Identification of plasmid- and integron-borne $\text{bla}_{\text{IMP}-1}$ and $\text{bla}_{\text{IMP}-10}$ in clinical isolates of Serratia marcescens

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The emergence of carbapenem-hydrolysing metallo-$\beta$-lactamasas (MBLs) is a serious threat to the clinical utility of carbapenems. This study identified plasmid- and integron-borne $\text{bla}_{\text{IMP}-1}$ and $\text{bla}_{\text{IMP}-10}$ in clinical isolates of Serratia marcescens. The $\text{bla}_{\text{IMP}-1}$ and $\text{bla}_{\text{IMP}-10}$ gene cassettes were carried by a class 1 integron and followed by the $\text{aac}(6')\text{-Ic}$ gene cassette. The $\text{bla}_{\text{IMP}-1}$ and $\text{bla}_{\text{IMP}-10}$ gene cassettes were preceded by a weak $\text{P}_{\text{ant}}$ promoter, TGGACA(N)$_{17}$TAAGCT, and an inactive P2 promoter, TGTTA(N)$_{17}$TACAGT. These genes were easily transferred to Escherichia coli by conjugation and transformation, indicating that they are located on transferable plasmids. Due to the acquisition of $\text{bla}_{\text{IMP}-1}$, the susceptibility of E. coli transconjugants to imipenem, meropenem, panipenem and biapenem decreased by 64-, 2048-, 256- and 64-fold, respectively. In comparison, after gaining $\text{bla}_{\text{IMP}-10}$, the susceptibility of E. coli transconjugants to the four carbapenems decreased by 32-, 256-, 64- and 128-fold, respectively. In comparison, after gaining $\text{bla}_{\text{IMP}-10}$, the susceptibility of E. coli transconjugants to the four carbapenems decreased by 64-, 2048-, 256- and 64-fold, respectively. Strains harbouring $\text{bla}_{\text{IMP}-10}$ showed higher-level resistance to imipenem, meropenem and panipenem than the strains harbouring $\text{bla}_{\text{IMP}-1}$, although the nucleotide sequences of the class 1 integrons carrying $\text{bla}_{\text{IMP}-10}$ and $\text{bla}_{\text{IMP}-1}$ were identical except for a single point mutation.

INTRODUCTION

Carbapenems are relatively stable agents that act against extended-spectrum $\beta$-lactamases produced by Gram-negative rods, and are one of the first-choice antibiotics for the treatment of Serratia marcescens infections (Gilbert et al., 2005). However, the occurrence and spread of genes encoding metallo-$\beta$-lactamases (MBLs) including IMP, VIM, SPM and GIM have been reported in a variety of clinical isolates of Enterobacteriaceae from 28 countries (Walsh et al., 2005). The clinical significance of MBLs is further highlighted by their ability to hydrolyse almost all $\beta$-lactams except for monobactams, and by the fact that to date there is no clinically available inhibitor (Bush, 1998; Livermore & Woodford, 2000; Nordmann & Poirel, 2002).

The $\text{bla}_{\text{IMP}-1}$ gene, which encodes IMP-1 MBL, was the first MBL determinant reported, initially in a clinical isolate of S. marcescens from Japan in 1994 (Ito et al., 1995; Osano et al., 1994). To date, the nucleotide sequences of 24 IMP variants have been determined (Lahey Clinic, 2008).

$\text{bla}_{\text{IMP}-10}$ is a point mutation derivative of $\text{bla}_{\text{IMP}-1}$ and has been identified in clinical isolates of Pseudomonas aeruginosa and Alcaligenes xylosoxidans (Iyobe et al., 2002; Zhao et al., 2008). To our knowledge, the $\text{bla}_{\text{IMP}-10}$ gene has not been identified among Enterobacteriaceae strains.

MBL genes are usually found as gene cassettes in integrons, mostly in class 1 integrons (Shibata et al., 2003). An integron is a mobile DNA element that can capture and carry genes, particularly antibiotic-resistance genes, by site-specific recombination (Bennett, 1999; Hall & Collis, 1995; Stokes & Hall, 1989). 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). Typical class 1 integrons contain two conserved segments (CSs), the 5'- and 3'-CS. The 5'-CS includes the $\text{intI1}$ gene encoding integrase, the $\text{attI}$ site for addition of the inserted gene cassette and a promoter(s). The 3'-CS is composed of the $\text{qacEA1}$ and $\text{sul1}$ genes, which are responsible for resistance to quaternary ammonium compounds and sulphonamides, respectively. Over 80 different gene cassettes carried by class 1 integrons have been described (Mazel, 2006).

Four different $\text{P}_{\text{ant}}$ and two different P2 promoters have been described (Bunny et al., 1995; Stokes & Hall, 1989).
and their relative strengths have been compared with that of the Escherichia coli tac promoter (Collis & Hall, 1995; Lévesque et al., 1994). TTGACA(N)₁₇TAAACT and TGGACA(N)₁₇TAAACT, are described as strong and weak $P_{\text{int}}$ promoters, respectively. Two other promoters, TTGACA(N)₁₇TAAGCT and TGGACA(N)₁₇TAAACT, are described as hybrid promoters. The P₂ promoters have an active version, TTGT(TA)₁₇TACAGT, and an inactive version, TTGT(A)₁₇TACAGT, in which the −35 and −10 regions are separated by 14 nt, a distance that is not optimal for promoter function.

In this study, we identified plasmid- and integron-borne $bla_{\text{IMP}}$ and $bla_{\text{IMP}-10}$ in clinical isolates of S. marcescens and compared the carbapenem susceptibilities of the E. coli transconjugants and transformants after acquiring $bla_{\text{IMP}}$ and $bla_{\text{IMP}-10}$.

**METHODS**

**Bacterial strains and reagents.** S. marcescens strains S₁, S₂ and S₃ were $bla_{\text{IMP}}$-positive clinical isolates recovered from unrelated inpatients at Showa University Hospital, Tokyo, Japan, in 2000. S. marcescens S₄, a susceptible isolate, was used as a control. Competent cells of E. coli strains HB101 and JM109 (Wako Pure Chemical Industries) were used as recipients for conjugation and transformation, respectively. The following reagents were purchased from commercial sources: imipenem (IPM) (US Pharmacopeia); meropenem (MEPM) and biapenem (BIPM) (Becton Dickinson); and sodium mercaptoacetic acid (SMA) discs (Eiken Chemical).

**Susceptibility testing.** The susceptibilities of bacteria to various antibiotics were expressed as MICs, determined by the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 2000). All experiments were performed at least three times to establish reproducibility.

**Confirmation of $bla_{\text{IMP}}$ expression.** Double-disc synergy testing was performed to confirm the expression of $bla_{\text{IMP}}$ by using discs containing CAZ, a third-generation cephalosporin, and SMA, a specific inhibitor of MBLs (Arakawa et al., 2000). Bacterial cells (2 x 10⁷) were inoculated onto a Mueller–Hinton agar plate (Becton Dickinson) and discs were then set with a distance of 1.5 cm between the CAZ and SMA discs. After incubating the plates at 35 °C for 24 h, zones of inhibition were measured.

**PFGE.** PFGE of SpeI-digested genomic DNA from S. marcescens isolates was carried out using a CHEF-DR III System (Bio-Rad) according to the manufacturer’s directions. The restriction patterns were evaluated according to a standard, as described previously (Tenover et al., 1995).

**Transfer of resistance determinants.** Resistance factors were transferred from S. marcescens to E. coli HB101 by conjugation with a donor:recipient ratio of 1:1 in Luria–Bertani (LB) broth at 37 °C for 2 h. Transconjugants were selected on LB agar plates containing ampicillin and streptomycin (64 µg ml⁻¹). E. coli JM109 was used as the recipient for transformation of purified plasmids from S. marcescens. Plasmid DNA was prepared using a QIAprep Spin Miniprep kit (Qiagen) and transformed into E. coli JM109 by electroporation with a Gene Pulser apparatus (Bio-Rad). The plasmids from S. marcescens S₁, S₂ and S₃ were designated pS₁, pS₂ and pS₃, respectively. Transforms were selected on LB agar plates containing 64 µg ampicillin ml⁻¹ and reisolated with 16 µg cefotaxime ml⁻¹. Transconjugants were designated E. coli HB101-pS₁, -pS₂ and -pS₃, and transformants were designated E. coli JM109-pS₁, -pS₂ and -pS₃, respectively.

**Identification of resistance gene cassettes and associated integrons by PCR and DNA sequencing.** The primers used for detecting resistance gene cassettes and associated integrons are listed in Table 1. Forward primers were coupled with the appropriate reverse primers: intI₁-F and imp-R for amplification of the 5'-CS and $bla_{\text{IMP}}$ genes, and imp-F and sul1-R for amplification of $bla_{\text{IMP}}$ genes and the 3'-CS. PCR was performed under the following conditions: 30 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s and extension at 72 °C for 60 s. The amplicons were purified using a QIAquick PCR purification kit, and sequenced on an Applied Biosystems 3730xl DNA analyser. Sequences were compared with the GenBank database via the BLAST network service.

**RESULTS**

**Transfer and expression of resistance determinants**

PCD analysis indicated that the S. marcescens clinical isolates S₁, S₂ and S₃ carry $bla_{\text{IMP}}$ genes. By PFGE

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’—3’)</th>
<th>PCR product (bp)</th>
<th>Target gene</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>imp-F</td>
<td>CTACGCCGACGAGTCTTGG</td>
<td>588</td>
<td>$bla_{\text{IMP}}$</td>
<td>D50438</td>
</tr>
<tr>
<td>imp-R</td>
<td>AACAGTTTGGCCTTACAT</td>
<td>309</td>
<td>aac(6’)-IIC</td>
<td>AF162771</td>
</tr>
<tr>
<td>aac(6’)-IIC-F</td>
<td>GGGTCTGGGAGATGTGATCG</td>
<td>484</td>
<td>intI₁</td>
<td>U49101</td>
</tr>
<tr>
<td>aac(6’)-IIC-R</td>
<td>GGGTCGCAGATCATGATCG</td>
<td>274</td>
<td>qacEA₁</td>
<td>U49101</td>
</tr>
<tr>
<td>intI₁-F</td>
<td>ACATCGCGTGTTAATCATCGTCG</td>
<td>784</td>
<td>sul1</td>
<td>U49101</td>
</tr>
</tbody>
</table>
analysis of chromosomal DNA restriction patterns, the three strains were confirmed to be different. The \( \text{bla}_{\text{IMP}} \) genes carried by these strains were easily transferred to \( E. \ coli \) HB101 by conjugation and to \( E. \ coli \) JM109 by electroporation. Expression of MBLs in the \( E. \ coli \) transconjugants and transformants was confirmed by double-disc (CAZ and SMA) synergy testing. An obvious inhibition zone (>12 mm) was observed around the side of the CAZ disc facing the SMA disc, suggesting that the \( E. \ coli \) transconjugants and transformants produced MBL and that the enzymic activity was blocked by the specific inhibitor SMA (data not shown).

**Structure of integrons carried by the plasmids derived from \( S. \ marcescens \) S\#1, S\#2 and S\#3**

To exclude a possible effect of chromosomal DNA in the wild-type \( S. \ marcescens \) S\#1, S\#2 and S\#3, plasmids pS\#1, pS\#2 and pS\#3 prepared from \( E. \ coli \) transconjugants were used as templates for PCR amplification. Amplicons of 1729 and 2791 bp were obtained when the primers intI1-F and imp-R, and imp-F and sul1-R were combined, respectively. These two amplicons were sequenced and the resultant sequences were joined to form a DNA fragment of 3933 bp. A BLAST search showed that the 3933 bp DNA fragments from the three plasmids belonged to class 1 integrons, and we designated these In-s1, In-s2 and In-s3.

These integrons contained the 5'-CS intI1, the 3'-CS qacE\( \Delta 1 \) and sul1, and a variable region where two gene cassettes, \( \text{bla}_{\text{IMP}} \) and \( \text{aac}(6')\text{-IIC} \), were inserted (Fig. 1). The \( \text{bla}_{\text{IMP}} \) genes in In-s1 and In-s2 were identical to the \( \text{bla}_{\text{IMP}-1} \) that was first identified in the \( S. \ marcescens \) AK9373 strain (GenBank accession no. D50438), but was carried by a class 3 integron (Arakawa et al., 1995). The \( \text{bla}_{\text{IMP}} \) gene in In-s3 was a point mutation derivative of \( \text{bla}_{\text{IMP}-1} \) with a single base replacement of G by T at nt 145 leading to an amino acid alteration of Val\(^{49}\)→Phe, identical to \( \text{bla}_{\text{IMP}-10} \) found in \( P. \ aeruginosa \) strains (GenBank accession no. AB074433) (Iyobe et al., 2002). The \( \text{bla}_{\text{IMP}-1} \) and \( \text{bla}_{\text{IMP}-10} \) gene cassettes were preceded by a weak \( P_{\text{int}} \) promoter, TGGACA(N)\(_{12}\)TAAGCT, and an inactive P2 promoter, TTGTTA(N)\(_{14}\)TACAGT, which were located at the 5'-end of the integrase 1 gene. The \( \text{aac}(6')\text{-IIC} \) cassettes were located downstream of the \( \text{bla}_{\text{IMP}-1} \) or \( \text{bla}_{\text{IMP}-10} \) cassette and were identical to the sequence derived from a \( P. \ aeruginosa \) isolate (GenBank accession no. AF162771) (Galani et al., 2005).

**Comparison of the resistance phenotypes of \( \text{bla}_{\text{IMP}-1} \) and \( \text{bla}_{\text{IMP}-10} \)**

\( S. \ marcescens \) strains S\#1, S\#2 and S\#3 were highly resistant to the carbapenems IPM, MEPM, PAPM and BIPM (MICs of 128–2048 \( \mu \)g ml\(^{-1}\)) and exhibited intermediate or low resistance to GEN and AMK (MICs of 4–64 \( \mu \)g ml\(^{-1}\)) (Table 2). \( E. \ coli \) transconjugants and transformants showed a reduced susceptibility to carbapenems. As a result of the acquisition of \( \text{bla}_{\text{IMP}-1} \), the susceptibility of \( E. \ coli \) transconjugants to IPM, MEPM, PAPM and BIPM decreased by 32-, 256-, 64- and 128-fold, respectively. Notably, after gaining \( \text{bla}_{\text{IMP}-10} \) the susceptibility of \( E. \ coli \) transconjugants to the four carbapenems decreased by 64-, 2048-, 256- and 64-fold, respectively. The same tendency was also observed when the susceptibilities of \( E. \ coli \) transformants were tested to IPM, MEPM, PAPM and BIPM (Table 2).

**DISCUSSION**

The clustering of several resistance genes in integrons favours the concerted acquisition of antibiotic-resistance
determinants. The \( \text{bla}_{\text{IMP-1}} \) and \( \text{bla}_{\text{IMP-10}} \) genes that we studied were inserted in class 1 integrons followed by the \( \text{aac(}6\text{)-IIc} \) cassette. The integrons \( \text{In-s1}, \text{In-s2} \) and \( \text{In-s3} \) had the weak promoter \( P_{\text{am}} \), \( \text{TGGACA(N)}_{12}\text{TAAAGCT} \), and the inactive promoter \( P_{2} \), \( \text{TTGTTA(N)}_{4}\text{TACAGT} \). Furthermore, the integrons were carried by a transferrable plasmid, allowing the rapid spread of multidrug-resistant genes among members of the \( \text{Enterobacteriaceae} \) in clinical settings.

To evaluate the effect of a plasmid-borne \( \text{bla}_{\text{IMP}} \) gene on susceptibility of bacterial cells to carbapenems, \( \text{E. coli} \) transconjugants and transformants are more suitable models than wild-type strains because factors such as the outer-membrane barrier and efflux system, possibly possessed by wild-type strains, can be excluded. Of note, it was observed that \( \text{E. coli} \) transconjugants and transformants harbouring \( \text{bla}_{\text{IMP-10}} \) showed higher levels of resistance to IPM, MEPM and PAPM than the strains harbouring \( \text{bla}_{\text{IMP-1}} \), although nucleotide sequences of the class 1 integrons harbouring \( \text{bla}_{\text{IMP-10}} \) and \( \text{bla}_{\text{IMP-1}} \) are identical except for a single point mutation. The mechanism of this phenomenon is unclear. One possible explanation is that IMP-10 MBL is more potent than IMP-1 MBL with respect to hydrolysis of IPM, MEPM and PAPM but not BIPM. In a previously published paper, the kinetic parameters of purified IMP-1 and IMP-10 MBL in hydrolysing IPM, MEPM and other \( \beta \)-lactams were determined (Iyobe et al., 2002). Compared with the rate of hydrolysis \( (K_{\text{cat}}) \) of IMP-1 MBL \( (130 \text{ and } 13 \text{ s}^{-1} \) for IPM and MEPM, respectively), IMP-10 MBL showed the much higher \( K_{\text{cat}} \) values of 220 and 64 s\(^{-1}\), respectively. However, no significant differences in the specificity constant \( (K_{\text{cat}}/K_{m}) \) were observed between IMP-1 and IMP-10 MBL. Further experiments are therefore needed to clarify the detailed mechanism(s).

In conclusion, nucleotide sequences of the class 1 integrons carrying \( \text{bla}_{\text{IMP-1}} \) and \( \text{bla}_{\text{IMP-10}} \) are identical except for a single point mutation. Strains harbouring \( \text{bla}_{\text{IMP-10}} \) showed higher-level resistance to IPM, MEPM and PAPM than strains harbouring \( \text{bla}_{\text{IMP-1}} \), and the location of the integrons on transferable plasmids makes the intra- and interspecies transfer of the resistance genes very efficient.

### ACKNOWLEDGEMENTS

We thank Dr Makito Kobayashi, Department of Biology, The College of Liberal Arts, International Christian University, for providing valuable suggestions.

### REFERENCES


### Table 2. Phenotypes and genotypes of wild-type \( S.\text{marcescens} \), \( E.\text{coli} \) HB101 and JM109 and their transconjugants and transformants

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg ml(^{-1}))</th>
<th>Genotype</th>
<th>CS of integron 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbapenem</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPM</td>
<td>MEPM</td>
<td>PAPM</td>
</tr>
<tr>
<td>Wild-type ( S.\text{marcescens} )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( S/1 )</td>
<td>256</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>( S/2 )</td>
<td>512</td>
<td>256</td>
<td>2048</td>
</tr>
<tr>
<td>( S/3 )</td>
<td>512</td>
<td>2048</td>
<td>2048</td>
</tr>
<tr>
<td>( S/4 )</td>
<td>1</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td>( E.\text{coli} ) HB101 and transconjugants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( E.\text{coli} ) HB101-pS#1</td>
<td>0.25</td>
<td>0.03125</td>
<td>0.125</td>
</tr>
<tr>
<td>( E.\text{coli} ) HB101-pS#2</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>( E.\text{coli} ) HB101-pS#3</td>
<td>16</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>( E.\text{coli} ) JM109 and transconjugants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( E.\text{coli} ) JM109-pS#1</td>
<td>0.0625</td>
<td>0.03125</td>
<td>0.0625</td>
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<tr>
<td>( E.\text{coli} ) JM109-pS#2</td>
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<td>16</td>
<td>8</td>
</tr>
<tr>
<td>( E.\text{coli} ) JM109-pS#3</td>
<td>4</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>IMP</td>
<td>MEPM</td>
<td>PAPM</td>
</tr>
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<td></td>
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</table>

The \( \text{bla}_{\text{IMP-1}} \) and \( \text{bla}_{\text{IMP-10}} \) genes that we studied were inserted in class 1 integrons followed by the \( \text{aac(}6\text{)-IIc} \) cassette. The integrons \( \text{In-s1}, \text{In-s2} \) and \( \text{In-s3} \) had the weak promoter \( P_{\text{am}} \), \( \text{TGGACA(N)}_{12}\text{TAAAGCT} \), and the inactive promoter \( P_{2} \), \( \text{TTGTTA(N)}_{4}\text{TACAGT} \). Furthermore, the integrons were carried by a transferrable plasmid, allowing the rapid spread of multidrug-resistant genes among members of the \( \text{Enterobacteriaceae} \) in clinical settings.

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