A conjugative plasmid carrying the carbapenem resistance gene bla\textsubscript{OXA-23} in AbaR4 in an extensively resistant GC1 Acinetobacter baumannii isolate

Mohammad Hamidian\textsuperscript{1}, Johanna J. Kenyon\textsuperscript{1}, Kathryn E. Holt\textsuperscript{2}, Derek Pickard\textsuperscript{3} and Ruth M. Hall\textsuperscript{1*}

\textsuperscript{1}School of Molecular Bioscience, The University of Sydney, NSW 2006, Australia; \textsuperscript{2}Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Victoria, Australia; \textsuperscript{3}Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

*Corresponding author. Tel: +61-2-9351-3465; Fax: +61-2-9351-5858; E-mail: ruth.hall@sydney.edu.au

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Objectives: To locate the acquired blaoxa-23 carbapenem resistance gene in an Australian A. baumannii global clone 1 (GC1) isolate.

Methods: The genome of the extensively antibiotic-resistant GC1 isolate A85 harbouring blaoxa-23 in Tn\textsubscript{2006} was sequenced using Illumina HiSeq, and the reads were used to generate a de novo assembly. PCR was used to assemble relevant contigs. Sequences were compared with ones in GenBank. Conjugation experiments were conducted.

Results: The sporadic GC1 isolate A85, recovered in 2003, was extensively resistant, exhibiting resistance to imipenem, meropenem and ticarcillin/clavulanate, to cephalosporins and fluoroquinolones and to the older antibiotics gentamicin, kanamycin and neomycin, sulfamethoxazole, trimethoprim and tetracycline. Genes for resistance to older antibiotics are in the chromosome, in an AbaR3 resistance island. A second copy of the \textit{ampC} gene in Tn\textsubscript{6168} confers cephalosporin resistance and the \textit{gyrA} and \textit{parC} genes have mutations leading to fluoroquinolone resistance. An 86335 bp repAci6 plasmid, pA85-3, carrying blaoxa-23 in Tn\textsubscript{2006} in AbaR4, was shown to transfer imipenem, meropenem and ticarcillin/clavulanate resistance into a susceptible recipient. A85 also contains two small cryptic plasmids of 2.7 and 8.7 kb. A85 is sequence type ST126 (Oxford scheme) and carries a novel KL15 capsule locus and the OCL3 outer core locus.

Conclusions: A85 represents a new GC1 lineage identified by the novel capsule locus but retains AbaR3 carrying genes for resistance to older antibiotics. Resistance to imipenem, meropenem and ticarcillin/clavulanate has been introduced into A85 by pA85-3, a repAci6 conjugative plasmid carrying Tn\textsubscript{2006} in AbaR4.

Keywords: Tn2006, conjugative resistance plasmids, A. baumannii

Introduction

Carbapenems are an important class of antibiotic used to treat Acinetobacter baumannii infections. However, many isolates are carbapenem resistant, most often due to the acquisition of an oxacillinase that is able to inactivate imipenem and meropenem. Several genes that confer resistance to carbapenems have been reported in this species.\textsuperscript{1} The \textit{blaoxa-23} gene has been seen in several different contexts.\textsuperscript{2} In the composite transposon Tn\textsubscript{2006}, the \textit{blaoxa-23} gene is flanked by inversely oriented copies of IS\textit{Amba}1,\textsuperscript{3} and Tn\textsubscript{2006} has been detected in several different genetic contexts in the chromosome or in a plasmid.\textsuperscript{2} One location is within the AbGRI1-type island, Tn\textsubscript{6167}, found in Australian global clone 2 (GC2) strains.\textsuperscript{4} Tn\textsubscript{2006} is also in AbaR4,\textsuperscript{5} a compound transposon made up of a Tn6022 backbone with Tn\textsubscript{2006} in the end of the sup gene.\textsuperscript{6} Hence, the blaoxa-23 gene can be disseminated by both of these transposon types. Since AbaR4 was first identified in the chromosome of a global clone 1 (GC1) isolate,\textsuperscript{5} it has also been found in the \textit{comM} gene of a distinct GC1 lineage.\textsuperscript{6} Transfer of blaoxa-23 into further strains by repAci6 conjugative plasmids has been reported,\textsuperscript{7} but little is known about these plasmids.

The blaoxa-23 gene is present in most Australian carbapenem-resistant A. baumannii isolates but carbapenem resistance is rare in the GC1 isolates.\textsuperscript{6,8–10} The GC1 isolate A85 is resistant to carbapenems (imipenem and meropenem), and to ceftazidime and cefotaxime as well as sulphonamides, tetracycline and gentamicin.\textsuperscript{6,11} Resistance to third-generation cephalosporins was recently traced
to the presence of Tn6168 \(^1\) and the carbapenem resistance is due to the bla\(\text{OXA-23}\) gene. \(^6\) A85 also carries AboR3, a Tn6019-based complex transposon located in com\(M\). \(^12\) Here, the whole genome sequence of A85 was determined and used to confirm the composition of the genomic antibiotic resistance islands and plasmids present, and to determine the location of Tn2006.

Materials and methods

DNA sequencing and sequence analysis

Genomic DNA isolated from A85 was sequenced using Illumina HiSeq at the Wellcome Trust Sanger Institute. Paired-end reads of 100 bp were assembled using Velvet, \(^12\) yielding 124 contigs with an average read depth of 60-fold. Contigs carrying parts of the AboR0/AboR3-type and AboR4 islands were recovered using standalone BLAST searches (www.ncbi.nlm.nih.gov/books/NBK526640) and assembled using sequences of fragments amplified using PCR with published primers. \(^5\), \(^11\) Parts of plasmids were identified as described previously, \(^10\) and junctions between contigs predicted by comparison with related plasmids were confirmed using published primers, \(^5\) and further primers listed in Table S1 (available as Supplementary data at JAC Online). The amplicons were sequenced. Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, MI, USA) was used for final sequence assembly. Gene clusters for the synthesis of the capsule and the outer core of lipooligosaccharide were found as described previously. \(^10\) Reading frames were predicted using ORF Finder (www.ncbi.nlm.nih.gov/projects/gorf/) and annotated manually. In addition, sequence type (ST) (in the Oxford (http://pubmlst.org/abaumannii/) and Institut Pasteur (http://www.pasteur.fr/recherche/genopole/PF8/mlst/ABAumannii.html) schemes were determined from the genome sequence as described previously. \(^11\)

Conjugation

Matings were performed as described previously, \(^14\) and transconjugants recovered on L-agar plates containing rifampicin (100 mg/L) to select for the recipient, a spontaneous rifampicin-resistant mutant of A. baumannii ATCC 17978 (resistant to sulphonamides), and ticarcillin (100 mg/L) to select for plasmid transfer. Spontaneous rifampicin-resistant derivatives of the donor were eliminated by screening purified potential transconjugants on L-agar containing ceftazidime (20 mg/L), to which the donor is resistant and the recipient susceptible. Ceftazidime-susceptible transconjugants were tested for resistance to antibiotics using disc diffusion.

GenBank accession numbers

The sequences of pA85-1, pA85-2 and pA85-3 were deposited in GenBank under accession numbers, KJ461963, KJ477078 and KJ493819, respectively. The Tn6168, AboR3, gyrA and parC, OCL3 and KL15 sequences are in GenBank under accession number KC118540.

Results and discussion

A85, a GC1 isolate exhibiting extensive resistance to antibiotics

In addition to the resistance phenotypes reported previously, A85 was found to be resistant to ticarcillin/clavulanate (due to bla\(\text{OXA-23}\)), sulbactam and fluoroquinolones, nalidixic acid and ciprofloxacin (Table 1). A85 was susceptible to colistin (MIC of 0.125 mg/L). Hence, using current definitions, \(^16\) A85 is extensively resistant. The genome sequence predicts a serine residue at 83 in GyrA and at 80 in ParC, explaining the fluoroquinolone resistance. A85 was previously shown by PCR mapping to carry the AboR3 resistance island in com\(M\). \(^12\) AboR3 \(^12\) includes aacCI, conferring resistance to gentamicin, and aadA1 (streptomycin and spectinomycin) in cassettes in the aacCI-orfP-orfQ-aadA1 cassette configuration in a class 1 integron with a sulI (sulphonamide resistance) gene, and the aphA1b gene (kanamycin and neomycin) in Tn6020, the tet(A) tetracycline resistance determinant and the bla\(\text{TEM}\) gene. These genes were found in the A85 genome and the complete 62989 bp island was assembled from 15 contigs using PCR primers published previously to link them.

Conjugative plasmid pA85-3 carries AboR4

The bla\(\text{OXA-23}\) carbapenem resistance gene of A85 is in Tn2006 but this transposon was not found in the AboR3 island or flanked by chromosomal genes. Instead, Tn2006 was in AboR4. A85 contains three plasmids. The largest, pA85-3 (Figure 1), includes the AboR4 surrounded by a 5 bp duplication (CCATT) that occurs only once in pA85-3 (GenBank accession number KF669606). pA85-3 resembles pAb-G7-2 \(^7\) in that it comprises three segments separated by a repeated segment (grey boxes in Figure 1; 2 and 3 are 423 bp and 1 is 225 bp).

pA85-3 carries the rep gene designated repAc6 \(^7\) and two separate regions with genes involved in conjugal transfer. The tra genes in Figure 1 are responsible for mating pair formation (MPF or type IV secretion system) and the second region contains the mobilization genes trwC and trwB. pA85-3 was shown to transfer ticarcillin/clavulanate, imipenem and meropenem resistance into a carbapenem-susceptible A. baumannii recipient strain, ATCC 17978 rif\(^\text{R}\) (Table 1). Recently, a sequenced repAc6 plasmid,

<table>
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<th>CIP 5</th>
<th>CTX 30</th>
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<td>5</td>
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<tr>
<td>ATCC 17978 rif(^\text{R})</td>
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<td>6</td>
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<td>3.5</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^6\)RIF, rifampicin; CIP, ciprofloxacin; CTX, cefotaxime; CAZ, ceftazidime; SAM, ampicillin/sulbactam; IPM, imipenem; MEM, meropenem; TIM, ticarcillin/clavulanic acid; SPT, spectinomycin; TET, tetracycline; TMP, trimethoprim; KAN, kanamycin; GEN, gentamicin.

\(^7\)Numbers next to the antibiotic names indicate the amounts of antibiotics in discs (in \(\mu\)g).
Figure 1. Map of pA85-3. Bold numbers outside the circle indicate positions in kb. The open boxes, with internal black arrows indicating the orientation of the trp gene, represent ISAbA1. Broad arrows represent genes (open reading frames with no known function are not shown). The IRs of AbaR4 are vertical bars and the extents of Tn2006 and AbaR4 are shown outside the circle. Grey boxes numbered 1, 2 and 3 represent the three repeat units. The location of PCR primers used to map the junctions of AbaR4 with the backbone and to link across the repeat units are shown within the circle. Primer sequences are given in Table S1.

pAb-G7-2, was shown to transfer TnaphA6, and this group appears to be responsible for the most recent resistance gene acquisition in A. baumannii.

Detection of AbaR4 in the pA85-3 context

The locations of transposons represent simple, useful markers for specific plasmids. Primers that amplify the left or right-hand boundaries of AbaR4 with the plasmid backbone found in pA85-3 (primer locations shown in Figure 1 and sequences listed in Table S1) were used to examine the additional carbapenem-resistant isolates in our collection that were found to carry AbaR4. Only two isolates, both GC1, met this criterion and the AbaR4 transposon was found to be in exactly the same location in one 2011 isolate, D108. In the second, AbaR4 was in comM. As this suggested that D108 carries a plasmid identical or closely related to pA85-3, the genome sequence of this isolate was determined and an essentially identical plasmid (five single-nucleotide polymorphisms) was assembled from it.

It would be interesting to know whether the conjugative repAc6 plasmids reported previously are identical to pA85-3 or whether they carry the blaOXA-23 gene in a different context. The eight European A. baumannii isolates that carried a repAc6 plasmid and blaOXA-23 may also contain pA85-3. This can now be tested using the PCR primers developed here.

The plasmid p2ABTDCO715 (70894 bp; GenBank accession number CP002524) from a GC2 isolate is closely related to pA85-3 but is cryptic. However, though the available sequence does not include AbaR4, part of each inverted repeat is present, flanked by the same 5 bp duplication as in pA85-3, and it is possible that AbaR4 has been omitted from the assembly. A similar omission was recently found for pACICU-2. Searches of the available A. baumannii draft genomes in GenBank (last searched March 2014) revealed contigs that contained a fragment of AbaR4 adjacent to the flanking sequence in pA85-3, indicating that they may also contain pA85-3 or a close relative. One of these isolates, Ab210, belonged to GC2, and was previously reported to have a deletion derivative of AbaR4 in the comM gene (GenBank accession number HQ700358). This conclusion now needs to be reassessed. One further draft genome for AC30 (a GC2 isolate from Malaysia) includes contigs for all of the components of pA85-3.

Other distinguishing features

A85 also includes two small cryptic plasmids. pA85-1 is 2726 bp and differs from p4ABAYE (GenBank accession number CU459139) at 66 positions. pA85-2 is an 8731 bp plasmid that differs from pA8-G7-1 (GenBank accession number KJ586856) by a single base substitution and from pAB0057 (GenBank accession number CP001183) by two additional bases.

From the genome sequence, A85 was ST1 (Institut Pasteur scheme), consistent with the GC1 assignment. Under the Oxford scheme A85 was known to be ST126 (10-53-4-11-4-64-5), differing by two alleles from ST109 (10-12-4-11-4-9-5) found in many Australian GC1 isolates, and in the 1984 GC1 reference isolate, A297 (RUH875). However the gpi allele is 200 rather than 64 when the genome sequence is used. As one of the differences between ST126 and ST109 is in the gpi allele, which was recently shown to be located in the gene cluster directing capsule biosynthesis, and two different gene clusters directing capsule biosynthesis have been reported at this locus in GC1 isolates, this region of the G7 genome was examined. A novel K locus, KL15, was identified (Figure S1, available as Supplementary data at JAC Online), indicating that this segment of the genome had been replaced again. KL15 has two sugar synthesis modules, dgaABC for 2,3-diacetamido-2,3-dideoxy-D-glucuroninic acid that is similar to a module in KL3, and qhbCB-gdr for a bacillosamine relative. The outer core locus was identified as OCL3.

Conclusions

The conjugative ability of the carbapenem resistance plasmid pA85-3 has allowed it to become widespread, disseminating the blaOXA-23 gene into the GC1 and GC2 clones. The PCR primers developed will allow it to be tracked globally.

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Transparency declarations
None to declare.

Supplementary data
Table S1 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References