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# Genetic and Biochemical Characterization of a Novel Metallo- $\beta$ -Lactamase, TMB-1, from an *Achromobacter xylosoxidans* Strain Isolated in Tripoli, Libya

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**An *Achromobacter xylosoxidans* strain from the Tripoli central hospital produced a unique metallo- $\beta$ -lactamase, designated TMB-1, which is related to DIM-1 (62%) and GIM-1 (51%).  $bla_{TMB-1}$  was embedded in a class 1 integron and located on the chromosome. The TMB-1  $\beta$ -lactamase has lower  $k_{cat}$  values than both DIM-1 and GIM-1 with cephalosporins and carbapenems. The  $K_m$  values were more similar to those of GIM-1 than those of DIM-1, with the overall  $k_{cat}/K_m$  values being lower than those for GIM-1 and DIM-1.**

Mobile metallo- $\beta$ -lactamases (MBLs) are becoming increasingly frequent and pose significant challenges to the treatment of Gram-negative infections worldwide, such that most MBL-producing organisms are only susceptible to colistin (2). These enzymes very efficiently hydrolyze all  $\beta$ -lactams, including carbapenems (with the exception of aztreonam), and the  $\beta$ -lactamase genes most often are located on transferable genetic platforms, namely, either ISCR elements or class 1 integrons sometimes embedded in Tn21- or Tn402-like transposons (22, 24). However, several recently characterized MBL genes have been flanked or associated with ISCR elements, namely,  $bla_{SPM-1}$  with ISCR4,  $bla_{NDM-1}$  with ISCR1, and  $bla_{AIM-1}$  with ISCR16 (6, 21).

Several different MBL-type enzymes have been described, with NDM, IMP, and VIM derivatives being the most widespread (2). The  $bla_{IMP}$ -like (17) and  $bla_{VIM}$ -like (4) genes have been identified in most clinically relevant bacteria belonging to the *Enterobacteriaceae* family, in *Pseudomonas* spp., and in *Acinetobacter* spp., while  $bla_{NDM-1}$  has mainly been found in *Enterobacteriaceae* (2, 6, 11, 27). Several other MBLs have been identified in specific geographical locations, including SIM-1 from *Acinetobacter baumannii* in Korea (8) and KHM-1 from *Citrobacter freundii* in Japan (16). SPM-1 in Brazil (10, 23), GIM-1 in Germany (3), and AIM-1 in Australia (T. R. Walsh, unpublished data) were all identified in *Pseudomonas aeruginosa*.

As hospitalized patients are subject to infections by Gram-negative bacteria, and because in Libya adherence to internationally accepted infection control policies is not optimal, we examined the hospital wards and immediate hospital environment for resistance to extended-spectrum cephalosporins. This study reports these findings and further describes the genetic and biochemical characterization of a novel MBL, TMB-1 (for Tripoli metallo- $\beta$ -lactamase), from Tripoli, Libya.

## MATERIALS AND METHODS

**Bacterial strains and susceptibility testing.** A total of 38 nonclinical environmental swabs (from hospital wards, cafes, corridors, ventilators, floors, bedside cabinets, oxygen cylinders, electrocardiograph machines, and toilets of intensive care unit wards) were collected from and near four major hospitals in Tripoli. The swabs were transferred to the laboratory in transferring charcoal media, and bacteria were selected by culturing on MacConkey agar (Oxoid, United Kingdom) supplemented with 100 mg/

liter vancomycin (to eliminate the growth of Gram-positive bacteria) and 2 mg/liter of ceftazidime to select for strains resistant to extended-spectrum cephalosporins. Isolates were initially identified by the use of Phoenix (Becton and Dickinson) and confirmed by API 20NE (bioMérieux, La Plaine, France). The susceptibility tests were performed by Phoenix 100 (Becton Dickinson, Oxford, United Kingdom) and Etest strips (bioMérieux, La Plaine, France) and were interpreted by the European Committee on Antimicrobial Susceptibility Testing ([http://www.eucast.org/eucast\\_disk\\_diffusion\\_test/breakpoints/](http://www.eucast.org/eucast_disk_diffusion_test/breakpoints/)).

**Phenotypic and molecular detection of MBLs.** Phenotypic MBL detection was carried out using the new MBL Etest strips with meropenem as a substrate (bioMérieux, La Plaine, France), and the results were interpreted according to the manufacturer's instructions.

**Identification of  $bla_{TMB-1}$ , PCR experiments, and cloning.** Molecular screening was performed on all isolates targeting the conserved region of class 1 integrons, ISCR elements, and Tn21- and Tn402-like transposons (Table 1). The PCR conditions were undertaken as previously described (24). Where possible, PCR products from the conserved region of class 1 integrons were run on 1% (wt/vol) agarose gels, purified, and subcloned into *Escherichia coli* TOP10 cells (Invitrogen, Life Technologies Ltd., Paisley, United Kingdom) and sequenced using the primers listed in Table 1. All of the PCR products were run on 1% (wt/vol) agarose gel, purified, and sequenced using an automated sequencer (AB 377; Perkin-Elmer, CT).

**Conjugation experiments.** The conjugational transfer of antibiotic resistance to the laboratory strain *E. coli* J53 (azide resistant) and *Pseudomonas aeruginosa* PAO1 (rifampin resistant) was carried out on blood agar without selection. After 18 h, the mixed cultures were washed from the plates, suspended in saline, and plated onto MacConkey agar containing sodium azide (100  $\mu$ g/ml) and ceftazidime (2  $\mu$ g/ml) or rifampin (50  $\mu$ g/ml) and ceftazidime (2  $\mu$ g/ml). Ceftazidime-resistant colonies were screened for  $bla_{TMB-1}$  by the primers listed in Table 1.

**Hybridization.** Gel plugs of chromosomal DNA were prepared and restricted with S1 nuclease as previously described (27). Hybridization

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TABLE 1 Primers used in this study

Gene target	Primer no. <sup>a</sup>	Primer name	Primer sequence	Reference or source
Integrase gene	1	VAF	GCC TGT TCG GTT CGT AAG CT	22
Quaternary ammonium compound	2	QacR	CGG ATG TTG CGA TTA CTT CG	22
TMB-1	3	Trip-1F61	GCC AAC GAA GAA ATA CCC GC	This study
TMB-1	4	Trip2-10	TGG GCT AGG TTA CAC TGG TG	This study
TMB-1	5	Trip617R	TTC TAG CGG ATT GTG GCC AC	This study
TMB-1	6	Trip2	CAA GGA GCT CAT TCA AAGG	This study
TMB-1	7	Trip1	GGA GCA GGC AAG GAG CT	This study
TMB-1	8	Trip75	ACC CGG ATT GGA AGT TGA GG	This study
TMB-1	9	Trip1 FF	TGA TCA GTG GCC ACA ATC CG	This study
TMB-1	10	Trip1 F	CGG ATT GTG GCC ACT GAT CA	This study
<i>dhfrA</i>	11	dhfrA 1F	CGA AGA ATG GAG TTA TCG GG	This study
<i>dhfrA</i>	12	dhfrA 1R	GTT AGA GGC GAA GTC TTG GG	This study
<i>dhfrA</i>	13	dhfrA 1FF	CCC AAG ACT TCG CCT CTA AC	This study
<i>aac6II</i>	14	aac6II R	GGC GTC GGC TTG AAT GAG TT	This study
<i>aac6II</i>	15	aac6II F	AAG TGG CAG CAA CGG ATT CG	This study
<i>aac6II</i>	16	aac6II FR	GAA TCC GTT GCT GCC ATC TG	This study
<i>aac6II</i>	17	aac6II FF	CAA CTC ATT CAA GCC GAC GC	This study
Oxa-4	18	oxa-4-FR	CAC TTA TGG CAT TTG ATG CG	This study
Oxa-4	19	oxa-4-F	CGC ATC AAA TGC CAT AAG TG	This study
Tn21	NA	Tn21 A	GGG GTC GTC TCA GAA AAC GG	22
Tn21	NA	Tn21 B	GGA AAA TAA AGC ACG CTA AG	22
Tn402	NA	Tn402F	GTC GTT TTC AGA AGA CGG C	24
Tn402	NA	Tn402R	CTA TGC TCA ATA CTC GTG TGC	24
ISCR1	NA	CRFF	GGT TGC AAC GAC TCA AGCG	6
ISCR1	NA	RECR1	CAC TCG TTT ACC GCT CAA GC	6

<sup>a</sup> The primer numbers are the same as those used in Fig. 1.

was performed in a gel. Briefly, the gel was dried for 5 h at 50°C and then rehydrated in double-distilled water for 5 min before 30-min incubations in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralizing solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) at room temperature. The gel was then prehybridized at 65°C using prehybridizing solution (20 ml; 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] polyvinylpyrrolidone 400, 0.1% [wt/vol] Ficoll, 0.1% [wt/vol] bovine serum albumin [BSA; Cohn fraction V], 0.5% [wt/vol] SDS, and 150 μg/ml denatured calf thymus DNA) for at least 4 h before adding the probe. The <sup>32</sup>P-labeled probe was added for a minimum of 8 h at 65°C before being removed with washes of 2× SSC, 0.1% SDS for 1 h, followed

by 0.1× SSC, 0.1% SDS for 3 h; both washes were performed at 65°C. *bla*<sub>TMB-1</sub> probes were labeled with <sup>32</sup>P by using a random primer technique (Stratagene, La Jolla, CA).

**Purification of TMB-1.** To negate posttranslational modification in an unnatural host, TMB-1 was purified directly from strain AES301, which was grown overnight at 37°C in Terrific broth (Sigma, St. Louis, MO). TMB-1 was purified from the periplasm (15), followed by ion exchange (Q-Sepharose HP column; Pharmacia, GE Healthcare, United Kingdom) and gel filtration (Superdex 200 column; GE Healthcare) using 50 mM Tris (pH 7.2), 100 μM ZnCl<sub>2</sub>, 0.02% (wt/vol) sodium azide, with or without NaCl, as the buffer system. Fractions containing TMB-1 were

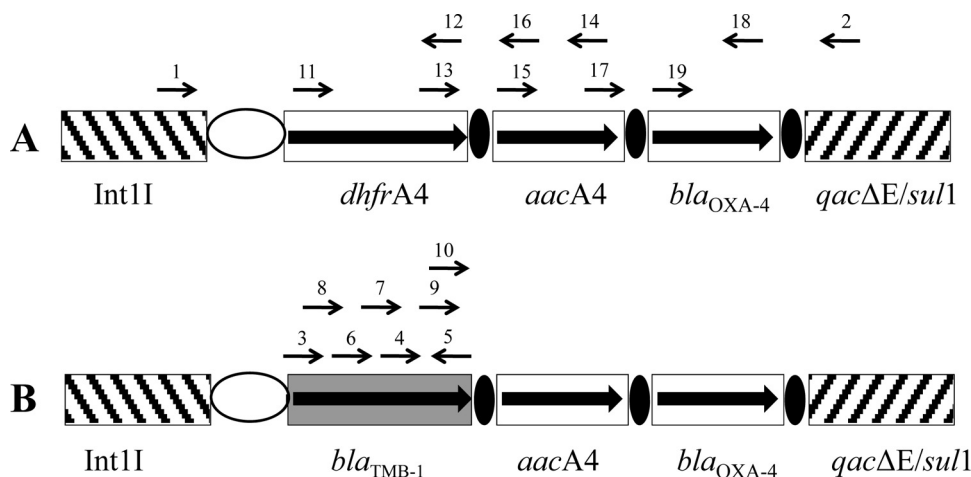


FIG 1 Genetic context of two class 1 integrons found in *A. xylosoxidans* and the primers used to sequence the structures. (A) Class 1 integron consisting of the gene cassettes containing *dhfrA4*, *aacA4*, *bla*<sub>OXA-4</sub>, and the *qacΔE/sulI* fusion. (B) Class 1 integron consisting of the gene cassettes containing *bla*<sub>TMB-1</sub>, *aacA4*, *bla*<sub>OXA-4</sub>, and *qacΔE/sulI*. The white ellipses represent the hybrid promoter from *intI1*. The black ellipses represent the 59-bp elements at the start of each gene cassette. The arrows depict primers used to amplify and sequence the integrons, and the sequences are given in Table 1.

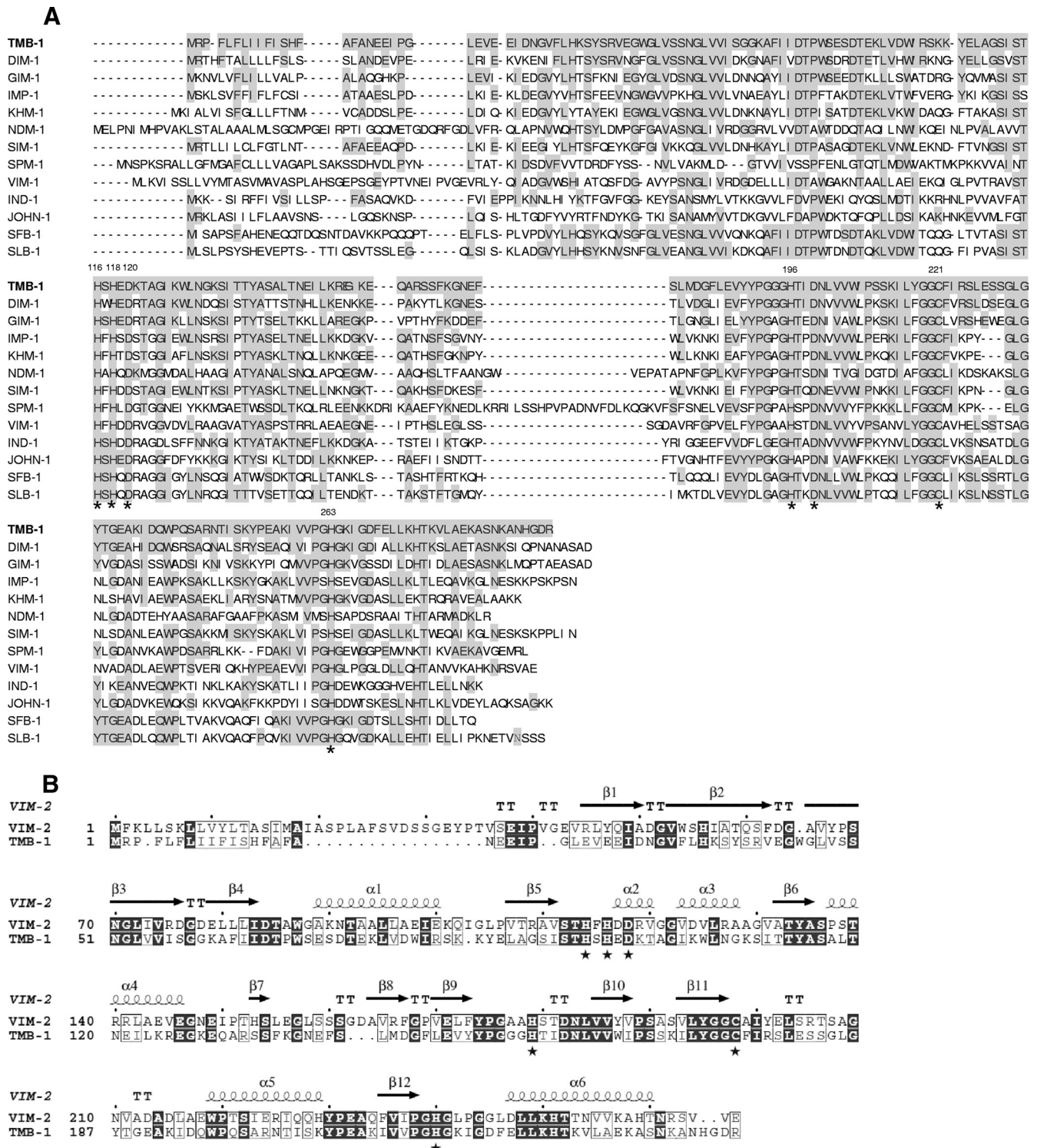


FIG 2 (A) Comparison of amino acid sequence of the  $\beta$ -lactamase TMB-1 to those of other acquired MBLs (DIM-1, GIM-1, IMP-1, KHM-1, NDM-1, VIM-1, SPM-1, and SIM-1) and several naturally occurring MBLs (IND-1 from *Chryseobacterium indologenes*, JOHN-1 from *Flavobacterium johnsoniae*, SLB-1 from *Shewanella livingstonensis*, and SFB-1 from *Shewanella frigidimarina*). Shaded amino acids are those conserved with TMB-1.  $\beta$ -Lactamase numbering was according to the BBL nomenclature (5). (B) Secondary structure of TMB-1 compared to that of VIM-2. The  $\beta$ -strands and  $\alpha$ -helices are indicated above the TMB-1 sequence. The conserved residues are indicated in black. The conservative amino acid substitutions are boxed. The figure was obtained using ESPript software (<http://esprict.ibcp.fr/ESPript/ESPript/>).



TABLE 2 Steady-state kinetic constants of TMB-1, DIM-1, and GIM-1

Compound	Steady-state kinetic constants of <sup>c</sup> :								
	TMB-1			DIM-1 <sup>a</sup>			GIM-1 <sup>b</sup>		
	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{\text{cat}}/K_m$ (s <sup>-1</sup> /μM)	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{\text{cat}}/K_m$ (s <sup>-1</sup> /μM)	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (μM)	$k_{\text{cat}}/K_m$ (s <sup>-1</sup> /μM)
Ampicillin	3.3	27	0.122	20	110	0.182	3.3	20	0.165
Piperacillin	3.3	72	0.046	NR	NR	NR	6.9	69	0.1
Cefoxitin	0.3	69	0.004	8	20	0.4	8.3	206	0.04
Cefuroxime	0.1	9	0.011	NR	NR	NR	5.9	7	0.843
Ceftazidime	0.07	31	0.002	3	50	0.06	18	31	0.58
Ertapenem	0.4	31	0.013	NR	NR	NR	NR	NR	NR
Imipenem	1.7	200	0.009	35	80	0.438	27	287	0.094
Meropenem	1.4	75	0.019	50	10	5	2.7	25	0.108
Aztreonam	<0.01	ND	ND	<0.01	ND	ND	ND	ND	ND

<sup>a</sup> Data are from Poirrel et al. (12).<sup>b</sup> Data are from Castanheira et al. (3).<sup>c</sup> NR, not reported. ND, not detected.

analyzed using nitrocefin with or without EDTA, and the fractions were analyzed by SDS-PAGE (Invitrogen, CA). TMB-1 was concentrated to 1.94 mg/ml using ultrafiltration (Millipore, MA).

**Kinetics assays.** Steady-state kinetics were performed at 25°C in a spectrophotometer (SpectramaxPlus; Molecular Devices) using 96-well plates (BD Falcon UV microplates; BD Biosciences) (15). All substrates were tested as duplicates using 50 mM HEPES buffer, pH 7.2, 100 μM ZnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, and 0.1 mg/ml bovine serum albumin (Sigma-Aldrich) as a buffer system. The kinetic data were analyzed by nonlinear regression (GraphPad Software, San Diego, CA).

**Nucleotide sequence accession number.** The full sequence of the 3-kb class 1 integron reported in the present study has been submitted to the EMBL/GenBank and assigned nucleotide sequence accession number [FR771847](https://www.ncbi.nlm.nih.gov/nuclot/FR771847).

## RESULTS AND DISCUSSION

**Analysis of swabs from Tripoli hospitals.** All swabs yielded isolates capable of growing on 2 μg/ml ceftazidime and 10 μg/ml vancomycin. All isolates were screened for class 1 integrons and mobile genetic elements (Tn21, Tn402, and ISCR elements), and 4 out of 38 isolates were positive for class 1 integrons: one *Achromobacter xylosoxidans* isolate (two integrons of ~3 kb), one *Stenotrophomonas maltophilia* isolate (2.5 kb), and two isolates of *Citrobacter freundii*, each positive for a class integron of 1 kb. None of the isolates were positive for Tn21, Tn402, and ISCR elements. *A. xylosoxidans* AES301 displayed the following MIC profile: imipenem, 2 μg/ml; meropenem, 4 μg/ml; cefepime, 16 μg/ml; ceftazidime, 8 μg/ml; cefotaxime, 32 μg/ml; aztreonam, 16 μg/ml; amikacin, 8 μg/ml; gentamicin, 8 μg/ml; ciprofloxacin, 1 μg/ml; and colistin, 0.5 μg/ml. All isolates that grew on media containing ceftazidime were screened by the MBL Etest strip to detect the presence of MBLs. Apart from the *S. maltophilia* isolate (which naturally carries the L1 MBL) (26), the only other MBL-positive isolate was an *A. xylosoxidans* strain, designated AES301, possessing the class 1 integron, and it was investigated further.

**Genetic analysis of carbapenem resistance in *A. xylosoxidans* strain AES301.** The sequencing analysis of the class 1 integron PCR products from *A. xylosoxidans* AES301 revealed two nearly identical integrons, the first possessing the gene cassette *dhfrA4-aacA4-bla<sub>OXA-4</sub>* and the second integron containing the gene cassette *bla<sub>TMB-1</sub>-aacA4-bla<sub>OXA-4</sub>* (Fig. 1). The carbapenem resistance could not be mated to either *E. coli* DH5α or *P. aeruginosa* PAO1 recipients (data not shown), suggesting that the *bla<sub>TMB-1</sub>* integron

is chromosomally located. This inference was supported by Southern hybridization data using the *bla<sub>TMB-1</sub>* gene as a probe, which was back blotted to the *A. xylosoxidans* AES301 chromosome (data not shown) even though it produced several plasmids.

The TMB-1 gene contains 735 nucleotides and encodes a protein of 245 amino acids possessing all of the key motifs of Ambler class B β-lactamase. At the amino acid level, TMB-1 was most closely related to DIM-1 (62%) and GIM-1 (51%) and showed only 48, 31, and 29% identity to IMP-1, VIM-2, and NDM-1, respectively (Fig. 2A). TMB-1 also possesses virtually the same key residues as DIM-1 that make up the zinc binding residues and the secondary residues supporting the active sites, including the putative loop used to facilitate the binding of β-lactams during hydrolysis (Fig. 2A). A secondary structural comparison of TMB-1 to VIM-2 shows that TMB-1 possesses the key zinc binding residues for B1 MBLs, namely, His116, His118, and His196 (zinc 1) and Asp120, Cys221, and His263 (zinc 2) (Fig. 2A). The most noticeable difference between TMB-1 and VIM-2 is a gap in the N terminus of the TMB-1 protein just before the beginning of the first β-sheet (β1 in Fig. 2B). This gap in TMB-1 is situated just prior to the flapping loop of VIM-2, which has been shown to facilitate the binding and hydrolysis of substrates (1). Further, there are several amino acid differences in this region, namely (comparing VIM-2 to TMB-1), Q60S, S61R, F62V, D63E, A66G, V67L, and a gap at position 65. This region is also diverse between VIM-2 and VIM-7, where it has been suggested that it contributes to a more flexible flapping loop (1). Interestingly, DIM-1 possesses the same sequence as TMB-1 in this region, with the exception of the gap and the amino acid changes N63E and F65W (12 and data not shown). An additional gap in TMB-1 between β7 and β8 compared to the VIM-2 sequence is also observed (Fig. 2B).

**Kinetic properties of TMB-1.** The kinetic properties of TMB-1 were compared to those of DIM-1 and GIM-1 (Table 2) and are broadly similar, with the exception of the rate of turnover of substrates ( $k_{\text{cat}}$  values) (Table 2). The  $K_m$  values for TMB-1 are similar to those of DIM-1 and GIM-1 for the penicillins and cephalosporins but are larger for meropenem, indicating that TMB-1 binds meropenem comparatively weakly. The  $k_{\text{cat}}$  values for TMB-1 are similar between the penicillins and GIM-1 but are markedly less (30- to 260-fold) than those for both DIM-1 and GIM-1 for ce-

foxitin, cefuroxime, and ceftazidime (Table 2). TMB-1 also possesses lower  $k_{\text{cat}}$  values for the carbapenems (2- to 36-fold) compared to those of DIM-1 and GIM-1. These data further show that the efficiency of the enzyme ( $k_{\text{cat}}/K_m$ ) is lower for the cephalosporins and carbapenems (Table 2). Such differences in kinetic values are interesting, given that TMB-1 and DIM-1 are similar and that their sequences in the VIM-2 flapping loop (15) are nearly identical, further suggesting that the reasons for these kinetic differences lie elsewhere in the TMB-1 structure (Fig. 2B).

*Achromobacter* is not the most important pathogen, although a growing number of reports indicate that it is capable of causing urinary tract infections (20), ocular infections (13), and the contamination of dialysis (25) and ultrasound equipment (9), and it can cause additional complications in cystic fibrosis patients (7, 14). Interestingly, although AES301 carrying TMB-1 was found in a ward surface swab, the same strain could not be identified from a clinical source; however, in Libya clinical diagnostic microbiology laboratories may not scrutinize strains to the species level. To date, only two cases of MBL genes (both *bla*<sub>VIM-2</sub>) have been reported from *Achromobacter* spp., from Greece (19) and Korea (18), and both were carried by class 1 integrons. This is the first MBL reported from Libya, and being a new subclass, it provides further evidence of the structural heterogeneity of this group of  $\beta$ -lactamases.

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