Diverse class 2 integrons in bacteria from beef cattle sources

Robert S. Barlow* and Kari S. Gobius

Food Science Australia, PO Box 3312, Tingalpa DC, Queensland 4173, Australia

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Objectives: The purpose of this study was to determine the diversity of class 2 integrons in bacteria isolated from beef cattle sources.

Methods: The variable regions of a subset of 11 class 2 integron-containing bacteria were analysed by PCR and DNA sequencing for the presence of novel rearrangements.

Results: A total of six different class 2 integron arrays were identified and four of these were fully characterized. Three of the four arrays characterized have been previously described; however the remaining array is unlike previously described class 2 integrons. The novel class 2 integron was found in Providencia stuartii and contains an apparently functional class 2 integrase. Examination of the variable region of the P. stuartii integron identified nine open reading frames, mostly of unknown function, and represents the first report of a class 2 integron without inserted antibiotic resistance gene cassettes.

Conclusions: This study has identified a novel class 2 integron found in P. stuartii that contains an apparently functional naturally occurring class 2 integrase. Further investigation of this novel class 2 integron is required to determine the impact of a functional class 2 integrase upon the evolution of class 2 integrons.

Keywords: Providencia, functional integrase, integron evolution

Introduction

Integrons are mobile genetic elements capable of gene capture and expression via site-specific recombination and the action of a promoter. The site-specific recombination reaction is catalysed by an integrase that is encoded within the 5′-conserved segment (5′-CS) of integrons. Numerous classes of integrase have been described with the type of integrase present lending itself to the class of integron i.e. class 1 integrase (intI1) defines class 1 integrons. However, the presence of antibiotic resistance genes within integrons is predominantly confined to class 1, 2 and 3 integrons with class 1 integrons having the greatest diversity of antibiotic resistance genes. In fact, genes encoding resistance to almost every class of antibiotic have been found associated with class 1 integrons. Class 2 and 3 integrons also possess antibiotic resistance genes; however the diversity of antibiotic resistance encoded by genes within these integrons is limited. The limited diversity observed with class 3 integrons is a representation of their low prevalence throughout the world with few examples reported in the literature or GenBank database.

Class 2 integrons exhibit decreased diversity, primarily because of the presence of a stop codon at amino acid 179 in the class 2 integrase (intI2). It is believed that the stop codon results in the production of a shorter and probably inactive polypeptide that is unable to catalyse the recombination reaction observed in other classes of integrons. Class 2 integrons are most often found on transposon Tn7 and its relatives and commonly carry the three antibiotic resistance genes dfrA1, sat2 and aadA1. However, studies into the variability of class 2 integrons have identified a number of novel rearrangements within class 2 integrons. Antibiotic resistance genes previously unassociated with class 2 integrons such as ereA and estX (GenBank accession no. AB161462) have been shown to be associated with Tn7-related class 2 integrons. Furthermore, Ramirez et al. recently described a novel rearrangement of a class 2 integron from non-epidemiologically related Acinetobacter baumannii isolates. This class 2 integron has the genes sat2, aadB and catB2 inserted upstream of the three conventional antibiotic resistance genes of Tn7 class 2 integrons. The resulting structure is a class 2 integron with a variable region comprising six antibiotic resistance genes and represents the first description of aadB and catB2 within a class 2 integron.

The interest in integron-associated antibiotic resistance has continued to escalate since they were first described in 1989.
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Table 1. Characteristics of class 2 integron carrying strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Organism</th>
<th>Source</th>
<th>Variable region size (bp)</th>
<th>Variable region content</th>
<th>tnsE</th>
<th>tnsD</th>
<th>Resistance phenotype</th>
<th>GenBank accession no.</th>
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</thead>
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<tr>
<td>ABR 88</td>
<td>Escherichia coli</td>
<td>faeces</td>
<td>1700</td>
<td>sat2-aadA1</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>DQ286458</td>
</tr>
<tr>
<td>ABR 128</td>
<td>Citrobacter freundii</td>
<td>faeces</td>
<td>1700</td>
<td>sat2-aadA1</td>
<td>+</td>
<td>+</td>
<td>AMP</td>
<td>DQ286459</td>
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<tr>
<td>ABR 224</td>
<td>Aeromonas veronii biovar sobria</td>
<td>faeces</td>
<td>2200</td>
<td>dfrA1-sat2-aaa1</td>
<td>+</td>
<td>+</td>
<td>SXT</td>
<td></td>
</tr>
<tr>
<td>ABR 226</td>
<td>E. coli</td>
<td>faeces</td>
<td>2200</td>
<td>dfrA1-sat2-aaa1</td>
<td>+</td>
<td>+</td>
<td>TMP</td>
<td></td>
</tr>
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<td>ABR 228</td>
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<td>2500</td>
<td>estX-sat2-aaa1</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>TIM</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
<td>ABR 23</td>
<td>Providencia stuartii U+</td>
<td>hide</td>
<td>?</td>
<td>9 ORFs</td>
<td>+</td>
<td>+</td>
<td>AMC, AMP, CEF, NIT</td>
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<tr>
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<td>?</td>
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<td>+</td>
<td>+</td>
<td>AMC, AMP, CEF, NIT</td>
<td>DQ533991</td>
</tr>
</tbody>
</table>

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cefalotin; NIT, nitrofurantoin; SXT, trimethoprim/sulfamethoxazole; TIM, ticarcillin/clavulanic acid; TMP, trimethoprim. ND = none detected.

*+ = evidence of gene presence.

†= variable region size not determined using primers hep74 and hep51.  
= no evidence of gene presence.

The progression of studies identifying novel antibiotic resistance genes harboured by integrons has confirmed initial suggestions that they play a major role in the development of antibiotic resistance in clinical settings. Integrons are also being used as a way of investigating the potential link between antibiotic resistance development in food production animals and the subsequent clinical treatment failure caused by exposure to humans of antibiotic-resistant bacteria in the food chain. A number of studies have determined the prevalence of class 1 and 2 integrons in food production systems as well as the antibiotic resistance genes that are carried by the integrons. In particular, surveys investigating class 2 integrons in Escherichia coli from meat and meat products, and class 2 integrons from poultry environments, have both identified class 2 integrons containing bacteria isolated from cattle faeces, cattle hides and retail ground beef.

Materials and methods

Bacterial strains

The bacterial strains used in this study were selected from a collection of class 2 integron-containing bacteria in the Food Science Australia culture collection. All strains had been isolated from beef cattle sources such as faeces and hides using the method of Barlow et al. The identity of bacterial isolates was determined using the VITEK Junior system (BioMerieux, Hazelwood, USA). The size of the class 2 integron variable region of each isolate was determined using the PCR primers hep74 and hep51. A subset of 11 isolates from the initial collection was chosen for more detailed comparison of their class 2 integron properties with that of Tn7. Within this subset, six isolates did not produce variable region amplicons, three isolates produced variable region amplicons of different size to Tn7 and two isolates produced variable region amplicons of similar size to Tn7. The characteristics of each isolate are shown in Table 1. A strain of E. coli carrying the class 2 integron containing transposon Tn7 (GenBank accession no. NC_002525) was used as a positive control in all PCRs. Bacterial isolate E. coli ATCC 25922 was used as a control organism for antibiotic susceptibility testing.

PCR

Class 2 integron variable regions were amplified using primers hep74 and hep51. Isolates that produced an amplicon of any size were further tested using primers specific for dfrA1 (D1 & D2), sat2 (sat top1 5'-GGAAAACATTGGATGCTGAA-3' and sar bot1 5'-CATGACAGTCATCATCCGAGA-3') and aadA1 (aadA1-5'-TATCCAGCTAAACGCGAATC-3' and aadA1-R 5'-ATTTGGCCGACTACCT-TGGTG-3'). PCR cartography, using a series of seven PCRs using combinations of the above-mentioned primers, was conducted on all amplicon-producing isolates. Isolates that failed to produce an amplicon by conventional PCR were further tested by long PCR for novel cassette arrangements using the attI2-specific primer RB 201 (5'-GCAAACGCAACCATCTTAA-3') and the tnsE-specific primer tnsE-5' (5'-GTTAGAGCGATGTTGCTAATG-3'). Purified genomic DNA was prepared using the Wizard Genomic DNA purification kit (Promega, USA) and 200 ng was added to each long PCR. Long PCR was performed in 50 μL reaction mixtures containing 1× reaction buffer (ABgene, UK), 2 mM MgCl2, 400 μM dNTP mix (Finnzymes, Finland), 25 pmol of each primer, 400 μg/mL BSA and 2 U of Red Hot DNA polymerase (ABgene). Distilled water was added to adjust the volume to 45 μL prior to the addition of DNA template. PCR was performed for 35 cycles of
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denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 68°C for 12 min. A final extension step of 68°C for 15 min was performed to complete the reaction. In addition to characterization of the variable region, each isolate was tested by PCR for the presence of tnsD (primers tnsD-f 5'-TGCACAGACTGGCTAACAGG-3' and tnsD-r 5'-CGACATCAATTTTGGGCTTT-3') and tnsE (primers tnsE-f 5'-GTGGGCTCAGGCTCTATGAC-3') and tnsE-r (as above). PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 40 s. A final extension step of 72°C for 7 min was performed to complete the reaction. The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

DNA sequencing
DNA sequencing of both the sense and antisense strands was performed using the BigDye terminator kit (Version 3.1; Applied Biosystems, USA) and a 9700 thermal cycler (Perkin Elmer, Norwalk, USA). Sequencing reactions were prepared in accordance with the instructions provided by the Australian Genome Research Facility (AGRF; http://www.agrf.org.au). The resulting products were purified using the magnesium sulphate cleanup method and submitted to AGRF for analysis using the ABI3730xl DNA analyser (Applied Biosystems). The resulting sequences were analysed by BLAST searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service. Sequence contigs were assembled using the ContigExpress component of the Vector NTI Advance software suite (version 10.0.1; Invitrogen, Australia). Additional primers required to complete DNA sequencing were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/prime3/primer3_www.cgi).

Antibiotic susceptibility testing
MICs of various antibiotics for the integron-containing organisms were determined using the VITEK Junior system (BioMerieux, Hazelwood, USA). VITEK cards for antibiotic susceptibility testing (GNS-424 cards) were inoculated and incubated according to the recommendations of the manufacturer. The following antibiotics were tested: amikacin, amoxicillin/clavulanic acid, ampicillin, cefotaxime, ceftazidime, cefalotin, ciprofloxacin, gentamicin, imipenem, meropenem, nitrofurantoin, norfloxacin, ticarcillin/clavulanic acid, tobramycin, trimethoprim/sulphamethoxazole and trimethoprim. A test for the detection of extended-spectrum β-lactamases (ESBLs) was also carried out, and interpretation of the results was based on comparison of the reduction in growth caused by ceftoxime/clavulanate and ceftazidime/clavulanate and that caused by cephalosporins alone. The outcome of the test was either ESBL positive or ESBL negative, as determined by the VITEK Junior.

Nucleotide sequence accession numbers
The nucleotide sequences reported in this study have been submitted to the EMBL/GenBank nucleotide sequence database under the accession numbers DQ286457, DQ286458, DQ286459, DQ533990 and DQ533991.

Results
PCR screening of class 2 integron-containing bacteria from the Food Science Australia culture collection identified a subset of 11 isolates that represented a variety of class 2 integrons warranting further investigation (data not shown). The isolates were selected based on the size of the variable region PCR amplicons they produced using the primers hep74 and hep51 or on the inability to produce a variable region PCR amplicon. The characterization of these isolates is described below.

Variable region size determination
The variable region of each of the 11 class 2 integrons was amplified by PCR to determine their respective size. Variable regions of five of the 11 isolates were identified using the primers hep74 and hep51. Two of these five strains produced a 2200 bp PCR-generated amplicon, which is consistent with the size expected to be produced by Tn7-like class 2 integrons. Two of the remaining three isolates produced amplicons of ~1700 bp with the final isolate producing a 2500 bp amplicon. The remaining six isolates were tested by long PCR to determine if novel cassette arrangements were present that could not be amplified using conventional PCR. Of the six tested, only two produced an amplicon and both of these amplicons were ~12 kb in size. Additionally, both 12 kb amplicons were generated from class 2 integrons carried by Providencia stuartii U+ strains. The remaining four isolates failed to generate PCR amplicons of any size even when tested using alternative primers (data not shown).

Characterization of class 2 integrons
Isolates ABR 224 and ABR 226 both produced a 2200 bp PCR-generated amplicon consistent with Tn7-like integrons. PCR cartography, using a series of seven PCRs, confirmed the presence of the three antibiotic resistance genes found in Tn7-like class 2 integrons and its organizational structure (Figure 1). Both strains produced the expected amplicons and are considered Tn7-like class 2 integrons. As many of the previously described novel class 2 integrons contain rearrangements of the classical Tn7 class 2 integron, all remaining strains were also tested by all seven PCRs. The strains possessing variable regions of 1700 or 2500 bp all tested positive for sat2 and aadA1 and it was confirmed that these genes were in the same configuration as observed in classical class 2 integrons. This suggested that only minor differences existed between these integrons and classical class 2 integrons. Sequencing of the 1700 bp amplicons from ABR 88 and ABR 128 identified a class 2 integron carrying the antibiotic resistance genes sat2 and aadA1. The structure of this integron is identical to that present in Tn1826.19 Sequencing of the variable region amplicon from ABR 228 identified a class 2 integron carrying the genes estX, sat2 and aadA1. This integron shares >99.6% nucleotide sequence identity with GenBank accession no AB161461. Isolates ABR 88, ABR 128 and ABR 228 were further analysed to determine if the class 2 integrase gene of each integron contained the internal stop codon. The internal stop codon was present in each of the integrases examined.

Testing of the P. stuartii isolates ABR 23 and ABR 130 by PCR for the presence of genes common to the variable regions of Tn7-like class 2 integrons determined that the genes dfrA1, sat2
and *aadA1* were not present in these isolates. Sequencing of the ~12 kb amplicons from upstream of *intI2* to *tnsD* identified a novel putative class 2 integron structure containing *intI2*, nine open reading frames (ORFs) (eight forward and one reverse orientation), *tnsE* and *tnsD* (Figure 2). The size of the integrons (*intI2* to *tnsE* inclusive) was 11,390 bp (ABR 23) and 11,354 bp (ABR 130), respectively. The 36 bp difference occurs at a single point in a non-coding region between *attI2* and ORF1 with the remainder of the array sharing 100% nucleotide sequence identity. Analysis of *P. stuartii intI2* revealed that it shares 99.4% identity with *intI2* of pR721 (GenBank accession no. NC_002525) at the nucleotide level and 98.4% predicted amino acid identity. Most importantly, *P. stuartii intI2* does not possess the internal stop codon at amino acid 179 as it has been replaced by glutamine as a result of a single base substitution (T!C) at nucleotide 444. The predicted translation of the *P. stuartii intI2* ORF produces an uninterrupted full-length (325 amino acid) polypeptide.

BLAST analysis of the nine ORFs comprising the novel variable region failed to identify genes in GenBank with significant homology at the nucleotide level. Analysis at the amino acid level identified a number of proteins that share homology with the ORFs from the novel variable region. The majority of the ORFs (CDS 1–6) are homologous (28–45%) to conserved hypothetical proteins with unknown functions. CDS 7 and 8 are homologous (41% and 54%) to the Type II *SalI* restriction-modification system of *Streptomyces albus* and CDS 9 is homologous (65%) to a putative transposase of *E. coli*. The ORF (CDS 1–9) homologues do not have any association with previously described super-integrons. The sequences of *tnsE* and *tnsD* from the *P. stuartii* class 2 integron were compared with those of pR721 (NC_002525) and share 90% and 92% homology, respectively. The class 2 integron from *P. stuartii* is the first example of a class 2 integron that does not harbour known antibiotic resistance genes.

**P. stuartii 59-be recombination sites**

Analysis of the *P. stuartii* integron array revealed a total of four complete 59-be recombination sites. The structure of the gene cassettes found in the *P. stuartii* integron is shown in Table 2. Despite there being only four recognizable 59-be within the array, they may account for the integration of six of the nine ORFs (ORFs 1–6). It appears that the proposed gene cassettes, PS01 and PS02 both contain two overlapping ORFs. Additionally, the sequences of the 59-be of PS01 and PS02 show significant homology (90.3%) with each other as well as a previously described 59-be from a class 1 integron in *Pseudomonas stutzeri*.20 The remaining 59-be of PS03 and PS04, respectively, do not match previously described 59-be. The remaining 3 ORFs (ORF 7–9) for which obvious 59-be sites were not determined were likely to have entered the integron array as a result of non-*intI2* associated recombination event(s).

**Antibiotic susceptibility testing**

The antibiotic resistance phenotype of each isolate is shown in Table 1. Phenotypic resistance to amoxicillin/clavulanic acid, ampicillin, cefalotin and nitrofurantoin was observed in the *P. stuartii* strains; however, the nucleotide sequences indicated that genes encoding resistance to these antimicrobials were not associated with the novel integrons. The Tn7-like class 2

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**Figure 1.** PCR targets used for the confirmation of Tn7-like class 2 integrons. The triangles represent primer location for each PCR. Large solid arrows indicate coding regions for the genes described. The arrowhead orientation indicates the direction of transcription for each gene. PCR amplicons are indicated by opposing arrowheads (representative of primers) joined by black lines.

**Figure 2.** Schematic representation of a novel class 2 integron from *Providencia stuartii* isolates. Large solid arrows indicate coding regions for the genes described. The arrowhead orientation indicates the direction of transcription for each gene. The proposed gene cassettes (Table 2) and their associated 59-be are shown by solid black lines linking small black block arrows.
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<table>
<thead>
<tr>
<th>Gene cassette</th>
<th>1 R sequence</th>
<th>Space 1 (bp)</th>
<th>ORF length (amino acids)</th>
<th>Space 2 (bp)</th>
<th>1 L sequence</th>
<th>59-be length (bp)</th>
<th>Homologous proteins</th>
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</thead>
<tbody>
<tr>
<td>PS01</td>
<td>GGTAGAC</td>
<td>16</td>
<td>167 (ORF1) &amp; 291 (ORF2)</td>
<td>1</td>
<td>GTCTAAC</td>
<td>72</td>
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<tr>
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<td>GTTATGC</td>
<td>29</td>
<td>142 (ORF3) &amp; 294 (ORF4)</td>
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<td>72</td>
<td>EAM45209 &amp; AAW40609</td>
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<td>-6</td>
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<td>72</td>
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<td>ACATAAC</td>
<td>75</td>
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</table>

*aSpace 1 refers to the region between 1R and the start codon of the associated ORF.

*bSpace 2 refers to the region between the stop codon of the associated ORF and 1L. A negative value indicates that the ORF extends into the 59-be."

Integrons of ABR 224 and 226 showed phenotypic resistance to trimethoprim and sulfamethoxazole/trimethoprim, respectively. The isolates lacking tnsD and tnsE (ABR 106 and 325) both showed resistance to trimethoprim and sulfamethoxazole/trimethoprim, suggesting that these isolates may carry a gene that confers resistance to trimethoprim other than *dfrA1*.

**Discussion**

The prevalence of antibiotic-resistant bacteria remains a major public health concern throughout the world. The role that integrons, particularly class 1 integrons, play in the development of antibiotic-resistant bacteria has been studied extensively during the last decade. Recent studies have demonstrated the presence of novel class 2 integrons in clinical settings and consequently the importance of this class of integron appears to be steadily increasing. Despite the increase in diversity of class 2 integrons, the presence of a stop codon at amino acid 179 of *intI2* has remained consistent and lends support to the suggestion that novel rearrangements of class 2 integrons arise from the action of a class 1 integrase from another element in *P. stuartii*. This possibility now provides an alternative hypothesis to the previous proposal that *trans* action of *intI1* leads to generation of novel rearrangements within *Tn7*-like class 2 integrons. It would now seem more likely that they would form by the *trans* action of a novel functional IntI2.

The uniqueness of the novel *P. stuartii* class 2 integrin is further highlighted upon examination of its variable region, which comprised nine ORFs. However, none of the ORFs encode known antibiotic resistance genes and therefore represents the first report of a class 2 integron lacking at least one antibiotic resistance gene. Four 59-be were identified and may account for the integration of six of the nine ORFs. Interestingly two of the four 59-be are associated with dual overlapping ORFs. The relevance of this finding is unclear but it is logical to infer that the gene cassette formed via excision of either 59-be would contain both ORFs. The variable region does not appear to have been formed solely from integrase-associated site-specific recombination as obvious 59-be could not be located for ORFs 7–9. It is likely that the potential restriction/modification system and the putative transposase (ORFs 7–9) found at the 3’ end of the array have been incorporated via an alternative recombination mechanism.

Sequence analysis of the transposition genes *tnsE* and *msD* identified considerable divergence from the published *Tn7* transposition genes. This finding raises questions about the transposition capability of this integron, given that *tnsE* is required for random site transposition and *msD* for *attTn7* transposition. It is possible that the presence of altered transposition genes may result in an integron that displays a high degree of host specificity due to an inability to transpose as readily as *Tn7*. Further investigation of this novel class 2 integrin is required to demonstrate the functionality of the *P. stuartii* *intI2* and to determine the impact of a functional class 2 integrase upon the evolution of class 2 integrons.

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**Transparency declarations**

None to declare.
References