22 Sun Y, Kong F, Zhao Z *et al.* Comparison of a 3-set genotyping system with multilocus sequence typing for *Streptococcus agalactiae* (group B *Streptococcus*). *J Clin Microbiol* 2005; **43**: 4704–7.

23 Bisharat N, Crook DW, Leigh J *et al.* Hyperinvasive neonatal group B *Streptococcus* has arisen from a bovine ancestor. *J Clin Microbiol* 2004; **42**: 2161–7.

24 Jones N, Oliver KA, Barry J *et al.* Enhanced invasiveness of bovine-derived neonatal sequence type 17 group B *Streptococcus* is independent of capsular serotype. *Clin Infect Dis* 2006; **42**: 915–24.

25 Van der Mee-Marquet N, Fourny L, Arnault L *et al.* Molecular characterization of human-colonizing *Streptococcus agalactiae* strains isolated from throat, skin, anal margin, and genital body sites. *J Clin Microbiol* 2008; **46**: 2906–11.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2011, p. 5143–5149 Vol. 55, No. 11
0066-4804/11/$12.00 doi:10.1128/AAC.05045-11

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▽

Jun-ichi Wachino,[1]* Hiroyuki Yoshida,[2] Kunikazu Yamane,[1,3] Satowa Suzuki,[1] Mari Matsui,[1] Takuya Yamagishi,[1] Atsuko Tsutsui,[1] Toshifumi Konda,[1] Keigo Shibayama,[1] and Yoshichika Arakawa[1,4]

Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan[1]; Department of Infection Control and Prevention, Kobe University Hospital, 7-5-2 Chuo-ku, Kobe, Hyogo 650-0017, Japan[2]; Department of Public Health, Kawasaki Medical University, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan[3]; and Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan[4]

Received 9 June 2011/Returned for modification 31 July 2011/Accepted 19 August 2011

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-

* Corresponding author. Mailing address: Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 3546. Fax: 81-42-561-7173. E-mail: wachino@nih.go.jp.

▽ Published ahead of print on 29 August 2011.

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

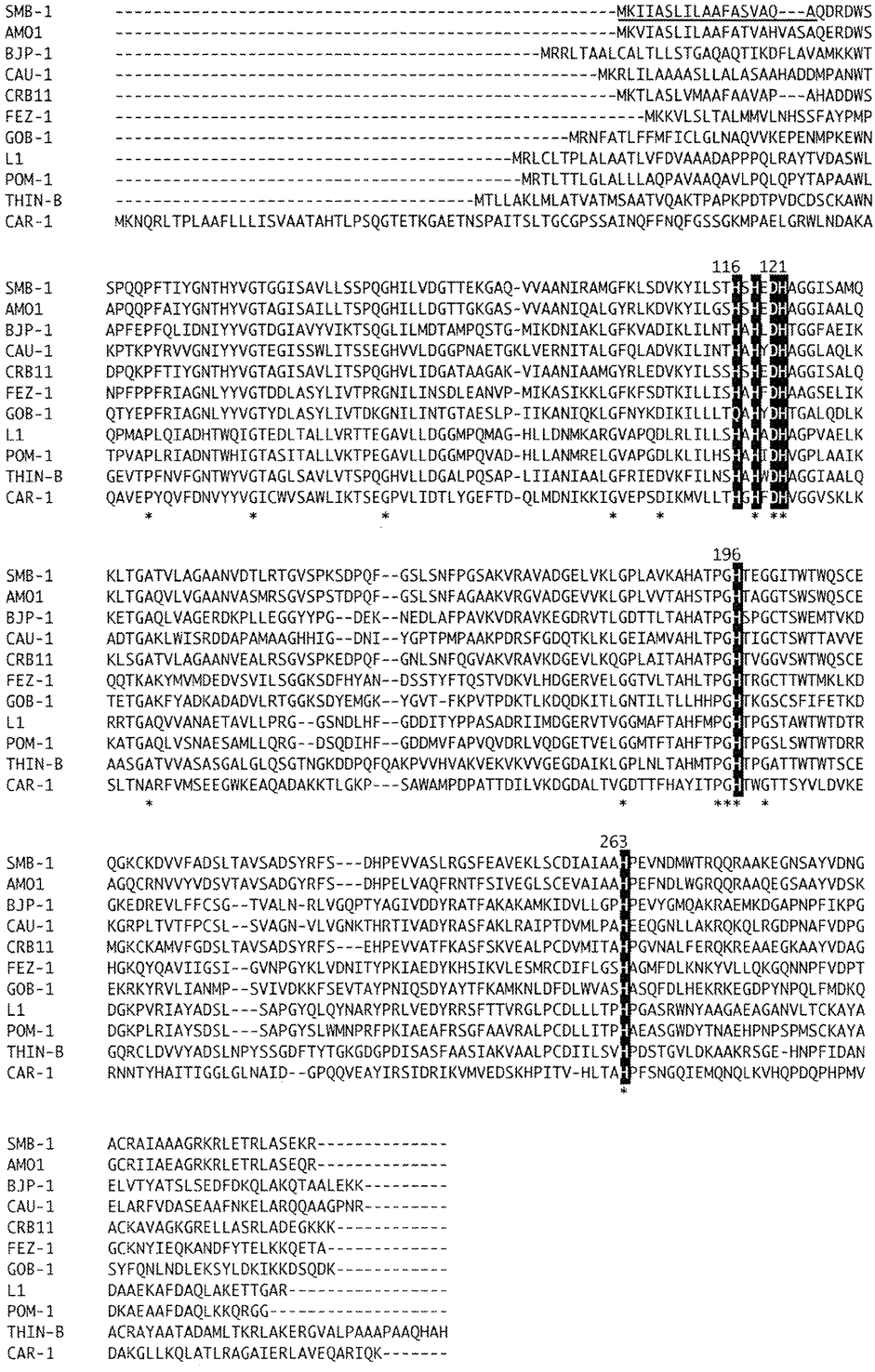

FIG. 1. Amino acid alignments of SMB-1 sequence with those of other subclass B3 MBLs. The residues involved in zinc binding are highlighted with a dark background. The signal peptide of SMB-1 is shown with an underline. An asterisk indicates amino acid residues conserved among all subclass B3 MBLs. Proteins (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).

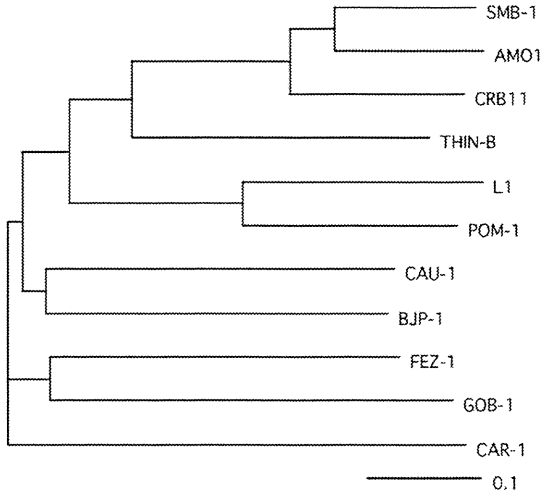

FIG. 2. Tree view exhibiting the similarity of SMB-1 with the other subclass B3 MBLs. The tree was constructed using ClustalW, version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html), and was provided by the DNA Data Bank of Japan (DDBJ). Sequences incorporated to draw the tree were the same as those 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.

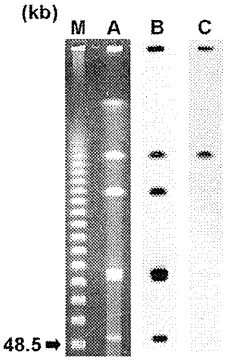

FIG. 3. Localization of the bla_{SMB-1} gene on I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 separated by PFGE. Lane: M, CHEF DNA size standard marker (Bio-Rad); A, I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 stained with ethidium bromide; B, hybridization of I-CeuI-digested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

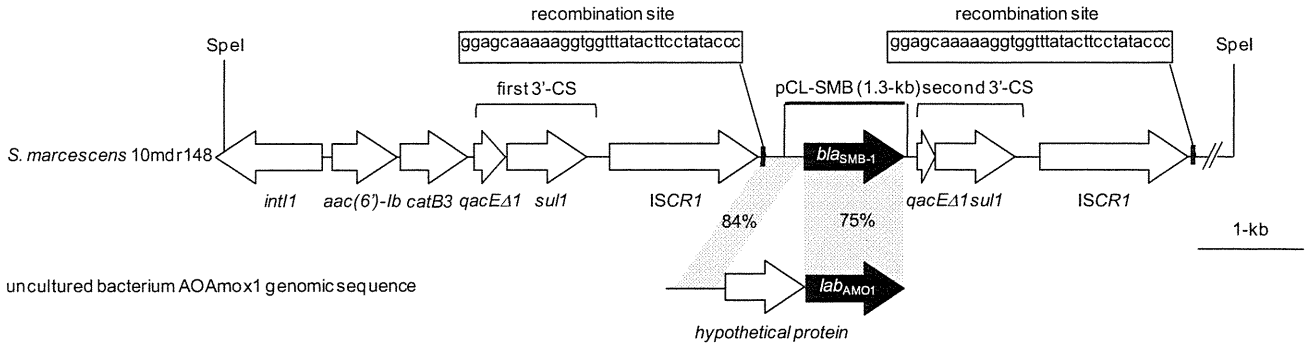

FIG. 4. Schematic representation of the generic environment of the bla_{SMB-1} gene. The 5′ end of the *qacEΔ1* gene in the second 3′-CS was deleted of 143 bp that are present in the first 3′-CS.

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.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Health, Labor, and Welfare of Japan (grant no. H21-Shinkou-Ippan-008).

We are grateful to Kai Kumiko, Yoshie Taki, and Yumiko Yoshimura for their technical assistance. We thank Tomofusa Tsuchiya, a professor of the faculty of Pharmaceutical Sciences, Okayama University, for donating *E. coli* KAM32. We thank the National BioResource Project (National Institute of Genetics, Japan) for providing the cloning vector pMCL210.

REFERENCES

1. **Bellais, S., D. Aubert, T. Naas, and P. Nordmann.** 2000. Molecular and biochemical heterogeneity of class B carbapenem-hydrolyzing ß-lactamases in *Chryseobacterium meningosepticum*. Antimicrob. Agents Chemother. **44:** 1878–1886.
2. **Bush, K.** 2010. Alarming ß-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*. Curr. Opin. Microbiol. **13:**558–564.
3. **Bush, K., and G. A. Jacoby.** 2010. Updated functional classification of ß-lactamases. Antimicrob. Agents Chemother. **54:**969–976.
4. **Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh.** 2004. Molecular characterization of a ß-lactamase gene, bla_{GIM-1}, encoding a new subclass of metallo-ß-lactamase. Antimicrob. Agents Chemother. **48:**4654–4661.
5. **Chihara, S., K. Okuzumi, Y. Yamamoto, S. Oikawa, and A. Hishinuma.** 2011. First case of New Delhi metallo-ß-lactamase 1-producing *Escherichia coli* infection in Japan. Clin. Infect. Dis. **52:**153–154.
6. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 8th ed. Document M07-A8. CLSI, Wayne, PA.

7. **Cornaglia, G., and G. M. Rossolini.** 2010. The emerging threat of acquired carbapenemases in Gram-negative bacteria. Clin. Microbiol. Infect. **16:**99–101.
8. **Docquier, J. D., et al.** 2004. Biochemical characterization of the THIN-B metallo-ß-lactamase of *Janthinobacterium lividum*. Antimicrob. Agents Chemother. **48:**4778–4783.
9. **Docquier, J. D., et al.** 2002. CAU-1, a subclass B3 metallo-ß-lactamase of low substrate affinity encoded by an ortholog present in the *Caulobacter crescentus* chromosome. Antimicrob. Agents Chemother. **46:**1823–1830.
10. **Donato, J. J., et al.** 2010. Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. Appl. Environ. Microbiol. **76:**4396–4401.
11. **Felici, A., and G. Amicosante.** 1995. Kinetic analysis of extension of substrate specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* metallo-ß-lactamases. Antimicrob. Agents Chemother. **39:**192–199.
12. **Felici, A., et al.** 1993. An overview of the kinetic parameters of class B ß-lactamases. Biochem. J. **291:**151–155.
13. **Garau, G., et al.** 2004. Update of the standard numbering scheme for class B ß-lactamases. Antimicrob. Agents Chemother. **48:**2347–2349.
14. **Kaase, M., et al.** 2011. NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. J. Antimicrob. Chemother. **66:**1260–1262.
15. **Kumarasamy, K. K., et al.** 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect. Dis. **10:**597–602.
16. **Lee, K., et al.** 2005. Novel acquired metallo-ß-lactamase gene, bla_{SIM-1}, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob. Agents Chemother. **49:**4485–4491.
17. **Mercuri, P. S., et al.** 2001. Biochemical characterization of the FEZ-1 metallo-ß-lactamase of *Legionella gormanii* ATCC 33297T produced in *Escherichia coli*. Antimicrob. Agents Chemother. **45:**1254–1262.
18. **Osano, E., et al.** 1994. Molecular characterization of an enterobacterial metallo-ß-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
19. **Poirel, L., E. Lagrutta, P. Taylor, J. Pham, and P. Nordmann.** 2010. Emergence of metallo-ß-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. Antimicrob. Agents Chemother. **54:**4914–4916.
20. **Poirel, L., J. M. Rodriguez-Martinez, N. Al Naiemi, Y. J. Debets-Ossenkopp, and P. Nordmann.** 2010. Characterization of DIM-1, an integron-encoded metallo-ß-lactamase from a *Pseudomonas stutzeri* clinical isolate in the Netherlands. Antimicrob. Agents Chemother. **54:**2420–2424.
21. **Rossolini, G. M., et al.** 2001. Metallo-ß-lactamase producers in environmental microbiota: new molecular class B enzyme in *Janthinobacterium lividum*. Antimicrob. Agents Chemother. **45:**837–844.
22. **Sekiguchi, J., et al.** 2008. KHM-1, a novel plasmid-mediated metallo-ß-lactamase from a *Citrobacter freundii* clinical isolate. Antimicrob. Agents Chemother. **52:**4194–4197.
23. **Shibata, N., et al.** 2003. PCR typing of genetic determinants for metallo-ß-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol. **41:**5407–5413.
24. **Stoczko, M., J. M. Frere, G. M. Rossolini, and J. D. Docquier.** 2008. Functional diversity among metallo-ß-lactamases: characterization of the CAR-1 enzyme of *Erwinia carotovora*. Antimicrob. Agents Chemother. **52:**2473–2479.
25. **Stoczko, M., J. M. Frere, G. M. Rossolini, and J. D. Docquier.** 2006. Postgenomic scan of metallo-ß-lactamase homologues in rhizobacteria: identification and characterization of BJP-1, a subclass B3 ortholog from *Bradyrhizobium japonicum*. Antimicrob. Agents Chemother. **50:**1973–1981.
26. **Tijet, N., et al.** 2011. New Delhi metallo-ß-lactamase, Ontario, Canada. Emerging Infect. Dis. **17:**306–307.
27. **Toleman, M. A., P. M. Bennett, and T. R. Walsh.** 2006. ISCR elements: novel gene-capturing systems of the 21st century? Microbiol. Mol. Biol. Rev. **70:**296–316.
28. **Toleman, M. A., et al.** 2002. Molecular characterization of SPM-1, a novel metallo-ß-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J. Antimicrob. Chemother. **50:**673–679.
29. **Wachino, J., et al.** 2006. Horizontal transfer of bla_{CMY}-bearing plasmids among clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates and emergence of cefepime-hydrolyzing CMY-19. Antimicrob. Agents Chemother. **50:**534–541.
30. **Wachino, J., et al.** 2006. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob. Agents Chemother. **50:**178–184.
31. **Yigit, H., et al.** 2003. Carbapenem-resistant strain of *Klebsiella oxytoca* harboring carbapenem-hydrolyzing ß-lactamase KPC-2. Antimicrob. Agents Chemother. **47:**3881–3889.
32. **Yong, D., et al.** 2009. Characterization of a new metallo-ß-lactamase gene, bla_{NDM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother. **53:**5046–5054.
33. **Zheng, B., et al.** 2011. An unexpected similarity between antibiotic-resistant NDM-1 and ß-lactamase II from *Erythrobacter litoralis*. Protein Cell **2:**250–258.

JOURNAL OF CLINICAL MICROBIOLOGY, June 2011, p. 2378–2379 Vol. 49, No. 6
0095-1137/11/$12.00 doi:10.1128/JCM.00278-11

Letters to the Editor

Practical Disk-Based Method for Detection of *Escherichia coli* Clinical Isolates Producing the Fluoroquinolone-Modifying Enzyme AAC(6′)-Ib-cr[▽]

Since a fluoroquinolone-modifying enzyme gene, *aac(6′)-Ib-cr*, was first reported in 2006, it has rapidly spread among *Enterobacteriaceae* clinical isolates worldwide (7). AAC(6′)-Ib-cr differs from AAC(6′)-Ib by two amino acids, Trp102Arg and Asp179Tyr, and these substitutions allow it to reduce the antibacterial activities of norfloxacin and ciprofloxacin through acetylation of their piperazinyl substituent (6). Detection of *aac(6′)-Ib-cr* has so far depended mainly on genotyping, PCR, and sequencing (2). Recently, simultaneous high-resolution melting analysis and pyrosequencing were developed for detection (1, 3). However, these methods are costly and need specialized equipment. Therefore, the availability is limited to highly advanced institutions, such as research laboratories and university hospitals. In the present study, we developed a cost-effective and practical disk-based method to screen for AAC(6′)-Ib-cr producers.

The *Escherichia coli* clinical isolates CR1 [*aac(6′)-Ib-cr* positive], N64 [*aac(6′)-Ib* positive], and BN41 [*aac(6′)-Ib-cr* and *aac(6′)-Ib* negative] were grown in LB broth containing norfloxacin (8 μg/ml), with shaking for 18 h at 35°C. The broth containing the same concentration of norfloxacin as the other tubes, but lacking any bacteria, was used as the control in this study. Ten microliters of each culture medium was applied on the blank disk set on a Mueller-Hinton agar plate inoculated with *E. coli* ATCC 25922 and incubated for 18 h at 35°C. The result is shown in Fig. 1. When the control medium was applied, an 18-millimeter growth-inhibitory zone (corresponding to 80 ng norfloxacin per disk) was observed. The significant decrease of a growth-inhibitory zone was observed when the culture medium of the *aac(6′)-Ib-cr*-positive *E. coli* strain CR1 was applied, while neither of the *aac(6′)-Ib-cr*-negative strains, N64 and BN41, showed a decrease in zone diameter. The decrease in zone diameter is an indicator of AAC(6′)-Ib-cr production and would be attributed to the inactivation of norfloxacin in the culture medium by the AAC(6′)-Ib-cr enzyme produced during growth (4).

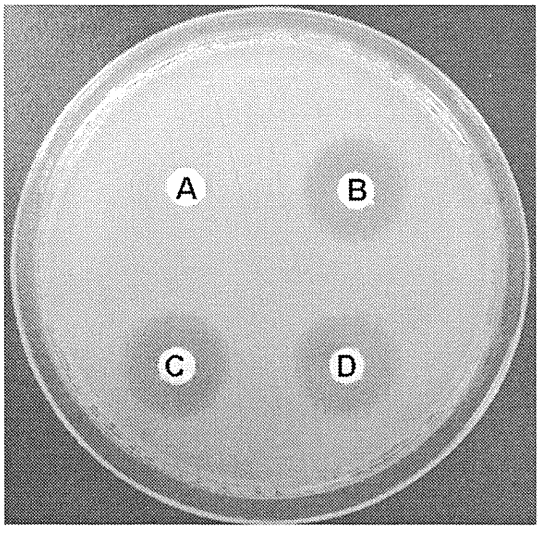

FIG. 1. Result of disk-based detection. (A) *E. coli* strain CR1 [*aac(6′)-Ib-cr* (+)]; (B) *E. coli* strain N64 [*aac(6′)-Ib* (+)]; (C) *E. coli* strain BN41 (negative for both genes); (D) control medium.

In this study, a total of 89 *E. coli* clinical isolates from our own strain collection, which were obtained from 49 facilities throughout Japan between 2002 and 2010, were subjected to the developed disk-based method. The MICs of norfloxacin for these strains were ≥16 μg/ml, and they showed visible growth in liquid broth containing 8 μg/ml of norfloxacin. The presence and absence of the *aac(6′)-Ib-cr* and *aac(6′)-Ib* genes in these isolates were preliminarily determined by PCR and nucleotide sequencing (5) but were not examined for the presence of other fluoroquinolone resistance mechanisms, such as *qnr* and mutations in DNA gyrase and topoisomerase IV, in the present study. As a result, all of the 19 *aac(6′)-Ib-cr*-positive strains showed a decrease in zone diameter of >10 mm, while all of the 70 *aac(6′)-Ib-cr*-negative strains, including 10 *aac(6′)-Ib*-positive ones, showed a growth-inhibitory zone that was the same size as that of the control medium. These results indicated that the new disk-based method developed here might have a specificity and sensitivity equivalent to those of genotyping using PCR and nucleotide sequencing. Therefore, the method seems to be effective for screening of AAC(6′)-Ib-cr producers, although application of the method is limited to bacterial strains that can grow in medium including norfloxacin.

We applied the method to *aac(6′)-Ib-cr*-positive clinical isolates of *Klebsiella pneumoniae* (n = 3), *Serratia marcescens* (n = 1), and *Citrobacter freundii* (n = 1), and we confirmed the >10-mm reduction in zone diameter seen in *aac(6′)-Ib-cr*-positive *E. coli*. This result indicates the possibility that the method could be applied not only to *E. coli* but also to the other bacterial strains belonging to the family *Enterobacteriaceae*. Extension of this method to members of the *Enterobacteriaceae* family other than *E. coli* would, however, require substantial additional experimentation aimed at demonstrating that the method is more broadly applicable to these other species.

We conclude that our method is simple and highly sensitive and specific for identification of AAC(6′)-Ib-cr producers and that it would be of great assistance in screening for AAC(6′)-Ib-cr producers and their epidemiology in clinical microbiology laboratories.

This study was supported by the Ministry of Health, Labor, and Welfare of Japan (grant H21-Shinkou-Ippan-008).

REFERENCES

1. **Bell, J. M., J. D. Turnidge, and P. Andersson.** 2010. *aac(6′)-Ib-cr* genotyping by simultaneous high-resolution melting analyses of an unlabeled probe and full-length amplicon. Antimicrob. Agents Chemother. **54:**1378–1380.
2. **Cano, M. E., et al.** 2009. Detection of plasmid-mediated quinolone resistance genes in clinical isolates of *Enterobacter* spp. in Spain. J. Clin. Microbiol. **47:**2033–2039.
3. **Guillard, T., et al.** 2010. Rapid detection of *aac(6′)-Ib-cr* quinolone resistance gene by pyrosequencing. J. Clin. Microbiol. **48:**286–289.
4. **Jung, C. M., T. M. Heinze, R. Strakosha, C. A. Elkins, and J. B. Sutherland.** 2009. Acetylation of fluoroquinolone antimicrobial agents by an *Escherichia*

coli strain isolated from a municipal wastewater treatment plant. J. Appl. Microbiol. **106:**564–571.

5. **Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahm, and D. C. Hooper.** 2006. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. **50:**3953–3955.
6. **Robicsek, A., et al.** 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat. Med. **12:**83–88.
7. **Strahilevitz, J., G. A. Jacoby, D. C. Hooper, and A. Robicsek.** 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin. Microbiol. Rev. **22:**664–689.

Jun-ichi Wachino*
Kunikazu Yamane
Yoshichika Arakawa
Department of Bacteriology II
National Institute of Infectious Diseases
4-7-1 Gakuen, Musashi-Murayama
Tokyo 208-0011, Japan

*Phone: 81-42-561-0771, ext.539
Fax: 81-42-561-7173
E-mail: wachino@nih.go.jp

▽ Published ahead of print on 6 April 2011.

Journal of Hospital Infection 78 (2011) 317–322

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevierhealth.com/journals/jhin

Genotypes and infection sites in an outbreak of multidrug-resistant *Pseudomonas aeruginosa*

A. Tsutsui [a,b,c], S. Suzuki [a,*], K. Yamane [a], M. Matsui [a], T. Konda [a], E. Marui [b], K. Takahashi [c], Y. Arakawa [a]

[a] *Department of Bacteriology 2, National Institute of Infectious Diseases, Tokyo, Japan*
[b] *Department of Public Health, Juntendo University Graduate School of Medicine, Tokyo, Japan*
[c] *Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan*

ARTICLE INFO

Article history:
Received 23 November 2010
Accepted 7 April 2011
by S.J. Dancer
Available online 20 June 2011

Keywords:
Multidrug resistance
Outbreak
Pseudomonas aeruginosa
Pulsed-field gel electrophoresis
VIM-2

SUMMARY

An outbreak of multidrug-resistant (MDR) *Pseudomonas aeruginosa* occurred in an acute care hospital in Japan, which lasted for more than three years. During January 2006 to June 2009, 59 hospitalised patients with MDR *P. aeruginosa* were mainly detected by urine culture in the first half, whereas isolation from respiratory tract samples became dominant in the latter half of the outbreak. Non-duplicate MDR *P. aeruginosa* isolates were available from 51 patients and all isolates were positive for bla_{VIM-2}. Pulsed-field gel electrophoresis (PFGE) analysis categorised the isolates into three major clusters; types A, B and C with eight, 19 and 21 isolates, respectively. The outbreak started with patients harbouring PFGE type A strains, followed by type B, and type C strains. Multivariate analysis demonstrated that patients with PFGE type C strains were more likely to be detected by respiratory tract samples (odds ratio: 11.87; 95% confidence interval: 1.21–116.86). Improved aseptic urethral catheter care controlled PFGE type A and type B strains and improvement in respiratory care procedures finally contained the transmission of PFGE type C strains.

Introduction

Pseudomonas aeruginosa is a major nosocomial pathogen which survives in moist environments and colonises the respiratory tract of mechanically ventilated patients.[1] It causes severe infections such as pneumonia in critically ill and immunocompromised patients, and especially multidrug-resistant (MDR) *P. aeruginosa* is associated with increased mortality because no adequate therapeutic option exists.[2]

In Japan, MDR *P. aeruginosa* is defined as *P. aeruginosa* resistant to carbapenems, fluoroquinolones and aminoglycosides. A nationwide clinical laboratory-based survey from 2003 to 2006 showed that 2.4% *P. aeruginosa* were MDR in medical facilities.[3] Previous studies showed how difficult and time-consuming it is to eradicate this organism from hospital settings. Crespo *et al.* described an outbreak caused by VIM-8-type metallo-β-lactamase (MBL)-producing *P. aeruginosa* lasting for more than six years.[4] The MDR *P. aeruginosa* isolates were recovered from sinks and stethoscopes, and reinforcement in disinfection and hygienic precautions reduced the number of patients with MDR *P. aeruginosa* infections and environmental contamination. However, it continued to colonise high risk inpatients.

Additionally, there are several reports describing high mortality in MDR *P. aeruginosa* infections. During the outbreak, 12 (50%) among 24 patients with MDR *P. aeruginosa* infections died in an intensive care unit (ICU).[5] A much higher mortality was observed in another outbreak in ICU, when 10 (77%) of 13 patients with MDR *P. aeruginosa* infections died.[6]

A four-fold increase in MDR *P. aeruginosa* isolates has been observed in an acute care hospital in Japan. According to the microbiology laboratory data from this hospital, 5% of *P. aeruginosa* were MDR between 2003 and 2005, but their incidence suddenly rose to 20.5% in 2006. Therefore, we conducted an outbreak analysis of this hospital to clarify how to eliminate MDR *P. aeruginosa* from hospital settings.

Methods

MDR P. aeruginosa patient isolates

This study took place in a general hospital located in central Japan. It is a secondary-care teaching hospital with 600 beds,

* Corresponding author. Address: Department of Bacteriology 2, National Institute of Infectious Diseases 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Tel.: +81 42 561 0771; fax: +81 42 561 7173.
E-mail address: suzukiss@nih.go.jp (S. Suzuki).

0195-6701/$ – see front matter © 2011 The Healthcare Infection Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.jhin.2011.04.013

including four beds in medical–surgical ICU. The microbiology laboratory data from this hospital from January 2006 to June 2009 were used to select inpatients infected or colonised with MDR *P. aeruginosa*. Identification and antimicrobial susceptibility tests for the isolates were carried out using Microscan WalkAway 96 Plus system (Siemens Japan K.K., Tokyo, Japan). In this study, MDR *P. aeruginosa* was defined as *P. aeruginosa* resistant to imipenem [minimum inhibitory concentration (MIC) $\geq$16 mg/L], levofloxacin (MIC $\geq$ 8 mg/L) and amikacin (MIC $\geq$ 64 mg/L). Additional testing of susceptibility to colistin was performed using E-test (Sysmex bioMérieux Co., Ltd, Tokyo, Japan).

Clinical information on MDR *P. aeruginosa* patient isolates was collected retrospectively from medical records. Information included age, gender, time at risk (number of days from admission to MDR *P. aeruginosa* isolation), prior admission within one year, history of transfer from another hospital, hospitalised departments and wards, basic activities of daily living (ADLs), clinical outcome, underlying diseases, medical care exposures prior to MDR *P. aeruginosa* isolation such as indwelling devices and antimicrobial use over 48 h, and microbiological data. The patients were assumed to have acquired MDR *P. aeruginosa* before hospitalisation when the microbe was isolated within 48 h of admission.[7]

Bacterial isolates

Non-duplicate MDR *P. aeruginosa* isolates obtained from inpatients from January 2006 to June 2009 were evaluated. Environmental cultures were conducted as part of infection control measures in September 2006, March 2007 and June 2009. Nalidixic acid centrimide (NAC) agar (Eiken Chemical Co., Ltd, Tokyo, Japan), a selective agar for *P. aeruginosa*, was stamped on the environmental surface.

Screening of MBL producers and polymerase chain reaction (PCR) detection of MBL genes

All MDR *P. aeruginosa* isolates were screened for MBL production by the double-disc synergy test. Two Kirby–Bauer discs containing 30 μg of ceftazidime and one disc containing 3 mg of sodium mercaptoacetic acid (SMA) (Eiken Chemical Co., Ltd) were used.[8] DNA template preparation and PCR amplification for the detection of bla_{IMP-1}-, bla_{IMP-2}- and bla_{VIM-2}-type MBL genes were performed using previously described primers and conditions.[9–11]

Pulsed-field gel electrophoresis (PFGE) analysis

The isolates were embedded in agarose plugs and digested with SpeI restriction enzyme overnight at 35°C. Electrophoresis was performed with a CHEF Mapper system (Bio-Rad Laboratories, Inc., Tokyo, Japan). The run time was 27 h at 6 V/cm with 14°C, and the switch times ranged from 3 to 31 s. The gel images in tagged image file format were analysed with Fingerprinting II software (Bio-Rad Laboratories, Inc., Tokyo, Japan). Correlation between band patterns was calculated with Dice coefficient, and a dendrogram was generated by the unweighted-pair group method with average linkages.

Multilocus sequence typing (MLST) analysis

MLST was performed for the representative isolates by previously published protocols.[12] Genomic DNA was extracted by using QIAmp DNA Mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's guidelines. The nucleotide sequences were compared to existing sequences in the online MLST database for assignment of allelic numbers and sequence type (ST).

Statistical analysis

All calculations were computed with Epi Info software version 6 (Centers for Disease Control and Prevention, GA, USA) and STATA software version 11 (StataCorp. LP, College Station, TX, USA). Univariate analysis was conducted for each of the variables. Categorical variables were compared by the χ^2 or Fisher's exact test and continuous variables were compared by Student's *t*-test or Wilcoxon rank-sum test. The candidate variables for the logistic regression model included clinically relevant variables and variables with $P < 0.05$ in the univariate analysis. Two-tailed $P < 0.05$ was considered statistically significant.

Results

Outbreak description

A total of 59 patients with MDR *P. aeruginosa* were identified on searching the microbiology laboratory database of the hospital from January 2006 to June 2009. Figure 1a details the wards to which those patients were first admitted on MDR *P. aeruginosa* isolation. There were 14 units in the hospital, nine (64%) of which admitted patients harbouring MDR *P. aeruginosa*. Figure 1b records the MDR *P. aeruginosa* isolate specimens of 59 patients. MDR *P. aeruginosa* was isolated from 35 (59%) urine samples and 16 (27%) respiratory tract samples; six of the 12 patients admitted to ICU before the first MDR *P. aeruginosa* isolation were detected by sputum cultures. Although the number of patients with MDR *P. aeruginosa* fluctuated, they were detected consistently during the study period.

During the first half of the outbreak, from July 2006 to June 2008, 41 patients with MDR *P. aeruginosa* were identified. Twenty-two (54%) of them were hospitalised in ward 3W, and five patients (12%) stayed in the ICU before MDR *P. aeruginosa* isolation. Among patients admitted to ward 3W, 14 (63.5%) were cared for by the department of urology, six (27%) by brain surgery and two (9%) by internal medicine. As most of the patients (73%, 30/41) had urinary tract infections (UTIs) with MDR *P. aeruginosa*, infection control measures were focused on urethral catheter care together with reinforcement in standard precaution and contact isolation since October 2006. Environmental culturing in 2007 yielded one MDR *P. aeruginosa* isolate from the lavatory of ward 3W. During this period, only 14.5% (6/41) of the patients had respiratory tract infection with MDR *P. aeruginosa*.

After July 2008, during the latter half of the outbreak, 16 patients with MDR *P. aeruginosa* were identified and only one of them was admitted to ward 3W. The rest were hospitalised in various units such as ward 6W (department of internal medicine) and 7E (department of cardiology and heart surgery). However, seven (44%) patients stayed in the ICU before detection of MDR *P. aeruginosa* and eight (50%) patients were cared for in cardiology. During the second half of the outbreak, respiratory MDR *P. aeruginosa* isolates increased to 56% (9/16), while urine samples decreased to 25% (4/16), which enhanced infection control in respiratory care procedures such as change to single use of tracheal suctioning tube. Surveillance cultures on urine and sputum samples of ICU patients were also introduced. Environmental culturing yielded one MDR *P. aeruginosa* isolate from a sink in the ICU where ventilator tubings were first cleaned. Moreover, a pot used to irrigate tracheal suction catheters was washed in the same sink. Supply and sterilisation of the respiratory care equipment were improved, and consequently the outbreak ended due to elevated awareness of the importance of infection control among the staff. Since October 2009, only one patient has remained in ward 7E and no new infected or colonised patients have been detected.

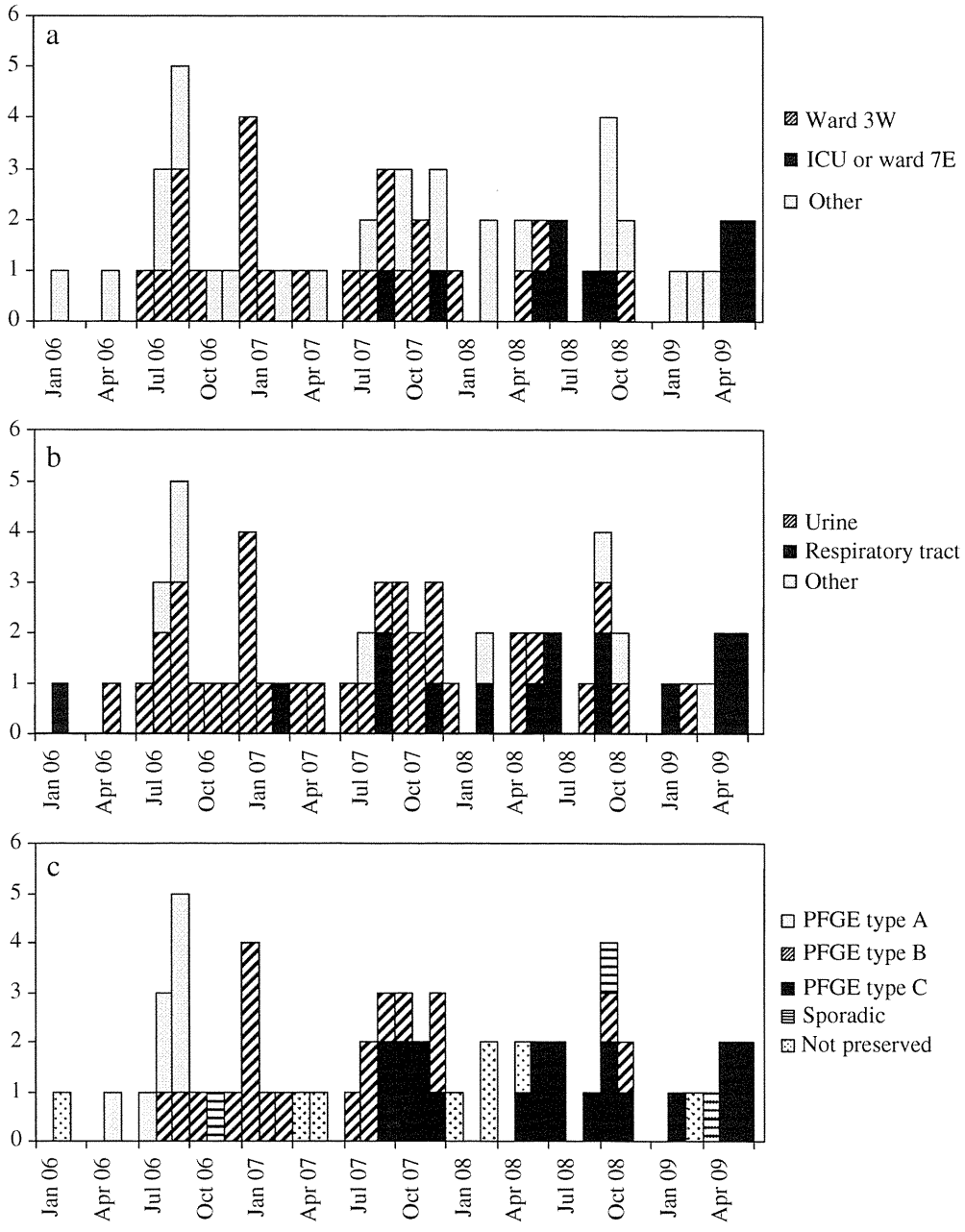

Figure 1. Epidemic curves of patients with multidrug-resistant (MDR) *P. aeruginosa*, January 2006 to June 2009: (a) hospitalised wards on first MDR *P. aeruginosa* isolation; (b) MDR *P. aeruginosa* isolate specimens; (c) pulsed-field gel electrophoresis (PFGE) genotypes of MDR *P. aeruginosa*. $N = 59$ for (a)–(c). ICU, intensive care unit.

Interestingly during the first half of the outbreak, when MDR *P. aeruginosa* was isolated from ICU patients' urine samples, spread to other patients did not occur. But when detected from respiratory tract samples, cross-transmission between severely ill patients in the ICU occurred.

Bacteriological profile of MDR P. aeruginosa isolates

Of 59 patients, 51 MDR *P. aeruginosa* isolates were available since isolates from eight patients were not preserved. All 51 isolates from inpatients and two isolates from the environment were positive for MBL production by the double-disc synergy test using the SMA disc, and the presence of VIM-2 MBL gene was confirmed by PCR amplification with the bla_{VIM-2}-specific primers. These isolates were sensitive to colistin according to Clinical and Laboratory Standards Institute guidelines.[13]

Analysis of MDR P. aeruginosa patients by molecular typing

The MDR *P. aeruginosa* isolates were classified into three large clusters by PFGE analysis; types A, B and C, which consisted of eight, 19 and 21 isolates, respectively (Figure 2). The restriction profiles of the remaining three sporadic isolates differed from one another. Although the band patterns within PFGE type A strains varied, they differed only by one to two bands. The band patterns of more than half (63%, 12/19) of PFGE type B strains were indistinguishable. All but two of PFGE type C strains revealed 100% identical band patterns, suggesting clonal expansion.[14] Although the isolates were categorised into three major clusters, the similarity of those clusters was more than 80%. The MDR *P. aeruginosa* isolate recovered from environmental cultures of ward 3W was PFGE type B, while the one from ICU was PFGE type C. All three representative isolates of PFGE types A, B and C belonged to ST233 by MLST analysis.

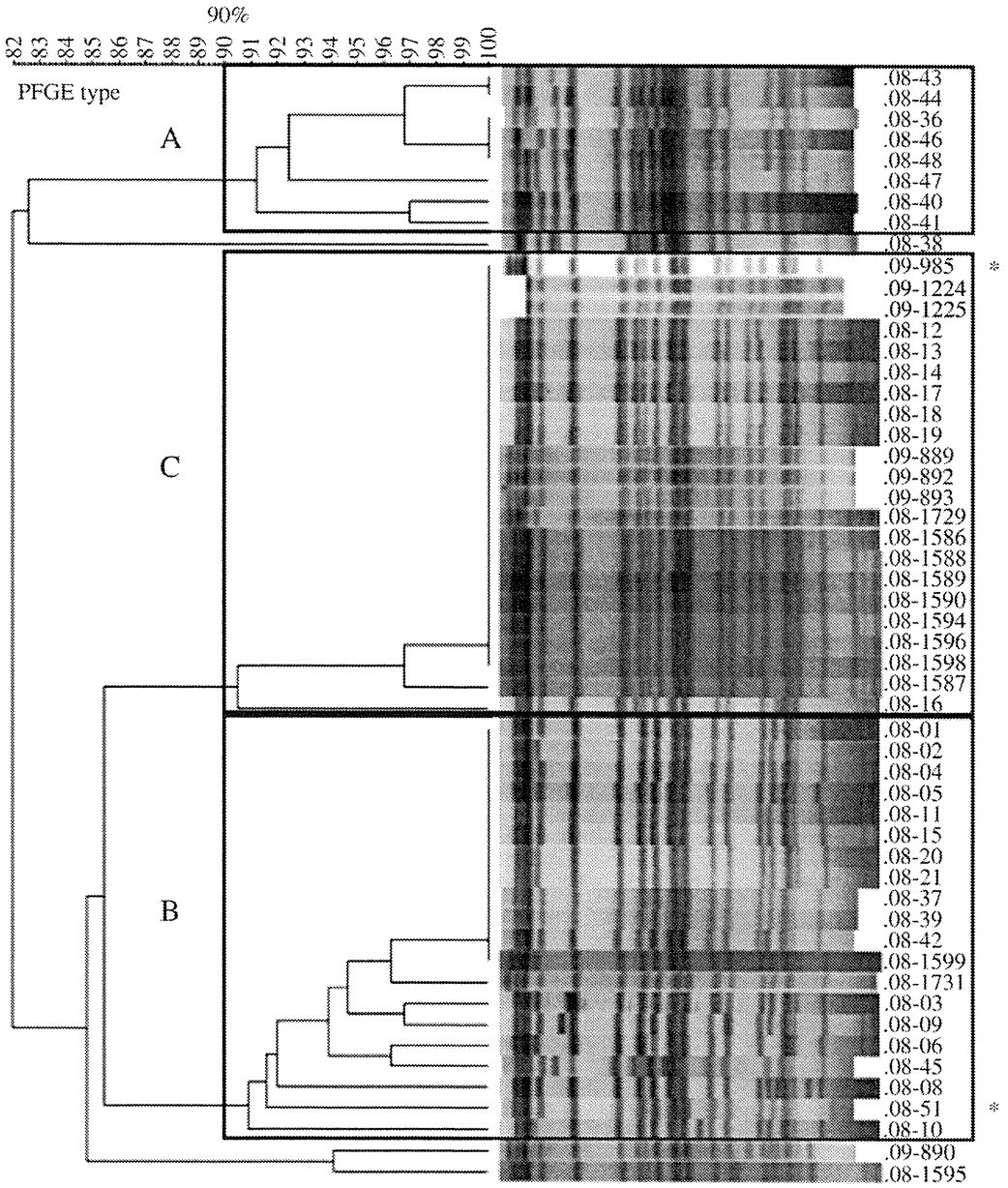

Figure 2. Dendrogram of pulsed-field gel electrophoresis (PFGE) band patterns among VIM-2-positive *P. aeruginosa* isolates based on unweighted-pair group method with average linkages; 51 isolates from inpatients and two isolates from the environment (*). These are categorised into three major clusters, in order of isolation. Isolate numbers are on the right.

Patients with PFGE type A, B, and C strains showed both temporal and spatial clustering (Figure 1c). The outbreak was initiated with patients harbouring PFGE type A strains in May 2006, followed by type B, and type C strains. PFGE type C strains were dominant for the last 12 months of the study period. There were two patients with PFGE type B strains during that period, and one of them was admitted to this hospital in the previous year when PFGE type B strains were predominant. PFGE type A and type B strains were mainly isolated from patients associated with ward 3W.

To identify the risk factor for acquiring PFGE type C strains in the hospital, we compared the characteristics and exposures of patients with PFGE type C strains to type non-C strains by univariate analysis. Three patients with sporadic isolates and six who were assumed to have acquired MDR *P. aeruginosa* before hospitalisation were excluded from this analysis. In all, 42 patients were included (Table I). There was no difference in terms of underlying diseases and medical care exposures (data not shown). Patients with PFGE type C strains were mostly admitted to the ICU or ward 7E before MDR *P. aeruginosa* isolation. ADLs were lower and overall mortality was higher in patients with PFGE type C strains than type non-C strains. Among 21 PFGE type C strains, 11 (52.5%) were recovered from respiratory tract samples. Multiple regression analysis demonstrated that patients with PFGE type C strains were more likely to be isolated from respiratory tract samples (odds ratio: 11.87; 95% confidence interval: 1.21–116.86).

Discussion

This study illustrates the successful containment of a three-and-a-half-year outbreak caused by bla_{VIM-2}-positive *P. aeruginosa*. The first half of the outbreak involved patients harbouring MDR *P. aeruginosa* mainly detected by tests of urine samples. By contrast, patients with MDR *P. aeruginosa* were detected by tests of respiratory tract samples during the latter half of the outbreak. Previous studies

Table I
Characteristics and exposures of patients with multidrug-resistant (MDR) *P. aeruginosa*

Variables	PFGE type C (N = 21)	PFGE type non-C (N = 21)	*P*-value
Age (mean, years)	73.4	72.1	NS
Male	15 (71.4%)	12 (57.1%)	NS
Time at risk (mean, days)	44.5	34.4	NS
Prior admission	5 (23.8%)	7 (33.3%)	NS
Transfer from another hospital	2 (9.5%)	2 (9.5%)	NS
Admission to ICU or ward 7E before MDR *P. aeruginosa* isolation	14 (66.7%)	4 (19.0%)	0.005
Basic activity of daily living: need assistance or bedridden	20 (95.2%)	14 (66.7%)	0.045
Overall death cases	15 (71.4%)	4 (19.0%)	0.002
Department of cardiology	11 (52.4%)	0	<0.001
Vasopressors	16 (76.2%)	4 (19.0%)	<0.001
Respiratory tract sample	11 (52.4%)	1 (4.8%)	0.002
Urine sample	9 (42.9%)	17 (81.0%)	0.026

PFGE, pulsed-field gel electrophoresis; ICU, intensive care unit; NS, non-significant.

have demonstrated that MDR *P. aeruginosa* was mostly isolated from urine samples, ranging from 44% to 73%.[3,15,16] The respiratory tract became the main source of MDR *P. aeruginosa* isolation in the ICU, accounting for around 50%.[5,17] In general, ICU patients are more exposed to respiratory care procedures than patients on other wards. High mortality ranging from 40% to more than 60% has been reported in bacterial nosocomial pneumonia and ventilator-associated pneumonia.[1] Therefore, once MDR *P. aeruginosa* has been introduced to ICU, it may be transmitted by respiratory care procedures among intubated patients, which could increase mortality. To prevent this from happening, one must intervene in respiratory care procedures even if MDR *P. aeruginosa* is mainly isolated from urine samples.

It is noteworthy that the genotypes changed with time during the outbreak. Since PFGE type A, B, and C strains evidenced the same sequence type by MLST analysis, we speculated that they have derived from a common origin and evolved from PFGE type A strains, followed by type B and type C strains. However, the results of multivariate analysis comparing patients with PFGE type C strains to type non-C strains indicated that respiratory tract samples were rather closely related to PFGE type C strains. PFGE type C strains seemed to spread in the ICU and ward 7E through respiratory care procedures. This may suggest that PFGE type C strains acquired better adaptation to the respiratory tract than other types during the outbreak. A study of isolates from cystic fibrosis patients revealed that the genotypes by random amplified polymorphic DNA (RAPD) analysis remained stable despite alterations in phenotypes such as bacterial motility for adaptation.[18] Accordingly, even if PFGE type C strains share a common sequence type with type non-C strains, probable alterations in phenotypes such as pili, non-pilus adhesion and bacterial motility, which are important in colonisation of *P. aeruginosa* to the respiratory tract, may well be considered.

Carbapenem-resistant *P. aeruginosa*, which produces MBL, was first discovered in Japan and then later reported in many different countries.[19,20] In Japan, the predominant type of MBL is IMP-1, whereas VIM-2 is rarely detected, accounting for less than 10%.[21–23] In this study, six patients were assumed to have acquired MDR *P. aeruginosa* before admission. These strains were either PFGE type A or type B, and each of them was the dominant type on isolation. We also analysed the bla_{VIM-2}-positive *P. aeruginosa* isolates from a medical centre located near the hospital in 2002; they were genetically related to PFGE type A strains and belonged to ST233 by MLST analysis. These suggest that bla_{VIM-2}-positive *P. aeruginosa*, especially PFGE type A and B strains, already exists in the local community. To our knowledge, PFGE type C strain has colonised only this hospital.

In conclusion, this study describes a persistent outbreak of MDR *P. aeruginosa*, characterised by change of predilection sites concomitant with genotype shifts of the outbreak strains. Infection control focus on aseptic urethral catheter care successfully controlled PFGE type A and B strains, and improvement in respiratory care procedures terminated the spread of PFGE type C strains. The lesson to be learned from this outbreak is that as soon as MDR *P. aeruginosa* is isolated from urine samples, infection control need not only target catheter care but also ventilator care on ICU.

Acknowledgements

We thank K. Kai, Y. Yoshimura and Y. Taki for technical assistance and T. Yamagishi for helpful discussions.

Conflict of interest
None declared.

Funding sources
This study was supported by H21-Shinkou-Ippan-008 from the Ministry of Health, Labour and Welfare.

References

1. Rossolini GM, Mantengoli E. Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 2005;**11**(Suppl. 4):17–32.
2. Giske CG, Monnet DL, Cars O, Carmeli Y. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother* 2008;**52**:813–821.
3. Kirikae T, Mizuguchi Y, Arakawa Y. Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan. *J Antimicrob Chemother* 2008;**61**:612–615.
4. Crespo MP, Woodford N, Sinclair A, *et al.* Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing VIM-8, a novel metallo-β-lactamase, in a tertiary care center in Cali, Colombia. *J Clin Microbiol* 2004;**42**:5094–5101.
5. Hota S, Hirji Z, Stockton K, *et al.* Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. *Infect Control Hosp Epidemiol* 2009;**30**:25–33.
6. Bukholm G, Tannaes T, Kjelsberg AB, Smith-Erichsen N. An outbreak of multidrug-resistant *Pseudomonas aeruginosa* associated with increased risk of patient death in an intensive care unit. *Infect Control Hosp Epidemiol* 2002;**23**:441–446.
7. Horan TC, Emori TG. Definitions of key terms used in the NNIS system. *Am J Infect Control* 1997;**25**:112–116.
8. Arakawa Y, Shibata N, Shibayama K, *et al.* Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. *J Clin Microbiol* 2000;**38**:40–43.
9. Shibata N, Doi Y, Yamane K, *et al.* PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *J Clin Microbiol* 2003;**41**:5407–5413.

10. Senda K, Arakawa Y, Ichiyama S, *et al.* PCR detection of metallo-beta-lactamase gene (bla_{IMP}) in gram-negative rods resistant to broad-spectrum β-lactams. *J Clin Microbiol* 1996;**34**:2909–2913.
11. Yagi T, Kurokawa H, Shibata N, Shibayama K, Arakawa Y. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. *FEMS Microbiol Lett* 2000;**184**:53–56.
12. Curran B, Jonas D, Grundmann H, Pitt T, Dowson CG. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J Clin Microbiol* 2004;**42**:5644–5649.
13. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; nineteenth informational supplement.* Document M100–S19. Wayne, PA: CLSI; 2009.
14. Tenover FC, Arbeit RD, Goering RV, *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;**33**:2233–2239.
15. Tsuji A, Kobayashi I, Oguri T, Inoue M, Yabuuchi E, Goto S. An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *Pseudomonas aeruginosa* isolated at medical institutes nationwide in Japan. *J Infect Chemother* 2005;**11**:64–70.
16. Sekiguchi J, Asagi T, Miyoshi-Akiyama T, *et al.* Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. *J Clin Microbiol* 2007;**45**:979–989.
17. Deplano A, Denis O, Poirel L, *et al.* Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. *J Clin Microbiol* 2005;**43**:1198–1204.
18. Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Speert DP. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol* 1996;**34**:1129–1135.
19. Watanabe M, Iyobe S, Inoue M, Mitsuhashi S. Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1991;**35**:147–151.
20. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-lactamases: the quiet before the storm? *Clin Microbiol Rev* 2005;**18**:306–325.
21. Kimura S, Alba J, Shiroto K, *et al.* Clonal diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in geographically diverse regions of Japan. *J Clin Microbiol* 2005;**43**:458–461.
22. Nishio H, Komatsu M, Shibata N, *et al.* Metallo-β-lactamase-producing gram-negative bacilli: laboratory-based surveillance in cooperation with 13 clinical laboratories in the Kinki region of Japan. *J Clin Microbiol* 2004;**42**:5256–5263.
23. Yatsuyanagi J, Saito S, Ito Y, *et al.* Identification of *Pseudomonas aeruginosa* clinical strains harboring the bla_{VIM-2} metallo-β-lactamase gene in Akita Prefecture, Japan. *Jpn J Infect Dis* 2004;**57**:130–132.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2011, p. 4352–4360 Vol. 55, No. 9
0066-4804/11/$12.00 doi:10.1128/AAC.01274-10

Genetic Organization and Mode of Action of a Novel Bacteriocin, Bacteriocin 51: Determinant of VanA-Type Vancomycin-Resistant *Enterococcus faecium*▽†

Hitoshi Yamashita,[1] Haruyoshi Tomita,[1,2] Takako Inoue,[1] and Yasuyoshi Ike[1,2]*

Department of Bacteriology[1] and Laboratory of Bacterial Drug Resistance,[2] Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Received 21 September 2010/Returned for modification 3 November 2010/Accepted 17 June 2011

Bacteriocin 51 (Bac 51) is encoded on the mobile plasmid pHY (6,037 bp), which was isolated from vancomycin-resistant *Enterococcus faecium* VRE38. Bacteriocin 51 is active against *E. faecium*, *E. hirae*, and *E. durans*. Sequence analysis of pHY showed that it encodes nine open reading frames (ORFs) from ORF1 to ORF9 (in that order). Genetic analysis suggested that ORF1 and ORF2, which were designated *bacA* and *bacB*, respectively, are the bacteriocin and immunity genes. *bacA* encodes a 144-amino-acid protein. The deduced BacA protein has a typical signal sequence at its amino terminus, and a potential signal peptidase-processing site corresponding to the V-E-A sequence is located between the 37th and 39th amino acids. The predicted mature BacA protein consists of 105 amino acids. A potential promoter sequence was identified upstream of the start codon. *bacB* encodes a 55-amino-acid protein. No obvious promoter or terminator sequence was identified between *bacA* and *bacB*. Northern blot analysis of *bacA* and *bacB* with a *bacA* RNA probe produced a transcript of approximately 700 nucleotides, which corresponded to the combined nucleotide sizes of *bacA* and *bacB*, indicating that transcription was initiated from the promoter upstream of *bacA*, continued through *bacB*, and was terminated at the terminator downstream of *bacB*. The transcription start site was determined to be the T nucleotide located 6 nucleotides downstream from the −10 promoter sequence. These results indicate that *bacA* and *bacB* constitute an operon and that *bacA* is the bacteriocin structural gene while *bacB* is the immunity gene. The purified C-terminally His tagged BacA protein of Bac 51 showed bacteriostatic activity against the indicator strain. The purified C-terminally His tagged BacA protein of Bac 32 (whose mature BacA protein has 54 amino acids) and the culture filtrates of the Bac 31- and Bac 43-producing *E. faecalis* strain FA2-2 showed bactericidal activity. Bac 31 and Bac 43 are pore-forming bacteriocins, unlike the newly characterized bacteriocin Bac 51.

Bacteriocins are produced by a wide variety of Gram-positive and Gram-negative bacteria. They are bacterial proteins that inhibit the growth of bacteria closely related to the producer strain, and they usually exhibit a relatively narrow spectrum of activity. Bacteriocins are thought to provide the producer strain with an ecological or selective advantage over other strains. Bacteriocin production has been described for several genera of lactic acid bacteria (LAB) (10). LAB bacteriocins can be divided into two main classes. Class I consists of modified bacteriocins (the lantibiotics), and class II consists of the small heat-stable nonlantibiotics (27). Class II bacteriocins are further divided into subgroup IIa, comprising pediocin-like bacteriocins with strong antilisterial effects, and subgroup IIb, comprising non-pediocin-like bacteriocins composed of two peptides and requiring the complementary activity of both peptides for full antimicrobial activity. In the genus *Enterococcus*, *Enterococcus faecalis* and *E. faecium* bacteriocins have been well characterized genetically and biochemically. *E. faecalis* bacteriocins include the beta-hemolysin/bacteriocin (cytolysin) (8, 9, 17–22), the peptide antibiotic AS-48 (26), bacteriocin 21 (Bac 21) (16, 33), and bacteriocin 31 (Bac 31) (32). These *E. faecalis* bacteriocins have been identified in clinical isolates (8, 26, 31–33). The well-characterized *E. faecium* bacteriocins have been identified in food-grade organisms (7). These include enterocins A (2), B (4), P (6), I (13), and L50A and L50B (5). These bacteriocins belong to LAB class II bacteriocins and are active against *Listeria monocytogenes* (27). Enterocins A and P are pediocin-like bacteriocins (27).

Little is known about the bacteriocins present in *E. faecium* clinical isolates. Previously, we were the first to report the isolation and characterization of bacteriocin 32 (Bac 32), which is encoded on mobile plasmid pTI1 (12.5 kbp) of VRE200, a vancomycin-resistant *E. faecium* (VRE) clinical isolate (23). Bac 32 is active against *E. faecium*, *E. hirae*, and *E. durans* but has shown no activity against *L. monocytogenes*, unlike the bacteriocins identified in food-grade *E. faecium* strains. A high frequency of clinical isolates (about 40%) encode Bac 32-type bacteriocins (23). In this study, we describe the genetic analysis of a novel bacteriocin, designated bacteriocin 51 (Bac 51), that was identified in a VRE clinical isolate. We show that Bac 51 is active against *E. faecium*, *E. hirae*, and *E. durans* but not against *L. monocytogenes*.

* Corresponding author. Mailing address: Department of Bacteriology and Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma 371-8511, Japan. Phone: 81-27-220-7990. Fax: 81-27-220-7996. E-mail: yasuike@med.gunma-u.ac.jp.

† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 27 June 2011.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Description	Reference or source
Strains			
Enterococcus faecalis			
FA2-2	Rif^r Fus^r	Derivative of JH2	31
OG1S	Str^r	Derivative of OG1	11
Enterococcus faecium			
BM4105RF	Rif^r Fus^r	Derivative of plasmid-free *E. faecium* BM4105	35
BM4105SS	Str^r Spc^r	Derivative of plasmid-free *E. faecium* BM4105	35
VRE38	pHY (Bac 51) Van^r Gm^r Em^r Tc^r	Bacteriocinogenic clinical isolate	This study
Enterococcus hirae ATCC 9790			
Enterococcus durans ATCC 49135			
Enterococcus raffinosus JCM8733			
Enterococcus gallinarum BM4174			
Staphylococcus aureus FDA209P			
Escherichia coli			
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44* Δ(*argE-lacZYA*)*U169*		Bethesda Research Laboratories
TH688	CSH57b *thr*::Tn*5*		
BL21	F$^-$ *ompT hsdS*(r_B^- m_B^-) *gal dcm* (DE3)	Protein expression	Novagen
Plasmids			
pHY	Bac 51	Mobilizable plasmid (6.0 kb)	This study
pTI1	Bac 32	Mobilizable plasmid (12.5 kb)	23
pYI17	Bac 31	Conjugative plasmid (57.5 kb)	32
pMG502	Bac 43	pAM401 containing ORF1 and ORF2 of pDT1	30
pAM401	Cm^r Tc^r	*E. coli-E. faecalis* shuttle vector	36
pMW119	Amp^r *lacZ*	*E. coli* cloning vector; low copy number	Nippon Gene Co.
pUC19	Amp^r *lacZ*	*E. coli* cloning vector	Nippon Gene Co.
pET22b(+)	Amp^r; His_6 affinity tag	Overexpression vector	Novagen

MATERIALS AND METHODS

Bacteria, media, and reagents. The laboratory strains and plasmids used in this study are listed in Table 1. Six VanA-type vancomycin-resistant *Enterococcus faecium* (VRE) clinical isolates were obtained from different patients who had been admitted to a hospital in Japan. A total of 138 vancomycin-sensitive *E. faecium* and 120 vancomycin-sensitive *E. faecalis* clinical isolates were included in the study. These strains were obtained from different patients at the Gunma University School of Medicine Hospital, Maebashi, Gunma, Japan. In addition, 87 VRE clinical isolates were obtained from different patients in Japanese hospitals in 2002, and 662 VRE isolates were obtained at the University of Michigan Medical School Hospital, Ann Arbor, between 1994 and 1999 (35). Enterococcal strains were grown in Todd-Hewitt broth (THB; Difco, Detroit, MI). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium. Solid or soft medium was prepared by the addition of 1.5 or 0.75% (wt/vol) agar, respectively. All cultures were grown at 37°C. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 500 μg/ml; spectinomycin, 500 μg/ml for enterococci and 50 μg/ml for *E. coli*; kanamycin, 40 μg/ml; tetracycline, 12.5 μg/ml; vancomycin, 5 μg/ml; and gentamicin, 200 μg/ml.

Antimicrobial susceptibility testing. The MICs of the antibiotics were determined by the agar dilution method. A pure overnight culture of each strain grown in Mueller-Hinton broth (Nissui, Tokyo, Japan) was diluted 100-fold with fresh broth. An inoculum of approximately 5×10^5 cells was plated on a series of Mueller-Hinton agar (Eiken, Tokyo, Japan) plates containing a range of concentrations of the test drug. The plates were incubated at 37°C, and the susceptibility results were finalized after 24 h of incubation. Susceptibility testing and interpretation of the results were in compliance with the standards of the Clinical and Laboratory Standards Institute (formerly NCCLS). *E. hirae* ATCC 9790 was used as a control strain.

Detection of bacteriocin production and immunity. To detect antimicrobial activity, a soft-agar assay was performed as described previously (20). The test for immunity to the bacteriocin was performed essentially as described previously (20). To examine the bacteriocin activities of liquid samples (e.g., cell-free supernatants or purified bacteriocin protein solutions), the metal-cup method was used. A 50-μl volume of the sample was dropped into a metal cup on the surface of soft (0.75%) THB agar inoculated with the indicator strain. After incubation, the plate was examined for inhibition zones below and around the metal cup.

Conjugative transfer and mobilization experiments. Solid-surface mating was performed on agar plates (19, 23, 34). The mating mixture of the donor and recipient was made with a donor/recipient ratio of 1:10, and 10 μl of the mixed culture was spotted onto THB agar without antibiotics. The plates were then incubated overnight at 37°C. After incubation, the bacteria that had grown on the agar plates were scraped off and transferred to 1 ml of fresh THB, and then 0.1 ml of the mixture was spread onto appropriate selective agar plates. The colonies were counted after 48 h of incubation at 37°C. The frequency of conjugative transfer was calculated as the ratio of the number of transconjugants to the number of donors. The frequency of mobilized transfer was calculated as the ratio of the number of transconjugants that showed bacteriocin activity to the number of donors.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method (28). Plasmid DNA was treated with restriction enzymes and was subjected to agarose gel electrophoresis for analysis of DNA fragments, etc. Restriction enzymes were obtained from Nippon Gene (Toyama, Japan), New England Biolabs, Inc., and Takara (Tokyo, Japan) and were used in accordance with the suppliers' specifications. Agarose was obtained from Wako Chemicals, Osaka, Japan. Electrophoresis on 0.8% agarose gels was used to determine the sizes of DNA fragments larger than 0.5 kb. The eluted fragments were ligated to dephosphorylated, restriction enzyme-digested vector DNA with

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Description
M13F	GTTTTCCCAGTCACGACGTT	Analysis of pHY sequence
M13R	GGAAACAGCTATGACCATGA	
D1	AAACAGATTTAACGGAGTAC	Analysis of pHY sequence
D2	CAAAGAAACGGAAAGAAGCAAC	
D3	AAAGACATTCATTGCATCAAC	
D4	GATTGCCTGGGCAATGTCTG	
D5	TAGCCACTTTGTAGCTCGTTG	
D6	TACAACAGTCCTAACGAAGA	
Tn*5*	AATTGGGCGGCGACGTTAAC	Analysis of Tn*5* insertion mutant
T1	ATTTTAGCTGTAATTATTTC	Analysis of Tn*5* insertion mutant
T2	TGAAAAAGCCAACCAACTAG	
T3	GGCTCATCTATAAAATTTTC	
T4	AGGATTTTTATCCAAGACTG	
T5	TCCTAAAAGTTATAAGCAAC	
T6	TTCTAGGCTTCAGTCCGATG	
T7-1F	ATATATTAATACGACTCACTATAGGGAAGGAGGAAGTAACAATGAA[a]	*bacA*-specific primer for RNA probe
T7-1R	TTATTTTATTACATAACGAG	
T7-2F	ATATATTAATACGACTCACTATAGGGTCAGAAAGTGAAACTATCTC[a]	*bacB*-specific primer for RNA probe
T7-2R	CTATTTAGAGTTTTTATTTC	
RT377	[Phos]ACATAATAGTTC[b]	Reverse transcription primer for 5′-RACE[c] method
A1-246	ACCGAACAAAAATAGACAAG	5′-RACE method
A2-201	CATTGTTACTTCCTCCTTAG	
S1-260	CAAGTACTCCGTTAAATCTG	
S2-288	TAGTGCTAGAGGTATAAAAG	
EP1	ACTTGTCTATTTTTGTTCGG	*bacA*-specific primer
EP2	GTTGTATTGCTAAAGTGAGC	
pHY-316F-*Nde*I	TTTTTTCATATGGCAAGTTCACGATATAATCATAATC[d]	Cloning into pET22b(+)
pHY-630R-*Xho*I	TTTTTTCTCGAGTTTTATTACATAACGAGCGTAATC[d]	
pTI1-183F-*Nde*I	TTTTTTCATATGGCTGCTCAAAGAGGATATATC[d]	Cloning into pET22b(+)
pTI1-344R-*Xho*I	TTTTTTCTCGAGAATGTAGTAATAATATTTGGC[d]	

[a] The T7 promoter sequence is underlined.
[b] [Phos], phosphorylated 5′ end.
[c] RACE, rapid amplification of cDNA ends.
[d] Restriction enzyme cloning sites are underlined.

T4 DNA ligase and were then introduced into *E. coli* by electrotransformation (15). Transformants were selected on Luria-Bertani agar containing the appropriate antibiotics.

PFGE analysis. Pulsed-field gel electrophoresis (PFGE) was carried out on a 1% agarose gel with 0.5% Tris-borate-EDTA buffer. The following settings were applied: 1 to 23 s, 6 V/cm, and 22 h (with the CHEF Mapper system [Bio-Rad]).

PCR methodology. The PCR program, with *Ex Taq* DNA polymerase (Takara), comprised 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C, and a final incubation at 4°C with a GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer).

DNA sequence analysis. Nucleotide sequence analysis was carried out as described previously (28). To determine the entire sequence of pHY, shotgun sequencing was performed. Fragmented DNA libraries were constructed by sonication of pHY, followed by ligation into the SmaI-digested pUC19 vector plasmid. pUC19 plasmids containing 0.5- to 1.0-kb inserts were used to transform *E. coli* DH5α. The resulting constructs were sequenced in both orientations with an ABI Prism 310 genetic analyzer. The BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems) and primers M13F and M13R were used for the sequencing reaction (Table 2). Open reading frame (ORF) analysis was performed with Genetyx, version 6.1 (Genetyx Corp., Tokyo, Japan). The DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan) and a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) were used for homology analysis of nucleotide and amino acid sequences.

Cloning of pHY plasmid DNA. To identify the bacteriocin determinant and the immunity determinant of the bacteriocin, the entire pHY plasmid DNA was digested with EcoRI (since there was a single EcoRI site in ORF9) and was cloned into vector pMW119. The spectinomycin resistance gene, which is expressed in both *E. coli* and *E. faecalis*, was cloned into the pMW119 XmaI site from pDL278 (12, 25). The cloned plasmid DNA was prepared from *E. coli* DH5α and was used either to transform *E. faecalis* OG1S in order to test for bacteriocin activity or to transform *E. hirae* ATCC 9790 in order to test for immunity to the bacteriocin.

Generation of transposon (Tn*5*) insertion mutants. Tn*5* (Km^r) was inserted into the cloned plasmid DNA as described elsewhere (29). The target plasmid pMW119 (Spc^r)::pHY was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) by electrotransformation. Transformants were spread onto selective plates containing kanamycin and spectinomycin, and the plates were left at room temperature for 10 days. The bacteria that grew on the selective plates were pooled, and the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin and spectinomycin for the selection of Tn*5*-mediated kanamycin resistance and plasmid-mediated spectinomycin resistance, respectively. The transformants were purified and examined to determine the location of Tn*5* within the plasmid. The precise locations of Tn*5* insertion were determined by DNA sequence analysis using a synthetic primer that hybridized to the end of Tn*5* (Table 2).

Northern blotting of *bacA* and *bacB* transcripts. The method for Northern blotting of *bacA* and *bacB* transcripts is described elsewhere (28). The *bacA* and *bacB* probes were prepared with a DIG Northern Starter kit (Roche Diagnostics GmbH, Mannheim, Germany). T7-1F and T7-1R were used as primers with *bacA*; T7-2F and T7-2R were used as primers with *bacB*; and pHY plasmid DNA was used as the template (Table 2). Total RNAs were prepared with a FastRNA Pro Red kit (MP Biomedicals, LLC). Northern blot analysis involved electrophoresis in a 1.2% agarose-morpholinepropanesulfonic acid (MOPS)-formaldehyde system. Hybridization and detection were performed with a DIG Northern Starter kit. The chemiluminescent substrate CDP-Star (Boehringer Mannheim) was used for visualization of the RNA bands. Chemiluminescence was detected using Lumi-Film (Boehringer Mannheim).

Identification of the bacteriocin 51 transcriptional initiation site. To determine the initiation site of the transcript, rapid amplification of cDNA ends (RACE) was used. Total RNA was isolated from *E. faecium* BM4105SS(pHY). The transcriptional initiation site was determined using the 5′-Full RACE core set (Takara Bio Inc.) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out in a reverse transcription reaction using the 5′-phosphorylated primer RT377, which is specific to the target RNA, and 5 μg

TABLE 3. Drug resistance of VanA-type vancomycin-resistant enterococci

E. faecium strain	Level of resistance (MIC [μg/ml]) to the following antimicrobial drug[a]:									
	VAN	TEC	GEN	KAN	STR	ERY	CHL	TET	AMP	CIP
VRE34	1,024	32	≥1,024	≥1,024	16	≥1,024	8	32	512	8
VRE35	1,024	16	1,024	≥1,024	16	≥1,024	4	64	512	8
VRE36	1,024	128	4	128	16	≥1,024	4	64	512	128
VRE37	1,024	32	≥1,024	≥1,024	≥1,024	≥1,024	8	32	512	8
VRE38	1,024	128	≥1,024	≥1,024	16	≥1,024	4	128	512	256
VRE39	1,024	32	≥1,024	≥1,024	16	≥1,024	4	64	256	8

[a] VAN, vancomycin; TEC, teicoplanin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CHL, chloramphenicol; TET, tetracycline; AMP, ampicillin; CIP, ciprofloxacin.

of total RNA (Table 2). The resulting purified cDNA was circularized with RNA ligase. The sequence of the unknown region containing the transcription initiation site was determined by two rounds of PCR; the first round used the circular cDNA as a template with primers A1-246 and S1-260, and the second round used the first PCR product as a template with primers A2-201 and S2-288 (Table 2).

DNA-DNA hybridization. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany). All procedures were based on the manufacturer's manual and standard protocols (28). The plasmid DNA was isolated and digested with EcoRI. Hybridization was performed overnight at 42°C in the presence of 50% formamide. The probes were generated by using a DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). The signals were detected with a nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate stock solution (Roche Diagnostics GmbH).

Construction of the plasmid producing C-terminally His tagged bacteriocin protein. The plasmid producing C-terminally His tagged bacteriocin (Bac) protein was constructed using the pET-22b(+) vector plasmid according to the manufacturer's instructions (Novagen). The *E. coli* vector pET-22b(+) (Amp^r), containing the C-terminal 6-amino-acid His tag sequence just downstream from the XhoI sequence, was used to introduce a His tag into the C terminus of the mature Bac protein. The DNA sequence of the predicted mature Bac protein was amplified by PCR using *E. faecium* BM4105SS(pHY) for Bac 51 and *E. faecalis* FA2-2(pTI1) for Bac 32. The PCR product obtained was cloned between the NdeI and XhoI sites of the pET-22b(+) vector. The resultant cloned plasmid was transformed into *E. coli* BL21. Ampicillin-resistant transformants (Amp^r) were selected on LB plates containing ampicillin, and the transformant was designated *E. coli* BL21[pET-22b(+)::*bac*].

Purification of His-tagged Bac 51 protein from *E. coli* BL21[pET22b(+)::*bac*]. The *bac* gene was expressed as a His-tagged fusion protein, which was purified on a Ni-nitrilotriacetic acid (NTA) column according to the manufacturer's protocol (Novagen, Madison, WI). LB broth (100 ml) containing 100 μg of ampicillin per ml was inoculated with 1 ml of an overnight culture of *E. coli* BL21[pET22b(+)::*bac*]. Cells were grown aerobically at 30°C to an optical density at 600 nm (OD_{600}) of 0.5, and then isopropyl-β-D-thiogalactoside (IPTG) was added to a concentration of 0.5 mM for 4 h at 30°C to induce protein expression. Cells were harvested from liquid medium by centrifugation for 10 min at 10,000 × *g*. The medium was decanted, and the pellet was allowed to drain so as to remove as much liquid as possible.

The pellet was completely resuspended in 10 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl) to give a 10× concentration factor (100 ml of culture to a 10-ml buffer volume), and lysozyme was then added to a 100-μg per ml concentration. Phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM was also added. The mixture was incubated at 25°C for 30 min prior to sonication. The resuspended pellet was mixed by swirling and was sonicated on ice using a sonicator (Ultrasonic Disruptor UD-201; Tomy) set at power level 6, at 40% duty, for 20 min. The entire lysate was centrifuged for 10 min at 14,000 × *g* to separate the soluble and insoluble fractions. The soluble supernatant was used for the isolation of His-tagged Bac. His-tagged Bac was isolated using the Ni-NTA nickel chromatography resin (Ni-NTA purification system; Invitrogen, CA) according to the manufacturer's instructions. The bacteriocin protein was eluted from the column by first loading 10 ml of imidazole at concentrations of 10 mM, 20 mM, 35 mM, and 50 mM and then loading 4 ml of imidazole at concentrations of 100 mM, 200 mM, 300 mM, and 400 mM.

SDS-PAGE. The purity of each His-tagged BacA (BacA-His) sample was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out as described previously (28). Electrophoresis was conducted on 18% (wt/vol) polyacrylamide separating gels at a constant current of 30 mA for 60 min. To visualize the proteins, the gel was stained with Silver Stain Plus (Bio-Rad Laboratories, CA) or a SeePico CBB stain kit (Benebiosis Co., Seoul, South Korea) according to the manufacturer's instructions. Precison Plus protein dual-color standards (Bio-Rad Laboratories, CA), consisting of a mixture of proteins with molecular masses ranging from 10 to 250 kDa, were electrophoresed beside the bacteriocin samples in each gel.

Preparation of a culture filtrate from *E. faecalis* FA2-2 carrying a bacteriocin plasmid. *E. faecalis* FA2-2 carrying a bacteriocin plasmid was grown to late-exponential phase. The culture was centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was filtered through a Millipore filter (pore size, 0.45 μm), and the filtrate was used to test for bacteriocin activity.

Bacteriocin activity. The bacteriocin activities of the eluates obtained throughout the purification process or of the culture filtrate were calculated by microtube assays. The test samples were 2-fold serially diluted with microtubes containing 5 μl imidazole. A 5-μl volume of each dilution was spotted onto the soft-agar plate containing the indicator strain, and the plates were incubated at 37°C for 18 h. Growth inhibition was examined, and the bacteriocin activity was calculated in bacteriocin units (BU). One bacteriocin unit was defined as the reciprocal of the highest dilution of bacteriocin causing growth inhibition. For example, if the highest dilution was 128-fold, the bacteriocin activity was 25 BU/μl (i.e., 128/5 μl).

Liquid bacteriocin assay. The bacteriocin indicator strain *E. hirae* ATCC 9790 was grown overnight in THB at 37°C. An overnight culture of the strain was diluted 10^2-fold with fresh THB. A diluted culture of the indicator strain containing about 10^7 cells per ml was incubated for 90 min at 37°C with gentle shaking. The culture was diluted 10^4-fold with fresh THB. The diluted culture, which contained about 10^3 bacterial cells per ml, was used as the indicator strain in the liquid bacteriocin assay. One portion (0.2 ml) of the test sample was added to nine portions (1.8 ml) of the diluted culture of the indicator strain. These cultures were incubated at 37°C with gentle shaking. Samples were removed at various intervals and were appropriately diluted, and the dilution was plated onto a THB agar plate to determine cell survival.

Effect of temperature on bacteriocin activity. Purified bacteriocin from the BacA-His samples was exposed to temperatures of 60°C, 70°C, 80°C, 90°C, and 100°C for 5 min or 10 min. After heat treatment, the samples were cooled rapidly, and then 5 μl of the sample was spread onto soft agar containing the indicator strain in order to carry out the soft-agar assay. Based on the initial results, BacA-His samples were then exposed to 70°C, 72°C, 74°C, 76°C, 78°C, and 80°C for 10 min, and the residual bacteriocin activities of the heat-treated samples was examined using the soft-agar assay. Untreated samples were used as controls in all cases.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article are available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB570326.

RESULTS

Bacteriocinogenic strains and the plasmid encoding the bacteriocin. Drug resistance of six VanA-type vancomycin-resistant *E. faecium* (VRE) isolates recovered in a hospital is shown in Table 3. Two strains, VRE34 and VRE35, were found to be identical, and the other four were found to be different, by PFGE analysis of their chromosomal DNAs (data not shown). Of these VRE strains, five (i.e., VRE34, -36, -37, -38, and -39)