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SMB-1, a Novel Subclass B3 Metallo-β-Lactamase, Associated with IS*CR1* and a Class 1 Integron, from a Carbapenem-Resistant *Serratia marcescens* Clinical Isolate▽

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A carbapenem-resistant *Serratia marcescens* strain, 10mdr148, was identified in a Japanese hospital in 2010. The carbapenem resistance of this strain was attributed to the production of a novel metallo-β-lactamase (MBL), named SMB-1 (*Serratia* metallo-β-lactamase). SMB-1 possessed a zinc binding motif, H(Q)XHXDH (residues 116 to 121), H196, and H263 and was categorized as a member of subclass B3 MBL. SMB-1 has 75% amino acid identity with the most closely related MBL, AMO1, of uncultured bacterium, recently identified through the metagenomic analysis of apple orchard soil. The introduction of bla_{SMB-1} into *Escherichia coli* conferred resistance to a variety of β-lactam antibiotics, penicillins, cephalosporins, and carbapenems, but not aztreonam, a resistance pattern consistent with those of other MBLs. SMB-1 demonstrated high k_{cat} values of $>500\ s^{-1}$ for carbapenems, resulting in the highest hydrolyzing efficiency (k_{cat}/K_m) among the agents tested. The hydrolyzing activity of SMB-1 was well inhibited by chelating agents. The bla_{SMB-1} gene was located on the chromosome of *S. marcescens* strain 10mdr148 and at the 3′ end of the IS*CR1* element in complex with a typical class 1 integron carrying *aac(6′)-Ib* and *catB3* gene cassettes. Downstream of bla_{SMB-1}, the second copy of the 3′conserved segment and IS*CR1* were found. To our knowledge, this is the first subclass B3 MBL gene associated with an IS*CR1* element identified in an *Enterobacteriaceae* clinical isolate. A variety of antibiotic resistance genes embedded with IS*CR1* have been widely spread among *Enterobacteriaceae* clinical isolates, thus the further dissemination of bla_{SMB-1} mediated by IS*CR1* transposition activity may become a future concern.

The emergence of carbapenem resistance in *Enterobacteriaceae* clinical isolates is becoming a substantial clinical concern, because carbapenem antibiotics remain important agents for the treatment of infectious diseases caused by pathogenic *Enterobacteriaceae* in clinical settings (2, 7). The carbapenem resistance of these bacterial strains is due mostly to the production of horizontally acquired β-lactamases that are capable of hydrolyzing carbapenems, like IMP-1 metallo-β-lactamase (MBL), which was first characterized in a *Serratia marcescens* clinical isolate in Japan (18), and a KPC β-lactamase was first identified from a *Klebsiella pneumoniae* clinical isolate in the United States (31). As for MBLs, the identification of IMP- and VIM-type MBL genes, mediated by specific genetic elements like integrons, have increasingly been reported worldwide. In addition, SPM-1, SIM-1, GIM-1, KHM-1, and DIM-1 MBL genes have been found sporadically in members of the family *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter baumannii* (4, 16, 20, 22, 28).

Recently, a novel MBL, NDM-1, was identified from a *K. pneumoniae* strain recovered from a Swedish patient who had come back from India (32). After that, several reports indicate the further worldwide dissemination of NDM-1 producers (5, 15, 19, 26), and it is becoming a great threat to human health together with the fact that NDM-1 producers also often possess a multidrug-resistant nature (14, 19). In consideration of the rapid worldwide spread of NDM-1 producers, a nationwide survey in Japan was performed to determine whether or not the NDM-1-producing bacterial strain resided in imipenem-nonsusceptible or ceftazidime-resistant *Enterobacteriaceae* strains collected from clinical facilities in Japan between September and December in 2010. In this survey, the presence of bla_{NDM-1}, bla_{KPC}, bla_{IMP-1}, bla_{IMP-2}, and bla_{VIM-2} was detected by PCR in the collected strains. As a result, a small number of bla_{NDM-1}-positive strains were identified, although most of the MBL genes found in the collected strains were IMP-1-type MBL genes (unpublished data).

Several strains, including *S. marcescens* strain 10mdr148, were found to be negative for the five carbapenemase genes described above, despite showing resistance to carbapenems. These results indicate the possibility that the carbapenem resistance of these isolates, including *S. marcescens* strain 10mdr148, depends on an unknown molecular mechanism, such as the production of a novel MBL. This study aimed to characterize the molecular mechanism underlying the carba-

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TABLE 1. Primers used in this study

Primer	Sequence[a]	Nucleotide position[b]
ampC-F	5′-ATG ACG AAA GTG AAC CGC-3′	1–18
ampC-R	5′-CCT GGA CGA TGT GGT AAG-3′	1103–1120
CTX-M-3F	5′-ACC GTC ACG CTG TTG TTA G-3′	47–64
CTX-M-3R	5′-CTT TCT GCC TTA GGT TGA G-3′	806–824
IMP-1F	5′-ACC GCA GCA GAG TCT TTG CC-3′	49–68
IMP-1R	5′-ACA ACC AGT TTT GCC TTA CC-3′	616–635
IMP-2F	5′-GTT TTA TGT GTA TGC TTC C-3′	16–34
IMP-2R	5′-AGC CTG TTC CCA TGT AC-3′	677–693
VIM-2F	5′-ATG TTC AAA CTT TTG AGT AAG-3′	1–21
VIM-2R	5′-CTA CTC AAC GAC TGA GCG-3′	784–801
NDM-1F	5′-TTG CCC AAT ATT ATG CAC CC-3′	7–26
NDM-1R	5′-ATT GGC ATA AGT CGC AAT CC-3′	407–426
16S rRNA-10F	5′-GTT TGA TCC TGG CTC A-3′	11–26
16S rRNA-800R	5′-TAC CAG GGT ATC TAA TCC-3′	785–802
SMB-F	5′-CAG CAG CCA TTC ACC ATC TA-3′	79–98
SMB-R	5′-GAA GAC CAC GTC CTT GCA CT-3′	551–570
SMB-CloF	5′-CCC AAG CTT TCC GCC GAC TTG GCG CAG-3′	
SMB-CloR	5′-GGG GTA CCA AGA CCG ATT TAG CCG GC-3′	
PET-1	5′-GGA ATT CCA TAT GAA AAT CAT CGC TTC CC-3′	
PET-2	5′-CCC AAG CTT TCA GCG TTT CTC GCT GGC C-3′	

[a] Underlines indicate the sites for restriction endonuclease.
[b] Position numbers correspond to the nucleotides of the coding sequences. Position numbers are assigned to the primers that amplify the internal region of the coding sequences.

penem resistance found in the *S. marcescens* 10mdr148 clinical isolate.

MATERIALS AND METHODS

Bacterial strain. The clinical isolate *S. marcescens* 10mdr148 was identified using the API-20E system (bioMérieux) and VITEK2 system (bioMérieux). The chromosomally encoded *ampC* β-lactamase gene and 16S rRNA gene of *S. marcescens* strain 10mdr148 was amplified with the primers ampC-F and ampC-R as well as 16S rRNA-10F and 16S rRNA-800R, respectively (Table 1). The sequence of the amplified products was determined.

PCR. The primers used for the detection of β-lactamase genes are listed in Table 1.

Susceptibility testing. The production of MBL was detected using a disk containing sodium mercaptoacetic acid (SMA) (Eiken) (23) and an Etest MBL IP/IPI strip (bioMérieux). The MICs of various β-lactam antibiotics were determined with the agar dilution method according to the CLSI guideline (6). The MICs of amikacin, gentamicin, ciprofloxacin, moxifloxacin, and tigecycline were determined by Etest.

Conjugation. *Escherichia coli* strain DH10B was used as the recipient. Conjugation was performed as described elsewhere (30). The conjugants were selected on LB agar plates containing streptomycin (100 μg/ml) and ceftazidime (2 μg/ml).

Cloning of bla_{SMB-1}. The total DNA of *S. marcescens* strain 10mdr148 was extracted using the Wizard genomic DNA purification kit (Promega) and partially digested with Sau3AI. The digested fragments were ligated to pCL1920 cloning vector previously digested with BamHI, dephosphorylated, and transformed into *E. coli* strain KAM32. The transformants were selected on LB agar plates supplemented with streptomycin (25 μg/ml) and ceftazidime (2 μg/ml). The fragments on the two obtained recombinant plasmids (pCL1 and pCL2) were sequenced. The bla_{SMB-1} gene and its putative promoter region were amplified with the primers SMB-CloF and SMB-CloR (Table 1) with total DNA of *S. marcescens* strain 10mdr148 as the template and then cloned into a pCL1920 vector. The constructed plasmid (pCL-SMB) was transformed into *E. coli* KAM32.

Overexpression and purification of SMB-1. The bla_{SMB-1} gene was amplified with the primers PET-1, which introduced an NdeI restriction site at the 5′ end, and PET-2, which introduced a HindIII site at the 3′ end (Table 1). The amplified fragments were digested with the endonucleases and ligated into pET30a vector (Novagen). The recombinant plasmid pET-SMB was introduced into *E. coli* BL21(DE3)pLysS by electroporation. The cells were cultured in 2 liters of LB broth supplemented with chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 0.5 mM) was added when the culture reached an optical density at 600 nm of 0.5, and the culture was incubated for an additional 3 h at 37°C. Cells were washed with 50 mM morpholineethanesulfonic acid (MES) buffer (pH 6.0), disrupted with a French press, and centrifuged at 100,000 × *g* for 30 min. The supernatant containing recombinant protein was loaded onto a HiTrap SP HP column (GE Healthcare) preequilibrated with 50 mM MES buffer (pH 6.0) and eluted with a linear gradient of 0 to 0.5 M NaCl. The fraction containing the protein was concentrated to a volume of 2 ml using an Amicon Ultra-15 Centricon (Millipore), loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare), and eluted with 20 mM Tris-HCl (pH 7.5) buffer containing 0.2 M NaCl. The fraction containing the protein was concentrated and further loaded onto a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.5) buffer containing 0.2 M NaCl. The eluted protein was stored at −80°C until use. The purity of the protein was estimated by SDS-PAGE and Coomassie brilliant blue (CBB) staining. The N-terminal sequence of the purified SMB-1 was obtained by Edman degradation with a model Shimadzu PPSQ-33A automated protein sequencer. The SMB-1 was subjected to isoelectric focusing (IEF) analysis with a CleanGel IEF (GE Healthcare).

Kinetic parameters. The kinetic parameters of the purified SMB-1 against various β-lactams were measured at 30°C in 50 mM HEPES-NaOH buffer (pH 7.5) with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The concentration of enzyme used was 1.6 nM to 1 μM in this assay. The values of the kinetic parameters K_m and k_{cat} were calculated from a Michaelis-Menten plot of the initial steady-state velocities (29). The kinetic values in this study are the means from at least three independent measurements. At least six different concentrations were used to determine the kinetic parameters for each substrate.

Inactivation of SMB-1 by chelating agents. The inactivation of SMB-1 by removing Zn^{2+} was carried out in the presence of EDTA (Wako), dipicolinic acid (Sigma), and 1,10-*o*-phenanthroline (Sigma) at different concentrations. Meropenem (100 μM) was used as the reporter substrate in the present study. The 50% inhibitory concentration (IC_{50}) was determined by preincubating 1.6 nM enzyme with various concentrations of chelating agents in HEPES-NaOH buffer (pH 7.5) for 5 min at 30°C before adding meropenem.

PFGE and Southern hybridization. Plugs containing total DNA of the *S. marcescens* 10mdr148 clinical isolate were digested with I-CeuI overnight. The digested DNAs were subjected to electrophoresis with a contour-clamped homogeneous electric field (CHEF) DRIII drive module (Bio-Rad), with pulses ranging from 2.9 to 33.7 s at 6 V/cm for 27 h at 14°C. The DNA was transferred to Zeta-Probe blotting membranes (Bio-Rad) and hybridized with two probes: a 492-bp probe specific for bla_{SMB-1} (primers SMB-F and SMB-R) and a 792-bp probe specific for the 16S rRNA gene (primers 16S rRNA-10F and 16S rRNA-800R) (Table 1). The generation of probes and signal detection were carried out using the DIG High Prime DNA labeling and detection starter kit II (Roche).

TABLE 2. Result of susceptibility testing

Antimicrobial agent	MIC (μg/ml) against strain:		
	S. marcescens 10mdr148	*E. coli* KAM32 (pCL-SMB)	*E. coli* KAM32 (pCL1920)
Ampicillin	>256	>256	2
Piperacillin	>256	>256	0.25
Cephalothin	>256	256	8
Cephaloridine	>256	64	2
Cefazolin	>256	256	1
Cefuroxime	>256	>256	0.25
Cefotaxime	>256	16	≤0.06
Ceftazidime	256	256	0.25
Cefepime	>256	0.5	≤0.06
Cefoxitin	>256	>256	2
Cefmetazole	>256	256	0.5
Flomoxef	>256	64	≤0.06
Aztreonam	>256	0.13	0.13
Imipenem	>32	8	0.25
Meropenem	>32	16	0.03
Panipenem	>32	32	0.5
Biapenem	>32	16	0.25
Amikacin	12	ND[a]	ND
Gentamicin	1.5	ND	ND
Ciprofloxacin	1	ND	ND
Moxifloxacin	0.5	ND	ND
Tigecycline	1	ND	ND

[a] ND, not determined.

Determination of genetic environment of bla_{SMB-1}. Plugs containing total DNA of the *S. marcescens* 10mdr148 clinical isolate were digested with SpeI. The plugs were completely melted with incubation at 70°C, and β-agarase (Takara) was added. After digestion, DNA was subjected to electrophoresis with a 1% agarose gel, and fragments of 9 to 23 kb were purified with a Wizard SV gel and PCR clean-up system (Promega). The purified fragments were ligated to the pMCL210 vector and transformed into *E. coli* DH10B by electroporation. The transformants were selected on LB agar plates supplemented with chloramphenicol (15 μg/ml) and ceftazidime (2 μg/ml). The cloned fragments were sequenced.

Nucleotide sequence accession number. The nucleotide sequence of bla_{SMB-1} presented in this study has been deposited in GenBank under accession no. AB636283.

RESULTS AND DISCUSSION

Clinical isolate. *S. marcescens* strain 10mdr148 was isolated in 2010 from the urine of an inpatient in a Japanese hospital. This strain showed a high level of resistance to various β-lactams, including penicillins, cephalosporins, carbapenems, and aztreonam, but it was susceptible to aminoglycosides, fluoroquinolones, and tigecycline (FDA breakpoints), as shown in Table 2. An apparent expansion of the growth-inhibitory zone around the imipenem disk was observed when the SMA disk was closely placed, and the >64-fold reduction in the MIC of imipenem was observed when using an Etest IP/IPI strip (data not shown). These results indicated the possibility that carbapenem resistance of *S. marcescens* strain 10mdr148 was attributed to the production of some kind of MBL. However, the preliminary PCR detection of the MBL genes bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-2}, and bla_{NDM-1} that have been found so far in clinical isolates in Japan gave no positive result.

The CTX-M-3-type β-lactamase gene and *ampC* β-lactamase gene were detected by PCR. The sequence of the amplified fragments (nucleotide positions 65 to 805) for the CTX-M-3-type β-lactamase gene was 100% identical to the corresponding region of $bla_{CTX-M-3}$ of *Enterobacter aerogenes* (GenBank accession no. AB432919). The sequence of the amplified fragments (nucleotide positions 19 to 1102) for the *ampC* β-lactamase gene was 99% identical to the corresponding region of the chromosomally encoded *ampC* β-lactamase gene of *S. marcescens* (GenBank accession no. AY524276).

Cloning and characterization of an MBL gene. Although the conjugation experiment was performed to transfer the β-lactam resistance of *S. marcescens* strain 10mdr148 to *E. coli* DH10B, a transconjugant could not be obtained under the experimental conditions in this study. Thus, we attempted to isolate the genes responsible for β-lactam resistance by a shotgun cloning experiment using total DNA of *S. marcescens* strain 10mdr148. As a result, two recombinant plasmids of different sizes were obtained: pCL1, carrying a ca. 2-kb fragment, and pCL2, carrying a ca. 6-kb fragment. Both plasmids carried the same 843-bp open reading frame (ORF) encoding a protein consisting of 280 amino acids. This protein was assigned to be a member of Ambler class B β-lactamases through database homology searching, and it was named SMB-1 (*Serratia* metallo-β-lactamase). SMB-1 possessed a zinc binding motif, H(Q)XHXDH (residues 116 to 121), H196, and H263, which were well conserved in MBLs belonging to the subclass B3 MBL group (Fig. 1) (13). Thus, SMB-1 could be categorized as a member of subclass B3 MBL. SMB-1 exhibited 75 and 71% amino acid identity to AMO1 and CRB11 MBLs, respectively, of uncultured bacterium recently identified through the metagenomic analysis of apple orchard soil (10), and it exhibited 42% identity to THIN-B MBL of *Janthinobacterium lividum* (Fig. 2) (21). The G+C content of bla_{SMB-1} was 63%, slightly higher than that (60%) of the *S. marcescens* strain Db11 genome (http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html).

Susceptibility testing of the transformant producing SMB-1. In the present study, the *E. coli* transformant that produces SMB-1, encoded on the recombinant plasmid (pCL-SMB) carrying bla_{SMB-1} and its putative promoter region, was subjected to susceptibility testing. The transformant producing SMB-1 showed resistance to a variety of β-lactams, except aztreonam (Table 2). The SMB-1 production yielded a >8-fold increase in the MIC of cefepime, but cefepime remained fully active against the SMB-1-producing transformant. This trend was observed in the transformant that produces the THIN-B subclass B3 MBL (21). On the one hand, most of the transformants producing the subclass B3 MBLs, such as CAR-1 and BJP-1, could not confer resistance to cefepime (24, 25). The high MICs of cefepime and aztreonam observed for the parent *S. marcescens* strain 10mdr148 probably were attributable to the production of CTX-M-3-type β-lactamase and/or AmpC β-lactamase, not to SMB-1 MBL.

Biophysical characterization of SMB-1. *E. coli* BL21(DE3)pLysS and the pET30a expression vector were used for the overexpression and purification of SMB-1. *E. coli* BL21(DE3)pLysS carrying pET30a was susceptible to ceftazidime (MIC, ≤0.06 μg/ml), while *E. coli* BL21(DE3)pLysS carrying pET-SMB showed a reduction in susceptibility to ceftazidime (MIC, 8 μg/ml). This result indicated that the recombinant SMB-1 produced was functional and responsible for β-lactam resistance in *E. coli* BL21(DE3)pLysS. An optimized culture condition yielded 22 mg of purified protein per 2 liters of bacterial

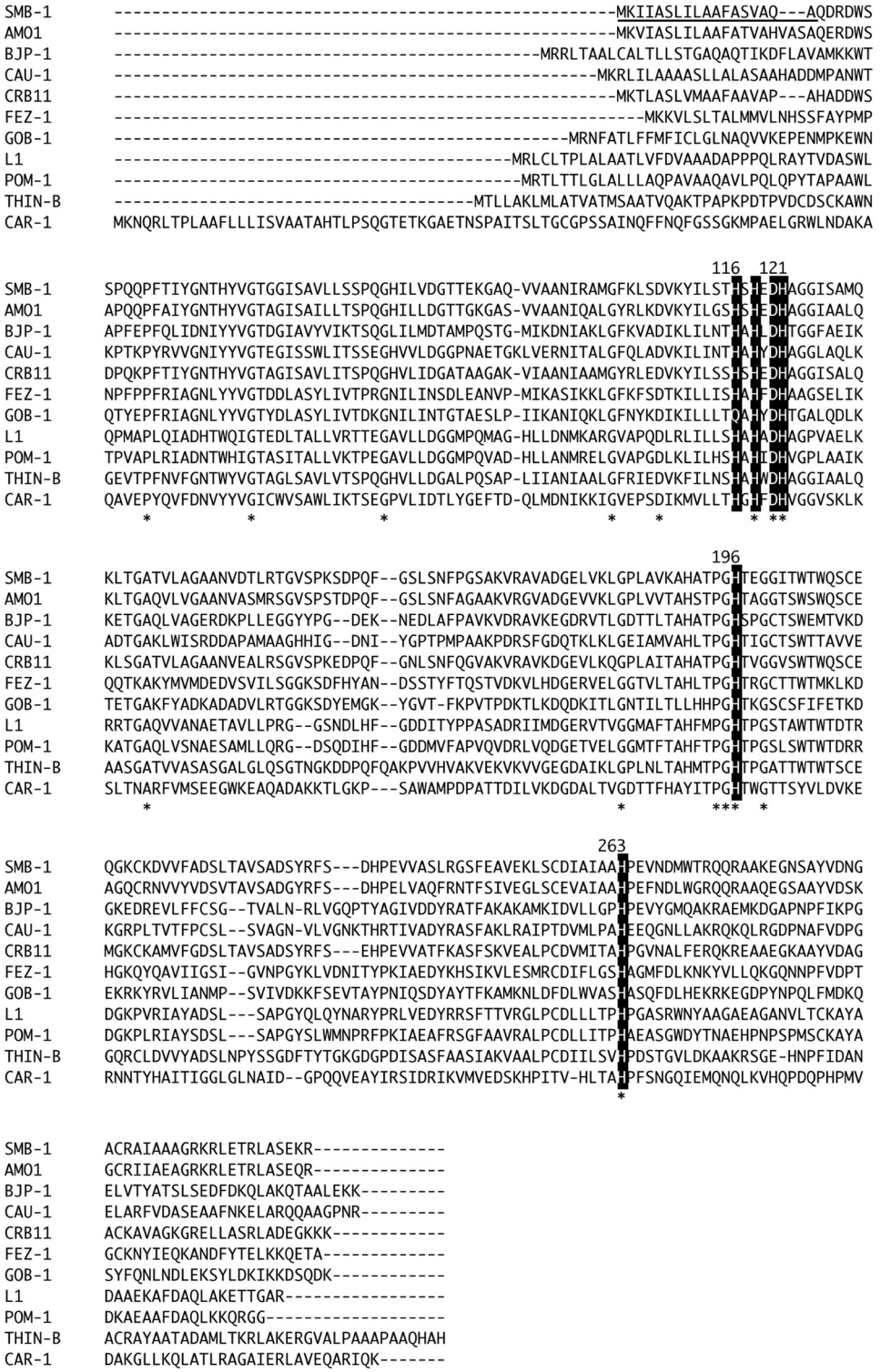

FIG. 1. Amino acid alignments of SMB-1 sequence with those of other subclass B3 MBLs. The residues involved in zinc binding are highlighted with a dark background. The signal peptide of SMB-1 is shown with an underline. An asterisk indicates amino acid residues conserved among all subclass B3 MBLs. Proteins (GenBank accession no.) are the following: SMB-1 (AB636283), AMO1 (ACS83721), BJP-1 (NP772870), CAU-1 (CAC87665), CRB11 (ACS83724), FEZ-1 (CAB96921), GOB-1 (ABO21417), L1 (ABO60992), POM-1 (ADC79555), THIN-B (CAC33832), and CAR-1 (Q6D395).

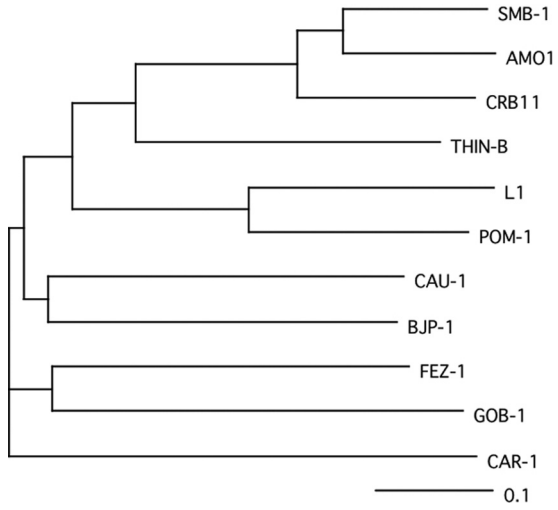

FIG. 2. Tree view exhibiting the similarity of SMB-1 with the other subclass B3 MBLs. The tree was constructed using ClustalW, version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html), and was provided by the DNA Data Bank of Japan (DDBJ). Sequences incorporated to draw the tree were the same as those used for Fig. 1. The 0.1 scale represents a genetic unit reflecting 10% of the amino acid substitutions, and it was calculated with the ClustalW program.

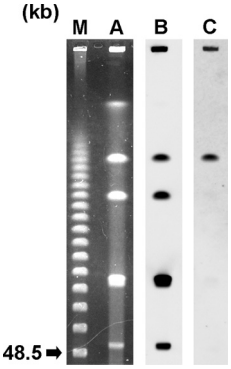

FIG. 3. Localization of the bla_{SMB-1} gene on I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 separated by PFGE. Lane: M, CHEF DNA size standard marker (Bio-Rad); A, I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 stained with ethidium bromide; B, hybridization of I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 with probe specific for 16S rRNA gene; and C, hybridization of I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 with probe specific for the bla_{SMB-1} gene.

culture, and the purified enzyme gave a single band on SDS-PAGE with CBB staining (data not shown). The N-terminal sequence of mature SMB-1 was determined to be QDRDW by Edman degradation, and this corresponds to the sequence after the cleavage of signal peptide predicted by GENETYX-MAC version 14.0.1. The native SMB-1 was determined to be a monomeric form by gel filtration. The pI of SMB-1 was estimated to be 7.4 by isoelectric focusing.

Kinetic parameters of SMB-1. The results of the kinetic parameters of SMB-1 against representative β-lactams are shown in Table 3. SMB-1 was capable of hydrolyzing penicillins, most cephalosporins (except cefepime), and carbapenems. The hydrolyzing efficiency (k_{cat}/K_m) of SMB-1 against cefepime was low due to its higher K_m value (747 μM) and the lower k_{cat} value (2.7 s^{-1}), corroborating the MIC of cefepime conferred by SMB-1 production (Table 2). The poor hydrolytic efficiency (k_{cat}/K_m, $<10^4$ $M^{-1} \cdot s^{-1}$) of SMB-1 against cefepime was very similar to those of other subclass B3 MBLs, such as BJP-1, FEZ-1, CAR-1, and THIN-B (8, 17, 24, 25). SMB-1 demonstrated higher k_{cat} values (>500 s^{-1}) against carbapenems, imipenem, and meropenem, resulting in high hydrolytic efficiency. The properties of high hydrolytic efficiency (k_{cat}/K_m, $>10^5$ $M^{-1} \cdot s^{-1}$) against carbapenems of SMB-1 are similar to those of BJP-1, L1, GOB-1, FEZ-1, CAU-1, and THIN-B (1, 8, 9, 11, 17, 25). Against carbapenems, a significant substrate preference, like meropenem over imipenem, as observed in BJP-1, L1, and GOB-1, was not identified in SMB-1 (1, 11, 12, 25). SMB-1 exhibited no measurable hydrolyzing activity against aztreonam, in accordance with previous biochemical studies of other MBLs (3). The inhibition profile determined, the IC_{50} with meropenem as the substrate, revealed that the activity of SMB-1 was well inhibited by the chelating agents dipicolinic acid, 1,10-*o*-phenanthroline, and EDTA, as shown in Table 3.

TABLE 3. Kinetic parameters and inhibition profile of SMB-1[a]

Substrate or chelating agent	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	Relative k_{cat}/K_m[d]	IC_{50} (μM)
Ampicillin	102	247	2.4×10^6	100	
Piperacillin	380	68	1.8×10^5	7.5	
Cephalothin	15	28	1.9×10^6	79	
Cefuroxime	22	30	1.4×10^6	58	
Cefotaxime	35	31	8.9×10^5	37	
Ceftazidime	57	4.4	7.7×10^4	3.2	
Cefepime	747	2.7	3.6×10^3	0.15	
Cefoxitin	26	39	1.5×10^6	63	
Aztreonam	NH[b]	ND[c]	ND	ND	
Imipenem	133	518	3.9×10^6	163	
Meropenem	144	604	4.2×10^6	175	
Dipicolinic acid					2.2
1,10-*o*-Phenanthroline					156
EDTA					14

[a] Standard deviations for each parameter were below 10%.
[b] NH, no measurable hydrolysis detected with 1 μM enzyme.
[c] ND, not determined.
[d] Relative k_{cat}/K_m value was expressed compared to that of ampicillin, which was assigned 100.

Localization of bla_{SMB-1} in *S. marcescens* strain 10mdr148. The I-CeuI digestion of total DNA of *S. marcescens* strain 10mdr148 yielded six fragments of different sizes under the experimental conditions employed in this study (Fig. 3). Southern hybridization revealed that five of the six fragments hybridized with the probes specific for the 16S rRNA gene (Fig. 3). The signal by the bla_{SMB-1} probe was detected with the ca. 730-kb fragment (Fig. 3), which also was hybridized with the probes specific for the 16S rRNA gene. Therefore, it was found that the bla_{SMB-1} gene was located on the chromosome of *S. marcescens* strain 10mdr148.

Genetic environment of bla_{SMB-1}. To characterize the genetic context of bla_{SMB-1}, the sequence of the SpeI fragment cloned from genomic DNA of *S. marcescens* strain 10mdr148 was partially determined. The genetic structure of flanking regions of bla_{SMB-1} is shown in Fig. 4. The bla_{SMB-1} gene was located downstream of the IS*CR1* element (previously called

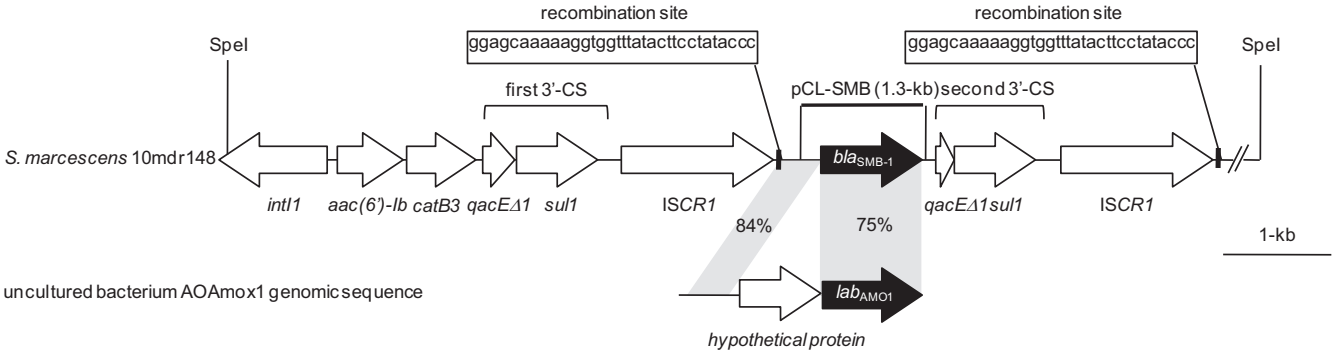

FIG. 4. Schematic representation of the generic environment of the bla_{SMB-1} gene. The 5′ end of the *qacEΔ1* gene in the second 3′-CS was deleted of 143 bp that are present in the first 3′-CS.

orf513) that was frequently linked to antibiotic resistance genes such as class A β-lactamase genes ($bla_{CTX-M-2}$, $bla_{CTX-M-9}$, and bla_{PER-1}), class C β-lactamase genes (bla_{CMY-9} and bla_{DHA-1}), a plasmid-mediated quinolone resistance gene (*qnrA*), and an aminoglycoside-resistant 16S rRNA methyltransferase gene (*armA*) (27). The IS*CR1* element upstream of bla_{SMB-1} typically was associated with a class 1 integron carrying the gene cassettes *aac*(*6′*)*-Ib* and *catB3*, which were involved in kanamycin and chloramphenicol resistance, respectively.

The bla_{SMB-1} gene was followed by the second 3′ conserved segment (CS) and another IS*CR1* element. The 3′-CS was made of *qacEΔ1* with a 143-bp deletion at the 5′ end and *sul1* (Fig. 4). Both IS*CR1* elements in the cloned SpeI fragment carried the same crossover recombination site containing *ori*IS at their 3′ ends. Toleman et al. proposed the model that IS*CR1* mobilizes an adjacent DNA sequence bearing an antibiotic resistance gene using *ori*IS and an alternative termination site (*ter*IS) (27). Therefore, it is suggested that the IS*CR1* element plays a role in spreading antibiotic resistance genes among pathogenic *Enterobacteriaceae* clinical isolates. Although the detailed mechanism of transferring antibiotic resistance genes mediated by IS*CR1* remains controversial, it is likely that the bla_{SMB-1} gene will be horizontally disseminated in the near future among pathogenic *Enterobacteriaceae* clinical isolates via the transposition activity of the second IS*CR1* element downstream of bla_{SMB-1} (Fig. 4). Furthermore, the finding of a horizontally acquired subclass B3 MBL gene like bla_{SMB-1} would imply that the subclass B3 MBL gene will spread and become a great clinical concern, as did the subclass B1 MBL gene bla_{NDM-1}.

The bla_{SMB-1} gene has 75% nucleotide identity with bla_{AMO1}, which was isolated as a β-lactamase gene through the functional metagenomic analysis of apple orchard soil (10). In addition, the genetic region upstream of start codon ATG of bla_{SMB-1} and downstream of the first IS*CR1* element shows 84% nucleotide identity with the 5′ end of the gene encoding a hypothetical protein upstream of bla_{AMO1} (Fig. 4). This genetic relatedness between bla_{SMB-1} and bla_{AMO1} presents the possibility that the bla_{SMB-1} gene found in *S. marcescens* strain 10mdr148 was derived from an environmental bacterial species that existed as a natural reservoir for this subclass B3 MBL gene. This is supported by the fact that subclass B3 MBL genes reported so far have been found mostly through the large-scale postgenomic analysis of environmental microbial genomes (9, 10, 21, 24, 25). For example, the origin of NDM-1 was speculated to be the genomic enzyme of some marine bacterium, such as *Erythrobacter* spp. or its family (33). Further metagenomic analyses of environmental microbial genomes will help identify the origin of the bla_{SMB-1} gene.

Conclusion. To our knowledge, this is the first subclass B3 MBL gene in complex with an IS*CR1* element to be identified in a human pathogenic *Enterobacteriaceae* clinical isolate. The majority of the horizontally acquired MBL genes identified so far in pathogenic Gram-negative microbes belong primarily to the subclass B1 MBL group, but here it is likely that pathogenic microbes producing subclass B3 MBLs, like SMB-1, which show high hydrolyzing activity against carbapenems, may emerge and become an actual concern in clinical settings. Therefore, special precautions must be taken continuously regarding the emergence of carbapenem-resistant pathogenic microbes in clinical settings and the molecular mechanisms underlying carbapenem resistance.

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