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Site-Specific Deletion and Rearrangement of Integron Insert Genes Catalyzed by the Integron DNA Integrase

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Deletion of individual antibiotic resistance genes found within the variable region of integrons is demonstrated. Evidence for gene duplications and rearrangements resulting from the insertion of gene units at new locations is also presented. Deletion, duplication, and rearrangement occur only in the presence of the integron-encoded DNA integrase. These events are precise and involve loss or gain of one or more complete insert units or gene cassettes. This confirms the recent definition of gene cassettes as consisting of the gene coding sequences, all except the last 7 bases of the 59-base element found at the 3′ end of the gene, and the core site located 5′ to the gene (Hall et al., Mol. Microbiol. 5:1941–1959, 1991) and demonstrates that individual gene cassettes are functional units which can be independently mobilized. Both deletions and duplications can be generated by integrase-mediated cointegrate formation followed by integrase-mediated resolution involving a different pair of sites. However, deletion occurs 10 times more frequently than duplication, and we propose that the majority of deletion events are likely to involve integrase-dependent excision of the gene unit to generate a circular gene cassette. The implications of these findings in understanding the evolution of integrons and the spread of antibiotic resistance genes in bacterial populations is discussed.

It is now well established that many antibiotic resistance genes found in the plasmids and transposons of gram-negative bacteria are located at a unique site within a conserved DNA segment (4, 9, 10, 20, 23, 26, 29). The conserved flanking sequences extend for 1.36 kb 5′ to the genes (10) and at least 2.0 kb on the 3′ side (26). These 5′ and 3′ conserved segments, together with the variable region which contains the resistance genes, constitute a novel family of potentially mobile DNA elements which have been named integrons (26).

The variable region of integrons includes one or more genes, which are most commonly antibiotic resistance genes. Genes found in the variable region all have the same orientation and are transcribed from a promoter in the 5′ conserved segment (see references 9 and 26). Each inserted gene is associated at its 3′ end with a short imperfect inverted repeat element, known as a 59-base element (4, 9). Despite differences in sequence and length, members of the 59-base element family are active as sites for site-specific cointegration events catalyzed by the integron DNA integrase (9, 16). The DNA integrase is encoded by the *int* gene, which is located in the 5′ conserved segment (10, 16, 21, 26). A shorter core recombination site, defined as the consensus GTTRRRY (R = purine, Y = pyrimidine), was predicted from comparisons of sequences at the boundaries of conserved and insert regions and at the boundaries between gene pairs (9, 26). Core recombination sites are found at the junction of the 5′ conserved segment and the first insert gene and as the last 7 bases of 59-base elements. The recombination crossover point, predicted from comparisons of naturally occurring sequences and determined from sequences of the junctions in experimentally derived cointegrates, is within or on either side of the GTT, which is conserved in all core sites (9).

From examination of the published sequences of integron insert genes, we have recently defined the insert units. For individual gene inserts, three features are conserved: the coding region of the gene, all except the last 7 bases of the specific 59-base element associated with the 3′ end of the gene, and the core site located 5′ to the gene (9); it was proposed that together these sequences constitute a gene cassette (Fig. 1). The core site found at the 3′ end of the 59-base element is excluded from this unit and constitutes either the 5′ core site of the following gene or the first 7 bases of the 3′ conserved segment. The simplest explanation for this arrangement is that prior to insertion of the gene, the 5′ core site and all except the last 7 bases of the 59-base element at the 3′ end of the gene were covalently associated to form a circle. We have therefore proposed that the primary insertion of genes into integrons involves circularized gene units, which are inserted by a single site-specific recombination event catalyzed by the integron DNA integrase (9). We have also proposed that the naturally occurring integron inserts which include only one gene have arisen by insertion of a circular gene cassette into an ancestral integron which contains neither a gene nor a 59-base element and that integrons with more than one insert arose by subsequent sequential insertion of further gene insert units. The ancestral integron should then include only the 5′ and 3′ conserved segments with a single core recombination site at the junction, and this structure has been identified (3).

An extension of the circular gene insertion model is that integron insert genes can also be excised by the integrase, and the circular gene cassette generated can be reinserted at a new location (9). If the circular gene insertion model for the evolution of integron inserts is correct, *int*-dependent deletion and reinsertion of gene cassettes should be observed, and excision and reinsertion should be precise and should involve a circular gene cassette consisting of the gene, its 59-base element, and its 5′ site (Fig. 1). We have recently demonstrated that the integron integrase catalyzes the reso-

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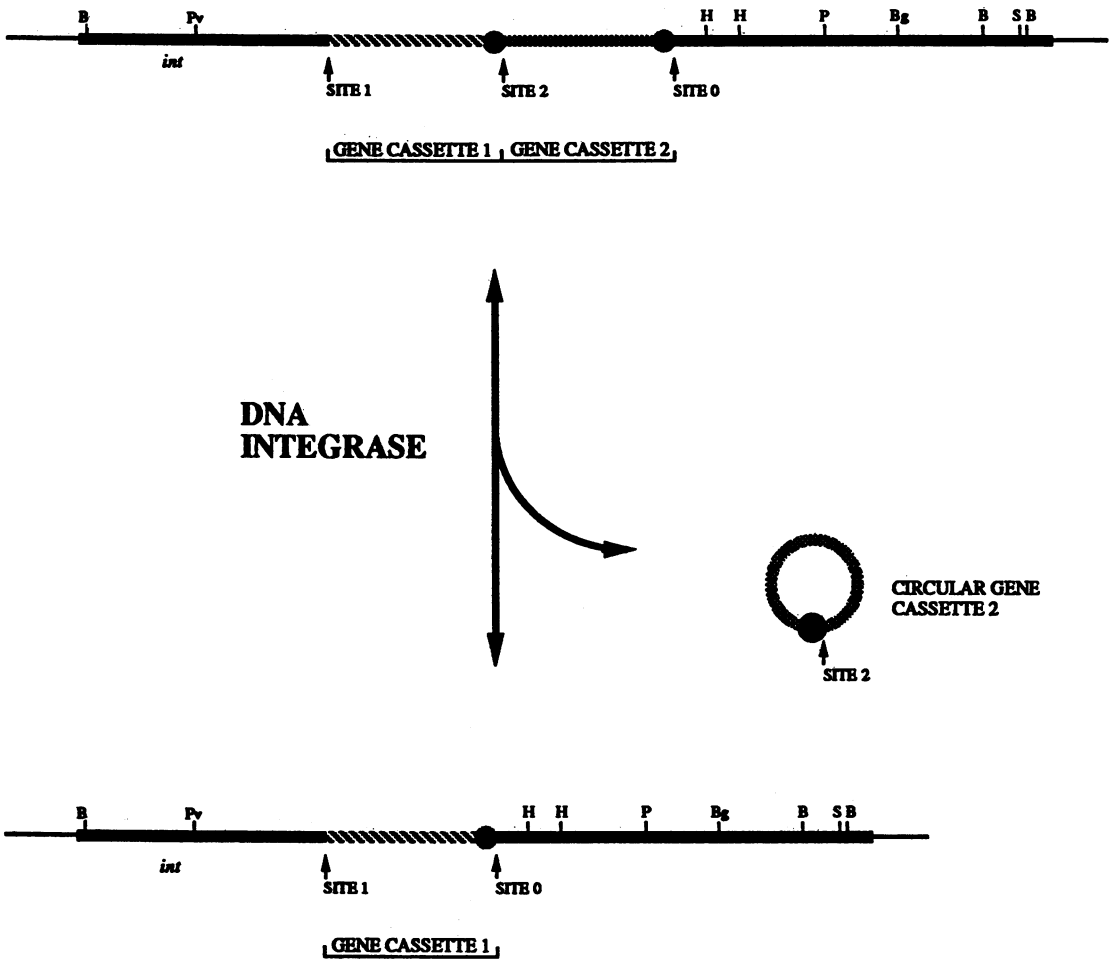

FIG. 1. Circular model for gene insertion. The conserved segments of the integrons are shown as thick lines, and the location of the *int* gene encoding the DNA integrase is indicated. Two gene cassettes are shown, each of which includes a core recombination site (GTTRRRY), the gene coding region, and all except the last 7 bases of a 59-base element (●). The recombination between core sites 2 and 0 would lead to loss of the gene cassette 2 and generate the circular gene cassette shown. The reverse reaction would lead to insertion of the gene cassette 2. Restriction sites in the conserved segments: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I.

lution of experimental cointegrates (9), and gene excision is thus a formal possibility. The *recA*-independent deletion of genes which are now known to be integron insert genes has been reported (23, 32), and the boundaries of the deleted regions were shown by electron microscopy to be close to the boundaries of an *aadA* gene (32). However, a requirement for the integron integrase has not been demonstrated, and the sequence of only a single deletion product has been reported (23). Though the authors' interpretation (23) of the location of the crossover which generated this deletion is incorrect, the deletion is precise in this case (see reference 9).

Here, we present experimental evidence which demonstrates that deletion of insert genes is an *int*-dependent, *recA*-independent process and that deletion involves precise loss of one or more gene cassettes. Evidence for the *int*-dependent rearrangement of gene cassettes to form insert regions with two copies of a gene cassette and other precise rearrangements is also presented. The independent mobilization of individual gene cassettes provides direct evidence that gene cassettes are functional units. Though both loss and duplication of gene cassettes can occur via formation and resolution of cointegrates, the relative frequencies of plasmids which have lost or gained the same insert gene suggest that the majority of deletion events have not occurred by this route. We propose that the major route for gene loss involves recombination between the pair of sites flanking the gene insert. Such excision events are formally identical to cointegrate resolution and should also generate a covalently closed circular gene cassette.

MATERIALS AND METHODS

Bacterial strains and plasmids. UB5201 is F^- *pro met recA56 gyrA* (6). Plasmids, containing integron insert fragments, used in this study are shown in Table 1 and Fig. 2. Plasmids were introduced into UB5201 by electroporation using a Gene-Pulser (Bio-Rad, Richmond, Calif.). pXS1 (11) was used as a source of the 3.64-kb *Bam*HI fragment of Tn*1696* which was cloned into the *Bam*HI site of pACYC184 (5) to generate pRMH52 (Cm^r Gm^r Sp^r Int^+). In pRMH52 the *Bam*HI site in the *cmlA* gene is closest to the *tet* gene promoter, and deletion of an *Sph*I fragment of pRMH52 generated the Int^- derivative pRMH57, which has lost part

TABLE 1. Plasmids

Plasmid	Description	Relevant phenotype	Source or reference
pACYC184		Cm^r Tc^r	5
pXS1	3.7-kb *Bam*HI fragment of Tn*1696* inserted into pAT153	Gm^r Sp^r Int^+	11
pSU2056	1.18-kb *Bam*HI-*Rsa*I fragment from Tn*21* inserted into pUC9	Ap^r Int^+	16
pUCD105	Contains 2.82-kb *Pst*I-*Pvu*II fragment from pSa (with the *Pvu*II site converted to a *Xba*I site)	Km^r Sp^r Int^-	29
pSU2718	pACYC184-derived cloning vector	Cm^r	15
pRMH52	3.7-kb *Bam*HI fragment of pXS1 inserted into *Bam*HI site of pACYC184 with *Sph*I site nearest to *Sph*I site of pACYC184	Cm^r Gm^r Sp^r Int^+	This study
pRMH57	*Sph*I deletion derivative of pRMH52	Cm^r Gm^r Sp^r Int^-	This study
pRMH58	2.82-kb *Pst*I-*Xba*I fragment from pUCD105 inserted into pSU2718	Cm^r Km^r Sp^r Int^-	This study
pRMH59	*Int*-mediated loss of *aadA* from pRMH52; integron insert *aacC1*-orfE-(*cmlA*)	Cm^r Gm^r Int^+	This study
pRMH61	*Int*-mediated loss of *aacC1* and orfE from pRMH52; integron insert *aadA2*-(*cmlA*)	Cm^r Sp^r Int^+	This study
pRMH62	*Int*-mediated rearrangement of pRMH52; integron insert *aadA2*-orfE-*aadA2*-(*cmlA*)	Cm^r Sp^r Int^+	This study
pRMH63	*Int*-mediated loss of *aadA2* and orfE from pRMH52; integron insert *aacC1*-(*cmlA*)	Cm^r Gm^r Int^+	This study
pRMH65	*Int*-mediated loss of orfE from pRMH52; integron insert *aacC1*-*aadA2*-(*cmlA*)	Cm^r Gm^r Sp^r Int^+	This study
pRMH75	*Int*-mediated rearrangement of pRMH52; integron insert *aadA2*-*aacC1*-orfE-*aadA2*-(*cmlA*)	Cm^r Gm^r Sp^r Int^+	This study
pRMH77	*Int*-mediated rearrangement of pRMH52; integron insert *aadA2*-*aacC1*-*aadA2*-(*cmlA*)	Cm^r Gm^r Sp^r Int^+	This study
pRMH82	*Int*-mediated rearrangement of pRMH52; integron insert *aadA2*-*aacC1*-orfE-(*cmlA*)	Cm^r Gm^r Sp^r Int^+	This study
pRMH67	*Int*-mediated loss of *aadA2* from pRMH58; integron insert *aacA4*	Cm^r Km^r Int^-	This study
pRMH69	*Int*-mediated loss of *aacA4* from pRMH58; integron insert *aadA2*	Cm^r Sp^r Int^-	This study
pRMH71	*Int*-mediated loss of *aacA4* and *aadA2* from pRMH58; no integron insert	Cm^r Int^-	This study

of the *int* gene. pUCD105 (30) contains the 2.8-kb *Pst*I-*Pvu*II fragment from pSa with the *Pvu*II site converted to an *Xba*I site. The *Pst*I-*Xba*I fragment of pUCD105 was transferred to the pACYC184-derived cloning vector pSU2718 (15) to generate pRMH58 (Cm^r Km^r Sp^r Int^-). pSU2056 (16) contains the 1,176-bp *Bam*HI-*Rsa*I fragment, from Tn*21*, which completely includes the *int* gene, inserted into pUC9.

Growth of cultures. Bacteria were cultured in L broth (17). Antibiotic concentrations used were 50 μg and 5 mg of spectinomycin per ml, 40 μg of gentamicin per ml, 25 μg of

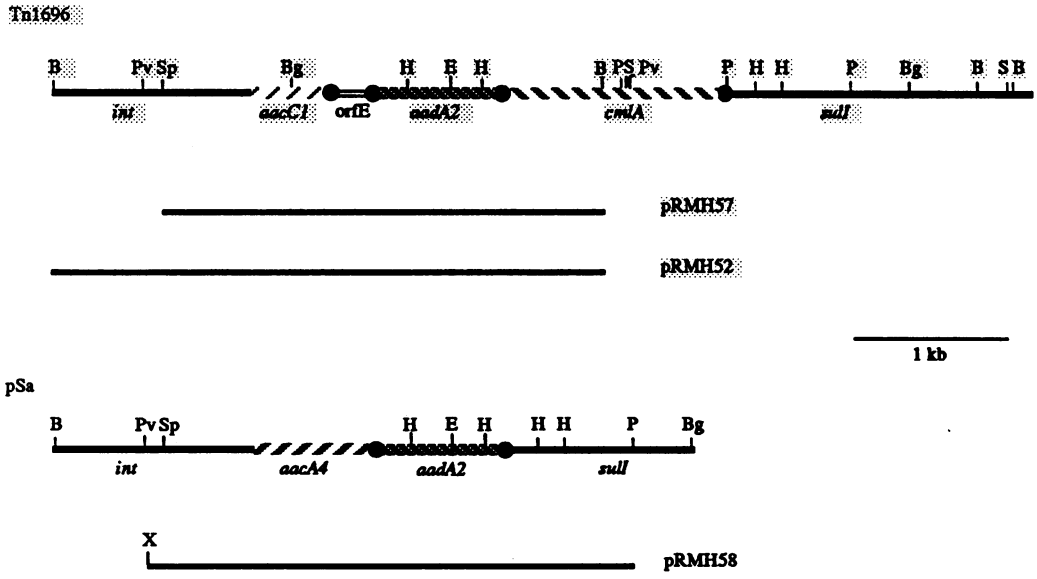

FIG. 2. Structures of insert regions and the cloned integron segments from In4 (Tn*1696*) and In6 (pSa). In4 includes four insert units, *aacC1*, orfE (formerly X), *aadA2*, and *cmlA*. The DNA fragment present in pRMH52 and pRMH57 does not include the complete *cmlA* insert. Only the part of the integron In6 which includes the two inserts *aacA4* and *aadA2* is shown. Both insert genes are present in pRMH58. Symbols and restriction sites are defined in the legend to Fig. 1 (E, *Eco*RI; Sp, *Sph*I; X, *Xba*I).

chloramphenicol per ml, 25 μg of kanamycin per ml, and 25 μg of ampicillin per ml. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Isolation and analysis of deletions and reinsertions. For deletion and duplication experiments, cells were initially grown in L broth supplemented with all antibiotics to which the strain was resistant, including integron- and vector-encoded determinants. After overnight incubation at 37°C, the culture was diluted 2,000-fold into 2 ml of fresh L broth containing only chloramphenicol and again grown to stationary phase. Plasmid DNA was isolated by the alkaline lysis method (1), followed by treatment with RNase A (100 μg/ml; Sigma) for 15 min, phenol extraction, and a further ethanol precipitation. DNA was dissolved in a final volume of 50 μl.

To isolate deletions, DNA was introduced into UB5201 under conditions in which fewer than 1 in 100 transformants received more than one DNA molecule. Transformants were selected on chloramphenicol plates, and colonies were then patched onto media containing appropriate antibiotics. Plasmids containing a duplication of the *aadA2* gene were isolated after transformation of UB5201 with the same DNA preparations and selection on spectinomycin (5 mg/ml). Those isolates were also screened for resistance to gentamicin, chloramphenicol, and ampicillin. Ampicillin-resistant colonies also contain pSU2056 and were not studied further.

DNA was prepared from stationary-phase cultures of cells containing plasmids with suspected deletions or reinsertions by the alkaline lysis method and subjected to digestion by restriction enzymes according to the manufacturer's instructions. Digestion products were analyzed by electrophoresis on 1% agarose gels.

DNA fragment isolation and DNA sequencing. The 517-bp *Hin*dIII fragment of the *aadA2* gene from pXS1 (In4) was purified from a 1% agarose gel by using GeneClean (Bio 101 Inc., La Jolla, Calif.), cloned into *Hin*dIII-digested M13mp18 (34) in both orientations, and sequenced. The polymerase chain reaction (PCR) product resulting from amplification by primers RH50 and RH51 was ethanol precipitated, digested with *Eco*RI and *Hin*dIII, and purified by electrophoresis through 4% acrylamide. The gel was stained with ethidium bromide, a single band was excised, and the DNA was eluted. The product was cloned into M13mp18 digested with *Eco*RI and *Hin*dIII. M13 clones were sequenced by the chain termination technique (22) with a Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio) and the −40 sequencing primer. For double-strand sequencing, plasmid DNA was isolated by using the miniprep procedure of Jones and Schofield (14) except that DNA was resuspended in a final volume of 5 μl. Sequencing was performed by using the procedure of Hsiao (13).

Calculation and measurement of fragment lengths. The sequence of the Tn*1696* integron insert region has been determined (2, 27, 33) except for the *Hin*dIII fragment internal to the *aadA2* gene which was determined in this study. An extra A residue present in the 59-base element of insert X was also identified in this study. This residue is located between bases 2105 and 2106 in the published sequence (33) and introduces a termination codon for the open reading frame which commences at 1902 to 1904 in this sequence. This reading frame has been designated orfE, and insert X (9) is thus renamed orfE. The sequences of the *aadA2* gene and part of *aacA4* from pSa have been determined (30), and the sequence of the 3′ end of the *aadA2* gene has been redetermined (31) and shown to be identical to the corresponding region of *aadA2* in Tn*1696* (27). The sequence of the complete *aacA4* insert found in Tn*1331* has been determined (18, 19), and this sequence was used to calculate the size of the *aacA4* cassette. Sizes of gene cassettes are measured from the conserved G in the 5′ core site to the base preceding the conserved G residue in the site located at the end of the 59-base element associated with the 3′ end of the gene. Insert sizes are 577 bp for *aacC1*, 262 bp for orfE, 637 or 638 bp for *aacA4*, and 856 bp for *aadA2*. Sizes of experimentally observed fragments were calculated by using the fragments from an *Eco*RI digest of bacteriophage SPP1 or a *Hpa*II digest of pUC19 (Bresatec Ltd., Adelaide, South Australia, Australia) as standards.

End labelling and hybridization. For end-labelling experiments, 10 μl of DNA isolated from cells grown without selection for insert resistances was digested with *Eco*RI for 2 h at 37°C, followed by the addition of 2 U of Klenow fragment of DNA polymerase I (Pharmacia Biotechnology, Uppsala, Sweden) and 10 μCi of [α-^{32}P]dATP. After 60 min at room temperature, the reaction was stopped by the addition of EDTA to 20 mM. End-labelled fragments were separated by electrophoresis at 45 V for 16 h on a 25-cm 1% agarose gel, then fixed in 1% cetyltrimethylammonium bromide–50 mM sodium acetate (pH 5.5) for at least 2 h and dried onto Whatman 3MM paper under vacuum at 60°C.

For hybridization experiments, *Eco*RI-digested DNA samples were separated by electrophoresis at 45 V for 16 h on a 25-cm 1% agarose gel. After treatment with 0.25 M HCl, the DNA was denatured with 1.5 M NaCl–0.5 M NaOH, transferred to a Zeta-Probe membrane (Bio-Rad) in 1 M ammonium acetate–50 mM NaOH by the method of Southern (24), and UV-cross-linked to the membrane. A ^{32}P-labelled probe was prepared from the 517-bp *Hin*dIII fragment of the *aadA2* gene by the random priming method (7) by using a Hexaprime DNA labelling kit (Bresatec). Membranes were prehybridized for at least 30 min in a solution containing 0.52 M Na phosphate (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin, and 1 mM EDTA and then hybridized for 16 h at 65°C in the same solution containing probe. Membranes were then washed at room temperature with two changes of 100 mM Na phosphate (pH 7.2)–5% SDS–0.5% bovine serum albumin–1 mM EDTA and six changes of 100 mM Na phosphate (pH 7.2)–1% SDS–1 mM EDTA; the last wash was at 60°C.

Primers and PCR conditions. PCR primers (see Table 3) were synthesized by using an Applied Biosystems 391 PCR-mate DNA synthesizer and purified by using an oligonucleotide purification cartridge (Applied Biosystems, Foster City, Calif.). PCR reactions were carried out in 100 μl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–3 mM $MgCl_2$ containing 200 μM each dATP, dGTP, dCTP, and dTTP, 1 μM each primer, 2.5 U of *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.), and 1 μl of a 1:10 dilution of DNA that had been digested with *Eco*RI (for primer pairs RH50-RH51, RH52-RH53, RH54-RH51, and RH56-RH53), *Bgl*I (for RH54-RH55), and *Bsp*1286 (for RH56-RH57 and RH55-RH56). After 32 cycles of sequential 1-min incubations at 92, 65, and 72°C in a Cherlyn Intelligent Heating Block (Cherlyn Electronics Ltd, Cambridge, United Kingdom), 10-μl samples of the reaction mixture were analyzed by electrophoresis through a 2% agarose–1% Nusieve (FMC BioProducts, Rockland, Maine) gel.

Nucleotide sequence accession number. The *Hin*dIII fragment internal to the *aadA2* gene which was determined in this study has been assigned GenBank DNA data base accession no. M 60454.

TABLE 2. Deletion of antibiotic resistance genes

Strain and plasmid(s)	No. of Cm^r transformants screened	No. with phenotype		
		Gm^r Sp^s or Km^r Sp^s	Gm^s Sp^r or Km^s Sp^r	Gm^s Sp^s or Km^s Sp^s
UB5201/pRMH52	3,600	0	0	0
UB5201/pRMH52/pSU2056	2,500	93	2	0
UB5201/pRMH58	1,000	0	0	0
UB5201/pRMH58/pSU2056	2,500	80	5	2

RESULTS

Integrase-dependent deletion of gene inserts. While *recA*-independent deletion of antibiotic resistance genes which are now known to be integron inserts has been reported (23, 32), this process has not been shown to be dependent on the integron DNA integrase. To facilitate the detection of specific deletion events, we have utilized the plasmid pSU2056 (16), which includes the *int* gene of the In2 (Tn*21*) integron coupled to the β-galactosidase promoter of pUC9. This construction increases the expression of the integron integrase and increases the frequency of cointegrate formation at least 1,000-fold (16). DNA segments containing insert genes from the integrons In4, found in Tn*1696*, and In6, found in the plasmid pSa, were cloned into pACYC184 or pSU2718, both of which are compatible with pUC9. The structure of the original integrons and the cloned DNA segments is shown in Fig. 2. The In4 integron includes four insert units, *aacC1*-orfE-*aadA2*-*cmlA* (9, 27), the first three of which are completely included in the cloned fragments in pRMH52 and pRMH57. The In6 integron includes two inserts, *aacA4*-*aadA2* (9, 30), both of which are included in the cloned fragment in pRMH58.

To isolate deletions, UB5201 (*recA* mutant) cells containing either pRMH52 or pRMH58, without or with pSU2056, were grown for 10 generations in the absence of selection for insert gene antibiotic resistances. DNA was isolated and used to transform UB5201. Transformants were selected on chloramphenicol (25 μg/ml), which selects for the vector marker of pACYC184 and pSU2718, and screened for loss of insert genes by using gentamicin (*aacC1*), spectinomycin (*aadA2*), or kanamycin (*aacA4*). Loss of orfE could not be monitored, as no phenotype has been ascribed to this insert. The results are shown in Table 2. Gene loss was observed only when pSU2056 was present, and the frequency of deletion was 3×10^{-2} to 4×10^{-2} for *aadA2*, 8×10^{-4} for *aacC1*, and 2.8×10^{-3} for *aacA4*. No deletions were observed in the absence of the overexpressed *int* gene, indicating that deletion is an integrase-mediated event. This interpretation is not complicated by the presence of the complete In4 *int* gene in pRMH52, as the frequency of deletion sustained by this gene in its natural configuration is too low to be detected here. The frequency of loss of *aadA2* reported by Schmidt et al. (23) was 4×10^{-4} when a plasmid which contains the In4 *int* gene was used. The substantial differences between the deletion frequencies for *aadA2* and for *aacC1* and *aacA4* may reflect the fact that the latter genes are each the first gene in their respective inserts and thus flanked on the 5′ side by only a core recombination site. In cointegration experiments, recombination between two core sites located at the end of a 59-base element is highly preferred over events involving the core site at the junction of the 5′ conserved segment and one 59-base element (9).

Deletion of gene inserts is precise. To confirm that the deletion of genes observed in these experiments was a precise process, restriction digestion of DNA isolated from deletion derivatives was performed. The structures of possible precise deletions with the phenotypes detected and the predicted size of the resulting fragments are shown in Fig. 3. DNA isolated from 46 spectinomycin-sensitive isolates from pRMH52 was digested with *Bam*HI, and in 37 isolates a 2.8-kb fragment replaced the 3.64-kb *Bam*HI fragment of pRMH52. This is consistent with the loss of the 856-bp *aadA2* insert unit. In the remaining nine plasmids, a *Bam*HI fragment of 2.5 kb was observed, consistent with the loss of both the *aadA2* and orfE inserts (1,118 bp). These assignments were confirmed by digestions with *Eco*RI and *Bam*HI and with *Bgl*I. Similarly, DNA from each of the three types of deletion derivatives isolated from pRMH58 was digested with *Pst*I and *Xba*I, and fragment sizes were consistent with precise excision of *aadA2* (856 bp), *aacA4* (637 bp), or both genes (1,493 bp).

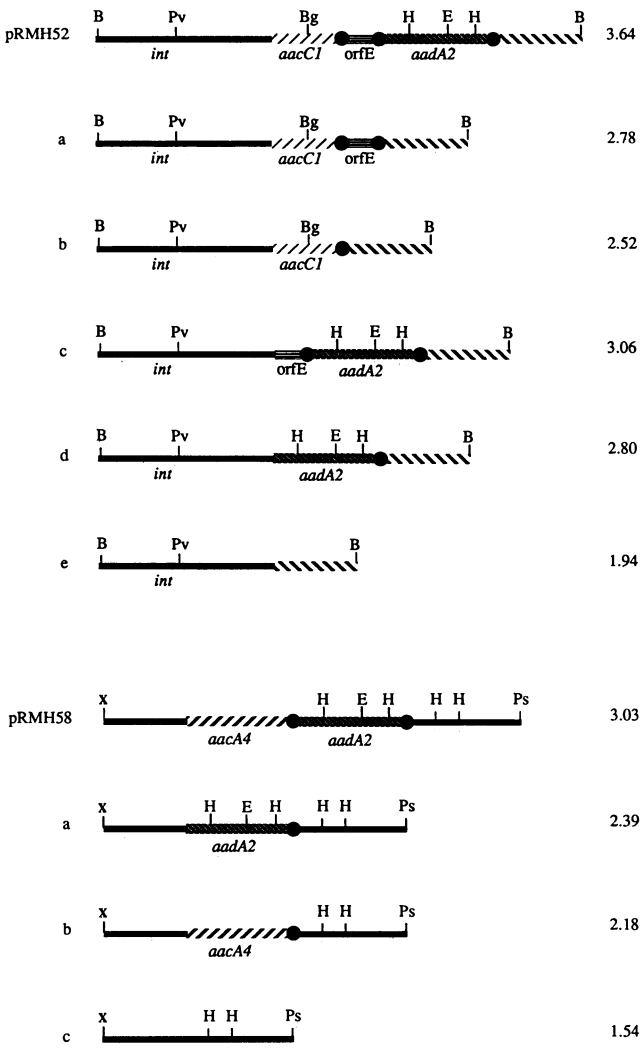

FIG. 3. Structures of deletion derivatives of pRMH52 and pRMH58. The structures of all possible deletions which can be formed by site-specific recombination between pairs of core sites flanking insert genes are shown. The predicted sizes of the *Bam*HI and *Xba*I-*Pst*I fragments are indicated. Symbols and restriction sites are defined in the legends to Fig. 1 and 2.

A. Deletions derived from pRMH52

	Insert Order	Sequence of Junction	Junction
Tn*1696*	*aacC1-orfE-aadA2-cmlA*	GTCGCCCTAAAACAAA **GTTAG**GT GGCTCAAGTATGGGCA	5'cons-*aacC1*
Tn*1696*		GCCGCTTACCTTGGCC **GTTAG**AC ATCATGAGGGTAGCGG	*orfE-aadA2*
pRMH61	*aadA2-(cmlA)*	GTCGCCCTAAAACAAA **GTTAG**AC ATCATGAGGGTAGCGG	5'cons-*aadA2*
Tn*1696*		CCGGCTTAACTCA**GGC GTT**AGTC CCATCACCCGCTTTAT	*aacC1-orfE*
Tn*1696*		GCGGCTTAACTCC**GGC GTT**GGGC GCACAATAAGGCTCCT	*aadA2-cmlA*
pRMH63	*aacC1-(cmlA)*	CCGGCTTAACTCA**GGC GTT**GGGC GCACAATAAGGCTCCT	*aacC1-(cmlA)*
Tn*1696*		GCCGCTTACCTTGGC**C GTT**AGAC ATCATGAGGGTAGCGG	*orfE-aadA2*
Tn*1696*		CCGGCTTAACTCCGG**C GTT**GGGC GCACAATAAGGCTCCT	*aadA2-cmlA*
pRMH59	*aacC1-orfE-(cmlA)*	GCCGCTTACCTTGGC**C GTT**GGGC GCACAATAAGGCTCCT	*orfE-(cmlA)*
Tn*1696*		CCGGCTTAACTCA**GGC GTTAG**TC CCATCACCCGCTTTAT	*aacC1-orfE*
Tn*1696*		GCCGCTTACCTTG**GCC GTTAG**AC ATCATGAGGGTAGCGG	*orfE-aadA2*
pRMH65	*aacC1-aadA2-(cmlA)*	CCGGCTTAACTCA**GGC GTTAG**AC ATCATGAGGGTAGCGG	*aacC1-aadA2*

B. Deletions derived from pRMH58

	Insert Order	Sequence of Junction	Junction
pSa	*aacA4-aadA2*	GTCGCCCTAAAACAAA **GTTAG**GC ATCACAAAGTACAGCA	5'cons-*aacA4*
pSa		CGCCCTCATGTCAAAC **GTTAG**AC ATCATGAGGGTAGCGG	*aacA4-aadA2*
pRMH69	*aadA2*	GTCGCCCTAAAACAAA **GTTAG**AC ATCATGAGGGTAGCGG	5'cons-*aadA2*
pSa		CGCCCTCATGTCAAA**C GTTAG**AC ATCATGAGGGTAGCGG	*aacA4-aadA2*
pSa		GCGGCTTAACTCCGG**C GTTAG**AT GCACTAAGCACATAAT	*aadA2*-3'cons
pRMH67	*aacA4*	CGCCCTCATGTCAAA**C GTTAG**AT GCACTAAGCACATAAT	*aacA4*-3'cons
pSa		GTCGCCCTAAAACAAA **GTTAG**GC ATCACAAAGTACAGCA	5'cons-*aacA4*
pSa		GCGGCTTAACTCCGGC **GTTAG**AT GCACTAAGCACATAAT	*aadA2*-3'cons
pRMH71	-	GTCGCCCTAAAACAAA **GTTAG**AT GCACTAAGCACATAAT	5'cons-3'cons

FIG. 4. Sequences of junctions between gene cassettes in deletions. The sequences of the boundaries between pairs of inserts or between insert cassettes and the conserved segment sequences in the parental insert regions of Tn*1696* (In4) or pSa (In6) are shown with the sequences determined for the new boundaries present in the deletions isolated in this study. The core site is indicated, and the GTT triplet is shown in boldface type. The crossover required to generate the deletion occurs within or on either side of the underlined bases.

For two representatives of each deletant type observed, the new boundary generated by deletion of the gene(s) was sequenced. These sequences are shown in Fig. 4, together with the sequences of the junctions flanking the insert in the parental configuration. In all cases the boundary generated was identical to that predicted by deletion of one or more complete gene cassettes. Thus deletion is precise, and the recombination crossover point can be confined to within or on either side of the GTT triplets which bound the gene cassette(s). This is identical to the crossover point identified from sequences of new junctions in experimental cointegrates and predicted from comparisons of naturally occurring insert boundaries (9). These data demonstrate directly that the gene cassettes defined by comparisons of sequences of natural insert regions (9) are functional units which can be independently lost.

Two deletions isolated from pRMH58 which have lost both the *aacA4* and *aadA2* genes contain no gene inserts and thus the 5′ and 3′ segments are fused as in the proposed ancestral integron. The sequence of the boundary between the 5′ and 3′ conserved segments in these plasmids is precisely that predicted for the ancestral integron. The sequence includes only the conserved core site GTTAGAT which is found as the first 7 bases of the 3′ conserved segment (site 0 in Fig. 1) in integrons which contain inserts (9, 26). The generation of these plasmids by gene loss demonstrates that the absence of gene inserts from integrons found in naturally occurring plasmids cannot necessarily be interpreted as indicating an early position for that particular integron in the evolution of integrons, as has been proposed for In0 found in plasmid pVS1 (3).

Detection of deleted gene cassettes is masked by the presence of gene duplications. If deletion occurs via a single *int*-mediated site-specific recombination event, the second product of the reaction should be a circularized gene unit. These products cannot be maintained stably, as they do not contain replication functions; however, the high frequency of deletion of the *aadA2* gene, observed in the presence of pSU2056, may allow direct detection of the circular gene cassettes. The DNA used to isolate the deletions described above was digested with *Eco*RI, which cleaves within the *aadA2* gene, to linearize the postulated circular molecules and either end labelled prior to separation on an agarose gel or separated on an agarose gel and hybridized with an *aadA2*-specific probe. The results obtained with pRMH52 are shown in Fig. 5. An end-labelled fragment of 0.86 kb, and thus corresponding in size to the *aadA2* gene cassette, was detected only in DNA samples isolated from cells containing both pSU2056 and pRMH52 (Fig. 5A), indicating that this fragment arises as the result of one or more *int*-mediated

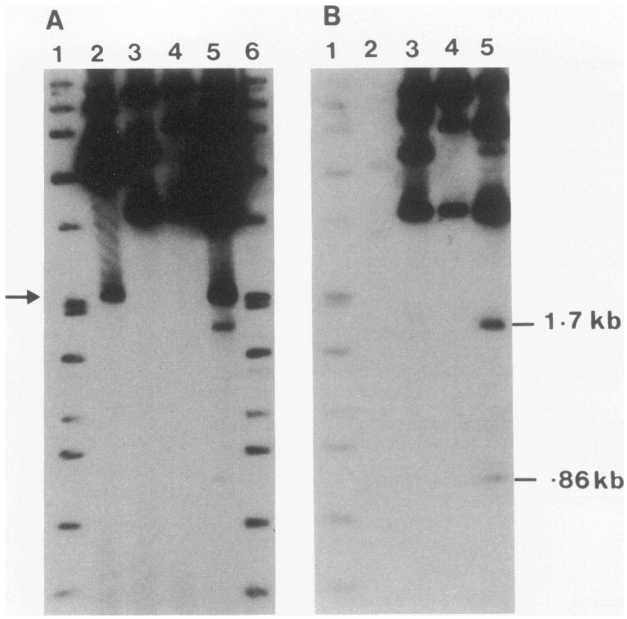

FIG. 5. End labelling and hybridization experiments. Plasmid preparations from cells containing one or more plasmids were digested with *Eco*RI and then divided into two, and one half was end labelled. Digestion products were separated on an agarose gel, and the unlabelled half was cut off, blotted to a membrane, and hybridized with the radioactively labelled 517-bp *Hin*dIII fragment of *aadA2*. (A) End-labelled samples. (B) Hybridized samples. Lanes 1 and 6, size markers of 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, and 0.48 bp from an *Eco*RI digest of bacteriophage SPPI DNA; lanes 2, pSU2056; lanes 3, pRMH57; lanes 4, pRMH52; lanes 5, pRMH52 with pSU2056. The positions of the 860- and 1,700-bp fragments are indicated. The band marked by the arrow (2.1 kb) is found only in DNA samples from cells containing pSU2056, and its origin has not been investigated.

events. This fragment was also detected by hybridization with an *aadA2* gene probe (Fig. 5B), confirming that it includes the *aadA2* gene. A second fragment of 1.7 kb is also detected in these experiments. This band corresponds in size to the combined length of *aacC1*, orfE, and *aadA2* and also hybridizes with the *aadA2* probe. Equivalent results were obtained when the plasmid used was pRMH58; fragments of 0.86 and 1.5 kb, corresponding in size to *aadA2* alone and *aacA4* and *aadA2* together, respectively, were detected (data not shown).

However, in both cases the larger band (1.7 or 1.5 kb) is more intense than the 0.86-kb band, and this does not correlate with the results of deletion experiments where excision of *aadA2* alone occurs at least 40-fold more frequently than deletion of both *aacC1* and *aadA2* or of both *aacA4* and *aadA2*. This discrepancy raised the possibility that the observed bands do not arise from excised circles. Bands of the observed sizes could also arise from plasmids containing two copies of *aadA2*, either adjacent (0.86 kb) or separated by the *aacC1* and orfE cassettes (1.7 kb) or the *aacA4* cassette (1.5 kb), and it is possible that such plasmids are present in the DNA sample. Indeed, when the same amount of undigested DNA from the strain containing pRMH52 and pSU2056 was separated on an agarose gel and hybridized with the *aadA2* probe, bands of a size corresponding to circular molecules of 0.86 or 1.7 kb were not detected (data not shown). Thus the bands observed in Fig. 5 are most likely to have arisen from plasmid molecules which contain two copies of the *aadA2* gene. A broad band of 2.1 kb is also observed in end-labelled *Eco*RI digests of all DNA samples isolated from cells containing pSU2056, including the control which contains only pSU2056. This band may represent a deletion product of pSU2056.

Isolation of plasmids with duplicated gene inserts. Plasmids with rearranged insert regions could arise either by reinsertion of excised gene cassettes at new locations or, as the integron integrase can catalyze both cointegration and resolution events (9, 16), by formation and resolution of cointegrates. The formation of plasmids with rearranged inserts, via cointegration of two plasmid molecules containing insert genes followed by resolution of the cointegrates, would necessarily involve different pairs of recombination sites for the two recombination events. In the study of deletants described above, one of the two Gm^s deletants derived from pRMH52 contained a *Bam*HI fragment larger than would be expected if the *aacC1* gene had been lost. Digestions with further enzymes revealed that this isolate had indeed lost the *aacC1* gene but now contained a second copy of the *aadA2* gene insert. This plasmid, designated pRMH62, has three cassettes, *aadA2*-orfE-*aadA2*, in the insert region. To confirm the presence of two copies of *aadA2*, the capacity for growth on high levels of spectinomycin was investigated. UB5201 cells containing pRMH62 grew on plates containing spectinomycin at 5 mg/ml, whereas UB5201 containing the parental plasmid pRMH52 did not form colonies at concentrations above 3 mg/ml. Growth on higher levels of antibiotics is consistent with the presence of two copies of the *aadA2* gene in pRMH62.

The selection for growth on spectinomycin (5 mg/ml) was then used to select for further plasmids potentially containing more than one copy of the *aadA2* gene cassette. The DNA prepared from UB5201 containing pRMH52 and pSU2056, which was used in the experiments shown in Table 2 and Fig. 5, was used to transform UB5201. The transformation frequency when 5 mg/ml of spectinomycin was used to select transformants was 150-fold lower than when spectinomycin (50 μg/ml) was used. This finding indicates that about 1 in 150 of the DNA molecules containing the *aadA2* gene may include two or more copies of this gene.

Analysis of DNA from such isolates by digestion with *Bam*HI, *Eco*RI, and *Bgl*I (Table 3) demonstrated that a second copy of the *aadA2* gene cassette had been inserted into the pRMH52 insert region in 41 of 55 isolates tested, and in 39 of these the second copy was inserted between the 5′ conserved segment and the *aacC1* gene to yield an insert region with the gene order *aadA2-aacC1-orfE-aadA2*. Both new boundaries in two such plasmids were sequenced, and insertion of the *aadA2* cassette was precise (Fig. 6A). The predominance of the insert arrangement *aadA2-aacC1*-orfE-*aadA2* correlates well with the fact that the intensity of the 1.7-kb *Eco*RI fragment (Fig. 5), which would arise from this arrangement, is far greater than that of the 0.86-kb fragment, which arises when the two *aadA2* genes are adjacent. Plasmids with adjacent *aadA2* inserts were not isolated. The remaining two plasmids with two copies of *aadA2* in the insert had lost the orfE insert, and the insert order was *aadA2-aacC1-aadA2*.

Seven further isolates also contained two copies of *aadA2* but were cointegrates formed between two pRMH52 molecules. Six of these cointegrates had the same structure with *aadA2* alone in one insert region and *aacC1*-orfE-*aacC1*-orfE-*aadA2* in the second insert region. The remaining seven isolates had only one copy of *aadA2*, and of these four had

TABLE 3. Properties of plasmids conferring resistance to spectinomycin (5 mg/ml)

Plasmids	Frequency	Total analyzed	No. with order of inserts				
			aadA2-aacC1-orfE-*aadA2*	*aadA2-aacC1-aadA2*	*aacC1-aadA2*	Other	Cointegrates[b]
pRMH52 + pSU2056[a]	6.7×10^{-3}	55	39	2	4	3	7
pRMH52 + pSU2056	1.1×10^{-2}	41	36	0	0	0	5
pRMH52	3.3×10^{-5}	10	2	6	0	1	1

[a] This DNA preparation was used to obtain the results shown in Table 2 and Fig. 5.
[b] Cointegrates with several different pairs of insert arrangements were found in this group.

lost the orfE insert (insert order *aacC1-aadA2*), one had the rearranged insert order *aadA2-aacC1*-orfE, and two had lost the *aacC1* and orfE cassettes (insert *aadA2*). The sequence of the new boundary in two plasmids which have lost the orfE insert is shown in Fig. 4, and the deletion has arisen by precise loss of the orfE cassette. As these isolates did not include two copies of *aadA2*, the increased level of resistance may result from increased production of the aminoglycoside adenylyl transferase due to the new position of the gene in the insert (see Discussion).

To determine whether the distribution of rearranged inserts reflects preferred integrase mediated events or simply events which have occurred early in the growth of the particular culture from which the plasmid DNA was isolated, plasmids conferring resistance to high levels of spectinomycin were isolated from DNA prepared from an independent culture and analyzed by digestion with restriction enzymes. The results are shown in Table 3. The predominant plasmid type was again that with a duplication of *aadA2* and the insert order *aadA2-aacC1*-orfE-*aadA2*. Plasmids with two copies of the *aadA2* gene cassette were also isolated from pRMH52 grown in the absence of pSU2056 (Table 3), albeit at much lower frequency.

Evidence for other rearranged products. The sensitive method of PCR amplification can also be used to detect DNA molecules present at low concentrations in the population. To demonstrate the existence of plasmids with adjacent duplications of the *aadA2* gene, pairs of primers were designed to PCR amplify the region of DNA which is continuous only in circular *aadA2* gene cassettes or when two correctly oriented copies of the *aadA2* gene are present. In these experiments, DNA samples were digested with *Eco*RI, which cleaves within the *aadA2* gene cassette. With the insert gene arrangement contained in pRMH52, pRMH57, and pRMH58, these primers face outwards towards the ends of the *aadA2* gene and no continuous target exists (Fig. 7A). In plasmids with rearranged inserts containing two copies of the *aadA2* gene, amplification of a DNA segment whose length is dependent on the order of genes in the insert should occur and the shortest product arises from adjacent *aadA2* genes (Fig. 7B). Circular intermediates would also be detected (Fig. 7C), but the data presented above indicate that duplications are substantially more abundant than circular intermediates. The sequences of the primers, RH50, RH51, RH52, and RH53, and their locations in the published sequences are shown in Table 4. When DNA isolated from cells containing pRMH52, pRMH57, or pRMH58 is used as a substrate, amplification of a DNA fragment with primer pairs RH50 and RH51 or RH52 and RH53 should occur only if a circular form of the *aadA2* gene cassette or a plasmid containing two copies of the *aadA2* gene is present in the population of DNA molecules. A band of the length predicted (Table 5) for a circular gene cassette or two adjacent *aadA2* cassettes was observed only with DNA preparations from cells containing pSU2056 with the plasmid containing integron insert genes (pRMH52, pRMH57, pRMH58) or with DNA from cells containing pRMH52 alone (Fig. 8; data for pRMH58 not shown). These bands cannot be interpreted as evidence for the precise *int*-mediated deletion of the *aadA2* gene as a circular gene cassette, as they are most likely to reflect the presence of plasmids with adjacent *aadA2* inserts. Plasmid pRMH52 contains the complete *int* gene from In4, and the production of a low level of integrase by this plasmid is detected by the more sensitive method of PCR amplification. When the number of PCR cycles was increased, longer products were also detected (data not shown).

The 309-bp RH50-RH51 amplification product from

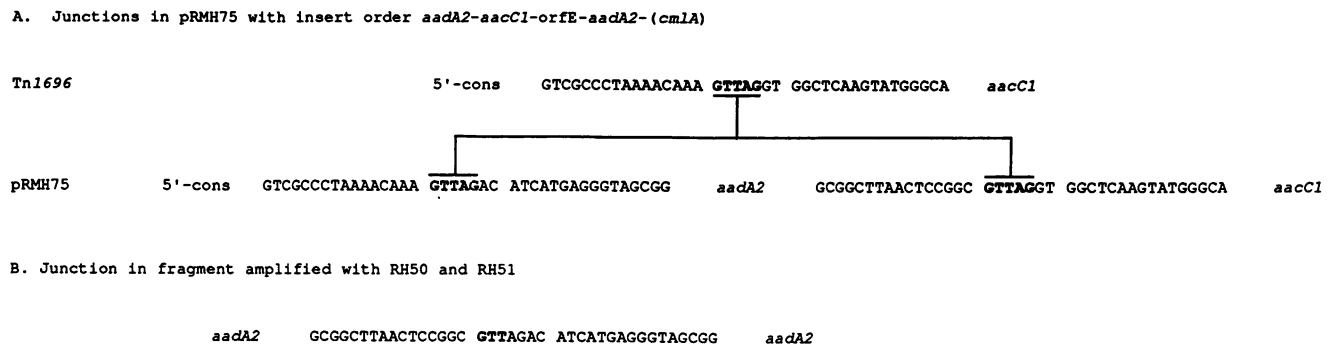

FIG. 6. Sequences of junctions from plasmids with duplicated *aadA2* genes. (A) The sequences of the boundary between the 5′ conserved segment and the *aacC1* gene cassette in Tn*1696* (In4) are shown with the sequences of the new boundary in plasmids with *aadA2* inserted at this position. The position of the insertion is indicated. (B) Sequence at the junction between adjacent *aadA2* inserts obtained by sequencing the PCR product amplified with primers RH50 and RH51 and plasmid DNA isolated from UB5201/pRMH52/pSU2056 as substrate.

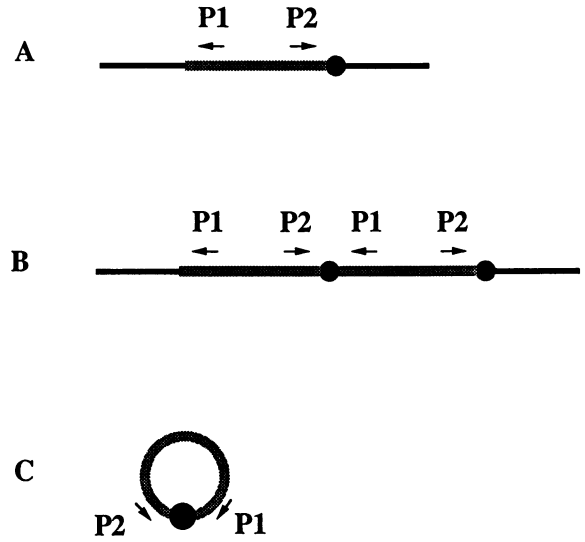

FIG. 7. Design of PCR amplification primers. (A) The two primers in each pair face outwards towards the ends of the gene insert, such that an amplification product will not be obtained from the parental insert configuration. A short fragment will be amplified from either a plasmid with two copies of the insert (B) or a circularized gene insert unit (C).

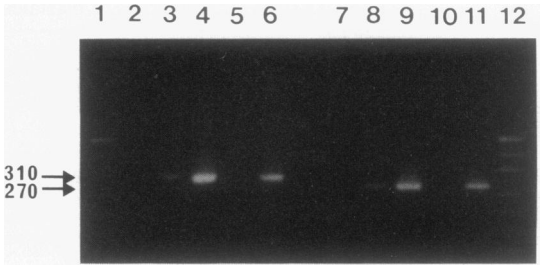

FIG. 8. PCR amplification of plasmid DNA. Lanes 1 and 12, size markers from *Hpa*II-digested pUC19 DNA of 501/489, 404, 331, 242, 189, 147, and 111/110 bp. The DNA samples used as substrates for amplification were isolated from cells containing the following plasmids: lanes 2 and 7, pSU2056; lanes 3 and 8, pRMH52; lanes 4 and 9, pRMH52 and pSU2056; lanes 5 and 10, pRMH57; lanes 6 and 11, pRMH57 and pSU2056. DNA fragments were amplified with primers RH50-RH51 (lanes 2 to 6) and RH52-RH53 (lanes 7 to 11). Measured lengths of the products are indicated in base pairs.

pRMH52 DNA propagated in the presence of pSU2056 was cloned into M13mp18 as described in Materials and Methods, and the sequence of six isolates was determined. The sequence of the junction between the two *aadA2* cassettes in the PCR products is shown in Fig. 6B. This junction is identical to that expected for either a circular DNA intermediate containing only the *aadA2* gene or for two precisely inserted, and correctly oriented, adjacent *aadA2* inserts.

PCR amplification can also be used to search for further rearranged products. Primers were originally designed to amplify the In4 circular excision products orfE alone, *aacC1* alone, *aadA2* with orfE, *aacC1* with orfE, and *aacC1*, orfE, and *aadA2* together. However, each of these primer pairs can also amplify a fragment from particular rearranged orders of gene inserts. The products amplified and the predicted and observed sizes of the smallest product are shown in Table 5. The sequences of these primers and their locations in the published sequences are shown in Table 4. In all cases a band of the expected size was obtained only when DNA from plasmids grown together with pSU2056 was used as a template for amplification (data not shown), confirming that many different insert arrangements have arisen as a result of integrase action. In all cases the sizes of the bands observed corresponded with those predicted (Table 5), indicating that these events are also precise.

DISCUSSION

The experimental data presented here demonstrate that precise deletion and rearrangement of four different integron insert units, *aadA2*, *aacC1*, orfE, and *aacA4*, is catalyzed by the integron DNA integrase. Thus a wide range of deletions and rearrangements, each involving one or more complete gene cassettes, can be catalyzed by this site-specific recombinase. Two features of the deletion and rearrangement process are noteworthy. First, all deletions and rearrangements involve complete gene cassettes and the sequences of the new boundaries confirm the definition of the sequences

TABLE 4. PCR primers

Primer[a]	Sequence	Position in published sequence
RH50	5′-CATC<u>AAGCTT</u>TACGGCCACAGTAACC-3′ *Hin*dIII	9–6 (this study), 2324–2303 (32)
RH51	5′-CGCAGATCACTTGGAAG<u>AATTC</u>ATTCGC-3′ *Eco*RI	41–68 (27)
RH52	5′-GATTCCAGATG<u>GAGCTC</u>AATGACGCT-3′ *Sac*I	2228–2202 (32)
RH53	5′-GCCCGTCTTACTTG<u>AAGCTT</u>AGCAAG-3′ *Hin*dIII	494–519 (This study)
RH54	5′-GGCTAGA<u>AAGCTT</u>TTGGAACAGGGCG-3′ *Hin*dIII	1954–1929 (32)
RH55[b]	5′-ATGC<u>AAGCTT</u>GTGCAGCAAGCGCCACCAGC-3′ *Hin*dIII	1970–1993 (32)
RH56	5′-GTCTCC<u>GAGCTC</u>ACGACCGAAAAG-3′ *Sac*I	1408–1385 (32)
RH57	5′-CTCCTC<u>AAGCTT</u>GAGGCCAACGCG-3′ *Hin*dIII	1634–1657 (32)

[a] For all primers except RH50 and RH55, a mismatch was introduced to create the restriction enzyme cleavage site shown.
[b] The *Hin*dIII site was created by addition of 6 bases at the 5′ end.

TABLE 5. PCR amplification products

Primer pair	Smallest target		Length of product (bp)	
	Excised circle	Rearranged insert	Predicted	Observed
RH50-RH51	*aadA2*	*aadA2-aadA2*	309	310
RH52-RH53	*aadA2*	*aadA2-aadA2*	270	270
RH54-RH55	orfE	orfE-orfE	253	250
RH56-RH57	*aacC1*	*aacC1-aacC1*	351	360
RH54-RH51	orfE + *aadA2*	*aadA2*-orfE	196	195
RH55-RH56	*aacC1* + orfE	orfE-*aacC1*	283	285
RH56-RH53	*aacC1* + orfE + *aadA2*	*aadA2-aacC1*	289	290

associated with each gene cassette, which was derived from comparisons of natural insert sequences (9). The recombination crossover points can be confined to within or on either side of the GTT triplet which is conserved in all core sites, and this location is identical to that found for experimental cointegrates (9, 16). Thus gene cassettes are functional units. Second, cassettes found in new locations always have the same orientation. We have previously shown that experimental cointegration events involving one plasmid which includes a 59-base element on a cloned fragment also give rise to cointegrates with only a single orientation (9) and concluded that the 59-base elements include features which determine the orientation of the site-specific recombination event.

The data presented here provide support for the recent model for the insertion, deletion, and reinsertion of integron insert gene units as circular gene cassettes (9). First, the demonstration that gene cassettes are functional units is a formal requirement of the model. Second, apart from deletion and reinsertion there is only one alternative route which can give rise to the products observed in this study. This is *int*-dependent formation of cointegrates between two plasmid molecules followed by *int*-dependent resolution of the cointegrates. In this case, the sites used for the cointegration and resolution events must differ and equal numbers of plasmids which have lost and gained the same gene insert unit(s) should be found in the population of DNA molecules. Though this is likely to be the mechanism which gives rise to a substantial fraction of rearrangements, the fact that the observed frequency of loss of the *aadA2* gene (3.7×10^{-2}) is about 10-fold higher than the frequency of plasmids carrying two copies of the *aadA2* gene cassette (4.5×10^{-3}) argues against this route as the major source of plasmids which have lost the *aadA2* gene insert. The majority of plasmids which have lost the *aadA2* gene cassette are thus likely to have arisen by direct excision of *aadA2* via recombination between the sites flanking this cassette, and this recombination event should generate a circular gene cassette. The possibility that plasmids with a second *aadA2* cassette inserted adjacent to *aadA2* in the pRMH52 insert region may not be detected by selection for resistance to high levels of spectinomycin (see below) does not weaken the argument that the majority of deletions arise from precise gene excision events. That the relative abundance of such molecules is substantially lower than that of plasmids with the insert arrangement *aadA2-aacC1*-orfE-*aadA2* is demonstrated by the experiments which directly detect the *Eco*RI fragments (Fig. 5), and the failure to isolate plasmids with this insert arrangement would not therefore significantly influence the estimate of the frequency of plasmids with an *aadA2* duplication. Furthermore, as direct excision of gene cassettes is formally equivalent to cointegrate resolution, and cointegrate resolution has been shown to occur (9), the only possible impediment to the occurrence of direct gene excision is a topological constraint when the distance between the two recombination sites is too small.

Attempts to obtain direct evidence for circular gene cassettes were not successful because of the presence of plasmids which contain two copies of the *aadA2* gene insert unit in the DNA extracts used. The bands observed in Fig. 5A and B and Fig. 8 are most likely to arise from plasmid molecules which contain duplications. Though no plasmids in which two copies of the *aadA2* cassette are located adjacent to one another in the insert region were isolated, their presence was detected by end labelling, hybridization, and PCR amplification. Similarly, the bands observed with other primer pairs designed to detect circular products (Table 5) are also likely to reflect the presence of plasmids with different rearranged insert regions. As excision and reinsertion as well as cointegration and resolution should be catalyzed by the same enzyme, the integron DNA integrase, it is not possible to devise experimental conditions where only excision or insertion is unequivocally known to be taking place. Others have recently interpreted the existence of PCR products equivalent to those shown in Fig. 8 as direct evidence for the existence of circular gene cassettes (28). Our data clearly demonstrate that this interpretation is invalid.

Several further conclusions can be made from the results presented. The *aacC1* gene, which is the first insert in the In4 (Tn*1696*) integron, is fused in frame with a short peptide encoded by the 5′ conserved segment sequences (33), and the N-terminal sequence of the AAC3-1 protein (12) confirms that the fused peptide is translated. Translation of *aacC1* from the first ATG codon within the *aacC1* insert unit would result in a protein 23 amino acids shorter. We have isolated plasmids which include a copy of the *aadA2* gene cassette inserted between the 5′ conserved segment junction and the *aacC1* cassette, and these plasmids express the gentamicin resistance phenotype even though the N-terminal fusion is not possible in this configuration. This result indicates that the N-terminal 23 amino acids derived from the fusion are not essential for the acetylase activity. However, it is possible that expression of *aacC1* in Tn*1696* is enhanced by the fusion, as a ribosome binding site GGAG located 7 bases prior to the translational start point is likely to be more efficient than the AGG located 10 bases prior to the first methionine codon within the insert (33). Indeed, the MIC of gentamicin for strains containing plasmid pRMH75, in which *aacC1* is the second insert unit, was threefold lower than for pRMH52, in which *aacC1* is the first insert gene.

It is also possible that the increased resistance to specti-

nomycin used in this study to isolate *aadA2* gene duplications also results from increased expression of the *aadA2* gene when it is positioned as the first insert unit. Precise insertion of the *aadA2* cassette as the first insert unit would also result in a translational fusion with the same peptide resulting in the addition of 20 amino acids to the N terminus of the protein. Indeed, of a total of 55 plasmids isolated by selection for resistance to high levels of spectinomycin, only four did not contain an insert region with *aadA2* as the first insert unit. In these plasmids the orfE insert had been lost, leading to the arrangement *aacC1-aadA2*. That isolation of these plasmids was not fortuitous was confirmed by demonstrating a high efficiency of plating for strains containing such a plasmid on spectinomycin (5 mg/ml). Increased expression of spectinomycin resistance is less simply explained in this case but may result in some way from increased proximity to the promoter in the 5′ conserved segment. One possibility is that the 59-base element found at the end of orfE acts as a transcriptional terminator, and loss of orfE leads to higher levels of *aadA2* in transcripts.

In this study we have isolated plasmids which contain no insert genes and are thus identical to the predicted ancestral integron (9) and the integron In0 found in pVS1 (3). In these plasmids the sequences of the 3′ and 5′ conserved segments are fused, but the GTT which is present at both the 3′ end of the 5′ conserved segment and at the 5′ end of the 3′ conserved segment (26) occurs only once. This confirms the conclusion that one of the GTT triplets, though conserved, is in fact part of the insert region. For simplicity, we have previously designated the GTT at the 3′ end of the 5′ conserved segment to be part of the insert unit (9). The extent of the 5′ conserved segment is thus revised to exclude this GTT triplet. The ability of the integrase to generate plasmids with no inserts argues that caution should be exercised in claiming that integrons with no gene inserts found in natural isolates are necessarily the ancestors of integrons with inserted genes. Integrons have been defined by identifying the conserved sequences associated with In1 in R46 and In2 in Tn*21* and comparison of restriction maps for In3 in R388 and In4 in Tn*1696* (26). Although the 5′ conserved segment of integrons is precisely defined (10, 25, 26), the full extent of the 3′ conserved segment sequences associated with integrons has not yet been established, as the sequences of In1, In2, In3, and In4 integrons diverge at different points from a common sequence (25). It is, however, unlikely that the argument can be sustained that the absence of insert genes in the integron found in pVS1 (3) indicates that the configuration of the 3′ conserved segment in this integron is also ancestral.

It has also been argued that integrons are not mobile DNA elements, because the configuration of insert genes in Tn*21* (In2) is not found in other locations (8). However, the results presented here demonstrate that integron insert regions are readily rearranged, and it is thus likely that the failure to detect identical arrangements in differently located integrons simply reflects the fact that insert rearrangements occur more frequently than movement of a complete integron. Furthermore, the different endpoints for 3′ conserved segment sequences in integrons with different locations (25) indicate that the complete integron sequences may not be present in many naturally occurring integrons, and this may preclude integron movement.

Some of the implications of the circular gene cassette model for the spread of antibiotic resistance genes in bacterial populations have been discussed elsewhere (9). However, other routes are also likely to participate in gene spread. One of these is integrase-mediated formation and resolution of cointegrates, and this mechanism is likely to have given rise to the majority of the duplications and rearrangements observed in this study. The formation or resolution of cointegrates can also be achieved by *recA*-dependent recombination within the 5′ or 3′ conserved segment sequences. Such events could not occur in this study, as all experiments were performed in *recA* mutant strains. A combination of *int*-dependent cointegrate formation followed by *recA*-dependent resolution has recently been proposed for the rearrangement of integron inserts (8). However, as *int*-dependent resolution of cointegrates has since been demonstrated, it is most likely that in wild-type cells both *recA*-dependent and *int*-dependent processes contribute to both the formation and the resolution of cointegrates. Nonetheless, it is difficult to envisage how the primary insertion of gene units into integrons and Tn*7*-like elements can occur by such mechanisms.

The insertion of circular gene cassettes can be invoked as the primary gene insertion event (9). The results presented here are consistent with the notion that integron insert genes can be excised as circular gene cassettes, and if this is so it seems likely that the excised genes can be reinserted at new locations in integrons or Tn*7*-like transposons. However, direct evidence for the existence of a circular gene cassette is necessary to finally establish the validity of this model. Such evidence is currently being sought. Molecules corresponding in size to those of covalently closed circles including only the *aadA2* or orfE gene cassettes have been detected by hybridization when large quantities of plasmid DNA are analyzed, and digestion of these molecules with restriction enzymes which cut once within the relevant gene yields a linear product of the correct size (unpublished observations). Further characterization of these molecules should establish if they are indeed circular gene cassettes.

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