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RESEARCH ARTICLE

An unexpected similarity between antibiotic-resistant NDM-1 and beta-lactamase II from *Erythrobacter litoralis*

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ABSTRACT

NDM-1 (New Delhi metallo-beta-lactamase) gene encodes a metallo-beta-lactamase (MBL) with high carbapenemase activity, which makes the host bacterial strain easily dispatch the last-resort antibiotics known as carbapenems and cause global concern. Here we present the bioinformatics data showing an unexpected similarity between NDM-1 and beta-lactamase II from *Erythrobacter litoralis*, a marine microbial isolate. We have further expressed these two mature proteins in *E. coli* cells, both of which present as a monomer with a molecular mass of 25 kDa. Antimicrobial susceptibility assay reveals that they share similar substrate specificities and are sensitive to aztreonam and tigecycline. The conformational change accompanied with the zinc binding visualized by nuclear magnetic resonance, Zn^{2+}-bound NDM-1, adopts at least some stable tertiary structure in contrast to the metal-free protein. Our work implies a close evolutionary relationship between antibiotic resistance genes in environmental reservoir and in the clinic, challenging the antimicrobial resistance monitoring.

KEYWORDS NDM-1, metallo-β-lactamase, *Erythrobacter litoralis*, similarity, antibiotics resistance

INTRODUCTION

Growing threat of antibiotic resistance has been recognized as one of the greatest challenges to treatment for clinical infections and human health (Levy and Marshall, 2004). The emergence of New Delhi metallo-β-lactamase (NDM-1) is just the latest twist in a global health nightmare, which has made the headlines recently for its ability of rapid dissemination worldwide and destroying the last-resort antibiotics known as carbapenems (Yong et al., 2009; Kumarasamy et al., 2010). The first NDM-1 infection was reported in a Swedish patient of Indian origin in 2009 (Yong et al., 2009). Subsequently, a sudden increase in the number of NDM-1 carrying bacteria all over the world set alarm bells ringing (Bush, 2010; Huo, 2010; Moellering, 2010; Poirel et al., 2010, 2011a, 2011b, 2011c; Rolain et al., 2010; Samuelsen et al., 2011; Walsh, 2010; Zhang, 2010). A Belgian patient became the first known fatality from NDM-1 who was infected in Pakistan following a car accident (Rolain et al., 2010). It is particularly disturbing that the gene for NDM-1 can jump between different bacterial species by horizontal gene transfer (HGT), and NDM-1 rampancy worldwide is considered a potential nightmare scenario, for the investigator warns that hardly any antibiotic is effective against the "superbug." (Kumarasamy et al., 2010)

The most tantalizing question is, of course, where did

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NDM-1 come from? The origin of the antibiotic resistance genes in clinic has long been a mystery (D'Costa et al., 2006; Wright, 2010). The widespread of these genes is usually recognized by scientists and the public owing to the overuse of antibiotic drugs (Wright, 2007). However, there are some increasing evidences that antibiotic resistance genes in pathogens have an environmental origin (Marshall et al., 1997; Bonten et al., 2001; Poirel et al., 2002; D'Costa et al., 2006; Martínez, 2008). To answer the question of where NDM-1 gene arose, here, we use a battery of tests to try to address this issue, and this work unexpectedly steers us to identify the NDM-1 homologue in seemingly unrelated marine bacteria species.

RESULTS

Similarity between NDM-1 and ElBla2

Earlier phylogenetic analysis has shown that NDM-1 shares some identity with VIM (Verona integron-encoded metallo-lactamase) group of metallo-β-lactamases (MBLs) (Yong et al., 2009). Intriguingly, according to our BLAST results, we found that NDM-1 gene has closest orthologues in marine bacteria. NDM-1 shares high identity with the β-lactamase II from *Erythrobacter litoralis* (ElBla2), lactamase from *Haliangium ochraceum* (Holactamase) and lactamase from *Hirschia baltica* (Hb lactamase), with the identities of 55%, 49% and 37%, respectively. We then assessed the multiple amino acid sequence alignment of NDM-1 and homologues. Figure 1 reveals notable homology in core region between NDM-1 and ElBla2 (57% identity and 72% similarity). NDM-1 and marine origin lactamases possess a type II signal peptide for Sec protein-translocation pathway (Hutchings et al., 2009), with a conserved LXXC like motif (Fig. 1; labeled yellow). However, other lactamases contain a type I signal peptide manifested by AXA motif (Fig. 1; labeled tan). NDM-1 and marine lactamases also share HAHXD motif (Fig. 1; labeled green); by contrast, other MBLs contain an HFHXD or HSHXD motif. It is worth noting that both NDM-1 and ElBla2 have the unique loop at positions 168 to 172 (Fig. 1; labeled red), unique amino acids at positions 195, 229, 246 and 259 (Fig. 1; labeled cyan). These data indicate that NDM-1 has highly genetic relationship with ElBla2, and suggest that they possess a novel structure (Yong et al., 2009). Nucleotide sequences analysis of NDM-1 and ElBla2 demonstrates that the identity between these two genes is 59.4%. The nucleotide composition reveals that *ndm-1* gene has closest relative to the *Elbla2* gene among homologues (Table 1).

To investigate the evolution of NDM-1 in more detail, we have conducted phylogenetic analysis on MBLs (Fig. 2). In our analysis, NDM-1 shows the closest relationship with ElBla2; they are in the same clade and constitute a new subgroup among the B1-β-lactamase, consistent with previous finding (Yong et al., 2009).

Both M-NDM-1 and M-ElBla2 proteins behave as a monomer

To further dissect the NDM-1, we expressed the mature protein of M-NDM-1 and M-ElBla2 in *E. coli* cells. After 6 h of induction under the condition of 0.1 mmol/L IPTG, high-level expression of the recombinant proteins was observed. Subsequently, the protein was purified using Resource Q column. An analytical FPLC profile of Superdex 200 showed that they elute as a monomer (Fig. 3). The purified proteins were confirmed by SDS-PAGE (Fig. 3; inset). Dynamic light-scattering (DLS) experiments were performed as a complementary method to characterize M-NDM-1 and M-ElBla2. The result was analyzed to yield the size distribution of hydrodynamic radius (Fig. 3B and 3C). The DLS studies are consistent with the theoretical molecular mass of 25 kDa.

Only one single peak was observed from the profile, and the hydrodynamic radius of the scattering particles is 2.7 nm. Accordingly, the number average of the weight average molar mass measurements across the elution peak is 35 kDa corresponding to a 13.1% degree of polydispersity. According to the number average molar mass, this suggests that TroA exists as an interacting mixture of monomers and dimers (theoretical molecular weight of 32 kDa).

Sensitivity to aztreonam and tigecycline

The overexpression plasmids NDM-1 and ElBla2 were transformed into *E. coli* BL21 (DE3) cells, and antibiotic sensitivity assay was conducted (Fig. 4). Aztreonam, ceftazidime, tigecycline, imipenem and meropenem disks were evenly placed on the plates, and the dishes were incubated at 37°C for 16 h. The *E. coli* cells containing plasmid NDM-1 grew well in the presence of ceftazidime, imipenem and meropenem; however, they were inhibited in the area near the disks containing aztreonam and tigecycline (Fig. 4). Similarly, the *E. coli* cells containing plasmid ElBla2 also grew well in the presence of ceftazidime, imipenem and meropenem with slight inhibition. Aztreonam and tigecycline inhibited the activity of ElBla2 as they did to NDM-1 (Fig. 4). This result demonstrates not only the close similarity at the amino acid level between NDM-1 and ElBla2, but also the similarity they share in substrate specificities. Moreover, the inhibition assay indicates that enzyme activity of NDM-1 is inhibited by EDTA. In addition, our data are consistent with the previous work (Yong et al., 2009) that when NDM-1 gene was expressed in *E. coli*, the host cell was highly sensitive to aztreonam.

Zn^{2+} stabilization of the NDM-1 tertiary structure

Metallo-β-lactamases utilize a metal-dependent pathway to inactivate β-lactam containing antibiotics (Hu et al., 2008). This metal was proved to be zinc in recombinant NDM-1 protein by inductively coupled plasma mass spectrometry

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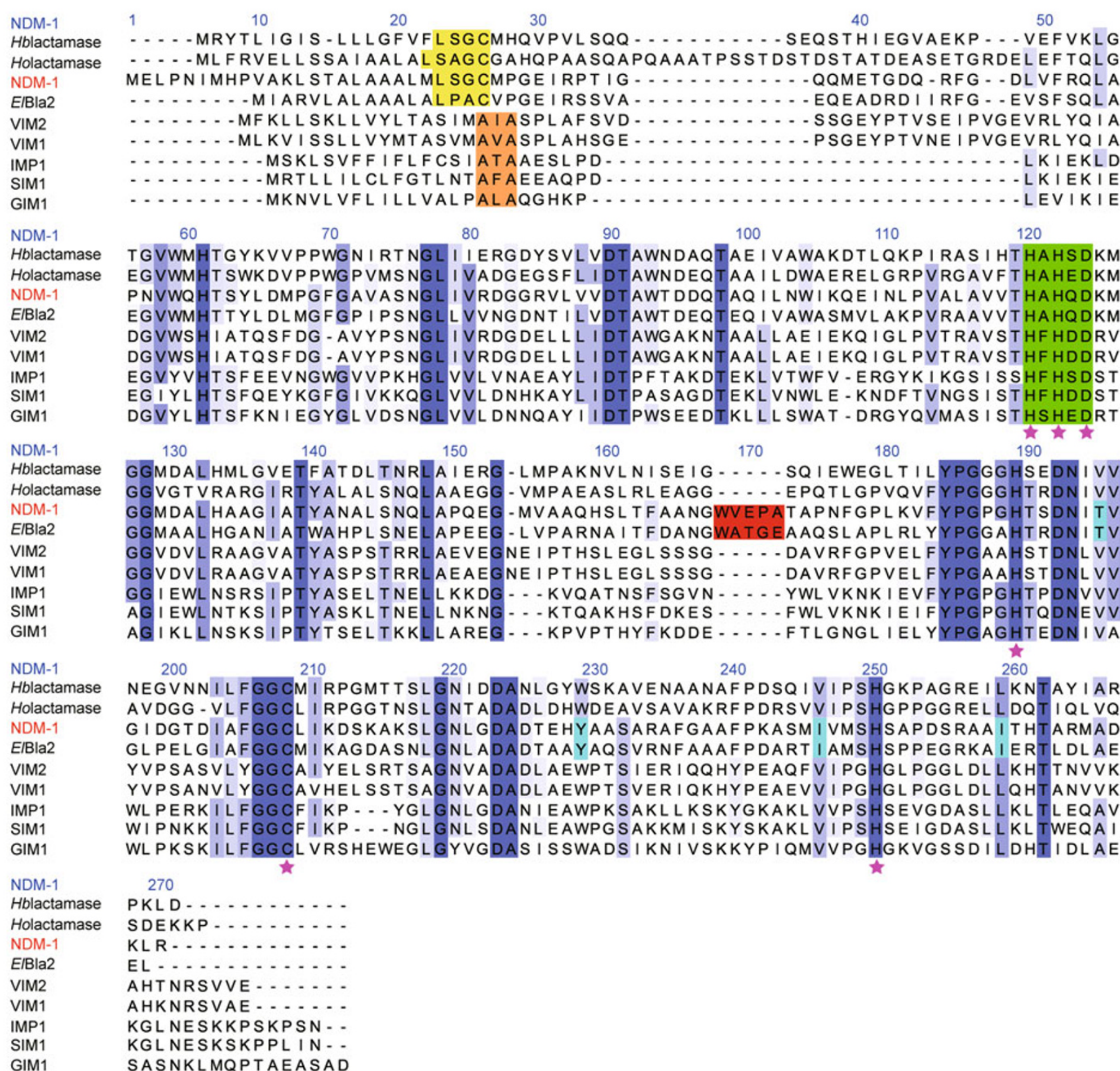

Figure 1. Multiple amino acid sequence alignment of NDM-1 homologues. Sequence overlaps in selected regions of MBLs. The most-conserved residues in all homologues are shaded in blue. The six metal-coordinating residues are highlighted in pink pentagram. The LXXC-like motif (type II signal peptides for Sec-dependent transport) (Hutchings et al., 2009) is shown in yellow, and the AXA motif (type I signal peptides for Sec-dependent transport) is highlighted in tan. Additional unique loops are colored in red. HXHXD motif is colored in green. NDM-1 and ElBla2 own a threonine at position 195, tyrosine at position 229, isoleucine at position 246 and isoleucine at position 257; in contrast, the other MBLs possess the conserved valine, tryptophan, valine and leucine in the respective positions. Unique amino acids are highlighted in cyan.

Table 1 Frequencies of nucleotides in NDM-1 homologues

Nucleotide	*ndm-1*	*Elbla2*	*Holactamase*	*Hblactamase*	*imp-1*	*imp-2*	*vim-2*
Adenine	0.198	0.178	0.148	0.264	0.314	0.310	0.201
Cytosine	0.315	0.337	0.344	0.265	0.170	0.166	0.257
Guanine	0.301	0.321	0.338	0.196	0.221	0.224	0.292
Thymine	0.186	0.164	0.170	0.275	0.294	0.300	0.250
C + G	0.616	0.658	0.682	0.461	0.391	0.390	0.549
A + T	0.384	0.342	0.318	0.539	0.609	0.610	0.451

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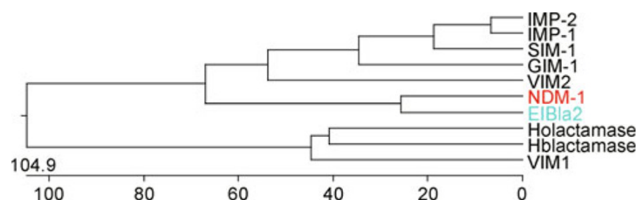

Figure 2. Phylogenetic tree of NDM-1 homologues. Sequences were aligned and a phylogenetic tree was constructed using the Clustal W method by DNAstar (Madison, WI). The GenBank accession numbers for the sequences are as follows: NDM-1, CAZ39946; ElBla2, YP_458405.1; Holactamse, NC_013440.1; Hblactamse, YP_003061440.1; IMP-1, S71932; IMP-2, AJ243491; VIM-1, Y18050; VIM-2, AF191564; SIM-1, AY887066; GIM-1, AJ620678.

(ICP-MS) experiments (data not shown). Inactivation assay showed that the activity of NDM-1 was significantly inhibited by EDTA (Fig. 4). Previous work indicated that a loop adjacent to the metal catalytic site exhibits significant structural variation upon the binding of metal ions in MBLs (Scrofani et al., 1999; Crawford et al., 2005; Periyannan et al., 2006). To probe the extent of protein conformational changes in apo-NDM-1 induced by Zn^{2+} binding, we recorded nuclear magnetic resonance (NMR) spectroscopy at 25°C. It is clear that the spectrum of apo-protein contains less amide cross-peaks in the HSQC spectrum than can be accounted for by the number of residues in the zinc-bound protein (Fig. 5). Zn^{2+} caused a lot of peaks to appear that were absent in apo-NDM-1 (highlighted in circles). The dramatic spectral changes indicated that the binding of Zn^{2+} induced conformational changes, demonstrating a high affinity of the apoprotein toward the zinc. Overlays with the spectrum of the apoenzyme and with the spectrum of the zinc-bound enzyme demonstrated that Zn^{2+}-bound NDM-1 adopts at least some stable tertiary structure in contrast to the metal-free protein. We attribute these behaviors to the binding of zinc ions that induce a local conformational change in the metal binding centers.

DISCUSSION

The NDM-1 encoding plasmids also harbor genes conferring resistance to almost all antibiotics (Yong et al., 2009); these antibiotic resistance genes are often too sophisticated to have arisen from the gradual evolution of precursors present within the genomes of pathogens in few decades (Wright, 2007). Therefore, it is more likely that NDM-1 appears via HGT from environmental reservoirs. Furthermore, *E. litoralis*, *H. ochraceum* and *H. baltica* are all isolated from the marine habitat (Schlesner et al., 1990; Fudou et al., 2002; Oh et al., 2009) and these β-lactamase genes are all chromosome-encoded. These data infer that marine originated β-lactamases may stringently be regulated as a protective mechanism against β-lactam molecule secreted by antibiotic producers that share the same ecological niche.

Two chromosome-encoded MBLs were isolated from a marine environment (Poirel et al., 2005). Vakulenko's group reported the existence of a β-lactamase from deep oceanic sources (Toth et al., 2010). These studies provide evidence that marine bacterial species may constitute a reservoir of β-lactam resistance genes, expanding our knowledge of the resistant gene source to the oceanic resistome. In the ocean, gene-transfer agents (GTAs) shuttle random fragments of the host chromosome from one species to another with unexpected ease and gene transfer frequency may occur 1900 to 459 million times more often than previously thought (McDaniel et al., 2010). Intriguingly, genes encoding GTAs are found in most marine bacteria including *E. litoralis* (Biers et al., 2008). Additionally, researchers found that carbohydrate active enzyme gene was transferred from marine bacteria to human gut microbiota by eating non-sterile seaweeds (Hehemann et al., 2010), providing the possibility for the incorporation of marine bacteria genes into human microbiota. These observations may help to explain how bacteria acquire resistance to antibiotics from marine environment.

Martínez (Martínez, 2008) laid out the scenario that most antibiotic resistance genes acquired through HGT, which have been originated in environmental microorganism, plasmid-encoded resistance determinants and human activities, are involved in the evolution and dissemination of resistance in pathogens. Thus, based on the close relationships between the NDM-1 and ElBla2 found in this study, we propose that antibiotic resistance genes may arise from marine resistome (Fig. 6). Physical and biological forces as described (Wright, 2007) mediate the dispersal of resistance genes from marine to terrestrial environment (Allen et al., 2010). After contacting with human populations, selective pressures would then lead to selection and dissemination in rampant bacteria via some bacterial intermediaries.

MATERIALS AND METHODS

Alignment, nucleotide composition analysis and phylogenetic analysis

To compare the relationships between the NDM-1 and other MBLs, representative MBLs amino acid sequences and DNA sequences were searched within NCBI database and downloaded from GenBank (Benson et al., 2010). They are NDM-1, ElBla2 (beta-lactamase II from *Erythrobacter litoralis* HTCC2594), Holactamase (beta-lactamase from *Haliangium ochraceum* DSM 14365), Hblactamase (beta-lactamase from *Hirschia baltica* ATCC 49814), IMP-1, IMP-2, VIM-1, VIM-2, GIM-1 and SIM-1. The amino acid sequences were aligned with the Clustal W program and subjected to a phylogenetic tree by DNAstar (Madison, WI). To further characterize NDM-1 and ElBla2, the nucleotide sequences of the two genes were exerted by CLC Main Workbench program (CLC.bio), and the different nucleotide compositions of NDM-1 homologues were determined (Table 1). The GenBank accession numbers for the

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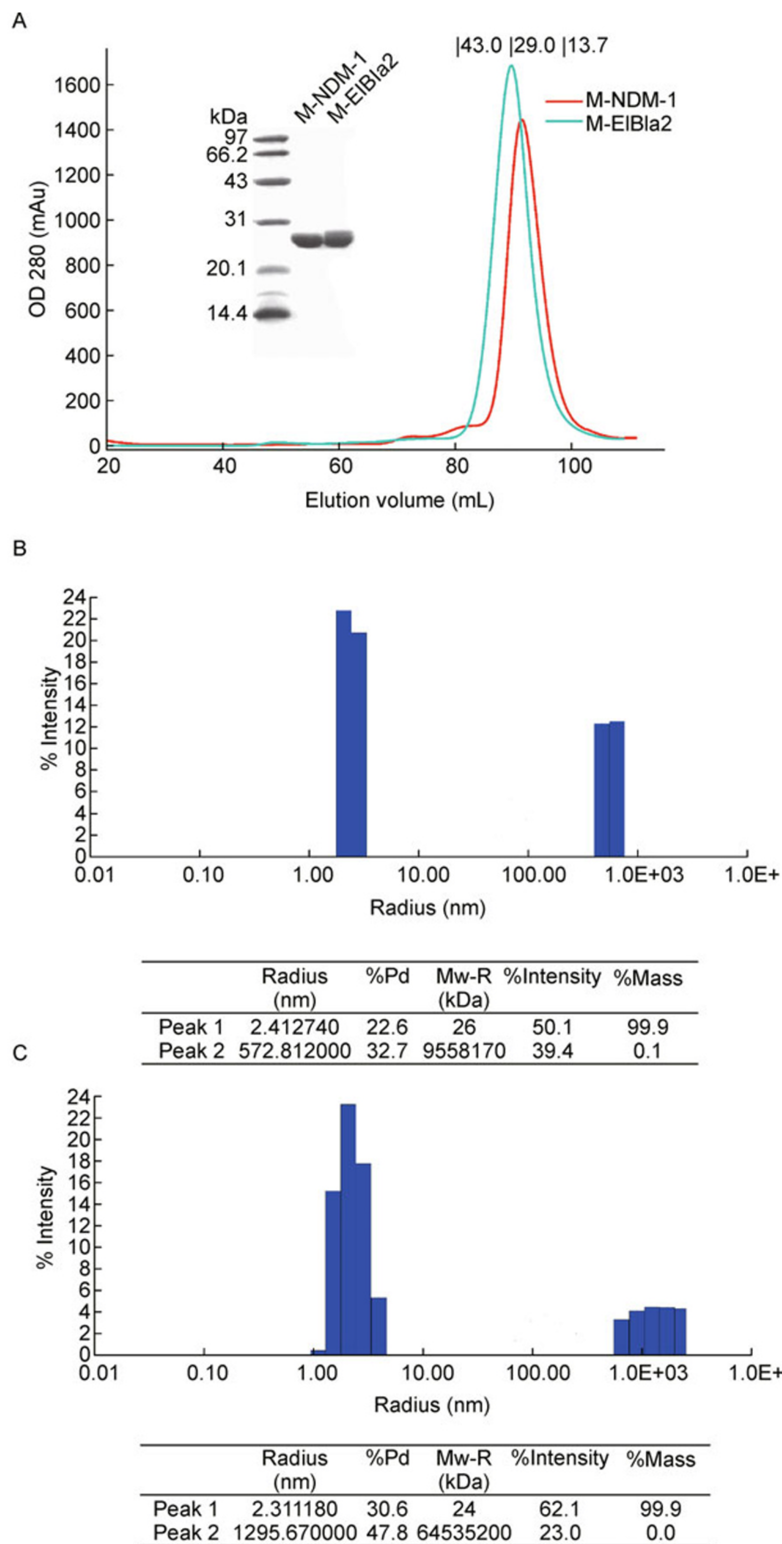

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	2.412740	22.6	26	50.1	99.9
Peak 2	572.812000	32.7	9558170	39.4	0.1

	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	2.311180	30.6	24	62.1	99.9
Peak 2	1295.670000	47.8	64535200	23.0	0.0

Figure 3. The oligomeric states of M-NDM-1 and M-ElBla2. (A) The size-exclusion chromatography profiles of M-NDM-1 and M-ElBla2, SDS-PAGE (left inset), and gel filtration chromatograms. The SDS-PAGE samples correspond to the peak in the elution profile. The profiles are marked, along with approximate positions of molecular mass standards of 43.0, 29.0 and 13.7 kDa. (B) Dynamic light scattering experiment of M-NDM-1. (C) Dynamic light scattering experiment of M-ElBla2. The results are presented as particle size distributions. The inset shows the results summary table.

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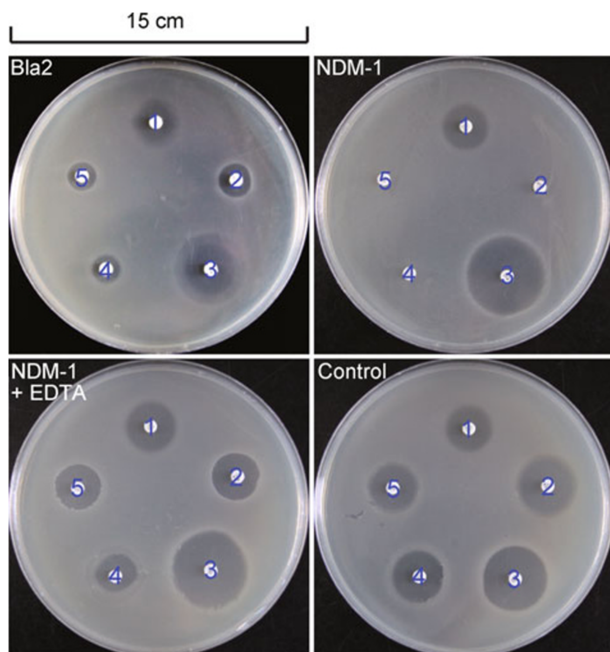

Figure 4. Antibiotic selection assay. (A) Plasmid ElBla2 in *E. coli* BL21; (B) Plasmid NDM-1 in *E. coli* BL21; (C) Plasmid NDM-1 in *E. coli* BL21 incubated with 2 mmol/L EDTA; (D) *E. coli* BL21 cells without any plasmids. Antibiotic disks used are as follow: 1, Tigercycline; 2, Meropenem; 3, Aztreonam; 4, Cetazidime; 5, Imipenem.

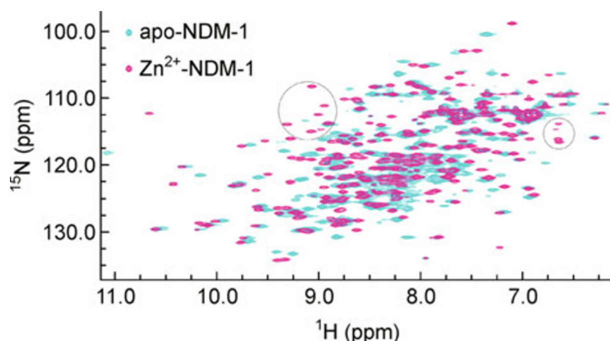

Figure 5. Superposition of two-dimensional $^1H^{15}N$-HSQC spectra comparing 0.5 mmol/L apo-M-NDM-1 (cyan) with 0.5 mmol/L Zn^{2+}-M-NDM-1 (pink). All spectra have been acquired at 25°C in 10 mmol/L sodium acetate, pH 6.5, and spectra were recorded at 600-MHz ^1H frequency. A number of the cross-peaks exhibiting significant changes are highlighted in circles.

sequences are as follows: NDM-1, CAZ39946; ElBla2, YP_458405.1; Holactamse, NC_013440.1; Hblactamse, YP_003061440.1; IMP-1, S71932; VIM-1, Y18050; VIM-2, AF191564; SIM-1, AY887066; GIM-1, AJ620678.

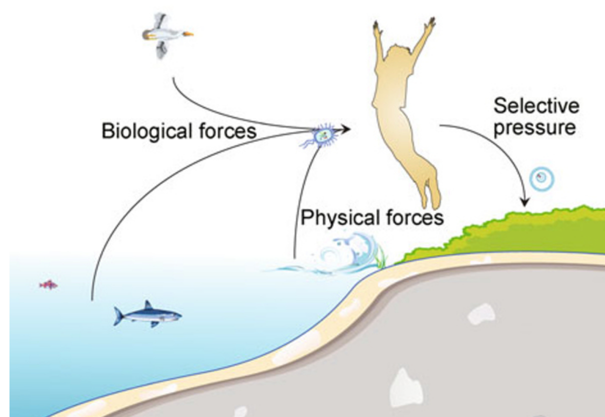

Figure 6. Proposed shift of the antibiotic resistance genes from marine to terrestrial environment. Many resistance genes acquired through horizontal gene transfer have been originated in natural environment. There are increasing evidences that marine bacterial species may constitute a reservoir of resistance genes (Poirel et al., 2005; Toth et al., 2010). The observation found that carbohydrate active enzyme gene was transferred from marine bacteria to human gut microbiota providing the possibility for the shift of resistance genes from marine to terrestrial environment (Hehemann et al., 2010). In their original host, these resistance genes are involved in metabolic networks. After their dissemination, their only role will be resistance. Physical and biological forces mediate the dispersal of resistance genes from marine to terrestrial environment (Martínez et al., 2007).

DNA cloning, protein expression, purification and analytical size-exclusion chromatography

DNA preparations and manipulations were performed according to the standard protocol. Plasmid NDM-1 (containing the entire *ndm-1* gene), truncated *ndm-1* gene (encoding amino acid residues 37–270), ElBla2 (containing the entire *bla2* gene) and truncated *bla2* gene (encoding amino acid residues 28–261) were generated by polymerase chain reaction (PCR) amplification from synthesized DNA. FL-NDM-1 (full-length NDM-1), M-NDM-1 (mature NDM-1, without his tag), FL-ElBla2 (full-length ElBla2) and M-ElBla2 (mature ElBla2) PCR products were amplified and cloned into the pET-21a vector (Novagen) to generate the recombinant plasmid (Table S1). The identity of the DNA constructs was verified by DNA sequencing. Full length gene plasmids were conducted to antimicrobial susceptibility assay and mature proteins were used in analytical size-exclusion chromatography.

E. coli BL21(DE3) cells transformed with the appropriate plasmid were grown in LB medium containing 100 μg/mL ampicillin at 37°C until an optical density at 600 nm of 0.4 to 0.6 was reached. Expression of protein was induced by the addition of 0.2 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture, and bacteria were grown at 16°C for 6 h. The induced cells were harvested by centrifugation and resuspended in a lysis buffer containing 20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl, 0.1%

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Triton X-100 and 1 mmol/L DTT. The cells were lysed by sonication (on ice), and the resulting supernatants were loaded onto a Resource-Q column (Amersham Biosciences) equilibrated with the same dialysis buffer, and then, the elution samples with target protein were collected and loaded onto a Superdex 200 fast protein liquid chromatography column (Amersham Biosciences). To produce uniformly ^{15}N-labeled M-NDM-1 sample for NMR studies, *E. coli* BL21 (DE3) were grown in M9 minimal medium containing 0.1% $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) as the sole source of nitrogen. The other steps of protein expression and purification were the same as described for unlabeled protein.

The oligomeric states of both purified M-NDM-1 and M-ElBla2 were determined by gel-filtration chromatography at 20°C in a HiLoad 16/60 Superdex 200 pg column with ÄKTA FPLC (GE Healthcare). The mobile phase was 20 mmol/L Tris-HCl, 50 mmol/L NaCl, pH 8.0, and the flow rate was 1.0 mL/min. The molecular mass standards were: ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; and ribonuclease-A, 13.7 kDa. The eluted proteins were detected by monitoring the absorbance at 280 nm.

Dynamic light scattering

Dynamic light scattering (DLS) studies were done using a DynaPro Titan (Wyatt Technology Inc.). The protein was dissolved at a concentration of 1 mg/mL in 20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl, and then, the protein was filtered through a 0.22 μm filter before detection. Temperature control was maintained at (25 ± 0.1)°C. At least ten scattering measurements were collected such that the root mean square deviation (RMSD) was less than 0.1%. The average molecular masses were calculated based on the graphical size analysis software, DYNAMICS software version 6, provided with the instrument.

Antimicrobial susceptibility assay

The *in vitro* activity of antibiotics against FL-NDM-1 and FL-ElBla2 that were overexpressed in *E. coli* BL21 (DE3) cells were determined by the Mueller-Hinton agar (OXDIO) plate dilution method as described previously with modification (Hu et al., 2008). Bacterial cultures were grown to midlog-phase, and protein production was induced by making the cultures containing 0.2 mmol/L IPTG. Cultures were then grown approximately 4 h at 16°C, and disk diffusion testing was performed by the NCCLS (National Committee for Clinical Laboratory Standards) methodology on agar plates. The antimicrobials evaluated and used in the agar plate dilution method are as follows: aztreonam, ceftazidime, tigecycline, imipenem and meropenem (OXDIO). For inhibition assay, bacterial cultures were incubated with 5 mmol/L EDTA.

Metal content assays

The metal content of recombinant NDM-1 samples exchanged into metal-free 20 mmol/L Tris-HCl (pH 8.0) and 50 mmol/L NaCl and buffer controls were determined. Metal ion analysis was determined by ICP-MS, at Analysis Center of Tsinghua University (Beijing, China). Metal contents were analyzed on an XSeries II (Thermo-Fisher) apparatus. Each sample was quantified three times and averaged.

Preparation of apo-NDM-1

Exhaustive dialyzing of the purified M-NDM-1 was carried out against 4×1 L of 50 mmol/L Hepes, 0.2 mol/L NaCl, pH 7.5, containing 10 mmol/L EDTA, at 4°C (4 h for each dialysis step). The chelator was removed by dialysis against 4×1 L of metal-free, 50 mmol/L Hepes, pH 7.5, at 4°C. Metal-free buffer solution prepared with metal-free water, and, pretreatment of the buffer solution with Chelex-100 were carried out. All plasticwares were treated for more than 2 h in 0.2 mol/L EDTA to remove the contaminating metals. Dialysis vessels were covered and well stirred.

Nuclear magnetic resonance spectroscopy

All NMR experiments were carried out at 25°C on a Bruker DMX 600 MHz spectrometer with a triple resonance cryo-probe. Uniformly ^{15}N-labeled NDM-1 was prepared as described above. Sample of apo-NDM-1 was prepared at 0.5 mmol/L protein concentration in 10 mmol/L sodium acetate buffer (pH 6.5). Metal bound NDM-1s were prepared by addition of 100 mmol/L zinc acetate stock to a final concentration of 1 mmol/L metal ions in 0.5 mmol/L protein, and the samples then were subjected to three rounds of concentration with an Amicon Ultra 15 centrifugal filter (Millipore) followed by dilution to remove unbound metal. Samples for NMR measurements contained 0.5 mmol/L ^{15}N labeled NDM-1, 95% H_2O/5% ^2H_2O in 10 mmol/L sodium acetate buffer (pH 6.5). All NMR spectra were processed and analyzed using Felix software (Accelrys Inc.).

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ABBREVIATIONS

DLS, dynamic light scattering; GTAs, gene-transfer agents; HGT, horizontal gene transfer; ICP-MS, inductively coupled plasma mass spectrometry; MBL, metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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