Table 1. STs and characteristics of the 28 PRGBS strains

Strain	ST	Serotype	Specimen	Year of isolation	Reference
B1	4[a]	III	sputum	1995	10
B6	1	VIII	sputum	1997	10
B7	23	III	sputum	1997	10
B8	458[a]	VI	sputum	1997	10
B10	297[a]	III	sputum	1997	10
B12	297[a]	III	sputum	1997	10
B40	297[a]	III	sputum	1997	10
B60	23	III	sputum	1998	10
B68	1	VI	sputum	1998	10
B502	458[a]	VI	sputum	2005	10
B503	458[a]	Ib	sputum	2005	10
B513	1	III	sputum	2005	10
B514	458[a]	VI	sputum	2005	10
B516	458[a]	III	sputum	2005	10
MRY06-238	1	VI	sputum	2006	this study
MRY06-241	458[a]	VI	sputum	2006	this study
MRY08-517	1	VI	sputum	2005	this study
MRY08-527	358[a]	Ib	sputum	2008	this study
MRY08-528	458[a]	VI	TTA	2005	this study
MRY08-1422	464	III	blood	2008	this study
R1	458[a]	VI	TTA	2003	11
R2	458[a]	VI	TTA	2003	11
R3	1	Ib	CS	2004	11
R4	1	VI	sputum	2004	11
R5	458[a]	VI	PHA	2003	11
R6	458[a]	VI	sputum	2004	11
R7	23	Ia	sputum	2004	11
R8	23	NT	sputum	2004	11

TTA, transtracheal aspirate; CS, conjunctival sac discharge; PHA, pharyngeal swab; NT, non-typeable.
[a]This ST belongs to the 'ST1 group', as it possesses no more than three allelic changes compared with ST1.

Table 2. STs, ST profiles, allelic profiles and numbers of PRGBSs

ST	ST profile	Allelic profile (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, *tkt*)	Number of PRGBSs (%)
ST1	ST1	1, 1, 2, 1, 1, 2, 2	7 (25)
ST458[a]	one allelic variant of ST1	1, 1, 2, 1, 1, 2, 3	11 (39)
ST297[a]	one allelic variant of ST1	1, 1, 2, 2, 1, 2, 2	3 (11)
ST4[a]	three allelic variant of ST1	1, 1, 4, 1, 1, 3, 4	1 (4)
ST358[a]	three allelic variant of ST1	1, 1, 4, 1, 3, 3, 2	1 (4)
ST23	ST23	5, 4, 6, 3, 2, 1, 3	4 (14)
ST464	three allelic variant of ST23	5, 4, 4, 3, 2, 3, 1	1 (4)
			total=28 (100)

[a]This ST belongs to the 'ST1 group', as it possesses no more than three allelic changes compared with ST1 (see the 'Allelic profile' column).

described previously.[10] After allelic profiling, the ST was assigned through the MLST website for *S. agalactiae* (http://pubmlst.org/sagalactiae). eBURST analysis was performed using eBURST version 3, available through the website http://eburst.mlst.net/.

Results

The STs and characteristics of the 28 PRGBSs are listed in Table 1. All were exactly confirmed as PRGBS by determination of the MIC of penicillin by the agar dilution method as recommended by the CLSI and sequence analysis of the PBP2X gene. The results were consistent with the results of the disc diffusion tests using ceftibuten discs.[17] The MICs of penicillin for the 28 isolates were in the range 0.25–1 mg/L. Among the 28 clinical isolates, 26 harboured both or either of the two PRGBS-specific amino acid substitutions, Q557E and V405A, in PBP2X. Although the two remaining clinical isolates, B7 and MRY08-1422, harboured neither the Q557E nor V405A substitution in PBP2X, these clinical isolates harboured several amino acid substitutions other than Q557E and V405A in the transpeptidase domain of PBP2X, although the contribution of those substitutions to the augmented MICs of several β-lactams remains to be elucidated.

The STs of the 28 isolates are shown in Tables 1 and 2. Eleven of the 28 isolates (39%) belonged to ST458, a new ST identified in the present study. The numbers of PRGBS isolates of ST1, ST23 and ST297 were 7 (25%), 4 (14%) and 3 (11%), respectively. The remaining three isolates belonged to ST4, ST358 and ST464.

By eBURST analysis, a single allelic difference was seen in both ST458 (allelic profile: 1, 1, 2, 1, 1, 2, 3) and ST297 (allelic profile: 1, 1, 2, 2, 1, 2, 2) when compared with ST1 (allelic profile: 1, 1, 2, 1, 1, 2, 2), therefore ST1, ST458 and ST297 formed clonal complex (CC) 1, as illustrated in Figure 1. There were 21 isolates of ST1, ST458 and ST297, accounting for 75% of the PRGBS tested. Thus CC1 was the predominant CC of PRGBS characterized in the present study. Moreover, ST4 (allelic profile: 1, 1, 4, 1, 1, 3, 4) and ST358 (allelic profile: 1, 1, 4, 1, 3, 3, 2) have three allelic differences compared with ST1 (allelic profile: 1, 1, 2, 1, 1, 2, 2); these two STs also belong to CC1 and form the ST1 group, together with ST1, ST458 and ST297. On the other hand, ST23 (four isolates) and ST464 (one isolate) belonged to a group far different from the ST1/ST19 group (Figure 1a and Figure S1). ST464 (allelic profile: 5, 4, 4, 3, 2, 3, 1) has three allelic differences compared with ST23 (allelic profile: 5, 4, 6, 3, 2, 1, 3) and forms the ST23 group (Figure 1b and Figure S1). Interestingly, ST17 was not found in the PRGBS from this study, despite the fact that ST17 has been reported as the most frequent ST among the GBS recovered from neonatal meningitis.[18–22]

Discussion

PRGBSs have thus far been mainly isolated from respiratory specimens of elderly people in Japan. In this study the STs of 28 PRGBSs were found predominantly to belong to the ST1 group, with a minority of isolates belonging to the ST23 group. ST1 and ST23 were reported as the major STs involved in carriage and invasive infections in neonates and non-pregnant adults,[16,23,24] and one strain, MRY08-1422, isolated from blood was assigned to ST464, and thus belonged to the ST23 group. Both ST1 and ST23 have been frequently identified among the isolates of throat flora.[25]

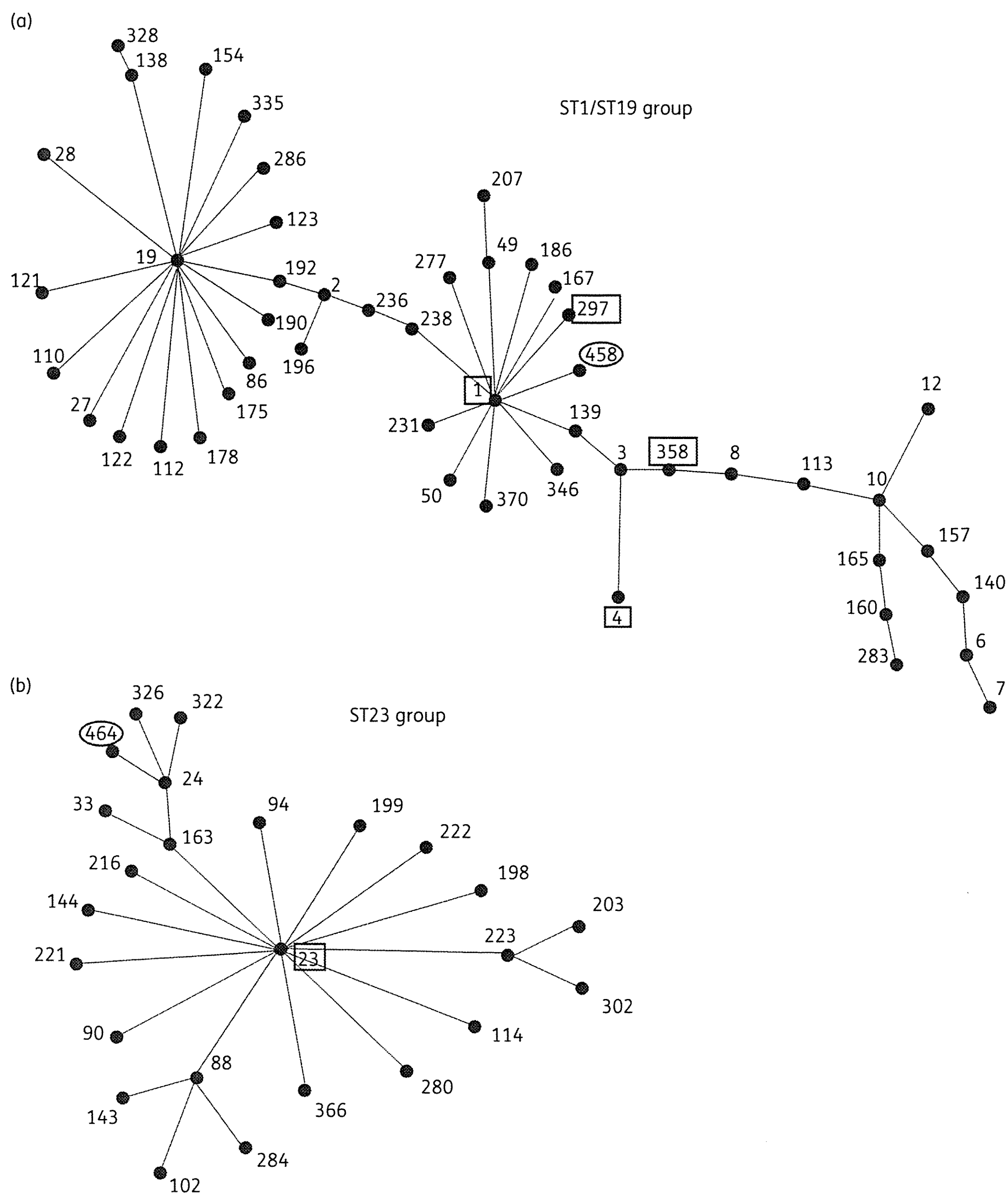

Figure 1. Rough sketch of eBURST analysis of STs of PRGBS. (a) ST1/ST19 group. (b) ST23 group. Numbers in the figure indicate STs. Numbers in squares or ellipses indicate STs of PRGBS. Numbers in ellipses indicate novel STs of PRGBS. Numbers that are not in squares or circles are STs of GBS other than the PRGBS in this study. The eBURST analysis connected two STs with one allelic profile difference by one line. The lengths of lines between two STs do not reflect the genetic distances between two STs. This rough sketch was created with random deletion of STs of GBS other than PRGBS from Figure S1 (available as Supplementary data at *JAC* Online) in order to simplify the original sketch. Therefore, this rough sketch does not contain all of the STs of GBS.

Although information concerning STs isolated from respiratory specimens of elderly people is limited, the STs of PRGBSs determined in this study might reflect the fact that most PRGBSs isolated so far have tended to be from respiratory specimens of elderly people.

The most frequent ST of PRGBS found in the present study was the novel type ST458. The eBURST analysis showed that ST458 is a single allelic variant of ST1. Interestingly, among the 11 ST458 and 7 ST1 strains, 9 and 4 were serotype VI, respectively (Table 1), suggesting a probable correlation between the ST1 group and

serotype VI in the PRGBSs tested. Limited information about the correlation between serotype VI and ST is available in GBS at present, but the ST of three serotype VI GBS strains deposited in the PubMLST database (http://pubmlst.org/sagalactiae/) are all ST1. Moreover, among six GBS strains with serotype VI, four strains were reported to be ST1 and the remaining two strains were ST14 (one allelic variant of ST1) and ST13 (five allelic variant of ST1), respectively.[16] Although ST458 clinical isolates with serotype VI may have biological characteristics similar to those of ST1, more information about the strains of ST458 will be needed to evaluate the correlation between serotype VI and the ST1-group PRGBS, together with its clinical significance.

As we reported previously, the PFGE analysis of PRGBS using ApaI showed different band patterns among the PRGBS clinical isolates tested. Moreover, phylogenetic analyses of the PBP genes of PRGBS suggested their genetically divergent origin.[10,11] However, an American study reported that four PRGBSs isolated in different states all belonged to ST19, suggesting a clonal expansion of PRGBS in the USA.[13] In the present study, however, ST19 was not found among the 28 PRGBSs isolated in Japan, and a greater variety of STs was observed among the PRGBS strains than in those isolated in the USA. We confirmed, therefore, that PRGBS in Japan could be classified into at least two ST groups, the ST1 group and the ST23 group, which are genetically different from the ST19 PRGBS isolated in the USA. However, two allelic similarities are found between ST1 and ST19, and these groups may well form the ST1/ST19 group as shown in Figure 1(a). This observation might suggest that the strains belonging to the ST1/ST19 group tend to have an ability to develop reduced penicillin susceptibility among various STs in GBS.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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

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

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

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

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

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TABLE 1. Primers used in this study

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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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

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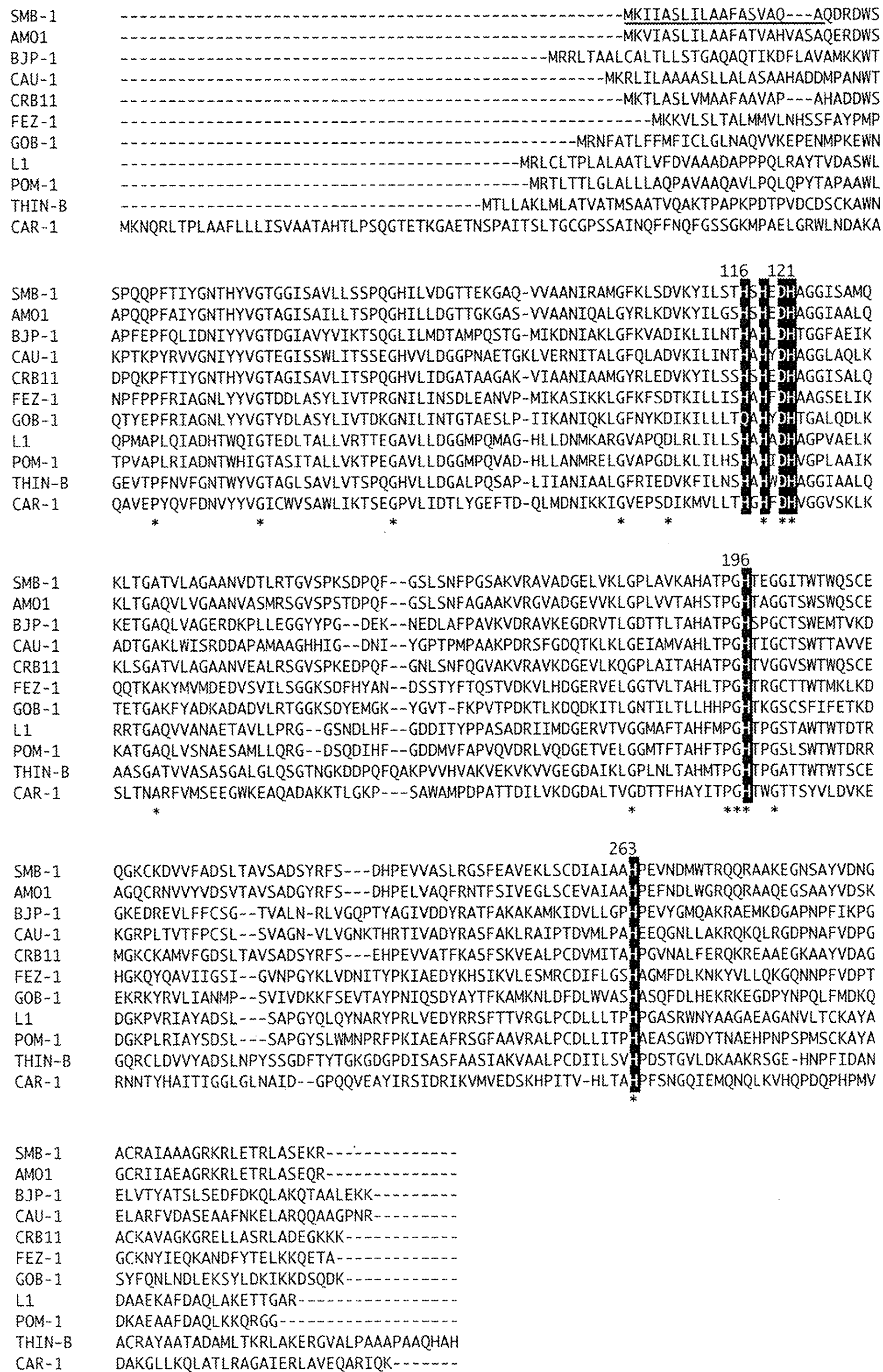

FIG. 1. Amino acid alignments of SMB-1 sequence with those of other subclass B3 MBLs. The residues involved in zinc binding are highlighted with a dark background. The signal peptide of SMB-1 is shown with an underline. An asterisk indicates amino acid residues conserved among all subclass B3 MBLs. Proteins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

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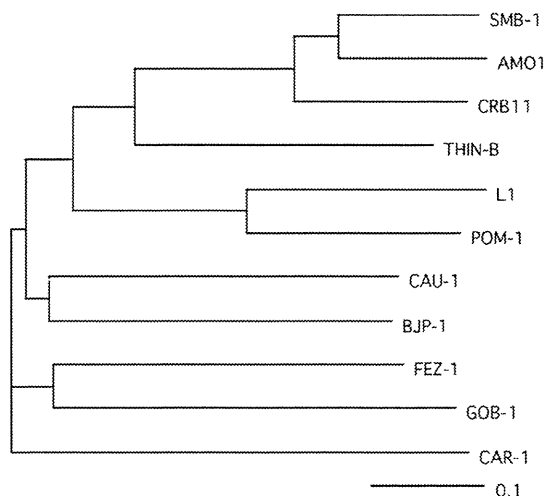

FIG. 2. Tree view exhibiting the similarity of SMB-1 with the other subclass B3 MBLs. The tree was constructed using ClustalW, version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html), and was provided by the DNA Data Bank of Japan (DDBJ). Sequences incorporated to draw the tree were the same as those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

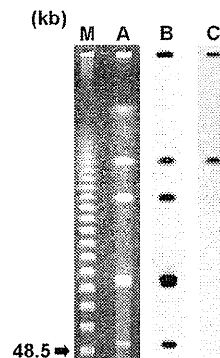

FIG. 3. Localization of the bla_{SMB-1} gene on I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 separated by PFGE. Lane: M, CHEF DNA size standard marker (Bio-Rad); A, I-CeuI-digested total DNA of *S. marcescens* strain 10mdr148 stained with ethidium bromide; B, hybridization of I-CeuI-digested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

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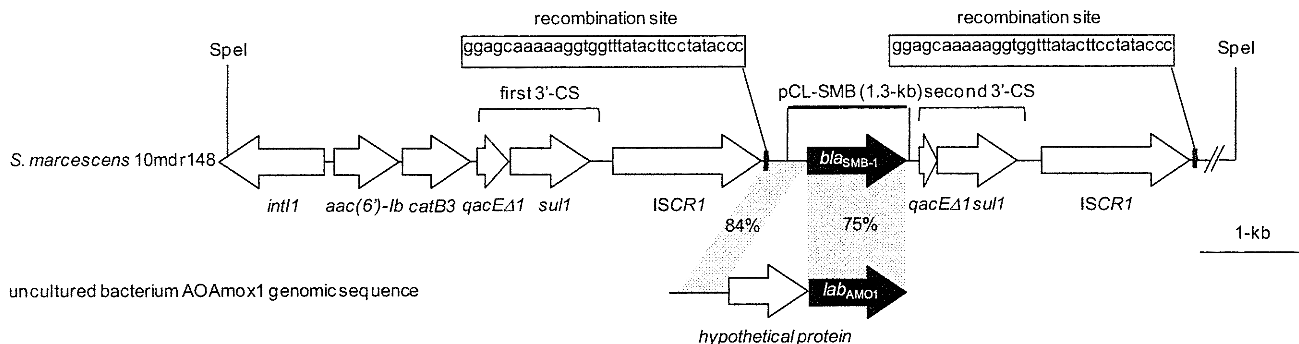

FIG. 4. Schematic representation of the generic environment of the bla_{SMB-1} gene. The 5′ end of the *qacEΔ1* gene in the second 3′-CS was deleted of 143 bp that are present in the first 3′-CS.

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

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

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

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

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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).

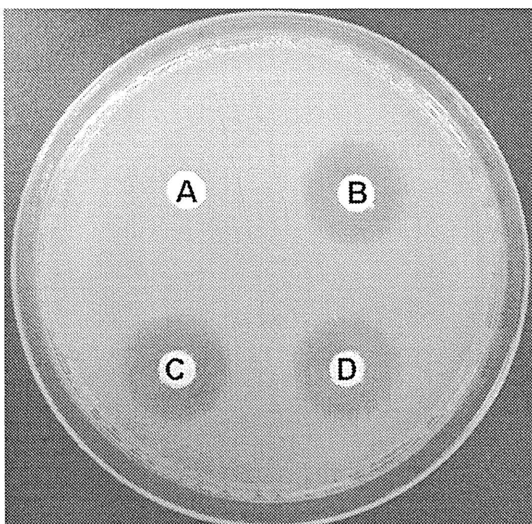

FIG. 1. Result of disk-based detection. (A) *E. coli* strain CR1 [*aac(6′)-Ib-cr* (+)]; (B) *E. coli* strain N64 [*aac(6′)-Ib* (+)]; (C) *E. coli* strain BN41 (negative for both genes); (D) control medium.

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.

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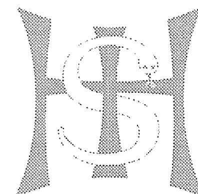

Genotypes and infection sites in an outbreak of multidrug-resistant *Pseudomonas aeruginosa*

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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.

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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,

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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 $bla_{\text{IMP-1}}$-, $bla_{\text{IMP-2}}$- and $bla_{\text{VIM-2}}$-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.

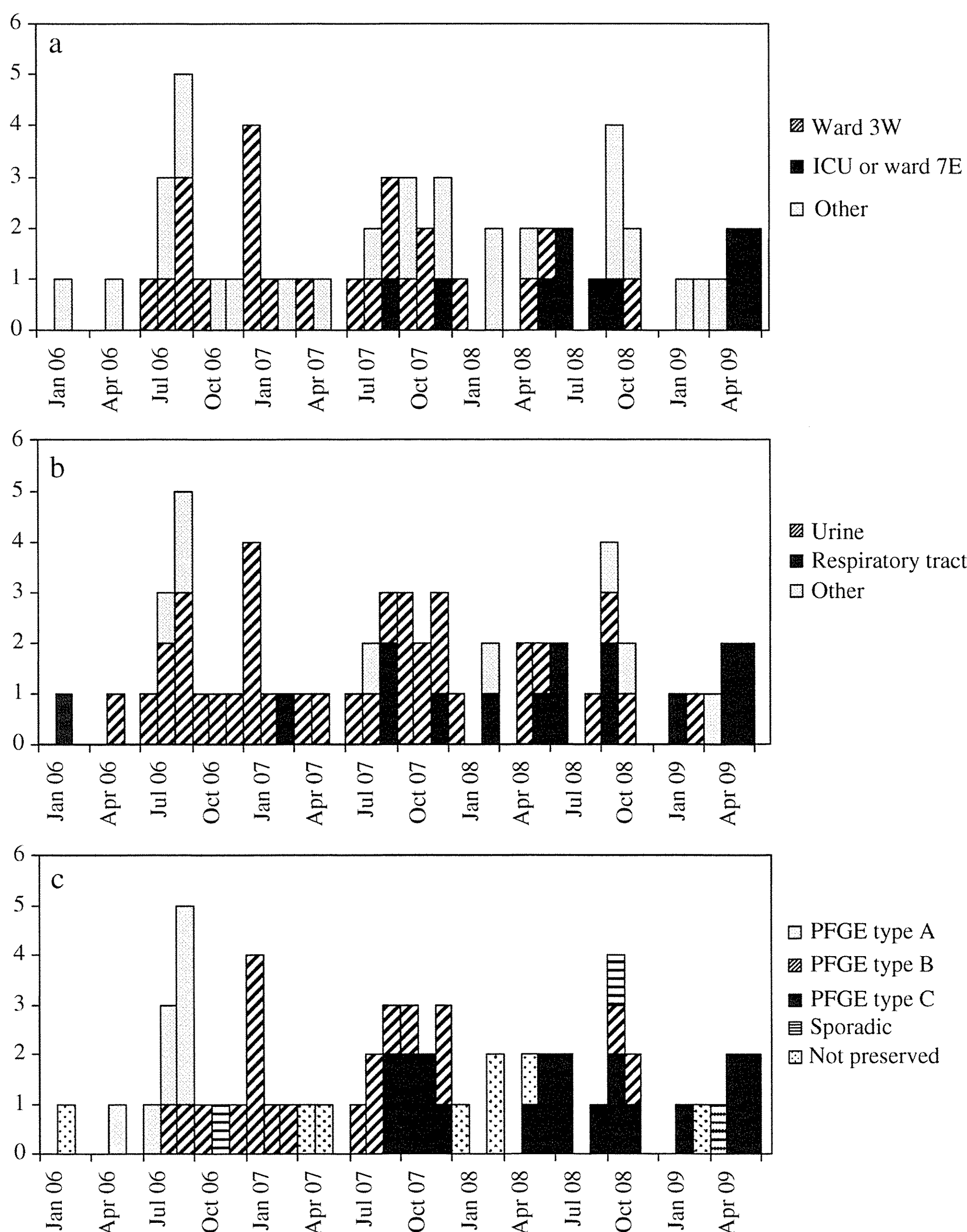

Figure 1. Epidemic curves of patients with multidrug-resistant (MDR) *P. aeruginosa*, January 2006 to June 2009: (a) hospitalised wards on first MDR *P. aeruginosa* isolation; (b) MDR *P. aeruginosa* isolate specimens; (c) pulsed-field gel electrophoresis (PFGE) genotypes of MDR *P. aeruginosa*. $N =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

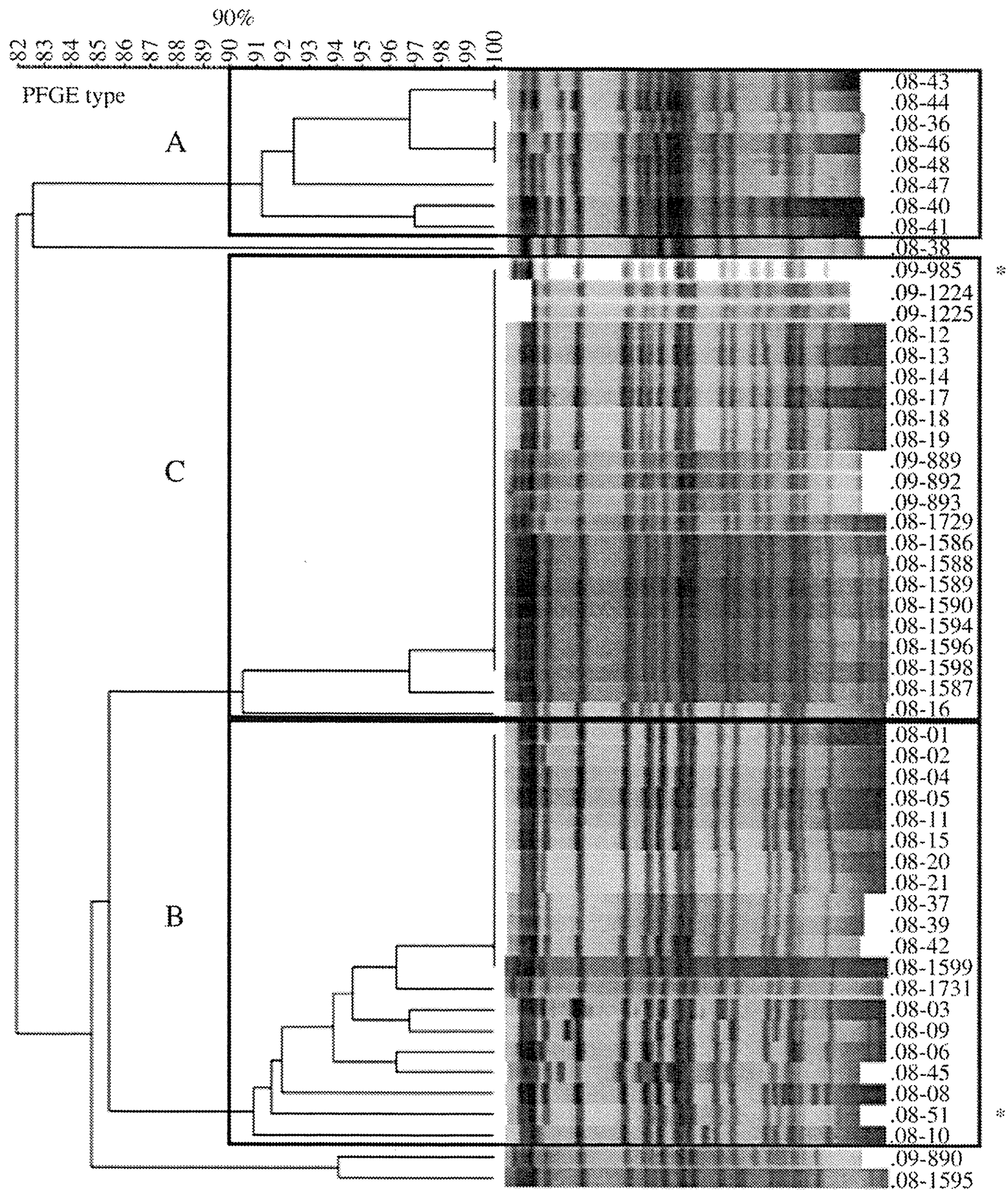

Figure 2. Dendrogram of pulsed-field gel electrophoresis (PFGE) band patterns among VIM-2-positive *P. aeruginosa* isolates based on unweighted-pair group method with average linkages; 51 isolates from inpatients and two isolates from the environment (*). These are categorised into three major clusters, 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	PFGE type non-C	*P*-value
	(*N* = 21)	(*N* = 21)	
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.

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Conflict of interest

None declared.

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RESEARCH LETTER

RmtC introduces G1405 methylation in 16S rRNA and confers high-level aminoglycoside resistance on Gram-positive microorganisms

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Abstract

Seven plasmid-mediated 16S rRNA methyltransferases (MTases), RmtA, RmtB, RmtC, RmtD, RmtE, ArmA, and NpmA, conferring aminoglycoside resistance have so far been found in Gram-negative pathogenic microorganisms. In the present study, by performing an RNase protection assay, primer extension, and HPLC, we confirmed that RmtC indeed methylates at the N7 position of nucleotide G1405 in 16S rRNA as found in ArmA and RmtB. RmtC has an MTase activity specific for the bacterial 30S ribosomal subunit consisting of 16S rRNA and several ribosomal proteins, but not for the naked 16S rRNA, as seen in ArmA, RmtB, and NpmA. All seven 16S rRNA MTases have been found exclusively in Gram-negative bacilli to date, and no plasmid-mediated 16S rRNA MTase has been reported in Gram-positive pathogenic microorganisms. Thus, we checked whether or not the RmtC could function in Gram-positive bacilli, and found that RmtC could indeed confer high-level resistance to gentamicin and kanamycin in *Bacillus subtilis* and *Staphylococcus aureus*. 16S rRNA MTases seemed to be functional to some extent in any bacterial species, regardless of the provenance of the 16S rRNA MTase gene responsible for aminoglycoside resistance.

Introduction

Large amounts of aminoglycosides have been used thus far for the treatment of infections caused by Gram-negative and Gram-positive bacteria in clinical settings and for growth promotion in livestock-farming settings. On the other hand, bacteria have acquired various resistance mechanisms to cope with aminoglycosides.

Plasmid-mediated 16S rRNA methyltransferases (MTases), which confer a high level of resistance to various aminoglycosides, especially to those containing 4,6-disubstituted 2-deoxystreptamine (2-DOS), have been widely distributed among pathogenic microorganisms belonging to the family *Enterobacteriaceae* and glucose nonfermentative Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical and livestock-farming environments (Chen *et al.*, 2007; Yamane *et al.*, 2007). RmtA (Yokoyama *et al.*, 2003), RmtB (Doi *et al.*, 2004), RmtC (Wachino *et al.*, 2006), RmtD (Doi *et al.*, 2007), RmtE (Davis *et al.*, 2010), ArmA (Galimand *et al.*, 2003), and NpmA (Wachino *et al.*, 2007) have so far been reported as plasmid-mediated 16S rRNA MTases conferring aminoglycoside resistance, but methylation sites have only been determined as G1405 for RmtB and ArmA, and A1408 for NpmA (Liou *et al.*, 2006; Perichon *et al.*, 2007; Wachino *et al.*, 2007). As for RmtA, RmtC, RmtD, and RmtE, the site of methylation in the 16S rRNA has not been described.

Plasmid-mediated 16S rRNA MTases have only been found in Gram-negative pathogenic bacteria, and not in Gram-positives. It remains controversial whether or not 16S rRNA MTase as described above is functional and confers aminoglycoside resistance in Gram-positives as well as in Gram-negatives, although it was revealed previously that *armA* controlled under the original promoter could confer aminoglycoside resistance in *Bacillus subtilis* (Liou *et al.*, 2006). Therefore, in this study, we aimed to determine exactly the residue modified by RmtC, and investigated whether RmtC can provide aminoglycoside resistance in Gram-positive pathogens.

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