EDITORIAL

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Lipidated β-lactamases: from bench to bedside



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Carbapenemases represent one of the largest clinical threats to the action of carbapenems; the last resort drugs for the treatment of healthcare-associated infections caused by Gram-negative bacteria [1]. Metallo-β-lactamases (MBLs) are Zn(II)-dependent enzymes that constitute the largest family of carbapenemases of clinical impact. Among them, the New Delhi Metallo-β-lactamase (NDM-1) is a plasmid-encoded carbapenemase that has experienced the fastest and widest geographical spread, having been detected in more than 80 countries worldwide since its identification in 2008 [2]. Remarkably, the clinical success of this resistance determinant does not seem to be associated with the dissemination of a particular clone or genetic structure [3]. We have recently suggested that this particular success is due to the cellular localization of NDM-1: while all other known MBLs are soluble periplasmic proteins, NDM-1 is a lipoprotein anchored to the inner leaflet of the outer membrane in Gram-negative bacteria [4]. Despite NDM-1 being reported as a lipidated protein in 2011 [5], this fact was regarded as a biochemical curiosity and deserved little attention until recently when we reported that lipidation and membrane anchoring confer unique evolutionary advantages to NDM-1 [4].

Protein lipidation in bacteria

The mechanism of bacterial protein lipidation is a well-characterized process and has been extensively reviewed elsewhere [6]. Protein lipidation takes place by covalent attachment of acyl moieties to a cysteine residue at the N-terminus, that enable anchoring of globular, hydrophilic proteins to the phospholipids of the bacterial

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membrane. This allows lipoproteins to be membrane-anchored but at the same time preserving their functionality in an aqueous environment. This post-translational modification, first identified in the *Escherichia coli* protein Lpp (also known as Braun's lipoprotein), is present in hundreds of bacterial proteins with diverse roles encompassing enzymatic activity, transport, virulence and structural integrity of the bacterial envelope [6]. Indeed, approximately 1–3% of the bacterial genome codes for lipoproteins, many of them with unknown function.

Bacterial protein lipidation depends on a processing system located in the cytoplasmic membrane, widely conserved in both Gram-negative and Gram-positive organisms [6]. Prelipoprotein precursors synthesized in the cytosol are generally exported by the secretion (Sec) pathway, though there are instances of export through the twin-arginine translocation (Tat) system. After translocation, proteins containing a lipidation signal (lipobox) are first modified by the addition of a diacylglycerol moiety to the sulfhydryl group of the conserved cysteine residue within the lipobox, catalyzed by the Lgt enzyme. This diacylated protein is then processed by the lipoprotein signal peptidase LspA, which cleaves the protein within the lipobox at the peptide bond immediately preceding the lipidated cysteine residue. The free amine moiety of the now N-terminal cysteine is acted upon by the transacylase Lnt, which binds an acyl group to it by an amide linkage.

In Gram-negative bacteria, lipoproteins are by default translocated to the outer membrane by the LolABCDE system [6]. LolCDE is an ATP-binding cassette which extracts lipidated proteins from the inner membrane and transfers them to LolA, a periplasmic protein which shuttles its acylated substrates to the outer membrane. Finally, LolB, itself an outer membrane lipoprotein, receives this cargo from LolA and deposits it into the inner leaflet of the outer membrane. Lipoproteins that remain in the inner membrane possess a signal to avoid the LolCDE system, generally located at positions +2 to +4 from the lipidated cysteine.

Lipidated lactamases

Lactamases bound to the cell surface were first reported in Gram-positive organisms more than 50 years ago [7], and were later demonstrated to be membrane-anchored proteins containing lipobox sequences within their leader peptides [8]. These lipidated enzymes are class A serine-β-lactamases: BLIII (also known as BlaZ) from *Bacillus cereus* [9], PC1 from *Staphylococcus aureus* [8] and PenP from *Bacillus licheniformis* [10], with numerous homologs in related organisms readily found in the GenBank database. In Gram-positive bacteria, lacking a periplasmic space, membrane anchoring would prevent the enzyme from being released into the growth medium, thus keeping the lactamase associated to the cell. Despite the advantage of this anchoring, up to 50% of the total activity of BLIII and PenP lactamases can be detected in culture supernatants [9,10], and a variable percentage of activity has been also observed for PC1 and its homologs in the soluble form [11]. These 'exoenzyme' forms are generated by proteolytic cleavage of the lipidated enzyme, resulting in a soluble lactamase of lower molecular weight than the membrane-anchored counterpart [8].

Lipidated lactamases are less common among Gram-negative bacteria, as may be expected from the presence of an outer membrane which maintains soluble enzymes associated to the cell. The most characterized is the serine β-lactamase BRO-1, from the opportunistic respiratory pathogen *Moraxella catarrhalis* [12]. Despite displaying a canonical lipobox, only 10% of BRO-1 is membrane-bound in its natural host. Instead, when expressed in *E. coli*, 45% of the enzyme is attached to the membrane [13]. In contrast, NDM-1 [4] and *Burkholderia pseudomallei* PenA [14] were recently described to be completely localized in membrane fractions.

The *bro-1* gene is characterized by a significantly lower G+C content (31%) than the *M. catarrhalis* genome (42%). Bootsma *et al.* suggested that β-lactamase lipoproteins derive from Gram-positive organisms, since all known enzymes at the moment belonged to that group of bacteria [12]. This is not the case for *penA* and $bla_{\text{NDM-1}}$, with much higher G+C contents (70 and 62%, respectively). In the case of *penA*, the G+C content matches that of the bacterial host genome.

While the membrane anchoring of β-lactamases in Gram-positive bacteria seems to provide an advantage in terms of enzyme localization and association of the lactamase activity to the cell, the role of lipidation from enzymes in Gram-negative organisms is less clear. Our recent work demonstrated that the membrane anchoring of NDM-1, which is a

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zinc-dependent enzyme, helped prevent its degradation in the periplasmic space upon metal starvation in the extracellular milieu, and occurs with a soluble mutant of the enzyme [4]. This gain in stability is of great importance for maintaining carbapenem resistance during pathogenesis, since the immune system secretes large amounts of a Zn(II)-chelator protein, calprotectin, in the sites of infection. In this way, lipid modification confers additional fitness to this MBL by augmenting its stability *in vivo*.

Membrane anchoring also favors secretion of the lactamases into outer membrane vesicles (OMVs) [4,15]. This has been shown to be the case for NDM-1 and possibly BRO-1. OMVs are released by both pathogenic and nonpathogenic Gram-negative bacteria in a ubiquitous process that occurs over the course of normal growth [16]. OMVs are formed by fission of outer membrane protrusions that enclose periplasmic components in a yet uncharacterized process. These membrane-enclosed 'bacterial packs' have been associated with cellular communication and bacterial pathogenesis. We showed that OMVs containing NDM-1 can protect nearby populations of bacteria from otherwise lethal antibiotic levels by vesicle-mediated transfer of the enzyme [4]. Inclusion into OMVs not only allows secretion of NDM-1, but also protects the enzyme from extracellular proteases or Zn(II) depletion as it resides in the lumen of the vesicles. We also showed that OMVs from clinical pathogens expressing NDM-1 can carry both the protein and the gene coding for it, supporting the notion that OMVs can expand the options for horizontal transfer. Overall, membrane anchoring is able to stabilize a protein scaffold and facilitate secretion of enzymes in the extracellular milieu.

Despite NDM-1 is the only characterized membrane-bound MBL, we have reported the existence of numerous endogenous MBL homologs containing a lipobox sequence sharing a high degree of homology with different groups of characterized soluble MBLs [4]. These putative lipidated MBLs derived mostly from functional metagenomics analysis of soil bacteria [17], suggesting that the environmental microbiota constitute a reservoir of membrane-anchored MBLs. As in the case of NDM-1, these lipidated MBLs may have an enhanced potential for dissemination, helped by the transfer of β-lactamase protein via OMVs, which protects antibiotic-sensitive bacteria.

Bacterial protein lipidation as a target

Bacterial protein lipidation remains an unexploited antibiotic target for clinical treatment of infections. Globomycin, a cyclic lipopeptide from *Streptomyces* sp., inhibits lipoprotein signal peptidase LspA, leading to accumulation of unprocessed prolipoproteins in the inner membrane of *E. coli*, followed by cell death [18]. Both globomycin and a functional analog compound from *Myxococcus xanthus* were proposed as promising leads for the development of a new class of antibiotics [19]. Apart from the LspA peptidase, compounds that target the Lol lipoprotein translocation system have also been identified (recently reviewed by Brown [20]). In both cases, the blocking of the lipidation pathway prevents lipoproteins from reaching the outer membrane, which is lethal to the cell. As a result, these drugs not only offer new effective antibiotic targets not present in eukaryotic organisms, but also provide a mechanism to retain membrane-anchored lactamases in the inner membrane, avoiding their secretion in OMVs.

Knowledge of β-lactamase lipidation has a direct impact in the detection of carbapenemases in clinical pathogens. Membrane anchoring thwarts NDM-1 detection using the modified Hodge test, by preventing the release of the enzyme into the extracellular medium where carbapenemase activity is detected [21]. This problem can be sorted out by addition of a detergent to the Petri dish, which dissolves the enzyme and enables NDM-1 detection without false positives [21]. This provides an excellent example of how basic knowledge of molecular features can be translated directly to a clinical test. We anticipate that lipidated β-lactamases may become increasingly prevalent, and efforts in developing new tests and drugs will be required.

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