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ZHO-1, an intrinsic MBL from the environmental Gram-negative species *Zhongshania aliphaticivorans*

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Objectives: Our aim was to characterize the putative MBL of the environmental strain *Zhongshania aliphaticivorans* isolated from a marine environment.

Methods: The putative MBL was identified *in silico* using the NCBI database. The β-lactamase gene was cloned into different *Escherichia coli* backgrounds. Kinetic parameters were determined using the purified enzyme.

Results: The enzyme named ZHO-1 shared 51% amino acid identity with the acquired class B carbapenemases IMP-1, KHM-1 and DIM-1. Expression of the bla_{ZHO-1} gene in a susceptible *E. coli* resulted in a carbapenemase phenotype. Kinetic parameters determined from purified ZHO-1 enzyme showed that it had significant hydrolytic activity against most β-lactams including penicillins, cephalosporins and carbapenems, with the exception of aztreonam and cefepime.

Conclusions: This study adds to the knowledge regarding environmental species as a reservoir of possible clinically relevant MBLs.

Introduction

MBLs are zinc-dependent enzymes capable of hydrolysing all classes of β-lactams except monobactams. They constitute a highly diverse family of enzymes and can be categorized into three subclasses, namely B1, B2 and B3. The subclass B1 enzymes are the most important clinically since they comprise MBLs such as IMP-1, NDM-1, SPM-1, KHM-1 and VIM-1/-2,[1] widely identified in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. The spread of MBL genes is associated with mobile genetic elements such as composite transposons and class 1 integrons. It is well known that environmental bacterial species constitute an important reservoir of antimicrobial resistance genes.[2] According to a recent metagenomic study, the marine environment in particular constitutes a significant reservoir of MBL producers.[3]

In this study, we characterized a novel subtype B1 MBL showing a significant relationship to the IMP-1 enzyme whose gene was identified on the chromosome of an environmental and marine species named *Zhongshania aliphaticivorans*.

Materials and methods

In silico sequence analyses

The putative bla_{ZHO-1} gene was identified using the NCBI blast alignment tool. Phylogenetic analysis was performed using the alignment software SeaView (Prabi, La Doua, France).

Bacterial isolates and susceptibility testing

Strain *Z. aliphaticivorans* SM-2^T was cultured on marine agar (Roth AG, Arlesheim, Switzerland) at 30°C for 36 h. Antimicrobial susceptibility testing was performed according to the standard disc diffusion method using marine agar plates and Mueller–Hinton (MH) agar plates (Bio-Rad, Cressier, Switzerland) for strain SM-2^T and the *Escherichia coli* recombinant strains, respectively, following CLSI recommendations.[4] MICs were determined by broth microdilution using MH broth.

Cloning and expression of the bla_{ZHO-1} gene

The bla_{ZHO-1} gene was cloned into the pTOPO cloning vector using the pCR-blunt TOPO cloning kit (Invitrogen, Illkirch, France) with specific primers

(ZHO-1-Fw: 5′-GCGCAGCCCAATGTCTATTG-3′ and ZHO-1-Rv: 5′-GCCGAGCGTCAATTA CTGTC-3′). The resulting recombinant plasmid was electroporated into *E. coli* TOP10 (pZHO-1-TOP) and into OmpC/OmpF porin-deficient *E. coli* HB4 (pZHO-1-HB4) strains to analyse the additional effect of porin deficiency on the acquired resistance phenotype.

Purification of β-lactamase ZHO-1

Four litres of LB broth supplemented with ampicillin (100 mg/L) was inoculated with *E. coli* TOP10 carrying pZHO-1-TOP for 24 h at 37°C with shaking. The bacterial culture was centrifuged and the pellet was resuspended in Tris–HCl buffer pH 7.5 (100 μM) and sonicated using a Vibra cell™ 75186 sonicator (Thermo Fisher Scientific). After filtration using a 0.22 μm nitrocellulose filter, the crude extract was loaded in a Q-Sepharose column connected to an ÄKTAprime chromatography system (GE Healthcare, Glattbrugg, Switzerland) and eluted with a linear NaCl gradient. The presence of the β-lactamase was monitored using nitrocefin (200 μM). All positive fractions were pooled and dialysed overnight at 4°C against HEPES buffer (0.1 M, pH 7.5) supplemented with $ZnSO_4$ (5 μM). The protein concentrations were measured using Bradford reagent (Sigma–Aldrich, Buchs, Switzerland). The purity of the enzyme was estimated by SDS–PAGE analysis (GenScript, NJ, USA).

Kinetic measurements

Kinetic measurements were performed at room temperature in HEPES buffer (0.1 M, pH 7.5) supplemented with $ZnSO_4$ (5 μM) using a UV/visible ULTROSPEC 2100 pro spectrophotometer (Amersham Biosciences, Buckinghamshire, UK).

Determination of the zinc concentration dependence of ZHO-1

In order to determine the effect of different concentrations of $ZnSO_4$ on the enzymatic activity of ZHO-1, three different conditions were tested: (i) a zinc-deprived condition in the presence of 50 mM EDTA; (ii) a standard condition in the presence of 5 μM $ZnSO_4$; and (iii) a zinc-excess condition in the presence of $ZnSO_4$ at 50 μM. These conditions were tested with three different substrates, namely benzylpenicillin, cefalotin and meropenem.

Results and discussion

Analysis of the *Z. aliphaticivorans* SM-2^T genome sequence (accession number: NZ_CP014544) revealed a putative chromosomally encoded MBL gene that was named *bla*$_{ZHO-1}$. The GC content of 42.6% of the gene corresponds to that of chromosomally encoded genes of that species. No mobile element was detected surrounding the gene, suggesting that the *bla*$_{ZHO-1}$ gene was constitutive for that species. The *bla*$_{ZHO-1}$ gene encoded a putative β-lactamase (ZHO-1) sharing 51% amino acid identity with the following acquired class B1 MBLs: IMP-1 identified originally in *Serratia marcescens* and *Pseudomonas aeruginosa*[5], KHM-1 in *Citrobacter freundii*[6] and DIM-1 in *Pseudomonas stutzeri*.[7] Protein alignments of the β-lactamase ZHO-1 with protein structures of these other class B MBLs revealed the conserved amino acid residues known to be involved in the hydrolytic activity of MBLs such as the histidine residues at positions 116, 118, 196 and 263, the aspartic acid residue at position 120 and the cysteine residue at position 221, respectively [class B β-lactamase (BBL) nomenclature] (Figure 1).[8]

Antimicrobial susceptibility testing performed with *Z. aliphaticivorans* strain SM-2^T demonstrated susceptibility to all β-lactams. However, once expressed in *E. coli* TOP10, ZHO-1 conferred high resistance levels to aminopenicillins, carboxypenicillins, and narrow- and broad-spectrum cephalosporins including cefalotin, cefoxitin, cefotaxime and ceftazidime, and intermediate susceptibility to ureidopenicillins (such as piperacillin). However, *E. coli* TOP10 carrying pZHO-1-TOP remained susceptible to cefepime and the monobactam aztreonam. Susceptibility to monobactams is a feature of class B MBL producers. Notably, expression of ZHO-1 in porin-deficient *E. coli* HB4 conferred additional resistance to meropenem and ertapenem, and an intermediate level of resistance to cefepime (Table 1).

The purification of the ZHO-1 enzyme was estimated to be >90% with a single dominant band visible on the SDS-polyacrylamide gel. Kinetic parameters of ZHO-1 showed a hydrolytic profile of a class B carbapenemase (Table 2). Actually, strong hydrolytic activities were observed for most β-lactams tested. The activity

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ZHO-1      MRPIFVALLL T LTLMSHAQ VWASEELPPL KIQQLTDSVY LHISHKVVDG FGLVDSNGLV VLIGSEAYIV DTPWSTQDTE
IMP-1      /////MSKLS VFFIFLFCSI ATAAESL-D- K-EKLDEGVY VHTSFEEVN- W-V-PKH--- -LVNAE-YLI ---FTAK---
KHM-1      MK///IALVI SFGLLLFTNM VCADDSL-E- D-QKIEDGVY LYTAYEKIE- W-L-GSN--- -LDNKN-YLI ---ISAT---
DIM-1      MRTHFTA/// //LLLLFSLS SLANDEV-E- R-EKVKENIF LHTSYSRVN- F-L-SSN--- -IDKGN-FIV ---WSDR---
                      90        116 118 120 110        120        130        140        150        160
ZHO-1      TLLQWINAQG FTLKSVVSTH FHEDRTAGIE YLNANAIPTY ASARTNKILQ FGLVDSNGLV RQGRPLAANT FNKDKFSLVK
IMP-1      K-VT-FVER- YKIKGSI-S- F-S-S-G--E W--SRS-PTY -SEL--EL-K W-V-PKH--- KDGKVQ-TNS FSGVNYWLVK
KHM-1      K-VK-IDAQ- FTAKASI-T- F-T-S-G--A F--SKS-PTY -SKL--QL-K W-L-GSN--- NKGEEQ-THS FGKNPYWLLK
DIM-1      T-VH-IRKN- YELLGSV-T- W-E-R-A--K W--DQS-STY -TTS--HL-K F-L-SSN--- ENKKEP-KYT LKGNESTLVD
                  170 196    180        190 221     200        210        220        230 263    240
ZHO-1      AHIEVFYPGA GHAQDNVVVW LPEQKLLFGG CLIRANAATS LGNTSDAVLS AWSASVEELQ SRYADAKLVV PGHGDVGDVS
IMP-1      NK--V--PGP -TP--VVVW --ER------ FIKPY///G --NLGD-NIE A-PK-AKLLK SK-GK-KL-V -S-SEV--AS
KHM-1      NK--A--PGA -TP--LVVW --KQ------ FVKPE///G --NLSH-VIA E-PA-AEKLI AR-SN-TM-V -G-GKC--AS
DIM-1      GL--V--PGG -TI--VVVW --KS------ FVRSLDSEG --YTGE-HID Q-SR-AQNAL SR-SE-QI-I -G-GKI--IA
                  250
ZHO-1      LLEHTRVLAT VGQAVSK*
IMP-1      --KL-LEQ-V KGLNESKKPS KPSN*
KHM-1      --EK-RQR-V EALAAKK*
DIM-1      --KH-KSL-E TASNKSIQPN ANASAD*
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Figure 1. Amino acid sequence comparison between ZHO-1 and its closest MBL relatives. Shaded areas show conserved amino acid residues corresponding to the binding site with the zinc ion required for the hydrolytic activity. Numbers in bold represent the BBL nomenclature.

Table 1. MICs (mg/L) of β-lactams for *E. coli* strains (a WT strain and a porin-deficient strain) with and without the ZHO-1 β-lactamase gene

Antibiotics	*E. coli* TOP10 (pZHO-1)	*E. coli* HB4[a] (pZHO-1)	*E. coli* TOP10 empty vector	*E. coli* HB4[a] empty vector	*Z. aliphaticivorans*
Amoxicillin	**128**	>**128**	2	16	0.5
Amoxicillin/clavulanic acid[b]	**128**	>**128**	1	16	0.5
Piperacillin	32	64	1	8	0.5
Cefalotin	**128**	>**128**	4	**128**	0.5
Cefoxitin	**128**	>**128**	2	64	0.5
Ceftazidime	**4**	**128**	0.12	1	0.06
Cefotaxime	**64**	**64**	0.06	0.5	0.06
Cefepime	0.5	4	0.12	0.5	0.06
Aztreonam	0.06	0.5	0.06	0.5	0.06
Imipenem	0.5	0.5	0.06	0.06	0.06
Meropenem	1	**4**	0.06	0.25	0.06
Ertapenem	1	**4**	0.06	1	0.06

Underlined MIC values correspond to intermediate susceptibility and bold MIC values correspond to resistance.
[a]*E. coli* HB4 is an OmpC/OmpF porin-deficient strain.
[b]Clavulanic acid was added at a fixed concentration of 2 mg/L.

Table 2. Kinetic parameters of the ZHO-1 MBL

Antibiotics	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Penicillin G	85	150	220
Piperacillin	50	1350	40
Cefalotin	4500	20	>10 000
Cefoxitin	2	150	15
Ceftazidime	1	150	5
Cefotaxime	25	35	750
Cefepime	0.5	850	0.5
Aztreonam	<0.01	>1000	ND
Imipenem	40	120	325
Meropenem	10	15	500

ND, not determined.

against broad-spectrum cephalosporins was particularly high, except for cefepime and ceftazidime. Notably, the weak activity observed against ceftazidime was not owing to a lack of affinity considering the very low K_m value but rather to a poor hydrolysis rate. Noticeably, ZHO-1 also showed significant activity against carbapenems. Finally, no enzymatic activity was detected for aztreonam, which is a common feature of MBLs.

Zinc dependence experiments showed that in the presence of EDTA, the enzymatic activity decreased by >50% for each substrate tested. However, no change in activity was observed when increasing the $ZnSO_4$ concentration. These results confirmed that ZHO-1 activity is zinc dependent, a common feature of B1 MBLs.

This study characterized a novel B1 MBL identified from an environmental species recovered from a marine sample. *Z. aliphaticivorans* SM-2^T is an environmental species recovered in 2013 from sea tidal flats in the Dangjin coastal area of South Korea.[9] Members of the *Zhongshania* genus are Gram-negative, oxidase-positive bacilli, all identified from a marine environment. ZHO-1 showed its ability to hydrolyse most β-lactams including carbapenems, but sparing aztreonam and cefepime. Such an enzyme might constitute a potential threat if acquired by clinically relevant species (such as *Klebsiella pneumoniae* or *P. aeruginosa*). Notably, the *Z. aliphaticivorans* SM-2^T isolate remained susceptible to all β-lactams despite the presence of the bla_{ZHO-1} gene on its chromosome. Such a discrepancy might be the consequence of poor expression of this gene in its progenitor. A similar phenomenon has been observed for several environmental species identified as progenitors of carbapenemases, such as *Shewanella xiamenensis* or *Acinetobacter radioresistens*, which are the progenitors of the $bla_{OXA-181}$ and bla_{OXA-23} genes, respectively.[10,11] Finally, these results raise the potential concern of the spread of a hidden reservoir of carbapenemase genes.

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Transparency declarations

None to declare.

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