kDa gonococcal plasmid was present (102). Two geographically distinct plasmids of different sizes emerged almost simultaneously, each of which encoded the bla_{TEM} gene in gonococci (103). Epidemiologically, this was attributed in part to the promiscuity of naval populations and prostitutes who were exchanging organisms with R-factor-associated TEM enzymes during trips between the Philippines and Europe or between western Africa and England (104). Prior to this, a single dose of penicillin had been demonstrated to cure >95% of the cases of gonorrhea (105), so the loss of an effective, safe, and inexpensive drug was devastating (106). To add insult to injury, the penicillin-resistant organisms originating from the Asia-Pacific region also were tetracycline resistant due to the *tet(M)* gene that appeared to travel on a different plasmid from bla_{TEM} . This loss of useful therapeutic approaches to a widespread venereal disease resulted in panic, not only from military organizations operating in the western Pacific, but also from operators of clinics designed to treat outpatients with a single injection of either penicillin or tetracycline. As a result of concerns from the medical community (107), pharmaceutical companies quickly took notice and increased research efforts to identify either inhibitors of the TEM β -lactamase or molecules that were stable to β -lactamases from Gram-negative pathogens (4).

Because of the relatively small number of known β -lactamases in the mid-1970s, TEM-stable molecules, or TEM inhibitors, were evaluated for antimicrobial activities against a few standard β -lactamases and β -lactamase-producing strains that were common in multiple pharmaceutical companies. The enzymes studied were those that were easily purified in large amounts and that could be assayed with relatively rapid spectrophotometric methods (79). Although TEM-1 was the primary target for many research groups, other enzymes in the testing panels often included a class A penicillinase from *S. aureus*, a class C cephalosporinase from *Enterobacter cloacae* (frequently the P99-hyperproducing strain), and the class A K1 enzyme from *Klebsiella pneumoniae* (now *Klebsiella oxytoca*), the earliest extended-spectrum β -lactamase (ESBL) that served as the most stringent enzyme to test the stability of oxymino-cephalosporins and monobactams (22, 108). As a result of this research, 24 new β -lactam-containing agents, including 15 cephalosporins, were approved by the U.S. Food and Drug Administration (FDA) in the 1980s (109). In addition to the TEM-stable oxymino-substituted cephalosporins (e.g., cefotaxime, cefuroxime, ceftriaxone, and ceftazidime), monobactam (aztreonam) and carbapenem (imipenem), two β -lactamase inhibitors targeting the TEM β -lactamase and staphylococcal penicillinases, were also approved between 1981 and 1986 in three different combinations, as follows: clavulanic acid with amoxicillin (orally administered) or ticarcillin (parenterally administered), and sulbactam with ampicillin as an intravenous drug (4).

1985 to 2000, new β -lactams as drivers of resistance. When microbiologists were asked to predict how resistance would develop to these new agents, the most common response involved the selection of derepressed AmpC mutants from the *Enterobacteriaceae* and *P. aeruginosa*, bacteria known to have inducible cephalosporinases whose hyperproduction could be selected clinically by the oxyimino-cephalosporins, or expanded-spectrum cephalosporins (110). *E. cloacae* in particular, as well as *Citrobacter* spp. and *P. aeruginosa* (111), were reported to be prone to this kind of selection, with clinical reports of 25% of patients treated with ceftazidime exhibiting resistance to the drug as a result of a derepressed AmpC enzyme in *E. cloacae* (112). However, outbreaks of cefotaxime- or ceftazidime-resistant *Enterobacteriaceae* reported in Clermont-Ferrand, France, in the mid-1908s heralded a different broad-based resistance to these new agents (113). These resistant isolates were the result of transferable β -lactamases, now known as ESBLs, which were capable of hydrolyzing the new β -lactams substituted with an oxyimino side chain, e.g., aztreonam and the third-generation or expanded-spectrum cephalosporins. Initially, ESBLs were derived from the common SHV-1, TEM-1, or TEM-2 β -lactamases and differed from the parental enzymes by no more than two or three amino acids in the coding region (<http://www.lahey.org/Studies/temtable.asp>) (5). In both Europe and the United States where these enzymes were identified almost simultaneously in the late 1980s, the prominent ESBLs were almost all SHV or TEM variants (114–117). Early ESBL-producing isolates were resistant to penicillins and most cephalosporins but susceptible to β -lactamase inhibitor combinations, a differentiating feature used to define ESBL producers phenotypically. As a result of the selective use of agents, such as amoxicillin-clavulanic acid and piperacillin-tazobactam, “inhibitor-resistant” TEM enzymes (IRTs) (118), followed by inhibitor-resistant SHV enzymes (119) emerged. Curiously, organisms that produced many of these enzymes were resistant to penicillin combinations with clavulanic acid or tazobactam but were susceptible to cephalothin (120). Although these enzymes have not played a major role in resistance to the inhibitor combinations in most parts of the world, a recent report from Spain indicates that IRTs are still prevalent in Spanish *E. coli* isolates, from both community and hospital sources (121).

Over the past decade, the common TEM, IRT, and SHV variants have diminished in numbers, only to be replaced by the CTX-M family of ESBLs, a dominating contributor to the multidrug-resistant profile in many Gram-negative bacteria (122, 123). In contrast to the TEM and SHV variants that arose from prevalent European and North American plasmid-encoded penicillinases, the CTX-M enzymes are closely related to chromosomal β -lactamases from the genus *Kluyvera* (124–126), a genus rarely associated with clinical disease (127). SHV ESBL variants are still identified, but few new TEM-related enzymes are now seen. Only recently have tazobactam-resistant CTX-M β -lactamases been identified, unusual enzymes that are still inhibited by clavulanic acid (128, 129). The family of GES enzymes was initially believed to represent another set of ESBLs, but some GES variants with single point mutations have acquired the ability to hydrolyze carbapenems (130, 131). The numbers of enzymes in major β -lactamase families are reflected in Table 3, where the high rate of increase in TEM and SHV novel naturally occurring variants from the 1990s/early 2000s has diminished over time compared to other families. In current isolates, multiple ESBLs may be produced in the same organism, together with enzymes from any of the other molecular classes (132–134).

Class C, AmpC-related, cephalosporinases also played a role in the resistance to cephalosporins, cephamycins, and, to a lesser extent, to carbapenems in both enteric bacteria and nonfermentative pathogens (135–137). Until the late 1980s, they were most worrisome when derepressed AmpC production resulted in high levels of chromosomal/species-specific enzymes. Even enzymes with poor hydrolysis rates for these β -lactams could inactivate enough of the drug to cause clinical resistance, particularly in strains with decreased permeability or increased efflux (138–140). However, when plasmid-encoded AmpC-type enzymes on high-copy-number plasmids began to emerge by 1990 (141), a new threat was introduced, as these enzymes were

TABLE 3 Increasing numbers of β -lactamases in well-characterized families^a

Enzyme type	Class	Functional group	No. in class by yr						
			1961	1995	2000	2005	2010	2015	2018
CMY	C	1	0	1	6	22	64	136	139
PDC ^b	C	1	0	(1)	(1)	(1)	10	30	226
ADC ^c	C	1	0	0	(1)	7	7	7	81
All TEMs	A	2b, 2be, 2br	0	36	86	153	178	219	224
All SHVs	A	2b, 2be, 2br	0	6	26	89	134	182	193
CTX-M	A	2be	0	2	9	51	103	172	182
KPC	A	2f	0	0	0	3	11	22	24
All OXAs	D	2d	0	18	28	88	202	498	520
IMP	B	3	0	1	3	23	29	48	53
VIM	B	3	0	0	2	12	27	41	46
NDM	B	3	0	0	0	0	1	12	14
Estimated total of all unique β -lactamases			(<13) ^d	217	309	584	1,003	1,855	2,771

^aSome data are from reference 224, as well as from <http://www.lahey.org/Studies/> and <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047>.

^bPDC, *Pseudomonas*-derived cephalosporinase family, first named in 2009 (136). AmpC pseudomonal cephalosporinases were described as early as 1965 (137). This family was not included in the Lahey database <http://www.lahey.org/Studies/>.

^cADC, *Acinetobacter*-derived cephalosporinase, family first named in 2005 (190). The name ADC-1 was assigned to an enzyme first described in 2000 (135). This family was not included in the Lahey database <http://www.lahey.org/Studies/>.

^dEstimated, based on data from references 18 and 92 that most likely included some of the same enzymes.

freely transferable among species, resulting in increased resistance to multiple β -lactam classes, including carbapenems (142).

As ESBL-producing isolates became a more prominent segment of the Gram-negative population in hospitalized patients, carbapenems were used more frequently in those health care centers with large outbreaks of cephalosporin-resistant infections (143). This often occurred when isolates produced multiple β -lactamases and a β -lactamase inhibitor combination was not potent enough to overcome all the enzymes in the resistant isolate (144). Selection of carbapenem-resistant pathogens was the fully predictable consequence. Resistance to carbapenems or alternative β -lactam-containing agents emerged due to a multiplicity of the following factors: altered PBPs in *Acinetobacter* spp. (145), overproduction of plasmid-encoded class C cephalosporinases together with porin deficiencies in the *Enterobacteriaceae* (144), and the production of carbapenemases, both chromosomal and plasmid encoded (132).

Carbapenemases remain the major resistance mechanism for carbapenems in the Gram-negative bacteria. Initially they were regarded as nontraditional clinically irrelevant zinc-containing β -lactamases occurring only in the occasional *Stenotrophomonas* (*Pseudomonas*) *maltophilia* clinical isolate (146) and in isolates of *Bacillus* spp. (other than *B. anthracis*) that are infrequent causes of nongastrointestinal human infections (147). However, class A carbapenemases, such as the species-specific chromosomal SME enzymes in *Serratia marcescens*, began to be identified intermittently in Europe (148, 149) and the United States (150) in the 1980s. Plasmid-encoded MBLs emerged in Japan in 1990 with the IMP family of enzymes (151) and in Italy in 1997 with the VIM β -lactamases (152), causing some to predict a global epidemic of multidrug-resistant MBL-producing Gram-negative bacteria. Although the IMP and VIM MBL families began to expand after 2000 (Table 3), outbreaks associated with these enzymes have tended to be small, limited in time, and localized to specific geographical regions (153–157).

2001 to 2018, major epidemic β -lactamases. Identification of the plasmid-encoded KPC serine carbapenemases in the early 2000s (158, 159) soon led to major epidemics caused by carbapenemase-producing *Enterobacteriaceae* in many areas of the world (160–162). These enzymes can appear in almost any Gram-negative pathogen (9, 163, 164), although they are predominantly identified in *K. pneumoniae*. The most prominent of the KPCs are KPC-2 and KPC-3, frequently found in *K. pneumoniae* clonal complex 258 (CC258) (165, 166). Within CC258, the common sequence type 258 (ST258) found in the United States and Europe, are two major clades, clade I associated with KPC-2 dissemination and clade II associated with KPC-3 (167). KPC-producing

organisms have been associated with high rates of mortality, as high as 51% in patients with infections caused by colistin-resistant *K. pneumoniae* strains (168). Other serine carbapenemases have not become quite as prolific or deleterious as the KPCs. Only the *Serratia*-specific chromosomal SME enzymes have occasionally caused small outbreaks (169). A recent unusual multidrug-resistant *S. marcescens* isolate was selected during therapy due to the selection of hyperproduction of both the AmpC and SME chromosomal β -lactamases (170).

Until recently, plasmid-encoded MBL dissemination had been a minor threat in most geographical regions, even in countries that have recorded sporadic outbreaks with enzymes, such as VIM-1 in Greece (171), IMP-8 in Taiwan (172), and IMP-1 in Japan (173, 174). However, MBLs became more menacing after the NDM-1 zinc-containing carbapenemase was identified in 2009 from an isolate originating from New Delhi, India (175). Retrospective studies have traced the origins of this MBL to at least 2006 (176). In contrast to the other MBLs, NDM-1 quickly spread worldwide, being the predominant carbapenemase in the Indian subcontinent, but with major outbreaks also reported in the Balkans and the Middle East (9, 166). Of great concern is the widespread occurrence of the *bla*_{NDM} gene that has been identified in environmental water samples in India (177, 178).

A third transferable carbapenemase associated with outbreaks is the OXA-48 enzyme, originally identified as a class D oxacillinase from Turkey in 2001 (179). This enzyme, found in multidrug-resistant *Enterobacteriaceae*, slowly hydrolyzes carbapenems and expanded-spectrum cephalosporins and is poorly inhibited by most β -lactamase inhibitors, with the exception of avibactam (180). It is most prevalent in the Mediterranean region and southern Europe. Outbreaks have been reported in France (181) and Spain (182), where 74% and 32% of the carbapenemases in *E. coli* and *Citrobacter* spp., respectively, were recently identified as OXA-48 (182, 183). Class D oxacillinases are also frequently found in *Acinetobacter* spp. and are the primary cause for carbapenem resistance in those organisms. As *Acinetobacter*-related infections increased during the early 2000s, the contributions of the chromosomally encoded OXA-51 in *Acinetobacter baumannii* (184) and plasmid-encoded OXA-23, OXA-24/33/40, and OXA-58 enzymes were more fully appreciated (185). Although multidrug-resistant *Acinetobacter* spp. have been associated with a number of outbreaks, the contribution of β -lactamases to these outbreaks has been contributory, but not necessarily the driving factor, due to the intrinsic resistance of these pathogens to most antibiotics (185, 186).

β -LACTAMASE CLASSIFICATIONS

With the variety of unique β -lactamases that have been identified in natural isolates now exceeding 2,770 (Table 3 and Fig. 3), it is important to have reliable and easily understandable nomenclature to refer to these enzymes. Beginning with Sawai and colleagues, who classified β -lactamases as penicillinases or cephalosporinases according to substrate profiles (18), other classification schemes arose (Table 2) based traditionally on the functional characteristics of these enzymes (19). As nucleotide and amino acid sequences became available, molecular relatedness was added as a defining characteristic (14–16). One of the most cited classification schemes using functional group designations was proposed by Bush, initially in 1988 (187), and then in collaboration with Jacoby and Medeiros (21), as discussed above. Their updated scheme described in 2010 (20) has been further expanded, as shown in Fig. 1, with the addition of avibactam as a differentiating inhibitor to separate serine carbapenemases from MBLs, potentially a useful diagnostic characteristic in whole-cell phenotypic assays (188). Fortuitously, assignments to functional groups generally aligned with molecular classes, although exceptions were noted, particularly with the intrinsically diverse OXA enzymes (189).

Numbering of variants within β -lactamase families has been a challenge, beginning with the emergence of numerous ESBLs resulting from point mutations in both nucleotide and amino acid sequences (5). As a result, in 1996, Jacoby offered to serve

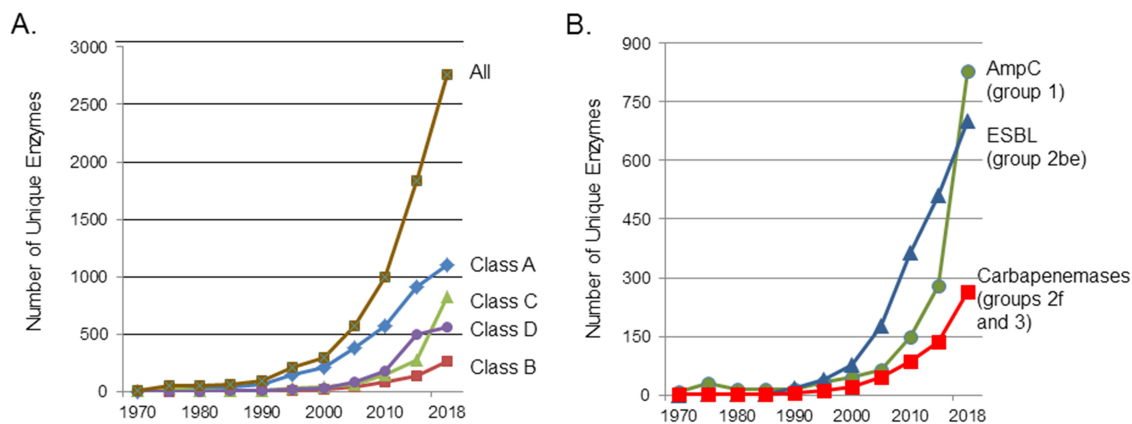


FIG 3 Increase in numbers of unique, naturally occurring β -lactamases (some data from reference 224 as well as from <http://www.lahey.org/Studies/> and <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047>). (A) β -Lactamases enumerated according to molecular classes A, B, C, and D, with the total number of enzymes (all) equal to 2,771. (B) β -Lactamases enumerated according to major functional groups with their trivial names, AmpC, group 1; ESBLs, group 2be; and carbapenemases, groups 2f and 3.

as the curator for assignments of new allele numbers for naturally occurring plasmid-encoded ESBLs, with the establishment of the website at <http://www.lahey.org/Studies/>. The site was later expanded to include assignments for natural alleles in all major β -lactamase families containing more than three known variants. Functional assignments were made for the TEM, SHV, and OXA enzymes if substrate and inhibitor profiles were available. In 2015, the task of assigning new numbers for novel β -lactamases was transferred to NCBI (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047>), with a request for phenotypic information and genotype data. Tracking of the LEN, OXY, and OKP enzyme families in *Klebsiella* spp. is being curated by investigators at the Institut Pasteur (<http://bigsdw.web.pasteur.fr/klebsiella/klebsiella.html>). Bonomo and colleagues have established numbering systems for the *Acinetobacter*-derived cephalosporinases (ADCs) (190), followed by similar numbering of *Pseudomonas*-derived cephalosporinases (PDCs) (136, 191), families whose numbers have increased greatly in the past 5 years as a result of whole-genome sequencing projects (185) (Table 3 and Fig. 3).

The agreement within the β -lactamase community to provide a unique designation for each unique (natural) allele has met with criticism from others in the antibiotic resistance field who advocate that there should be at least a 2% change in nucleotide or predicted amino acid sequence before a new enzyme or gene name is assigned (192). However, a single amino acid substitution in a TEM or SHV β -lactamase can alter its biochemical properties, such that the substrate spectrum is dramatically altered to provide for an ESBL phenotype (5), or resistance to β -lactamase inhibitors can occur (118). These enzymes clearly have different functional characteristics and as such warrant a unique designation (193). A recent attempt to lump ESBLs and carbapenemases into a single ESBL category (194) was challenged by many in the β -lactamase community (195). Although the original intention was to simplify β -lactamase nomenclature for the practicing physician, many thought that this would be confusing, especially in situations in which carbapenems, which are routinely used to treat traditional ESBL-producing pathogens (196), would not be effective against infections caused by carbapenemase-producing organisms if carbapenemases were also named ESBLs.

More recent classification schemes are based on the association between 3-dimensional structures and functional information, particularly for the class A/group 2 β -lactamases (197–200). As crystallographic analyses become available for additional β -lactamases, determination of structure-function relationships will become more common. However, for this approach to be successful, high-quality phenotypic data must be provided for novel β -lactamases. The numbers of β -lactamases continue to increase almost exponentially (see Fig. 3 and reference 201), partly because of easy access to inexpensive and rapid

gene sequencing. Laboratories now have the ability to identify dozens of “new” β -lactamase sequences (185), with no functional information provided. Unfortunately, whole-genome sequencing provides more data than we perhaps need to know. Some of the genes that are identified as β -lactamase-encoding genes are incomplete, some are misannotated, and some are not expressed, so that assignments for new alleles are not aligned with functionality (202). In addition, “ β -lactamase” may be included in the annotation for a new protein sequence that has no β -lactamase hydrolytic capability. An example is the NCBI annotation for sequences in the metallo- β -lactamase superfamily, a family of more than 6,000 enzymes that includes oxidoreductases, as well as enzymes which hydrolyze thiol-ester, phosphodiester, and sulfuric ester bonds (203) but <300 verified MBLs (Fig. 3). Naive investigators may incorrectly assume that this commonly identified set of sequences signals the presence of an MBL, potentially triggering aggressive medical treatment in the hospital laboratory. Even for legitimate enumerations of new β -lactamases based only on sequence data, a lack of functional data minimizes the usefulness of the information. Attempts by structural biologists to align structures with function will suffer due to the lack of phenotypic information to correlate with novel sequences. Although minimally, it is recommended that any novel β -lactamase gene be transferred to a non- β -lactamase-producing strain and its resistance phenotype confirmed, it is preferable that a novel enzyme be purified and its biochemical properties determined and then correlated with microbiological properties in the producing organism (20, 193). Ideally, both genomic and biochemical information should be available before classifying enzymes into families (20).

DISCUSSION

Selective pressure from both naturally occurring β -lactams and clinically overused β -lactam-containing drugs has created an environment in which new β -lactamases readily emerge, together with maintenance of some of the older, more fit, enzymes. Recent laboratory studies have demonstrated that conjugal plasmids with antibiotic resistance genes are stable in multispecies communities over long periods of time, even in the absence of antibiotic pressure (204). This partially explains the continued presence of antibiotic resistance genes in apparently naive environments (45, 46) and the perpetuation of β -lactamase genes encoding common TEM, SHV, and OXA enzymes. It is notable that TEM-1 and, to a lesser extent, OXA-1, two of the first known plasmid-encoded β -lactamases, have not disappeared and are frequently identified in contemporary, multidrug-resistant, clinical, and environmental isolates (132, 205, 206), perhaps because of their exquisite ability to hydrolyze inexpensive penicillins that are still used therapeutically in community settings. However, there is great concern about environmental contamination in public water supplies by β -lactamase-producing antibiotic-resistant pathogens. A recent study in Hyderabad, India, in both urban and rural areas, found that 100% of all water samples collected from bulk drug manufacturing facilities and multiple water sources, including those contaminated by sewage treatment plants, were positive for ESBL genes, and 95% of them contained carbapenemase genes, often with multiple genes per sample (e.g., *bla*_{OXA-48}, *bla*_{KPC}, and *bla*_{NDM}) (178). Several studies have identified environmental sources of carbapenem-resistant organisms carrying primarily *bla*_{KPC} and *bla*_{VIM} genes in hospital drains and sinks, thereby providing sources for these pathogens to enter water supplies outside health care centers (153, 207).

One of the most worrisome threats described by the Centers for Disease Control and Prevention (CDC) is carbapenemase production in recent Gram-negative clinical isolates, especially those enzymes with genes carried on mobile genetic elements (208). Fortunately, serine carbapenemases are readily inhibited by new β -lactamase inhibitors, including the diazabicyclooctanone (DBO) inhibitors, such as avibactam (209), and the boronic acid derivative vaborbactam (210), both of which have been recently approved for marketing in combination with ceftazidime or meropenem, respectively. These inhibitor combinations allow relatively effective clinical treatment of infections caused by organisms producing serine carbapenemases (211), thus lowering the risk of

clinical failures. A combination of avibactam with the MBL-stable monobactam aztreonam is currently in phase 3 clinical trials to treat Gram-negative bacterial infections, including those with MBL-producing pathogens (<https://clinicaltrials.gov/ct2/show/NCT03329092?term=avibactam&draw=2&rank=19>). Although resistance has been reported in patients treated with avibactam-ceftazidime, the numbers are currently small (212). As can be expected, however, resistance will increase as the new agents are used. At this time, a β -lactam-containing agent inhibiting a broad spectrum of pathogens, including nonfermentative and anaerobic pathogens, is still to be identified. This is due in part to the increase in multidrug-resistant organisms producing MBLs and/or OXA-48 carbapenemases in combination with β -lactamases of different classes and the lack of an effective inhibitor of all the enzymes (213). Thus, it is possible that MBLs rather than serine β -lactamases may become the predominant carbapenemases in multidrug-resistant bacteria. It is also possible that β -lactamase-stable β -lactams, such as cefiderocol (S-649266), or non- β -lactam-containing agents, such as plazomicin, omadacycline, or eravacycline, will serve as reliable and safe therapeutic alternatives against the most resistant carbapenem-resistant pathogens (214).

In conclusion, β -lactamases, some of our oldest enzymes, have emerged as perhaps the most studied, and most troublesome, of the antibiotic resistance determinants. The fact that many β -lactamase-encoding genes travel together on mobile elements with transmissible resistance factors for other antibiotic classes means that resistance can arise due to a multiplicity of selection pressures. One can anticipate that additional unique β -lactamases with unusual properties will be identified due to the widespread existence of these genes in both environmental and clinical sources and to continued pressure from the use of β -lactam antibiotics. It will be up to the scientific community to monitor their presence with detailed structural and functional studies to provide a scientific basis for the design of new antimicrobial agents that may evade these emerging enzymes, at least for the immediate future.

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