Published in "Antimicrobial Agents and Chemotherapy 61(3): e02413-16, 2017"
which should be cited to refer to this work.

Characterization of BRP_{MBL}, the Bleomycin Resistance Protein Associated with the Carbapenemase NDM

Laurent Dortet,[a,b,c] Delphine Girlich,[b] Anne-Laure Virlouvet,[a] Laurent Poirel,[d,e] Patrice Nordmann,[d,e,f] Bogdan I. Iorga,[g] Thierry Naas[a,b,c]

Bacteriology-Hygiene unit, Assistance Publique/Hôpitaux de Paris, Bicêtre Hospital, Le Kremlin-Bicêtre, France[a]; EA7361 (Structure, dynamic, function and expression of broad spectrum β-lactamases), Paris-Sud University, LabEx Lermit, Faculty of Medicine, Le Kremlin-Bicêtre, France[b]; Associated French National Reference Center for Antibiotic Resistance: Carbapenemase-producing Enterobacteriaceae, Le Kremlin-Bicêtre, France[c]; Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Switzerland[d]; INSERM European Unit (LEA Paris, IAME, France), University of Fribourg, Switzerland[e]; University hospital and University of Lausanne, Switzerland[f]; Institut de Chimie des Substances Naturelles, CNRS UPR 2301, LabEx LERMIT, Gif-sur-Yvette, France[g]

ABSTRACT The metallo-β-lactamase NDM-1 is among the most worrisome resistance determinants and is spreading worldwide among Gram-negative bacilli. A bleomycin resistance gene, ble_{MBL}, downstream of the bla_{NDM-1} gene has been associated with resistance almost systematically. Here, we characterized the corresponding protein, BRP_{MBL}, conferring resistance to bleomycin, an antitumoral glycopeptide molecule. We have determined whether the expression of the bla_{NDM-1}-ble_{MBL} operon is inducible in the presence of carbapenems and/or bleomycin-like molecules using quantitative reverse transcription-PCR (qRT-PCR), determination of imipenem and zeocin MICs, and carbapenemase-specific activity assays. We showed that the bla_{NDM-1}-ble_{MBL} operon is constitutively expressed. Using electrophoretic mobility shift and DNA protection assays performed with purified glutathione *S*-transferase (GST)-BRP_{MBL}, we demonstrated that BRP_{MBL} is able to bind and sequester bleomycin-like molecules, thus preventing bleomycin-dependent DNA degradation. *In silico* modeling confirmed that the mechanism of action required the dimerization of the BRP_{MBL} protein in order to sequester bleomycin and prevent DNA damage. BRP_{MBL} acts specifically on bleomycin-like molecules since cloning and expression of ble_{MBL} in *Staphyloccoccus aureus* did not confer cross-resistance to any other antimicrobial glycopeptides such as vancomycin and teicoplanin.

KEYWORDS antimicrobial resistance, cancer treatment, mechanism of action, structure

Address correspondence to Laurent Dortet, laurent.dortet@aphp.fr.

Bleomycin, initially discovered from *Streptomyces verticillus*, is an antimitotic-antibiotic compound of the glycopeptide family (1), causing cell death as a result of multiple-strand scissions by direct interaction with DNAs through a mechanism that is still not completely understood (2). It is a medication used to treat several cancers. It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system (http://www.who.int/medicines/publications/essentialmedicines/en/).

Resistance to bleomycin can be mediated by three different types of proteins or mechanisms: (i) bleomycin hydrolases; (ii) bleomycin N-acetylating enzymes that inactivate bleomycin-like molecules; and (iii) bleomycin-binding proteins (BMLA and BMLT) that act by trapping the bleomycin-like molecules (2, 3). Bleomycin hydrolase from rabbit lungs is an aminopeptidase B-like enzyme that is inhibited by N-ethylmaleimide, leupeptin, puromycin, and divalent cations but is unaffected by chelating agents (4).

The bleomycin hydrolase from *Saccharomyces cerevisiae* (encoded by an open reading frame [ORF] consisting of 483 amino acids [AA]) is a thiol protease with an aminopeptidase activity (5). In *S. verticillus*, bleomycin resistance is related to the expression of *blmA* and *blmB* genes encoding two proteins, BLMA (122 AA) and BAT (301 AA). BLMA is a binding protein with a high affinity for bleomycin, whereas BAT is an acetyltransferase that catalyzes the acetylation of bleomycin, leading to subsequent loss of antibacterial activity, i.e., of the DNA-cleaving activities of bleomycin (6, 7). In methicillin-resistant *Staphylococcus aureus* (MRSA), acquired bleomycin resistance has been reported to be mediated by another bleomycin-binding protein, named BLMS (8). In Gram-negative bacteria, transposon Tn*5* carries a bleomycin resistance gene, named *ble*. The *ble* gene product, also called BMLT, is a 126-amino-acid-long bleomycin-binding protein of 126 AA sharing low identity with BLMA (from *S. verticillus*) and BLMS (from *S. aureus*).

Recently, a new bleomycin resistance gene, named ble_{MBL}, has been characterized from carbapenem-resistant *Enterobacteriaceae* and *Acinetobacter baumannii* (9). The ble_{MBL} gene is located downstream of the bla_{NDM-1} gene, as part of the same operon, and the deduced protein, named BRP_{MBL}, showed 54% amino acid identity with the most closely related Tn*5*-encoded bleomycin resistance protein, BMLT (9, 10). In addition, it was recently demonstrated that BRP_{MBL} is functional and yields decreased susceptibility to bleomycin and bleomycin-like molecules, such as zeocin, when expressed in *Enterobacteriaceae* and *A. baumannii* (9).

The aims of the study were (i) to monitor the expression of the bla_{NDM-1}-ble_{MBL} operon under conditions of carbapenem and bleomycin induction, (ii) to elucidate the bleomycin resistance mechanism related to BRP_{MBL} and its resulting role in DNA protection against bleomycin DNA degradation, and (iii) to identify any possible cross-resistance to other antimicrobial glycopeptides such as vancomycin and teicoplanin that is related to ble_{MBL} expression.

RESULTS

Expression of bla_{NDM-1}-ble_{MBL} is not inducible. Both the bla_{NDM-1} and ble_{MBL} genes have been shown to be expressed from the same promoter located upstream of the operon (9). Induction of this promoter in the presence of imipenem or bleomycin-like molecule subinhibitory concentrations was assessed using quantitative reverse transcription-PCR (qRT-PCR) with the NDM-1-producing *Klebsiella pneumoniae* 419 clinical isolate (11) and with *Escherichia coli* TOP10 harboring the recombinant plasmid pCR2.1 P_{NDM}-bla_{NDM-1}-ble_{MBL} (9). As shown in Fig. 1, no significant modification of the expression of the bla_{NDM-1}-ble_{MBL} operon was observed in the presence of subinhibitory concentrations of imipenem or zeocin (a bleomycin analogue). Similarly, addition of zeocin to the culture medium of NDM-1-producing enterobacterial clinical isolates did not induce higher levels of resistance to imipenem (Table 1). Furthermore, the absence of NDM-1 induction in the presence of zeocin was confirmed by measuring the specific activity of the carbapenemase on crude protein extracts of an *E. coli* TOP10 (pCR2.1 P_{NDM}-bla_{NDM-1}-ble_{MBL}) clone and of three NDM-1-producing clinical isolates (*E. coli* 471, *K. pneumoniae* 419, and *K. pneumoniae* DIN) (Table 1). Moreover, the addition of imipenem at different concentrations (0.01, 0.1, and 1 mg/liter) in the culture medium of *E. coli* TOP10 (pCR2.1 P_{NDM}-bla_{NDM-1}-ble_{MBL}) did not induce zeocin resistance (see Fig. S1 in the supplemental material). However, an additive effect of the two molecules was observed (Fig. S1).

BRP_{MBL} binds to bleomycin-like molecules. The purified soluble glutathione *S*-transferase (GST)-BRP_{MBL} fusion protein had a molecular mass corresponding to the expected size of 40 kDa, as assessed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). An electrophoretic mobility shift assay (EMSA) using purified GST-BRP_{MBL} (20 μg) and 20 μg of bleomycin or zeocin showed that the GST-BRP_{MBL} protein migration was delayed in the presence of bleomycin-like molecules (Fig. 2). This result indicates that BRP_{MBL} could bind the bleomycin-like molecules. In addition, the observed shift of migration was approximately 50 to 60 kDa, correspond-

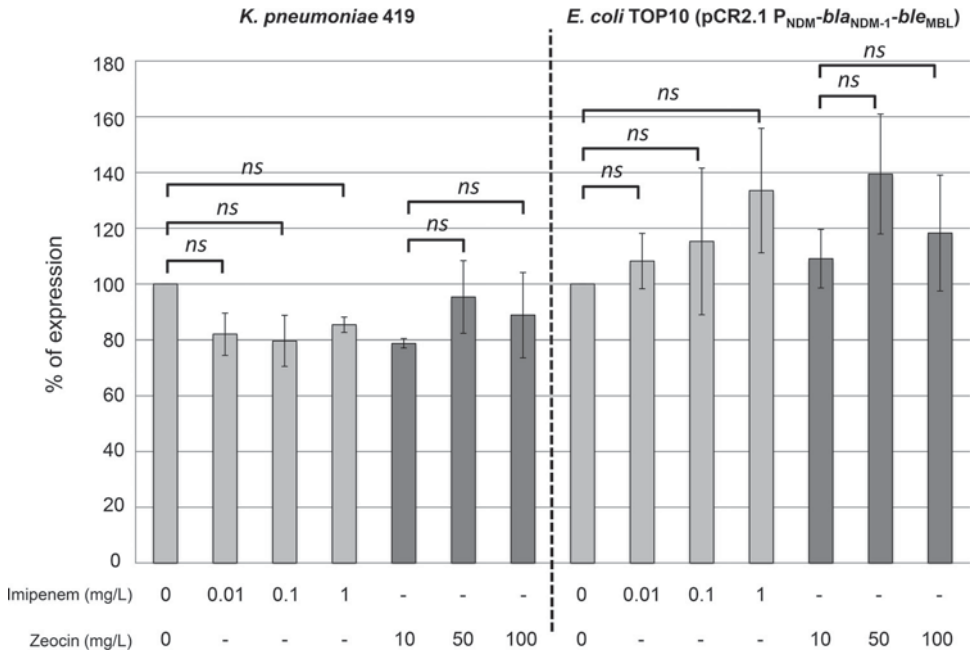

FIG 1 Relative expression levels of the operon bla_{NDM-1}-ble_{MBL} under conditions of imipenem or zeocin induction. Statistical analysis was performed on the results of 3 independent experiments using Student's *t* test. *P* values of <0.05 were considered to represent statistically significant differences. *ns*, not significant.

ing to the molecular mass of the GST-BRP_{MBL} (40 kDa) plus that of bleomycin or zeocin (~14 kDa), suggesting a dimerization of the BRP_{MBL} in the presence of bleomycin-like molecules.

BRP_{MBL} inhibits bleomycin-induced DNA damages. Resistance to DNA-damaging agents was assayed using EcoRI-linearized pUC19 (1 μg), used as probe DNA, in the presence of a mixture of bleomycin and the GST-BRP_{MBL} used for the EMSA. The DNA protection assay demonstrated that bleomycin degraded the linearized plasmid DNA (as the DNA band disappeared) and that the excess of BRP_{MBL} prevented the DNA cleavage induced by bleomycin from occurring (Fig. 3). The specificity was assessed with 5 μg of GST-MVP used as a control protein, and no protection of the DNA damage was observed (12).

***In silico* three-dimensional model of the BRP_{MBL}-bleomycin complex.** The three-dimensional model of BRP_{MBL} in complex with bleomycin was built by homology with the BLMT structure (PDB code 1EWJ) (13) as the template (see Fig. 4A for the alignment used in this process). The model needed to be constructed directly as a dimer, in the presence of the bleomycin ligand, in order to avoid important clashes in the resulting

TABLE 1 MIC of and specific enzymatic activity in response to imipenem after induction with increasing concentrations of zeocin

Strain	Genotype[a]		MIC of imipenem (mg/liter) on Mueller-Hinton agar supplemented with zeocin at the indicated concn (mg/liter)[b]					Specific activities (mU/mg of protein) using imipenem as the substrate after induction with zeocin at the indicated concn (mg/liter)[c]		
	bla_{NDM-1}	ble_{MBL}	0	10	50	100	500	0	10	50
E. coli TOP10 (pCR2.1)	−	−	0.12	−	−	−	−	ND	ND	ND
E. coli TOP10 (pCR2.1 P$_{NDM}$-bla$_{NDM-1}$-ble$_{MBL}$)	+	+	>32	>32	>32	>32	16	2.92 ± 0.26	3.07 ± 0.14^{ns}	2.84 ± 0.21^{ns}
E. coli 271	+	+	6	6	6	4	2	4.67 ± 1.33	7.17 ± 1.15^{ns}	5.24 ± 0.65^{ns}
K. pneumoniae 419	+	+	1.5	1	1	1	0.5	1.31 ± 0.73	2.09 ± 0.52^{ns}	3.27 ± 0.88^{ns}
K. pneumoniae DIN	+	+	4	2	2	1.5	1.5	6.21 ± 1.08	3.88 ± 1.26^{ns}	7.91 ± 1.45^{ns}

[a] −, absence of the corresponding gene; +, presence and expression of the corresponding gene.
[b] −, no growth.
[c] ND, not determined; ^{ns}, not significant. Statistical analysis was performed on the results of 3 independent experiments using Student's *t* test. *P* values of <0.05 were considered significantly different.

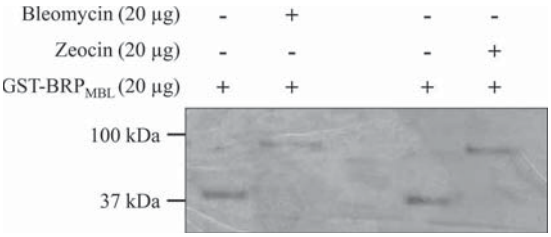

FIG 2 SDS-PAGE profiles of GST-BRP$_{MBL}$, preincubated with bleomycin or zeocin. Lanes 1 and 3, SDS-PAGE profiles in the presence of GST-BRP$_{MBL}$ (20 μg); lane 2, SDS-PAGE profile in the presence of bleomycin (20 μg); lane 4, SDS-PAGE profile in the presence of zeocin (20 μg).

structure (Fig. 4B; see also Fig. S2). A comparison of the BLMT template to the BRP$_{MBL}$ model (Fig. 4C) showed very similar conformations for the proteins and for the bleomycin ligands, with the exception of the region consisting of AA 34 to 42, which represents an 8-amino-acid deletion in the BRP$_{MBL}$ protein sequence. That region contains one of the two tryptophan (Trp) residues that interact with the bithiazole moiety of bleomycin in the bleomycin-BLMT complex (Fig. 4C).

The interactions between bleomycin and the binding site residues of BRP$_{MBL}$ are shown in Fig. 4D. All the fragments present in the structure of bleomycin formed a large number of hydrogen bonds with the surrounding residues, with the exception of the terminal γ-aminopropyldimethylsulfonium, located at the end of the long groove, which showed no particular interaction and was probably highly flexible, in agreement with the crystallographic data previously obtained for the complex bleomycin-BLMA (3). It is also noteworthy that the three intramolecular hydrogen bonds maintained the relative rigidity of the core of the bleomycin structure (Fig. 4D). The π-π interactions observed between the bithiazole moiety of bleomycin and Trp35 and Trp99 from BLMT are lost in the BRP$_{MBL}$-bleomycin complex. Indeed, Trp35 is part of the 8-AA deletion in the region AA 35 to 42 and Trp99 is replaced by a Ser residue in BRP$_{MBL}$ (Ser91) (Fig. 4A). However, the bithiazole fragment is solidly anchored in the BRP$_{MBL}$ binding site groove through hydrogen bond interactions with the side chains of Arg57 and Ser91 (Fig. 4D).

BRP$_{MBL}$ does not confer resistance to other antimicrobial glycoproteins. In order to identify any cross-resistance to other antimicrobial glycopeptides such as vancomycin and teicoplanin that is related to *ble*$_{MBL}$ expression, the *ble*$_{MBL}$ gene was cloned into a shuttle vector (pPRT plasmid) that allows replication and expression in Gram-negative (*E. coli* TOP10) and Gram-positive (*S. aureus* CIP658) species. Although the MICs of bleomycin and zeocin were >256 mg/liter for the recombinant *S. aureus* CIP658 strain (pPRT-*ble*$_{MBL}$) (versus 8 mg/liter and 2 mg/liter, respectively, for the wild-type *S. aureus* CIP658 strain), no decreased susceptibility could be observed for

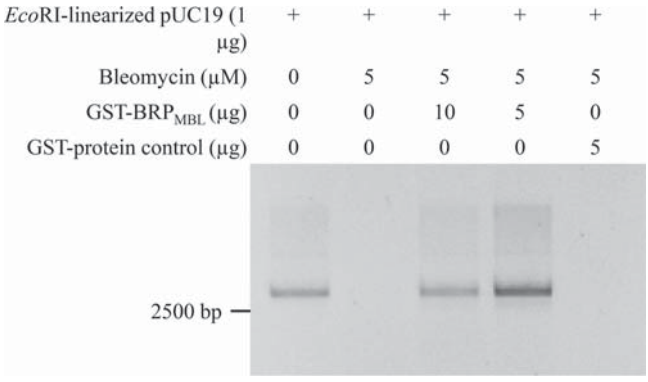

FIG 3 Electrophoretic profile of EcoRI-linearized pUC19 after incubation with bleomycin alone or with bleomycin and GST-BRP$_{MBL}$.

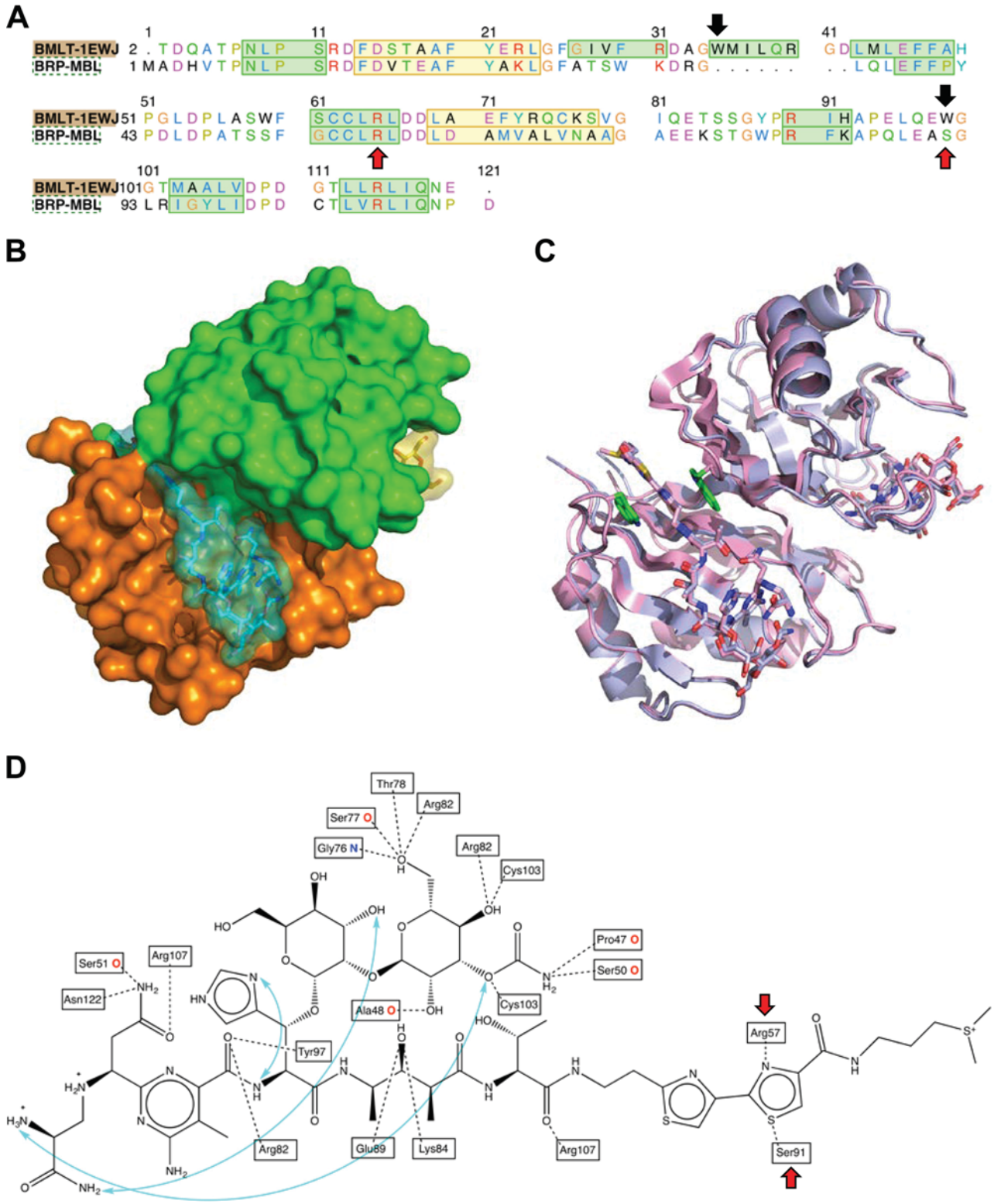

FIG 4 BRP_{MBL} interactions with a bleomycin substrate. (A) Sequence alignment between BRP_{MBL} and BMLT that was used for building the three-dimensional model of BRP_{MBL}. Black arrows indicate Trp35 and Trp99 from BLMT (which are lost in the BRP_{MBL}) involved in the π-π interactions with the bithiazole moiety of bleomycin. Red arrows indicate Arg57 and Ser91 of BRP_{MBL} involved in the bleomycin bithiazole fragment interaction. (B) Surface representation of the BRP_{MBL} model (chains A and B colored in orange and green, respectively) in complex with two bleomycin molecules (colored in cyan and yellow). (C) Superposition of BRP_{MBL} (blue) and BMLT (magenta) dimers in complex with the corresponding bleomycin molecules. Two Trp residues from BMLT that interact with the bithiazole moiety from bleomycin are colored in green. (D) Chemical structure of bleomycin. Hydrogen bond interactions with the BRP_{MBL} binding site are represented as dashed lines. Most of these interactions are established with the side chains of binding site residues, but where the backbone is involved, an "O" or "N" follows the residue name. Intramolecular hydrogen bonds in bleomycin are represented as arrows colored in cyan. Red arrows indicate Arg57 and Ser91 of BRP_{MBL} involved in the bleomycin bithiazole fragment interaction.

glycopeptides (vancomycin and teicoplanin) compared to the susceptibility of the parental strain (data not shown).

DISCUSSION

NDM is a recently described carbapenemase that has rapidly disseminated worldwide, with the Indian subcontinent being an important reservoir. NDM is currently among the most threatening antimicrobial resistance traits in *Enterobacteriaceae* (14). The bla_{NDM} genes have been almost systematically found in association with the ble_{MBL} gene encoding BRP_{MBL}, a putative bleomycin resistance protein. Despite its low (54%) amino acid sequence identity with the other known BRPs, BRP_{MBL} was shown to be a functional protein conferring resistance to bleomycin-like molecules. The genes encoding BRP_{MBL} and NDM are coexpressed from the same promoter, located upstream of the bla_{NDM} gene and in the right extremity of insertion sequence IS*Aba125* (9). This coexpression suggested coselection by either of the two molecules with carbapenems and bleomycin. Thus, not only the usage of carbapenem but also that of bleomycin-like molecules might be responsible for the selection of NDM producers. We have demonstrated here that neither carbapenems nor bleomycin-like molecules have any impact on the expression of the bla_{NDM}-ble_{MBL} operon. This result is in agreement with the fact that, most often, promoter elements brought by insertion sequences lead to constitutive expression of genes located downstream, as observed for other carbapenemases (15, 16).

As observed for other BRPs (17), the role of BRP_{MBL}, once produced in a hypermutable *E. coli* strain (9), in DNA stabilization has been demonstrated. However, the exact mechanism of action of BRP_{BML} has still not been elucidated. Here, we have shown that BRP_{MBL} prevents the occurrence of bleomycin-mediated DNA damage by directly binding bleomycin-like molecules. Several three-dimensional structures of bleomycin-binding proteins are available in the Protein Data Bank (18): bleomycin resistance protein from *Streptoalloteichus hindustanus ble* (Sh*ble*) (PDB codes 1BYL, 1XRK, and 2ZHO), transposon Tn*5*-carried bleomycin resistance determinant BLMT (PDB codes 1ECS, 1EWJ, 1MH6, and 1NIQ), and bleomycin-binding protein from *Streptomyces verticillus* BLMA (PDB codes 1JIE, 1JIF, and 1QTO). Those proteins, even though sharing, respectively, 18%, 52%, and 16% amino acid identity with BRP_{MBL} only, have similar three-dimensional (3D) structures, suggesting that they may be used as bases for BRP_{MBL} modeling. The crystal structures are dimers, and a few of them are complexed with two bleomycin molecules, which are located symmetrically at the monomer-monomer interfaces (13). *In silico* modeling suggested that the three-dimensional model of the BRP_{MBL} needed to be constructed directly as a dimer, in the presence of the bleomycin ligand. These results were confirmed by EMSA, which showed that the BRP_{MBL} protein dimerized in the presence of bleomycin-like molecules.

Finally, although the terminal γ-aminopropyldimethylsulfonium, located at the end of the long groove of the BRP_{MBL} protein, was found to be highly flexible, we demonstrated that BRP_{MBL} acts specifically on bleomycin-like molecules and thus that the results explain why other antimicrobial glycopeptides, such as vancomycin and teicoplanin, could not be sequestered by BRP_{MBL}.

MATERIALS AND METHODS

Bacterial strains. The previously described pCR2.1 P_{NDM}-bla_{NDM-1}-ble_{MBL} *E. coli* TOP10 clone (9) and clinical isolates *K. pneumoniae* 419 (11), *E. coli* 271 (19), and *K. pneumoniae* DIN (9) expressing the bla_{NDM-1}-ble_{MBL} operon were used for the monitoring of bla_{NDM-1}-ble_{MBL} operon expression upon induction with imipenem and bleomycin-like molecules. The BL21(DE3) *E. coli* strain (Life Technologies, Cergy-Pontoise, France) was used as the host for BRP_{MBL} expression and purification. The *S. aureus* CIP658 strain was used as the host for cloning experiments with pAT28-ble_{MBL} plasmid.

Susceptibility testing. MICs of imipenem were determined on Mueller-Hinton agar (bioMérieux, La Balme-les-Grottes, France) using the Etest technique (bioMérieux) following the manufacturer's instructions.

Zeocin susceptibility testing was performed using the disc diffusion method following the European Society of Clinical Microbiology and Infectious Diseases (EUCAST) recommendations (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Manual_v_5.0_EUCAST_Disk_Test.pdf) on Mueller-Hinton agar supplemented with imipenem (Sigma-Aldrich, Saint Quentin Fallavier, France) at various concentrations (0, 0.01, 0.1, and 1 mg/liter). Discs impregnated with various amounts of zeocin

(20 μg, 50 μg, 100 μg, 200 μg, 500 μg, and 1,000 μg) were extemporaneously prepared by adding 10 μl of diluted zeocin solutions (ThermoFisher, Villebon, France) to sterile dried discs.

Carbapenemase-specific activity. The specific activities with 100 μM imipenem as the substrate of the crude protein extracts obtained from overnight cultures of *bla*$_{NDM-1}$-*ble*$_{MBL}$-expressing isolates, namely, *E. coli* TOP10 (pCR2.1 P$_{NDM}$-*bla*$_{NDM-1}$-*ble*$_{MBL}$), *K. pneumoniae* 419, *E. coli* 271, and *K. pneumoniae* DIN, were determined with an Ultrospec 2000 UV spectrophotometer (Amersham Pharmacia Biotech, Orsay, France) as previously described (20). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per min. Bacterial isolates were grown overnight in brain heart infusion medium supplemented with zeocin at 0, 2, or 200 mg/liter.

qRT-PCR. The pCR2.1 P$_{NDM}$-*bla*$_{NDM-1}$-*ble*$_{MBL}$ *E. coli* TOP10 clone and *K. pneumoniae* 419 isolate were grown overnight in BHI medium supplemented with 0, 0.01, 0.1, and 1 mg/liter of imipenem or 0, 10, 50, and 100 mg/liter of zeocin. RNAs were recovered using an RNeasy minikit (Qiagen, Courtaboeuf, France). In order to quantify expression of the *ble*$_{MBL}$ gene, one-step qRT-PCR (SYBR green Rotor gene; Qiagen, France) was used as previously described (21) with primers rtBleoFw (5′-TATCCTGACCTCGACCCAGC-3′) and rtBleoRv (5′-ATCAGGTAGCCGATCCTCAGG-3′). The levels of expression of the *ble*$_{MBL}$ gene and the 16S rRNA gene were quantified with the same RNA extracts as described previously (21). Expression levels were standardized relative to the transcription level of the constitutively expressed 16S rRNAs with primers rt16SFw (5′-GGACGGGTGAGTAATGTCTG-3′) and rt16SRv (5′-TCTCAGACCAGCTAGGGATCG-3′). The transcriptional levels, determined by qRT-PCR, were interpreted using the threshold cycle ($2^{\Delta\Delta CT}$) method as described previously (22).

BRP$_{MBL}$ purification. In order to overexpress and purify BRP$_{MBL}$, the *ble*$_{MBL}$ gene from *E. coli* 271 was amplified (19) with primers pET41b-*ble*-SpeI-F (5′-GAGTCACTAGTGCTGACCACGTCACCCC-3′) and pET41b-*ble*-XhoI-R (5′-GAGTCCTCGAGTCAGTCGGGGTTCTGGATC-3′). The resulting product was introduced into PCRblunt pTOPO (Life Technologies) and expressed in *E. coli* TOP10 (Life Technologies) for amplification and subcloning into SpeI- and XhoI-restricted pET41b+ (Novagen, Darmstadt, Germany). BRP$_{MBL}$ was expressed at a high level as a fusion protein with 220-AA glutathione *S*-transferase (GST). GST-BRP$_{MBL}$ fusion constructs were expressed in *E. coli* BL21(DE3) upon induction with IPTG (isopropyl-β-D-thiogalactopyranoside) at 0.1 mM during 16 h at 25°C, and the crude protein extract was prepared using NaCl (100 mM)- and lysozyme (50 mg/liter)-containing phosphate buffer (50 mM, pH 7), sonication, DNase (5 mg/liter), RNase (10 mg/liter), and high-speed centrifugation as recommended by the manufacturer (Qiagen, les Ulis, France). The resulting GST-BRP$_{MBL}$ was purified by glutathione chelate affinity (Qiagen) on an Akta purifier (GE Healthcare, Les Ulis, France) and dialyzed in 50 mM Tris-HCl (pH 8).

BRP$_{MBL}$-bleomycin-like molecule binding assay. The capacity of binding of the GST-BRP$_{MBL}$ to bleomycin was assayed by electrophoretic mobility shift assay (EMSA) by using purified GST-BRP$_{MBL}$ (20 μg) and 20 μg bleomycin or zeocin in 30 μl of 10 mM Tris-HCl (pH 7.5) at 16°C. After 3 h of incubation, 30 μl of the reaction mixture was loaded onto a 10% nondenaturing polyacrylamide gel, as previously described (13, 23, 24).

DNA protection assay. The DNA protection assay was performed as previously described (24, 25). Briefly, GST-BRP$_{MBL}$ (0, 5, and 10 μg) or a GST-protein control, named GST-major vault protein (GST-MVP) (12) (5 μg), was mixed with 5 μM bleomycin–10 mM Tris-HCl (pH 7.5). After incubation at 25°C for 5 min, 0.1 μg of EcoRI-linearized pUC19, 0.1 mM $FeSO_4$, and 1 mM dithiothreitol were added to achieve a total volume of 20 μl. The mixture was incubated at 25°C; after 10 min, 40 mM EDTA was added. The solution (10 μl) was separated by agarose (1%) gel electrophoresis.

Generation of BRP$_{MBL}$-overexpressing *S. aureus*. To express BRP$_{MBL}$ in *S. aureus*, the pPRT-*ble*$_{MBL}$ derivative plasmid was constructed. The *ble*$_{MBL}$ gene was amplified from *E. coli* 271 with primers pAT18-BamHI-bleF (5′-GAGTCGGATCCATGGCTGACCACGTCA-3′) and pAT18-XbaI-bleR (5′-GAGTCTCTAG ATCAGTCGGGGTTCTGG-3′) and introduced into the BamHI- and XbaI-restricted pPRT plasmid, a pAT18 derivative plasmid (26). In the pPRT-*ble*$_{MBL}$ plasmid, BRP$_{MBL}$ was expressed under the control of the promoter region of the protease gene from *Lactococcus lactis* subsp. *cremoris*, which is active in Gram-positive bacterial species (26). The pPRT-*ble*$_{MBL}$ plasmid was introduced into *S. aureus* CIP658 by electroporation using erythromycin (5 mg/liter) as the selective agent as previously described (27).

Molecular modeling. The three-dimensional model of BRP$_{MBL}$ was built using Modeler v9.16 (28), with the structure of BMLT (PDB code 1EWJ) as the template (13). The structure was constructed as a dimer, with two bleomycin molecules positioned at the interface of the monomers. The protein-ligand interactions were analyzed with UCSF Chimera (29), and the images for sequence alignment and protein-ligand complexes were generated using UCSF Chimera and Pymol, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02413-16.

TEXT S1, PDF file, 1 MB.

ACKNOWLEDGMENTS

We have no conflicts of interest to declare.

This work was partially funded by a grant from the University Paris-Sud and INSERM (U914), France. L.D., D.G., B.I.I., and T.N. are members of the Laboratory of Excellence LERMIT supported by a grant from ANR (ANR-10-LABX-33).

REFERENCES

1. Umezawa H, Maeda K, Takeuchi T, Okami Y. 1966. New antibiotics, bleomycin A and B. J Antibiot (Tokyo) 19:200–209.
2. Sugiyama M, Kumagai T. 2002. Molecular and structural biology of bleomycin and its resistance determinants. J Biosci Bioeng 93:105–116. https://doi.org/10.1016/S1389-1723(02)80001-9.
3. Sugiyama M, Kumagai T, Hayashida M, Maruyama M, Matoba Y. 2002. The 1.6-A crystal structure of the copper(II)-bound bleomycin complexed with the bleomycin-binding protein from bleomycin-producing *Streptomyces verticillus*. J Biol Chem 277:2311–2320. https://doi.org/10.1074/jbc.M103278200.
4. Sebti SM, DeLeon JC, Lazo JS. 1987. Purification, characterization, and amino acid composition of rabbit pulmonary bleomycin hydrolase. Biochemistry 26:4213–4219. https://doi.org/10.1021/bi00388a006.
5. Enenkel C, Wolf DH. 1993. BLH1 codes for a yeast thiol aminopeptidase, the equivalent of mammalian bleomycin hydrolase. J Biol Chem 268:7036–7043.
6. Sugiyama M, Kumagai T, Shionoya M, Kimura E, Davies JE. 1994. Inactivation of bleomycin by an N-acetyltransferase in the bleomycin-producing strain *Streptomyces verticillus*. FEMS Microbiol Lett 121:81–85. https://doi.org/10.1111/j.1574-6968.1994.tb07079.x.
7. Sugiyama M, Thompson CJ, Kumagai T, Suzuki K, Deblaere R, Villarroel R, Davies J. 1994. Characterisation by molecular cloning of two genes from *Streptomyces verticillus* encoding resistance to bleomycin. Gene 151:11–16. https://doi.org/10.1016/0378-1119(94)90626-2.
8. Sugiyama M, Kumagai T, Matsuo H, Bhuiyan MZ, Ueda K, Mochizuki H, Nakamura N, Davies JE. 1995. Overproduction of the bleomycin-binding proteins from bleomycin-producing *Streptomyces verticillus* and a methicillin-resistant *Staphylococcus aureus* in *Escherichia coli* and their immunological characterisation. FEBS Lett 362:80–84. https://doi.org/10.1016/0014-5793(95)00218-X.
9. Dortet L, Nordmann P, Poirel L. 2012. Association of the emerging carbapenemase NDM-1 with a bleomycin resistance protein in *Enterobacteriaceae* and *Acinetobacter baumannii*. Antimicrob Agents Chemother 56:1693–1697. https://doi.org/10.1128/AAC.05583-11.
10. Blot M, Meyer J, Arber W. 1991. Bleomycin-resistance gene derived from the transposon Tn*5* confers selective advantage to *Escherichia coli* K-12. Proc Natl Acad Sci U S A 88:9112–9116. https://doi.org/10.1073/pnas.88.20.9112.
11. Poirel L, Al Maskari Z, Al Rashdi F, Bernabeu S, Nordmann P. 2011. NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. J Antimicrob Chemother 66:304–306. https://doi.org/10.1093/jac/dkq428.
12. Dortet L, Mostowy S, Samba-Louaka A, Gouin E, Nahori MA, Wiemer EA, Dussurget O, Cossart P. 2011. Recruitment of the major vault protein by InlK: a *Listeria monocytogenes* strategy to avoid autophagy. PLoS Pathog 7:e1002168. https://doi.org/10.1371/journal.ppat.1002168.
13. Maruyama M, Kumagai T, Matoba Y, Hayashida M, Fujii T, Hata Y, Sugiyama M. 2001. Crystal structures of the transposon Tn*5*-carried bleomycin resistance determinant uncomplexed and complexed with bleomycin. J Biol Chem 276:9992–9999. https://doi.org/10.1074/jbc.M009874200.
14. Dortet L, Poirel L, Nordmann P. 2014. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. BiomedRes Int 2014:249856. https://doi.org/10.1155/2014/249856.
15. Hu WS, Yao SM, Fung CP, Hsieh YP, Liu CP, Lin JF. 2007. An OXA-66/OXA-51-like carbapenemase and possibly an efflux pump are associated with resistance to imipenem in *Acinetobacter baumannii*. Antimicrob Agents Chemother 51:3844–3852. https://doi.org/10.1128/AAC.01512-06.
16. Poirel L, Decousser JW, Nordmann P. 2003. Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a bla_{CTX-M} β-lactamase gene. Antimicrob Agents Chemother 47:2938–2945. https://doi.org/10.1128/AAC.47.9.2938-2945.2003.
17. Bostock JM, Miller K, O'Neill AJ, Chopra I. 2003. Zeocin resistance suppresses mutation in hypermutable *Escherichia coli*. Microbiology 149:815–816. https://doi.org/10.1099/mic.0.C0111-0.
18. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. 2000. The Protein Data Bank. Nucleic Acids Res 28:235–242. https://doi.org/10.1093/nar/28.1.235.
19. Poirel L, Lagrutta E, Taylor P, Pham J, Nordmann P. 2010. Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. Antimicrob Agents Chemother 54:4914–4916. https://doi.org/10.1128/AAC.00878-10.
20. Bernabeu S, Poirel L, Nordmann P. 2012. Spectrophotometry-based detection of carbapenemase producers among *Enterobacteriaceae*. Diagn Microbiol Infect Dis 74:88–90. https://doi.org/10.1016/j.diagmicrobio.2012.05.021.
21. Naas T, Cuzon G, Truong HV, Nordmann P. 2012. Role of IS*Kpn7* and deletions in bla_{KPC} gene expression. Antimicrob Agents Chemother 56:4753–4759. https://doi.org/10.1128/AAC.00334-12.
22. Yuan JS, Reed A, Chen F, Stewart CN, Jr. 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7:85. https://doi.org/10.1186/1471-2105-7-85.
23. Jeong MS, Hwang EY, Kim HT, Yoo MA, Jang SB. 2009. Purification of caudal-related homeodomain transcription factor and its binding characterization. J Microbiol Biotechnol 19:1557–1564. https://doi.org/10.4014/jmb.0905.05021.
24. Mori T, Mizuta S, Suenaga H, Miyazaki K. 2008. Metagenomic screening for bleomycin resistance genes. Appl Environ Microbiol 74:6803–6805. https://doi.org/10.1128/AEM.00873-08.
25. Gatignol A, Durand H, Tiraby G. 1988. Bleomycin resistance conferred by a drug-binding protein. FEBS Lett 230:171–175. https://doi.org/10.1016/0014-5793(88)80665-3.
26. Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. 1991. Shuttle vectors containing a multiple cloning site and a *lacZ* alpha gene for conjugal transfer of DNA from *Escherichia coli* to gram-positive bacteria. Gene 102:99–104. https://doi.org/10.1016/0378-1119(91)90546-N.
27. Trieu-Cuot P, Derlot E, Courvalin P. 1993. Enhanced conjugative transfer of plasmid DNA from *Escherichia coli* to *Staphylococcus aureus* and *Listeria monocytogenes*. FEMS Microbiol Lett 109:19–23. https://doi.org/10.1111/j.1574-6968.1993.tb06137.x.
28. Webb B, Sali A. 2016. Comparative protein structure modeling using MODELLER. Curr Protoc Bioinformatics 54:5.6.1–5.6.37. https://doi.org/10.1002/cpbi.3.
29. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612. https://doi.org/10.1002/jcc.20084.