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Identification and characterisation of colistin-resistant *Acinetobacter colistiniresistens* co-producing IMP-1 and OXA-58 carbapenemases

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ABSTRACT

Background: Carbapenem-resistant *Acinetobacter* is of increasing global concern because infections are challenging to treat with standard antibiotics. Here, we identified a previously uncharacterised *Acinetobacter* sp. clinical isolate as *Acinetobacter colistiniresistens* co-producing IMP-1 and OXA-58. We also examined expression of genes related to antibiotic susceptibility and drug resistance, including bla_{IMP}.
Methods: The isolate was deposited at the National Institute of Technology and Evaluation (NITE) as *Acinetobacter* sp. NBRC 110496. Susceptibility was defined according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints. Genomic and clonal analyses were performed to identify species and resistance genes.
Results: The isolate was resistant to β-lactams, including broad-spectrum cephalosporins and carbapenems, polymyxins, and trimethoprim/sulfamethoxazole. Genomic analysis identified the isolate as *A. colistiniresistens* harbouring bla_{IMP-1}, bla_{OXA-58}, $bla_{OXA-670}$, *aac(6′)-Ib*, *aac(6′)-Ij*, *ant(3″)-II*, *aph(3′)-VI*, *msrE*, *mphE*, and *sul1*. Colistin resistance was associated with the *eptA*-like gene, which encodes a lipid A-modifying enzyme. SNP-based phylogenetic analysis revealed that the strain clustered with other strains isolated in Japan. The IMP-1/OXA-58-producing strain described in this study has a novel integron structure surrounding bla_{IMP-1}, *aacA* and *sul1*.
Conclusions: Colistin-resistant IMP-1/OXA-58-co-producing *A. colistiniresistens* was identified in a patient. This isolate could serve as a reservoir for carbapenemase-producing organisms. This study suggests that screening for colistin-resistant isolates is crucial to preserve colistin as a therapeutic agent for multidrug-resistant bacteria. Identification of this MDR isolate in Asia, and the danger of it spreading worldwide, should raise serious concerns.

1. Introduction

Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. comprise ESKAPE pathogens that frequently acquire multidrug resistance [1]. Multidrug-resistant (MDR) organisms, including carbapenemase- and/or extended-spectrum β-lactamase (ESBL)-producing organisms, are highly prevalent worldwide [2,3]. Carbapenem-resistant *Acinetobacter* is an increasing global concern [4–6]; indeed, infections are a severe clinical issue because they are challenging to treat using standard antibiotics. The Centers for Disease Control and Prevention has ranked carbapenem-resistant *Acinetobacter* as an urgent threat, and The World Health Organization has ranked carbapenem-resistant *Acinetobacter baumannii* (CRAB) as a critical pathogen for the research and development of new antibiotics [7]. Although CRAB are highly prevalent in low- and middle-income countries, as well as in high-income countries [8], carbapenem-resistant *Acinetobacter* clinical isolates account for less than 1.5% of infections identified by the Japan Nosocomial Infection Surveillance (JANIS); however, sporadic MDR *Acinetobacter* (MDRA) infections have been reported [9,10]. Carbapenem resistance is usually mediated by imipenemase (IMP)-type metallo-β-lactamase (MBL), overproduction of intrinsic oxacillinase (OXA)-type carbapenemase, or overexpression of AmpC cephalosporinases along with porin mutations. Three large carbapenemases, *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), and oxacillinase-48 (OXA-48), are increasingly common, but detection remains sporadic [11]. By contrast, IMP-type MBLs are endemic in Japan. Carbapenem-resistant *Acinetobacter* strains, which are limited to East Asia, including Southeast Asia [12], are often MDR, and so pose severe problems with respect to clinical treatment and infection

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Table 1
Acinetobacter colistiniresistens clinical isolates.

Strain	Year	Location	Source	Genbank Accession Number
CIP 64.2	NA	Germany	Conjunctivitis	APRT00000000.1
NIPH 1859	1980/1981	Sweden: Malmo	Conjunctiva	APRZ00000000.1
NIPH 2036	1990	Belgium	Catheter	ATGK00000000.1
NBRC 110496	2009	Japan: Osaka	Blood	BBTF00000000.1
TUM15188	2012	Japan: Kanagawa	Sputum	BKKF00000000.1
TUM15205	2012	Japan: Kanagawa	Sputum	BKKW00000000.1
TUM15240	2013	Japan: Kanagawa	Sputum	BKMF00000000.1
TUM15258	2014	Japan: Kanagawa	Sputum	BKMX00000000.1
TUM15271	2011	Japan: Tokyo	Sputum	BKNK00000000.1
OCU_Ac7	2014	Japan: Osaka	Blood	BHGD00000000.2
NR1165	2015	Japan: Sendai	Sputum	BGNT00000000.1
C-214	2018	Malaysia	Faecal	CP102099.1

control. *Acinetobacter colistiniresistens* has been classified as belonging to the genome species 13BJ/14TU due to intrinsic colistin resistance [13]. Here, we describe the identification and characterisation of a clinical isolate of colistin-resistant *Acinetobacter colistiniresistens* co-producing IMP-1 and OXA-58, which is a previously uncharacterised *Acinetobacter* sp.

2. Materials and methods

2.1. Culture and isolation of the bacterial strains

Acinetobacter sp. NBRC 110496 was isolated initially from human blood at the Clinical Laboratory, Kindai University Hospital in Osaka, Japan, in 2009. The strain was deposited at the National Institute of Technology and Evaluation (NITE) in 2014 and obtained in our laboratory. This study was exempted by the Ethical Review Board for Medical and Health Research Involving Human Subjects. Carbapenem-resistant strains were cultured on CROMagar mSuper CARBA (Kanto Chemical Co., Tokyo, Japan) [14].

2.2. Antimicrobial susceptibility testing

Susceptibility was defined according to breakpoints listed in the Clinical and Laboratory Standards Institute (CLSI) guidelines [15,16]. Breakpoints undefined for *Acinetobacter* sp. were applied to other Gram-negative species. Testing was performed using a Dry plate and Mullar Hinton broth (Kanto Chemical, Tokyo, Japan). The minimum inhibitory concentrations were confirmed using the broth-microdilution method [10].

Azithromycin, cefotaxime and ceftriaxone were purchased from Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan). Doripenem was purchased from Shionogi & Co., Ltd. (Osaka, Japan). Ertapenem, colistin, polymyxin B, nalidixic acid, and tetracycline were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Ticarcillin/clavulanic acid was purchased from Carbosynth (Berkshire, UK). Tobramycin was purchased from J-Dorph Pharmaceutical Co., Ltd. (Shiga, Japan). Chloramphenicol, erythromycin and tigecycline were purchased from Wako Chemicals (Osaka, Japan). Netilmicin was purchased from Namiki Shoji Co., Ltd., Tokyo, Japan. Cefepime was purchased from Bristol-Myers Squibb (New York, NY, USA). Cefozopran and doxycycline were generously provided by Takeda Pharmaceutical (Osaka, Japan) and Pfizer (New York, NY, USA), respectively. Ciprofloxacin and meropenem were generously provided by Sumitomo Dainippon Pharma (Osaka, Japan).

2.3. Detection and identification of resistance genes

Multiplex PCR to detect carbapenemase and ESBL genes was performed using a Cica Geneus genotype detection kit (Kanto Chemical, Tokyo, Japan). DNA fragments from cultured bacterial colonies were amplified by PCR using KOD FX Neo (Toyobo, Tokyo, Japan) and

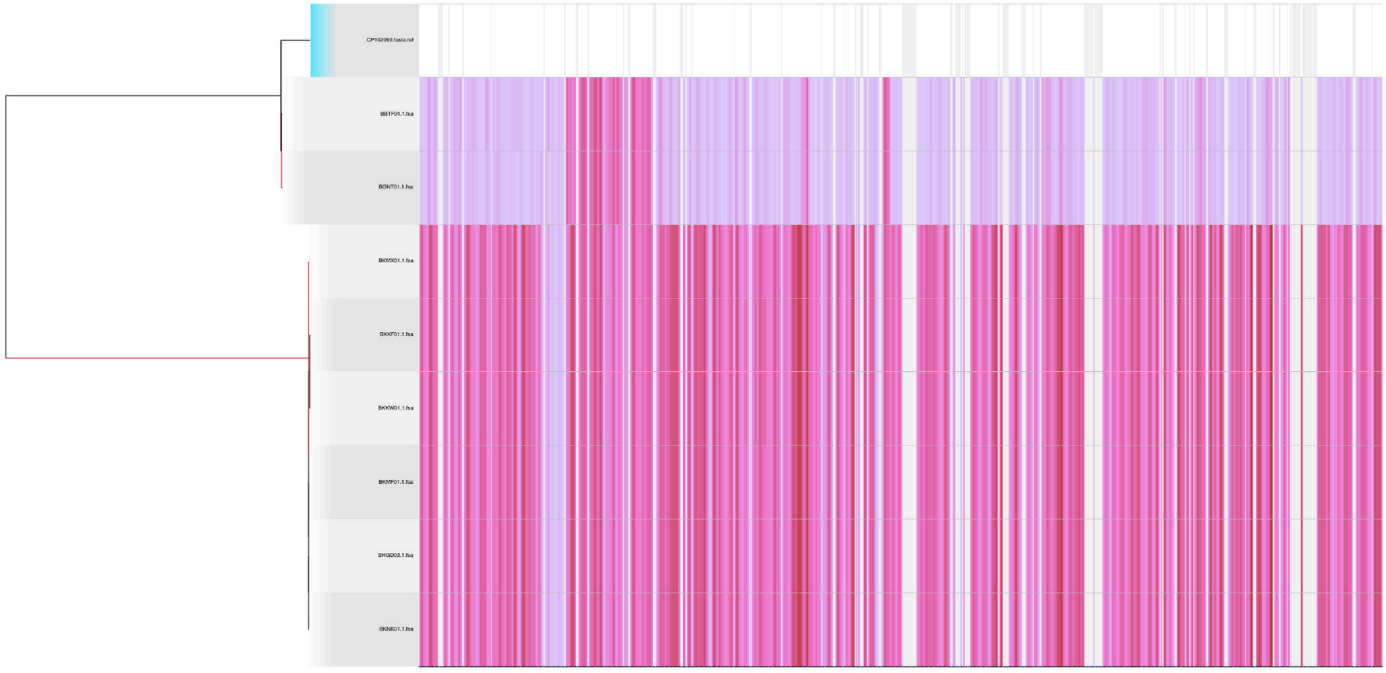

Fig. 1. Phylogenetic analysis of *Acinetobacter colistiniresistens* genomes. Phylogenetic tree of *A. colistiniresistens* chromosome sequences. Pink lines indicate single-nucleotide polymorphisms (SNPs) identified using the Harvest suite. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Susceptibility profiles of *Acinetobacter colistiniresistens* clinical isolates.

Antimicrobial agent	NBRC 110496		NR1165[a]	
	MIC (mg/L)	Genes	MIC (mg//L)	Genes
Penicillins				
Ampicillin	**> 32**	bla_{IMP-1}, bla_{OXA-58}	**32**	bla_{IMP-34}, bla_{OXA-58}
Piperacillin	**32**	bla_{IMP-1}		
Cephalosporins				
Cefotaxime	**> 32**	bla_{IMP-1}	**32**	bla_{IMP-34}
Ceftazidime	**32**	bla_{IMP-1}	**64**	bla_{IMP-34}
Ceftriaxone	**64**	bla_{IMP-1}		
Cefoxitin			**> 256**	bla_{IMP-34}
Cefpirome	**16**	bla_{IMP-1}		
Cefepime	**64**	bla_{IMP-1}	**16**	bla_{IMP-34}
Cefozopran	**64**	bla_{IMP-1}		
Cefmetazole	**> 32**	bla_{IMP-1}		
Cefpodoxime	**> 32**	bla_{IMP-1}		
Flomoxef	**> 32**	bla_{IMP-1}		
Latamoxef	**> 32**	bla_{IMP-1}		
Faropenem	**> 2**	bla_{IMP-1}		
Carbapenems				
Doripenem	**16**	bla_{IMP-1}		
Ertapenem	**64**	bla_{IMP-1}	**128**	bla_{IMP-34}
Imipenem	**8**	bla_{IMP-1}	**8**	bla_{IMP-34}
Meropenem	**> 16**	bla_{IMP-1}	**32**	bla_{IMP-34}
Monobactams				
Aztreonam	8		8	
BL/BLI				
Ticarcillin/clavulanic acid	**128/2**	bla_{IMP-1}, bla_{OXA-58}		
Sulbactam/ampicillin	≤2/4			
Sulbactam/ cephoperazon	≤8/8			
Tazobactam/ piperacillin	≤4/16			
Ceftazidime/dipicolic acid	4	bla_{OXA-58}		
Imipenem/dipicolic acid	≤0.5	bla_{OXA-58}		
Meropenem/dipicolic acid	≤0.5	bla_{OXA-58}		
Quinolones				
Nalidixic acid	2	*adeF*		
Ciprofloxacin	≤0.125	*adeF*		
Levofloxacin	≤0.125	*adeF*	**8**	
Aminoglycosides				
Gentamicin	1	*aph(3'')-VIa*	1	*aph(3'')-VIa*
Amikacin	4	*aac(6′)-Ib9, aac (6′)-Ijant(3″)-IIc, aph(3′)-VIa*	**128**	*aac(6′)-Ib, aac (6′)-Ij*
Netilmicin	1	*aac(6′)-Ij*		
Tobramycin	1	*aac(6′)-Ib9, aac (6′)-Ij*		
Streptomycin		*ant(3'')-IIc*		
Tetracyclines				
Tetracycline	2	*adeF*		
Doxycycline	≤0.25			
Minocycline	≤0.125			
Tigecycline	8		4	
Polymyxins				
Colistin	**16**	*eptA*-like	**16**	
Polymyxin B	**4**	*eptA*-like		
Macrolide				
Erythromycin	**> 256**	*mphE, msrE*		
Azithromycin	**128**	*msrE*		
Other				
Chloramphenicol	**8**			
Fosfomycin	**64**			
Sulfamethoxazole/ Trimethoprim	**19/1**	*sul1*		

MIC, minimum inhibitory concentration; BL/BLI, β-lactam and β-lactamase inhibitor; a, data based on reference [35]. Values in bold denote resistant or non-susceptible.

primers specific for resistance genes [17–21]. DNA was subjected to direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (ThermoFisher Applied Biosystems, Foster City, CA, USA). Carbapenemase and ESBL production were confirmed using lateral flow immunoassays (LFIA) (NG-test CARBA 5 (NG biotech, Guipry, France) and Quick chaser IMP/AAC (Mizuho Medy, Saga, Japan)) [22–25].

2.4. Genomic analysis

Whole genome shotgun sequencing was performed on the NITE Biological Resource Center using Illumina HiSeq 1000 platform; the draft genome had 89 × coverage and was assembled using Newbler v. 2.6 (BioProject number PRJDB1754). The genomic sequences of the analysed strains were obtained from the GenBank database, and the accession numbers are listed in Table 1. Sequence similarity was analysed using BLASTN version 2.8.1+ [26]. Antimicrobial resistance genes were detected using the Comprehensive Antibiotic Resistance Database (CARD) [27]. OrthoANI was used for average nucleotide identity (ANI) analysis [28,29], and Harvest was used for SNP-based phylogenomic analysis [30]. Easyfig was used for synteny analysis [31], and Gview was used to compare megaplasmids [32].

3. Results

3.1. Identification of Acinetobacter sp. NBRC 110496

OrthoANI revealed that the draft genome sequence of *Acinetobacter* sp. NBRC 110496 showed an ANI of 98.50% with the reference genome (GenBank accession number APRT00000000.1) of *A. colistiniresistens* type strain CIP 64.2, and 98.43% with the complete reference genome (GenBank accession number CP102099) of *A. colistiniresistens* strain C-214 isolated from a human faecal sample in Malaysia [33]. By contrast, the NBRC 110496 genome showed 99.78% ANI with the reference genome (GenBank accession number BGNT00000000.1) of IMP-34/OXA58-producing *A. colistiniresistens* strain NR1165. Next, we performed an SNP-based phylogenetic analysis of strains isolated in Japan and compared the results with the complete reference genome (GenBank accession number CP102099) of *A. colistiniresistens* strain C-214 (Fig. 1). SNP-based phylogenomic analysis using Harvest resulted in a phylogenetic tree that revealed two clusters: one containing isolates of IMP-producing *A. colistiniresistens* strains and one containing IMP-non-producing *A. colistiniresistens* strains. These data suggest that both clusters were dependent on IMP production, but were independent of geographic location and source of isolation.

3.2. Antimicrobial susceptibility and resistance genes

The antimicrobial susceptibility tests revealed resistance to ampicillin, piperacillin, ceftazidime, cefepime, doripenem, imipenem, meropenem, colistin, polymyxin B, chloramphenicol, fosfomycin and sulfamethoxazole/trimethoprim; and sensitivity to aminoglycosides, amikacin, gentamicin, aztreonam, quinolones, nalidixic acid, ciprofloxacin, levofloxacin, sulbactam/ampicillin and tetracyclines (Table 2). These data suggest that *A. colistiniresistens* co-producing IMP-1, OXA-58, and OXA-670 are sensitive to sulbactam and tazobactam, both of which are β-lactamase inhibitors. Since resistance to ceftazidime, imipenem and meropenem was reduced by the MBL inhibitor dipicolic acid (DPA), it seems that IMP-1 mainly contributes to resistance to cephalosporins and carbapenems. Draft genome analysis using CARD identified multiple carbapenemase genes (bla_{IMP-1}, bla_{OXA-58} and $bla_{OXA-670}$), aminoglycoside resistance genes (*aac(6′)-Ib9*, *aac(6′)-Ij*, *ant(3'')-IIc* and *aph (3′)-VIa*), macrolide resistance genes (*msrE* and *mphE)*, the sulphonamide resistance gene *sul1*, and the disinfectant resistance genes *adeF*, *amvA* and *qacG*. Colistin resistance was associated with the *eptA* homologue gene, which encodes phosphoethanolamine transferase-

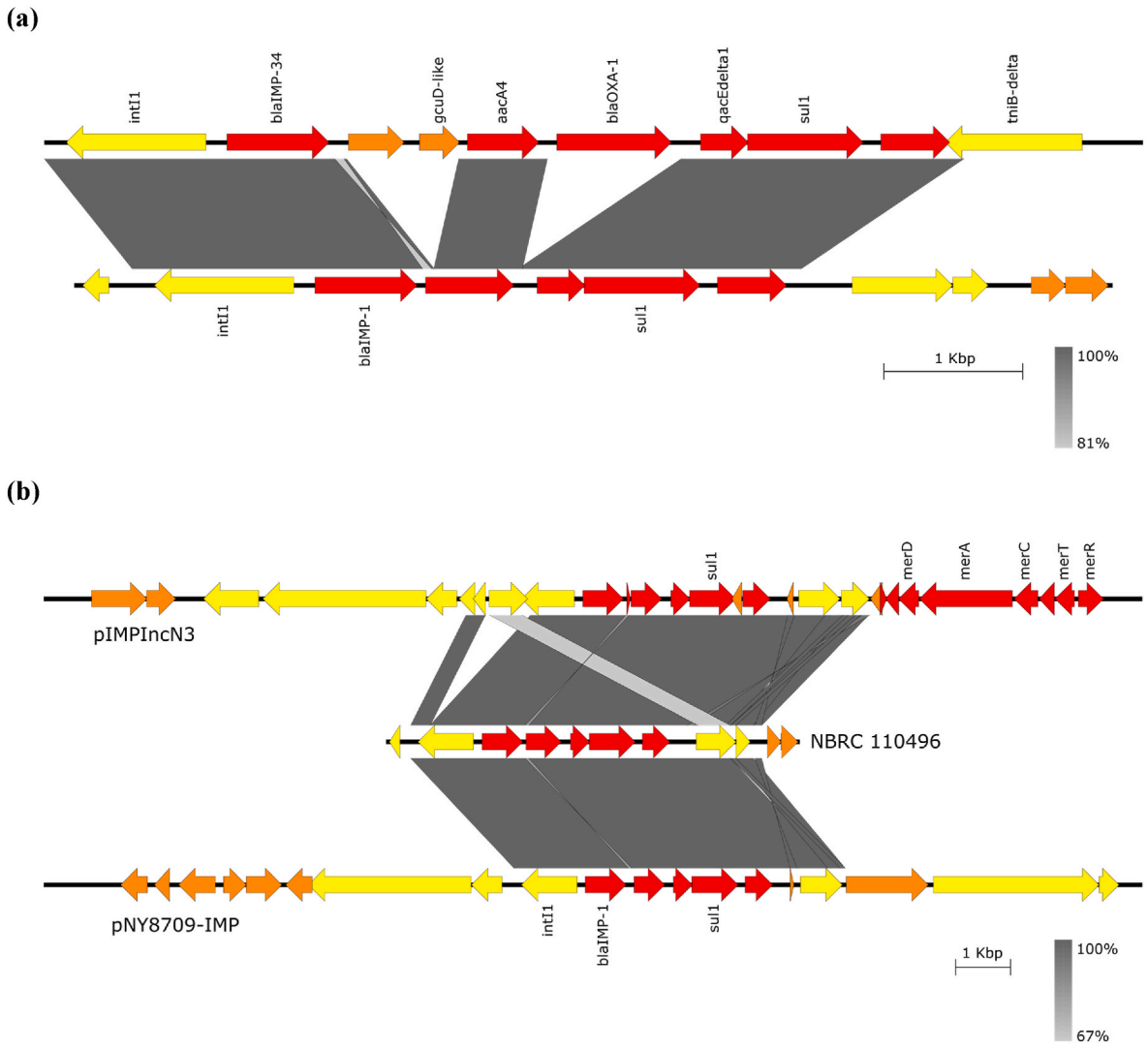

Fig. 2. Comparative analysis of the integron structures of *Acinetobacter colistiniresistens*. (a) The integron structures of strains NR1165 (top, Japan) and NBRC 110496 (bottom, Japan) were compared using Easyfig. (b) Comparative analysis of the integron structures of NBRC 110496 strain (middle, Japan), plasmid pIMPIncN3 (top, UK) and plasmid pNY8709-IMP (bottom, USA) was conducted using Easyfig.
Arrows indicate open reading frames. The red and yellow arrows denote antimicrobial resistance genes and genes encoding mobile genetic elements, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

modifying lipid A (Supplemental Fig. 1) [34]. The *mcr* genes in the NBRC 110496 genome sequence were not detected by either the CARD Resistance Gene Identifier or multiplex PCR [20,21]. The $bla_{\text{OXA-670}}$ and *eptA*-like genes in IMP-34/OXA58-producing *A. colistiniresistens* strain NR1165 were identified by CARD; neither of these genes have been reported previously [35]. The $bla_{\text{OXA-670}}$ gene was only detected in ten *Acinetobacter* spp., including *A. colistiniresistens*, by BLAST analysis (GenBank accession numbers NG_062219, NG_050611, BK010548, NG_049598, CP102099, CP098479, CP098478, NG_049588, NG_049602, NG_050610), suggesting the presence of the intrinsic β-lactamase of *A. colistiniresistens*. Discrepancies between drug susceptibility data and expression of resistance genes suggest that the latter are either non-functional or show insufficient expression. The LFIA NG-test CARBA 5 confirmed IMP production, with that of other major carbapenemases (KPC, OXA-48, NDM, VIM) being negative. Another LFIA, the Quick chaser IMP/AAC, also confirmed IMP production, but not that of AAC(6′)-Iae and AAC(6′)-Ib, again suggesting either non-functional genes or insufficient expression.

3.3. Integron structure harbouring the IMP carbapenemase gene

$bla_{\text{IMP-1}}$ was located in a novel class 1 integron structure following *aac(6′)-Ij*, *qacEΔ1* and *sul1*. This integron structure differs from the class 1 integron in NR1165, which produces IMP-1 and OXA-58 (In1413, GenBank accession number LC276939). In particular, the *fosX/fosE/fosI* family gene, *gcuD*-like gene, and the $bla_{\text{OXA-1}}$ gene were absent from the integron of NBRC 110496 (Fig. 2a). By contrast, the integron structure of NBRC 110496 was similar to that of the plasmids pIMPIncN3 (GenBank accession number CP043856) from *Enterobacter hormaechei* EB_P6_L3_02.19, and pNY8709-IMP (GenBank accession number CP096823) from *Pseudomonas aeruginosa* NY8709 (Fig. 2b). These data suggest that integrons are mobilised in multiple Gram-negative species.

Next, we performed a comparative genomic analysis to identify a mobile genetic element in NBRC 110496. BLAST analysis of the draft genome mapped sequences onto a 323 kb megaplasmid, p2014N21-145-1, from *Acinetobacter pitti* 2014N21-145 [36]. Gview was then used to map the draft genomes onto the reference plasmid (GenBank accession number CP033569) (Fig. 3). Interestingly, the draft genome of *A. colistiniresistens* NR1165 was also mapped to the plasmid. The

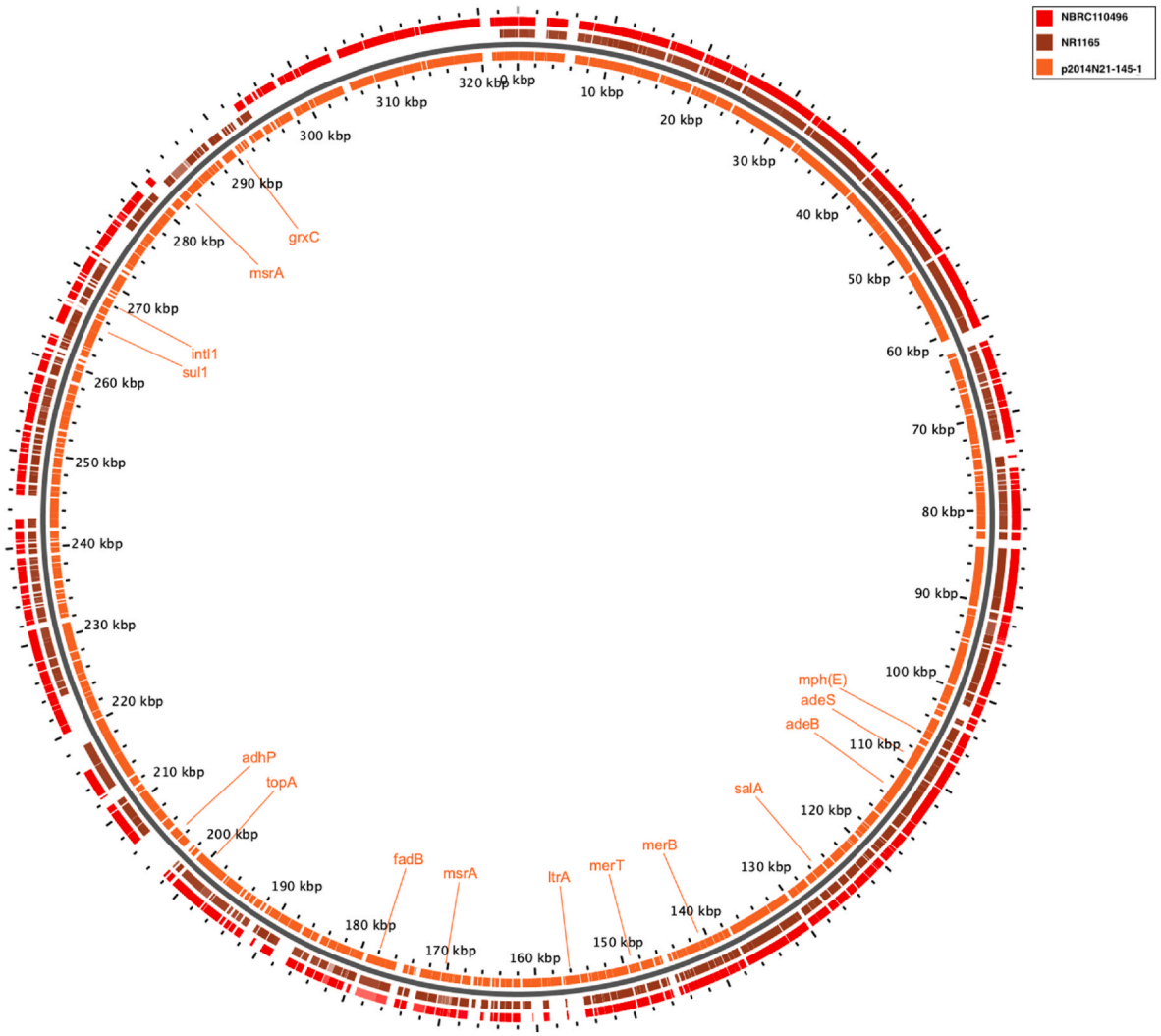

Fig. 3. Comparative analysis of the plasmid structures of *Acinetobacter colistiniresistens*.
GView was used to compare the draft sequences of NR1165 and NBRC 110496 with the sequence of plasmid p2014N21-145-1.

complete sequence of the megaplasmid was not confirmed because of the draft genome sequence analysis. Further investigation is needed to identify the megaplasmid sequences.

4. Discussion

To the best of our knowledge, this is the first report of IMP-1/OXA-58/OXA-670-producing *A. colistiniresistens* isolated from a patient in 2009, although an IMP-34/OXA-58-producing *A. colistiniresistens* isolate was isolated in 2015 [35]. These findings suggest that IMP/OXA-co-producing *A. colistiniresistens* evolved divergent carbapenemase and integron structures. The IMP-1/OXA-58/OXA-670-producing *A. colistiniresistens* strain described in this study can serve as a reservoir for MBL-producing plasmids. MBL-non-producing *A. colistiniresistens* strains have been reported in Europe and Asia (Table 1) [13]. Tetracyclines, sulbactams, and quinolones are viable antimicrobial options for IMP-1/OXA-58/OXA-670-producing *A. colistiniresistens* (Table 2); however, the emergence of IMP/OXA-coproducing *A. colistiniresistens* threatens clinical therapy and infection control. The discovery of this MDR isolate in Asia, as well as the danger of worldwide spread, should raise serious concerns. Recently, bloodstream isolates of IMP-1-producing *A. colistiniresistens* and OXA-58/OXA-67-producing *A. colistiniresistens* were reported independently in Brazil [37,38]. Former isolates caused outbreaks of bloodstream infections in a neonatal intensive care unit.

It is essential to have high standards for patient screening, reliable laboratory detection, and feedback reporting of resistant strains to reduce the prevalence and dissemination of carbapenemase-producing organisms. The results presented herein suggest that isolation of colistin-resistant and carbapenemase-producing organisms from a patient is rare; however, they also suggest that Japan suffers a heavy burden imposed by a wide variety of MBL-producing organisms. Previously, we isolated IMP-1-producing Enterobacterales species from patients [39]. Recently, it was reported that IMP-producing isolates comprised 15.1% of Enterobacteriaceae isolates obtained from patients in hospital settings in Japan, and 85.5% of carbapenemase-producing Enterobacteriaceae isolates [40]. Screening patients for colistin-resistant and carbapenemase-producing organisms is necessary to reduce the spread of IMP-producing organisms to other countries. Hospital laboratories require sensitive tests that can identify MDR organisms rapidly, followed by immediate application of infection control measures.

Recently, ESKAPE pathogens have become highly abundant in low- and middle-income countries (in addition to high-income countries) [8,41,42]. *Acinetobacter* spp. is a nosocomial pathogen frequently isolated in hospital and community settings [4,8–10,43,44]. Its characteristics include antimicrobial resistance, environmental persistence (e.g., desiccation tolerance), virulence (e.g., secreted enzymes; factors that facilitate evasion of immune responses), biofilm formation, and motility

[4–6,45–47]. Our study was limited to antimicrobial resistance mechanisms; therefore, the virulence and biological characteristics related to nosocomial infection in medical settings should be considered in further studies.

In conclusion, we identified and characterised an IMP-1/OXA-58/OXA-670-co-producing *A. colistiniresistens* clinical isolate. Draft genome analysis led us to assume that this isolate has a novel integron structure surrounding the bla_{IMP-1}, *aacA* and *sul1* genes. The integron could be located on a megaplasmid co-harbouring the *aph(3')-VIa* and *msrE* genes. Further molecular epidemiological studies are needed to reduce the spread of colistin-resistant and carbapenemase-producing *A. colistiniresistens*.

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Transparency declaration

The authors declare no conflicts of interest associated with this manuscript.

CRediT authorship contribution statement

Satoshi Nishida: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yasuo Ono:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2024.101484.

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