Relevance of resistance levels to carbapenems and integron-borne *bla*_{IMP-1}, *bla*_{IMP-7}, *bla*_{IMP-10} and *bla*_{VIM-2} in clinical isolates of *Pseudomonas aeruginosa*

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Molecular detection and surveillance of the resistance genes harboured by *Pseudomonas aeruginosa* are becoming increasingly important in assessing and controlling spread and colonization in hospitals, and in guiding the treatment of infections. This study analysed the resistance mechanisms of carbapenem-resistant clinical isolates of *P. aeruginosa* and identified the associated integron-borne metallo- β -lactamase (MBL)-encoding genes. Twenty-seven imipenem (IPM)-resistant clinical isolates of *P. aeruginosa* were divided into three groups according to their resistance levels to carbapenems. Strains bearing *bla*_{IMP-10} showed extremely high-level resistance to IPM, with MICs of 512–2048 $\mu\text{g ml}^{-1}$. By comparison, strains bearing *bla*_{IMP-1}, *bla*_{IMP-7} and *bla*_{VIM-2} showed an intermediate level of resistance, with MICs of 32–256 $\mu\text{g ml}^{-1}$. The non-MBL-producing strains showed a low level of resistance, with MICs of 8–32 $\mu\text{g ml}^{-1}$. The same trend in resistance levels was also observed when resistance to other carbapenems, such as meropenem and panipenem, was determined. DNA sequencing showed that the MBL-encoding gene cassettes were carried by class 1 integrons. The *bla*_{IMP-1}, *bla*_{IMP-7} and *bla*_{IMP-10} gene cassettes were preceded by a hybrid P_{ant} promoter, TGGACA-N₁₇-TAAACT, and the *bla*_{VIM-2} gene cassette was preceded by a weak promoter, TGGACA-N₁₇-TAAGCT. Most of the MBL-encoding genes were linked to one or two resistance genes encoding aminoglycoside-modifying enzymes, such as *aac*(6')*lae*, *aac*(6')*II*, *aacA7*, *aacC4*, *aadA1*, *aadA2* and *aadA6*, highlighting the multidrug-resistant properties of these clinical isolates.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and shows high intrinsic resistance to many antimicrobial agents. Carbapenems are one of the first-choice antibiotics for the treatment of *P. aeruginosa* infections (Gilbert *et al.*, 2005). However, acquired metallo- β -lactamases (MBLs), including the types IMP, VIM, SPM and GIM, have been reported in a variety of clinical isolates of *Enterobacteriaceae* worldwide (Bush, 1998; Livermore & Woodford, 2000; Walsh *et al.*, 2005). MBLs are becoming increasingly important clinically because the enzymes hydrolyse almost all β -lactams, including carbapenems, and to date there is no clinically available inhibitor (Walsh *et al.*, 2005).

IMP- and VIM-type enzymes are two dominant groups of MBLs, and the nucleotide sequences of 24 IMP variants and 22 VIM variants have been determined (Lahey Clinic, 2008). The *bla*_{IMP-1} gene was the first MBL determinant reported, initially in a clinical isolate of *Serratia marcescens* (Ito *et al.*, 1995; Osano *et al.*, 1994). The *bla*_{IMP-7} gene is a *bla*_{IMP} allele and IMP-7 shares 91 % amino acid identity with IMP-1 (Gibb *et al.*, 2002). The *bla*_{IMP-10} allele is a point mutation derivative of *bla*_{IMP-1} with a single base replacement of G by T at nt 145 leading to an amino acid alteration of Val49 to Phe (Iyobe *et al.*, 2002). The *bla*_{VIM-2} gene is a *bla*_{VIM} allele and VIM-2 shares 90 % identity with VIM-1, but only 31 % identity with IMP-1 (Poirel *et al.*, 2000).

Five classes of integron are known to play a role in the dissemination of antibiotic resistance, and class 1 integrons are the most extensively studied (Mazel, 2006). MBL-

Abbreviations: BIPM, biapenem; CAZ, ceftazidime; CS, conserved segment; IPM, imipenem; MBL, metallo- β -lactamase; MEPM, meropenem; PAPM, panipenem; SMA, sodium mercaptoacetic acid.

encoding genes are usually found as gene cassettes in class 1 integrons (Shibata *et al.*, 2003). Typical class 1 integrons contain two conserved segments (CSs), the 5'-CS and the 3'-CS (Stokes & Hall, 1989; Bennett, 1999). The 5'-CS includes the *intI1* gene encoding integrase, the *attI* site for addition of the inserted gene cassette and a promoter(s). The 3'-CS is composed of the *qacEΔ1* and *sul1* genes, which are responsible for resistance to quaternary ammonium compounds and sulphonamides, respectively. Four different *P*_{ant} promoters have been described (Bunny *et al.*, 1995; Stokes & Hall, 1989), and their relative strengths have been compared with the *Escherichia coli* *tac* promoter (Collis & Hall, 1995; Lévesque *et al.*, 1994). TTGACA-N₁₇-TAAACT and TGGACA-N₁₇-TAAGCT have been described as strong and weak *P*_{ant} promoters, respectively, whilst TTGACA-N₁₇-TAAGCT and TGGACA-N₁₇-TAAACT are described as hybrid promoters. In this study, we analysed the relevance of resistance determinants (*bla*_{IMP-1}, *bla*_{IMP-7}, *bla*_{IMP-10} and *bla*_{VIM-2}) and resistance levels to carbapenems in imipenem (IPM)-resistant clinical isolates of *P. aeruginosa*, and identified the associated integrons.

METHODS

Bacterial strains and reagents. Twenty-seven IPM-resistant strains of *P. aeruginosa* (MIC ≥ 8 μg ml⁻¹) and one susceptible strain of *P. aeruginosa* P01 were used. They were isolated from unrelated inpatients at Showa University Hospital, Tokyo, Japan, from 2005 to 2008. Mueller–Hinton (MH) broth (Becton Dickinson) supplemented with 25 mg Ca²⁺ l⁻¹ and 12.5 mg Mg²⁺ l⁻¹ was used for bacterial cell culture and antibiotic-susceptibility testing. The following reagents were purchased from commercial sources: IPM (US Pharmacopeia); meropenem (MEPM) and biapenem (BIPM) (both from LKT Laboratories); panipenem (PAPM; Sankyo Organic Chemicals); ceftazidime (CAZ) discs (Becton Dickinson) and sodium mercaptoacetic acid (SMA) discs (Eiken Chemical).

PFGE. PFGE of *SpeI*-digested genomic DNA from *P. aeruginosa* isolates was carried out using a CHEF-DRII system (Bio-Rad) according to the manufacturer's instructions. The restriction patterns

were evaluated according to a standard, as described previously (Tenover *et al.*, 1995).

Susceptibility testing. The susceptibilities of bacteria to the various antibiotics were expressed in terms of their MICs. MICs were determined by a broth microdilution method according to Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines (NCCLS, 2000). Bacterial cells (5 × 10⁵ ml⁻¹) were inoculated into 100 μl MH broth containing twofold serial dilutions of the carbapenems, and MICs were determined after incubation at 35 °C for 18 h. All experiments were performed at least three times to establish reproducibility.

Detection of MBL production. Double-disc synergy testing was performed to detect the production of MBLs by using discs containing CAZ, a third-generation cephalosporin, and SMA, a specific inhibitor of MBLs (Arakawa *et al.*, 2000). Bacterial cells (2 × 10⁷) were inoculated on an MH agar plate (Becton Dickinson) and discs were then set on the plate with a distance of 1.5 cm between the CAZ and SMA discs. After incubating the plates at 35 °C for 24 h, strains that had an inhibition zone (>12 mm) around the CAZ disc towards the SMA disc were considered to be MBL-producing strains.

Identification of resistance gene cassettes and associated integrons by PCR and DNA sequencing. Bacterial genomic DNA was extracted using a commercial kit (Qiagen). The primers used for detecting resistance genes and other associated genes are listed in Table 1. The primer pairs *intI1*-F/*qacEΔ1*-R and *intI1*-F/*sul1*-R were used for amplification of the integrons containing *bla*_{IMP}, and the primer pair *intI1*-F and *vim2*-R was used for amplification of the integron containing *bla*_{VIM-2}. The amplicons were purified using a QIAquick PCR purification kit and sequenced on an Applied Biosystems 3730 × 1 DNA analyser. Sequences were compared with those in GenBank by using the BLAST network service.

RESULTS AND DISCUSSION

Relevance of resistance levels and resistance determinants

As all of the strains were from the same hospital, we performed PFGE to exclude the possibility that the isolates

Table 1. Primers used for detecting resistance genes and sequence identification of the amplified genes

Gene	Primer	Sequence (5'→3')	PCR product size (bp)	GenBank accession no.
<i>bla</i> _{IMP}	<i>imp</i> -F	CTACCGCAGCAGAGTCTTTG	588	D50438
	<i>imp</i> -R	AACCAGTTTTGCCTTACCAT		
<i>bla</i> _{VIM-2}	<i>vim2</i> -F	AAAGTTATGCCGCACTCACC	865	AF191564
	<i>vim2</i> -R	TGCAACTTCATGTTATGCCG		
<i>intI1</i>	<i>intI1</i> -F	ACATGCGTGTAATCATCGTCG	484	U49101
	<i>intI1</i> -R	GGGTCAAGGATCTGGATTTTCG		
<i>qacEΔ1</i>	<i>qacEΔ1</i> -F	GGCTGGCTTTTCTTGTTATCG	274	U49101
	<i>qacEΔ1</i> -R	TGAGCCCCATACCTACAAAGC		
<i>sul1</i>	<i>sul1</i> -F	TGGTGACGGTGTTTCGGCATTC	784	U49101
	<i>sul1</i> -R	GTTTCCGAGAAGGTGATTGCG		
<i>intI2</i>	<i>intI2</i> -F	CACGGATATGCGCAAAAAGGT	789	L10818
	<i>intI2</i> -R	GTAGCAAACGAGTGACGAAATG		
<i>intI3</i>	<i>intI3</i> -F	GCCTCCGGCAGCGACTTTCAG	980	AF416297
	<i>intI3</i> -R	ACGGATCTGCCAAACCTGACT		

might be identical. Only isolates confirmed as being different were used in this study. The different patterns of MICs, growth speeds and abilities to produce the pigments, and the different gene cassettes in integron 1, as described below, also indicated that the studied isolates were clonally unrelated.

Of 27 IPM-resistant clinical isolates of *P. aeruginosa*, 15 strains were putative MBL producers. Of these 15 MBL producers, the *bla*_{IMP-1}, *bla*_{IMP-7}, *bla*_{IMP-10} and *bla*_{VIM-2} genes were harboured by 2, 5, 7 and 1 of the strains, respectively. The 27 isolates could be divided into three groups according to their resistance levels to carbapenems (Table 2). Group I was highly resistant to carbapenems with MICs of 512 to 2048, 2048 to >4096, 1024 to 4096 and 128 to 2048 µg ml⁻¹ for IPM, MEPM, PAPM and BIPM, respectively. By comparison, group II showed an intermediate level of resistance with MICs of 32 to 256, 128 to 512, 256 to 512 and 16 to 512 µg ml⁻¹, respectively. Group III showed a low level of resistance with MICs of 8 to 32, 4 to 64, 8 to 64 and 4 to 32 µg ml⁻¹, respectively. Double-disc (CAZ and SMA) synergy testing confirmed

that the strains in group I (*n*=7) and II (*n*=8) produced MBLs, whilst the strains in group III (*n*=12) did not. Furthermore, it was confirmed by PCR and DNA sequencing analysis that all of the strains in group I harboured *bla*_{IMP-10}, whilst those in group II harboured *bla*_{IMP-1}, *bla*_{IMP-7} or *bla*_{VIM-2}. MBL-encoding genes were not detectable in group III. Of the group II strains carrying *bla*_{IMP-1}, *bla*_{IMP-7} or *bla*_{VIM-2}, no significant differences were observed in their resistance to carbapenems.

The above results indicated that the production of MBLs was a crucial factor in conferring high and intermediate levels of resistance and that the *bla*_{IMP-10} allele was responsible for the observed cases of extremely high resistance to carbapenems. These data were also supported by the experiment using the specific MBL inhibitor SMA, which greatly reduced the resistance level of MBL-producing strains. For example, the MIC of IPM against strain P0701 decreased from 512 to 32 µg ml⁻¹ in the presence of 0.5 mM SMA. The low-level resistance to carbapenems exhibited by the non-MBL-producing strains was probably caused by some non-MBL factors, such as the

Table 2. Phenotypes and genotypes of IPM-resistant *P. aeruginosa* clinical isolates

Group	Strain	MIC (µg ml ⁻¹)				MBL production	<i>bla</i> gene			
		IPM	MEPM	PAPM	BIPM		IMP-1	IMP-7	IMP-10	VIM-2
I	P0701	512	4096	2048	512	+	–	–	+	–
	P0706	1024	>4096	2048	512	+	–	–	+	–
	P0709	512	4096	2048	512	+	–	–	+	–
	P0711	1024	4096	2048	512	+	–	–	+	–
	P0713	1024	>4096	4096	1024	+	–	–	+	–
	P0716	512	2048	1024	128	+	–	–	+	–
	P0717	2048	>4096	4096	2048	+	–	–	+	–
II	P0708	256	512	512	512	+	+	–	–	–
	P0710	128	512	512	256	+	+	–	–	–
	P0705	128	512	512	256	+	–	+	–	–
	P0712	32	128	256	16	+	–	+	–	–
	P0714	256	256	256	256	+	–	+	–	–
	P0715	128	256	512	256	+	–	+	–	–
	P0720	128	512	512	256	+	–	+	–	–
III	P0501	128	128	512	64	+	–	–	–	+
	P0702	32	8	32	16	–	–	–	–	–
	P0703	32	16	32	16	–	–	–	–	–
	P0704	16	8	32	16	–	–	–	–	–
	P0707	32	64	64	32	–	–	–	–	–
	P0718	16	4	32	16	–	–	–	–	–
	P0719	16	8	32	16	–	–	–	–	–
	P0721	32	32	64	16	–	–	–	–	–
	P0801	16	8	32	16	–	–	–	–	–
	P0802	16	8	32	8	–	–	–	–	–
	P0803	32	8	64	16	–	–	–	–	–
	P0805	16	4	16	4	–	–	–	–	–
Susceptible strain	P0806	8	4	8	4	–	–	–	–	–
	P01	≤0.5	0.25	4	0.5	–	–	–	–	–

Table 3. GenBank sequences used for identification of the amplified genes

Gene	Gene size (bp)	GenBank accession no.
<i>bla</i> _{IMP-1}	741	D50438
<i>bla</i> _{IMP-7}	741	AF318077
<i>bla</i> _{IMP-10}	741	AB074433
<i>bla</i> _{VIM-2}	801	AF191564
<i>aac</i> (6') <i>Iae</i>	552	AB104852
<i>aac</i> (6') <i>II</i>	555	M29695
<i>aacA7</i>	459	DQ143913
<i>aacC4</i>	555	AF318077
<i>aadA1</i>	792	AB104852
<i>aadA2</i>	792	FJ042874
<i>aadA6</i>	846	AY444814
<i>orfD</i>	279	AY444814
<i>orfI</i>	432	DQ092497

low permeability of the outer membrane due to loss of OprD, the activity of the efflux system due to over-expression of the MexAB–OprM and MexEF–OprN pumps, or the production of chromosomal AmpC β -lactamases, as well as the cooperation of such factors (Li *et al.*, 2000; Masuda *et al.*, 1999; Nikaido, 1989, 1996; Pai *et al.*, 2001; Quale *et al.*, 2006). Further experiments are needed to clarify the details of the underlying mechanism(s).

Structure of integrons associated with MBL-encoding genes from IPM-resistant strains of *P. aeruginosa*

By sequencing the PCR products, the class 1 integron gene cassettes were identified from 15 MBL-producing strains; no class 2 or class 3 integrons were detected. Based on GenBank sequences (Table 3), five of the integrons were a typical class 1 integron, containing a 5'-CS *intI1*, a 3'-CS *qacE Δ 1* and *sul1*, and a variable region (Fig. 1a, b, c, d, e). One integron carried only part of *qacE Δ 1*, the 132 bp at the 3'-end of the total 348 bp gene (Fig. 1f). The integron carrying *bla*_{VIM-2} did not possess the 3'-CS (Fig. 1g). The *bla*_{IMP-1}, *bla*_{IMP-7} and *bla*_{IMP-10} gene cassettes were preceded by a hybrid *P*_{ant} promoter, TGGACA-N₁₇-TAAACT, located at the 5' end of the integrase 1-encoding gene. The *bla*_{VIM-2} gene cassette was preceded by a weak *P*_{ant} promoter, TGGACA-N₁₇-TAAGCT. Except for in the integron shown in Fig. 1(a), the *bla*_{IMP} and *bla*_{VIM-2} genes were linked to resistance genes encoding aminoglycoside-modifying enzymes, such as *aac*(6')*Iae*, *aac*(6')*II*, *aacA7*, *aacC4*, *aadA1*, *aadA2* and *aadA6* (Fig. 1).

We have reported previously that *S. marcescens* strains harbouring *bla*_{IMP-1} and *bla*_{IMP-10} genes showed quite different patterns of MICs, as with the strains of *P. aeruginosa* described in this study. Using *E. coli* transconjugants and transformants, it was also observed that the *bla*_{IMP-10}-positive strains showed much higher levels of resistance to IPM, MEPM and PAPM than the strains

Fig. 1. Schematic representation of the structures of integrons containing *bla*_{IMP} or *bla*_{VIM}. The class 1 integrons were identified from 15 MBL-producing clinical isolates of *P. aeruginosa*. Arrows represent genes and their transcriptional direction. Short lines represent *attI* sites. Circles represent *attC* sites (e.g. the 59 base element).

harbouring *bla*_{IMP-1} (Hu & Zhao, 2009). The *E. coli* transconjugants and transformants are more suitable models than wild-type strains of *S. marcescens* and *P. aeruginosa* because factors such as the outer-membrane barrier, the efflux system and the chromosomal AmpC β -lactamases that are possibly possessed by these wild-type strains can be excluded. A strain of *bla*_{IMP-10}-positive *P. aeruginosa* has also been reported to show extremely high-level resistance against IPM (Takeda *et al.*, 2008).

The mechanism for this phenomenon is unclear at present. One possible explanation is that IMP-10 MBL might be more potent than IMP-1 MBL in hydrolysing IPM, MEPM and PAPM. In a published paper, the kinetic parameters of purified IMP-1 and IMP-10 MBL in hydrolysing IPM, MEPM and other β -lactams were described (Iyobe *et al.*, 2002). Compared with the rate of hydrolysis (K_{cat}) of IMP-1 MBL (130 and 13 s⁻¹ for IPM and MEPM, respectively), IMP-10 MBL showed higher values of K_{cat} (220 and 64 s⁻¹). The higher K_{cat} values of IMP-10 MBL compared with those of IMP-1 MBL could partly explain the phenomenon described in this study. However, no significant differences of the specificity constant (K_{cat}/K_m) were observed between IMP-1 and IMP-10 MBLs. Further experiments are therefore needed to reveal the detailed mechanism(s) involved.

Conclusion

The molecular detection and surveillance of resistance genes harboured by *P. aeruginosa* is becoming increasingly important for assessment and control of their spread and colonization in hospitals, and for guiding treatment of infections. In the IPM-resistant clinical isolates of *P. aeruginosa*, the MBL-producing strains showed much higher resistance to carbapenems than non-MBL-producing strains. Of the MBL producers, the strains bearing *bla*_{IMP-10} showed extremely high resistance compared with strains bearing *bla*_{IMP-1}, *bla*_{IMP-7} and *bla*_{VIM-2}. In addition to the MBL-encoding genes, the class 1 integrons generally harboured one or two aminoglycoside-resistance genes, highlighting the multidrug-resistant properties of these clinical isolates.

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