Variants of the gentamicin and tobramycin resistance plasmid pRAY are widely distributed in Acinetobacter

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Objectives: To determine the cause of resistance to the aminoglycosides gentamicin and tobramycin in Acinetobacter isolates and the location of the resistance genes.

Methods: Australian Acinetobacter baumannii isolates were screened for resistance to aminoglycosides. PCR followed by restriction digestion of amplicons was used to detect genes and plasmids. Plasmids were isolated and examined by restriction digestion. Plasmid DNA sequences were determined and bioinformatic analysis was used to identify features. The sequence of the bla_{OXA-Ab} gene and multilocus sequence typing were used to determine strain types.

Results: Isolates that exhibited resistance to gentamicin, kanamycin and tobramycin were of diverse strain types. These isolates all carried the aadB gene cassette, and in all but one the cassette was in a 6 kb plasmid similar to pRAY. The three plasmid sequences determined revealed multiple frame-shift differences in the available pRAY sequence that altered the reading frames. In pRAY*, mobA and mobC mobilization genes were identified, but no potential replication initiation protein was found. pRAY*-v1 differed from pRAY* by 66 single-base differences, and pRAY*-v2 included two insertion sequences, ISAba22, located upstream of the aadB gene cassette, and IS18-like, within ISAba22.

Conclusions: The plasmid pRAY* and variants are widely distributed in Acinetobacter spp. and are the most common cause of resistance to gentamicin and tobramycin. Mobilization genes should assist in the dissemination of pRAY* and its variants.

Keywords: aadB gene cassettes, gentamicin resistance, tobramycin resistance

Introduction

The aadB gene, which encodes an aminoglycoside adenyltransferase and confers resistance to the aminoglycosides gentamicin, kanamycin and tobramycin, is part of a 591 bp gene cassette and is usually associated with a class 1 integron.1,2 However, in an Acinetobacter sp. from South Africa the aadB gene cassette was found in a secondary site in a 6 kb plasmid, pRAY (GenBank accession no. AF003958). pRAY includes several open reading frames (orf) (Figure 1a), and orf 3 was identified as an mbeA gene encoding a mobilization protein related to that found in CoIE1,3 and belonging to the MOBHEN family in the CoIE1 superfamily.4

pRAY or closely related plasmids are globally distributed and have been isolated in South Africa, Australia, Europe and the USA. For Acinetobacter baumannii AB058, a global clone 1 (GC1) isolate from the USA,5 part of its sequence is in GenBank accession number ADHA00000000. It was also detected in 18 GC2 A. baumannii isolates from four Australian hospitals that were characterized by resistance to gentamicin, kanamycin and tobramycin, but did not contain a class 1 integron.6 This study detected the aadB cassette in pRAY using a PCR assay. The sequence of the PCR amplicon (GenBank accession number JF343536) and restriction digestion of plasmid DNA from a representative isolate, C2, confirmed the presence of pRAY.7 The aadB cassette in the pRAY context was also found in GC1 isolates RUH875(A297) from the Netherlands7 and D36 from Australia.8

The combination of gentamicin, kanamycin and tobramycin resistance is indicative of the presence of the aadB gene, and here all of the remaining isolates in our Acinetobacter collection that exhibited this phenotype were examined for the presence of the aadB gene and the plasmid pRAY.
Methods

Gentamicin- and tobramycin-resistant Acinetobacter isolates

A collection of 100 Acinetobacter isolates obtained from various Australian hospitals was screened for resistance to 20 antibiotics including the aminoglycosides amikacin, gentamicin, kanamycin, netilmicin, neomycin and tobramycin as described previously.6 Isolates resistant to tobramycin are listed in Table 1. A single isolate representing ones reported in a previous study of GC2 isolates,6 and GC1 isolates D36 and RUH875/A297, which is the GC1 reference strain, were also included. These isolates had previously been shown to include the aadB gene in the context found in pRAY.7,8 Isolates were classified as A. baumannii if the chromosomal blaOXA-Ab gene could be amplified from whole-cell DNA as described previously,9 and the amplicon was sequenced. Multilocus sequence typing (MLST) according to the scheme hosted at Oxford University10 was performed using modifications described previously.8 The sequence type (ST) was assigned using the Acinetobacter baumannii MLST Databases web site (http://pubmlst.org/abaumannii/).

Plasmid analysis and sequencing

Plasmid DNA was extracted using Wizard plus SV Miniprep kits (Promega), and digested with restriction enzymes using the manufacturer’s instructions. Primers used (Figure 1a) are listed in the footnotes to Table 1. PCR products were amplified, separated and purified for sequencing as described previously.8 Sequences were analysed as described previously. ISAba22 was submitted to IS-Finder (http://www-is.biotoul.fr).

GenBank accession numbers

The sequences of pRAY* from D36 (6078 bp) and pRAY*-v2 (8433 bp) from E7 were submitted to GenBank under accession numbers JQ904627 and JX076770. The remainder of the pRAY*-v1 sequence from isolate C2 was added to JF343536.

Results and discussion

All isolates in our collection that were gentamicin-, kanamycin- and tobramycin-resistant, and had not previously been reported to carry the aadB gene in pRAY, were found to harbour the aadB gene using published primers.6 These isolates were screened for the presence of aadB in a class 1 integron or in pRAY (Table 1). Only one, isolate D4, carried the aadB cassette in a class 1 integron. The pRAY amplicon containing aadB was detected in the remaining isolates, but in E7 it was larger than predicted (see further analysis of E7 below). Two overlapping PCRs that amplify all of pRAY (PCR1, RH561 with RH1359 and PCR2, RH1360 with RH1361) were used to demonstrate that the complete pRAY plasmid was present, and all (except E7) yielded bands of the expected sizes (3314 and 3279 bp, respectively). These PCR products yielded fragments of the predicted sizes (1407, 910, 525, 396 and 76 bp for PCR1 and 1697, 1545 and 37 bp for PCR2) after digestion with HindIII.

Plasmid DNA was extracted from all isolates and digested with BamHI, which linearized pRAY yielding a 6 kb band. All isolates contained additional plasmids. Digestion with HindIII generates fragments of 2808, 1851, 910, 396, 76 and 37 bp from pRAY and the larger bands could be distinguished in digests from all isolates except E7.

Strain types

All but one of the isolates, PCH49, were identified as A. baumannii based on the presence of the chromosomal blaOXA-Ab gene. An MLST type could not be assigned to PCH49 as the gdhB gene could not be amplified. The sequence of the blaOXA-Ab gene designated allele 1 in Table 1 was consistent with the assignment of RUH875 and D36 to GC17,8 and allele 2 in C2 is found in other
GC2 strains. Two isolates, D4 and D46, shared allele 3, predicting OXA-64. However, D4 and D46 carry different aminoglycoside resistance genes. D4 carries only aadB, but in a class 1 integron. D46 was resistant to all aminoglycosides tested and was resistant (amikacin, kanamycin and neomycin resistance) in Tn4001. 

1LUMC, Leiden University Medical Centre; RNSH, Royal North Shore Hospital; CRGH, Concord Repatriation General Hospital; RWBH, Royal Brisbane and Women’s Hospital; WVH, Woden Valley Hospital; RCH, Royal Children’s Hospital; PCH, Prince Charles Hospital.
2Detected with primers RH561 (5′-GGGAGAAATCAATTACCGAAA-3′) and RH562 (AATTTCACCCAAACATCG); amplicon size 999 bp.
3PCR1 uses RH561 with RH1359 (5′-ATTCAAAACAGGCCACAG-3′); amplicon size 3314 bp. PCR2 uses RH1360 (5′-GACGAGACCAGCGGAAAA-3′) with RH1361 (5′-GGCCCTCCAGAAGTGCA-3′); amplicon size 3279 bp. Annealing temperature 62°C.
4Oxford MLST scheme, which uses gatA, gyrB, qdhB, recA, cpn60, gpi and rapO genes.
5blOXA-Al alleles have been numbered arbitrarily. 1 predicts the OXA-69 protein, 2 predicts OXA-66, 3 predicts OXA-64, 4 predicts OXA-88, 5 predicts OXA-70.
6OxaB alleles have been numbered arbitrarily. 1 predicts the OXA-69 protein, 2 predicts OXA-66, 3 predicts OXA-64, 4 predicts OXA-88, 5 predicts OXA-70.
7Also known as A297 (GC1 reference strain).
8D4 has aadB in a class 1 integron.
9Also known as RH2B.
10gadH primers did not produce a product for E7 and PCH49.
11Not known.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year</th>
<th>Hospital</th>
<th>City</th>
<th>aadB in pRAY</th>
<th>PCR1/PCR2</th>
<th>MLST</th>
<th>bla&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>RUH875&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1984</td>
<td>LUMC</td>
<td>Dordrecht</td>
<td>+</td>
<td>+/-</td>
<td>ST109 (10-12-4-11-4-9-5)</td>
<td>1</td>
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<tr>
<td>D36</td>
<td>2008</td>
<td>RNSH</td>
<td>Sydney</td>
<td>+</td>
<td>+/-</td>
<td>ST247 (10-12-4-11-4-58-5)</td>
<td>1</td>
</tr>
<tr>
<td>C2</td>
<td>2007</td>
<td>CRGH</td>
<td>Sydney</td>
<td>+</td>
<td>+/-</td>
<td>ST92 (1-3-3-2-2-7-3)</td>
<td>2</td>
</tr>
<tr>
<td>F2&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1999</td>
<td>RBWH</td>
<td>Brisbane</td>
<td>+</td>
<td>+/-</td>
<td>ST125 (1-52-59-12-1-18-44)</td>
<td>4</td>
</tr>
<tr>
<td>E7</td>
<td>2008</td>
<td>WVH</td>
<td>Canberra</td>
<td>+</td>
<td>+/-</td>
<td>ST... (1-34-*-28-22-55-5)</td>
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<tr>
<td>RCH51</td>
<td>NK&lt;sup&gt;i&lt;/sup&gt;</td>
<td>RCH</td>
<td>Brisbane</td>
<td>+</td>
<td>+/-</td>
<td>ST253 (1-52-29-28-18-24-7)</td>
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<tr>
<td>PCH49</td>
<td>NK&lt;sup&gt;i&lt;/sup&gt;</td>
<td>PCH</td>
<td>Brisbane</td>
<td>+</td>
<td>+/-</td>
<td>ST... (11-27-*-21-16-22-15)</td>
<td>12</td>
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</table>

**aadB and pRAY in Acinetobacter**

Plasmids containing the aadB gene cassette recovered from D36 (GC1) and C2 (GC2) were sequenced. Both were 6078 bp long. However, when these sequences were compared with the available pRAY sequence (GenBank accession number AF003958), a total of 14 additions or deletions of one to six bases were revealed. These differences may represent errors in the original pRAY sequence. As none of the differences alter the reading frame within a predicted orf in pRAY (Figure 1b), the sequence was re-annotated. The plasmid recovered from D36 was designated pRAY<sup>*</sup> and the reading frames found in it are shown on Figure 1b. The mobA gene was much longer than mbeA seen in pRAY (orf3 in Figure 1a) and predicted a 604 amino acid (aa) MobA protein. The N-terminal portion of MobA was 43% identical to the equivalent part of Mbe from ColE1, consistent with placement in the ColE1 superfamily (MOB<sub>HEN</sub> family) of MobA proteins. A further reading frame, mobC in Figure 1(b), overlaps mobA by 11 bp, and predicts a 135 aa protein homologous to MobC proteins in the GenBank protein database, and the region previously identified as the putative origin of transfer (oriT) identified previously as bom<sup>+</sup> is located upstream of mobC. DNA segments related to this mobilization segment and containing mobA and mobC genes and oriT were found in the draft genomes of Acinetobacter johnsonii (91% identical) and Acinetobacter radioresistens (83% identical) (GenBank accession numbers ACPL0100000 and ACVR0100000, respectively). Though two additional orfs overlap the mobA gene and three further orfs were found elsewhere, none had homologues in the GenBank protein database, and no potential replication initiation protein could be found. Hence, the region required for plasmid replication remains to be established.

The sequence of pRAY<sup>*-v1</sup> from C2 differed from pRAY<sup>*</sup> by 66 single-base differences, 65 of which were clustered between bp 3344 and 4702, covering the C-terminal end of the mobC gene and the N-terminal end of the mobA gene. The differences generate four aa substitutions in the 135 aa MobC protein and 15 substitutions in the 604 aa of MobA.

**pRAY<sup>*</sup>-v2 in isolate E7**

E7 was resistant only to gentamicin, tobramycin and kanamycin. However, the RH561 with RH562, RH561 with RH1359, and RH1360 with RH1361 PCR<sub>s</sub> all yielded amplicons that were ~2.5 kb larger than predicted from the sequence of pRAY<sup>*</sup>, indicating the presence of an additional segment located between primers RH561 and RH1361 (Figure 1a). The 8433 bp sequence of pRAY<sup>*-v2</sup>, the plasmid from E7, was identical to that of pRAY<sup>*</sup> except for the presence of two insertion sequences (IS),
one inside the other, in the location indicated by the arrow in Figure 1(b). ISAba22 is a 1274 bp insertion sequence with 36 bp inverted repeats (26 bp matching) that belongs to the IS3 transposon family. It is flanked by a 4 bp (GGTG) duplication in pRAY*-v2. An uninterrupted single copy of ISAba22 flanked by a 3 bp direct duplication (TTA) was found in the same position in the chromosome of A. baumannii isolate MDR-ZJ06 (GenBank accession number CP001937). Direct duplications of either 3 or 4 bp are typical of IS3 family IS. ISAba22 is interrupted by an IS that is 97.8% identical to IS18, which belongs to the IS30 family, and was previously isolated from an Acinetobacter spp. IS18-like is flanked by an imperfect 3 bp duplication (CAG and CAT) at 1136-8 in the uninterrupted ISAba22.

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Transparency declarations
None to declare.

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