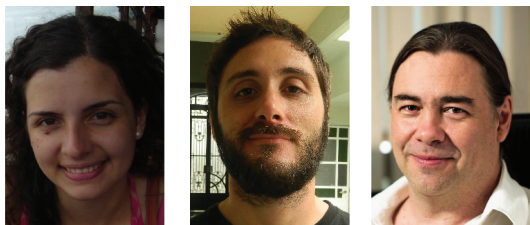


Antibiotic resistance in Zn(II)-deficient environments: metallo- β -lactamase activation in the periplasm



“Evolution favors selection of metallo- β -lactamase variants with high Zn(II) affinity.”

María-Rocío Meini^{†1}, Lisandro J González^{†1} & Alejandro J Vila^{*1}

[†]Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR) Ocampo y Esmeralda, Predio CONICET Rosario, 2000 Rosario, Argentina & Área Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

^{*}Author for correspondence: Tel.: +54 341 4237070 ext. 632 ■ Fax: +54 341 4390465 ■ vila@ibr-conicet.gov.ar

[†]Authors contributed equally

Zn(II) is an essential metal ion in living organisms, playing a wide variety of roles as a structural, regulatory or catalytic cofactor in proteins, that is able to interact with approximately 10% of the entire proteome in humans [1]. As is the case for most transition metal ions, high Zn(II) levels are toxic. Therefore, organisms have developed a series of mechanisms to regulate Zn(II) concentrations and to ensure proper metal uptake by metalloproteins [2]. These mechanisms involve specific metal sensor proteins, import and export machineries that allow subcellular compartmentalization and a pool of small molecules and/or proteins that are able to bind excess Zn(II) [2]. As a result, there is rarely free Zn(II) within cells and biological fluids [3].

Bacterial pathogens require transition metal ions during infection to achieve an optimum colonization level and to activate a variety of virulence factors. This condition is exploited by the human host, which sequesters these metal ions in a process generally termed ‘nutritional immunity’, originally coined to account for the role of iron ions [4]. This concept has been more recently extended to also describe the competition for Mn(II) and Zn(II) [5]. The latest mechanism is based on the action of the neutrophil protein calprotectin, which tightly binds Mn(II) and Zn(II), thus inhibiting bacterial growth [6]. Therefore, a tight competition takes place between the host and the pathogen for capturing Zn(II) ions, which can significantly affect bacterial infection processes.

In addition to that, antibiotic resistance can also be affected by Zn(II) sequestration,

as recently reported for a multidrug-resistant *Acinetobacter baumannii* strain, whose susceptibility to carbapenems was increased in the presence of a Zn(II)-chelating agent [7]. Multiple resistance mechanisms against β -lactam antibiotics involve Zn(II) ions as essential factors. For example, resistance to imipenem in *Pseudomonas aeruginosa* is Zn(II)-dependent through the downregulation of porin OprD [8]. However, the most outstanding resistance mechanism towards β -lactam antibiotics involving Zn(II) ions is the expression of metallo- β -lactamases (M β LS). M β LS, unlike classical serine- β -lactamases, are metalloenzymes requiring one or two Zn(II) ions for their activity [9,10]. M β LS gained importance since the 1990s as the principal mechanism of resistance against carbapenems, one of the most valuable antibiotics nowadays for treating multiresistant pathogens. M β LS are actually broad-spectrum enzymes, being able to degrade almost all classes of β -lactams (penicillins, cephalosporins and carbapenems). M β L genes have been detected in a wide variety of environmental bacteria as endogenous genes. However, their association with mobile genetic elements (often with other resistance cassettes) prompted the dissemination of M β LS genes into clinically relevant pathogens, such as *P. aeruginosa* or members of *Enterobacteriaceae*, which possess nearly pan-resistant phenotypes. Moreover, unlike most other β -lactamases, M β LS are not susceptible to any of the therapeutic β -lactamase inhibitors available, which converts them into a serious clinical threat. Outbreaks of pathogens producing the M β LS NDM-1, IMPs, VIMs or

Keywords

- antibiotic resistance
- metallo- β -lactamases
- nutritional immunity
- periplasm ■ Zn(II)

SPM-1 are increasingly common worldwide, with high rates of mortality and morbidity.

***In vitro* structural & mechanistic studies of Zn(II) binding to MβLs**

MβLs share a common protein fold and a highly conserved Zn(II) binding motif forming the active site, albeit being highly divergent in terms of sequence [10]. According to sequence and structural homology, MβLs are classified in three subclasses: B1, B2 and B3 [11]. We will focus on B1 MβLs, a group that includes the clinically relevant and plasmid-encoded MβLs.

Crystal structures and enzymatic studies of different B1 MβLs have provided insightful information on their catalytic mechanism [12,13]. Most of the debate in the literature regarding these issues has been focused on the metal content of the active species of MβLs. Despite it being well accepted that the metal ions are essential for substrate binding and catalysis, contrasting evidence has supported suggestions that either B1 enzymes could be active with only one Zn(II) ion, or that they require two Zn(II) ions in their active sites. This is not a trivial issue, since any attempt at inhibitor design requires knowledge of the active species to be targeted. It was also proposed that these enzymes exist *in vivo* as the apo (nonmetallated) forms and would be able to bind Zn(II) ions only in the presence of substrates [14].

“...*in vitro* studies should be extrapolated with caution to infer results about antibiotic resistance.”

These hypotheses were supported by *in vitro* studies, in other words, with purified proteins, spanning a wide variety of different conditions, none of them able to mimic the natural environment of these enzymes (the periplasmic space). Moreover, these studies were in general not accompanied by *in vivo* assays. Given the tight control of available Zn(II) on cells, this picture should be put into an organismal context.

Biogenesis & metal binding *in vivo* of MβLs

MβLs in Gram-negative bacteria are synthesized as cytoplasmatic precursors that are then exported into the periplasmic space. Recently, Viale and coworkers have elegantly shown that MβLs cross the inner membrane as unfolded polypeptides through the Sec system, in a

process involving chaperone DnaK [15]. This conclusion implies that MβLs fold and acquire the essential Zn(II) ions in the periplasmic space. The process defining the binding preference for Zn(II) over other divalent cations is strictly linked to their cellular localization. MβLs L1 and GOB-18 accumulate as inactive iron species when overexpressed in the cytoplasm of *Escherichia coli* [16,17]. However, when isolated from periplasmic extracts, both enzymes exclusively bind Zn(II).

“Novel strategies should consider Zn(II) uptake as a limiting step on metallo-β-lactamase activation.”

Folding and metal acquisition of MβLs in the periplasm might be assisted by specific chaperones. However, although metallochaperones insert the correct metal into some proteins, no Zn(II) chaperones have been reported so far. Therefore, Zn(II) proteins in the periplasm are expected to acquire the metal cargo directly from cellular pools. According to this, variation in the metal composition of *E. coli* periplasm drastically alters the metal content and composition of MβL L1 [16]. This work also showed that, in contrast with the situation found in the cytoplasm, the periplasmic concentration of metals, at least in *E. coli*, is highly sensitive to the metal composition of the growth medium. These conclusions highlight the relevance of Zn(II) availability in the external milieu in regulating antibiotic resistance.

The amount of available Zn(II) in biological fluids is an issue still under debate, which results from a complex equilibrium among different chelating agents (proteins such as albumin and transferrin, and small molecules) [18]. As already pointed out, during the infective processes, the host sequesters Zn(II) to compete with the pathogen. The cytoplasmic Zn(II) pool in bacteria is mainly maintained by the action of an avid Zn(II) importer (ZnuABC), which scavenges Zn(II) from the periplasm [19]. Thus, periplasmic Zn(II) levels are limited by the external medium and by the strict requirements from the bacterial cytoplasm. MβLs are therefore under selection pressure to compete for the available periplasmic Zn(II).

Evolution favors selection of MβL variants with high Zn(II) affinity. Indeed, MβL variants with high catalytic efficiencies *in vitro*, but displaying antibiotic resistance profiles sensitive to Zn(II) availability, have not been fixed during evolution [20]. Thus, *in vitro* studies should be

extrapolated with caution to infer results about antibiotic resistance.

Similar results have been observed in artificially evolved mutants, which possess higher tolerance to Zn(II) limiting conditions [MEINI M-*ET AL.*, UNPUBLISHED DATA].

Inhibitor design strategies for MβLs have been largely unsuccessful, mostly due to the structural diversity of their active sites. Many efforts have relied on a structural and biochemistry bases, but *in vivo* inhibition has not been effective. Novel strategies should consider Zn(II) uptake as a limiting step on MβL activation. Selective Zn(II) chelating schemes, reinforcing human host Zn(II) sequestering strategies, should be put into focus.

Financial & competing interests disclosure

M-R Meini and LJ González are recipients of doctoral fellowships from Consejo Nacional de Investigaciones Científicas y Técnicas. AJ Vila is a staff member from Consejo Nacional de Investigaciones Científicas y Técnicas. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica and the US NIH (1R01AI100560) to AJ Vila. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

- Andreini C, Bertini I, Rosato A. Metalloproteomes: a bioinformatic approach. *Acc. Chem. Res.* 42(10), 1471–1479 (2009).
- Ma Z, Jacobsen FE, Giedroc DP. Coordination chemistry of bacterial metal transport and sensing. *Chem. Rev.* 109(10), 4644–4681 (2009).
- Outten CE, O'Halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* 292(5526), 2488–2492 (2001).
- Hood MI, Skaar EP. Nutritional immunity: transition metals at the pathogen-host interface. *Nat. Rev. Microbiol.* 10(8), 525–537 (2012).
- Kehl-Fie TE, Skaar EP. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr. Opin. Chem. Biol.* 14(2), 218–224 (2010).
- Brophy MB, Hayden JA, Nolan EM. Calcium ion gradients modulate the zinc affinity and antibacterial activity of human calprotectin. *J. Am. Chem. Soc.* 134(43), 18089–18100 (2012).
- Hood MI, Mortensen BL, Moore JL *et al.* Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. *PLoS Pathog.* 8(12), e1003068 (2012).
- Diepinois G, Ducret V, Caille O, Perron K. The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. *PLoS ONE* 7(5), e38148 (2012).
- Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm? *J. Am. Chem. Soc.* 127(2), 306–325 (2005).
- Crowder MW, Spencer J, Vila AJ. Metallo-beta-lactamases: novel weaponry for antibiotic resistance in bacteria. *Acc. Chem. Res.* 39(10), 721–728 (2006).
- Galleni M, Lamotte-Brasseur J, Rossolini GM, Spencer J, Dideberg O, Frère JM. Standard numbering scheme for class B beta-lactamases. *Antimicrob. Agents Chemother.* 45(3), 660–663 (2001).
- King DT, Worrall LJ, Gruninger R, Strynadka NC. New Delhi metallo-beta-lactamase: structural insights into beta-lactam recognition and inhibition. *J. Am. Chem. Soc.* 134(28), 11362–11365 (2012).
- Tioni MF, Llarrull LI, Poeylout-Palena AA *et al.* Trapping and characterization of a reaction intermediate in carbapenem hydrolysis by *B. cereus* metallo-beta-lactamase. *J. Am. Chem. Soc.* 130(47), 15852–15863 (2008).
- Wommer S, Rival S, Heinz U *et al.* Substrate-activated zinc binding of metallo-beta-lactamases: physiological importance of mononuclear enzymes. *J. Biol. Chem.* 277(27), 24142–24147 (2002).
- Moran-Barrio J, Limansky AS, Viale AM. Secretion of GOB metallo-beta-lactamase in *Escherichia coli* depends strictly on the cooperation between the cytoplasmic DnaK chaperone system and the Sec machinery: completion of folding and Zn(II) ion acquisition occur in the bacterial periplasm. *Antimicrob. Agents Chemother.* 53(7), 2908–2917 (2009).
- Hu Z, Gunasekera TS, Spadafora L, Bennett B, Crowder MW. Metal content of metallo-beta-lactamase L1 is determined by the bioavailability of metal ions. *Biochemistry* 47(30), 7947–7953 (2008).
- Moran-Barrio J, Gonzalez JM, Lisa MN *et al.* The metallo-beta-lactamase GOB is a mono-Zn(II) enzyme with a novel active site. *J. Biol. Chem.* 282(25), 18286–18293 (2007).
- Harris WR, Keen C. Calculations of the distribution of zinc in a computer model of human serum. *J. Nutr.* 119(11), 1677–1682 (1989).
- Berducci G, Mazzetti AP, Rotilio G, Battistoni A. Periplasmic competition for zinc uptake between the metallochaperone ZnuA and Cu,Zn superoxide dismutase. *FEBS Lett.* 569(1–3), 289–292 (2004).
- Gonzalez JM, Meini MR, Tomatis PE, Medrano Martin FJ, Cricco JA, Vila AJ. Metallo-beta-lactamases withstand low Zn(II) conditions by tuning metal-ligand interactions. *Nat. Chem. Biol.* 8(8), 698–700 (2012).