Aminoglycoside resistance in multiply antibiotic-resistant Acinetobacter baumannii belonging to global clone 2 from Australian hospitals

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Received 17 February 2011; returned 24 March 2011; revised 24 March 2011; accepted 25 March 2011

Objectives: To examine the distribution and context of aminoglycoside resistance genes in multiply antibiotic-resistant Acinetobacter baumannii isolates from Australia that are members of the global clone 2 and carry the \( \text{bla}_{\text{OXA-23}} \) gene conferring resistance to carbapenems.

Methods: Sixty-one multiply antibiotic-resistant \( A. \text{baumannii} \) strains isolated between 2000 and 2010 at six Australian hospitals that belonged to global clone 2 and carried the \( \text{bla}_{\text{OXA-23}} \) gene were studied. Various molecular techniques were used to determine their relatedness and to detect antibiotic resistance genes and insertion sequences. Structures surrounding the aminoglycoside resistance genes were sequenced.

Results: The isolates all shared several antibiotic resistance genes, including the \( \text{sul2} \) sulphonamide resistance gene, but varied in their pattern of resistance to aminoglycosides. The aminoglycoside resistance profiles of isolates were accounted for by four resistance genes—\( \text{aadB} \), \( \text{aacC1} \), \( \text{aphA1b} \) and \( \text{aphA6} \)—in various combinations. The \( \text{aadB} \) gene cassette was located at a secondary site on a 6 kb plasmid similar to \( \text{pRAY} \). The \( \text{aphA6} \) gene was in a transposon, \( \text{TnaphA6} \), bounded by directly oriented copies of \( \text{ISAba125} \). The \( \text{aacC1} \) gene cassette in a class 1 integron and \( \text{Tn6020} \) carrying \( \text{aphA1b} \) were always present together, but were not linked.

Conclusions: Imipenem-resistant global clone 2 \( A. \text{baumannii} \) isolates containing \( \text{bla}_{\text{OXA-23}} \) have been present in Australian hospitals for at least 10 years. Variation in this global clone 2 type has occurred with the introduction of various aminoglycoside resistance genes carried on a small plasmid or within transposons.

Keywords: multiply antibiotic-resistant \( A. \text{baumannii} \), aminoglycoside resistance genes, Australia

Introduction

Acinetobacter baumannii isolates resistant to all or most of the antibiotics currently used for treatment have been observed, posing therapeutic problems.\(^1\)–\(^3\) In Europe and the Mediterranean, most multiply antibiotic-resistant \( A. \text{baumannii} \) isolates were known to belong to the European clonal lineages, ECI and ECII,\(^4\)–\(^5\) but recently the presence of these two clones in Australia\(^6\)–\(^7\) and elsewhere has been reported.\(^8\)–\(^12\) These two groups are hereafter referred to as global clones 1 and 2 (GC1 and GC2). Although resistance to carbapenem antibiotics—imipenem and meropenem—challenges current therapeutic approaches, resistance to other antibiotics has rarely been addressed in recent studies. As a consequence, we know relatively little about the complete resistance gene complement in members of these clones.

\( A. \text{baumannii} \) isolates from four Sydney hospitals (2002–07) that were resistant to at least 12 antibiotics were initially shown to fall into two groups based on repetitive extragenic palindromic sequence-based PCR (REP-PCR) typing\(^13\) and subsequently these two groups were shown to correspond to the GC1 and GC2 lineages.\(^7\) The isolates in the GC2 group were all resistant to imipenem, ceftazidime and ciprofloxacin and all carried a characteristic set of antibiotic resistance genes, namely \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{OXA-23}} \), \( \text{strB} \) and \( \text{tetA(B)} \). They also carried an \( \text{ISaba1} \) insertion sequence (IS) upstream of the \( \text{bla}_{\text{OXA-23}} \) gene and upstream of the chromosomally located \( \text{ampC} \) gene.\(^13\) These shared features suggested that they were closely related. However, they varied in their resistance to aminoglycosides. Some had been shown to carry a class 1 integron, with the \( \text{aacC1} \) gene cassette conferring resistance to gentamicin and the \( \text{aphA1} \) gene conferring resistance to kanamycin and neomycin.\(^7\)\(^13\) However, the genes responsible for aminoglycoside resistance in the remaining isolates, which do not contain a class 1 integron, were not identified.
Here, we have identified these genes and examined their context. We have also enlarged the collection by examining further more recent isolates from one of the original Sydney hospitals and a Canberra hospital as well as a collection of older isolates (2000–04) from Sydney and Brisbane.

### Materials and methods

#### Bacterial isolates

The multiply antibiotic-resistant isolates examined (Tables 1 and 2) comprised 29 isolates recovered from four Sydney hospitals described

<table>
<thead>
<tr>
<th>Group 4</th>
<th>Isolates</th>
<th>Year</th>
<th>City</th>
<th>Aminoglycoside resistance</th>
<th>phenotype</th>
<th>genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11, F36</td>
<td>2001</td>
<td>Brisbane</td>
<td>GmKmNm</td>
<td>aacC1, apha1b</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>A74–A82, A84</td>
<td>2002</td>
<td>Sydney</td>
<td>GmKmNm</td>
<td>aacC1, apha1b</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>A91, A93, A94, A96, A97</td>
<td>2005</td>
<td>Sydney</td>
<td>GmKmNm</td>
<td>aacC1, apha1b</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 5</th>
<th>Isolates</th>
<th>Year</th>
<th>City</th>
<th>Aminoglycoside resistance</th>
<th>phenotype</th>
<th>genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20</td>
<td>2002</td>
<td>Sydney</td>
<td>AkGmKmNm</td>
<td>aacC1, apha1b, apha6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>D72</td>
<td>2010</td>
<td>Sydney</td>
<td>AkGmKmNm</td>
<td>aacC1, apha1b, apha6</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Ak, amikacin; Gm, gentamicin; Km, kanamycin; Nm, neomycin.

Letters designate different hospitals. Numbers in bold indicate the isolates used for sequencing of recA and blaOXA-Ad.

Table 1. Properties of GC2 isolates containing a class 1 integron

<table>
<thead>
<tr>
<th>Isolatesa</th>
<th>Year</th>
<th>City</th>
<th>phenotype</th>
<th>genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13, C14</td>
<td>2006</td>
<td>Sydney</td>
<td>AkKmNm</td>
<td>apha6</td>
</tr>
</tbody>
</table>

Ak, amikacin; Gm, gentamicin; Km, kanamycin; Nm, neomycin.

Letters designate different hospitals. Numbers in bold indicate the isolates used for sequencing of recA and blaOXA-Ad.

Table 2. Properties of GC2 isolates without integrons

<table>
<thead>
<tr>
<th>Isolatesa</th>
<th>Year</th>
<th>City</th>
<th>phenotype</th>
<th>genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13, C14</td>
<td>2006</td>
<td>Sydney</td>
<td>AkKmNm</td>
<td>apha6</td>
</tr>
</tbody>
</table>

Ak, amikacin; Gm, gentamicin; Km, kanamycin; Nm, neomycin.

Letters designate different hospitals. Numbers in bold indicate the isolates used for sequencing of recA and blaOXA-Ad.
previously\textsuperscript{7,13} and 32 additional isolates (Tables 1 and 2). Six additional isolates recovered in the years 2000–2002 at a Brisbane hospital (designated hospital B) were among those examined in a recent study.\textsuperscript{14} Two additional isolates were recovered at a Canberra hospital (hospital E) and were the only multiply antibiotic-resistant and imipenem-resistant \textit{A. baumannii} isolated there between 2006 and 2009. A single additional isolate was obtained from hospital B in 2010. Additional isolates from Sydney hospital D were part of collections recovered over short time periods in 2008, 2009 and 2010 that were screened in the course of this study. Two isolates resistant to imipenem (MICs of 32 and 16 mg/L) and meropenem (MICs of 32 and 8 mg/L) were recovered from the 2008 collection (midstream urine; medical ward) and one was resistant to gentamicin (MIC of 32 mg/L). Six isolates were from an outbreak in the burn unit in late 2009 (various sites including wound swab and midstream urine). Isolate D72 was from midstream urine from a patient in a separate ward. Additional isolates from hospital A predate those from earlier studies.\textsuperscript{7,13}

MICs of 12 antibiotics have been reported previously for the original collection.\textsuperscript{13} MIC or resistance profiles for some antibiotics were supplied with the remaining strains. However, all isolates were re-screened for resistance to 18 antibiotics using a disc diffusion method as described previously.\textsuperscript{7} Zone sizes were assessed relative to the members of the collection with previously determined MICs and relative to a collection of antibiotic-susceptible \textit{A. baumannii}. Zone sizes varied very little (<1 mm in the annular radius) within the collection, except for the aminoglycosides amikacin, gentamicin, tobramycin, kanamycin, neomycin and netilmicin. For these antibiotics, isolates matched one of the types reported previously.\textsuperscript{13}

### DNA extraction

Whole-cell (genomic) DNA used as template for PCR amplifications was isolated from single colonies using a boiled prep procedure or from cultures grown overnight at 37°C using an alkaline lysis procedure. PCR products were purified for sequencing by precipitation with ethanol or after ultraviolet (UV) light and imaged using a GelDoc \textsuperscript{TM} XR image analysis station (Bio-Rad, Hercules, CA, USA). Product sizes were estimated using 100 bp and 1 kb DNA ladders (New England Biolabs) as molecular size markers and, where possible, compared with sequenced amplification products as standards. The identity of PCR amplicons was confirmed by either digestion with restriction enzymes (PstI, BamHI, NheI or HindIII) or DNA sequencing or both.

### PCR

Amplification was carried out in 12.5 \textmu L of PCR buffer (New England BioLabs, Ipswich, MA, USA) containing each deoxynucleoside triphosphate at 200 \textmu M with 12.5 pmol of each primer, approximately 10–50 ng of template DNA and 1 unit of Taq DNA polymerase (New England BioLabs). Thermal cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of amplification, consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s to 3 min, and a final cycle of amplification at 72°C for 5 min. Primer pairs and expected product sizes are listed in Table 3. Products were separated by electrophoresis on 1%–2% (w/v) agarose gels, stained with ethidium bromide (5 mg/L), visualized with ultraviolet (UV) light and imaged using a GelDoc\textsuperscript{TM} XR image analysis station (Bio-Rad, Hercules, CA, USA). Product sizes were estimated using 100 bp and 1 kb DNA ladders (New England Biolabs) as molecular size markers and, where possible, compared with sequenced amplification products as standards. The identity of PCR amplicons was confirmed by either digestion with restriction enzymes (PstI, BamHI, NheI or HindIII) or DNA sequencing or both.

### Determination of clonal type

GC2 isolates were identified using the criteria described previously,\textsuperscript{7} namely assignment to GS1 using allele-specific multiplex PCRs\textsuperscript{15} and the sequence of \textit{recA} and \textit{blaOXA-23} amplicons for representative isolates (bold in Tables 1 and 2). REP-PCR was performed as described previously\textsuperscript{13} and random amplified polymorphic DNA (RAPD) PCRs were performed using primers M13 and DAF4 as described elsewhere.\textsuperscript{16}

### Identification of resistance genes and mapping

Resistance determinants, class 1 integrons, insertion sequences and \textit{Tn}6020 were detected by PCR using primers and annealing temperatures described previously\textsuperscript{6,13} or listed in Table 3. The boundaries of the insertion in \textit{comM} were detected using primer pairs described previously.\textsuperscript{7} PCR mapping of the resistance gene contexts was performed using primer pairs listed in Table 3.

### DNA sequencing and sequence analysis

Automated sequencing was performed at the Australian Genome Research Facility, Westmead, NSW, on an Applied Biosystems 3730xl DNA Analyser using the Big Dye system. Sequences were assembled using Sequencher version 4.9 (Gene Codes Corp., Ann Arbor, MI, USA). Sequences were compared with known sequences using the BLAST facility (http://blast.ncbi.nlm.nih.gov). Multiple alignments were performed using ClustalW (www.ebi.ac.uk/tools/clustalw2). Gene Construction Kit version 2.5 (Textco, West Lebanon, NH, USA) was used to draw figures to scale.
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Figure 1. TnaphA6. The transposon is drawn to scale. ISaba125 are open boxes with an arrow below indicating the position and orientation of the transposase gene. The central region containing aphA6 is a thin line. The amplicons used to detect the transposon are shown below with the positions of primers indicated as vertical bars with the primer name adjacent.

GenBank accession numbers

The sequences of part of pRAY, TnaphA6 and Tn6020 have been deposited in GenBank with the accession numbers JF343536, JF343537 and JF343535, respectively. Sequences of chromosomal blaOXA-Ab (blaOXA-51-like) and recA genes from representative GC2 strain B1 have been deposited in GenBank with the accession number GQ914991, and sequences of these genes from other isolates were identical.

Results

A collection of GC2 isolates with shared features

Additional isolates were included in the study after comparison to members of the original collection13 that had been shown to belong to GC2.7 All isolates were assigned to GC2 using allelespecific multiplex PCR,15 and representative isolates (indicated in bold in Tables 1 and 2) were also shown to carry allele 2 of the recA gene (Oxford multilocus sequence typing (MLST) scheme) and the OXA-66 determining allele of the blaOXA-Ab (formerly blaOXA-51-like) gene. They contained the blaOXA-23 gene with ISaba1 upstream and were of the same REP-PCR and RAPD type. They were also shown to carry an copy of ISaba1 upstream of the chromosomally located ampC gene and to include all of the additional resistance genes previously described as shared by the members of this group,13 namely blaTEM, strB and tetA(B). All isolates in this collection were additionally shown to carry the strA gene located adjacent to the strB gene and the complete tet(B) determinant. The isolates were all resistant to high levels of sulfamethoxazole (>250 mg/L), but the cause of sulphonamide resistance in these GC2 isolates that did not contain an integron and the sul1 gene had not previously been investigated. This resistance was traced to the sul2 sulphonamide resistance gene.

Aminoglycoside resistance in integron-containing isolates

Variation within this collection of isolates was confined to differences in the pattern of aminoglycoside resistance, with five different patterns detected (groups 1–5 in Tables 1 and 2). The only relevant genes detected previously were the aacC1 gene (gentamicin resistance) located in a cassette in a class 1 integron6,13 and the aphA1 gene (kanamycin and neomycin resistance).13 Additional isolates were screened for the presence of class 1 integrons and the sul1 gene, which were found in two 2001 isolates from hospital F (Brisbane) and 10 2002 isolates from hospital A (Sydney). These isolates carried the aac1-orfP-orfQ-orfQ-aacA1 cassette array and also carried the aphA1 gene (Table 1). Using linkage PCR and digestion of the products, the aphA1b gene in these isolates was shown to be part of a transposon closely related to Tn6020, which is bounded by IS26 and was previously found in the AbaR region of GC1 isolates.6,7 The sequence was determined for isolate A94 and was identical to Tn6020 (GenBank accession number FJ172370). Tests for linkage between Tn6020 and the class 1 integron, as seen in the AbaR, did not yield a product, indicating that they are not in the same configuration.

Other aminoglycoside resistance genes

All isolates were screened for the presence of several aminoglycoside resistance determinants by PCR using primers listed in Table 3. In all cases, the aminoglycoside resistance profiles matched those expected from the genes detected (Tables 1 and 2). However, no gene was found to account for the netilmicin resistance of D72. None of the aminoglycoside-susceptible group (group 1 in Table 2) carried any of these resistance genes. Two isolates (group 3 in Table 2) carried only the aphA6 gene. The aphA6 gene was also seen in combination with aacC1 and aphA1 in two isolates (group 5 in Table 1) from different hospitals, one from 2002 and one from 2010. Group 2 carried the aadB gene (resistance to gentamicin, kanamycin and tobramycin), but did not contain a class 1 integron.

Location of the aphA6 gene

The aphA6 gene was first reported in 1988 in A. baumannii18 and its sequence can be found in two GenBank entries (accession numbers X07753 and DQ315788). A careful analysis of these sequences revealed that there was part of an ISaba125 on one side in X07753 and on the other side in DQ315788, suggesting that there may be a transposon with an ISaba125 on each side. Using primers in ISaba125 combined with ones in aphA6 (Figure 1 and Table 3), the aphA6 gene was shown to be flanked by two directly oriented copies of ISaba125 in all four isolates that carried aphA6 (Tables 1 and 2). The 914 bp central part of the sequence determined from these PCR products from isolate D72 (GenBank accession number JF343537) was identical to that obtained by combining accession numbers X07753 and DQ315788. This transposon was designated TnaphA6.
Figure 2. Map of pRAY. Circular map of the Acinetobacter plasmid pRAY drawn to scale using the sequence in GenBank accession number AF091755. The aadB gene cassette is shown as an open box with a closed box representing the attC site. B, H and Hc indicate sites for the restriction enzymes BamHI, HindIII and HincII, respectively. The positions of PCR fragments amplified are shown with the names of the primers used to make them at each end. Primers are listed in Table 3.

The aadB gene cassette in pRAY

Because the aadB gene is normally associated with a gene cassette, but a class 1 integron was not present, we examined the possibility that the cassette was in a secondary site, as has been reported before. Primers were designed to detect aadB in pRAY (Figure 2), a small 6 kb plasmid that was originally recovered from an Acinetobacter spp. isolate of unknown clonal origin, and they yielded products of the predicted size (999 bp) for all aadB-containing isolates (Table 2). The sequence of this amplicon was determined for isolates C2 and D8 and was identical to that of pRAY (GenBank accession number AF091755). Plasmid DNA recovered from these two isolates was digested with BamHI, HincII and HindIII and yielded fragments of the sizes predicted from the available sequence of pRAY (restriction sites are shown in Figure 2). Thus pRAY or a closely related plasmid is responsible for the observed gentamicin resistance.

Discussion

It has been reported that imipenem- and meropenem-resistant A. baumannii first appeared in Australia at Westmead Hospital in Sydney in 1999, and shortly thereafter in 2001–02 they appeared in two other eastern Australian hospitals corresponding to our hospitals A and F. These isolates carried the blaOXA-23 gene and most harboured a class 1 integron carrying the aacC1-orfP-orfP-orfQ-aadA1 gene cassettes, but other resistances and resistance genes were not examined. The Brisbane isolates (hospital F) used in the current study are derived from the same collection that has been examined in two other recent studies; the earliest of the additional isolates from hospital A were also part of the original collection studied by Valenzuela et al. One of the recent studies determined the MLST type of a subset of Brisbane isolates and the most abundant type that carried the blaOXA-23 gene was ST92 (formerly ST22; Oxford MLST scheme), which is the most abundant GC2 type in the Oxford MLST database. Hence it is likely that the isolates in our study also belong to the ST92 group. However, it cannot be assumed that the five types detected in the current study all arose from a common precursor that already contained the blaOXA-23 gene. The ST92 type is common in Asia, but ST92 isolates from Korea do not all carry blaOXA-23, and it is possible that the blaOXA-23-carrying type has arisen on more than one occasion, as several locations for blaOXA-23 in GC2 have been reported.

Most of the aminoglycoside resistance subtypes detected in the current study were found in more than one location. However, the pRAY-containing strain was the most widely distributed, being found in five different hospitals in three cities. It has either spread amongst hospitals on the eastern seaboard of Australia or been repeatedly imported from elsewhere. After this work was completed others reported pRAY and the TnpA6 transposon in single GC1 (ECI) isolates, indicating that they have spread amongst different A. baumannii strains. The small size of pRAY may allow it to be readily taken up by transformation and TnpA6 may also be spread by plasmid movement. Earlier studies have also reported the presence of the aacC1, aadB, aphA1 and aphA6 aminoglycoside resistance genes in isolates recovered prior to 1998 and in both GC1 and GC2 isolates. In the latter study, the aadB gene, which is usually only found in an integron gene cassette, was not always detected in a class 1 integron context, particularly in GC1 isolates, suggesting that some of the isolates used in that study may also carry pRAY.

Conclusions

Variation in the aminoglycoside resistance patterns in the blaOXA-23, blaTEM, strAB, sul2 and tet(B) containing lineage of the GC2 clonal complex circulating on the eastern seaboard of Australia can be explained by the acquisition of different resistance genes, aadB in pRAY, aphA6 in transposon TnpA6 or the aacC1 gene cassette in a class 1 integron together with Tn6020 carrying aphA1b.

Acknowledgements

Part of this work was reported previously at the Forty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009 (Abstract C2-624).

Dr M. Katouli, R. Huggett, K. Varettas, I. Carter, P. Huntington and Dr C. Fernandez are thanked for supplying isolates used in this study.

Funding

No specific funding was received for this study. S. J. N. is supported by a University of Sydney Postgraduate Award. V. P. was supported by a University of Sydney International Research Scholarship. R. M. H. was supported by NHMRC Fellowship grant 358713.

Transparency declarations

None to declare.
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References


