Structural and Biochemical Characterization of Rm3, a Subclass B3 Metallo- β -Lactamase Identified from a Functional Metagenomic Study

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β -Lactamase production increasingly threatens the effectiveness of β -lactams, which remain a mainstay of antimicrobial chemotherapy. New activities emerge through both mutation of previously known β -lactamases and mobilization from environmental reservoirs. The spread of metallo- β -lactamases (MBLs) represents a particular challenge because of their typically broad-spectrum activities encompassing carbapenems, in addition to other β -lactam classes. Increasingly, genomic and metagenomic studies have revealed the distribution of putative MBLs in the environment, but in most cases their activity against clinically relevant β -lactams and, hence, the extent to which they can be considered a resistance reservoir remain uncharacterized. Here we characterize the product of one such gene, *bla*_{Rm3}, identified through functional metagenomic sampling of an environment with high levels of biocide exposure. *bla*_{Rm3} encodes a subclass B3 MBL that, when expressed in a recombinant *Escherichia coli* strain, is exported to the bacterial periplasm and hydrolyzes clinically used penicillins, cephalosporins, and carbapenems with an efficiency limited by high K_m values. An Rm3 crystal structure reveals the MBL superfamily $\alpha\beta/\beta\alpha$ fold, which more closely resembles that in mobilized B3 MBLs (AIM-1 and SMB-1) than other chromosomal enzymes (L1 or FEZ-1). A binuclear zinc site sits in a deep channel that is in part defined by a relatively extended N terminus. Structural comparisons suggest that the steric constraints imposed by the N terminus may limit its affinity for β -lactams. Sequence comparisons identify Rm3-like MBLs in numerous other environmental samples and species. Our data suggest that Rm3-like enzymes represent a distinct group of B3 MBLs with a wide distribution and can be considered an environmental reservoir of determinants of β -lactam resistance.

The continued efficacy of β -lactam antibiotics is threatened by the dissemination of β -lactamases, hydrolytic enzymes that inactivate these important drugs by cleavage of the scissile β -lactam amide bond (1). In the 70 years since β -lactams were first introduced into the clinic, repeated mobilizations of β -lactamase genes from a variety of bacterial sources have led to their rapid propagation in opportunistic Gram-negative pathogens, such as the *Enterobacteriaceae* and nonfermenting species, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (2). Notably, some of the most successful β -lactamases, in particular, the CTX-M extended-spectrum β -lactamase (ESBL), which is associated with resistance to third-generation cephalosporins, such as cefotaxime, and which is now distributed worldwide (3), find their origins in environmental organisms, illustrating how transfer of antibiotic resistance genes from environmental to pathogenic species can have profound clinical consequences (4). In the case of CTX-M enzymes, it is now accepted that these originated in *Kluyvera* spp. (5, 6), which are Gram-negative rod bacteria that are found in both the human intestinal microbiome and the wider natural environment (7).

β -Lactamases are divided, primarily on the basis of their amino acid sequences, into four main classes (8). Of these, three (classes A, C, and D) are active-site nucleophilic serine β -lactamases (SBLs), and the remaining class, class B, consists of zinc metalloenzymes that are structurally and mechanistically unrelated to the SBLs. The metallo- β -lactamases (MBLs) are themselves divided into a further three groups (B1, B2, and B3) on the basis of sequence differences that are manifest as variations in the number (1 or 2) of zinc ions required for full activity and as structural differences that include variations in the coordination of the active-site zinc ions (9, 10). MBLs are a growing clinical concern, as

they effectively hydrolyze all β -lactam classes except the monobactams and escape the action of SBL inhibitors (11) that are approved (clavulanate, tazobactam) or close to being approved (avibactam, relebactam) for use in the clinic. B1 MBLs, such as the NDM (12) and VIM (13) enzymes, are now encountered with increasing frequency on mobile genetic elements in organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *A. baumannii*.

While B3 family members, such as AIM-1 (14) and SMB-1 (15), have been identified on mobile genetic elements, the majority of these enzymes are chromosomal. However, in addition to their presence in opportunist pathogens, such as *Stenotrophomonas maltophilia* (16) and *Elizabethkingia meningosepticum* (17), the B3 MBLs also have a very wide distribution in environmental organisms and sequences. Compared to the B1 enzymes, the B3 MBLs are less well studied, display a greater degree of structural and sequence diversity, and are more closely related to other

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FIG 1 Sequence alignment of subclass B3 metallo-β-lactamases. Alignment of selected subclass B3 MBLs. Sequences were aligned using the Clustal Omega program (66). Invariant residues are highlighted with a red background, and conservative substitutions (positions boxed) are in red text. Residue numbering is according to the BBL standard numbering scheme (52); discontinuities (e.g., between residues 5 and 70, 80 and 90, and 150 and 170) are due to omission from the figure of other MBL subclasses. Secondary structure assignments (*Dictionary of Protein Secondary Structure* [67]) are from the Rm3 structure (this work). Red triangles, zinc binding residues. Cysteine pair 208 and 213 and cysteine pair 256 and 290 are labeled 1 and 2, respectively. The positions of key Rm3 residues and of Rm3 loops 1 and 2 are labeled below the alignment. This figure was prepared using the EsPrpt program (68).

branches of the wider metallohydrolase superfamily to which the MBLs belong (18). Investigations of B3 MBLs from environmental sources will thus expand our understanding of activity and structure within this group of enzymes and provide insights into the nature of MBLs in the environment and the extent to which such enzymes provide a reservoir of determinants of resistance to the most clinically important β-lactam antibiotics. Furthermore, identifying how the distribution of such sequences changes in response to human activity (i.e., exposure to antimicrobials within the environment) can also provide evidence of the effect of human activity upon the environmental resistance reservoir (19).

Technological advances have transformed our ability to sample and identify antibiotic resistance genes in the natural environment. In particular, combining sequence-based methodologies (metagenomics) with functional methodologies (construction and analysis of large libraries) can both establish the prevalence and distribution of putative resistance genes and identify those

that confer a resistance phenotype, i.e., that are able to alter antibiotic susceptibility in a model organism (e.g., *E. coli*) (20–22). This study provides a biochemical and structural characterization of a B3 MBL, Rm3, that was identified by application of this functional metagenomics approach to study the distribution of determinants of resistance to third-generation cephalosporins in environmental sources selected on the basis of different degrees of human impact. (Full details of the identification of Rm3 will be presented elsewhere.) The *bla*_{Rm3} gene (GenBank accession no. [KF485393.2](#)) was isolated from a metagenomic library derived from soil from a reed bed used to bioremediate effluent from a textile mill with high levels of usage of quaternary ammonium compounds (QACs). Screening of this library identified *bla*_{Rm3} to be one of a number of novel β-lactamase genes able to decrease the susceptibility of recombinant *E. coli* strains to third-generation cephalosporins.

The amino acid sequence of Rm3 (Fig. 1) most closely re-

FIG 2 Phylogenetic tree of selected subclass B3 metallo- β -lactamases. Sequences were aligned using the Clustal Omega program (66), and the phylogenetic tree was visualized using the Drawgram (version 3.67) component of the PHYLIP package (69).

sembles that of other putative B3 MBLs from environmental bacteria, in particular, sequences from the soil bacterium *Janthinobacterium* (e.g., GenBank accession no. [KKO63914.1](#); 89% sequence identity [23]) and *Solimonas* spp. (e.g., NCBI accession no. [WP_020650668.1](#); 56% identity). Rm3 also resembles (54% sequence identity) a novel B3 MBL, LRA-8, identified from a metagenomics study of the Tanana river in central Alaska (20) (Fig. 1) and a related sequence (GenBank accession no. [AIA12579.1](#); 56% identity) identified from a grassland soil sample from Minnesota, USA, as part of a functional metagenomics study of environmental antibiotic resistance genes (24). Of the biochemically characterized B3 MBLs, Rm3 shares the highest sequence identity with THIN-B (25) (49%) and is between 43% (SMB-1) (15) and 27% (BJP-1) (26) identical to enzymes of known structure. On this basis (Fig. 1 and 2), Rm3 can be considered a representative of a group of uncharacterized novel B3 MBLs that appear to be widely distributed within the environmental microbiome. Here we present the biochemical and structural characterization of recombinant Rm3.

MATERIALS AND METHODS

Identification of *bla*_{Rm3}. Full details of the identification of Rm3 will be presented elsewhere. Briefly, core samples were obtained from reed beds used for the remediation of effluent from a textile mill in Yorkshire, United Kingdom (27), and total DNA was purified as previously described (21). A metagenomic library was generated by cloning purified DNA fragments into plasmid pCF430 (28) and transforming into *E. coli* strain EC100 (Epicentre, Madison WI, USA) by electroporation. Recombinants were passaged over 10 to 20 generations, and clones resistant to third-generation cephalosporins were selected by plating on ceftazidime (1 μ g/ml). Putative resistance genes were identified by sequencing positive

clones, and their contribution to the resistance phenotype was confirmed by inactivation using transposon mutagenesis (EZ-Tn5 kit; Epicentre), allowing selection by loss of phenotype (21).

MIC determination for metagenomic clones. MIC values for metagenomic clones were determined by agar dilution on Iso-Sensitest agar (Oxoid) with an inoculum of 10^5 CFU per spot (29).

Recombinant Rm3 expression and purification. The complete Rm3 open reading frame (including the putative periplasmic export sequence) was amplified from metagenomic clone Rm3 by PCR with primers RM3F (AAGGCATATGATGTCCTCACACCACCGCGCG) and RM3R2 (AATGGGATCCTTACTGCTGTTTTCTGGT) with proofreading *Pfu* DNA polymerase. The product was ligated into the T7 expression vector pET26b (30) using the *Nde*I and *Bam*HI restriction sites, and the integrity of the resulting plasmid, pLHZRM3, was confirmed by DNA sequencing. *E. coli* ArcticExpress(DE3) competent cells (Agilent, Stockport, United Kingdom) transformed with pLHZRM3 were grown (at 30°C with shaking at 160 rpm in Power Broth [Athena Enzyme Systems, Baltimore, MD, USA]) to an optical density at 600 nm of ≈ 0.6 , and expression was induced overnight (1 mM isopropyl- β -D-thiogalactopyranoside [Melford Laboratories, Ipswich, United Kingdom], 13°C). Cells were harvested by centrifugation ($7,205 \times g$, 30 min, 4°C) and lysed in a Constant Systems (Daventry, United Kingdom) cell disruptor ($25,000 \text{ lb/in}^2$). Debris was removed by centrifugation ($38,724 \times g$, 1 h), and the supernatant was exchanged into buffer A (50 mM potassium phosphate, pH 7.0, 1 M ammonium sulfate) by extensive dialysis using a 3,000-Da-cutoff membrane (Medicell International, London, United Kingdom).

Protein for crystallography was purified by the following method. Twenty milliliters of the dialysate was loaded on a 1-ml Phenyl Sepharose fast-flow (FF) high-sub (HS) column (GE Healthcare Life Sciences, Little Chalfont, United Kingdom), and the column was washed consecutively with buffer B (buffer A plus 10 mM MgCl_2 , 5 mM ATP, 50 mM KCl) and buffer A prior to elution on a gradient of 0 to 50% buffer C (50 mM potassium phosphate, pH 7.0). Rm3-containing fractions were identified by SDS-PAGE (31) and concentrated to a volume of ~ 2 ml by centrifugal ultrafiltration using an Amicon concentrator with a 3,000-Da-molecular-mass cutoff (Millipore, Watford, United Kingdom). Protein was loaded onto a 300-ml Superdex S75 size exclusion column (GE Healthcare) and eluted at a flow rate of 1 ml/min in buffer D (20 mM Tris, pH 7.5, 200 mM NaCl). Rm3-containing fractions were pooled and concentrated as described above.

For enzyme kinetic experiments, Rm3 was purified by a modified version of the protocol described above, where recombinant protein was produced in *E. coli* SoluBL21 cells (AMS Biotechnology, Abingdon, United Kingdom) that were grown overnight in autoinduction Terrific broth (Formedium, Hunstanton, United Kingdom) at 25°C. The hydrophobic interaction chromatography step utilized a 40-ml Phenyl Sepharose FF HS column, omitted the ATP wash, and eluted bound protein on a 0 to 100% buffer C gradient; and size exclusion chromatography utilized a 120-ml HiLoad 16/60 Superdex 75 pg column (GE Healthcare).

Verification of recombinant Rm3 by mass spectrometry. Electrospray ionization mass analyses were performed (as described previously [32]) in the positive ion mode using a Waters (Elstree, United Kingdom) LCT Premier instrument equipped with a time of flight (TOF) analyzer. An LCT Premier mass spectrometer (Waters) was coupled to an Agilent 1100 series high-performance liquid chromatograph (HPLC) using a Chromolith FastGradient RP-18 end-capped column equipped with a 50-2 HPLC column made of monolithic silica (C-18; 2 by 50 mm; macropore diameter, 1.6 μ m; Merck, Beeston, United Kingdom). The instrument was connected to a CTC autosampler inlet system. A multistep gradient (solvent A, 94.9% H_2O , 5% acetonitrile [CH_3CN], and 0.1% formic acid; solvent B, 99.9% CH_3CN and 0.1% formic acid; 0 to 1 min 5% solvent B for equilibration, followed by a linear gradient to 100% solvent B over 4 min and then 100% solvent B for an additional 3 min, followed by a linear gradient over 2 min back to 5% solvent B to reequilibrate the column) was run over 10 min to separate the protein samples at

TABLE 1 Effect of Rm3 expression on β -lactam MICs for recombinant *Escherichia coli* EC100

Construct	MIC ^c (μ g/ml)							
	AMP	AMX	CAR	TMC	ATM	CTX	CAZ	IPM
pCF430 ^a	8	8	32	16	0.25	0.25	0.5	0.5
pCF430-Rm3 ^b	16	8	32	32	0.5	0.25	8	1

^a Empty pCF430 in *Escherichia coli* EC100.^b pCF430 carrying an 8-kb Rm3 metagenomic fragment.^c AMP, ampicillin; AMX, amoxicillin; CAR, carbenicillin; TMC, temocillin; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem.

flow rates of 0.4 ml/min for the first 5 min and then 1.0 ml/min for the remaining time. The electrospray ionization source used a capillary voltage of 3.2 kV and cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a flow rate of 600 liters/h. Protein typically eluted as a peak between 3 and 5 min under these conditions. Calculated masses were obtained using the ExPasy ProtParam tool (<http://web.expasy.org/protparam/>) (33).

Steady-state kinetics of β -lactam hydrolysis by recombinant Rm3.

Hydrolysis of selected β -lactams by recombinant Rm3 was investigated under steady-state conditions. The buffer was 50 mM HEPES, pH 7.0, supplemented with 100 μ M ZnCl₂ and 100 μ g/ml bovine serum albumin, and the protein concentration was 10 nM. Measurements used either a Polarstar Omega plate reader (BMG LabTech, Aylesbury, United Kingdom) or, for complete hydrolysis curves, a Lambda 35 spectrophotometer (Perkin-Elmer, Seer Green, United Kingdom). The extinction coefficients and wavelengths used (34) were as follows: $-775 \text{ M}^{-1} \text{ cm}^{-1}$ at 235 nm for penicillin G, $-820 \text{ M}^{-1} \text{ cm}^{-1}$ at 235 nm for ampicillin, $-7,700 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm for cefoxitin, $-9,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm for ceftazidime, $-7,500 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm for cefotaxime, $-6,500 \text{ M}^{-1} \text{ cm}^{-1}$ at 300 nm for meropenem, $-9,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 300 nm for imipenem, and $-700 \text{ M}^{-1} \text{ cm}^{-1}$ at 320 nm for aztreonam.

Data were analyzed by fitting to the Michaelis-Menten equation: $V = k_{\text{cat}} \cdot [E] \cdot [S]/(K_m + [S])$, where V is the measured initial velocity at substrate concentration $[S]$, and $[E]$ is the concentration of enzyme. Where high apparent K_m values precluded data collection under the conditions required to achieve saturation of the hydrolysis rate, the value of k_{cat}/K_m was measured by fitting progress curves (absorbance versus time) for a complete hydrolysis reaction to the following exponential: $A_t = A_{\infty} + (A_0 - A_{\infty}) \cdot e^{-k t}$, where A_t is the absorbance at time t , A_0 is the initial absorbance, A_{∞} is the final absorbance, and k is the first-order rate constant. The observed first-order rate constant (k) was then $(k_{\text{cat}}/K_m) \cdot [E]$ (35). Curve fitting was undertaken using Prism software (GraphPad, La Jolla, CA, USA).

Rm3 crystallization and structure determination. Purified Rm3 protein in buffer D was concentrated to $\sim 13 \text{ mg/ml}$ by centrifugal ultrafiltration as described above and supplemented with 100 μ M ZnCl₂ and 5 mM Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (Fisher Scientific). Initial crystallization hits were obtained from commercial sparse matrix screening kits (Proplex; Molecular Dimensions, Newmarket, United Kingdom) (36) using a Phoenix crystallization robot (Art Robbins Instruments, Sunnyvale, CA, USA) to set 100-nl plus 100-nl sitting drops in 96-well MRC plates (Molecular Dimensions) using a reservoir volume of 100 μ l. Conditions were optimized using 1- μ l plus 1- μ l hanging drops in 24-well XRL plates (Molecular Dimensions) with a 500- μ l reservoir volume. Diffraction data were collected from a single crystal grown in a hanging drop from 14% (wt/vol) polyethylene glycol 8000, 0.1 M Tris, pH 8, 0.15 M LiCl. All crystallization experiments were carried out at 18°C.

The Rm3 crystal was cryoprotected for $\sim 30 \text{ s}$ by exposure to reservoir solution supplemented with 25% ethylene glycol, mounted in a Spine standard pin (Molecular Dimensions), and flash frozen in liquid nitrogen. Diffraction data were collected on beamline I04 of the Diamond Light Source (DLS), United Kingdom, using a Pilatus 6M-F detector. A total of 934 images of 0.15° oscillation (exposure, 0.15 s per image; beam intensity, 20%) were collected at a wavelength of 0.9795 Å. Diffraction data were

integrated using XDS (37), the space group was identified using Pointless (38), and the data were scaled and merged using Aimless (38), as implemented in the Xia2 pipeline (39). The structure was solved by molecular replacement using Phaser (40), with Chainsaw (41) being used to create a search model based upon *S. maltophilia* L1 (chain A of the structure with PDB accession no. 2QDT [42]) by pruning side chains of nonidentical amino acids to their C- γ atoms. Models were built in Coot (43), and refinement was carried out using Refmac 5 (44). Final refinement and model validation (MolProbity [45]) took place in Phenix (46).

Accession number(s). Coordinates and structure factors have been deposited in the Protein Data Bank (PDB; www.rcsb.org/pdb) under accession no. 5IQK.

RESULTS AND DISCUSSION

Identification of Rm3 as a subclass B3 metallo- β -lactamase.

*bla*_{Rm3} was identified by selecting ceftazidime-resistant clones from a metagenomic library constructed from DNA purified from samples originating from a reed bed used to bioremediate effluent from a textile mill with high levels of usage of quaternary ammonium compounds (QACs). QACs are disinfective agents with wide industrial application and have been implicated in the selection of co- and cross-resistance to a variety of antibiotic classes, including β -lactams (47, 48). *bla*_{Rm3} was situated on an 8-kb DNA fragment (metagenomic clone Rm3; GenBank accession no. KF485393.2) that exerted variable effects upon susceptibility to β -lactam antibiotics but that resulted in a 16-fold elevation of the MIC of *E. coli* EC100 to ceftazidime compared to that for the vector-only control (Table 1). This effect was abolished by insertional inactivation of *bla*_{Rm3} by transposition (data not shown). The amino acid sequence of the *bla*_{Rm3}-encoded protein, Rm3, showed properties (the presence of a His116-X-His118-X-Asp120-His121 sequence motif and similarity to previously characterized enzymes) characteristic of a subclass B3 MBL (Fig. 1).

Expression and kinetic characterization of recombinant Rm3. The *bla*_{Rm3} gene encodes a 302-residue polypeptide that includes an N-terminal leader peptide of 23 residues that was identified by the SignalP program (49) to be a periplasmic export sequence. The complete Rm3 open reading frame, including the putative export sequence, was expressed in either *E. coli* ArcticExpress or SoluBL21 and was purified to apparent homogeneity by hydrophobic interaction and size exclusion chromatography. Quadrupole time of flight (QTOF) mass spectrometry under denaturing conditions gave a mass of 29,805 Da for the purified protein, consistent with a predicted mass of 29,808.5 Da for the Rm3 fragment resulting from removal of the predicted precursor polypeptide after residue 23. Thus, these data confirm that the leader peptide is removed from recombinant Rm3 by posttranslational processing in *E. coli* and strongly indicate that, as is the case for other β -lactamases of Gram-negative bacteria, the protein is exported to the bacterial periplasm.

Steady-state kinetic experiments indicate that Rm3 is able to hydrolyze a range of penicillin, cephalosporin, and carbapenem antibiotics with various degrees of efficiency (Table 2). Notably, it was possible to obtain accurate K_m estimates for only two substrates, meropenem and ampicillin, of the eight that were evaluated. For the other substrates tested, it proved difficult to saturate the Michaelis-Menten (i.e., rate-versus-substrate concentration) plots, indicating high K_m values and, likely, low affinity. For these substrates, values for catalytic efficiency (k_{cat}/K_m) only are reported. Overall catalytic efficiencies approaching $10^5\text{ M}^{-1}\text{ s}^{-1}$ were achieved for substrates from all classes except the monobactam aztreonam, against which Rm3, as is the case for other MBLs, showed no hydrolytic activity. These data show Rm3, in common with most other B3 MBLs, to be an enzyme with a broad spectrum of activity. The relatively low catalytic efficiencies that are achieved by Rm3 compared to those that are achieved by other characterized B3 MBLs, where values for k_{cat}/K_m in excess of $10^7\text{ M}^{-1}\text{ s}^{-1}$ have been reported for some favorable enzyme-substrate combinations (e.g., AIM-1-catalyzed imipenem hydrolysis [14]), arise primarily from the relatively high K_m values. For all substrates tested, K_m values were 10^{-4} M or above; in contrast, with most other B3 MBLs for more favored substrates, K_m values of 10^{-5} M or better were obtained. Some other enzymes from environmental sources, such as *Janthinobacterium lividum* BJP-1 (26), *Erwinia carotovora* CAR-1 (50), and *Caulobacter crescentus* CAU-1 (51), are also notable for their comparably high K_m values across the range of β -lactams. However, Rm3 was distinguished from many of these by an apparent lack of discrimination against oxyminocephalosporins (e.g., ceftazidime) or 7- α -methoxy cephalosporins (e.g., cefoxitin), which are poor substrates for the B3 enzymes CAR-1 and CAU-1, respectively. The k_{cat}/K_m values for the hydrolysis of these substrates by Rm3 were in line with those for the other β -lactams tested.

Crystal structure of Rm3. Rm3 crystallized in space group P2₁ with two molecules in the asymmetric unit. A single 1.75-Å resolution data set was collected at the Diamond Light Source synchrotron radiation facility, and phases and an initial electron density map were calculated by molecular replacement. Data collection and refinement statistics are given in Table 3. The final structure contained 268 (chain A) and 269 (chain B) residues, with electron density not being observed for the 10 (chain A) or 9 (chain B) N-terminal amino acids or for the C-terminal glutamine residue of either polypeptide chain. We note that the N terminus of processed Rm3 is formed by a proline-rich sequence (QTPAP ATPP) that is likely to be unstructured in solution. Of all residues, 96.6% were in the most favored regions of the Ramachandran plot, with no residues being classified as outliers. The overall structure (Fig. 3) is that of the MBL superfamily, comprising an $\alpha\beta/\beta\alpha$ fold in which the N- and C-terminal halves of the protein form central seven- and five-stranded β -sheets, respectively, that are flanked by α -helices. The interface of these two sheets provides the location for the active site. The active-site environment is defined by three loop regions that connect elements of secondary structure: residues 150 to 164 (loop 1) connecting helix α 4 and strand β 7, residues 192 to 201 connecting strands β 9 and β 10, and residues 222 to 239 (loop 2) connecting strand β 11 and helix α 5. (The BBL numbering scheme [52] is used throughout this report.)

The presence of disulfide bonds also serves to define the overall architecture of the Rm3 structure. The processed Rm3 polypeptide contains a total of six Cys residues, of which two (residues 256

TABLE 2 Kinetic parameters for hydrolysis of selected β -lactams by Rm3 and selected B3 MBLs

β -Lactam	Rm3				L1				FEZ-1 ^a				BJP-1 ^b				AIM-1 ^c				SMB-1 ^d			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{ s}^{-1}$)
Penicillin G	ND ^e	ND	4.1×10^4	75 ± 10^f	410 ± 20	5.5×10^6	590 ± 70	70 ± 5	1.1×10^5	130	18	1.3×10^5	31	778	2.6×10^7	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ampicillin	$1,600 \pm 260$	33.6 ± 3	2.1×10^4	300 ± 15	580 ± 20	1.9×10^6	>5000	>5.5	1.1×10^4	670	13	1.9×10^4	41	594	1.4×10^6	102	247	2.4×10^6	102	247	2.4×10^6	102	247	2.4×10^6
Cefoxitin	ND	ND	1.5×10^4	3.3 ± 0.4^f	2.2 ± 0.1	6.7×10^5	11 ± 1	3 ± 0.5	2.7×10^5	140	10	7.1×10^4	26	145	5.7×10^6	26	39	1.5×10^6	26	39	1.5×10^6	26	39	1.5×10^6
Ceftazidime	ND	ND	2.1×10^4	145 ± 13^g	27 ± 3	2.0×10^5	>1000	>4	4.0×10^3	>700	>3	4.3×10^3	148	7	4.9×10^4	57	4.4	7.7×10^4	57	4.4	7.7×10^4	57	4.4	7.7×10^4
Cefotaxime	ND	ND	7.1×10^4	160 ± 20^f	140 ± 9	8.8×10^5	70 ± 8	165 ± 15	2.4×10^6	300	41	1.4×10^5	49	609	1.2×10^7	35	31	8.9×10^5	35	31	8.9×10^5	35	31	8.9×10^5
Meropenem	232 ± 9	8.9 ± 0.2	3.8×10^4	13^h	77	5.9×10^6	85 ± 3	45 ± 2	5.0×10^5	190	156	8.3×10^5	163	$1,000$	6.8×10^6	144	604	4.2×10^6	144	604	4.2×10^6	144	604	4.2×10^6
Imipenem	ND	ND	1.0×10^4	48 ± 8^g	384 ± 6	8×10^6	>1000	>200	2.0×10^5	260	15	6.0×10^4	97	$1,700$	1.7×10^7	133	518	3.9×10^6	133	518	3.9×10^6	133	518	3.9×10^6
Aztreonam	NH	ND	ND	ND	ND	ND	>1000	$<10^{-2}$	<10	NH ⁱ	ND	ND	NH	ND	ND	NH	ND	ND	NH	ND	NH	ND	ND	ND

^a Kinetic data for FEZ-1 are from reference 70.

^b Kinetic data for BJP-1 are from reference 26.

^c Kinetic data for AIM-1 are from reference 14.

^d Kinetic data for SMB-1 are from reference 15.

^e ND, not determined.

^f Kinetic data are from reference 71.

^g Kinetic data are from reference 72.

^h Kinetic data are from reference 73.

ⁱ NH, no hydrolysis.

TABLE 3 Crystallographic data collection and refinement statistics

Parameter	Value(s) for Rm3 ^a
Data collection statistics	
Beamline	DLS (I04)
Wavelength (Å)	0.9795
Space group	P2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	45.88, 74.45, 77.46
α , β , γ (°)	90, 99.48, 90
No. of molecules/asymmetric unit	2
Resolution (Å)	53.32–1.75 (1.78–1.75)
No. of unique reflections	50,630 (2,761)
Redundancy	2.4 (2.3)
<i>R</i> _{merge}	0.055 (0.363)
<i>CC</i> _{1/2}	0.997 (0.821)
<i>I</i> / σ	9.1 (2.1)
Completeness (%)	97.6 (97.2)
Refinement statistics	
Resolution (Å)	53.32–1.75 (1.78–1.75)
No. of reflections	50,593 (2,780)
<i>R</i> _{work} / <i>R</i> _{free} ^b	20.28/22.94 (31.15/32.18)
No. of:	
Protein atoms	2,022 ^c /2,029 ^d
Zinc ions	7
Water molecules	302
<i>B</i> factor	
Protein	25.58 ^c /26.65 ^d
Zinc	19.38
Water	29.25
RMSD	
Bond length (Å)	0.007
Bond angle (°)	1.09

^a Statistics for the highest-resolution shell are shown in parentheses.^b *R*_{free} was calculated with 5% of reflections omitted from the refinement.^c Chain A.^d Chain B.

and 290) form a disulfide bond between helices $\alpha 5$ and $\alpha 7$ that is common to all B3 MBLs of known structure except BJP-1 (53). In chain A of the current structure, a second disulfide between Cys208 and Cys213 constrains the short loop between strands $\beta 10$ and $\beta 11$. However, in chain B this disulfide bond is not present, Cys208 and Cys213 are reduced, and a zinc ion is positioned between them. This zinc ion is also coordinated by His246 and Glu249 of an adjacent chain and thus occupies a site that is formed at the interface of two Rm3 monomers in adjacent asymmetric units in the crystal. The final pair of Cys residues (positions 32 and 35) also contributes to a further zinc site at the interface between the two Rm3 molecules present in the crystallographic asymmetric unit, in which zinc coordination is completed by His158 of the opposing chain and by a crystallographic water molecule. However, as Rm3 eluted from the size exclusion chromatography column at a volume consistent with a molecular mass of approximately 30,000 Da (data not shown), indicating that the protein likely exists as a monomer in solution, we consider both of these interface sites to be crystallization artifacts that are unlikely to exert a physiological function.

Inspection of difference electron density maps from the early stages of refinement unambiguously identified the presence of two metal ions in the Rm3 active site. These were refined as zinc ions, on the basis of the presence of excess zinc in the crystallization

experiment and the absence of other metal ions in the crystal, as adjudged by the lack of additional peaks in an X-ray fluorescence excitation spectrum collected at the synchrotron beamline (data not shown). Both sites were refined to 100% occupancy with *B* factors similar to those of the adjacent protein atoms (Table 3). Consistent with assignment of Rm3 as a member of the B3 MBL subfamily, the two zinc ions (Zn1 and Zn2) occupy, respectively, the two binding sites that are defined by conserved residues of the MBL superfamily, i.e., a trihistidine (Zn1) site formed by His116, His118, and His196 and an Asp-His-His (Zn2) site formed by Asp120, His121, and His263 (Fig. 4). In both subunits, the two zinc ions lie approximately 3.5 Å apart (distances, 3.46 Å and 3.51 Å in chains A and B, respectively) and are connected by a bridging water molecule (Wat1; which likely exists as a hydroxide ion [54]) that is positioned asymmetrically with respect to the two metal ions and lies closer to Zn1 (1.81 to 1.90 Å) than to Zn2 (2.04 to 2.11 Å). Metal coordination is completed by a second water molecule (Wat2) that lies closer to Zn2 but also coordinates Zn1 (Wat2-Zn1 distances, 2.57 and 2.68 Å in chains A and B, respectively) and can thus also be considered to bridge the two metal ions. As a consequence, both Rm3 metal ions are five coordinated.

Five-coordinate metal ion systems can be described using the structural parameter τ [where $\tau = (\beta - \alpha)/60$] to discriminate between trigonal ($\tau = 1$) and square ($\tau = 0$) pyramidal geometries (55). For the Rm3 Zn1 site, the two angles α and β , which represent distortion from square to trigonal bipyramidal coordination, can be defined as His116-Zn1-His196 (103.5°) and Wat2-Zn1-His118 (167.4°), respectively (56), yielding a value for τ of 1.07 and indicating that coordination is best described as trigonal bipyramidal. For the Zn2 site, α and β are defined as Wat1-Zn2-His263 (127.4°) and Wat2-Zn2-Asp120 (155.9°), respectively (57), giving a value of τ of 0.475 and a coordination geometry intermediate between trigonal bipyramidal and square pyramidal. In chain B, Wat2 is less well defined by the experimental electron density but occupies a similar position, with values for τ of 0.97 for the Zn1 site and 0.41 for the Zn2 site. Thus, zinc coordination is similar in both Rm3 molecules.

Comparison with other B3 MBL structures. The PDBeFold program (58) was used to generate superpositions of chain B of Rm3 with the chains B of five other B3 MBLs of known crystal structure: L1 (PDB accession no. 1SML [59]; root mean square deviation [RMSD], 1.47 Å over 240 C- α atoms), FEZ-1 (PDB accession no. 1K07 [60]; RMSD, 1.65 Å over 254 C- α atoms), BJP-1 (PDB accession no. 3LVZ [53]; RMSD, 1.73 Å over 251 C- α atoms), AIM-1 (PDB accession no. 4AWY [61]; RMSD, 1.04 Å over 247 C- α atoms), and SMB-1 (PDB accession no. 3VPE [57]; RMSD, 0.87 Å over 245 C- α atoms). Thus, the Rm3 structure most closely resembles those of AIM-1 and SMB-1, consistent with the closer sequence relationship to these enzymes than to other structurally characterized B3 MBLs. Superposition of the B3 MBL structures (Fig. 5) identifies three regions where there is variation between the various structures: the extreme N terminus, the loop connecting helix $\alpha 4$ and strand $\beta 7$ (sometimes termed loop 1), and the loop connecting strand $\beta 11$ and helix $\alpha 5$ (loop 2 [57]). Together these three regions substantially define the active-site groove in B3 MBLs. Notably, the N-terminal region of Rm3 is poorly defined in the crystal structure, with no electron density being evident for the proline-rich sequence (QTPAPATPP) that forms the N terminus of the processed polypeptide after cleavage of the signal peptide. However, unlike the AIM-1 and SMB-1

FIG 3 Overall structure of Rm3. Stereo view of Rm3, with the protein backbone being color ramped from blue (N terminus) to red (C terminus). Active-site residues and disulfide bonds are rendered as sticks (green, carbon atoms; standard (CPK) colors were used for the other atoms). Zinc ions (gray) and water molecules (red) are shown as spheres. This figure was generated using the PyMOL program (www.pymol.org).

structures, where a turn preceding the conserved Trp41 forces relatively short N termini away from the active site, in Rm3, Trp41 is part of an α -helix (Fig. 3, $\alpha 1$) that defines one wall of a deeper active-site groove (Fig. 5). Thus, in this regard Rm3 more closely resembles BJP-1, where an extended helical N terminus creates an active site that is much narrower than the active sites of other B3 MBLs of known structure.

Loop 1 (residues 150 to 164) of B3 MBLs also contributes substantially to the active-site architecture. Hydrophobic residues (Phe156 and Ile162) in loop 1 of L1 were proposed to participate in binding of substrate (59), but subsequent directed mutagenesis investigations of L1 (62) and FEZ-1 (63) did not identify these individual positions to be essential to activity. However, rapid kinetic experiments (64) have demonstrated that this loop can adjust its position during the turnover of β -lactams by the L1 enzyme, indicating that the structure as a whole may have some mechanistic role. In addition, both AIM-1 and SMB-1 feature a Gln at position 157, where models of bound cephalosporin substrates suggest that it may interact with the carboxylate group at C-7/C-8 formed on hydrolysis of the β -lactam amide (57, 61). In Rm3, Gln157 is present as part of a DPQ motif that is also found in SMB-1, AIM-1, and THIN-B, and the organization of loop 1 closely resembles that found in AIM-1 and SMB-1 (Fig. 5). By way of contrast, loop 1 in the L1, FEZ-1, and BJP-1 structures adopts a conformation more open than is the case here.

Loop 2 (residues 224 to 230) is the third region of variability between B3 MBL structures. In common with AIM-1 and SMB-1, loop 2 of Rm3 is longer by 2 residues than its equivalent in other B3 enzymes, with the apex of this loop extending away from the active site. In L1 and FEZ-1, residues such as Asn225 (FEZ-1) and Tyr228 (both enzymes) on loop 2 are proposed to contribute to β -lactam hydrolysis through interaction with the C-7/C-8 carboxylate group of hydrolyzed species (see above) (59, 60, 62). Consis-

tent with the presence of Gln157 on loop 1 (see above), which could act as a functional replacement for these residues, the equivalent positions of Rm3 loop 2 are occupied by amino acids (Val and Pro) that are unable to replicate these proposed interactions, and the conformation of loop 2 is also incompatible with a contribution to β -lactam binding and/or hydrolysis. Loop 2 of BJP-1 also differs from the equivalent regions of L1 and FEZ-1, but in this case it is positioned in a more closed conformation nearer the zinc center. Taken together, these comparisons indicate that in both the overall fold and the specific architecture of variable regions (loops 1 and 2) adjacent to the active site, the Rm3 structure more closely resembles the structures of the mobile B3 enzymes AIM-1 and SMB-1 than it does the structures of the chromosomal B3 MBLs L1, FEZ-1, and BJP-1.

In contrast to these clear differences in overall structure between different B3 MBLs, comparison of the respective active sites indicates that the principal features of the Rm3 metal center are common between all structurally characterized B3 MBLs. Specifically, all B3 MBLs of known structure feature a binuclear zinc center with a five-coordinate ion in the Zn2 site and geometry intermediate between trigonal bipyramidal and square pyramidal, and (for structures that do not contain bound ligands) the zinc-zinc distance (3.46 Å and 3.51 Å in Rm3 chains A and B, respectively [see above]) varies between 3.40 and 3.58 Å (for structures determined at resolutions of between 1.40 Å and 1.80 Å, compared to a resolution of 1.75 Å for the structure of Rm3 presented here). With respect to other B3 enzymes, the main difference in the Rm3 active site is the positioning of the Wat2 water molecule (Fig. 4B and C), which is notably closer to both Zn1 (distances, 2.57 Å and 2.68 Å in Rm3 chains A and B, respectively) and Wat1 (distances, 2.33 Å and 1.96 Å in Rm3 chains A and B, respectively) than is the case in, e.g., L1 (Wat2-Zn1 and Wat2-Wat1 distances, 2.80 Å and

FIG 4 Rm3 active site. (A) Stereo view. Green, carbon atoms; gray, zinc ions; red, water molecules. Standard (CPK) colors were used for the other atoms. The electron density map is $2|F_o| - |F_c| \cdot \phi_{\text{calc}}$ (where F_o and F_c are observed and calculated structure factor amplitudes, respectively, and ϕ_{calc} is the calculated phase), contoured at 1.5 σ . (B) Active site of Rm3 showing the position of Wat2 relative to that of Zn1 (distance in black) and Wat1 (distance in white). (C) Active site of L1 (PDB accession no. 1SML [59]) showing the position of Wat2 relative to that of Zn1 and Wat1. This figure was generated using the PyMOL program.

3.04 Å, respectively, for the structure with PDB accession no. 1SML).

Implications of Rm3 structure for activity. Despite much effort, the precise mode of binding of β -lactams to the active site of B3 MBLs remains incompletely understood. In fact, only one crystal structure has so far been determined for a B3 MBL complexed with an antibiotic, that of L1 bound to the hydrolysis product of the oxacephem moxalactam (65); docking and quantum mechanics/molecular mechanics (QM/MM) approaches have been used to investigate the interactions of AIM-1 with hydrolyzed cefoxitin (61). We therefore used superposition of the Rm3 and L1-moxalactam structures to consider possible interactions of hydrolyzed moxalactam with Rm3 (Fig. 6A and B) in an effort to investigate determinants of β -lactamase activity and the basis for the high K_m values for β -lactam hydrolysis by Rm3 that were observed.

Consistent with the ability of Rm3 to hydrolyze most classes of β -lactams, these comparisons imply that the enzyme can replicate many of the interactions with substrates made by L1 or AIM-1. In addition to interactions involving the two metal ions (Zn1 with the C-7/C-8 carbonyl/carboxylate of the β -lactam amide and Zn2 with the C-3/C-4 carboxylate of the second ring), the Rm3 active site contains conserved residues at positions previously implicated in β -lactam binding. In particular, Ser221, a residue that is highly

conserved in B3 MBLs, and Asn223 (Ser or Thr in most other B3 enzymes) are well positioned to contact the C-3/C-4 carboxylate of bound β -lactam. Notably, in the Rm3 crystal structure, the anticipated positions adopted by the β -lactam carboxylate oxygen atoms are occupied by Wat2 and by a second water molecule (Wat3) positioned between the Ser221 and Asn223 side chains. As noted earlier and as has been proposed for AIM-1 (61) and SMB-1 (57), the Gln157 side chain is positioned to contact the C-7/C-8 carboxylate generated by β -lactam hydrolysis. Furthermore, the conserved Trp41 side chain is able to make hydrophobic interactions with the β -lactam core. Rm3 is thus able to make productive interactions with the core components common across the different classes of β -lactams.

Given this apparent availability of productive modes of substrate binding, we then considered why Rm3 hydrolyzes β -lactams with a relatively low efficiency. Inspection of molecular surfaces in the vicinity of the active site (Fig. 6C and D) indicates that, compared to other B3 enzymes in which the active site sits in a relatively shallow groove, the Rm3 active site is positioned at the bottom of a much deeper channel that runs across one side of the structure. Notably, the extended N terminus forms one wall of this cleft in the region that would be expected to form the binding site for the C-6/C-7 (R1) substituent of β -lactams, either requiring

FIG 5 Comparison of Rm3 with other B3 MBLs. The superposition of Rm3 structure upon the structures of other B3 MBLs is shown. (A to D) Overall fold of Rm3 (chain A; color-ramped from N (blue) to C (red) terminus (A), SMB-1 (PDB accession no. [3VPE](#) [57]) (B), L1 (PDB accession no. [1SML](#) [59]) (C), and BJP-1 (PDB accession no. [3LVZ](#) [53]) (D). Residues discussed in the text are rendered as sticks. This figure was generated using the PyMOL program.

substrates to adopt specific conformations on binding to avoid steric clashes or necessitating significant conformational changes of the enzyme to render the active site more accessible to β -lactams, particularly those such as later-generation cephalosporins (e.g., ceftazidime) with bulky C-7 substituents. Interestingly, for the B3 MBL BJP-1, where in the unliganded enzyme the active site is occluded by the extended N-terminal α -helix, the crystal structure of a complex with a 4-nitrobenzenesulfonamide inhibitor showed that inhibitor binding involved displacement of this entire helix from its position in the native structure in order to make the active site accessible (53). We thus propose that the high K_m values for Rm3-catalyzed hydrolysis of β -lactams arise in large part from the steric constraints upon substrate binding that are imposed by the extended N terminus. It is possible that the additional proline-

rich N-terminal sequence, comprising a further 10 amino acids that could not be modeled in our final crystal structure, could impose further restrictions upon substrate binding.

Concluding remarks. The increasing availability of sequence information from genomic and metagenomics projects has begun to establish the extent to which antibiotic resistance genes are distributed in the wider environment. It is now clear that MBLs, and the B3 subclass in particular, are frequently present on the chromosomes of environmental organisms that include, but are not limited to, opportunist human pathogens, such as *S. maltophilia* or *E. meningosepticum*. Accumulating evidence shows that the antibiotic era has been characterized by repeated instances of the mobilization of resistance determinants from environmental species, such as *Kluyvera* or *Shewanella* spp., into clinically significant pathogens

FIG 6 Proposed interactions of Rm3 with substrates. (A) Crystal structure of L1 bound to hydrolyzed moxalactam (PDB accession no. [2AIO](#) [65]). (B) Superposition of hydrolyzed moxalactam from the structure with PDB accession no. [2AIO](#) on structure of Rm3 (this work). Note that superposition places the moxalactam C-4 carboxylate over Wat2 and Wat3, N-5 and the C-4 carboxylate in proximity to Zn2, and the C-8 carboxylate close to Zn1. (C and D) Space-filling representations of the L1 complex (PDB accession no. [2AIO](#)) and Rm3 structure (this work) with bound moxalactam superposed in stick form. The extended N terminus of Rm3 (residues 32 to 43) is highlighted in pale green. This figure was generated using the PyMOL program.

and their subsequent global dissemination on multiresistance plasmids. It is also becoming apparent that exposure to detergents and biocides, as well as antibiotics, may also be implicated in the mobilization of resistance genes and coselection of multiresistance elements. In this work, we describe the properties of the product of a novel resistance gene, *bla*_{Rm3}, that was identified from an environment with high levels of biocide exposure.

*bla*_{Rm3} encodes a B3 MBL that is active against most β -lactam classes *in vitro* and is able to reduce the cephalosporin susceptibility of recombinant *E. coli*, thus replicating characteristics of enzymes of clinical importance. Sequence-based phylogeny indicates that Rm3 is representative of a distinct clade of B3 MBLs that differs from the L1 and FEZ-1/GOB groups (Fig. 2). It is likely that, given their occurrence in environmental samples from sites that differ greatly in their geographical location and level of hu-

man impact, these enzymes have a wide distribution in the environment. With increasing use of broad-spectrum β -lactams and the associated increase in selection pressure, there is thus considerable potential for future mobilization of MBLs of this type into the clinic. The structure of Rm3 demonstrates an overall resemblance to the structures of mobilized AIM-1 and SMB-1 enzymes and provides a basis for both the β -lactamase activity of Rm3 and the limited efficiency with which it hydrolyzes most substrates. However, the architecture of the active site that is created by the extended N terminus distinguishes Rm3 from other B3 MBLs that have been studied so far, suggesting both that (as has been suggested for other B3 MBLs [50]) β -lactams may not necessarily be the natural substrates for these enzymes and that there is a capacity for β -lactamase activity to be improved by mutation. Future experiments will investigate these possibilities.

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