The \textit{aadB} Gene Cassette Is Associated with \textit{bla\textsubscript{SHV}} Genes in \textit{Klebsiella} Species Producing Extended-Spectrum \(\beta\)-Lactamases

Louisa A. Jones,\textsuperscript{1,2} Christopher J. McIver,\textsuperscript{1,2,3} Mi-Jurng Kim,\textsuperscript{2} William D. Rawlinson,\textsuperscript{1,2,3} and Peter A. White\textsuperscript{1,2,4}\textsuperscript{*}

\textit{School of Biotechnology and Biomolecular Sciences, Faculty of Science,} \textit{University of New South Wales,} \textit{Sydney, Australia.}

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Received 30 March 2004/Returned for modification 26 June 2004/Accepted 25 October 2004

\textbf{Integrons were detected in 37 (72.5\%) of 51 \textit{Klebsiella} spp. producing extended-spectrum beta-lactamases by PCR with primers that targeted integrase genes and cassette regions. PCR and amplicon sequencing of the cassette regions revealed \textit{aadB} and \textit{aadA2} gene cassettes that confer resistance to a range of aminoglycosides. \textit{aadB} was associated with a class 1 integron on a 28-kb plasmid, \textit{pES1}, that also contained \textit{bla\textsubscript{SHV-12}} and \textit{IS26}.}

Multiple-antibiotic-resistant \textit{Klebsiella} spp. are important nosocomial pathogens (6, 18) and commonly express extended-spectrum \(\beta\)-lactamase (ESBL) enzymes belonging to the SHV family, encoded by \textit{bla\textsubscript{SHV}} genes (7, 8, 15, 19). In a previous study, we noted that a number of ESBL-producing \textit{Klebsiella} spp. in Sydney, Australia, were resistant to gentamicin by virtue of an \textit{aadB} gene cassette (confering resistance to gentamicin, tobramycin, and kanamycin) contained within a class 1 integron (25). Recently, integrons have been recognized as significant contributors to the acquisition of antibiotic resistance in gram-negative bacteria (5, 9, 11, 25). In the present study, we explored the association of \textit{bla\textsubscript{SHV}} genes and integrons in a collection of 51 ESBL-producing clinical isolates collected over a 10-year period in Sydney, Australia. Genetic linkage between integron-borne gentamicin resistance genes and \textit{bla\textsubscript{SHV}} genes was investigated.

\textbf{Bacterial strains and antibiotic susceptibility testing.} ESBL-producing \textit{Klebsiella} spp. comprising 45 \textit{K. pneumoniae} and 6 \textit{K. oxytoca} from 20 different wards in three hospitals in Sydney, Australia, were collected from 1989 to 1999. Susceptibility to antimicrobial agents was determined by using the calibrated dichotomous sensitivity method (1), available at http://www.medic.unsw.edu.au/pathology-cds/, to a panel of 20 antibiotics in seven different classes (Table 1). The diversity of the isolates was calculated by using Simpson’s index with antibiotic profiles. The probability of selecting two strains with different profiles was 95.4\%. All 51 isolates were resistant to ampicillin, cephalxin, and cefoxime; 47.1\% of bacteria were resistant to amikacin and trimethoprim, while 84.3\% were resistant to kanamycin, 82.4\% were resistant to tobramycin, and 72.5\% were resistant to gentamicin (Table 1). All isolates tested were susceptible to cefotetan and imipenem. Multiresistant ESBL-producing bacteria, particularly those that are resistant to aminoglycosides, have been reported with increasing frequency (6, 7, 14, 22). From a therapeutic perspective, the varied scope of antibiotic resistance displayed by the isolates of this study highlights the difficulties faced by those treating infections with these bacteria.

\textbf{Integron screening.} To determine if resistance in the \textit{Klebsiella} collection was conferred by integrons, DNA was extracted and screened by PCR as previously described with primers that anneal to conserved regions of integron-encoded integrase genes \textit{intI1}, \textit{intI2}, and \textit{intI3} (25) (Fig. 1). Integron cassette regions were amplified with primers that target the conserved flanking regions (24) (Fig. 1). Thirty-seven of 51 ESBL-producing strains (72.5\%) contained one or more class 1 integrons as determined by an analysis of integrase PCR amplicons by restriction fragment length polymorphism with Hin\textit{II} (Promega) and RsaI (Promega) (25). Class 2 and 3 integrons were not detected among these strains. Gene cassettes within integron cassette regions were characterized by restriction fragment length polymorphism and sequencing (24, 25). Of the 37 integron-positive strains, 20 contained two integrons and 16 contained one integron (Table 1). The cassette region from the remaining integron-positive strain could not be amplified (Table 1). Each cassette region amplified contained a single gene cassette. Three types of gene cassettes were found (Table 1), the most common being \textit{aadB} (confering resistance to gentamicin, tobramycin, and kanamycin) (4). This cassette was found in 33 (89.1\%) of 37 bacteria containing integrons (Table 1). The presence of \textit{aadB} correlated with phenotypic resistance to gentamicin, tobramycin, and kanamycin in all 33 strains with this gene cassette. Interestingly, 20 strains with \textit{aadB} (54.0\% of integron-positive bacteria) also contained a second integron with \textit{aadA2} (confering resistance to streptomycin and spectinomycin) (3). The integron with \textit{aadA2} correlated with streptomycin resistance in all strains containing this gene cassette (Table 1). Three strains contained a single integron with an \textit{aadA1} cassette (also confering resistance to streptomycin and spectinomycin) (21) (Table 1). No further gene cassettes were identified, a finding which implied that resistances not accounted for by integron-borne gene cassettes were encoded by non-integron-associated resistance genes.

\textbf{Detection of \textit{bla\textsubscript{SHV}} by PCR.} The \textit{bla\textsubscript{SHV}} gene is commonly associated with ESBL activity in \textit{Klebsiella} spp. (7, 8, 19);
hence, isolates were screened by PCR for \(\text{bla}_{\text{SHV}}\) genes with primers A (5'\text{H11032 CA}CTCAAGGATGTATTGTG 3'\text{H11032})\) and B (5'\text{H11032 TTAGCGTTGCCAGTGCTCG 3'\text{H11032}}) (14), which amplify a 883-bp product (Fig. 1). Fifty of 51 strains (98%) were PCR positive (Table 1), demonstrating the widespread prevalence of \(\text{bla}_{\text{SHV}}\) genes in \(\text{Klebsiella}\) spp. in Australia.

**Horizontal transfer of the aadB gene cassette.** The high incidence of the \(\text{aadB}\) gene cassette suggested that a common resistance element, which could also harbor a \(\text{bla}_{\text{SHV}}\) gene, may be prevalent in the collection. To determine whether the integron carrying \(\text{aadB}\) was located on a transferable plasmid, transconjugation assays were performed on two strains, \(\text{K. oxytoca}\) INS22K and \(\text{K. oxytoca}\) INS23K, chosen on the basis of susceptibility to nalidixic acid. Conjugal transfer of kanamycin resistance was observed at average frequencies of between \(3.8 \times 10^{-7}\) and \(6.1 \times 10^{-6}\) transconjugants/donor. Antibiotic susceptibility testing of the transconjugants revealed phenotypic resistance to cephalexin, cefotaxime, ticarcillin-clavulanate potassium (Timentin), ampicillin, gentamicin, tobramycin, sulfonamide, trimethoprim, and nalidixic acid. Donor strains were additionally resistant to tetracycline, norfloxacin, chloramphenicol, and nitrofurantoin.

**TABLE 1. Resistance determinants and phenotypes of 51 ESBL-producing** \(\text{Klebsiella}\) **spp.**

<table>
<thead>
<tr>
<th>Cassette array</th>
<th>No. of organisms</th>
<th>Total</th>
<th>With IS26</th>
<th>With (\text{bla}_{\text{SHV}}) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aadB</strong></td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td><strong>aadB/aadA2</strong></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><strong>aadA1</strong></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Undetermined</strong></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total no.</strong></td>
<td>51</td>
<td>44</td>
<td>50</td>
<td>24</td>
</tr>
</tbody>
</table>

The cassette array is shown as integron1/integron2 in the case of a strain containing more than one integron. Cassette arrays could not be amplified from one integron-positive strain. AMK, amikacin; AMP, ampicillin; AMC, amoxicillin-clavulanate potassium; CTX, cefotaxime; CT, cefotetan; CHL, chloramphenicol; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; LEX, cephalxin; NAL, nalidixic acid; NET, netilmicin; NFT, nitrofurantoin; NOR, norfloxacin; STR, streptomycin; SUL, sulfonamide; TET, tetracycline; TIC, ticarcillin-clavulanate potassium; TOB, tobramycin; TMP, trimethoprim.

**FIG. 1.** Structure of two drug-resistant regions of pES1 isolated from \(\text{Klebsiella oxytoca}\) INS23K. Binding sites for primers used to screen the collection of 51 ESBL-producing \(\text{Klebsiella}\) spp. for integrase genes (primer pair hep35-hep36), class 1 cassette regions (primer pair hep58-hep59), \(\text{IS}\) \(_{26}\) (primer pair hep121-hep122), and \(\text{bla}_{\text{SHV}}\) (primer pair A-B) are shown. The dashed line indicates the regions sequenced in this study; the lengths of those regions are shown. (A) The 5'-CS, aadB cassette, and 3'-CS were amplified by PCR with a combination of primers, and the products were sequenced. Analysis of the assembled 2,366-bp sequence revealed a class 1 integron with its associated \(\text{intI1}\) gene (open box) and \(\text{attI1}\) site (hatched box). The resistance genes identified, \(\text{aadB}\) (conferring resistance to gentamicin, tobramycin, and kanamycin), \(\text{qacE}\), and \(\text{suI}\), are shown as thick black lines, and the \(\text{aadB}\)-associated 59-base element is shown as a filled box. The 2,366-bp nucleotide sequence differs by two nucleotides from the sequence of pDGO100 that contains integron \(\text{In7}\) (GenBank accession number L06418; position 203 to 2,568). (B) The \(\text{bla}_{\text{SHV-12}}\) gene was amplified with primers A and B and sequenced. Sau3A fragments from pES1 were cloned into pUC18. pVRL98 was selected with cefotaxime and contained a 6-kb fragment of pES1. Approximately 1,400 bp of the 6-kb fragment were sequenced to reveal \(\text{IS}\) \(_{26}\) and the downstream \(\text{bla}_{\text{SHV-12}}\). IR indicates the position of the 14-bp terminal inverted repeats of IS26. The sequence matches that found in GenBank (accession number X84314; position 629 to 2,382), with the exception of a single point mutation that accounts for the difference between the SHV2a and SHV-12 enzymes at amino acid residue 240.
indicating that the majority of the resistance genes were transferred.

Plasmid extractions of the transconjugants isolated a plasmid (designated pES1) approximately 28 kb in size, while plasmid extractions of both donor strain INS22K and donor strain INS23K revealed the presence of pES1 and an additional plasmid (designated pES2) of approximately 4 kb in size (data not shown).

**Resistance genes identified for pES1.** PCR screening of pES1 extracted from transconjugants revealed a class 1 integron with an aadB gene cassette. The presence of both a 3’ conserved segment (3’-CS) and a 5’-CS was confirmed by PCR amplification and sequencing (Fig. 1). Sulfonamide resistance was conferred by the presence of a sulfonamide resistance gene, sul1, found within the 3’-CS (20). The aadB gene cassette accounts for the gentamicin, kanamycin, and tobramycin resistance profile of the transconjugants. Transconjugants displayed ESBL activity by the characteristic “keyhole” interaction, in the form of a keyhole between cefotaxime and amoxicillin-clavulanic acid (Augmentin), which was demonstrable by a disk diffusion susceptibility test (1). Transconjugants were screened by PCR for blaSHV genes as described above (14), and the products were sequenced. Sequencing revealed that pES1 harbored blaSHV-12. The presence of this gene accounts for the resistance to cephalaxin, ampicillin, and cefotaxime seen in the transconjugants. Thus, a class 1 integron carrying aadB, sul1, and a blaSHV-12 gene was identified on pES1, and these genes together account for its multiple-resistance phenotype. Future research will focus on mapping the distance between aadB and blaSHV-12 and sequencing the intervening region. Our findings could help explain the recently reported nosocomial outbreak of multiresistant _K. pneumoniae_ in The Netherlands (7), where 25 of 30 ESBL-producing bacteria (83.3%) were shown to carry blaSHV-12 and aadB. Consistent with our findings, eight strains also harbored aadA2 (7). The present study provides a possible explanation for the joint presence of aadB and blaSHV-12 on a single transmissible plasmid in _Klebsiella_ spp. The genetic determinant mediating trimethoprim resistance for pES1 was not identified.

**blaSHV genes on transmissible plasmids are associated with integrons and IS26 elements.** To isolate resistance genes carried on pES1, Sau3A fragments were cloned into pUC18 and transformants were selected on Luria-Bertani agar containing ampicillin (50 μg/ml) and subsequently selected on Luria-Bertani agar–cefotaxime (0.5 μg/ml) plates. This procedure led to the isolation of a plasmid, pVRL98, containing a fragment approximately 6 kb in size. Sequencing revealed a blaSHV-12 gene and an upstream insertion sequence, IS26 (Fig. 1). BLAST searches revealed that the fragment displayed identity to several plasmids from diverse locations worldwide (12, 13, 15, 17, 23). These plasmids all contain genes that encode for either SHV-2a or SHV-5 and that differ from SHV-12 (identified on pES1) by only one amino acid each, and all plasmids contain IS26. Moreover, class 1 integrons encoding aminoglycoside resistance have been reported for two of these plasmids (17, 23). The fact that IS26 is commonly found upstream of SHV-encoding genes suggests that it is involved in the creation of antibiotic resistance islands by promoting the integration of resistance genes (2, 8, 10).

**Prevalence of IS26 and blaSHV in the ESBL-producing _Klebsiella_ spp.** Primers hep121 (5’ AGCGGTTAATCCTGGGAG TGA 3’) and hep122 (5’ TTGTCCCTTTTACTGGC 3’) were designed for a region 35 bp upstream of the _blaSHV-12_ gene and for a portion of the IS26 _tnpA_ gene to determine the prevalence of this sequence in the collection of _Klebsiella_ spp. (Fig. 1). PCR screening resulted in the amplification of a positive 250-bp product in 44 of 51 strains (86.2%). It is worthy to note that the seven IS26-negative strains were among the oldest in the collection, mostly dating from before 1991, and none harbored an integron. All 20 strains that harbored both _aadA2_ and _aadB_ gene cassettes and all 13 strains with a single _aadB_ cassette had a _blaSHV_ gene with its associated upstream IS26 (Table 1), suggesting that these strains are likely to harbor a plasmid with features similar to those of pES1.

**Conclusion.** We and others (7) have demonstrated the close association between the _aadB_ gene cassette with _blaSHV_ genes in ESBL-producing _Klebsiella_, and in the present study, we show that both resistance genes are located on a single 28-kb conjugative plasmid. Our findings and those of others (12, 15–17, 23) also demonstrate that an IS26 insertion element upstream of a _blaSHV_ gene is commonly associated with conjugative plasmids in ESBL-producing organisms. _Klebsiella pneumoniae_ strains carrying IS26 upstream of _blaSHV-12_ or _blaSHV-2a_ have also been recently reported in Korea (8). Taken together, these findings suggest that the multiresistant phenotype of ESBL-producing _Klebsiella_ spp. is likely to be mediated by a conjugal plasmid that contains an integron, IS26, and either _blaSHV-2a_, _blaSHV-5_, or _blaSHV-12_. The potential of the integron-encoded site-specific recombination system to capture new cassettes means that continued selection of pES1-like plasmids is likely. Further studies concerning the evolution and dissemination of these plasmids are therefore warranted.

We thank Sydney Bell, Porl Reinbott, Jeanette Pham and Barrie Gatus, Antibiotic Reference Laboratory, Department of Microbiology (SEALS), Prince of Wales Hospital, for the provision of strains used in this study.

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