Supplementary Information

Title

Membrane-anchoring stabilizes and favors secretion of New Delhi Metallo-β-lactamase

Authors

Lisandro J. González[1], Guillermo Bahr[1], Toshiki G. Nakashige[2], Elizabeth M. Nolan[2], Robert A. Bonomo[3] and Alejandro J. Vila[1*]

Affiliations

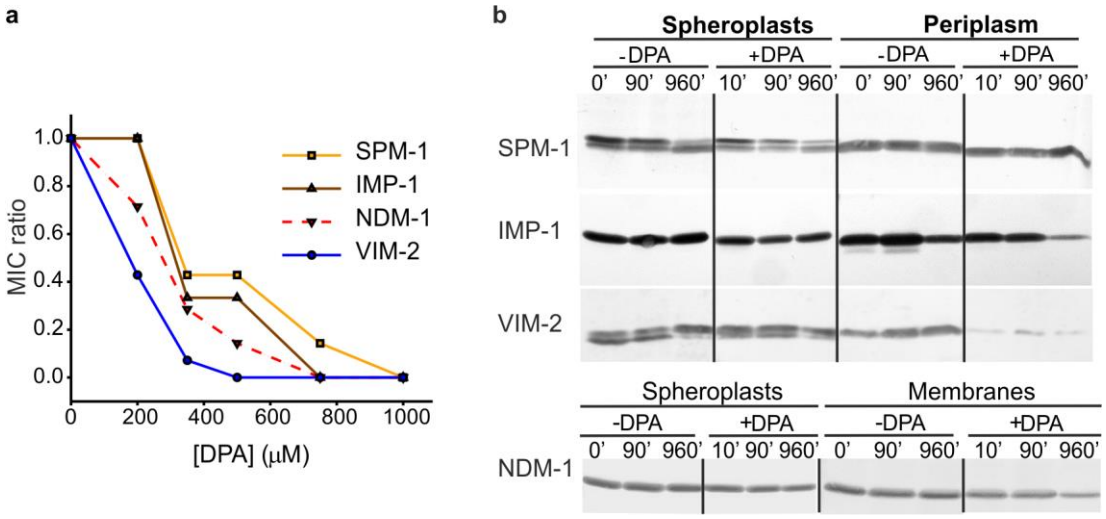
[1]Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR) and Área Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

[2]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

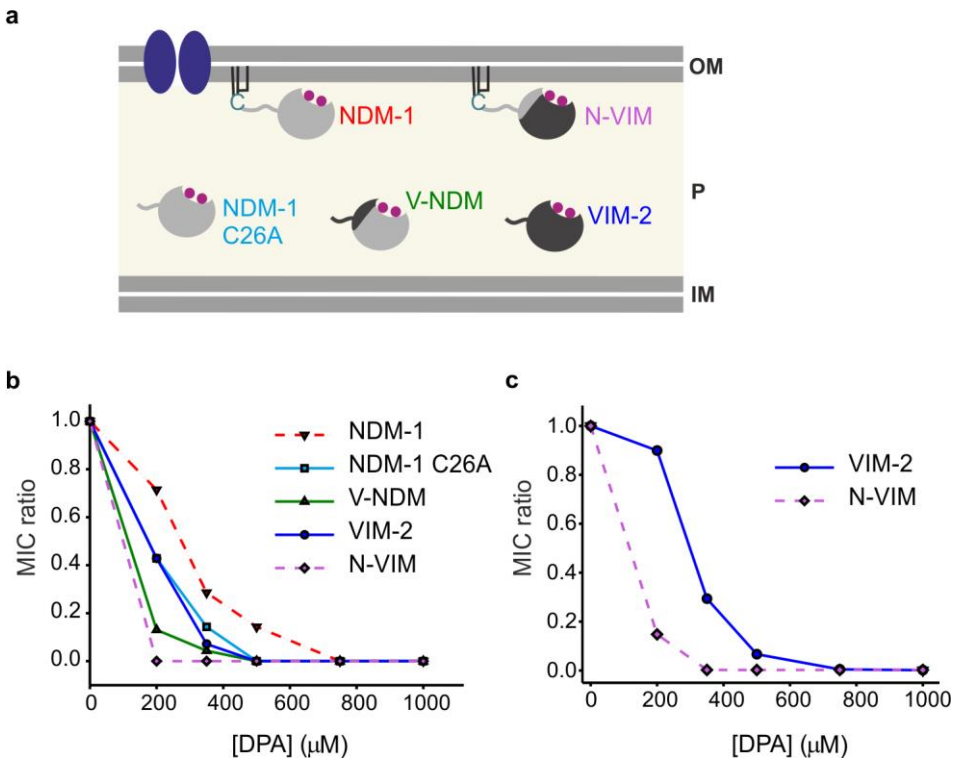
[3]Research Service, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, OH; Departments of Medicine, Pharmacology, Microbiology and Molecular Biology; Case Western Reserve University, Cleveland, OH, USA

*Corresponding author. E-mail: vila@ibr-conicet.gov.ar

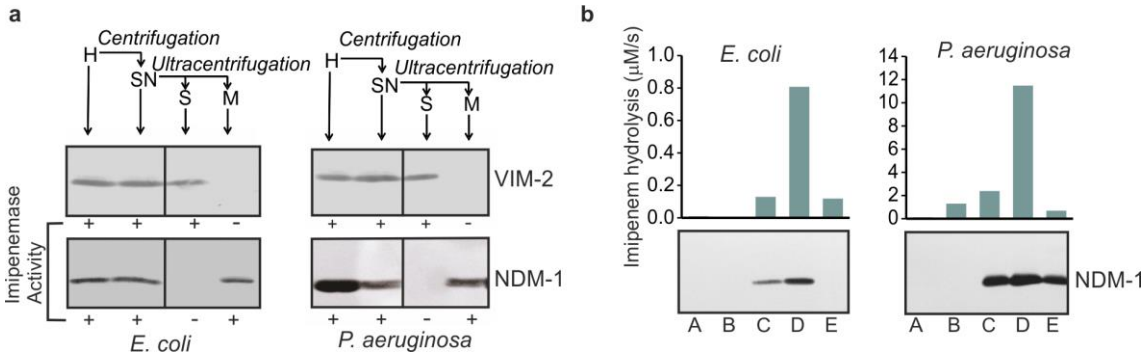
Supplementary Results



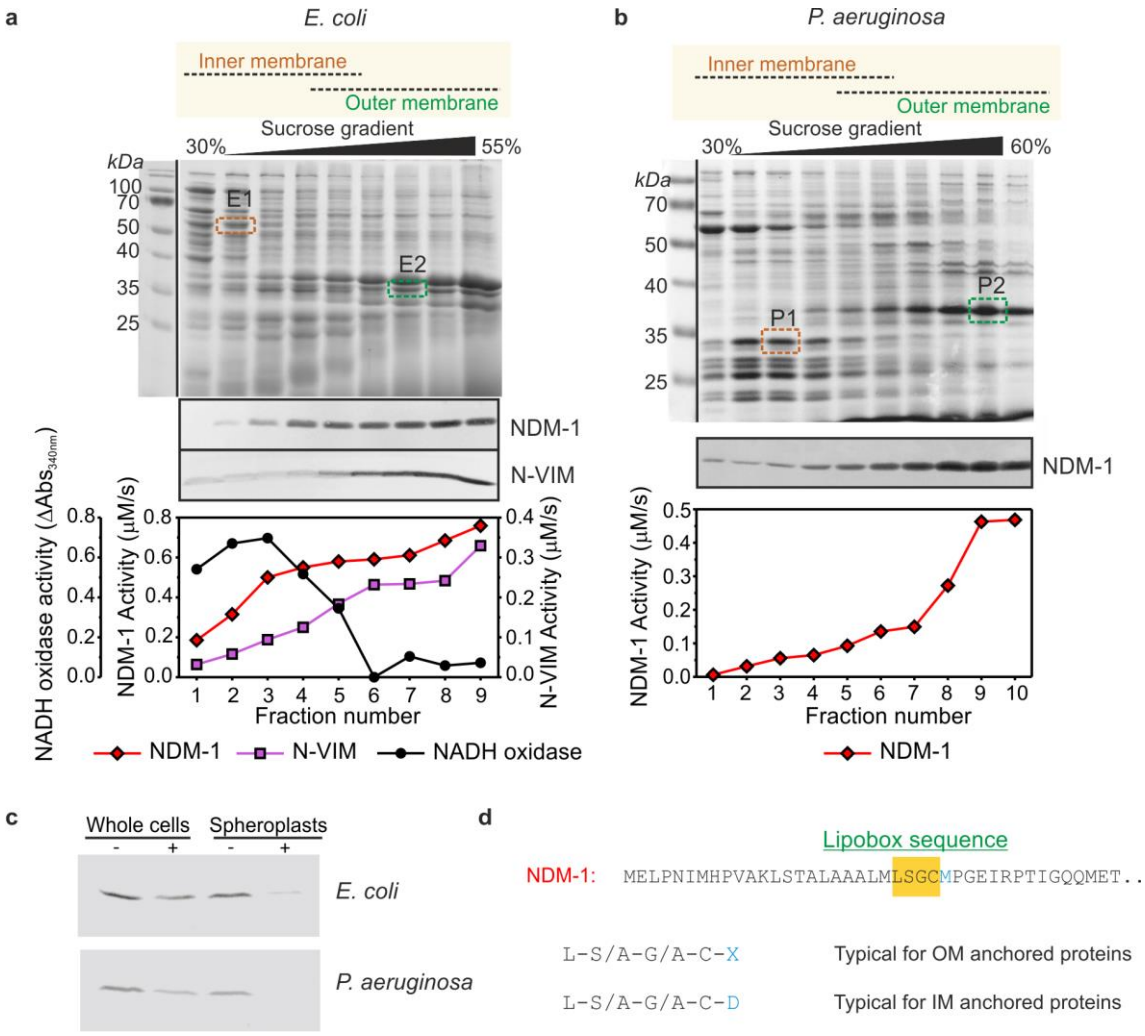
Supplementary Figure 1. Effect of Zn(II) availability on β-lactam resistance and MBL levels. (**a**) Relative MIC values of imipenem for *E. coli* cells expressing SPM-1, IMP-1, NDM-1 or VIM-2 in growth medium supplemented with different concentrations of the metal chelator DPA. MIC ratio values were calculated as described in the Methods section. Data correspond to three independent determinations, with standard errors ≤16% of each data point. (**b**) MBL levels in different cellular fractions as a function of time after addition of 1000 μM DPA. Equal amounts of total proteins were loaded on gels for each sample. Full Western Blots are displayed in Supplementary Fig. 12.



Supplementary Figure 2. Effect of Zn(II) availability on β-lactam MICs of *E. coli* strains expressing different MBLs and variants. (**a**) Schematic representation of the cellular localization of NDM-1, NDM-1 C26A, V-NDM, VIM-2 and N-VIM variants (see Supplementary Figure S4 for further details). (**b**) Relative MIC values of imipenem for *E. coli* cells expressing NDM-1, VIM-2, NDM-1 C26A, V-NDM and N-VIM variants in growth medium supplemented with different concentrations of DPA. (**c**) Relative MICs values of cefotaxime for *E. coli* cells expressing wild-type VIM-2 or N-VIM in growth medium supplemented with different concentrations of DPA. MIC ratio values were calculated as described in the Methods section. Data correspond to three independent determinations, with standard errors ≤16% of each data point.

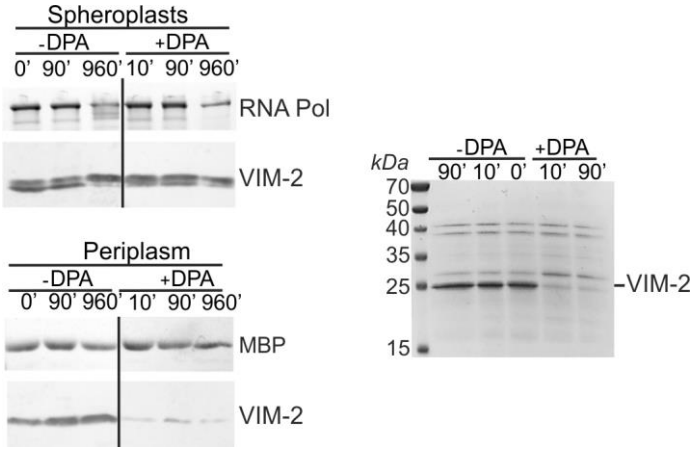


Supplementary Figure 3. NDM-1 is bound to the bacterial membranes of *E. coli* and *P. aeruginosa* through hydrophobic interactions. (**a**) Western blot detection of NDM-1 or VIM-2 in different cell fractions of *E. coli* or *P. aeruginosa*. After growth, cells were disrupted by sonication. The resulting homogenate (H) was then centrifuged 10 min at 12,800x*g* at 4ºC to remove cell debris, and a supernatant (SN) was separated. Soluble cells contents (S) and membrane vesicles (M) present in this fraction were separated through ultracentrifugation at 150,000x*g* 4ºC for 1 h. NDM-1 is a membrane-associated protein in both hosts, whereas VIM-2 is a soluble periplasmic MBL. (**b**) Membrane preparations of *E. coli* and *P. aeruginosa* expressing NDM-1. Membranes were sequentially treated with different reagents to determine the type of interaction that binds NDM-1 to membranes: (A) KCl 1M, (B) Na_2CO_3 0.1 M, (C) Triton X-100 0.0016% w/v, (D) Triton X-100 0.23% w/v and (E) remaining membranes after final treatment. Solubilization of NDM-1 after each step was analyzed by Western blot and imipenem hydrolysis. The results show that NDM-1 is extracted only in the presence of Triton X-100, indicating that this MBL is bound to bacterial membranes through hydrophobic interactions. Full Western Blots are displayed in Supplementary Fig. 12.

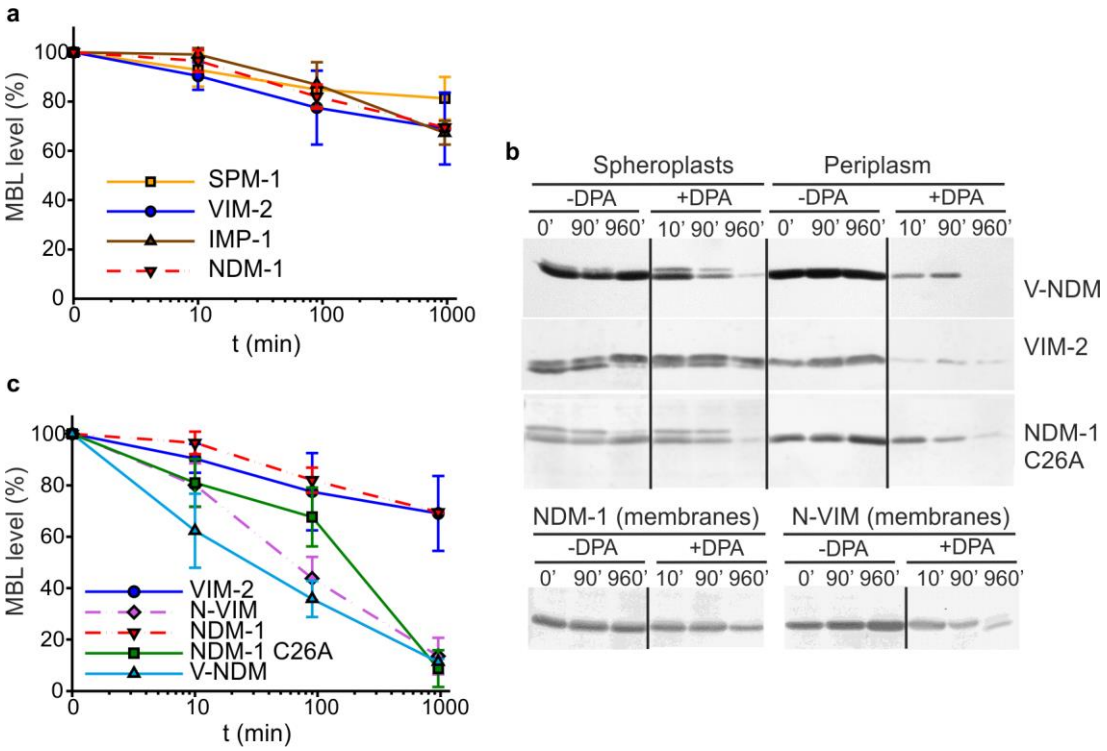


Supplementary Figure 4. Localization and orientation of NDM-1 and N-VIM within the bacterial outer membrane. (**a**) Membranes from *E. coli* expressing NDM-1 or N-VIM were subjected to ultracentrifugation in a 30 to 55% w/v sucrose gradient for separation of outer (OM) and inner (IM) membranes. Fractions along the gradient were analyzed by SDS-PAGE and Western blot detection of NDM-1 and N-VIM (anti StrepTag antibodies), imipenemase and NADH oxidase activity. In addition, protein bands highlighted with rectangles were excised from the gels and analyzed by mass spectrometry to confirm the OM (green) or IM (orange) character of the fractions (E1, protein-export membrane protein SecD; E2, OmpA). NDM-1 is concentrated in the OM fractions from both bacterial hosts, indicating OM localization. (**b**) Separation of OM and IM fractions from *P. aeruginosa* expressing NDM-1 along a 30 to 60% w/v sucrose gradient. OM or IM character was determined by mass spectrometry of excised bands

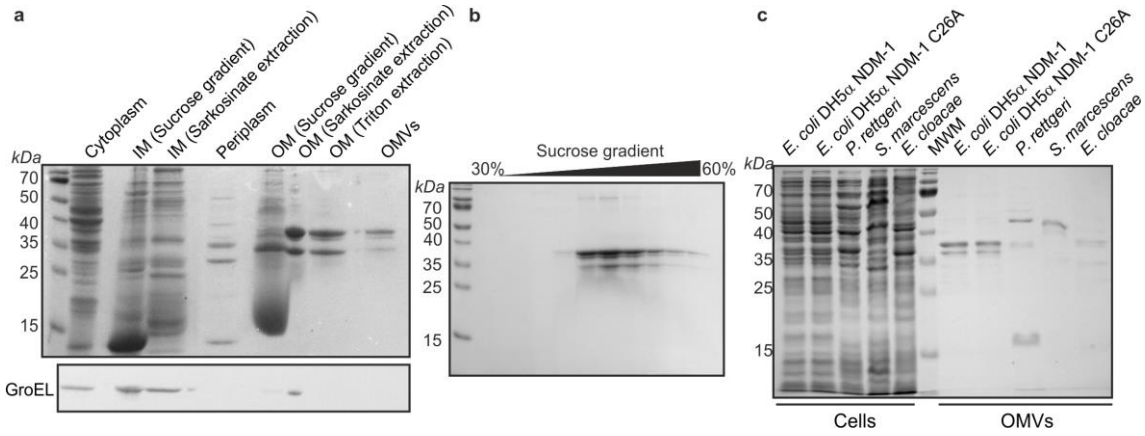
(P1, ATP synthase subunit b and Signal Peptidase I; P2, OmpF), and the presence of NDM-1 was detected as in (**a**). (**c**) Whole cells (W) and permeable spheroplasts (S) of *E. coli* and *P. aeruginosa* expressing NDM-1 were subjected to limited proteolysis with proteinase K. Comparison of NDM-1 levels detected by Western blot in protease treated samples (+) versus untreated controls (–) shows that NDM-1 is resistant to proteolysis in whole cells, while being degraded in spheroplasts under identical conditions. These results demonstrate that NDM-1 is inaccessible to external proteases, and thus located in the inner leaflet of the outer membrane. (**d**) The N-terminus of NDM-1 contains a canonical lipobox sequence (highlighted in orange), which includes a cysteine residue that undergoes lipidation followed by peptide leader processing in the bacterial IM[1]. The identity of the residue immediately posterior to the C terminal end of posterior the lipobox determines whether the lipoprotein will be localized to IM or OM, with an aspartic acid being required for retention in IM. The presence of a methionine residue at this position in NDM-1 is suggestive of targeting to the OM. Full Western Blots are displayed in Supplementary Fig. 12.



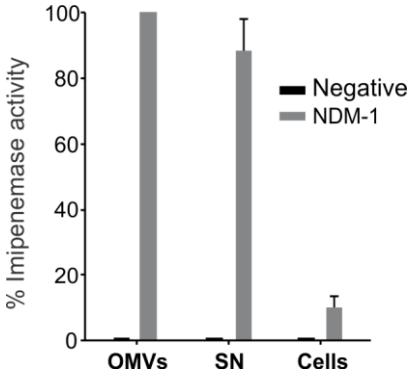
Supplementary Figure 5. Zn(II) deprivation causes selective degradation of MBLs in the periplasm. Left, immunodetection of VIM-2 in spheroplasts and periplasmic extracts of *E. coli* cells exposed to 1000 μM DPA (or no DPA) at 20ºC for different time-periods. Western blots of periplasmic maltose binding protein (MBP) and cytoplasmic RNA polymerase (RNA pol) were performed as loading controls for periplasmic extracts and spheroplasts, respectively. Right, Coomassie-stained SDS-PAGE of periplasmic extracts. There is a clear and selective reduction in the intensity of the band corresponding to VIM-2. The rest of the protein pattern remains unchanged. Full Western Blots are displayed in Supplementary Figs. 12 and 13.



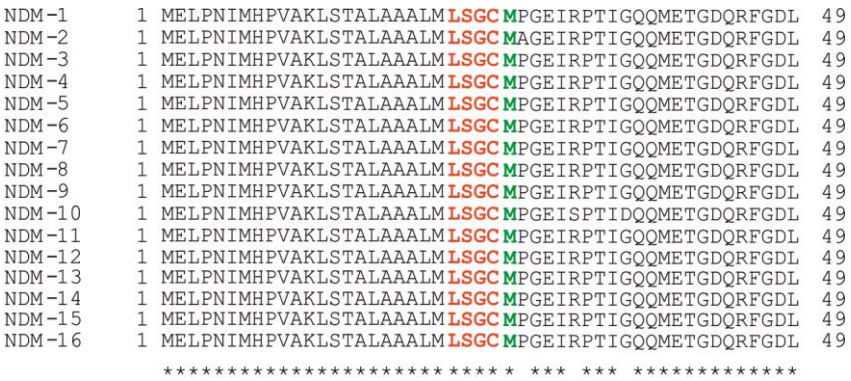
Supplementary Figure 6. Protein levels of MBLs in different cellular fractions under conditions of Zn(II) deprivation. (**a**) Spheroplast MBL levels (full-length and processed forms) determined by Western blot from *E. coli* expressing SPM-1, VIM-2, IMP-1 or NDM-1 at different times after addition of 1000 μM DPA .(**b**) MBL levels of NDM-1, VIM-2 and related mutants in periplasm and spheroplasts as a function time after addition of 1000 μM DPA. Full Western Blots are displayed in Supplementary Figs. 12 and 13. (**c**) Spheroplast MBL levels determined by Western blot from *E. coli* cultures VIM-2, N-VIM, NDM-1, NDM-1 C26A or V-NDM at different times after addition of 1000 μM DPA. Data correspond to three independent experiments and are shown as mean ± s.e.m.



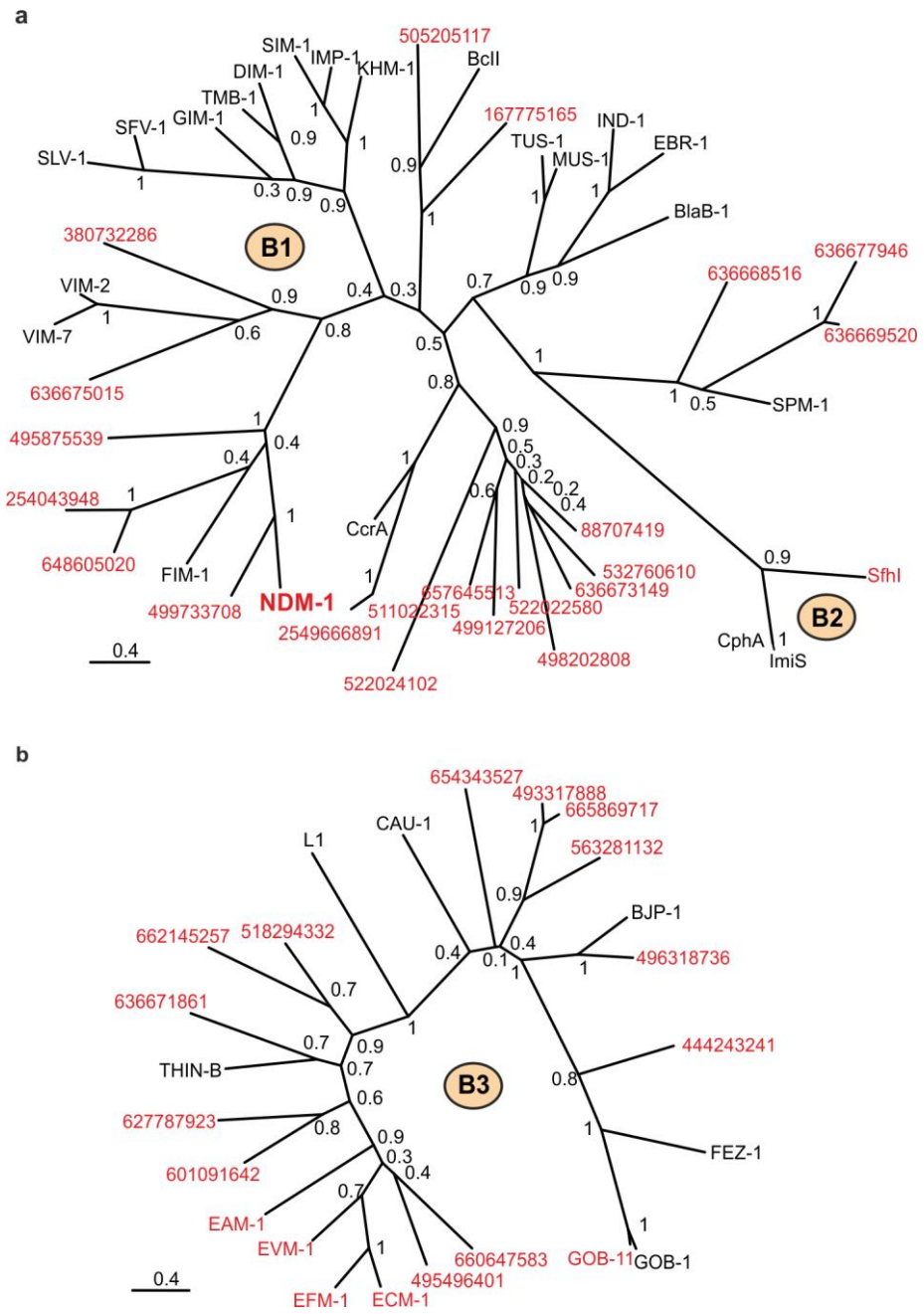
Supplementary Figure 7. Purification and quality control of OMVs. (**a**) Subcellular fractions from *E. coli* cells expressing NDM-1 were analyzed by SDS-PAGE and Western blot with anti-GroEL antibodies. Full Western Blot is displayed in Supplementary Fig. 13. (**b**) OMVs purified from *E. coli* expressing NDM-1 were subjected to ultracentrifugation in a 30 to 60% w/v sucrose gradient, and fractions along the gradient were analyzed by SDS-PAGE. (**c**) Whole cell extracts and OMVs purified from *E. coli* expressing NDM-1 or NDM-1 C26A, and three clinical isolates expressing NDM-1, were analyzed by SDS-PAGE.



Supplementary Figure 8. NDM-1 OMVs endow cells with β-lactamase activity. Imipenemase activity of β-lactam sensitive *E. coli* cells after incubation with 8 µg of NDM-1-containing (NDM-1) or non-MBL-producing OMVs (Negative), compared to OMVs and supernatant (SN) obtained after pelleting cells. Imipenem hydrolysis rates of SN and Cells were measured and normalized to OMVs for comparison. Data correspond to three independent experiments and are shown as mean ± s.e.m.



Supplementary Figure 9. All NDM variants have a conserved lipobox. Sequence alignment of N-terminus of NDM-1 to NDM-16. Conserved LSGC lipobox sequences and residues at +2 position are shown in red and green, respectively. Alignment was performed with the T-Coffee tool, available at www.tcoffee.org.



Supplementary Figure 10. Lipoproteins are present in all MBL subclasses. Phylogenetic trees of B1+B2 (**a**) and B3 (**b**) characterized MBLs, together with homologues harbouring a lipidation site in their N-termini (gi accession numbers). Lipoproteins are shown in red and characterized soluble MBLs in black. Trees were calculated from structure-assisted multiple sequence alignments with LG substitution model and 100 bootstraps, using the maximum likelihood algorithm PhyML (see Methods section for details).

Figure 1b

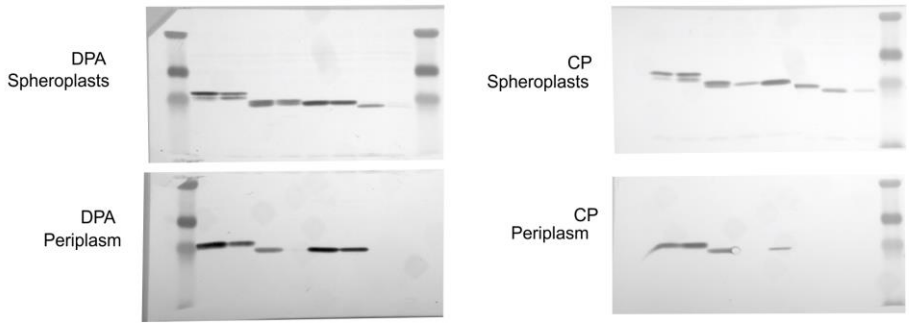


Figure 2a

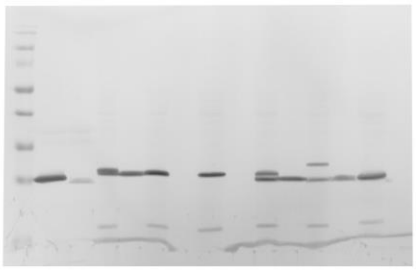


Figure 2c

Figure 3d

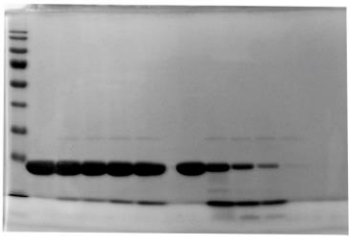


Figure 4b

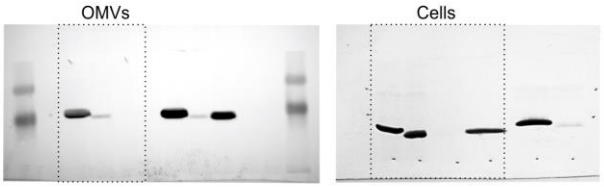


Figure 4c

Figure 4f

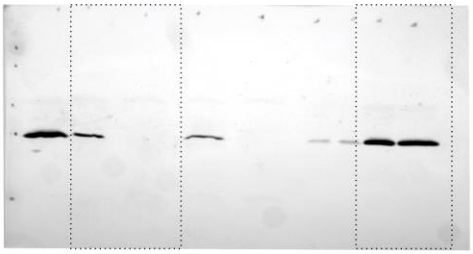


Figure 5c

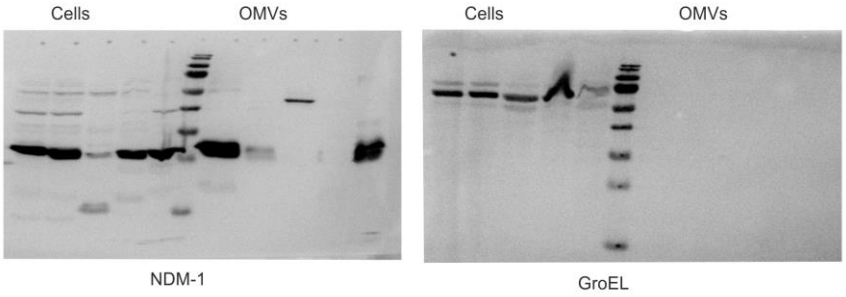
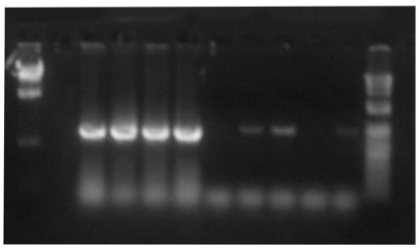
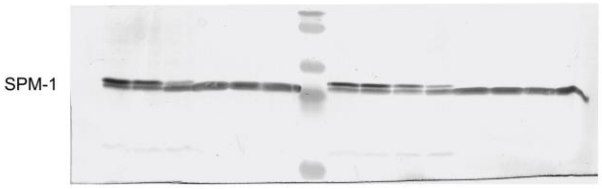


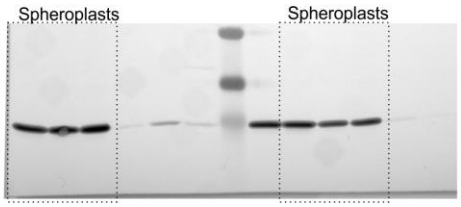
Figure 5d

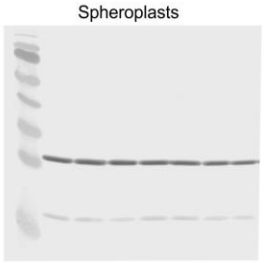


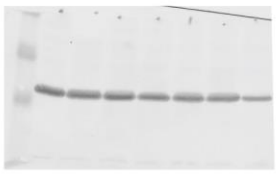
Supplementary Figure 11. Full, uncut gel images for Figures 1, 2, 3, 4 and 5.

Supplementary Figure 1b

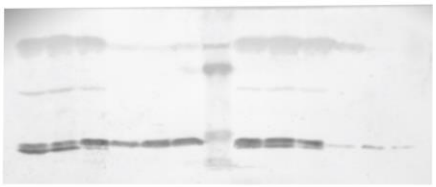


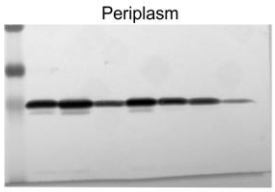






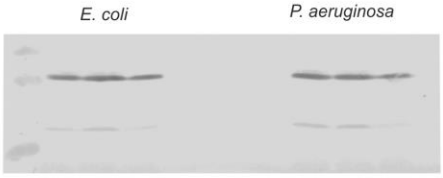
Supplementary Figures 1b, 5 and 6b

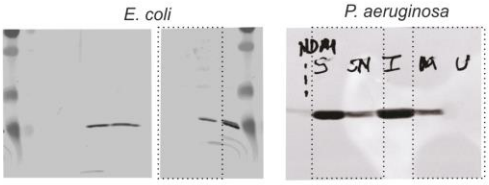




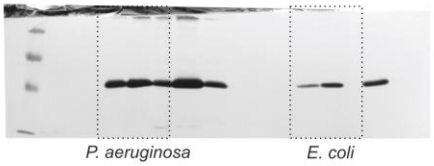
Supplementary Figures 1b and 6b

Supplementary Figure 3a

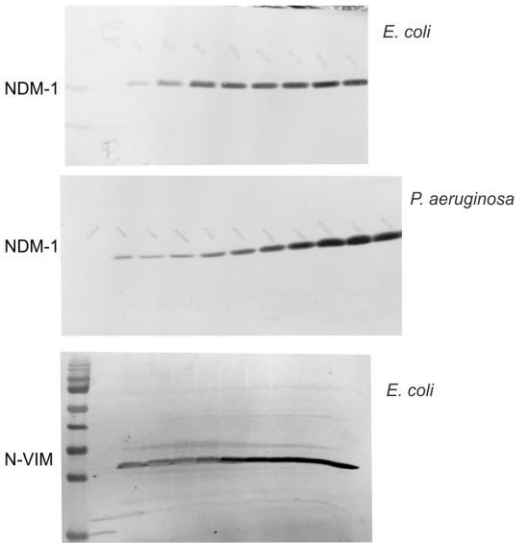




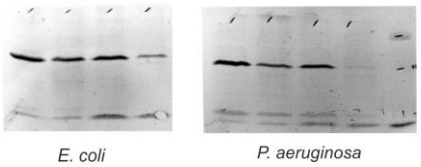
Supplementary Figure 3b



Supplementary Figure 4a-b

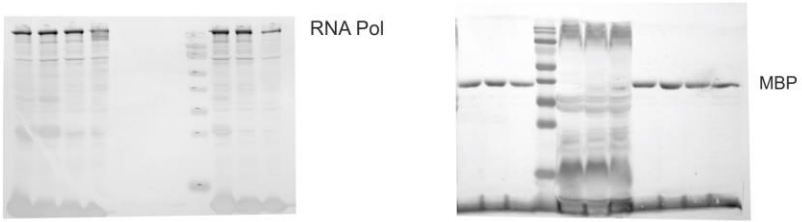


Supplementary Figure 4c

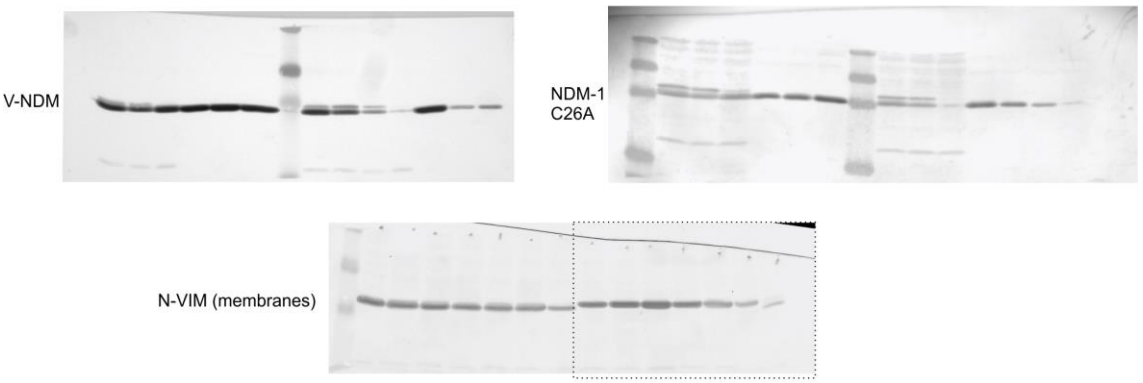


Supplementary Figure 12. Full, uncut gel images for Supplementary Figures 1, 3, 4, 5 and 6.

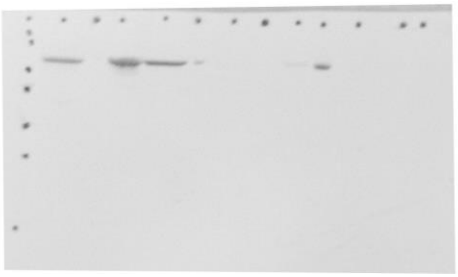
Supplementary Figure 5



Supplementary Figure 6b



Supplementary Figure 7



Supplementary Figure 13. Full, uncut gel images for Supplementary Figures 5, 6 and 7.

Imipenem MIC (μg/mL)												
	0 μM DPA		200 μM DPA		350 μM DPA		500 μM DPA		750 μM DPA		1000 μM DPA	
	WT	+ST	WT	+ST	WT	+ST	WT	+ST	WT	+ST	WT	+ST
pMBLe (control)	0.25		0.25		0.25		0.25		0.25		0.125	
NDM-1	2	2	1-2	1-2	0.5-1	0.5-1	0.5	0.5	0.25	0.25	0.125	0.125
VIM-2	2	2	1	1	0.25-0.5	0.25-0.5	0.25	0.25	0.25	0.25	0.125	0.125
IMP-1	1	1	1	1	0.5	0.5	0.5	0.5	0.25	0.25	0.125	0.125
SPM-1	2	2	2	2	1	1	1	1	0.5	0.5	0.125	0.125

Supplementary Table 1. Imipenem MICs of *E. coli* cells expressing wild type (WT) and C-terminal strep-tagged (+ST) variants of MBLs, as a function of extracellular Zn(II) availability. Values were determined by triplicate.

Imipenem MIC (μg/mL)						
	0 μM DPA	200 μM DPA	350 μM DPA	500 μM DPA	750 μM DPA	1000 μM DPA
pMBLe (control)	0.25	0.25	0.25	0.25	0.25	0.125
NDM-1	2	1-2	0.5-1	0.5	0.25	0.125
NDM-1 C26A	2	1	0.5	0.25	0.25	0.125
V-NDM	4-8	1	0.5	0.25	0.25	0.125
VIM-2	2	1	0.25-0.5	0.25	0.25	0.125
N-VIM	0.5	0.25	0.25	0.25	0.25	0.125
IMP-1	1	1	0.5	0.5	0.25	0.125
SPM-1	2	2	1	1	0.5	0.125

Cefotaxime MIC (μg/mL)						
	0 μM DPA	200 μM DPA	350 μM DPA	500 μM DPA	750 μM DPA	1000 μM DPA
pMBLe (control)	0.06	0.06	0.06	0.06	0.06	0.06
NDM-1	8	8	2	2	0.25	0.06
NDM-1 C26A	8-16	8	2	1	0.125	0.06
V-NDM	64	8	2	0.5	0.06-0.125	0.06
VIM-2	4-8	4-8	2	0.5	0.06-0.125	0.06
N-VIM	0.5	0.125	0.06	0.06	0.06	0.06
IMP-1	2-4	2-4	2	1-2	0.5	0.125
SPM-1	128	128	128	64-128	32	4

Supplementary Table 2. Imipenem and cefotaxime MICs of *E. coli* cells expressing different MBLs as a function of extracellular Zn(II) availability. Values were determined by triplicate.

	MIC (µg/mL)				
	Imipenem	Cefotaxime	Piperacillin	Cefepime	Ceftazidime
pMBLe (control)	0.25	0.06	1	0.03	0.125-0.25
VIM-2	2	4-8	32- 64	0.25 -0.5	8
N-VIM	0.5	0.5	8	0.03 - 0.06	0.5
NDM-1	2	8	32 - 64	4 - 8	256
V-NDM	4 - 8	64	256	16 - 32	1024-2048
NDM-1 C26A	2	16	64	8 - 16	512

Supplementary Table 3. β-lactam antibiotics MIC values for *E. coli* expressing VIM-2, NDM-1 and mutant variants. Values were determined by triplicate.

	Imipenem MIC (µg/mL)		
	0 µg/mL CP	100 µg/mL CP	300 µg/mL CP
pMBLe (control)	0.125	0.125	0.125
NDM-1	1	1	0.125
NDM-1 C26A	2	1	0.125
V-NDM	4	2	0.125
N-VIM	0.125-0.25	0.125	0.125
VIM-2	2	2	0.125

Supplementary Table 4. Imipenem MICs for *E. coli* expressing VIM-2, NDM-1 and mutant variants, in presence of calprotectin. Values correspond to three independent replicates.

MBL subclass	Name	Genbank GI number	Confirmed lactamase activity?	Lipobox sequence (including +2)	Organism
B1	NDM-1	GI:255031063	yes	LSGCM	Klebsiella pneumoniae
	380732286	GI:380732286	no	LTACA	Corallococcus coralloides
	636675015	GI:636675015	yes[2]	IVACA	Uncultured organism
	648605020	GI:648605020	no	LSGCA	Hirschia marítima
	254043948	GI:254043948	no	LSGCM	Hirschia báltica
	499733708	GI:499733708	yes[3]	LPACV	Erithrobacter litoralis
	495875539	GI:495875539	no	VAGCT	Alpha proteobacterium JLT2015
	505205117	GI:505205117	no	LIACS	Clostridium saccharoperbutylacetonicum
	167775165	GI:167775165	no	VSGCQ	Leptospira biflexa
	511022315	GI:511022315	no	LTGCS	Bacteroides massiliensis
	254966891	GI:254966891	yes[4]	LTGCT	Uncultured organism
	522024102	GI:522024102	no	LVGCS	Lewinella cohaerens
	88707419	GI:88707419	no	LIIGC	Maribacter sp.
	498202808	GI:498202808	no	LTSCK	Mesoflavibacter zeaxanthinifaciens
	657645513	GI:657645513	no	ITGCK	Cellulophaga baltica
	657642553	GI:657642553	no	ITGCK	Cellulophaga baltica
	499127206	GI:499127206	no	LAGCS	Cyclobacteriaceae bacterium AK24
	522022580	GI:522022580	no	IIGCT	Flexithrix dorotheae
	532760610	GI:532760610	no	LSSCI	Uncultured organism
	636673149	GI:636673149	yes[2]	IVACC	Uncultured organism
	636669520	GI:636669520	yes[2]	LASCA	Uncultured organism
	636669920	GI:636669920	yes[2]	LASCA	Uncultured organism
	636669520	GI:636669520	yes[2]	LASCA	Uncultured organism
	636677946	GI:636677946	yes[2]	LAGCT	Uncultured organism
	636668516	GI:636668516	yes[2]	LSSCS	Uncultured organism
B2	SfhI	GI:639192216	yes[5]	LIACE	Serratia fonticola
B3	EAM-1	GI:396086049	yes[6]	IAACA	Erythrobacter aquimaris
	EVM-1	GI:396086043	yes[6]	LSGCA	Erythrobacter vulgaris
	ECM-1	GI:396086047	yes[6]	LAGCA	Erythrobacter citreus
	EFM-1	GI:396086041	yes[6]	LAACA	Erythrobacter flavus
	627787923	GI:627787923	no	LAACR	Stenotrophomonas rhizophila
	601091642	GI:601091642	no	LAACA	Lysobacter capsici AZ78
	518294332	GI:518294332	no	LSACL	Dyella japonica
	662145257	GI:662145257	no	VAGCT	Mycobacterium abscessus
	GOB-11	GI:49798147	yes[7]	LSACL	Elizabethkingia meningoseptica
	444243241	GI:444243241	yes[8]	VIGCA	Uncultured organism
	496318736	GI:496318736	no	LAACP	Bradyrhizobium sp. ORS 375
	654343527	GI:654343527	no	LAACS	Mastigocoleus testarum
	563281132	GI:563281132	no	VSSCA	Blastomonas sp. CACIA14H2
	665869717	GI:665869717	no	LVGCA	Asticcacaulis sp. AC460
	493317888	GI:493317888	no	LAGCT	Asticcacaulis biprosthecium
	636671861	GI:636671861	yes[2]	VSSCA	Uncultured organism
	495496401	GI:495496401	no	LAGCS	Rheinheimera nanhaiensis
	660647583	GI:660647583	no	LAGCS	Erythrobacter sp. JL475

Supplementary Table 5. MBL lipoproteins from B1, B2 and B3 subclasses. Predicted MBL lipoproteins harbouring a consensus lipobox sequence [LVI][ASTVI][GAS][C] in the N-terminus, obtained as described in Methods section.

Supplementary Information References

1 Kovacs-Simon, A., Titball, R. W. & Michell, S. L. Lipoproteins of bacterial pathogens. *Infect. Immun.* **79**, 548-561 (2011).

2 Forsberg, K. J. *et al*. Bacterial phylogeny structures soil resistomes across habitats. *Nature* **509**, 612–616 (2014).

3 Zheng, B. *et al*., An unexpected similarity between antibiotic-resistant NDM-1 and β-lactamase II from *Erythrobacter litoralis*. *Protein Cell* **2**, 250-258 (2011).

4 Sommer, M. O. A., Dantas, G. & Church, G. M. Functional Characterization of the Antibiotic Resistance Reservoir in the Human Microflora. *Science* **325**, 1128-1131 (2009).

5 Saavedra, M. J. *et al*. Sfh-I, a subclass B2 metallo-β-lactamase from a *Serratia fonticola* environmental isolate. *Antimicrob. Agents Chemother.* **47**, 2330-2333 (2003).

6 Girlich, D., Poirel, L. & Nordmann, P. Diversity of naturally occurring Ambler class B metallo-β-lactamases in *Erythrobacter spp*. *J. Antimicrob. Agents Chemother.* **67**, 2661-2664 (2012).

7 Yum, J. H. *et al.* Genetic diversity of chromosomal metallo-β-lactamase genes in clinical isolates of *Elizabethkingia meningoseptica* from Korea*. J. Microbiol.* **48**, 358-64 (2010).

8 Terrón-González, L., Medina, C., Limón-Mortés, M. C. & Santero, E. Heterologous viral expression systems in fosmid vectors increase the functional analysis potential of metagenomic libraries*. Sci. Rep.* **3**, 1107 (2013).