Received: 19 July 2019 | Revised: 9 September 2019 | Accepted: 10 September 2019

DOI: 10.1002/pro.3728

Check for updates

METHODS AND APPLICATIONS

THE PROTEIN SOCIETY WILEY

A simple protocol to characterize bacterial cell-envelope lipoproteins in a native-like environment

Estefanía Giannini[1] | **Lisandro J. González**[1,2] | **Alejandro J. Vila**[1,2]

[1]Laboratorio de Metaloproteínas, Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Rosario, Argentina

[2]Área Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Correspondence
Lisandro J. González and Alejandro J. Vila, Laboratorio de Metaloproteínas, Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Rosario, Argentina.
Email: lgonzalez@ibr-conicet.gov.ar (L. J. G.) and vila@ibr-conicet.gov.ar (A. J. V.)

Funding information
Agencia Nacional de Promoción Científica y Tecnológica, Grant/Award Number: PICT-2016-1657; National Institute of Allergy and Infectious Diseases, Grant/Award Number: 2R01AI100560-06A1; Consejo Nacional de Investigaciones Científicas y Técnicas

Abstract

Physiological conditions in living cells are strictly regulated to allow, optimize, and coordinate biological processes. The bacterial cell envelope is the compartment where the communication with the external environment takes place. This involves membrane proteins, key players in many biological processes that ensure bacterial survival. The biochemical characterization of membrane proteins, either integral, lipidated or peripheral is challenging due to their mixed protein-lipid nature, making it difficult to purify and obtain considerable amounts of samples. In contrast to integral membrane proteins, lipidated proteins are usually purified as truncated soluble versions, neglecting the impact of the membrane environment. Here we report a simple and robust protocol to characterize bacterial lipidated proteins in spheroplasts from *Escherichia coli* using a β-lactamase as a model. The Metallo-β-lactamase NDM-1 is an enzyme anchored to the inner leaflet of the outer membrane of Gram-negative bacteria. Kinetic parameters and stability of the lipidated NDM-1 and the soluble unbound version (NDM-1 C26A) were measured in spheroplasts and periplasm, respectively. These studies revealed that membrane anchoring increases the K_M of the enzyme, consequently decreasing the catalytic efficiency, while not affecting its kinetic stability. This approach can be used to characterize lipidated proteins avoiding the purification step while mimicking its native environment. This approach also helps in filling the gap between *in vitro* and *in vivo* studies.

KEYWORDS

bacterial outer membrane, kinetic stability, lipidated proteins, lipidic environment, spheroplasts

1 | INTRODUCTION

Biological environments have a large impact in protein folding, stability and function.[1] Consequently, proteins are highly sensitive to environmental influences.[2] The choice of the optimum conditions for the *in vitro* characterization of a protein is challenging, since they may fail in reproducing accurately the native environment.[3–5] This situation is further aggravated in the case of membrane-bound proteins, since the membrane lipids strongly condition their structure, dynamics, and function.[6–9]

Biological membranes are dynamic cellular envelopes resulting from a complex network of proteins and lipids. The integrity of these membranes is mainly governed by lipid–lipid, protein–lipid, and protein–protein interactions.[10] These membranes have diverse lipid compositions and are active participants in essential biological processes.[11] The outer membrane of Gram-negative bacteria is the first of the three layers that surround the bacterial cytoplasm.[12] This bilayer is highly asymmetric: the inner leaflet is mainly built by various species of phospholipids, while the outer leaflet is largely composed of lipopolysaccharides (LPS).[13] Different bacterial

species indeed display a large chemical heterogeneity in their membrane composition, specially adapted to the specific living habitats.[10]

Biological processes taking place in the bacterial cell envelope are mainly related to the communication of the cell with the external medium, which includes sensing and providing feedback to external conditions.[13] Proteins located within the bacterial cell envelope, including integral membrane proteins, lipidated proteins, peripheral membrane proteins, and soluble proteins, play key roles in these communication events.[12,14] Lipidated proteins are anchored to biological membranes through a lipid moiety covalently bound to their N-termini and represent 2–8% of the total predicted proteome in bacteria.[15–17]

The biochemical characterization of membrane proteins is challenged by its mixed protein–lipid nature, making it difficult to purify considerable amounts of protein. Lipidated proteins are generally studied in their truncated soluble variants lacking the lipid moiety,[18,19] but this approach overlooks the cellular localization of these proteins, and the impact of the membrane interface.

We have recently reported that New Delhi Metallo-β-lactamase-1 (NDM-1) is a lipidated protein anchored to the inner leaflet of the outer membrane of Gram-negative bacteria, in contrast to all characterized Metallo-β-lactamases (MBLs) which are soluble proteins located in the periplasmic space of these bacteria.[20] Thus, we chose NDM-1 as a model lipoprotein to optimize a system that mimics the membrane environment and is amenable for performing biochemical studies.

Here we used spheroplasts from *Escherichia coli*, a frequent host of NDM-1, as a mimic of an *in vivo* system to characterize NDM-1 and analyze the effect of its cellular localization. Spheroplasts are bacterial cells lacking its peptidoglycan wall and with a permeabilized outer membrane that allows the free diffusion of different types of molecules into the periplasmic space.[21] In this work, we report two protocols to evaluate the activity and kinetic stability of NDM-1. Spheroplasts expressing lipidated NDM-1 and periplasm extracts expressing its soluble version were studied to assess the impact of lipidation in the activity and stability of NDM-1.

The spheroplasts' system, together with protocols to study the activity and kinetic stability of the lipidated MBL NDM-1 reported in this work, can be easily adapted to study other proteins located within the bacterial cell envelope. This system also allows the comparison of membrane proteins, located either in the outer or internal membrane of Gram-negative bacteria, with their soluble isoforms or with engineered variants designed in the laboratory. Mimicking the native environment of the membrane proteins without the need of purification allows performing medium-throughput biochemical characterizations of membrane proteins.

2 | RESULTS

2.1 | Spheroplasts as a system to characterize lipidated proteins

In order to devise an experimental protocol to study a lipidated enzyme in spheroplasts, we chose the following enzymes: the MBL NDM-1 (anchored to the outer membrane of Gram-negative bacteria), NDM-1 C26A (a mutant that lacks the lipidation site and is expressed as a soluble enzyme in the periplasm of *E. coli*)[20] and the allelic variant NDM-8[18] (Figure 1). The latter is a natural variant reported in the clinic, also anchored to the outer membrane of Gram-negative bacteria, previously reported to be more stable than NDM-1 *in vitro*.[18]

The genes coding for these enzymes were cloned with their peptide leaders in a plasmid designed for the controlled expression of MBLs.[20] This system allows the induction of MBL expression with IPTG, in such a way that the enzymes are expressed at levels similar to those found in clinical strains with their native promoter, avoiding artifacts due to overexpression.[20,22]

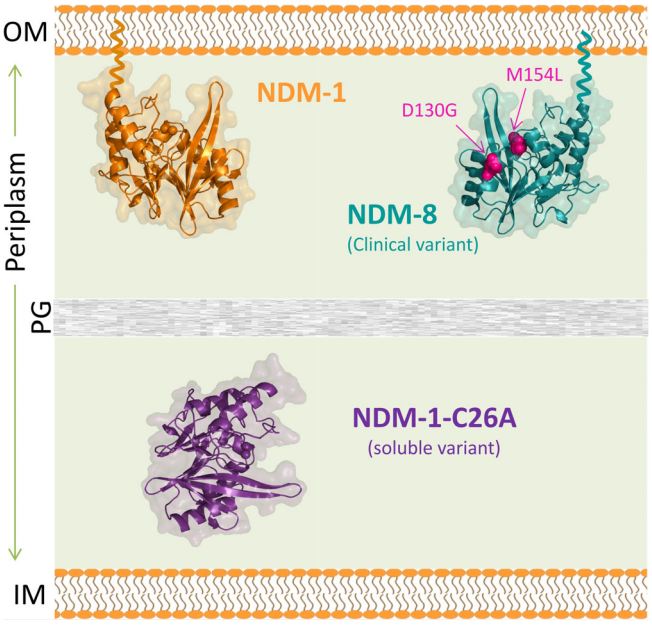

FIGURE 1 Localization of NDM-1, NDM-1 C26A and NDM-8 in the periplasm of *Escherichia coli*. The New Delhi Metallo-β-lactamase (NDM-1) has a lipobox sequence in its N-terminal, which is recognized by the lipidation machinery of Gram-negative bacteria. After being lipidated NDM-1 is directed to the inner leaflet of the outer membrane. The soluble variant (NDM-1 C26A) was engineered in the laboratory by mutating the essential Cys of the lipobox that is modified by lipidation. This mutation results in a periplasmic soluble MBL. The clinical variant NDM-8 is a double mutant: D130G–M154L (mutations, shown as pink spheres), which conserves the lipobox and is anchored to the outer membrane as well as NDM-1. OM, outer membrane, IM, inner membrane, PG, peptidoglycan

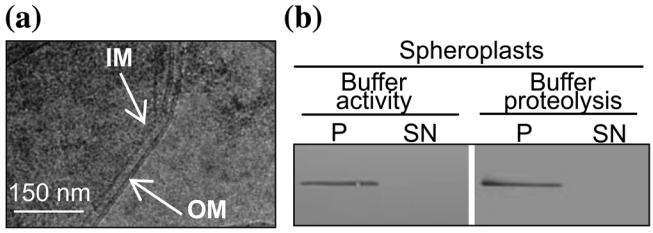

FIGURE 2 Spheroplasts of *Escherichia coli* remain intact in the experimental conditions and conserve their cellular compartments. (a) Cryo-TEM image of *E. coli* spheroplasts. *E. coli* spheroplasts were observed by Cryo-TEM to assess the integrity of their cellular compartments. Both membranes and the periplasmic space between them can be distinguished in the image. (b) *E. coli* spheroplasts were incubated in the experimental buffers for 15 min or 2 hr depending the duration of each experiment. After centrifugation, the pellet fraction (P) containing the spheroplasts and the supernatant fraction (SN) of each preparation were analysed by Western Blot using primary antibodies for RNA polymerase-α (RNApol). In both preparations RNA-polymerase was detected only in the pellet fraction confirming the integrity of spheroplasts in the experimental buffer conditions

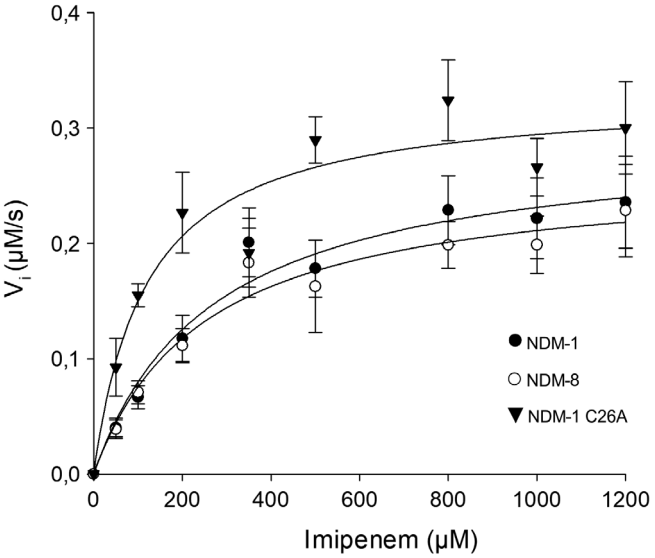

FIGURE 3 Michaelian curves obtained for NDM variants in *Escherichia coli* spheroplasts and periplasmic extracts. Initial reaction rates were measured with spheroplasts for NDM-1 and NDM-8, and periplasmic extracts for NDM-1 C26A toward imipenem and fitted to the Michaelis–Menten equation. Reaction conditions: 10 mM HEPES pH 7.5, 200 mM NaCl, 5 mM $ZnSO_4$ at 30°C

E. coli spheroplasts expressing the proteins of interest were obtained by treatment of cells with lysozyme.[23] The quality of the spheroplasts was assessed by Cryo-TEM (Figures 2a, and S1). Microscopy images account for the integrity of the spheroplasts, disclosing the presence of the outer membrane and the periplasmic space. Then we evaluated the stability of spheroplasts in the buffers used for activity and stability assays along the experimental time. Spheroplasts integrity was assessed by immunoblotting of cytosolic RNA-polymerase. The Western blots showed in Figure 2b confirm that spheroplasts were not damaged in the experimental conditions as RNA-polymerase was only found in the pellet containing the spheroplasts, and was not released to the medium.

2.2 | Activity measurements in spheroplasts

We obtained spheroplasts of *E. coli* cells expressing NDM-1 and NDM-8, and periplasmic extracts of *E. coli* cells expressing the soluble variant NDM-1 C26A. Spheroplasts/periplasmic extracts were diluted in a buffer solution usually employed for activity measurements of MBLs (see Materials and Methods for details).

We determined the enzymatic parameters of NDM-1 in spheroplasts toward three β-lactam antibiotics: imipienem, piperacillin, and cefepime. They all followed a Michaelian behavior, enabling an estimation of apparent V_{max} and K_M values (Figure S2a). The stability of the antibiotics and their binding to other cellular components were tested by incubation of each antibiotic with spheroplasts from cells that did not express NDM-1. All antibiotics were stable and no hydrolysis was observed [Figure S2(B)], allowing us to discard the possible hydrolytic activity of other endogenous beta-lactamase, or irreversible binding to PBPs. To discard the non-specific binding of the antibiotics to other cellular components, we also measured the effective concentrations of the antibiotics after being incubated with spheroplasts. The concentration of imipenem decreased by only 5%, while the concentrations of piperacillin and cefepime remained unaltered. These experiments indicate that the observed hydrolysis is exclusively due to the presence of NDM-1 in the spheroplasts.

The enzymatic parameters of the three enzymes toward imipenem are shown in Figure 3 and Table 1. Protein concentrations in spheroplast/periplasmic extracts were determined by Western Blot assays employing specific antibodies against NDM-1 and an internal calibration curve performed with known quantities of the purified recombinant enzyme (Figure S3). This allowed us to estimate apparent k_{cat} values for each enzyme (Table 1). The concentration of enzymes was between 5 and 20 nM, thus ensuring pseudo first-order kinetics.

The calculated kinetic parameters of the soluble NDM-1 C26A variant in periplasmic extracts are very similar to those previously reported for the recombinant soluble enzyme.[18,24] Both membrane-bound variants in spheroplasts (NDM-1 and NDM-8) were active against imipenem, showing K_M values that reveal that the substrate can easily reach the enzymes in spheroplasts, validating the employed approach. Soluble NDM-1 C26A displayed lower K_M and higher k_{cat} values than the lipidated NDM-1, and similar to the recombinant soluble enzyme, resulting in a fourfold more efficient enzyme for imipenem hydrolysis. Overall, the current data suggests

TABLE 1 Kinetic parameters of NDM variants

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})
NDM-1 (spheroplasts)	280 ± 63	270 ± 30	1.0 ± 0.3
NDM-8 (spheroplasts)	260 ± 52	250 ± 50	1.0 ± 0.4
NDM-1 C26A (periplasm)	340 ± 46	110 ± 20	3.1 ± 0.9
NDM-1 (recombinant)[a]	570 ± 30	150 ± 30	4.0 ± 1.0
NDM-1 (recombinant)[b]	600	78 ± 4	7.6
NDM-8 (recombinant)[b]	54	20 ± 3	2.9

Note: The activity of NDM-1, NDM-8 and NDM-1 C26A against Imipenem was measured in spheroplasts or in periplasmic extracts, according to the enzyme localization. The enzymes displayed a Michaelis–Menten behavior which allowed the determination of kinetic parameters (Figure 3). The values shown are the average of at least three independent determinations ± *SE*.

[a]Experimental condition: 10 mM HEPES pH 7.5, 200 mM NaCl, 20 μM $ZnSO_4$, 20 μg/ml bovine serum albumin; 30°C.[24]

[b]Experimental condition: 50 mM HEPES pH 7.2, 1 mg/ml BSA, 1 μM $ZnSO_4$ and 0.01% Triton X-100; 25°C.[18]

that membrane binding slightly impairs the catalytic efficiency, mostly by increasing the K_M value in both cases.

2.3 | Stability in spheroplasts

Protein stability is usually determined in purified enzymes using different biophysical techniques that monitor the impact of external perturbations such as temperature, pressure or the action of chaotropic agents on protein folding, providing thermodynamic stability data.[25] However, the biological function within the cell requires also kinetic stabilization, which means that the protein needs to remain in the native state during the biologically-relevant time-scale.[25] Proteins can explore different conformations in crowded intracellular environments; some of them being unstable and as such susceptible to aggregation or degradation by proteases, among other phenomena.[25] We, therefore, designed a protocol to evaluate the protein kinetic stability by exposing the spheroplasts containing the lipidated proteins (or the periplasmic extracts) to a protease, a common phenomenon to which proteins are exposed inside the cells. Local fluctuations of the protein structure under native conditions, that is, without addition of a denaturant, can lead to conformations that are more susceptible to proteolysis.[26] Thus, this approach allows obtaining information of the protein kinetic stability in native condition. Proteolysis was followed by quantitating the remaining amount of protein by immunoblotting after addition of the protease.

Proteinase K (PK) was added to spheroplasts preparations expressing each NDM variant at a final concentration of 0.75 mg/ml. Protein degradation was monitored for 120 min and the proteolysis reaction was stopped by addition of the protease inhibitor PMSF. The amount of intact protein at different times was quantified by immunodetection with specific

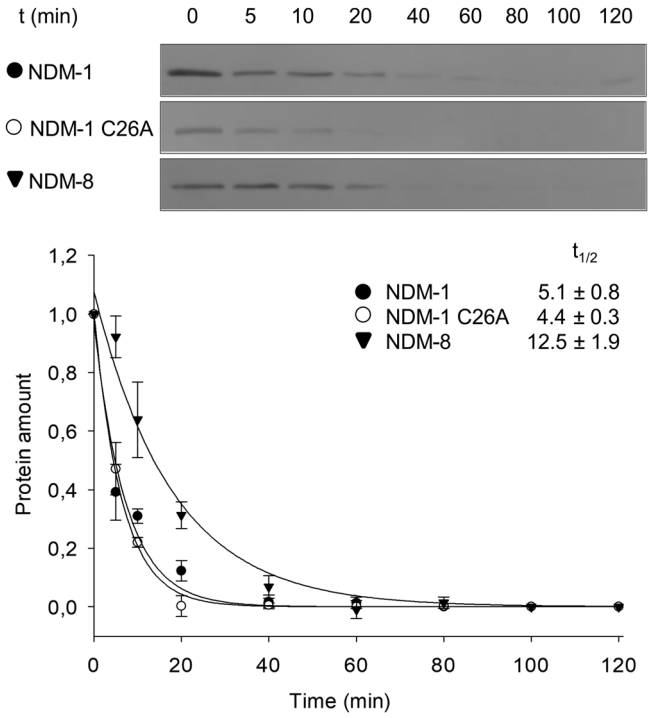

FIGURE 4 Membrane localization does not protect NDM-1 from proteinase K degradation. Degradation of the three NDM variants in spheroplasts or in periplasm after the addition proteinase K was followed by Western Blot. The half-life lives ($t_{1/2}$) of each variant were determined by fitting the curves to an exponential decay: $P = P_0e^{(-\lambda t)}$, and then calculated from: $t_{1/2} = \ln(2)/\lambda$. These results correspond to the average of at least three independent determinations ± *SE*

antibodies against NDM-1. The degradation phenomenon was fitted to an exponential decay curve which allowed an estimation of the half-life time ($t_{1/2}$) for each protein. NDM-1 and NDM-1 C26A displayed similar $t_{1/2}$ values within the experimental error (Figure 4). These results evidence that membrane anchoring does not increase the kinetic stability of NDM-1. In contrast, the clinical variant NDM-8 showed a threefold increase $t_{1/2}$ value (Figure 4).

3 | DISCUSSION

There is plenty of evidence about the effects of cellular environments on protein function and the need to take them into account while performing *in vitro* studies.[1–5] Here we report a simple and robust protocol to characterize bacterial lipidated proteins in spheroplasts from *E. coli* DH5α using a β-lactamase as a model. There are several advantages in this approach. For instance, *E. coli* DH5α is one of the most popular and widely used laboratory strain, and is sensitive to antibiotics, allowing the introduction of different plasmids conferring resistance. In addition, spheroplasts are easy to obtain, they retain both membranes after murein digestion and they are stable under the assayed experimental conditions.

Despite the loss of peptidoglycan and periplasmic components, this system retain the complete membrane environment (including native lipid rafts and other membrane proteins) rendering a system with conditions close to the native ones. In spheroplasts, substrate molecules freely diffuse into the periplasm, thus overriding the transit throughout the outer membrane in entire cells, and enabling the determination of K_M values that are within the same range of those measured in purified enzymes and in periplasmic extracts, which validates this approach.[21] Moreover, this system also enables determination of the kinetic stability by exposing the assayed enzyme to a commercial protease.

The enzymatic activities in spheroplasts displayed a Michaelis–Menten behavior (Figure 3, S2), which allowed us to determine apparent kinetic parameters for each variant. We report that membrane anchoring decreases the catalytic efficiency by a factor of 3–4, mostly due to an increase in the K_M values (Table 1). This difference allows us to hypothesize that the active site of the soluble variant is more accessible, while for the lipidated one, the proximity to the membrane may impair substrate binding. These results are in agreement with the observation that membrane binding decreases the Minimum Inhibitory Concentration (MIC) in NDM-1.[18,20] A comparison of the hydrolytic parameters of NDM-1 and NDM-8 both in the soluble and in the membrane-bound forms reveals that substitutions D130G and M154 L do not improve the catalytic efficiency, in agreement with previous reported data.[18]

The kinetic stabilities were estimated by calculating a half-life time for each enzyme upon exposure to proteinase K in the periplasm and in spheroplasts. This approach revealed that membrane-anchoring does not increase the kinetic stability of NDM-1, while mutations present in the variant NDM-8 gives rise to a substantial kinetic stabilization of the enzyme. NDM-8 has also been reported to be more stable thermodynamically than NDM-1.[18] We conclude that mutations D130G and M154 L indeed give rise to a more stable scaffold; increasing the thermal stability of NDM-1, while ensuring the kinetic stability of the native enzyme conformation.

This approach can be applied to different systems, also including integral or peripheral membrane proteins, and can be exploited as a strategy for medium-throughput evaluation of activity and stability parameters of multiple proteins in their native membrane environments, avoiding the requirement of protein purification and providing bona fide data.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains and reagents

E. coli DH5α was used for the expression of plasmid pMBLe and biochemical studies. *E. coli* DH5α cells were grown aerobically at 37°C in lysogeny broth (LB) medium supplemented with gentamicin 20 μg/ml when necessary. Chemical reagents were purchased from Sigma-Aldrich (USA) and Proteinase K from Invitrogen (USA).

4.2 | Preparation of spheroplasts and periplasmic extracts

Extraction of periplasmic proteins and spheroplasts was performed as previously described.[23] Briefly, 10 ml of *E. coli* pMBLe-NDM cultures were pelleted and cells were washed once with 20 mM Tris, 150 mM NaCl, pH 8.0. The washed cells were suspended in 20 mM Tris, 0.1 mM EDTA, 20% wt/vol sucrose, 1 mg/ml lysozyme (from chicken egg white, Sigma-Aldrich, protein ≥90%), 0.5 mM PMSF, pH 8 (resuspension volume was normalized according to the formula V = 100 μL × OD600 × Vc, where Vc is the starting volume of culture sample), incubated with gentle agitation at 4°C for 30 min, and finally pelleted, with the periplasmic extract in the supernatant. The pellet consisting of spheroplasts was washed in 20 mM Tris, 0.1 mM EDTA, 20% wt/vol sucrose, pH 8 and suspended in the same volume of this buffer. Western blots with antibodies detecting cytoplasmic RNA polymerase (BioLegend, 662903) were performed as integrity controls for spheroplasts. For Cryo-TEM images the spheroplast sample was spread in an EM grid and submerged in liquid ethane (Plunge freezing technique). The sample was analysed in a FEI Tecnai F20 Cryo.

4.3 | β-lactamase activity measurements in spheroplasts and MBL quantification

β-lactamase activity was measured in a Jasco V-670 spectrophotometer at 30°C in 10 mM HEPES, 200 mM NaCl pH 7.4, 5 μM $ZnSO_4$ for spheroplasts and periplasm, in 0.1 cm cuvettes, using imipenem, cefepime, and piperacillin as substrates. Antibiotic hydrolysis was monitored at 300 nm for imipenem, 260 nm for cefepime and 235 nm for piperacillin. The following differential extinction coefficients were used, imipenem: $\Delta_{300nm} = -9{,}000\ M^{-1}\ cm^{-1}$, cefepime: $\Delta\varepsilon_{260nm} = -10{,}000\ M^{-1}\ cm^{-1}$ and piperacillin: $\Delta\varepsilon_{235nm} = -820\ M^{-1}\ cm^{-1}$. MBL protein levels were determined by SDS-PAGE followed by Western blot with NDM-1 antibodies at 1:1,000 dilution from a 700 μg/ml solution (Instituto de Salud y Ambiente del Litoral, ISAL, UNL-CONICET, Argentina) and immunoglobulin G-alkaline phosphatase conjugates at 1:3,000 dilution (Biorad, 1706520). Protein band intensities were quantified from Nitrocellulose membranes with ImageJ software[18] and converted to protein amounts through a calibration curve constructed under the same experimental conditions with quantified recombinant NDM-1_Δ38.[27]

The plots of the dependence of initial rates on substrate concentration were fitted to the Michaelis–Menten equation using SigmaPlot 12.0. Reported kinetic parameters correspond to the average of at least three determinations with independent spheroplasts or periplasm preparations. Imipenem and cefepime were purchased from USP Pharmacopeia, and piperacillin was purchased from Sigma.

4.4 | Evaluation of β-lactams stability in spheroplasts and calculation of their effective concentration

The hydrolysis of imipenem, cefepime and piperacillin in the presence of spheroplasts not expressing NDM-1, that is, containing the empty plasmid pMBLe, was assay to discard unspecific hydrolysis by other cellular components. It was performed in the same conditions as the activity measurements described before, and was compared to the hydrolysis in the presence of spheroplasts harboring the pMBLe-*bla*$_{NDM-1}$ plasmid.

A fresh stock of each β-lactam antibiotic was prepared and quantified. Then, different amounts of each antibiotic (below, around and above the K_M value) were incubated with spheroplasts containing empty pMBLe, which were then harvested by centrifugation at 10,000 rpm during 2 min. The antibiotic present in the supernatant was quantified and compared with a negative control in the absence of.

4.5 | Evaluation of kinetic stability by limited proteolysis in Spheroplasts

Spheroplasts expressing NDM-1, NDM-8 and NDM C26A were suspended in buffer 10 mM Tris, 5 mM CaCl2, pH 8 and treated with 0,75 μg/ml of proteinase K (PK) in ice. Aliquots were taken at different times during 2 hr, the reaction was quenched with 5 mM PMSF. MBL remaining protein levels after PK treatment, were determined by SDS-PAGE followed by western blot with NDM-1 antibodies at 1:1,500 dilution from 700 μg/ml solution and immunoglobulin G-alkaline phosphatase conjugates at 1:3,000 dilution (Biorad, 170-6520). Protein band intensities were quantified from Nitrocellulose membranes with ImageJ software.[28]

Calculation of $t_{1/2}$. Curves adjust to an exponential decay: $\mathbf{P=P_0e^{(-\lambda t)}}$, being $\mathbf{P_0}$: initial amount of protein, **P**: amount of protein at time **t**, and **λ**: decay constant. The half-life time ($t_{1/2}$) can be expressed in terms of the decay constant as following: $\mathbf{t_{1/2} = \ln(2)/\lambda}$.

ACKNOWLEDGMENTS

E.G. is recipient of a doctoral fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). A.J.V. and L.J.G. are Staff members from CONICET. This work was also supported by funds from: National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number 2R01AI100560-06A1 and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) under award number PICT-2016-1657 to A.J.V. We thank Dr. Guillermo Bahr and the Interdisciplinary Centre for Electron Microscopy at EPFL-Swiss for Cryo-TEM images.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Estefanía Giannini https://orcid.org/0000-0001-6084-1395
Lisandro J. González https://orcid.org/0000-0002-0575-1810
Alejandro J. Vila https://orcid.org/0000-0002-7978-3233

REFERENCES

1. Cohen RD, Pielak GJ. A cell is more than the sum of its (dilute) parts: A brief history of quinary structure. Protein Sci. 2017;26:403–413.
2. Cheng JT, Hale JD, Elliott M, Hancock RE, Straus SK. The importance of bacterial membrane composition in the structure and function of aurein 2.2 and selected variants. Biochim Biophys Acta. 2011;1808:622–633.
3. Binolfi A, Theillet FX, Selenko P. Bacterial in-cell NMR of human alpha-synuclein: A disordered monomer by nature? Biochem Soc Trans. 2012;40:950–954.
4. Meini MR, Llarrull LI, Vila AJ. Evolution of metallo-beta-lactamases: Trends revealed by natural diversity and in vitro evolution. Antibiotics. 2014;3:285–316.
5. Monteith WB, Cohen RD, Smith AE, Guzman-Cisneros E, Pielak GJ. Quinary structure modulates protein stability in cells. Proc Natl Acad Sci U S A. 2015;112:1739–1742.
6. Ray A, Jatana N, Thukral L. Lipidated proteins: Spotlight on protein-membrane binding interfaces. Prog Biophys Mol Biol. 2017;128:74–84.
7. Brugger B. Inconspicuous little allies: How membrane lipids help modulate protein function. ACS Cent Sci. 2018;4:530–531.
8. Corradi V, Mendez-Villuendas E, Ingolfsson HI, et al. Lipid-protein interactions are unique fingerprints for membrane proteins. ACS Cent Sci. 2018;4:709–717.
9. Cross TA, Sharma M, Yi M, Zhou HX. Influence of solubilizing environments on membrane protein structures. Trends Biochem Sci. 2011;36:117–125.
10. Simons K, Gerl MJ. Revitalizing membrane rafts: New tools and insights. Nat Rev Mol Cell Biol. 2010;11:688–699.
11. Egan AJF. Bacterial outer membrane constriction. Mol Microbiol. 2018;107:676–687.
12. Konovalova A, Kahne DE, Silhavy TJ. Outer membrane biogenesis. Annu Rev Microbiol. 2017;71:539–556.

13. Ruiz N, Kahne D, Silhavy TJ. Advances in understanding bacterial outer-membrane biogenesis. Nat Rev Microbiol. 2006;4:57–66.
14. Laloux G, Collet JF. Major tom to ground control: How lipoproteins communicate extracytoplasmic stress to the decision center of the cell. J Bacteriol. 2017;199:e00216–e00217.
15. Kovacs-Simon A, Titball RW, Michell SL. Lipoproteins of bacterial pathogens. Infect Immun. 2011;79:548–561.
16. Allison JR. Let the powers combine. Structure. 2016;24:5–6.
17. Konovalova A, Silhavy TJ. Outer membrane lipoprotein biogenesis: Lol is not the end. Philos Trans R Soc Lond Ser B Biol Sci. 2015;370:20150030.
18. Makena A, Brem J, Pfeffer I, et al. Biochemical characterization of New Delhi metallo-beta-lactamase variants reveals differences in protein stability. J Antimicrob Chemother. 2015;70:463–469.
19. Takeda K, Miyatake H, Yokota N, Matsuyama S, Tokuda H, Miki K. Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. EMBO J. 2003;22:3199–3209.
20. Gonzalez LJ, Bahr G, Nakashige TG, Nolan EM, Bonomo RA, Vila AJ. Membrane anchoring stabilizes and favors secretion of New Delhi metallo-beta-lactamase. Nat Chem Biol. 2016;12:516–522.
21. Amro NA, Kotra LP, Wadu-Mesthrige K, Bulychev A, Mobashery S, G-y L. High-resolution atomic force microscopy studies of the *Escherichia coli* outer membrane: Structural basis for permeability. Langmuir. 2000;16:2789–2796.
22. Pasteran F, Gonzalez LJ, Albornoz E, Bahr G, Vila AJ, Corso A. Triton Hodge test: Improved protocol for modified Hodge test for enhanced detection of NDM and other carbapenemase producers. J Clin Microbiol. 2016;54:640–649.
23. 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. 2009;53:2908–2917.
24. Palacios AR, Mojica MF, Giannini E, et al. The reaction mechanism of metallo-beta-lactamases is tuned by the conformation of an active-site mobile loop. Antimicrob Agents Chemother. 2019;63:e01754–e01718.
25. Sanchez-Ruiz JM. Protein kinetic stability. Biophys Chem. 2010;148:1–15.
26. Park C, Marqusee S. Probing the high energy states in proteins by proteolysis. J Mol Biol. 2004;343:1467–1476.
27. Gonzalez MM, Kosmopoulou M, Mojica MF, et al. Bisthiazolidines: A substrate-mimicking scaffold as an inhibitor of the NDM-1 carbapenemase. ACS Infect Dis. 2015;1:544–554.
28. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671–675.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Giannini E, González LJ, Vila AJ. A simple protocol to characterize bacterial cell-envelope lipoproteins in a native-like environment. *Protein Science*. 2019;28:2004–2010. https://doi.org/10.1002/pro.3728