Deciphering the evolution of metallo-β-lactamases: A journey from the test tube to the bacterial periplasm

Received for publication, September 22, 2021, and in revised form, January 13, 2022 Published, Papers in Press, February 1, 2022,
https://doi.org/10.1016/j.jbc.2022.101665

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Edited by Wolfgang Peti

Understanding the evolution of metallo-β-lactamases (MBLs) is fundamental to deciphering the mechanistic basis of resistance to carbapenems in pathogenic and opportunistic bacteria. Presently, these MBL-producing pathogens are linked to high rates of morbidity and mortality worldwide. However, the study of the biochemical and biophysical features of MBLs *in vitro* provides an incomplete picture of their evolutionary potential, since this limited and artificial environment disregards the physiological context where evolution and selection take place. Herein, we describe recent efforts aimed to address the evolutionary traits acquired by different clinical variants of MBLs in conditions mimicking their native environment (the bacterial periplasm) and considering whether they are soluble or membrane-bound proteins. This includes addressing the metal content of MBLs within the cell under zinc starvation conditions and the context provided by different bacterial hosts that result in particular resistance phenotypes. Our analysis highlights recent progress bridging the gap between *in vitro* and *in-cell* studies.

The scourge of antimicrobial resistance represents a growing threat for public health. The indiscriminate use and abuse of antibiotics has fostered the selection of bacteria resistant even to our most potent antibiotics such as carbapenems (Fig. 1*A*) (1–7). β-Lactamases represent the most important mechanism of resistance against these "life-saving" drugs, because of the dissemination of genes coding for enzymes with carbapenemase activity (8–12). Metallo-β-lactamases (MBLs) are Zn(II)-dependent enzymes, that represent one of the largest groups of carbapenemases for which clinical inhibitors are not yet commercially available (13–16). Since MBLs are highly divergent in sequence, metal ligands at the active site, and Zn(II) stoichiometry, they have been divided into three subclasses: B1, B2, and B3 (Fig. 1*B*) (14, 17–19). Most acquired, clinically relevant MBLs present in Gram-negative pathogens belong to subclass B1 (16, 20–23). B1 enzymes are active with two metal ions: Zn1 coordinated to three His ligands: His116, His118, and His196 (3H site) and Zn2 bound to Asp120, Cys221, and His263 (also called DCH site) (Fig. 1*B*) (15, 19, 24, 25). The coordination sphere is completed by strategically positioned water molecules (Fig. 1*B*). B3 enzymes are also binuclear, with a 3H ligand set in the Zn1 site, but with different ligands at the Zn2 site: Asp120, His121, and His263 (DHH site) (Fig. 1*B*). Finally, B2 MBLs are active in their mono-Zn(II) form, in which the metal ion is bound to the DCH site (Fig. 1*B*). In these enzymes, uptake of a second Zn(II) ion inhibits their activity (15). For a more comprehensive description of the structural and biochemical features of MBLs, the reader is referred to a recent review covering these aspects (15).

β-Lactamases are a unique model to study protein evolution, since the survival of the bacterial cell challenged with antibiotics can be correlated with the biochemical and biophysical traits of a single protein (26). The serine-β-lactamase (SBL) TEM (named for patient Temoneira) has been an excellent model for the study of protein evolution (27–33). The essential Zn(II) ions in MBLs represent an additional constraint for protein fitness that does not allow to extrapolate evolutionary studies on SBLs to MBLs (15, 34–37).

The study of the biochemical and biophysical features of purified proteins has provided clues for understanding the impact of substitutions in the evolution of β-lactamases (27–33). However, it provides an incomplete and sometimes biased picture, since the impact of the cellular environment is disregarded. In the case of Gram-negative organisms, survival of the bacterial cell in the presence of β-lactam antibiotics depends on the successful expression, translocation, processing, and the appropriate localization of these enzymes. Moreover, MBLs can be present in different bacterial hosts with different resistance phenotypes. The *in vitro* study of the purified mature protein also overlooks the impact of mutations in the signal peptide (SP) that may affect the translocation and levels of functional protein in the periplasm (38, 39), their cellular localization, and even the correct

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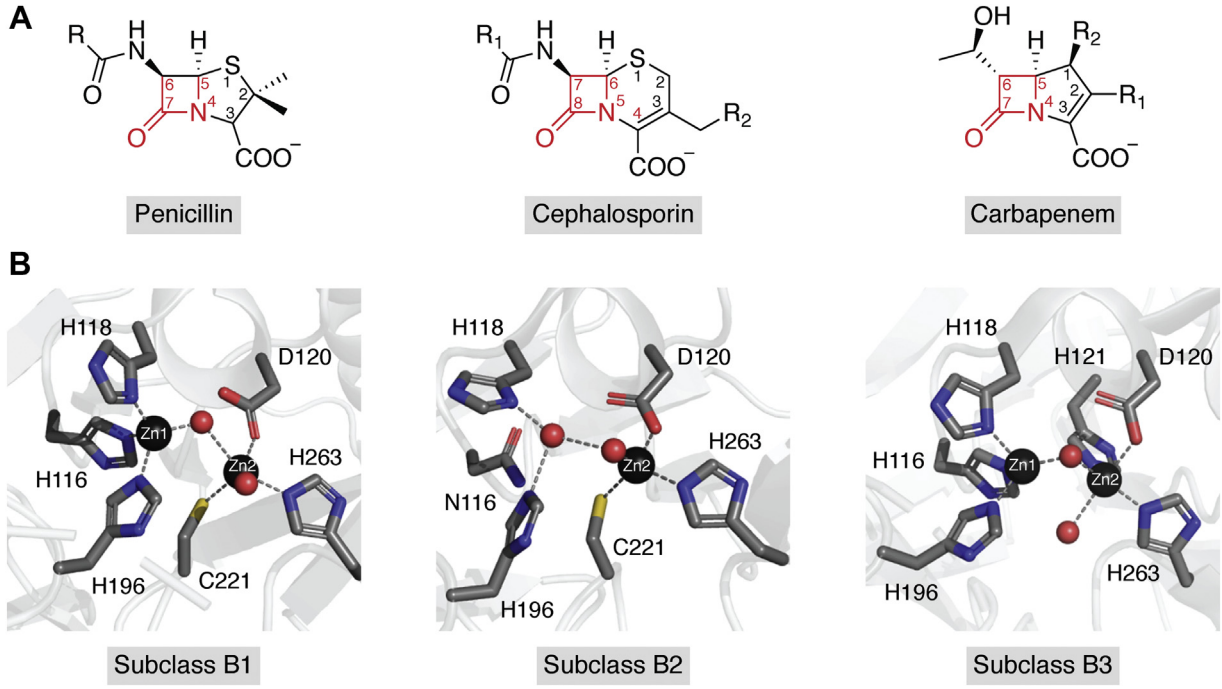

Figure 1. *A*, chemical scaffold of the main classes of β-lactam antibiotics hydrolyzed by MBLs. The β-lactam ring is highlighted in *red*. *B*, representative active site for subclasses B1, B2, and B3 MBLs. The Zn(II) ions are represented by *black spheres*, and water molecules are represented by *red spheres*. In each panel, metal ligand are highlighted in *sticks* and the *gray dashed lines* show coordination geometry spheres for the Zn1 and Zn2 sites. The Protein Data Bank files used are: 3SPU (B1—NDM-1), 3SD9 (B2—SfhI), and 3LVZ (B3–BJP-1). BJP-1, MBL from *Bradyrhizobium japonicum*; MBL, metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase 1; SfhI, MBL from *Serratia fonticola*.

folding or stability. The description of protein evolution based solely on *in vitro* studies in purified proteins is an example of the "streetlight effect," in which a person who is intoxicated is looking under a lamppost for the keys lost somewhere else because the light is there. The understanding of the differences between the features displayed by a protein *in vitro* and within the cell is crucial for disentangling the driving forces in evolution.

Evolution results from the accumulation of mutations and the selection of particular traits under defined conditions. However, bacteria are continuously exposed to changes in their environment. Two significant factors are usually disregarded when describing MBL evolution: the exposure of bacteria to antibiotics is not permanent, and bacteria cycle between restrictive and permissive conditions (with and without antibiotics, respectively) (2, 39–41) at the sites of infection, the immune system response elicits a massive metal starvation, limiting the amount of available Zn(II), which impacts on MBL-mediated resistance (42–45). These conditions define the driving forces in the evolutionary landscape of MBLs.

Here, we present and discuss recent advances and new challenges in the study of the evolution of MBLs, encompassing methodological approaches that enable a quantitative measurement of biochemical and biophysical parameters in cell-like environments. We also review the "state of the art" regarding the physiology (and life cycle) of MBLs in bacterial cells, which is intimately related to their evolutionary landscape. In the last section, we also discuss the biochemical and biophysical traits that have been optimized in the clinical evolution of different MBLs.

MBLs: A journey from the periplasm to the test tube

The first challenge in extrapolating *in vitro* to *in cell* data is the attempt to correlate the catalytic activities of the β-lactamases with the resistance phenotype in different bacteria. The resistance phenotype is quantitated based on the minimum inhibitory concentration (MIC) of an antibiotic that inhibits bacterial growth. Most B1 MBLs exhibit catalytic efficiencies (k_{cat}/K_M) within a range of 10^5–10^6 $M^{-1}s^{-1}$ (15, 17), differing at the most by one order of magnitude. In contrast, MIC values are quite variable, particularly among different hosts (38, 39). These different resistance phenotypes are due in most cases to changes in the cell permeability and the presence of other, complementary, and resistance mechanisms (15). In this review, we ask the reader to also appreciate the different environments present in each host that impact on the expression levels, processing, and activity of the MBLs. This notion regards the so-called "quinary structure of proteins," a term coined by McConkey (46), who considers macromolecular interactions within the cell, and it has been addressed by Pielak, Gruebele, Shekhtman, and others (47–49) by measuring the biochemical and biophysical properties of proteins under conditions mimicking the physiological environment.

In Gram-negative bacteria, MBLs are synthesized as cytoplasmic precursors with an N-terminal signal sequence

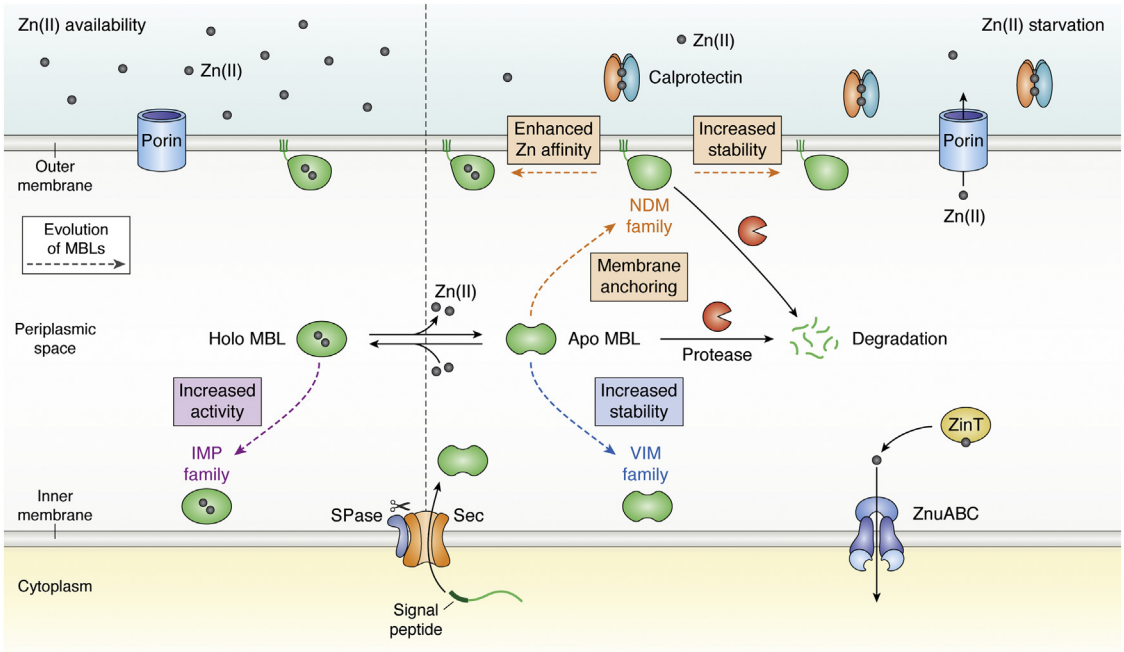

Figure 2. Mechanisms of MBL evolution. Periplasmic Zn(II) levels depend on the passive diffusion through porins in *Escherichia coli*. In the presence of Zn(II), represented by *gray spheres* (*left panel*), the external concentration is in equilibrium with periplasmic Zn(II) levels (62, 63), and MBLs bind the cofactor and hydrolyze β-lactams. The *dashed arrows* highlight the different mechanism of MBL evolution (79, 130, 139). IMP families have evolved increasing their activity in response of antibiotic evolutionary pressure. When the innate immune response is active (*right panel*), neutrophils secrete calprotectin (CP) that chelates Zn(II) ions (43), reducing the periplasmic Zn(II) levels and activating expression of the Zn(II) uptake mechanisms to the *E. coli* cytosol (ZnuABC system and ZinT chaperone). MBLs lose the Zn(II) cofactor leading to formation of apo-MBLs. The nonmetallated form is susceptible to degradation in the periplasm by proteases (shown as Pac-Man-like *orange* objects) (44). Different mechanisms of evolution are indicated with *dashed arrows* of different colors, such as outer membrane anchoring (44), stabilization toward degradation, or enhancement of the Zn(II) affinity (15, 73). IMP, imipenemase; MBL, metallo-β-lactamase; SPase, signal peptidase.

(the SP), which directs them to the secretion machinery for their translocation into the cell envelope, where they perform their hydrolytic activity (Fig. 2). So far, all known MBLs are exported by the SecA–SecYEG system as unfolded polypeptides, recognized and processed by type I signal peptidase (SPase I) or by type II lipoprotein signal peptidase (SPase II), depending on whether their final localization is the periplasm or the outer membrane, respectively (Figs. 2 and 3) (50–54). Finally, the enzymes fold and bind Zn(II) in the periplasmic space (55). Most MBLs are soluble periplasmic enzymes, except for New Delhi metallo-β-lactamase (NDM) enzymes, which are lipoproteins anchored to the inner leaflet of the outer membrane (Fig. 2) (44, 56). The functional levels of MBLs in the periplasmic space vary considerably among different bacterial hosts, finally impacting on the resistance phenotype (38, 44).

Based on this knowledge, one simple strategy to fill this gap is to measure the catalytic efficiency of the enzymes in periplasmic extracts, using immunoblotting quantitation to retrieve apparent k_{cat} values. k_{cat}/K_M values measured in the periplasm show a significant correlation with MIC values (57–59). This approach has been useful to describe the stepwise evolution of an enzyme evolved in the laboratory (57), to account for differences in the activities *in vitro* and MICs in MBL mutants (57–59), and to study the resistance phenotype of a series of MBLs (either chromosomal or acquired) in different bacterial hosts (38). In all these cases, the activity measured in these extracts showed a tight correlation with MIC data. In the case of membrane-bound lactamases, such as NDM-1, similar experiments have been optimized for the measurement of kinetic parameters in spheroplasts (60).

This approach can also be applied to quantitate the thermodynamic stability by measuring the enzymatic activity after incubating the periplasmic extracts at different temperatures (57, 59), providing an apparent T_m value that reflects the thermal stability of the MBLs in conditions mimicking the physiological ones (57, 59). This parameter describes how the equilibrium between the folded and unfolded forms of the protein is affected by the temperature. However, this information does not provide clues regarding the kinetic stability of the protein. The kinetic stability, instead, is related to the energetic barrier separating the native functional state from nonfunctional forms that can accumulate within the cell because of irreversible cellular processes (proteolysis, aggregation, etc.). In this regard, the kinetic stability is more relevant to describe the *in-cell* behavior, since it may anticipate possible events leading to the loss of the native functional state (61).

Impact of Zn(II) availability on the kinetic stability of MBLs in the periplasm

MBLs bind Zn(II) in the periplasm, since they are secreted as unfolded polypeptides (55). To date, specific metallochaperones for MBLs have not been identified (15), and different studies suggest that the metalation state of MBLs depends mostly on the Zn(II) availability in the external milieu (15, 57–59, 62–64).

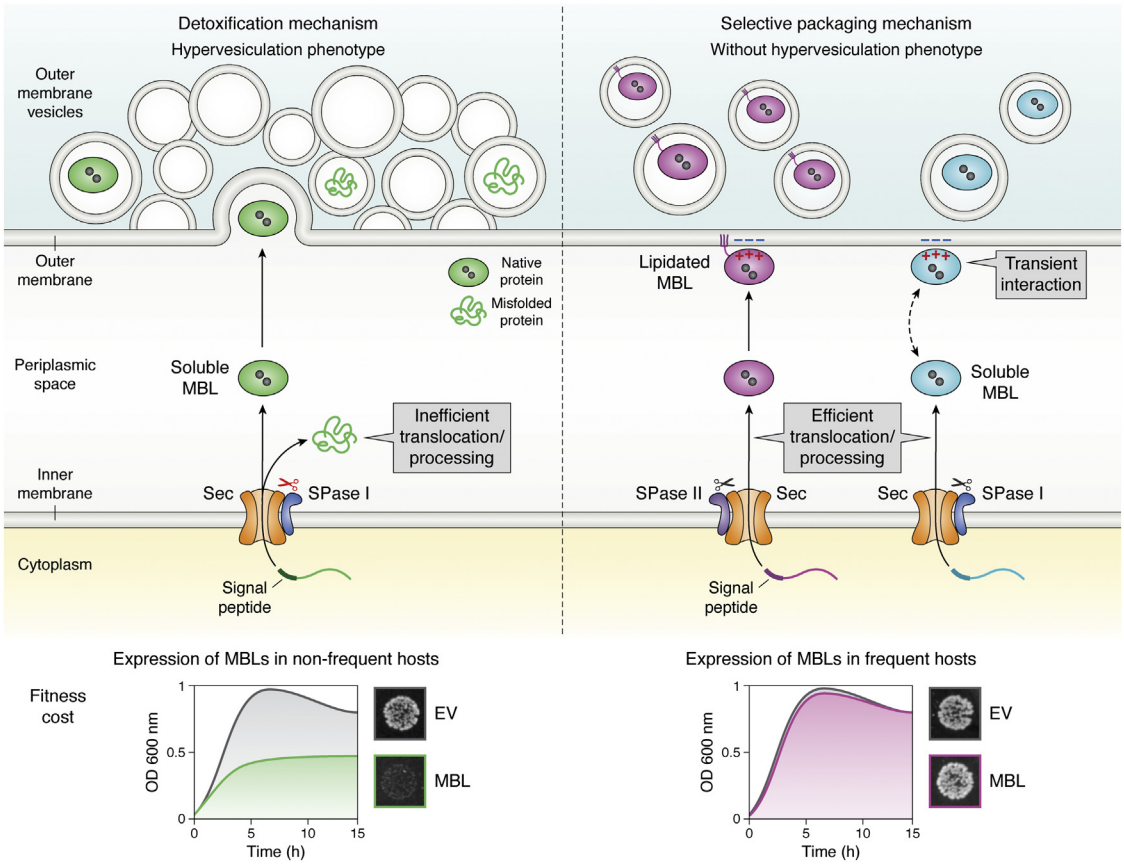

Figure 3. The adaptability of MBLs to different bacterial hosts depends on the impact of protein expression on biological fitness. This model describes the adaptability of MBLs to different bacterial hosts based on the processing of their SPs (39). An MBL is confined to a narrow range of bacteria (*left panel*) when its expression in nonfrequent hosts causes the accumulation of toxic precursor forms (shown as *green* misfolded protein) in the periplasmic face of the inner membrane because of an inefficient translocation and processing that compromises the bacterial growth (*bottom left panel*). This toxicity correlates with an increase in the production of outer membrane vesicles (OMVs) that incorporate both the mature (folded protein) and the precursor protein (misfolded) to alleviate the envelope stress; thus acting as a vesicle-mediated detoxification mechanism. By contrast, a broad host MBL (*right panel*) is produced by different bacteria without fitness cost (*bottom right panel*). These MBLs are efficiently translocated and processed. In these cases, the level of selective packaging into OMVs depends on the interaction of the MBL with the outer membrane, either by membrane anchoring (*violet* folded protein) or by specific electrostatic interactions (*cyan* folded protein) through a positively charged patch. The *double-headed dashed arrow* represents the binding equilibrium of soluble periplasmic MBL with the outer membrane, which favors protein packaging into OMVs. EV, cells carrying the empty vector; MBL, metallo-β-lactamase; SP, signal peptide.

During an infection, the immune system in mammals elicits a nutritional immunity response, in which metal-binding proteins are targeted to the infection site, withholding essential metal ions at the host–pathogen interface (65). Calprotectin (CP) is the most relevant protein in this process, by sequestering Zn(II) with a subnanomolar affinity (Fig. 2) (42, 43, 45, 66, 67). In *Escherichia coli*, this process triggers derepression of the Zur regulon and expression of high-affinity transporters in the inner membrane (ZnuABC) and periplasmic metallochaperones that deliver Zn(II) ions to ZnuA (such as ZinT) (Fig. 2), among other proteins (15, 68). However, active zinc importers at the outer membrane in *E. coli* are not reported. Thus, Zn(II) influx to the periplasm is assumed to occur mainly by passive diffusion through nonselective porins: OmpF and OmpC in *E. coli* (Fig. 2) (61, 62). The mechanisms for Zn(II) accumulation and transport in the bacterial periplasm have not been fully dissected yet. An in-depth discussion on these mechanisms is provided in these reviews (68, 69).

In the presence of a strong metal chelator, ($pK_d > 8$), such as EDTA, dipicolinic acid, or CP (70–72), MBLs are inhibited by removal of the metal ions, giving rise to stable apo (non-metalated) variants *in vitro*. The Zn(II)-binding affinities of B1 and B3 MBLs are in the nanomolar range, whereas B2 MBLs display picomolar binding affinities, based on *in vitro* measurements (15). The impact of a chelator in the resistance phenotype of MBL producers generally shows a trend that follows the *in vitro* metal-binding affinities, but there are clearly many other factors that determine the outcome of the treatment with chelators. In the bacterial cell, external Zn(II) depletion results in reduced resistance by eliciting metal dissociation from the MBLs in the periplasm (Fig. 2) (44, 73, 74). This phenomenon raised many questions regarding the level of antibiotic resistance conferred by MBLs expressed in clinical strains depending on the Zn(II) availability in infection models (75) (this discussion is beyond the scope of our review). In any case, infection models and clinical studies should consider that the Zn(II) availability impacts differently depending on the infection site, the bacterial host, and the MBL being expressed (76, 77). The optimization of the conditions to test MIC values in physiological and relevant

conditions is currently debated (77). Assessing the metal content of MBLs in the periplasm is essential to better understand these processes. The recent development of a selective fluorescent sensor has been a major advance in this direction since it enables a dynamic monitoring of the metalation state of NDM-1 in the periplasm of *E. coli* cells (78).

Apo-MBLs have been historically regarded as stable proteins, based on their high thermal stability (57, 74, 79, 80) that has enabled different biophysical studies (81), including resolution of several crystal structures (82). In stark contrast to this *in vitro* stability, apo-MBLs are not as stable in the periplasm, being subjected to degradation or (possibly) aggregation (Fig. 2) (44, 73). The kinetic stability of apo-MBLs can be quantitated by measuring the half-life time of decay in the periplasm upon zinc deprivation, which follows a single-exponential behavior (73). Different MBLs also show distinct kinetic stabilities in the periplasm: São Paulo metallo-β-lactamase 1 (SPM-1) and imipenemase 1 (IMP-1) are much more stable than Verona imipenemase 2 (VIM-2) (44). The situation of NDM-1 is unique since membrane anchoring stabilizes the apoenzyme (Fig. 2), as evidenced by the rapid degradation of an engineered soluble variant of NDM-1 (44). Overall, this supports the importance of studying the kinetic stability of MBLs under physiological conditions. The understanding of the factors governing the distinct kinetic stabilities of apo-MBLs is crucial to identify the evolutionary traits operative in clinical variants (61).

Evolution of MBLs in different bacterial hosts: Genetic and biochemical determinants of specificity

Table 1 summarizes the different families of acquired MBLs. Among them, IMP, VIM, and NDM types (from B1 subclass) are the most clinically relevant enzymes produced by human pathogens. Although MBLs associated with mobile genetic elements can spread rapidly among different bacteria, they have different host specificities. For example, NDM and IMP enzymes have spread rapidly in Enterobacterales and non-fermenters, whereas SPM-1, Adelaide imipenemase 1 (AIM-1), and VIM-2 are mainly found in *Pseudomonas aeruginosa* (Table 1) (13, 83). The elucidation of the molecular determinants of host specificity has been subject of recent studies.

NDM-1 is more prevalent than IMP-1 in carbapenem-resistant Enterobacterales despite exhibiting similar activities *in vitro* (15, 17). Avison *et al.* (84) have shown that this is because of higher expression levels of NDM-1, since the $bla_{\text{NDM-1}}$ gene is found downstream an IS*Aba*125 insertion sequence, which provides a conserved strong promoter. In contrast, $bla_{\text{IMP-1}}$ genes are located downstream of variable promoter sequences with different strengths, resulting in lower protein levels and, consequently, reduced resistance (84). An *E. coli* isolate carrying the $bla_{\text{IMP-6}}$ gene was shown to be susceptible to imipenem and meropenem, as the result of the transcriptional repression of $bla_{\text{IMP-6}}$. This repression was due to the binding of a protein-encoded upstream of $bla_{\text{IMP-6}}$ (85). However, the mechanisms regulating the production of MBLs warrant further studies, since there is little known about this aspect on acquired MBLs (15).

The finding of a strong promoter can account for the higher frequency of NDM-1 in Enterobacterales based on genetic determinants (84). However, this cannot explain why some bla_{MBL} genes are confined to one host, whereas others can adapt to a wide variety of bacteria (Table 1). A high-throughput analysis of the expression of 200 antibiotic resistance genes in *E. coli* revealed that the protein–host compatibility depends mostly on the biochemical interaction between the resistance mechanism and the physiology of the host, rather than on genetic factors (86). This insight has been addressed recently for MBLs in two studies, from Tokuriki's (38) and Vila's (39) groups. With complementary approaches, these studies have disclosed a key role of the SP processing in host specificity.

Table 1
Differential distribution of plasmid-associated MBLs among bacterial hosts

MBL host range	Acquired MBL	Ambler subclass	Bacterial hosts[a]
MBLs found in a narrow range of bacteria	VIM-2	B1	*Pseudomonas* spp. (*P. aeruginosa*, *P. putida*, *P. stutzeri*, *P. juntendi*, *P. asiatica*), *Serratia marcescens*, *Acinetobacter baumanii*
	SPM-1	B1	*Pseudomonas aeruginosa*
	AIM-1	B3	*Pseudomonas aeruginosa*
	SMB-1	B3	*Serratia marcescens*
	DIM-1	B1	*Pseudomonas aeruginosa*, *Pseudomonas stutzeri*
	FIM-1	B1	*Pseudomonas aeruginosa*
	KHM-1	B1	*Citrobacter freundii*, *Enterobacter hormaechei* subsp. *hoffmannii*
	SIM-1	B1	*Acinetobacter* spp. (*A. baumannii*, *A. bereziniae* and *A. baylyi*)
MBLs found in a broad range of bacteria	VIM-1	B1	Enterobacterales (*Enterobacter hormaechei*, *Klebsiella pneumoniae*, *Klebsiella michiganensis*, *Escherichia coli*, *Kluyvera cryocrescens*, *Enterobacter cloacae*, *Salmonella enterica*), *Pseudomonas aeruginosa*
	IMP-1	B1	*Pseudomonas aeruginosa*, Enterobacterales (*Serratia marcescens*, *Enterobacter cloacae*, *Enterobacter roggenkampii*, *Klebsiella pneumoniae*, *Escherichia coli*), *Acinetobacter baumannii*
	NDM-1	B1	Enterobacterales (*Klebsiella pneumoniae*, *Salmonella enterica*, *Escherichia coli*, *Enterobacter hormaechei*, *Enterobacter cloacae*, *Proteus mirabilis*, *Proteus vulgaris*, *Citrobacter freundii*, *Providencia rettgeri*), *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Vibrio cholera*
	GIM-1	B1	*Pseudomonas* spp. and Enterobacterales (*Enterobacter cloacae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Escherichia coli*, *and Citrobacter freundii*)
	TMB-1	B1	*Achromobacter* spp., *Acinetobacter* spp., Enterobacterales (*Enterobacter hormaechei* and *Citrobacter freundii*)

MBLs are shown according to their bacterial host range: in the first block, those confined to one or a few bacterial hosts are indicated, and in the second block, the enzymes widely distributed among different bacteria are indicated.
[a] Bacterial strains frequently producing each MBL are shown and ordered by decreasing frequency in cases where the MBL is not confined to a single bacterium host.

The efficiency of each step in the journey from the gene to the periplasm (transcription, translation, and translocation of an MBL) impacts on the resistance phenotype in a given bacterial host, mostly by defining the functional periplasmic levels of MBLs within the cell (38). In contrast, the guanine–cytosine content, codon usage, or the mRNA stability (87) are crucial in determining neither the resistance phenotype nor the expression levels in different hosts.

Expression of different MBLs (the chromosomal enzymes *Bacillus cereus* type II [BcII], cefoxitin and carbapenem resistant [CcrA], and IND-1 [Chryseobacterium (Flavobacterium) indologenes], and the acquired MBLs, NDM-1, VIM-1, VIM-2, IMP-1, and SPM-1) with a common SP (PelB from *Erwinia carotovora*) in different hosts provided a better correlation between MICs and the catalytic efficiencies *in vitro*, suggesting that the SP and its processing could give rise to the host-dependent phenotypes (38). A deep mutational study on full-length bla_{VIM-2} by Tokuriki *et al.* (88) revealed that its SP is highly tolerant to substitutions, except for residue Lys3, which is conserved in all but three VIM allelic variants (88). Synonymous mutation on the SP affected the resistance *in vivo*. These experiments also revealed that the SP of VIM-2 is not optimized for expression in *E. coli*, an observation in agreement with the fact that this bacterium is a nonfrequent host for VIM-2 (see later).

The fitness cost elicited in a particular bacterial host upon expression of a resistance determinant is a main driver of enzyme–host adaptability. López *et al.* (39) showed that the expression of MBLs in less frequent hosts under permissive conditions (in the absence of antibiotics) compromised the biological fitness by impairing bacterial growth (Fig. 3). The expression of SPM-1 and VIM-2 is well tolerated in *P. aeruginosa* (a frequent host of these enzymes). Instead, both MBLs induced a significant fitness cost when expressed in *E. coli* or *Acinetobacter baumannii* (nonfrequent hosts of these proteins). The expression of NDM-1 (an enzyme ubiquitously found in Enterobacterales and nonfermenters) does not cause a fitness cost in any of these three strains (39). The observed growth defects in nonfrequent or less common hosts were accompanied by the accumulation of the precursor proteins, triggering a strong stress envelope response. Thus, MBL expression in nonfrequent hosts saturates the Sec translocon, leading to the accumulation of toxic precursor species that eventually impact negatively on biological fitness (Fig. 3). These toxic species are eliminated from the cell as partially inactive species in outer membrane vesicles (39) (OMVs, see later) (Fig. 3).

These studies reveal that the adaptation of MBLs is not exclusively governed by the genetic determinants, since there are many biochemical factors playing key roles. In this regard, the SP sequence dictates this adaptability based on the processing efficiency (see model in Fig. 3). SP swapping between different MBLs led to a change in the fitness cost and adaptability in different hosts, being able to revert the deleterious impact of the inefficient processing (39). More studies are required to identify sequence patterns in the SPs that can assist in predicting the enzyme adaptability to different hosts.

MBL resistance is strongly dependent on the efficient processing by the corresponding signal peptidases. Therefore, signal peptidases can be also considered as targets to combat carbapenem resistance. Inhibitors of the translocation systems, such as arylomycins (89) or globomycins (90), as well as optimized derivatives (91), can be successfully combined with carbapenems to tackle MBL-mediated resistance.

MBLs beyond the bacterial cell: Protein sorting into vesicles

We have discussed the impact of Zn(II) binding and processing linked to the translocation of MBLs to their native environment, the periplasm. Interestingly, the periplasm is not necessarily the final localization of MBLs, since some of them can be packed into OMVs. All Gram-negative bacteria produce OMVs in response to a wide variety of environments (92–96). Bacterial OMVs, possessing a diameter of 20 to 250 nm, are spherical portions of the outer membrane that protrude and detach from growing cells. The content of the vesicles resembles that of the producing bacteria, but there are cargo selection mechanisms that results in the enrichment or exclusion of certain molecules in the vesicles (97, 98) (proteins, metabolites, and even nucleic acids) (99–101). Bacteria employ OMVs to improve their chances of survival and induce changes in their immediate environment (92, 102–104), such as the detoxification mechanism discussed previously. Cell envelope stress generated by the accumulation of toxic species (such as MBLs processed inefficiently in nonfrequent hosts) leads to a hypervesiculating phenotype, and these enzymes are eliminated by OMVs, mostly in misfolded and inactive forms (Fig. 3) (39).

OMVs can also transport β-lactamases in their folded and active form. The encapsulation of these enzymes in vesicles protects them from degradation in the extracellular environment maintaining a favorable milieu in which the enzymes can exert their catalytic activity beyond the cell boundaries. OMVs transport SBLs and MBLs in their active form as well as plasmids coding for these enzymes (39, 44, 99, 100, 105–108). OMVs incorporating β-lactamases can protect populations of susceptible bacteria against antibiotics (44, 106, 109), as recently shown *in vivo* (110, 111), and have been postulated as a novel mechanism of plasmid transfer (112, 113). While the secretion of toxic species is nonspecific, the packaging of folded and active MBLs is selective. In this regard, there is an emerging picture of the cargo selection of MBLs into vesicles.

The membrane-anchored enzyme NDM-1 is packaged in vesicles produced by different bacteria in an active form because of its cellular localization (39, 44). In addition to the lipid anchor, a positively charged patch on the surface of the soluble domain of NDM-1 makes attractive electrostatic interactions with the bacterial membrane (114) and favors their inclusion into vesicles (115). The same mechanism is valid for soluble MBLs: a positively charged patch in the surface of IMP-1 favors its inclusion into OMVs (115). These residues are conserved among NDM and IMP enzymes, suggesting that the phenomenon is common to most allelic variants from

these families. Instead, the lack of electrostatic interactions of VIM-2 with the membrane accounts for the small levels of this protein into OMVs under nonstressful conditions (115). This reveals that some MBLs are tuned or poised to interact with the bacterial membrane and therefore are selectively exported into OMVs in a folded, metalated, and active form (Fig. 3). The conserved nature of residues that favor the interaction with the membrane suggests that export of MBLs is common to many enzymes and could be a trait selected in evolution.

Biochemical and biophysical traits selected in the evolution of acquired MBLs

The three largest families of acquired MBLs (IMP, VIM, and NDM) comprise almost 200 allelic variants (83, 74, and 38, respectively) now (15, 116). This large diversity reflects the impact of antibiotic prescription in selecting different variants according to the evolutionary pressures in different environments (such as host, infection sites, and type of antibiotic). In the case of β-lactamase-mediated resistance, selection takes place at the level of bacterial cell survival, depending on the ability of these enzymes to hydrolyze the β-lactam antibiotics (26). This depends not only on the catalytic efficiency but also on the amount of folded and active lactamase in the periplasm (39, 44). Thus, issues discussed before, such as the optimization of expression levels and host adaptability, play a role in evolution, together with the efficacy of the secretion and processing that leads to the ultimate steps of folding and Zn(II) binding in the periplasm. Substitutions are pleiotropic in nature, able to act on the enzyme activity, stability, cofactor binding, among other factors (117). Moreover, when mutations accumulate, epistatic effects are usually observed and determine the possibility of incorporating more substitutions (31, 57, 117, 118). Thus, identification of the biochemical and biophysical traits influenced by the substitutions within the periplasm is crucial to understand the driving forces of evolution in each type of enzyme.

As previously discussed, the thermodynamic stability and kinetic parameters *in vitro* under nonphysiological conditions can be misleading. Here, we will discuss the current knowledge of the driving forces of evolution in the IMP, VIM, and NDM families. Active-site residues acting as metal ligands are fully conserved within these enzymes, and substitutions are found in second sphere residues (those interacting with the zinc ligands), in the active-site loops L3 and L10 (that flank the active site and define the substrate profile); and in more distant locations in the protein structure (Fig. 4*A*) (15, 116, 119). The latter substitutions are usually more difficult to rationalize, but (as discussed later), many of them are highly relevant in defining the resistance phenotype.

IMPs

IMP-1 was the first acquired MBLs to be identified in Japan (120). As such, it has been used as a model to assess the role of active-site loops and second sphere residues on the activity of MBLs (Fig. 4*A*) (121–123). At the moment, 83 natural IMP variants are divided into six subgroups (124), as the result of the accumulation of more than 54 substitutions distributed in different regions of the protein. The large diversity within this family suggests a polyphyletic origin of IMP enzymes (125), thus limiting a global comparison among variants.

Randomization of selected positions on IMP-1 revealed that second sphere residues (such as Ser262) and residues present in the mobile loop L3 (like Val67) (Fig. 4) shape the substrate profile (122). This pioneering randomization experiment by the Palzkill laboratory also identified positions 67 and 262 as "hot spots" that were found replaced in the clinical variants (126–129). This study did not explore newer antibiotics, such as ertapenem, or more recent combination treatments that may result in distinct requirements.

A comprehensive study of 20 variants from the IMP-1 subgroup (the largest one) revealed that substitutions Ser262Gly (214 in IMP-1) and Val67Phe (49 in IMP) increased the resistance against certain carbapenems at the expense of ampicillin resistance in *E. coli* (130). This improvement is the result of an increment in the k_{cat} values for carbapenems and a decrease for ampicillin in agreement with previous reports on isolated variants (127, 128, 131–133). Ser262Gly is present in other subgroups of the IMP family, eliciting the same effect, that is, without showing epistatic effects with other mutations (cf. IMP-7 and IMP-51 or IMP-11 and IMP-68 that differ only by the Ser262Gly substitution) (134, 135). The Ser262Gly substitution disrupts a hydrogen-bonding interaction with the base of L3 loop, altering its conformation (Fig. 4*B*) (136). Intriguingly, this substitution is not present in any variant from the second largest subgroup, that of IMP-2. The Val67Phe substitution, instead, is more distributed among the IMP family, and it is present within the subgroup IMP-2. This suggests that different IMP subgroups could be evolving against carbapenems.

The resistance profile of IMP variants under zinc starvation conditions revealed that occurring substitutions do not improve the enzyme ability to resist metal limitation (130). This can be attributed to the observation that IMP-1 is more resistant to zinc limitation than VIM-2 and NDM-1 (44, 76, 80, 130), despite the Zn(II) ions in IMP-1 are kinetically labile (137). It is not unlikely, however, that some mutations that improve the carbapenemase activity may reduce the zinc-binding affinity and require a compensatory mutation. This phenomenon has been reported in a dissection of the evolutionary pathways of the *in vitro* evolution of BcII (57).

More studies on IMP variants from different subgroups are required to better understand the impact of mutations, their adaptation to different conditions, and eventually relate this information with reported outbreaks (23).

VIMs

The VIM family presents a different evolutionary landscape compared with IMP enzymes. First, the 74 reported alleles form a monophyletic group (125) divided into five clusters (15). The main clusters are the ones related to VIM-1/4 and to VIM-2. Second, VIM alleles accumulate mutations in different regions of the structure, many of them in the active-site loop

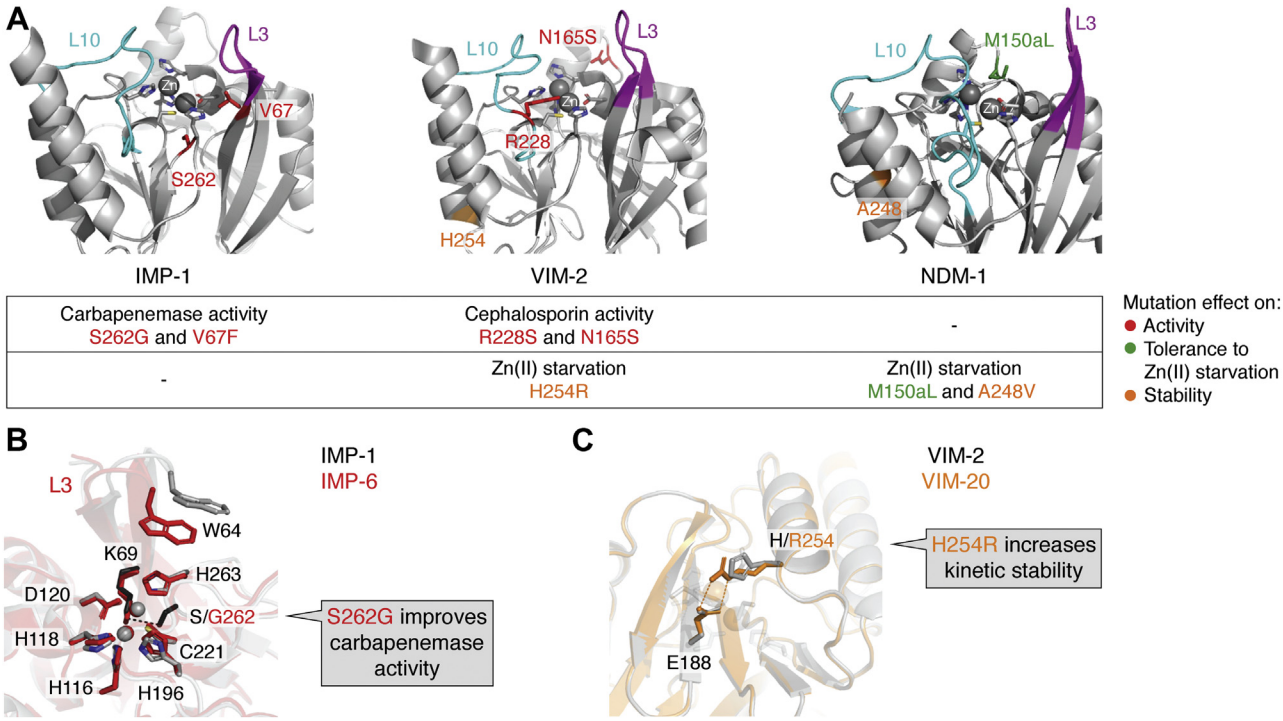

Figure 4. *A*, key mutations and evolutionary traits in the main families of acquired MBLs: IMP-1, VIM-2, and NDM-1. Figures were rendered with PyMol based on the PDB IDs 4C1G, 4NQ2, and 3SPU, respectively. The active-site loops L10 and L3 are shown in *cyan* and *magenta*, respectively; the metal ligands as *sticks*, and the Zn(II) ions as *spheres*. The mutational "hot spots" are indicated in different colors according to their impact on catalytic activity (*red*) (79, 130, 139) or in the tolerance against Zn(II) starvation by improving metal binding (*green*) or the kinetic stability (*orange*) (73, 74, 79). *B*, a conformational change in loop L3 introduced by substitution S262G in IMP improves carbapenemase activity. The interaction between S262 and K69 marked with *black dashes* in IMP-1 (*gray structure*) is disrupted by the substitution in IMP-6 (*red*; PDB: 6LVJ). *C*, the substitution H254R increases the kinetic stability of VIM-20 (PDB: 6OP6) by the formation of a salt bridge between R254 and E188. IMP-1, imipenemase 1; MBL, metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase 1; PDB, Protein Data Bank; VIM-2, Verona imipenemase 2.

L10, which determine the substrate profile of MBLs (Fig. 4*A*) (15). Third, analysis of the resistance profile of different variants suggests that mutations such as R228S and N165S have been selected to optimize the activity against some cephalosporins, as shown by the Galán, Bonomo, and Crowder groups (79, 138, 139). In particular, the substitution R228S was identified as the responsible of the diversification event that distinguishes VIM-1 from VIM-4. Both the VIM-2 and VIM-1/4 subgroups have evolved resistance against cephalosporins with bulky C-3 substituents such as ceftazidime. In contrast to the IMP family, these mutations do not seem to enhance the carbapenemase activity (79, 139–141). A deep mutational scanning study of VIM-2 (88) identified the essential positions and those tolerant to mutagenesis and identified beneficial mutations occurring in the natural VIM variants (79). Some single mutations giving rise to deleterious effects present in natural mutants may be compensated by other mutations in clinical alleles. *In vitro* studies comparing phylogenetically distant VIM variants (VIM-2, VIM-1, and VIM-5) suggested that the thermal stability could have been improved upon evolution of this family (142, 143). However, it is not known how this observation could impact on the kinetic stability of the VIM MBL in the periplasm.

A recent study on VIM variants revealed that many of them (10 of 45 studied) exhibit an increased tolerance to zinc starvation conditions (79). Preservation of enzymatic activity under these conditions was observed for VIM-20 (VIM-2 His254Arg) (Fig. 4), VIM-28 (VIM-4 His224Leu), VIM-37 (VIM-4 Ala57Ser), and VIM-59 (VIM-1 His254Arg). Notably, VIM-20 is much less susceptible to proteolysis *in vivo* than VIM-2 under zinc deprivation. This has been accounted for by the formation of the salt bridge between Arg254 (229 in VIM sequence) and Glu188 (171 in VIM) (Fig. 4*C*) (79). VIM-20 exhibits a higher thermal stability than VIM-2, but the most relevant trait optimized upon this mutation is the stabilization of the apo form in the periplasm (79). This study by the Crowder and Bonomo laboratories suggests that a group of VIM variants is evolving to better endure zinc starvation (79).

More studies on VIM variants are required, including aspects previously discussed such as the analysis of the expression levels and the impact of the SP in the processing in different hosts, to obtain a complete description of the effect of the occurring mutations in the resistance phenotype and the adaptation to the environmental challenges. Of note, VIM-2 is mostly present in *P. aeruginosa*, but most evolutionary studies in the laboratory use *E. coli* as host. As previously highlighted, it is important to interrogate the behavior of the proteins in a context as close as possible to the native one, such as in their most frequent host.

NDMs

The 38 known NDM alleles reveal amino acid substitutions outside the active site, distributed over all the protein

structures. The number of substitutions is smaller than those present in the IMP and VIM families, since in most of the cases, they differ by three amino acid changes (the exceptions are NDM-10, NDM-18, and NDM-36) (116). In general, substitutions are located outside the active site and the second sphere. All NDM variants contain the *lipobox* sequence in the SP that targets them to the outer membrane (44). Residues Arg39 (45 in NDM) and Arg46 (52 in NDM) form a positively charged patch that favors the electrostatic interaction with the membrane and are conserved in all NDM variants (114, 115).

Notably, the activity and resistance profile of NDM alleles does not suggest an adaptation to a particular antibiotic, in contrast with the IMP and VIM families (73, 74, 144). So far, the kinetic characterization and antimicrobial resistance profile of the first 17 variants is very similar in Zn(II)-replete conditions (73, 74, 144). A deep mutational study on NDM-1 by the Palzkill laboratory identified essential residues as well as other residues contributing to substrate specificity. This in-depth analysis revealed that carbapenem hydrolysis by NDM involves more stringent conditions than penicillin or cephalosporin hydrolysis. Based on these findings, the Palzkill group designed the Lys224Arg/Gly232Ala/Asn233Gln (211/219/220 in NDM numbering) triple mutant that lost the imipenemase activity, while retaining the ability to hydrolyze penicillins (145). The authors conclude that, if an inhibitor of NDM-1 is developed, its use in combination with a carbapenem (instead of penicillins or cephalosporins) would be more efficient in restricting the possible escape mutations.

Most of NDM variants are able to confer more resistance than NDM-1 under Zn(II) limitation conditions, suggesting that this enzyme, instead of optimizing the catalytic efficiency, is evolving to endure the metal restriction imposed by the immune system during the infection (73, 74). The variants that proved to be more refractory to Zn(II) starvation were double mutants, all of them containing the substitution Met150aLeu (154 in NDM numbering), which is the most frequent mutation, present in almost one-third of the variants. This reveals that overcoming Zn(II) deprivation is a trait selected in the evolution of NDMs (73, 74).

This evolution has taken place by two independent mechanisms: an increase of the Zn(II)-binding affinity to maintain high levels of active metalated enzyme elicited by the substitution Met150aLeu (Fig. 4*A*) as in NDM-4 (73, 74); and enhancement of the kinetic stability of the apo form in the periplasm, by substitutions Ala248Val (233 in NDM numbering) in NDM-6 (Fig. 4*A*) or Glu149Lys (152 in NDM numbering) in NDM-9 (73). Both mechanisms are independent, since the effects are additive, with no epistatic interactions. This is the case of NDM-15 (M150aL-A248V), exhibiting enhanced tolerance to Zn(II) starvation compared with most alleles, including single mutants NDM-4 and NDM-6 (73). Interestingly, NDM-15 retains high catalytic efficiency at subnanomolar Zn(II) levels against ampicillin, whereas NDM-1 did not show activity under these conditions (74). *In vitro* studies reported an improved thermal stability for most NDM variants (74, 144, 146). However, this stabilization does not correlate with the kinetic stability of the apo variants in the periplasm (73), which seems to be a trait selected in evolution.

Direct competition of *E. coli* cells expressing NDM-15 or NDM-4 *versus* NDM-1 at sub-MIC concentrations in Zn(II)-rich medium led to a prevalence of NDM-1. However, in the presence of the chelator dipicolinic acid or CP, at concentrations that do not impair bacterial growth, NDM-15 prevailed over NDM-1 or the single mutants (73). This result suggests that mutations enhancing the zinc-binding affinity or the kinetic stability of apo-NDM variants are shaping the evolution of this MBL family providing more fitness upon metal starvation. The available crystal structures have revealed no clue accounting neither for the increase in zinc-binding affinity because of the Met150aLeu substitution in NDM-4 nor for the kinetic stability provided by the Ala248Val substitution in NDM-6.

Gly63Ser (69 in NDM numbering) and Ala68Thr (74 in NDM numbering) are the only substitutions in the active-site loop 3, present in variant NDM-10 (both) and NDM-21 (G63S V82L M150aL). However, deep scanning mutagenesis revealed that a Gly residue was not essential for ampicillin, imipenem, and cefotaxime hydrolysis (145). The impact of other substitutions in NDM variants as well as the possible epistatic effects among them remains to be studied.

In conclusion, Zn(II) deprivation is shaping the adaptive landscape of the NDM family of proteins, through the accumulation of mutations that either increase the Zn(II) affinity or the kinetic stability of the apoenzyme in the periplasm. At the moment, zinc-binding affinities in the low nanomolar range seem to be required for MBLs to provide resistance (57, 58).

Other MBLs

SPM-1 is a B1 MBL restricted to *P. aeruginosa* (39), intriguingly with a single variant after almost 2 decades of its first report (147). However, SPM-1 is endowed with a unique set of second sphere residues (differing from most B1 enzymes) that optimize its resistance under Zn(II) deprivation and were selected to confer resistance against anti-pseudomonal β-lactam antibiotics in detriment of the catalytic efficiency against other β-lactams (59). This case reveals an evolutionary tradeoff in the catalytic profile in which residues surrounding the active site were selected to match the requirements of a particular bacterial host.

B2 enzymes represent the smallest group of MBLs, with a reduced number of allelic variants (116, 148). This may be because B2 enzymes are not present in primary human pathogens (23), being less exposed to prescribed antibiotics. The B2 enzyme CphA (carbapenem-hydrolyzing *Aeromonas hydrophila*) has the strongest affinity toward Zn(II) among MBLs (149) (K_d = 6 pM), suggesting that their possible dissemination to pathogenic bacteria could be a concern based on their ability to resist metal starvation.

A large allelic diversity (118 variants) was recently reported for the B3 enzyme L1 (150), from the opportunistic pathogen *Stenotrophomonas maltophilia*. L1 is unique among MBLs in

being a tetrameric enzyme (15, 151). Residues involved in tetramerization are conserved in all alleles, confirming that the quaternary arrangement is essential for the enzyme activity (150). Second sphere, loop residues, and positions surrounding the tetramerization patch vary among the different alleles. Zn(II) is essential for the tetramerization of L1 in the periplasm of *E. coli* (152). This result prompts for the study of the impact on Zn(II) starvation on L1 in *S. maltophilia* as well as for the evolutionary pathways leading to this large allelic diversity.

Concluding remarks

The early characterization of BcII, the MBLs from *Bacillus cereus* as a zinc-dependent lactamase in 1966 (153), spurred the curiosity of talented biochemists such as Professor Stephen Waley (154) to describe the features of this enzyme as an outlier within the realm of β-lactamases. Three decades later, genes coding for MBLs were identified in mobile genetic elements, leading to a new era in which efforts were devoted to understanding the genetics of dissemination, their diversity, and mechanism of action with the final aim to design an MBL inhibitor. In recent years, the finding of membrane-bound MBLs, their secretion into vesicles, and understanding that their metal load in the periplasm is variable has required a different approach that involves the description of their physiology, their bacterial host specificity, and the identification of different driving forces of evolution operative in different MBL families. A new era has started in the study of MBLs that requires a more comprehensive approach, integrating clinical and basic microbiology, biochemistry, biophysics, and cell biology, which we expect will provide a better understanding in the evolution of these fascinating enzymes in their native context or closely approaching their natural environment. Each family of acquired MBLs accumulates mutations in different positions of their structure and experiences different evolutionary trajectories with distinct traits being selected. These traits cannot always be described based on studies on purified proteins. The study of MBL evolution in a context as close as possible as the native one requires: (1) studying the resistance phenotype in model host systems related to the more frequent bacterial hosts in the clinics, instead of resorting only to *E. coli* as a model organism; (2) express each MBL with their native SPs; (3) quantitate the expression levels of active protein in the periplasm, either in the soluble form or in the membrane-bound form; (4) explore the impact of Zn(II) deprivation in the resistance phenotype and in the kinetic stability of the apovariant; (5) correlate these data with *in vitro* studies of catalytic efficiency and zinc-binding affinity; and (6) determine apparent kinetic parameters in periplasmic extracts or spheroplast to attempt filling the gap between *in vitro* and *in cell* experiments. In addition, insights into MBL folding linked to metal binding in the periplasm will require the use of new experimental (78, 155–157) and computational tools, particularly those that encompass different timescales (158, 159). We hope that these efforts can provide to a faster "bench-to-bedside" translation of novel therapies.

Acknowledgments—We are grateful to our colleagues of the Metalloprotein laboratory and collaborators for scientific contributions and discussions. We thank the support of our home institutions, CONICET and the University of Rosario.

Author contributions—C. L., J. D., and A. J. V. conceptualization; C. L., J. D., R. A. B., and A. J. V. writing–original draft; R. A. B. and A. J. V. funding acquisition.

Funding and additional information—This work was supported by a grant from ANPCyT (grant no.: PICT-2016-1657; to A. J. V.) and a National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant (grant no.: 2R01AI100560-06A1; to A. J. V. and R. A. B). C. L. and J. D. are recipients of a postdoctoral and doctoral fellowships from CONICET, respectively. A. J. V. is a staff member from CONICET. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases (to R. A. B.) under Award number R01AI100560. This study was also supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs (Award number: 1I01BX001974; to R. A. B.) from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center (Veterans Integrated Service Networks 10). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BcII, *Bacillus cereus* type II; CP, calprotectin; IMP-1, imipenemase 1; k_{cat}, β-lactamase's turnover rate; K_d, dissociation constant; K_M, Michaelis-Menten constant; MBL, metallo-β-lactamase; MIC, minimum inhibitory concentration; NDM, New Delhi metallo-β-lactamase; OMV, outer membrane vesicle; SBL, serine-β-lactamase; SP, signal peptide; SPM-1, São Paulo metallo-β-lactamase 1; VIM-2, Verona imipenemase 2.

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