Genomic characterization of triple-carbapenemase-producing Acinetobacter baumannii

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Objectives: To characterize Acinetobacter baumannii OCU_Ac16a, a clinical isolate co-harbouring three acquired carbapenemase genes, \( \text{bla}_{\text{NDM-1}} \), \( \text{bla}_{\text{TMB-1}} \), and \( \text{bla}_{\text{OXA-58}} \), and assess the clinical significance of so-called multiple-carbapenemase producers.

Methods: OCU_Ac16a and its close relative, OCU_Ac16b, which lacks the \( \text{bla}_{\text{NDM-1}} \), were isolated from sputum cultures of a patient at Osaka City University Hospital. We subjected these strains to whole-genome analysis, particularly focusing on the genetic context of each carbapenemase gene. The transmissibility and functionality of each carbapenemase gene were analysed by conjugation and transformation experiments and antimicrobial susceptibility tests.

Results: \( \text{bla}_{\text{TMB-1}} \) was located in a class 1 integron on the chromosome, whereas \( \text{bla}_{\text{NDM-1}} \) and \( \text{bla}_{\text{OXA-58}} \) were found on plasmids named pOCU_Ac16a_2 and pOCU_Ac16a_3, respectively. pOCU_Ac16a_2 (which exhibited highly efficient self-transmissibility) and pOCU_Ac16a_3 (which did not show transmissibility but could be introduced into another A. baumannii strain via electroporation) could both confer carbapenem resistance (MICs \( \geq 512 \) and \( \geq 32 \) mg/L, respectively) on the recipient strain. The functionality of \( \text{bla}_{\text{TMB-1}} \) was evident from the high resistance of OCU_Ac16b to ceftazidime and cefepime (MICs \( \geq 256 \) and \( 48 \) mg/L, respectively), and the high resistance of OCU_Ac16a to cefiderocol (MIC 32 mg/L) could be explained by the additive effect of \( \text{bla}_{\text{NDM-1}} \) and \( \text{bla}_{\text{TMB-1}} \).

Conclusions: Our data revealed the genomic organization of OCU_Ac16a and demonstrated that all the carbapenemase genes are functional, each contributing to the extremely high broad-spectrum resistance of OCU_Ac16a to \( \beta \)-lactams. As multiple-carbapenemase producers can be serious health threats as drug-resistant pathogens and disseminators of carbapenemase genes, close attention should be paid to their emergence.

Introduction

The increase in antimicrobial-resistant (AMR) bacteria is posing a serious threat to human health worldwide. One such AMR bacterial species is Acinetobacter baumannii, which has acquired clinically relevant AMR genes, such as carbapenemase genes, owing to the horizontal gene transfer of mobile genetic elements, such as plasmids. The carbapenem antimicrobials are used as a last resort against Gram-negative bacterial infections. However, they have become less effective due to the global spread of carbapenemase genes. In Acinetobacter spp., the most common group of carbapenemases is Ambler’s class D, which consists of enzymes referred to as oxacillinases (OXAs). In addition, Ambler’s class B, consisting of metallo-\( \beta \)-lactamases [e.g. New Delhi metallo-\( \beta \)-lactamase (NDM) and Tripoli metallo-\( \beta \)-lactamase (TMB)], is also prevalent. Notably, over the past decade, a significant number of studies have reported the emergence of bacterial strains that simultaneously possess two different carbapenemase genes. While the
emergence of triple-carbapenemase producers has also been reported.\(^6\)\(^7\) No studies have attempted to perform a thorough characterization including complete genome sequencing and assessment of the functionality of each carbapenemase gene. Multiple-carbapenemase producers pose a more serious health risk than others given that they are more difficult to combat and can act as a reservoir of carbapenemase genes for other pathogens. Therefore, evaluating the consequences of their prevalence and addressing the mechanism of how and why they emerge in clinical settings is necessary.

We recently identified a carbapenem-resistant \(A.\) \(baumannii\) strain, \(OCU\_\)\(Ac16a\) that was isolated from the intratracheal aspirate of a patient with oesophageal cancer at Osaka City University Hospital in Japan in 2015.\(^8\) The analysis of this strain via draft genome sequencing and subsequent multi locus sequence typing (MLST) revealed that this strain belonged to sequence type 412 (ST412) and co-harboured three acquired carbapenemase genes, namely, \(\text{bla}\_\text{NDM-1}\), \(\text{bla}\_\text{TMB-1}\), and \(\text{bla}\_\text{OXA-58}\), in addition to the intrinsic \(\text{bla}\_\text{OXA-51}\)-like and \(\text{bla}\_\text{OADC-25}\)-like \(\beta\)-lactamase genes. It should be noted that two of these genes encode metallo-\(\beta\)-lactamases (NDM-1 and TMB-1). Post isolation of \(OCU\_\)\(Ac16a\), a possible variant of \(OCU\_\)\(Ac16b\) (named \(OCU\_\)\(Ac16b\)), co-harbouiring \(\text{bla}\_\text{TMB-1}\) and \(\text{bla}\_\text{OXA-58}\) but not \(\text{bla}\_\text{NDM-1}\), was isolated from the same patient. In this study, we aimed to elucidate their genomic organization and also assess the impact of each carbapenemase gene on carbapenem resistance in order to evaluate the clinical significance of the emerging strains called multiple-carbapenemase producers.

### Materials and methods

#### Ethics

The study conformed to the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Review Board (approval no. 3568, 9/30/2016). Informed consent was waived according to the ethical guidelines for human research in Japan.

#### Clinical setting and isolation of bacterial strains

The \(A.\) \(baumannii\) strains \(OCU\_\)\(Ac16a\) and \(OCU\_\)\(Ac16b\) were isolated from a patient with type 3 oesophageal cancer in the middle thoracic oesophagus. The patient underwent transthoracic oesophagectomy followed by gastric tube reconstruction. \(OCU\_\)\(Ac16a\) was isolated from suctioned sputum culture on postoperative day (POD) 32, whereas \(OCU\_\)\(Ac16b\) was isolated from sputum obtained by bronchoscopy on POD 35 (for more detail on the methods see Supplementary data, available at JAC Online).

#### Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were conducted in accordance with the criteria specified by the Clinical and Laboratory Standards Institute\(^7\) using the broth microdilution method or Etest (bioMérieux Inc., Marcy-l’Etoile, France). Cation-adjusted Mueller Hinton (CAMH) broth or agar plates were used for all the tests except broth microdilution tests with cefiderocol, which were performed using iron-depleted CAMH broth. \(OCU\_\)\(Ac16b\) susceptibility to cefiderocol could not be determined because of a growth defect that occurred in the iron-depleted CAMH broth. Therefore, we additionally performed a disc diffusion assay with MASTDISCS AST Cefiderocol 30 μg (Mast Group Ltd., Merseyside, UK) using non-iron-depleted CAMH agar plates.

### Genome sequencing and analyses

Whole-genome sequencing of \(OCU\_\)\(Ac16a\) and \(OCU\_\)\(Ac16b\) were performed using the MiSeq system (Illumina, San Diego, CA) as previously described.\(^8\) \(OCU\_\)\(Ac16a\) was further sequenced using the PacBio RS II system (Pacific Biosciences, Menlo Park, CA) so as to construct the complete genome sequence, including the plasmid sequences (for method details, see Supplementary data). The sequences of complete chromosomal DNA, \(pOCU\_\)\(Ac16a\)\(_1\), \(pOCU\_\)\(Ac16a\)\(_2\), \(pOCU\_\)\(Ac16a\)\(_3\), and \(pOCU\_\)\(Ac16a\)\(_4\) have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AP023077–AP023080. Whole-genome shotgun assembly of the \(OCU\_\)\(Ac16b\) genome has been deposited under accession numbers BLWH01000001–BLWH01000743.

**MLST** was performed using the Institut Pasteur MLST scheme (http://pubmlst.org/abaumannii/). Antimicrobial resistance genes were detected using ResFinder v3.2 (http://cge.cbs.dtu.dk/services/ResFinder/). Genetic elements related to plasmid mobility were detected using the web-based tool, oriFinder.\(^9\) The classification of relaxases and mating pair formation systems was realized based on a scheme that was previously described by Smillie et al.\(^10\) Plasmid replicon typing was performed based on the rep gene sequence.\(^11\)

#### Conjugation experiments

Conjugation experiments were performed using \(OCU\_\)\(Ac16a\) as the donor and spontaneous rifampicin-resistant mutants (RFP50R) of \(A.\) \(baumannii\) ATCC 19606\(^7\), \(Acinetobacter ursingii\) \(OCU\_\)\(Ac4\), \(Acinetobacter soli\) \(OCU\_\)\(Ac8\) and \(OCU\_\)\(Ac9\), and \(Acinetobacter pittii\) \(OCU\_\)\(Ac12\) \(\text{as recipients (refer to Supplementary data for details). Transconjugants were selected on CAMH plates containing rifampicin and/or meropenem (50 mg/L each). The conjugal transfer frequency was defined as the ratio of the number of transconjugant cells that grew on plates containing both rifampicin and meropenem to the total number of recipient cells that grew on rifampicin-containing plates.**

#### Results and discussion

The complete genome of \(OCU\_\)\(Ac16a\) consisted of a chromosome and three plasmids, named \(pOCU\_\)\(Ac16a\)\(_1\), \(pOCU\_\)\(Ac16a\)\(_2\), and \(pOCU\_\)\(Ac16a\)\(_3\), with characteristics as summarized in Table 1. It was shown that \(pOCU\_\)\(Ac16a\)\(_1\) and \(pOCU\_\)\(Ac16a\)\(_3\) belonged to homology groups 6 and 4, respectively. However, \(pOCU\_\)\(Ac16a\)\(_2\) was untypeable owing to the lack of an apparent rep-like gene. \(\text{bla}\_\text{NDM-1}\) and \(\text{bla}\_\text{OXA-58}\) were found to be located on \(pOCU\_\)\(Ac16a\)\(_2\) and \(pOCU\_\)\(Ac16a\)\(_3\), respectively, whereas \(\text{bla}\_\text{TMB-1}\) was found to be located on the chromosome (Table 1). All the remaining AMR genes were found to be located on the chromosome, except for \(\text{aph(3’)-VIa}\), which was located on \(pOCU\_\)\(Ac16a\)\(_2\). The \(OCU\_\)\(Ac16b\) draft genome consisted of 743 contigs, and MLST analysis revealed that this isolate belonged to the same sequence type (ST412) as \(OCU\_\)\(Ac16a\), indicating their clonality. Additionally, a comparative analysis involving the genome sequences showed that \(OCU\_\)\(Ac16a\) was nearly identical to \(OCU\_\)\(Ac16a\), except that it lacked the whole \(pOCU\_\)\(Ac16a\)\(_2\) plasmid.

\(OCU\_\)\(Ac16a\) was highly resistant to all the \(\beta\)-lactam antimicrobials tested, including cefiderocol, a novel siderophore cephalosporin (MICs \(\geq\) 256 mg/L for pipercillin, ceftazidime, and ceftimepime, \(\geq\) 512 mg/L for imipenem and meropenem, and 32 mg/L for cefiderocol; 6 mm zone of inhibition with cefiderocol discs). However, it was susceptible to gentamicin, amikacin, levofloxacin, colistin, minocycline, and tigecycline (MICs 1, 8, 1, 0.094, 0.031, and 0.25 mg/L, respectively) (Table 2). The antimicrobial
susceptibility pattern of OCU_Ac16b was very similar to that of OCU_Ac16a, although OCU_Ac16b was more susceptible to cefepime, cefiderocol, and amikacin (MICs 48 and 3 mg/L for cefepime and amikacin, respectively; 21 mm zone of inhibition with cefiderocol discs) (Table 2).

Subsequently, we investigated the genetic contexts of the three acquired carbapenemase genes (Figure S1). blaTMB-1 was found to be located in a class 1 integron and was similar to its counterpart in A. baumannii strain A1, which was the first Acinetobacter strain clinically isolated in Japan in 2009 reported to possess blaTMB-1.14 blaNDM-1 was found to be located in a cluster with high overall identity with species previously reported to carry this gene (Figure S2).15-17 The complete conservation of the promoter sequence, which is partly contributed from the right end of ISaba125 located upstream of the blaNDM-1 structural gene, was confirmed. The cluster containing blaOXA-58 was similar to the plasmid, pTVICU14, from the Acinetobacter nosocomialis strain TVICU14 that was clinically isolated in Taiwan in 2004,18 whereas pOCU_Ac16a_3 showed no overall identity with any plasmid in the NCBI database.

A promotor-like sequence that is composed of the right end of IS1008 and a part of 5’ISaba3-like sequence, highly similar to that in pTVICU14, was observed in the upstream region of blaOXA-58 in OCU_Ac16a (Figure S1).

pOCU_Ac16a_1 and pOCU_Ac16a_2 were possibly self-transmissible given that they contained a set of mobilization elements. Our conjugation experiments further demonstrated the transmissibility of pOCU_Ac16a_2 (Table 1). pOCU_Ac16a_2 caused a significant increase in resistance to β-lactams, including carbapenems, and amikacin in A. baumannii ATCC 19606r RFP50R (MICs ≥256 mg/L for piperacillin, ceftazidime, and cefepime, ≥512 mg/L for imipenem and meropenem, and 192 mg/L for amikacin) (Table 2). Notably, however, this transconjugant was susceptible to cefiderocol (MIC 0.5 mg/L; 20 mm zone of inhibition with cefiderocol discs), an observation that is consistent with previous studies that reported the efficacy of cefiderocol against NDM-1-producing bacteria.19

Although we did not observe the transfer of pOCU_Ac16a_3 or blaOXA-58 in our conjugation experiments, we successfully transformed the plasmid into A. baumannii ATCC 19606r RFP50R via electroporation. The transformant showed significant resistance to piperacillin, imipenem, and meropenem (MICs ≥256, 64, and 32 mg/L, respectively) (Table 2). These data clearly demonstrate that blaNDM-1 and blaOXA-58 in pOCU_Ac16a_2 and pOCU_Ac16a_3 are functional. Additionally, we reasoned that blaTMB-1 is functional because OCU_Ac16b shows significant resistance to ceftazidime and cefepime, which are β-lactams to which blaOXA-58 does not usually confer resistance.20 Of note, no insertion sequence was detected upstream of the blaADC-25-like gene, which rules out the theory that overexpression of this intrinsic cephalosporinase caused the high resistance to ceftazidime and cefepime in OCU_Ac16b.

We found it particularly interesting that OCU_Ac16a was resistant to cefiderocol despite the fact that oxacillinases and metallo-β-lactamases do not generally confer resistance to this drug.19 We assume this was caused by an additive effect of NDM-1 and TMB-1, both of which have a certain level of ability to increase resistance of A. baumannii to cefiderocol. This is a good example that explains how challenging it is to combat multiple-carbapenemase producers. Although several promising β-lactamase inhibitors with efficacy against carbapenemases have been or are being developed, including avibactam and vaborbactam, they are less promising as a weapon against bacteria such as OCU_Ac16a because there are only a few candidate compounds that can inhibit metallo-β-lactamases and no one compound can universally inhibit multiple classes of β-lactamases.21 To prevent future public health crises, the appropriate use of antimicrobials is important and further

| Replicon      | Size (bp) | Antimicrobial resistance genes | rep gene group of plasmids | Elements related to plasmid mobility | Frequencies of plasmid transfer<sup>a</sup> |
|---------------|----------|--------------------------------|---------------------------|--------------------------------------|-------------------------------------------|
| Chromosome    | 3 992 063| bla<sub>TMB-1</sub>, bla<sub>ADC-25</sub>-like, bla<sub>OXA-51</sub>-like, strA, strB, mph(ɛ), msr(ɛ), sul1, sul2, gyrA (S81L) | –                         | –                                    | –                                         |
| pOCU_Ac16a_1  | 73 028   | ND                             | GR6                       | MOB<sub>F</sub> family relaxase, TrbA-like T4CP, MPF<sub>F</sub> | 2.5 × 10<sup>−5</sup>–1.5 × 10<sup>−2</sup> (A. baumannii ATCC 19606<sup>T</sup> RFP50R)<sup>b</sup>; 2.2 × 10<sup>−2</sup>–5.4 × 10<sup>−1</sup> (Acinetobacter strains OCU_Ac4, 8, 9, and 12 RFP50R) |
| pOCU_Ac16a_2  | 41 087   | bla<sub>NDM-1</sub>, aph(3′)-VIIa | ND                       | oriT, MOB<sub>B</sub> family relaxase, TrwB-like T4CP, MPF<sub>T</sub> | ND (A. baumannii ATCC 19606<sup>T</sup> RFP50R) |
| pOCU_Ac16a_3  | 13 096   | bla<sub>OXA-58</sub>           | GR4                       | ND                                   | ND (A. baumannii ATCC 19606<sup>T</sup> RFP50R) |

ND, not detected; –, not applicable or not tested; GR, homology group; MOB, mobilization; T4CP, type IV coupling protein; MPF, mating pair formation; oriT, origin of transfer.

<sup>a</sup>The recipient strains used are indicated in parentheses.

<sup>b</sup>Results from four independent experiments.
studies on the mechanism behind multiple occurrences of AMR genes are necessary.

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Transparency declarations

None to declare.

Author contributions

K.O., M.S., and Y.K. designed the study. M.S. conducted the genome assembly; K.O., M.S., and T.T. analysed and interpreted the obtained data. K.O., A.S., K. Saeki, and Y.K. performed conjugation and transformation experiments and antimicrobial susceptibility tests. K. Sato, K.Y., and H.K. conducted the clinical analysis and interpretation. K.O., M.S., and Y.K. drafted the manuscript, and all the other authors critically revised it. All authors contributed to the final version of the manuscript and approved its submission. The authors declare that there is no conflict of interest.

Supplementary data

Figures S1 and S2 and additional Methods details are available as Supplementary data at JAC Online.

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Table 2. Antimicrobial susceptibility of strains used in this study

| Strain                  | Antimicrobials | MICs (mg/L) | Diameters of the zone of inhibition (mm) |
|-------------------------|----------------|-------------|------------------------------------------|
| OCU_Ac16a/C21           | FDC            | ≥512 (R)    | 192 (R)                                  |
| OCU_Ac16b/C21           | FDC            | ≥512 (R)    | 192 (R)                                  |
| ATCC 19606T RFP50R      | FDC            | ≥512 (R)    | 192 (R)                                  |
| ATCC 19606T RFP50R with pOCU_Ac16a_2 | FDC            | ≥512 (R)    | 192 (R)                                  |
| ATCC 19606T RFP50R with pOCU_Ac16a_3 | FDC            | ≥512 (R)    | 192 (R)                                  |

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