An outbreak of \textit{bla}\textsubscript{OXA-51-like}- and \textit{bla}\textsubscript{OXA-66}-positive \textit{Acinetobacter baumannii} ST208 in the emergency intensive care unit

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A series of clinical isolates of drug-resistant (DR) \textit{Acinetobacter baumannii} with diverse drug susceptibility was detected from eight patients in the emergency intensive care unit of Tokai University Hospital. The initial isolate was obtained in March 2010 (\textit{A. baumannii} Tokai strain 1); subsequently, seven isolates were obtained from patients (\textit{A. baumannii} Tokai strains 2–8) and one isolate was obtained from an air-fluidized bed used by five of the patients during the 3 months from August to November 2011. The isolates were classified into three types of antimicrobial drug resistance patterns (RRR, SRR and SSR) according to their susceptibility (S) or resistance (R) to imipenem, amikacin and ciprofloxacin, respectively. Genotyping of these isolates by multilocus sequence typing revealed one sequence type, ST208, whilst that by a DiversiLab analysis revealed two subtypes. All the isolates were positive for \textit{bla}\textsubscript{OXA-51-like} and \textit{bla}\textsubscript{OXA-66}, as assessed by PCR and DNA sequencing. \textit{A. baumannii} Tokai strains 1–8 and 10 (RRR, SRR and SSR) had quinolone resistance-associated mutations in \textit{gyrA}/\textit{parC}, as revealed by DNA sequencing. The IS\textsubscript{Aba1} upstream of \textit{bla}\textsubscript{OXA-51-like} and aminoglycoside resistance-associated gene, \textit{armA}, were detected in \textit{A. baumannii} Tokai strains 1–7 and 10 (RRR and SRR) as assessed by DNA sequencing. The genes encoding resistance–nodulation–division family pumps (\textit{adeB}, \textit{adeG} and \textit{adeJ}) and outer-membrane porins (\textit{oprD} and \textit{carO}), overexpression of \textit{adeB} and \textit{adeJ} and suppression of \textit{oprD} and \textit{carO} were seen in isolates of \textit{A. baumannii} Tokai strain 2 (RRR), as assessed by real-time PCR. Thus, the molecular characterization of a series of isolates of DR \textit{A. baumannii} revealed the outbreak of ST208 and diverse antimicrobial drug susceptibilities, which almost correlated with differential gene alterations responsible for each type of drug resistance.

INTRODUCTION

\textit{Acinetobacter baumannii} is emerging as a nosocomial pathogen, particularly in intensive care units, including burn care units (Bayram \textit{et al.}, 2013; Guzek \textit{et al.}, 2013; Ohashi \textit{et al.}, 2013). Hospitalized patients at a greater risk of \textit{Acinetobacter} infections are those particularly ill on a ventilator, those with a prolonged hospital stay, those who have open wounds and those with invasive devices, such as urinary catheters (Wendt \textit{et al.}, 1997; Wisplinghoff \textit{et al.}, 2013).
cefepime (CFPM) was from Bristol-Myers Squibb, CPFX was from Eizai, ceftazidime (CAZ) was from Glaxo SmithKline, AMK and IPM were from Banyu Pharmaceutical, aztreonam (AZT) was from Taisho Toyama Pharmaceutical and tobramycin (TOB) was from Towa Pharmaceutical.

Molecular typing. DNA templates were extracted using a ZR-Duet DNA/RNA MiniPrep kit (Zymo Research). Multilocus sequence typing (MLST) was performed as described previously (Bartual et al., 2005; Fu et al., 2010). MLST sequences were uploaded into the A. baumannii MLST Type Database (http://pubmlst.org/abauumannii/) to determine the alleles and sequence types. A. baumannii isolates were screened for gene homology by a repetitive-element-based PCR (rep-PCR) DiversiLab Microbial Typing System (Sysmex bioMérieux), which amplified the regions between the non-coding repetitive sequences in bacterial genomes, as described previously (Carretto et al., 2006; Higgins et al., 2012). The annealing temperature of the PCR amplification used in this study was 55 °C for gltA, gyrB, recA and cpxR60, and 50 °C for dghB, gpi and rpoD. The amplification products were purified with a DNA purification kit (Qiagen). The DNA sequencing was performed using an ABI3500xL Genetic Analyzer (Applied Biosystems).

Evaluation of the mechanisms of resistance
Screening for metallo-β-lactamase (MBL). A. baumannii isolates were screened for the production of MBL by a double-disc synergy test with discs containing sodium mercaptoacetic acid as described previously (Arakawa et al., 2000).

PCR assay for β-lactamase and armA. The following resistance genes were examined by PCR: *bla*<sub>IMP-1</sub>, *bla*<sub>TEM</sub>, *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-24-like</sub>, *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-58-like</sub> and * ISAAb1*, as described previously (Turton et al., 2006; Woodford et al., 2006). The *armA* gene, which encodes 16S rRNA methylases and confers high resistance to aminoglycosides, was screened by PCR using primers that were described previously (Yamane et al., 2005).

Sequencing of OXA-type β-lactamase, and *gyrA* and *parC*. Sequencing of OXA-type β-lactamase was performed as described previously (Endo et al., 2012). The quinolone resistance-determining regions of *gyrA* and *parC* were amplified and analysed as described previously (Liu et al., 2012). DNA sequencing of the amplified DNA products was performed using an ABI3500xL Genetic Analyzer (Applied Biosystems).

Quantitative real-time (qRT)-PCR. RNA templates were extracted by a ZR-Duet DNA/RNA MiniPrep kit (Zymo Research). The expression levels of three different genes encoding resistance-nodulation–division (RND) family pumps (*adeB*, *adeG* and *adeD*) and two different genes encoding outer-membrane porins (*oprD* and *carO*) were analysed by qRT-PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems) (Peleg et al., 2008; Fernando & Kumar, 2012; Zander et al., 2013). The primers used for the analysis are listed in Table 1. The housekeeping gene 16S rRNA was used as a control (Coyne et al., 2010; Srinivasan et al., 2011; Hou et al., 2012). Reactions (20 µl) were set up using 400 nM primers and 2 µl CDNA template (diluted 1 : 10) with SYBR Premix Ex Taq II (Tli RNaseH Plus) and ROX plus (Takara Bio). The data analysis was carried out using StepOne software. The expression of each target gene was normalized based on the level of the 16S rRNA mRNA gene and was expressed as a relative rate compared with that in the susceptible isolate of each pair (the expression of A. baumannii Tokai strain 9 was taken as 1.0). Experiments were conducted at least three times independently and all reactions were carried out in triplicate.
RESULTS

Bacterial strains and antibiotic susceptibility

The characteristics of the A. baumannii Tokai strains are shown in Table 2. In March 2010, a DR A. baumannii Tokai strain 1 was detected initially from the wound of a patient with a severe burn injury. After 1.5 years, during a period of 3 months from August to November 2011, another seven clinical isolates of DR A. baumannii strains from patients (A. baumannii Tokai strains 2–8) were obtained. The DR A. baumannii Tokai strains were classified into three types according to their susceptibility to three drugs (IPM, AMK and CPFX) as RRR, SRR or SSR (R, resistant; S, susceptible; Tables 2 and 3). They were obtained from sputum, wounds and bile drains.

As the interval between the first patient and the others was long (>18 months), the environment of the ward was suspected to be a possible reservoir of the pathogen. Based on the results of the bacteriological surveillance of environmental surfaces, A. baumannii Tokai strain 10 was isolated from the cracks of a rubber frame and a lump of beads in an air-fluidized bed that was used by five patients during their hospitalization (A. baumannii Tokai strains 1, 2 and 4–6).

Molecular typing

The molecular genotyping of isolates by a MLST analysis revealed a sequence type of ST208 for A. baumannii Tokai strains 1–8 and 10 (ST profile, gltA-gyrB-gdhB-recA-cpn60-gpi-rpoD: 1-3-3-2-2-97-3) and another type for A. baumannii Tokai strain 9 (ST profile, gltA-gyrB-gdhB-recA-cpn60-gpi-rpoD: 15-48-58-42-36-54-41). The molecular genotyping of isolates by rep-PCR showed the same pattern (>97% similarity) as one type for eight of the isolates (A. baumannii Tokai strains 1–7 and 10) (Fig. 1), and the other isolates (A. baumannii Tokai strains 8 and 9) had different patterns (85 and <70% similarity, respectively).

Expression of resistance-related genes

The MBL assay of the clinically isolated A. baumannii Tokai strains revealed no apparent MBL production and all isolates showed expression of OXA-51-like carrying OXA-66 β-lactamase (Table 4). The expression of IMP-1, VIM, OXA-23-like, OXA-24-like and OXA-58-like was negative. Expression of ISAb1 and armA was found in A. baumannii Tokai strains 1–7 and 10. The DNA sequencing of gyrA and parC revealed that Ser83 (TCA) was changed to TTA (Leu) and that Ser80 (TCG) was changed to TTT (Phe) or TTG (Leu) in A. baumannii Tokai strains 1–8 and 10.

Our analysis of genes encoding RND pumps included an analysis of the expression of three previously characterized genes, adeB, adeG and adeJ, which encode the RND pump in the adeABC, adeFGH and adeIJK operons, respectively. The result of A. baumannii Tokai strains 1, 2, 8 and 9 as representative strains from each group with the same susceptibility pattern is shown in Table 5. Overexpression of adeB and adeJ was seen in A. baumannii Tokai strain 2. The expression of oprD was decreased in A. baumannii Tokai strains 2 and 8. Underexpression of carO was seen in isolates with A. baumannii Tokai strains 1, 2 and 8.

DISCUSSION

We investigated a series of clinical isolates of DR A. baumannii ST208 in the EICU of Tokai University Hospital. In order to elucidate the diversity of the drug resistance patterns in the same sequence type in these isolates, we studied the molecular characteristics of these isolates and their relationship with the resistance pattern.

A. baumannii Tokai strains 1–7 and 10 were positive for OXA-51-like and OXA-66 β-lactamase and ISAb1. A. baumannii strains with resistance to AMK (A. baumannii Tokai strains 1–7 and 10) were positive for armA. These results are consistent with the idea that ISAb1 regulates the expression of OXA-51-like carrying OXA-66 β-lactamase and that armA is related to aminoglycoside
Table 2. Cases and *A. baumannii* Tokai strains

One hundred and fifty nurses and five nurse-aids worked in the EICU and Burn centre, and they were not fixed as a team. ER, Critical care and emergency medicine; NR, neurosurgery; OP, orthopaedics; R, resistant; S, susceptible.

| Strain/disease | Ward        | Day detected after hospitalization | Source | Susceptibility pattern of IPM, AMK and CPFX* | Doctor team | Use of air-fluidized bed |
|----------------|-------------|-----------------------------------|--------|---------------------------------------------|-------------|--------------------------|
| 1. 74% total body surface area burn | Burn centre | 31 (3 March 2010) | Sputum | IPM-S, AMK-R, CPFX-R (SRR) | ER-a | Yes |
| 2. 85% total body surface area burn | Burn centre | 9 (29 August 2011) | Wound | IPM-R, AMK-R, CPFX-R (RRR) | ER-b | Yes |
| (Ohashi et al., 2013) | | | | | | |
| 3. 40% total body surface area burn | Burn centre | 33 (19 September 2011) | Wound | IPM-R, AMK-R, CPFX-R (RRR) | ER-c | No |
| (Ohashi et al., 2013) | | | | | | |
| 4. 70.5% total body surface area burn | Burn centre | 7 (19 September 2011) | Wound | IPM-R, AMK-R, CPFX-R (RRR) | ER-c | Yes |
| (Ohashi et al., 2013) | | | | | | |
| 5. Traffic injury | EICU | 44 (23 September 2011) | Bile drain | IPM-S, AMK-R, CPFX-R (SRR) | ER-d | Yes |
| 6. Traffic injury | EICU | 13 (26 October 2011) | Wound | IPM-R, AMK-R, CPFX-R (RRR) | ER-d | Yes |
| 7. Iliopsoas muscle abscess | EICU | 45 (15 November 2011) | Sputum | IPM-S, AMK-R, CPFX-R (SRR) | OP | No |
| 8. Subcortical haemorrhage | EICU | 240 (15 November 2011) | Sputum | IPM-S, AMK-S, CPFX-R (SSR) | ER-d | No |
| 9. Subarachnoid haemorrhage | EICU | 50 (17 November 2011) | Sputum | IPM-S, AMK-S, CPFX-S (SSS) | NS | No |
| 10. Air-fluidized bed | EICU | (20 November 2011) | Beads | IPM-S, AMK-R, CPFX-R (SRR) | ER-a, ER-b, ER-c, ER-d | |

Table 3. Susceptibility patterns of *A. baumannii* Tokai strains

| Strain | MIC (µg ml⁻¹) |
|--------|---------------|
|        | β-Lactams | Aminoglycosides | Fluoroquinolones | Other agents |
|        | IPM | PIPC | CAZ | CFPM | S/C | AZT | MEPM | CZOP | GM | TOB | AMK | LVFX | CPFX | MINO | FOM | S/T |
| 1, 5, 7, 10 | 2 | >64 | >16 | 16 | <16 | >8 | 16 | >8 | >8 | >32 | 4 | >2 | 4 | >16 | >2 |
| 2, 3, 4, 6 | >8 | >64 | >16 | 16 | <16 | >8 | 16 | >8 | >8 | >32 | >4 | >2 | ≤2 | >16 | >2 |
| 8 | ≤1 | ≤8 | ≤2 | <4 | <16 | 8 | ≤1 | 4 | ≤1 | ≤1 | ≤4 | >4 | >2 | ≤2 | >16 | ≤2 |
| 9 | ≤1 | ≤8 | 4 | 16 | <16 | 8 | ≤1 | 8 | 8 | 2 | 8 | ≤0.5 | 1 | ≤2 | >16 | ≤2 |

S/C, sulbactam/cefoperazone; S/T, sulfamethoxazole/trimethoprim.
resistance. The five isolates (*A. baumannii* Tokai strains 1, 2 and 4–6) could have been derived from the same source and/or transmitted horizontally, because the same air-fluidized bed had been used by those patients. Among them, *A. baumannii* Tokai strains 2, 4 and 6 showed multidrug resistance (RRR). These patients were treated with carbapenem (MEPM or DRPM) prior to sampling for at least 1 week, which may have played a role in the overexpression of *adeB* and *adeJ* in *A. baumannii*Tokai strain 2. *A. baumannii* Tokai strain 10 was detected from the cracks of the rubber frame and a lump of beads in an air-fluidized bed, even though the bed had been cleaned and disinfected every time after use. Although a few nosocomial outbreaks of *A. baumannii* ST2 have been reported (Suzuki et al., 2013; Yamada & Suwabe, 2013), an outbreak of *A. baumannii* ST208 has not been reported previously in Japan.

As the pattern of the rep-PCR and sequence type of MLST in the eight isolates was the same as that in the initial case, it was suggested that the strain survived for 1.5 years in the environmental reservoir. As infection control procedures, careful attention to environmental cleaning and disinfection in order to reduce the risk of transmission is suggested. *A. baumannii* Tokai strain 8 (SSR) was also ST208, but had a different pattern as shown by rep-PCR. During the transmission from the same original organism, the presence of a transposon or the insertion of a different plasmid might have led to the different pattern. During the period of an outbreak, *A. baumannii* with different drug susceptibility patterns appeared depending on the various resistance mechanisms.

Nine isolates (*A. baumannii* Tokai strains 1–8 and 10) had resistance to CPFX, which can be explained by the mutations of *gyrA* and *parC*. Another major factor contributing to the resistance of this organism was the overexpression of the RND pumps (Fernando & Kumar, 2012; Amin et al., 2013; Zander et al., 2013). Our analysis of genes encoding RND pumps included the expression of three previously characterized genes, *adeB*, *adeG* and *adeJ*, which encode the RND pumps in the *adeABC*, *adeFGH* and *adeIJK* operons, respectively. Efflux pumps such as AdeABC have been reported to be involved in multidrug resistance (Vila et al., 2007; Hou et al., 2012). In our study, *A. baumannii* Tokai strain 2 (RRR) showed overexpression of *adeB* and *adeJ*. *A. baumannii* Tokai strain 8 showed better sensitivity to some *β*-lactams (CAZ, CFPM and CZOP) than that of *A. baumannii* Tokai strain 9 (SSS). This phenomenon might be associated with underexpression of *adeB*. Two pumps,

| Table 4. Expression of resistance-related genes as assessed by PCR and qRT-PCR in *A. baumannii* Tokai strains |
|---------------------------------------------------------------|
| **Strain(s)** | **Susceptibility pattern** | **Gene expression** | **Mutation** |
|----------------|-----------------------------|----------------------|--------------|
|                |                             | OXA-type *β*-lactamase | ISAb1 | armA | gyrA (Ser83) | parC (Ser80) |             |
|                |                             | OXA-23-like | OXA-24-like | OXA-51-like | OXA-58-like | OXA-66-like | Leu | Ser |
| 1, 5, 7, 10    | SRR                         | –         | –         | +         | –         | +         | +   | Leu |
| 2, 3, 4, 6     | RRR                         | –         | –         | +         | –         | +         | +   | Leu |
| 8              | SSR                         | –         | –         | +         | –         | +         | –   | Leu |
| 9              | SSS                         | –         | –         | +         | –         | +         | –   | Ser |
|                |                             |           |           |           |           |           |      |     |

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such as adeB and adeJ, have been related to the acquisition of multidrug resistance. As for porins, the overexpression of genes encoding RND pumps and the downregulation of genes encoding porins is known to be common in clinical isolates of Acinetobacter spp. (Fernando et al., 2013). Our findings also suggest that the underexpression of carO in combination with or without oprD does not result in resistance to carbapenem in A. baumannii Tokai strains 1 and 8 (SRR and SSR). This observation is consistent with previous findings showing that a decrease in porins among Acinetobacter strains is not associated with resistance to carbapenems in the presence of β-lactamases (Rumbo et al., 2013; Singh et al., 2013).

In conclusion, we demonstrated that drug resistance is associated with the expression of ISAba1 and armA, and mutations in gyrA and parC, and that the overexpression of adeB and adeJ plays a role in the multidrug resistance of A. baumannii Tokai strain ST208.

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**Table 5.** Relative expression of efflux pumps and outer-membrane porins in A. baumannii Tokai strains by qRT-PCR

The results for A. baumannii Tokai strains 1, 2, 8 and 9 are shown as a representative strain from each group with the same susceptibility pattern.

| Strain | Susceptibility pattern | Relative expression | Efflux pump (ratio) | Outer-membrane porin |
|--------|------------------------|---------------------|---------------------|----------------------|
|        |                        | adeB    | adeG    | adeJ    | oprD    | carO    |
| 1      | SRR                    | 0.91    | 0.46    | 0.94    | 1.23    | 0.02    |
| 2      | RRR                    | 2.28    | 1.02    | 2.41    | 0.49    | 0.01    |
| 8      | SSR                    | 0.10    | 0.81    | 0.84    | 0.88    | 0.003   |
| 9      | SSS                    | 1.00    | 1.00    | 1.00    | 1.00    | 1.00    |
Drug resistance of *A. baumannii*

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