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 16S rRNA methylases.
of chromosome-encoded carbapenemases as a result of insertion of ISAba1 into the upstream region of the blaOXA-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 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 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 6.12) 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., 2006; 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 armA, rmtA, rmtB, rmtC and rmtD by PCR using primers described elsewhere (Doi & Arakawa, 2007).

PCR screening was performed for OXA-type carbapenemases (blaOXA-23, 24, 51 and -58-like) and MBLs (blaIMP-1, -2 and -3-like) (Shibata et al., 2003; CLSI, 2009).

Overexpression of OXA-51 β-lactamase and ADC β-lactamase is regulated by ISAba1, which is located upstream of the gene. The presence of ISAba1 correlates strongly with increased expression of the blaOXA-51 and bladADC genes. ISAba1, ISAba1-blaOXA-51-like and ISAba1-blaADC 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 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 blaOXA-51-like (Woodford et al., 2006), bladADC (Bratu et al., 2008) and 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 × 10^6 cells of 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 mRNA 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 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 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>CFPMP</th>
<th>IPM</th>
<th>MEPMP</th>
<th>S/C</th>
<th>GM</th>
<th>AMK</th>
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<th>CPFX</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>
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<td>4</td>
</tr>
<tr>
<td>1</td>
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<td>Sputum</td>
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<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>4</td>
<td>&gt;8</td>
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<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>16</td>
<td>2</td>
<td>≤1</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;8</td>
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<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2b</td>
<td>17/01/2011</td>
<td>Sputum</td>
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<td>&gt;16</td>
<td>8</td>
<td>&gt;16</td>
<td>4</td>
<td>&gt;8</td>
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<td>4</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>18/01/2011</td>
<td>Sputum</td>
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<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>2</td>
<td>4</td>
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<td>&gt;8</td>
<td>&gt;32</td>
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<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>3b</td>
<td>08/02/2011</td>
<td>Blood culture</td>
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<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;2</td>
<td>&gt;4</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</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 \( \text{bla}_{\text{IMP-1}}, \text{bla}_{\text{IPM-2}}, \text{bla}_{\text{VIP-2}}, \text{bla}_{\text{OXA-23}}, \text{bla}_{\text{OXA-24}} \) and \( \text{bla}_{\text{OXA-58}} \) genes were not detected from these six isolates. The \( \text{bla}_{\text{OXA-51}} \) gene and IS\( \text{Abal} \) were positive in all isolates, but IS\( \text{Abal} \)-\( \text{bla}_{\text{OXA-51}} \) was positive only in nos. 1a, 1b and 2b (Table 2). IS\( \text{Abal} \)-\( \text{bla}_{\text{ADC}} \) was positive in all six isolates (Table 2). The \( \text{armA} \) gene, which confers high resistance to aminoglycosides, was positive in all six isolates (Table 2). The \( \text{carO} \) gene, which encodes an OMP, was positive in these six isolates. However, a PCR amplicon for the \( \text{carO} \) gene (approx. 1700 bp), which was larger than the expected size of 741 bp, was obtained from one isolate (2b) (Table 2).

**Expression of \( \text{bla}_{\text{OXA-51}}, \text{bla}_{\text{ADC}} \) and \( \text{adeB} \)**

In the first case, both isolates were positive for IS\( \text{Abal} \)-\( \text{bla}_{\text{OXA-51}} \), but the expression of the \( \text{bla}_{\text{ADC}} \) 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 IS\( \text{Abal} \)-\( \text{bla}_{\text{OXA-51}} \), resulting in 21.5-fold increased expression of \( \text{bla}_{\text{OXA-51}} \) 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 \( \text{adeB} \) 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 \( A. \text{baumannii} \) 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 ( AdeR ). Overexpression of \( \text{adeB} \) has been attributed to mutations in the \( \text{ades} \) and \( \text{adeR} \) genes and also to insertion of IS\( \text{Abal} \) into the \( \text{adeS} \) gene, thereby resulting in multidrug resistance in \( A. \text{baumannii} \) (Coyne et al., 2011). However, the reduced permeability alone is not sufficient to cause carbapenem resistance (Peleg et al., 2008).

The insertion sequence element IS\( \text{Abal} \) provides a promoter in the upstream region of the \( \text{bla}_{\text{OXA-51}} \) and \( \text{bla}_{\text{ADC}} \) genes. In this study, all strains were positive for IS\( \text{Abal} \). Furthermore, in all strains, IS\( \text{Abal} \) was inserted in the upstream region of \( \text{bla}_{\text{ADC}} \), suggesting potential overproduction of the ADC \( \beta \)-lactamase. The change from carbapenem susceptibility in isolate 1a to carbapenem resistance in isolate 1b was ascribed to an increased expression of ADC \( \beta \)-lactamase (Table 2). In strain 2b, in which IS\( \text{Abal} \)-\( \text{bla}_{\text{OXA-51}} \) was positive, the expression of \( \text{bla}_{\text{OXA-51}} \) was increased (Table 2). The \( \text{carO} \) gene encoding an OMP was positive in these six isolates. However, a PCR amplicon for the \( \text{carO} \) 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 \( A. \text{baumannii} \). Bratu et al. (2008) reported that the loss of the CarO OMP did not correlate with carbapenem resistance. However, resistance to

<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>( \text{bla}_{\text{OXA-51}} )</th>
<th>IS( \text{Abal} )-( \text{bla}_{\text{OXA-51}} )</th>
<th>IS( \text{Abal} )-( \text{bla}_{\text{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 1.0 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 3.1 1.2</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 1.0 1.0</td>
</tr>
<tr>
<td>2</td>
<td>2b</td>
<td>II</td>
<td>+</td>
<td>–</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Lost</td>
<td>1.4 0.9 21.5</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 1.0 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>4.6 1.5 1.8</td>
</tr>
</tbody>
</table>

**Table 2. Epidemiological and genotypic characteristics of \( A. \text{baumannii} \) isolates**
Carbapenem in our strain 2b was inferred to be caused by the loss of CarO OMP, and also by overproduction of blaOXA-51 due to ISAba1. In strain 3b, which was negative for carbapenemases despite showing carbapenem resistance, the expression of the adef gene was increased (Table 2). Higgins et al. (2010) reported that both adef gene mutation and 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 carbapenems based on the criteria of CLSI (2009).

Three carbapenem-resistant strains (1b, 2b and 3b) were isolated after treatment with carbapenem and other antimicrobial agents (data not shown). These results suggested that A. baumannii has the propensity to develop resistance by rapid mutation upon exposure to antimicrobial agents. However, the relations between treatment with carbapenems and the mechanism of carbapenem resistance still needed to be clarified.

Three outbreak clones of multidrug-resistant A. 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 has 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 observing the epidemic spread of 16S rRNA methylase-producing A. baumannii infections in a hospital setting.

In summary, ArmA methylase-producing A. 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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