Diverse carbapenem-resistance mechanisms in 16S rRNA methylase-producing Acinetobacter baumannii

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Multidrug-resistant Acinetobacter baumannii has become a global problem. This study characterized amikacin-resistant A. baumannii isolated from eight patients during April 2010–March 2011 in our university hospital and examined the possible mechanisms in three cases in which carbapenem susceptibility changed to carbapenem resistance during treatment of the patients. The armA gene, which is one of the 16S rRNA methylase genes and is associated with high MICs of aminoglycosides, was positive in all isolates. The blaOXA-51 gene and ISAba1-blaADC were positive in all isolates, but ISAba1-blaOXA-51 was positive in only three isolates. The CarO outer-membrane protein was lost in one isolate. In the first case, both the susceptible and the resistant isolates were positive for ISAba1-blaOXA-51, but the expression of the blaADC gene was increased 3.1-fold in the carbapenem-resistant isolate of the pair. In the second case, the carbapenem-resistant strain became positive for ISAba1-blaOXA-51, resulting in 21.5-fold increased expression of blaOXA-51, compared to the carbapenem-susceptible strain of the pair. In the third case, the carbapenemase genes remained negative despite the carbapenem resistance, but the expression of the adeB gene was increased 4.6-fold after acquisition of carbapenem resistance. Multilocus sequence typing analysis of two isolates showing representative pulsed-field gel electrophoresis patterns demonstrated that both isolates were classified to sequence type 2 (ST2). These results showed that the 16S rRNA methylase-producing A. baumannii, initially susceptible to carbapenem, acquired carbapenem resistance via diverse mechanisms.

INTRODUCTION

Acinetobacter baumannii is an important pathogen causing ventilator-associated pneumonias, bloodstream infections and wound infections in severely ill patients. Recently, hospital-acquired infection caused by multidrug-resistant A. baumannii that has acquired resistance to antibiotic agents such as carbapenems, fluoroquinolones and aminoglycosides has become a global problem (Peleg et al., 2008; Perez et al., 2007).

Amikacin (AMK), an aminoglycoside agent, has retained good activity against A. baumannii. Consequently, it has become difficult to treat A. baumannii if they show resistance to AMK. Resistance to aminoglycosides has been attributed mainly to enzymic inactivation by acetyltransferases, nucleotidyltransferases and phosphotransferases. In recent years, however, methylation of 16S rRNA has been reported to play an important role in aminoglycoside resistance by blocking the binding of these antibiotics to 16S rRNA. 16S rRNA methylases are often responsible for the phenotype of Gram-negative pathogens that show high MICs to most aminoglycosides. Seven types of 16S rRNA methylases have been identified: ArmA, RmtA, RmtB, RmtC, RmtD, RmtE and NpmA (Wachino et al., 2010). The ArmA methylase was first reported in a strain of Klebsiella pneumoniae in France (Galimand et al., 2003). Subsequently, ArmA methylase-producing A. baumannii were detected in Korea (Lee et al., 2006). Carbapenem-resistant ArmA methylase-producing A. baumannii strains have more recently been reported in the USA (Adams-Haduch et al., 2008) and in China (Yu et al., 2007). The dissemination of such clinical isolates with multidrug resistance has become a global threat. Yamane et al. (2007) reported that four (0.13%) ArmA methylase-positive isolates were found out of 3116 A. baumannii in Japan, but these were susceptible to carbapenems.

The mechanisms of carbapenem resistance of A. baumannii have been explained by production of plasmid-encoded carbapenemases such as the OXA carbapenemases and metallo-β-lactamases (MBLs), and by increased production...
of chromosome-encoded carbapenemases as a result of insertion of \textit{ISAba1} into the upstream region of the \textit{bla}_{OXA-51} gene, providing a strong promoter. Aside from carbapenemases, other mechanisms include decreased expression of outer-membrane proteins (OMPs), overexpression of multidrug efflux pumps and alteration in the affinity of penicillin-binding proteins (Dijkshoorn et al., 2007).

The purposes of this study were to characterize six isolates of AMK-resistant \textit{A. baumannii} from three patients in our university hospital during April 2010–March 2011 and to examine possible mechanisms of carbapenem resistance in three of six isolates in which carbapenem susceptibility changed to carbapenem resistance during treatment of the patients.

**METHODS**

**Bacterial isolates and susceptibility testing.** Six isolates of AMK-resistant \textit{A. baumannii} obtained from three patients during April 2010–March 2011 were used for this study. Biochemical identification and antimicrobial susceptibility testing of isolates were conducted using a Neg Com 6.12J and a WalkAway-96 SI system (both from Siemens Healthcare Diagnostics). Criteria for antimicrobial susceptibility were based on guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2009).

**Phenotypic confirmatory test of carbapenemases.** Carbapenem-resistant isolates were screened by the modified Hodge test (Lee et al., 2003; CLSI, 2009). MBL production was assessed using sodium mercaptopropionic acid (SMA) (3 mg) (Eiken Chemical) as an inhibitor, based on the method described by Arakawa et al. (2000).

**DNA amplification analysis.** The isolates were screened for genes encoding 16S rRNA methylases such as \textit{armA}, \textit{rmtA}, \textit{rmtB}, \textit{rmtC} and \textit{rmtD} by PCR using primers described elsewhere (Doi & Arakawa, 2007).

PCR screening was performed for OXA-type carbapenemases (\textit{bla}_{OXA-23, 24, 51} and \textit{bla}_{SM}) and MBLs (\textit{bla}_{IMP-1, IMP-2 and VIP-2-like}) (Shibata et al., 2003; Turton et al., 2006).

Overexpression of OXA-51 β-lactamase and ADC β-lactamase is regulated by \textit{ISAba1}, which is located upstream of the gene. The presence of \textit{ISAba1} correlates strongly with increased expression of the \textit{bla}_{OXA-51} and \textit{bla}_{ADC} genes. \textit{ISAba1}, \textit{ISAba1-bla}_{OXA-51-like} and \textit{ISAba1-bla}_{ADC} were detected by PCR using primer sets and conditions previously described (Bratu et al., 2008; Turton et al., 2006; Woodford et al., 2006). Insertion into the \textit{carO} gene was screened as described previously (Lee et al., 2011; Lu et al., 2009).

**Reverse transcription PCR (RT-PCR).** Real-time RT-PCR was performed to quantify gene expression of \textit{bla}_{OXA-51-like} (Woodford et al., 2006), \textit{bla}_{ADC} (Bratu et al., 2008) and \textit{adeB} (Higgins et al., 2004) in six isolates obtained from three patients in which carbapenem susceptibility changed to carbapenem resistance. Total RNA was extracted from 1 x 10^6 cells of \textit{A. baumannii} using a High Pure RNA Isolation kit (Roche Diagnostics) according to the manufacturer’s instructions. RT-PCR was performed using Transcriptor Reverse Transcriptase (Roche Diagnostics). In the resistant isolates (1b, 2b and 3b), the expression of each target gene was normalized based on that of the 16S rRNA gene and expressed as a relative rate compared to that in the susceptible isolate of each pair (isolates 1a, 2a and 3a; expression taken as 1.0). Experiments were conducted three times independently; representative data are shown.

**Pulsed-field gel electrophoresis (PFGE) analysis.** Chromosomal DNAs were digested with Smal restriction enzyme (Takara BIO). DNA fragments were electrophoresed using the GenePath system (Bio-Rad). The banding pattern was interpreted according to the methods described by Tenover et al. (1995).

**Multilocus sequence typing (MLST) analysis.** We conducted MLST analysis using the Institut Pasteur’s MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html), as previously described (Diancourt et al., 2010). Among the isolates showing the same PFGE pattern, representative isolates (1a and 3a) were chosen for MLST analysis.

**RESULTS**

**Susceptibility testing**

Sixty-three unique isolates of \textit{A. baumannii} were detected in our clinical microbiology laboratory during the study period. Among these, the isolates from eight patients were AMK-resistant but were initially susceptible to carbapenems regardless of antimicrobial agent use. Carbapenem-resistant strains were, however, isolated later from three patients. Susceptibility testing results of these isolates are presented in Table 1. All isolates were resistant to cephalosporins,

**Table 1. Susceptibility tests of \textit{A. baumannii} isolates**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Strain</th>
<th>Date of isolation (dd/mm/yyyy)</th>
<th>Source</th>
<th>PIPC</th>
<th>CAZ</th>
<th>CZOP</th>
<th>CFPM</th>
<th>IPM</th>
<th>MEPM</th>
<th>S/C</th>
<th>GM</th>
<th>AMK</th>
<th>TOB</th>
<th>CFIX</th>
<th>LVFX</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>12/07/2010</td>
<td>Sputum</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>≤16</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>1b</td>
<td>01/09/2010</td>
<td>Sputum</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>4</td>
<td>&gt;8</td>
<td>&gt;16</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;2</td>
<td>&gt;4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2a</td>
<td>13/12/2010</td>
<td>Central venous catheter</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>8</td>
<td>&gt;16</td>
<td>2</td>
<td>≤1</td>
<td>32</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;2</td>
<td>&gt;4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2b</td>
<td>17/01/2011</td>
<td>Sputum</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>8</td>
<td>&gt;16</td>
<td>4</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;32</td>
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<td>4</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>18/01/2011</td>
<td>Sputum</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>2</td>
<td>4</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>3b</td>
<td>08/02/2011</td>
<td>Blood culture</td>
<td>&gt;64</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;2</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>4</td>
</tr>
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</table>
fluoroquinolones and aminoglycosides (gentamicin, AMK, tobramycin). Carbapenem-resistant strains developed from isolates 1b, 2b and 3b. In addition, these strains were negative both for the modified Hodge test and for the confirmatory test using the SMA disk for MBL.

**DNA amplification analysis**

The $bla_{IMP-1}$, $bla_{IMP-2}$, $bla_{VIM-2}$, $bla_{OXA-23}$, $bla_{OXA-24}$ and $bla_{OXA-58}$ genes were not detected from these six isolates. The $bla_{OXA-51}$ gene and ISAbal were positive in all isolates, but ISAbal-$bla_{OXA-51}$ was positive only in nos. 1a, 1b and 2b (Table 2). ISAbal-$bla_{ADC}$ was positive in all six isolates (Table 2). The armA gene, which confers high resistance to aminoglycosides, was positive in all isolates (Table 2). The carO gene, which encodes an OMP, was positive in these six isolates. However, a PCR amplicon for the carO gene (approx. 1700 bp), which was larger than the expected size of 741 bp, was obtained from one isolate (2b) (Table 2).

**Expression of $bla_{OXA-51}$, $bla_{ADC}$ and adeB**

In the first case, both isolates were positive for ISAbal-$bla_{OXA-51}$, but the expression of the $bla_{ADC}$ gene was increased 3.1-fold in the carbapenem-resistant isolate of the pair. In the second case, the carbapenem-resistant strain became positive for ISAbal-$bla_{OXA-51}$, resulting in 21.5-fold increased expression of $bla_{OXA-51}$ compared to the carbapenem-susceptible strain of the pair. In the third case, the carbapenemase genes remained negative despite the carbapenem resistance, but the expression of the adeB gene was increased 4.6-fold after acquisition of carbapenem resistance (Table 2).

**PFGE and MLST analyses**

Six isolates were classified to type I (isolates 1a and 1b) and type II (isolates 2a, 2b, 3a and 3b) based on the PFGE pattern. Type I and type II were demonstrated to be closely related clones (data not shown).

MLST analysis for two isolates (1a and 2a) of type I and II, showing representative PFGE patterns, demonstrated that both isolates belonged to sequence type 2 (ST2).

**DISCUSSION**

This study investigated the mechanisms by which ArmA methylase-producing *A. baumannii* developed carbapenem resistance in three isolates. The clones from two patients (cases 2 and 3) were proven to be identical by PFGE analysis, but the mechanisms of carbapenem resistance in the three isolates differed.

The loss of a 29 kDa OMP, also known as CarO, is reportedly associated with carbapenem resistance (Peleg et al., 2008). Efflux pumps such as AdeABC are also reportedly involved in multidrug resistance (Vila et al., 2007). AdeABC, a resistance-nodulation-division (RND) family-type pump, has a three-component structure of AdeA, AdeB and AdeC, which form the inner membrane fusion protein, the transmembrane component and the OMP, respectively. AdeABC is chromosomally encoded and is normally regulated by a two-component system with a sensor kinase (AdeS) and its associated response regulator (AdeK). Overexpression of adeB has been attributed to mutations in the adeS and adeR genes and also to insertion of ISAbal into the adeS gene, thereby resulting in multidrug resistance in *A. baumannii* (Coyne et al., 2011). However, the reduced permeability alone is not sufficient to cause carbapenem resistance (Peleg et al., 2008).

The insertion sequence element ISAbal provides a promoter in the upstream region of the $bla_{OXA-51}$ and $bla_{ADC}$ genes. In this study, all strains were positive for ISAbal. Furthermore, in all strains, ISAbal was inserted in the upstream region of $bla_{ADC}$, suggesting potential overproduction of the ADC β-lactamase. The change from carbapenem susceptibility in isolate 1a to carbapenem resistance in isolate 1b was ascribed to an increased expression of ADC β-lactamase (Table 2). In strain 2b, in which ISAbal-$bla_{OXA-51}$ was positive, the expression of $bla_{OXA-51}$ was increased (Table 2). The carO gene encoding an OMP was positive in these six isolates. However, a PCR amplicon for the carO gene of approximately 1700 bp, which was larger than the expected size of 741 bp, was obtained from strain 2b, suggestive of a CarO OMP defect. Lee et al. (2011) and Lu et al. (2009) reported that the loss of the CarO OMP had only a minor effect on carbapenem resistance in *A. baumannii*. Bratu et al. (2008) reported that the loss of the CarO OMP did not correlate with carbapenem resistance. However, resistance to

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**Table 2. Epidemiological and genotypic characteristics of *A. baumannii* isolates**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Strain</th>
<th>PFGE</th>
<th>ArmA</th>
<th>IMP-1</th>
<th>IMP-2</th>
<th>VIM-2</th>
<th>$bla_{OXA-51}$</th>
<th>ISAbal</th>
<th>$bla_{ADC}$</th>
<th>Status of CarO</th>
<th>Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>I</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>1b</td>
<td>I</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Intact</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>2a</td>
<td>II</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>2b</td>
<td>II</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>II</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>3b</td>
<td>II</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Intact</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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**adeB bla_{ADC} bla_{OXA-51}**

<table>
<thead>
<tr>
<th>Status of CarO</th>
<th>Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>Intact</td>
<td>0.9</td>
</tr>
<tr>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>Lost</td>
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</tr>
<tr>
<td>Intact</td>
<td>1.0</td>
</tr>
<tr>
<td>Intact</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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**Expression of $bla_{OXA-51}$, $bla_{ADC}$ and adeB**

In the first case, both isolates were positive for ISAbal-$bla_{OXA-51}$, but the expression of the $bla_{ADC}$ gene was increased 3.1-fold in the carbapenem-resistant isolate of the pair. In the second case, the carbapenem-resistant strain became positive for ISAbal-$bla_{OXA-51}$, resulting in 21.5-fold increased expression of $bla_{OXA-51}$ compared to the carbapenem-susceptible strain of the pair. In the third case, the carbapenemase genes remained negative despite the carbapenem resistance, but the expression of the adeB gene was increased 4.6-fold after acquisition of carbapenem resistance (Table 2).

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carbapenem in our strain 2b was inferred to be caused by the loss of CarO OMP, and also by overproduction of \( \text{bla}_{OXA-51} \) due to IS\( \text{Ab}a1 \). In strain 3b, which was negative for carbapenemases despite showing carbapenem resistance, the expression of the \( \text{adeB} \) gene was increased (Table 2). Higgins et al. (2010) reported that both \( \text{adeR} \) gene mutation and \( \text{adeB} \) gene overexpression might be mechanisms for carbapenem resistance following exposure to antimicrobial agents, including carbapenem.

The first isolates (1a, 2a and 3a) from our three patients were all resistant to fluoroquinolones and aminoglycosides, but were susceptible to carbapenem. However, the relations between treatment with carbapenems and other antimicrobial agents, including carbapenem.

Three carbapenem-resistant strains (1b, 2b and 3b) were isolated after treatment with carbapenems and other antimicrobial agents, including carbapenem. Furthermore, these isolates acquired carbapenem resistance following exposure to antimicrobial agents, including carbapenem.

Three outbreak clones of multidrug-resistant \( A. \text{baumannii} \) have been described, namely international clones I, II and III (Diancourt et al., 2010). In this study, the isolated clones were demonstrated to be ST2, which belongs to international clone II. Such ST2 clones have been reported in Italy, Greece (Di Popolo et al., 2011), the Czech Republic (Nemec et al., 2008), the USA (Adams-Haduch et al., 2011) and the UK (Zarrilli et al., 2011). We need to continue observations of the epidemic spread of 16S rRNA methylase-producing \( A. \text{baumannii} \) infections in a hospital setting.

In summary, ArmA methylase-producing \( A. \text{baumannii} \) showed susceptibility to carbapenem initially, but acquired multi-antibiotic resistance immediately after the use of antibacterial agents. Furthermore, these isolates acquired carbapenem resistance via mechanisms of various kinds.

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