**Tn6168, a transposon carrying an ISaba1-activated ampC gene and conferring cephalosporin resistance in Acinetobacter baumannii**

Mohammad Hamidian and Ruth M. Hall*

School of Molecular Bioscience, The University of Sydney, NSW 2006, Australia

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

Received 30 April 2013; returned 25 June 2013; revised 2 July 2013; accepted 9 July 2013

**Objectives:** To explore the cause of third-generation cephalosporin resistance in Australian *Acinetobacter baumannii* isolates belonging to global clone 1 (GC1).

**Methods:** GC1 isolates from Australia were tested for resistance to ceftazidime and cefotaxime using disc diffusion and MICs. PCR was used to determine the context of ISaba1-ampC configurations and amplicons were sequenced. The level of transcripts was measured using quantitative real-time PCR. Multilocus sequence typing was performed.

**Results:** All ceftazidime- and cefotaxime-resistant isolates carried an appropriately oriented ISaba1 adjacent to the *ampC* gene and ISaba1 increased *ampC* transcripts 8–12-fold. In three isolates, the *ampC* gene next to ISaba1 was not in the normal chromosomal position. Instead, ISaba1 was 7 bp upstream of an additional copy of *ampC* located in a 3155 bp duplicated segment of the chromosome that differs from the resident GC1 segment by 2.3% but is almost identical to the corresponding region in several non-GC1 draft genomes. The duplicated segment is bounded by directly oriented copies of ISaba1 and flanked by a 9 bp direct duplication. This 5.5 kb transposon, named Tn6168, is in the same position in the chromosome of the three Australian isolates and the GC1 isolate AB0057. Tn6168 was also detected in an unrelated *A. baumannii* strain, where it was in a different location. The central part of Tn6168 was probably acquired from a sequence type ST32 (Institut Pasteur scheme) *A. baumannii* strain.

**Conclusions:** The ISaba1-ampC configuration, which increases *ampC* expression, can be part of a composite transposon Tn6168.

**Keywords:** *A. baumannii*, cephalosporin resistance transposon, composite transposon, ISaba1, *ampC* activation

**Introduction**

In *Acinetobacter baumannii*, resistance to third-generation cephalosporins such as ceftazidime and cefotaxime can arise as a consequence of increased expression of the chromosomal *ampC* gene. When the insertion sequence (IS) ISaba1 is present in the appropriate orientation upstream of *ampC*, an outward-facing promoter in ISaba1 directs transcription of *ampC*, increasing its expression.1–3 This mechanism appears to be widespread and ISaba1 is generally found upstream of the *ampC* gene in isolates that are resistant to third-generation cephalosporins.4–8 Another IS, ISaba125, has also been detected upstream of *ampC* and it also increases *ampC* expression leading to cephalosporin resistance.9 A surprising finding was that an identical ISaba125-ampC segment was found in both ACICU a global clone 2 (GC2) isolate and a global clone 1 (GC1) isolate from Greece, and that this resulted from the transfer of a 10 kb chromosomal DNA segment from the GC1 lineage to the GC2 lineage.9 Mobilization of an *ampC* gene, believed to have originated in *A. baumannii*, into *Oligella urethralis* has also been reported.10 However, to date, mobilization of an *Acinetobacter* *ampC* gene via a transposon has not been reported.

We previously examined a small collection of GC1 *A. baumannii* isolates from Australian hospitals and showed that the presence of ISaba1 upstream of *ampC* correlated with cephalosporin resistance.7 Here, we have measured the MIC and the level of *ampC* expression in these isolates. In isolates carrying an ISaba1-ampC configuration, we examined whether the *ampC* gene was in the normal location in the chromosome. This led to the identification of a transposon carrying an *A. baumannii* *ampC* gene.

**Methods**

**Bacterial isolates**

The strains used are listed in Table 1. Most have been described previously.7,11–13 Melissa Brown (Flinders University, Adelaide, Australia) supplied strain 677216614 from Royal Adelaide Hospital. G7 and RBH3 (previously referred to as F3)12 were detected among representatives of collections reported previously7,16 and supplied by Anton Peleg (Monash University,
Melbourne, Australia) and Mohammad Katouli (University of the Sunshine Coast, Sippy Downs, Queensland, Australia), respectively. J9, representing pulsotype II, was supplied by Jon Iredell (Westmead Hospital, Sydney, Australia). Robert Bonomo (Case Western Reserve University, Cleveland, OH, USA) supplied AB005718 and A119 was supplied by Kevin Towner (Nottingham Universities NHS Trust, UK).

### Strain characterization

Isolates were all screened for resistance to ceftazidime and cefotaxime using disc diffusion and MICs determined using microbroth dilution. Multi-locus sequence typing (MLST) was performed using the Oxford scheme, modified as described previously.

### DNA procedures

DNA was isolated and short- and long-range PCR conducted as described previously. Primers and their locations are listed in Table S1 (available as Supplementary data at JAC Online). PCR amplicons were digested with restriction enzymes to confirm their identity. Amplicons were purified, sequenced and sequences analysed as described previously.

### Quantitative real-time PCR

The level of ampC gene transcripts relative to 16S rRNA was measured using quantitative real-time PCR (qRT-PCR). Total RNA was isolated using the Isolate RNA Mini Kit (Bioline, Australia) and its integrity checked by electrophoresis. RNA samples were treated with Amplification Grade DNase I (Sigma–Aldrich, St Louis, MO, USA) to remove contaminating genomic DNA. Then, cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). cDNA levels were quantified on an ABI7500 Real-Time PCR System using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers designed for ampC and 16S rRNA, described previously. PCR was for 40 cycles with an annealing temperature of 60°C. Triplicate determinations were averaged.

### GenBank accession numbers

The DNA sequences of Tn6168 and surrounds and of the folE-ampC region from strain A85 have been submitted to GenBank under accession number KC118540. The sequence of the chromosomal ampC allele of isolate J9 has been submitted to GenBank under accession number KF002790.

### Results and discussion

#### Cephalosporin-resistant isolates carry ISAb1 upstream of ampC

The MIC of ceftazidime and cefotaxime determined for GC1 isolates recovered over the period 1997–2010 (Table 1) correlated well with predictions from the annular radius around discs containing 30 μg of ceftazidime or 30 μg of cefotaxime, if a radius ≥4 mm (corresponds to a 14 mm diameter) was interpreted as susceptible. The early GC1 isolate A1 from the UK was susceptible and AB005718 was resistant. ISAb1 was detected upstream of an ampC gene in all ceftazidime- and cefotaxime-resistant isolates, but not in any of the

---

### Table 1. Properties of GC1 strains

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Year</th>
<th>CTX (mm)</th>
<th>CAZ (mm)</th>
<th>MIC (mg/L)</th>
<th>CTX</th>
<th>CAZ</th>
<th>R/S</th>
<th>ISAb1-ampC</th>
<th>Relative transcript level</th>
</tr>
</thead>
<tbody>
<tr>
<td>A83</td>
<td>S</td>
<td>2002</td>
<td>5</td>
<td>5.5</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A92</td>
<td>S</td>
<td>2005</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3208</td>
<td>S</td>
<td>1997</td>
<td>6</td>
<td>7</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>S</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>D2</td>
<td>S</td>
<td>2006</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>1.2</td>
</tr>
<tr>
<td>D3</td>
<td>S</td>
<td>2006</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>D6</td>
<td>S</td>
<td>2006</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D7</td>
<td>S</td>
<td>2006</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D27</td>
<td>S</td>
<td>2008</td>
<td>4</td>
<td>5</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D62</td>
<td>S</td>
<td>2010</td>
<td>12</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D30</td>
<td>S</td>
<td>2008</td>
<td>4</td>
<td>5</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D36</td>
<td>S</td>
<td>2008</td>
<td>0</td>
<td>2</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>8.2</td>
<td>–</td>
</tr>
<tr>
<td>D13</td>
<td>S</td>
<td>2009</td>
<td>0</td>
<td>1</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>8.5</td>
<td>–</td>
</tr>
<tr>
<td>D78</td>
<td>S</td>
<td>2010</td>
<td>0</td>
<td>3</td>
<td>&gt;128</td>
<td>32</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D81</td>
<td>S</td>
<td>2010</td>
<td>0</td>
<td>3</td>
<td>&gt;128</td>
<td>32</td>
<td>R</td>
<td>+</td>
<td>11.6</td>
<td>–</td>
</tr>
<tr>
<td>G7</td>
<td>M</td>
<td>2002</td>
<td>0</td>
<td>4.5</td>
<td>128</td>
<td>32</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A85</td>
<td>S</td>
<td>2003</td>
<td>0</td>
<td>0</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>7.7</td>
<td>–</td>
</tr>
<tr>
<td>RH3</td>
<td>B</td>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6777166</td>
<td>A</td>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>7.75</td>
<td>–</td>
</tr>
<tr>
<td>AB0057</td>
<td>USA</td>
<td>2004</td>
<td>0</td>
<td>0</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A1</td>
<td>UK</td>
<td>1982</td>
<td>4.5</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>S</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

---

*aCity or country of isolation, A, Adelaide; B, Brisbane; S, Sydney; M, Melbourne.
*Annular radius; CTX, cefotaxime, CAZ, ceftazidime.
*R, resistant; S, susceptible.
*The transcript level was measured relative to strain 3208. —, not determined.
susceptible isolates. Three ceftazidime- and cefotaxime-susceptible isolates (A1, 3208 and D30) did not include any copies of ISAba1 and therefore belong to a lineage predating the entry of ISAba1 into the GC1 clonal complex.

**Location of ampC genes associated with ISAba1**

For all susceptible isolates, a PCR designed to detect any IS upstream of the ampC gene in its normal location in the chromosome adjacent to the folE gene (Figure 1a) yielded the 1667 bp amplicon predicted when no IS is present. A larger fragment of 2853 bp was amplified from most of the ceftazidime- and cefotaxime-resistant isolates, consistent with the presence of ISAba1 between the folE and ampC genes. However, three sporadic ceftazidime-resistant isolates (RBH3, A85 and 6772166), recovered in different cities over the period 2002–03, had ISAba1 upstream of ampC but yielded an amplicon of the size expected if there is no IS between folE and ampC. The sequence of this amplicon from each of the three isolates was identical to that found in other GC1 isolates (3208 and D2) reported previously or determined here for A83.

It was concluded that the ISAba1-ampC amplicon detected in RBH3, A85 and 6772166 derives from a second ampC gene. The sequence of this amplicon was the same for all three isolates. The sequence was also identical to that of a region in the genome of the GC1 isolate AB0057 (GenBank accession number CP001182).

**Tn6168, a composite transposon carrying the ampC gene**

The chromosome of GC1 isolate AB0057 was known to contain two copies of ampC, one in the normal location that is not preceded by an IS and a second copy adjacent to an ISAba1 and located near the origin of replication. We examined this region more closely and found that the additional ampC is part of a 3155 bp continuous segment that is derived from an A. baumannii chromosome and is flanked by directly oriented copies of ISAba1 (Figure 1b). This structure is flanked by a 9 bp direct duplication, as expected for ISAba1. It is between open reading frames AB57_0006 and AB57_0013 (in CP001182), which both predict products that are resistance-nodulation–division efflux pumps, and these genes are adjacent in other available GC1 genomes. Hence, the structure is a composite transposon that was named Tn6168.

A85, RBH3 and 6772166 were screened for the presence of Tn6168 using primers listed in Table S1. AB0057 was the control. In all three, Tn6168 was present and the sequence of the amplicon linking ampC to the flanking sequence (RH741 to RH582) was identical, indicating that the transposon in A85, RBH3 and 6772166 is in the same location as it is in AB0057. The complete sequence of the transposon and surroundings from A85 was determined and was identical to that found in AB0057, except for an additional base in the right-hand ISAba1 of AB0057. A further GC1 isolate carrying Tn6168 in the same position was detected via searches of the GenBank whole genome sequence database (last searched 27 June 2013). The sequence type (ST) of isolate Canada BC-5 (GenBank accession number AFDN01000002) was found to be ST1 (Institut Pasteur MLST scheme) and hence this isolate is also a GC1 member.

**Origin of Tn6168**

The sequences of the central portion of Tn6168 and the resident chromosomal copy in GC1 strains differ at 72 positions. In Tn6168, 15 bases of the ampC gene (bp 729–743) were duplicated, causing a duplication of the last five amino acids of the Ω loop. Sixty-eight differences were single base substitutions, including 22 that were in ampC causing five amino acid substitutions and 20 that were in the adjacent open reading frame (orf in Figure 1b). The amino acid differences in AmpC did not affect any of the critical residues identified in previous studies.

A potential bidirectional ρ-independent terminator was found in this location and the stem–loop structure was not disrupted by the substitutions located within it.

A possible origin for this segment was found in several draft genomes, which had sequences differing by 1–2 bp from that in Tn6168 and lacking the 15 bp duplication (Table S2, available as Supplementary data at JAC Online). The ST of the isolates listed in Table S1 was determined from the genome data. Those without an ISAba1 upstream of ampC were identified as ST32 (Institut Pasteur scheme) or ST463/ST472 (Oxford MLST scheme). The remaining genomes were from ST3 (Institut Pasteur MLST scheme) isolates (corresponding to European clone III) and these carried an ISAba1 9 bp from the ATG of ampC, whereas, in Tn6168, ISAba1 is only 7 bp from the ATG codon.

**Transposon Tn6168 in other A. baumannii isolates**

Only a few ceftazidime- and cefotaxime-resistant isolates were found among the collection of >400 Australian Acinetobacter isolates, both sporadic and outbreak, we have screened to date. This collection included 89 isolates that are not GC1 or GC2 and 65 of these did not include a detectable oxa-Ab gene, indicating that they are probably not A. baumannii. Two cephalosporin-resistant isolates that were not GC1 or GC2 but included ISAba1 upstream
of ampC were described recently.\textsuperscript{8} In 7 further isolates, all
members of a group of 64 isolates that caused an outbreak at
Westmead Hospital extending from 1996 to 1999,\textsuperscript{17} ISAba1 was
linked to ampC, but the ISAba1 was not between folE and ampC.
PCR mapping of one isolate, J9, coupled with restriction enzyme
digestion of the amplicons, showed that it contained Tn6168.
However, the transposon was not between the AB57_0006 and
AB57_0013 genes as in the GC1 isolates. The MLST profile (Oxford
scheme) of this isolate could not be determined, because four of
the seven genes failed to produce an amplicon using a variety of
primers. However, the sequence of the chromosomal ampC gene
next to folE (GenBank accession number KF002790) differed by
17/1152 bp from the GC1 allele and by 20 bp from ampC in Tn6168.

\textit{Relationships among GC1 isolates carrying Tn6168}

Although isolated in different cities, the Australian isolates were all
found to belong to ST126 (Oxford scheme), suggesting that they
are from a specific lineage within the GC1 clonal complex.
Canada BC-5 differed from them only by a single base in the
gdhB allele. However, AB0057 carries a different gpi allele, suggest-
ing a difference in the capsule biosynthesis locus,\textsuperscript{2,3} as well as dif-
ferent gyrB and gdhB loci. Using a published PCR mapping
strategy,\textsuperscript{12} the AbaR resistance island in A85, RBH3 and 6772166
were described recently.\textsuperscript{8} In 7 further isolates, all
members of a group of 64 isolates that caused an outbreak at
Westmead Hospital extending from 1996 to 1999,\textsuperscript{17} ISAba1 was
linked to ampC, but the ISAba1 was not between folE and ampC.
PCR mapping of one isolate, J9, coupled with restriction enzyme
digestion of the amplicons, showed that it contained Tn6168.
However, the transposon was not between the AB57_0006 and
AB57_0013 genes as in the GC1 isolates. The MLST profile (Oxford
scheme) of this isolate could not be determined, because four of
the seven genes failed to produce an amplicon using a variety of
primers. However, the sequence of the chromosomal ampC gene
next to folE (GenBank accession number KF002790) differed by
17/1152 bp from the GC1 allele and by 20 bp from ampC in Tn6168.

\textit{Conclusions}

Tn6168, an ISAba1-bounded composite transposon, has mobilized
the ampC gene from an ST32 A. baumannii strain. The PCR strategy
developed will facilitate screening for Tn6168 in further isolates.

\textbf{Acknowledgements}

Dr Dale Hancock (School of Molecular Bioscience, The University of Sydney,
Sydney) is thanked for assistance with qRT-PCR.

\textbf{Funding}

Funding for this study was received from the School of Molecular Bioscience,
the University of Sydney and NHMRC Project Grant APP1026189. M. H. was
supported by a University of Sydney Postgraduate Research Award.

\textbf{Supplementary data}

Tables S1 and S2 are available as Supplementary data at JAC Online (http://
jac.oxfordjournals.org/).

\textbf{References}

\textsuperscript{1} Héritier C, Poirel L, Nordmann P. Cephalosporinase over-expression
resulting from insertion of ISAba1 in Acinetobacter baumannii. Clin Microbiol
Infect 2006; \textbf{12}: 123–30.

\textsuperscript{2} Segal H, Nelson EC, Elisha BG. Genetic environment and transcription of
ampC in an Acinetobacter baumannii clinical isolate. Antimicrob Agents

\textsuperscript{3} Corvec S, Caroff N, Espaze E et al. AmpC cephalosporinase hyperproduction
in Acinetobacter baumannii clinical strains. J Antimicrob Chemother 2003; \textbf{52}:
629–35.

\textsuperscript{4} Rodriguez-Martinez JM, Poirel L, Nordmann P. Genetic and functional
variability of AmpC-type \textit{\beta}-lactamases from \textit{Acinetobacter baumannii}. Antimicrob Agents
Chemother 2010; \textbf{54}: 4930–3.

\textsuperscript{5} Mak JK, Kim MJ, Pham J et al. Antibiotic resistance determinants in
nosocomial strains of multidrug-resistant \textit{Acinetobacter baumannii}. J

\textsuperscript{6} Nigo SJ, Post V, Hall RM. Aminoglycoside resistance in multiply
antibiotic-resistant \textit{Acinetobacter baumannii} belonging to global clone 2

\textsuperscript{7} Hamidian M, Hall RM. AbaR4 replaces AbaR3 in a carbapenem-resistant
\textit{Acinetobacter baumannii} isolate belonging to global clone 1 from an

\textsuperscript{8} Hamidian M, Hall RM. ISAba1 targets a specific position upstream of the
intrinsic ampC gene of \textit{Acinetobacter baumannii} leading to cephalosporin

\textsuperscript{9} Hamidian M, Hancock DP, Hall RM. Horizontal transfer of an ISAba125-
activated ampC gene between \textit{Acinetobacter baumannii} strains leading to

\textsuperscript{10} Mammeri H, Poirel L, Mangeney N et al. Chromosomal integration of a
cephalosporinase gene from \textit{Acinetobacter baumannii} into \textit{Oligella urethralis}
as a source of acquired resistance to \textit{\beta}-lactams. Antimicrob Agents Chemother
2003; \textbf{47}: 1536–42.

\textsuperscript{11} Post V, Hall RM. AbaR5, a large multiple antibiotic resistance region found
in \textit{Acinetobacter baumannii}. Antimicrob Agents Chemother 2009; \textbf{53}:
2667–71.

\textsuperscript{12} Post V, White PA, Hall RM. Evolution of AbaR-type genomic resistance
islands in multiply antibiotic-resistant \textit{Acinetobacter baumannii}. J Antimicrob
Chemother 2010; \textbf{16}: 1162–70.

\textsuperscript{13} Post V, Hamidian M, Hall RM. Antibiotic-resistant \textit{Acinetobacter baumannii}
variants belonging to global clone 1. J Antimicrob Chemother 2012; \textbf{67}:
1039–40.

\textsuperscript{14} Eikelkamp BA, Stroeher UH, Hassan KA et al. Adherence and motility
characteristics of clinical \textit{Acinetobacter baumannii} isolates. FEMS Microbial
Lett 2011; \textbf{323}: 44–51.

\textsuperscript{15} Peleg AY, Franklin C, Bell JM et al. Emergence of carbapenem resistance
in \textit{Acinetobacter baumannii} recovered from blood cultures in Australia.

\textsuperscript{16} Long YB, Fooagali J, Bodman J et al. Persistence of multiple antibiotic
resistant strains of \textit{Acinetobacter baumannii} carrying class 1 integron in a

\textsuperscript{17} Valenzuela JK, Thomas L, Partridge SR et al. Horizontal gene transfer in a
polyclonal outbreak of carbapenem-resistant \textit{Acinetobacter baumannii}. J

\textsuperscript{18} Adams MD, Goglin K, Molyneaux Net al. Comparative genome sequence
analysis of multidrug-resistant \textit{Acinetobacter baumannii}. J Bacteriol 2008;
\textbf{190}: 8053–64.

\textsuperscript{19} Hamouda A, Evans BA, Towner KJ et al. Characterization of epide-
miologically unrelated \textit{Acinetobacter baumannii} isolates from four continents
by use of multilocus sequence typing, pulsed-field gel electrophoresis, and
sequence-based typing of bla\textsubscript{OXA-51-like} genes. J Clin Microbiol 2010; \textbf{48}:
2476–83.

\textsuperscript{20} Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to
determine the minimal inhibitory concentration (MIC) of antimicrobial

\textsuperscript{21} Kenyon JJ, Hall RM. Variation in the complex carbohydrate biosynthesis
loci of \textit{Acinetobacter baumannii} genomes. PLoS ONE 2013; \textbf{8}: e62160.