ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2002, p. 2400–2408 Vol. 46, No. 8
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.8.2400–2408.2002

Class 1 Integron Containing a New Gene Cassette, *aadA10*, Associated with Tn*1404* from R151

Sally R. Partridge,[1,2] Christina M. Collis,[1] and Ruth M. Hall[1]*

CSIRO Molecular Science, North Ryde, NSW 2113,[1] and Department of Biological Sciences, Macquarie University, Sydney, NSW 2109,[2] Australia

Received 19 November 2001/Returned for modification 12 March 2002/Accepted 23 April 2002

The carbenicillin, gentamicin, kanamycin, streptomycin, spectinomycin, sulfonamide, and tobramycin resistance determinants found on *Pseudomonas aeruginosa* plasmid R151 have previously been shown to translocate to another plasmid, R388, and it was inferred that a transposon, Tn*1404*, carried the resistance determinants. Sequencing of the cassette array from the plasmid known as R388::Tn*1404* revealed two known gene cassettes, *oxa10* and *aadB*, and a previously unidentified cassette determining resistance to streptomycin and spectinomycin, here designated *aadA10*, in the order *oxa10-aadB-aadA10*. These cassettes replaced the *dfrB2-orfA* cassette array of R388, indicating that movement of the resistance determinants from R151 to R388 resulted from recombinational exchange between two class 1 integrons rather than transposition. The AadA10 protein is most closely related to AadA6 (85% identical) and AadA7 (80% identical). The *aadA10* cassette found here has only a simple site containing a 7-bp spacer derived from *attI1* in place of a 59-base element and is likely to represent a derivative of the complete cassette. IntI1-mediated deletion of the *aadA10* cassette was not detected, indicating that this single simple site is either inactive or only weakly active.

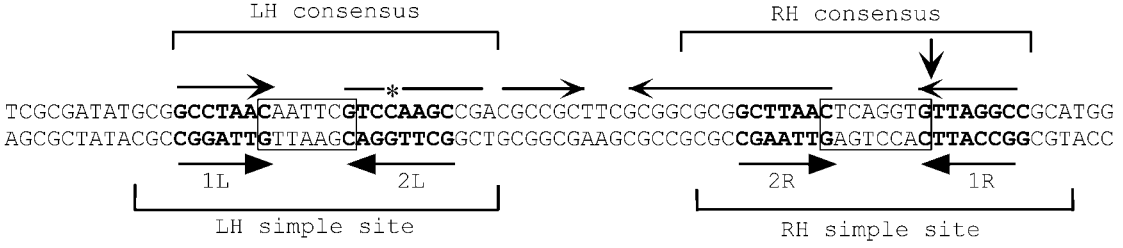
Multiresistance plasmid R151 was originally identified in *Pseudomonas aeruginosa* strain POW 151, isolated in Chicago, Ill., in or prior to 1973 (5). R151 is an IncP-11 plasmid that confers resistance to carbenicillin, gentamicin, kanamycin, streptomycin, spectinomycin, sulfonamides, and tobramycin (5, 38). The carbenicillin resistance of R151 is due to the PSE-2 β-lactamase (38), since renamed OXA-10. Philippon et al. (38) used recombinant plasmids of unknown structure derived from R151 and broad-host-range plasmid pUZ8 (IncP-1; tetracycline resistant [22]) to move the R151 resistance markers into *Escherichia coli*. Trimethoprim and sulfonamide resistance plasmid R388 (IncW) (1, 14) was introduced as the target for transposition. Ampicillin-resistant transconjugants were obtained and were also resistant to gentamicin, kanamycin, streptomycin, spectinomycin, sulfonamides, and tobramycin but not to tetracycline. The resistance determinants were then found to be associated with an IncW plasmid, suggesting that they had been translocated to R388, and the unit carrying them was named Tn*1404*. However, although the plasmid was designated R388::Tn*1404*, it appeared to be the same size as R388 and did not confer the trimethoprim resistance of R388, implying that this determinant had been lost or inactivated.

The sequence of the *oxa10* gene originating from R151 (GenBank accession no. J03427) (23) indicated that it was part of the first gene cassette in a class 1 integron (17, 23). Further sequencing (GenBank accession no. U37105.1) (40) revealed the beginning of an *aadB* cassette adjacent to the *oxa10* cassette, accounting for the gentamicin-adenylating activity (5) and the gentamicin, kanamycin, and tobramycin resistances of strain POW 151. Class 1 integrons usually contain two conserved segments (CS) flanking the antibiotic resistance gene cassettes (45). The 5′-CS (20, 45) includes the *intI1* gene, which encodes the IntI1 integrase (27); the *attI1* recombination site (36); and the P_c promoter, which directs transcription of cassette genes (10, 45). The 3′-CS can vary in length but usually includes the *sul1* sulfonamide resistance determinant (4, 18, 45). Gene cassettes can be inserted between the two CS of the integron by IntI1-catalyzed site-specific recombination between the 59-base element (59-be) of the cassette and the *attI1* site of the integron (9), and IntI1 also catalyzes cassette excision (11, 12). As R388 also includes the class 1 integron In3, which contains the *dfrB2* (trimethoprim resistance) and orfA (unknown function) cassettes (45), the resistance genes of R151 could have been acquired by R388 via cassette exchange, with the simultaneous loss of trimethoprim resistance.

Gene cassettes are small, mobile genetic elements consisting of a single gene (or occasionally two genes) plus a recombination site called the 59-be (6, 12, 17, 40). At least 75 different antibiotic resistance gene cassettes have now been identified; and the genes that they contain confer resistance to aminoglycosides, β-lactams, chloramphenicol, trimethoprim, erythromycin, and rifampin (28, 40; S. R. Partridge and R. M. Hall, unpublished data). The 59-be found in different gene cassettes vary in length (55 to 141 bp) and sequence, but they all contain consensus regions of about 25 bp at each end (11, 17, 46) (Fig. 1A). The consensus regions are imperfect inverted repeats of one another and are separated by a region of variable length that is generally also an inverted repeat (17). Each consensus region contains a simple site (Fig. 1A) made up of a pair of inversely oriented core sites (GTTRRRY) separated by a 7- or 8-bp spacer that overlaps the core sites by 1 bp at each end (46). The extents of these simple sites are inferred from integrase binding data (13, 16). The core sites are designated 1L and 2L at the left-hand (LH) end and 2R and 1R at the right-hand (RH) end (46), and the recombination crossover occurs between the G and the first T in the 1R core site (46). Hence, in

* Corresponding author: Mailing address: CSIRO Molecular Science, Sydney Laboratory, P.O. Box 184, North Ryde, NSW 1670, Australia. Phone: 61-2-9490 5162. Fax: 61-2-9490 5005. E-mail: ruth.hall@csiro.au.

A



B

aadA10/3'-CS	GAT**GTCTAAA**ACAAAG**tt-agat**gca-3'-CS
oxa10/aacA4	GGT**GGCTAAA**ACAAAG**tt-aggc**atc-*aacA4* cassette
	::::::::: : :::
oxa10 59-be	GGT**GGCTAAC**AAGTC**GCTCAAGG**TCG-65 bp-GCC**CCTTAGC**TCCAAC**Gttrrry**nnn
oxa10/aadA1	GGT**GGCTAAA**ACAAAG**tt-aaac**atc-*aadA1* cassette
	::::::::: : :::
oxa10 59-be	GGT**GGCTAAC**AAGTC**GCTCAAGG**TCG-65 bp-GCC**CCTTAGC**TCCAAC**Gttrrry**nnn
aadA1/oxa9	AAT**GTCTAAA**ACAAAG**tt-atgc**acc-*oxa9* cassette
	::::::::: : :::
aadA1 59-be	AAT**GTCTAAC**AATTC**GTTCAAGC**CGA-14 bp-GCG**GCTTAAC**TCAAGC**Gttrrry**nnn
bla$_{GES-1}$*/aacA4*	CCC**GTCTAAA**ACAAAG**tt-aggc**atc-*aacA4* cassette
	::::::::: : :::
bla$_{GES-1}$ 59-be	CCC**GTCTAAC**AATTC**GTTCAAGC**CGA-64 bp-GCG**GCTTAAC**TCAGGC**Gttrrry**nnn
aadA6/aacA4	GAT**GTCTAAA**ACAAAG**tt-aggc**atc-*aacA4* cassette
aadA6/aacA4	GAT**GTCTAAC**AATTC**ATTCAAGC**CGA-14 bp-GCG**GCTTAAT**TCAGGC**Gttrrry**nnn
	2 1
attI1 simple site	TCG**CCCTAAA**ACAAAG**tt-rrry**nnn
	simple site

FIG. 1. Recombination sites associated with gene cassettes. (A) Structure of 59-be. The 59-be from the circular form of the *aadB* cassette (GenBank accession no. L06418) is shown. Core sites, labeled as described by Stokes et al. (46), are in boldface type, with their relative orientations indicated by the arrows below the sequence. Spacer regions are boxed (note that one base at each end of the spacers is also part of the adjacent core site). Inverted repeat regions are indicated by the arrows immediately above the sequence, and an asterisk indicates the extra base present in 2L compared with the sequence of 2R. The extents of the LH and RH simple sites and consensus regions are also indicated. The vertical arrow shows the position of the recombination crossover point. (B) Simple sites with an *attI1* spacer found in derivatives of gene cassettes. The simple site at the end of the R151-derived *aadA10* cassette is compared with those of all known cassettes with single simple sites containing the *attI1* spacer. The latter are aligned with the 59-be associated with the complete cassette, and colons indicate identity. The *attI1* simple site, with the 7-bp core sites labeled as described by Partridge et al. (36) in boldface type and the 7-bp spacer boxed (note that A and G belong both to the spacer and the core site), is shown below. The sequence from the adjacent cassette or 3′ CS is indicated with lowercase letters. The sources of the sequences are as follows: *oxa10* and *aacA4*, GenBank accession no. Z22590; *oxa10*, GenBank accession no. U37105; *oxa10* and *aadA1*, GenBank accession no. AF205943; *aadA1* and *oxa9*, GenBank accession no. M55547; *aadA1*, GenBank accession no. X12870; *bla*$_{GES-1}$ and *aacA4*, GenBank accession no. AF156486; *bla*$_{IBC-1}$ (equivalent to *bla*$_{GES-1}$), reference 15; *aadA6* and *aacA4*, GenBank accession no. AF453998; and *aadA6*, GenBank accession no. AF140629.

the linear, integrated form of a cassette the 1R site, except for the initial G residue, is situated at the start of the cassette. Occasionally, incomplete versions of known cassettes containing a single simple site in place of a 59-be have been found (7, 31, 33, 36, 39, 41). The central part of the 59-be has been lost or replaced by the spacer from the *attI1* site of the integron (36). However, the effects of these changes on cassette mobility have not been investigated.

TABLE 1. Plasmids used in the study

Plasmid	Description	Relevant phenotype[a]	Reference
R388	33-kb IncW plasmid containing In3; cassette order, *dfrB2*-orfA	Su^r Tp^r Tra^+	1
R388::Tn*1404*	"Tn*1404*" transposed into R388	Ap^r Cb^r Gm^r Km^r Sm^r Sp^r Su^r	38
pSU2056	1.2-kb *Bam*HI-*Rsa*I fragment from Tn*21* inserted into pUC9	Ap^r $IntI1^+$	27
pRMH260	2.3 kb of In6 from pSa in pACYC184; cassette order, *aacA4-aadA2*	Cm^r Km^r Sm^r Sp^r	10
pRMH328	2.3 kb of In6 from pSa in pACYC184 in opposite orientation to pRMH260; cassette order, *aacA4-aadA2*	Cm^r Km^r Sm^r Sp^r	This work
pRMH549	5.3-kb *Bam*HI fragment of R388::Tn*1404* in pACYC184	Ap^r Cb^r Cm^r Gm^r Sm^r Sp^r Su^r	This work
pRMH857	5.3-kb *Bam*HI fragment of R388::Tn*1404* in pACYC184	Ap^r Cb^r Cm^r Gm^r Sm^r Sp^r Su^r	This work
pRMH549Δ*oxa10*	IntI1-mediated deletion of *oxa10* cassette from pRMH549; cassette order, *aadB-aadA10*	Cm^r Gm^r Sm^r Sp^r Su^r	This work
pRMH549Δ*aadB*	IntI1-mediated deletion of *aadB* cassette from pRMH549; cassette order, *oxa10-aadA10*	Ap^r Cb^r Cm^r Sm^r Sp^r Su^r	This work

[a] Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline; Tp, trimethoprim.

In the study described here, we have investigated the possibility that the loss of trimethoprim resistance observed on formation of R388::Tn*1404* could be due to replacement of the R388 cassette array by the one that is present on pUZ8-R151. The sequence of the cassette array in R388::Tn*1404* was determined, and a new *aadA* gene cassette was identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α [*supE44* Δ*lacU169* (ϕ*80 lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used to propagate plasmid DNA. R388::Tn*1404* in *E. coli* strain C600 was kindly supplied by G. A. Jacoby and was confirmed to confer resistance to ampicillin, gentamicin, kanamycin, streptomycin, spectinomycin, and sulfamethoxazole. It was transformed into DH5α with selection for sulfamethoxazole and spectinomycin. Descriptions of the plasmids used in the study are provided in Table 1. The 5.3-kb *Bam*HI fragment from R388::Tn*1404* was cloned in pACYC184 (8) by standard procedures (42), and plasmids with the insert in opposite orientations were designated pRMH549 and pRMH857. pRMH549Δ*oxa10* and pRMH549Δ*aadB* were obtained by IntI1-mediated deletion of the *aadB* cassette or the *oxa10* cassette, respectively, from pRMH857 to give the cassette orderes *aadB-aadA10* and *oxa10-aadA10*. pRMH260 contains part of integron In6 from pSa, consisting of 567 bp of the 5′ CS, the *aacA4-aadA2* cassette array, and 193 bp of the 3′ CS, in pACYC184 (10). A *Sal*I-*Cla*I fragment containing this truncated integron was end filled and ligated to *Eco*RV-digested pACYC184, and a plasmid with the integron region in the orientation opposite that in pRMH260 was designated pRMH328. Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium or on LB agar, with Mueller-Hinton agar being used to select for resistance to trimethoprim and sulfamethoxazole. The following antibiotics (Sigma) were added as appropriate at the indicated concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; gentamicin, 2 or 8 μg ml^{-1}; kanamycin, 15 μg ml^{-1}; streptomycin, 25 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; trimethoprim, 25 μg ml^{-1}; and sulfamethoxazole, 25 μg ml^{-1}.

DNA isolation and restriction mapping. Plasmid DNA for restriction analysis and cloning was isolated by an alkaline lysis method (2). Restriction enzymes were used in accordance with the manufacturers' instructions. Fragments were separated by electrophoresis on 1% (wt/vol) agarose gels and visualized by staining with ethidium bromide. *Eco*RI-digested bacteriophage SPP1 DNA (Geneworks) and *Hin*dIII-digested bacteriophage λ DNA (Progen) were used as size markers. Plasmid DNA for sequencing was purified with Wizard maxiprep (Promega) or Jetstar midi prep (Genomed) kits.

DNA sequencing and analysis. The DNA sequence on at least one strand of the insert in pRMH857 was determined. The sequences on both strands of the entire *aadA10* gene cassette and of regions where there were differences from standard or prototype sequences were determined. The sequences of the integron boundaries were obtained by sequencing R388::Tn*1404* directly. Automated sequencing was performed by the sequencing facility at the Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia, on an ABI-PRISM 377 sequencer with the Big Dye system. DNA sequences were assembled by using MacVector (version 6.5) software and AssemblyLIGN software (Oxford Molecular). GenBank searches were performed by using the BLASTN and FastA programs available through WebANGIS (Australian National Genomic Information Service). Programs in the Genetics Computer Group Wisconsin package (version 8.1.0) were used via WAG (WebANGIS GCG) to align and analyze the DNA and protein sequences.

Cassette deletion experiments. Strains for deletion experiments were constructed by introducing each of pRMH857, pRMH549Δ*aadB*, pRMH549Δ*oxa10*, or pRMH328 into DH5α containing plasmid pSU2056, which contains the *intI1* gene under the control of the *lac* promoter (27). Cassette deletion experiments were carried out as described by Collis and Hall (12), except that prior to DNA isolation the strains were grown in LB medium containing only chloramphenicol and ampicillin. The DNA recovered was used to transform DH5α by electroporation with selection on plates containing chloramphenicol, and the resultant colonies were patched onto plates containing chloramphenicol alone and chloramphenicol plus gentamicin, ampicillin, or spectinomycin, as appropriate. The patches were scored for resistance, and plasmid DNA prepared from selected patches was digested with restriction enzymes to confirm that a cassette had been lost.

Nucleotide sequence accession number. The sequence with GenBank accession no. U37105.1 has been extended to include the sequence of the whole cassette array in the R388–R151 hybrid plasmid and is now GenBank accession no. U37105.2.

RESULTS

Movement of resistance determinants. The possibility that an exchange of gene cassettes was responsible for the loss in R388 of the trimethoprim resistance accompanying the acquisition of R151 resistance markers (38) was investigated. Integron In3 in R388 contains characteristic *Bam*HI sites present in the 5′-CS and the 3′-CS of class 1 integrons plus an additional *Bam*HI site in the orfA cassette (Fig. 2). As no other *Bam*HI sites are present in R388 (1), *Bam*HI digestion gives a large fragment, comprising the bulk of the plasmid, plus fragments of 2.1 and 1.8 kb derived from integron In3 (Fig. 2). In *Bam*HI digests of R388::Tn*1404*, the 2.1- and 1.8-kb fragments of R388 were replaced by a single band of 5.3 kb (Fig. 2). This indicates that only a single integron is present in R388::Tn*1404* and that replacement of the R388 cassette array (*dfrB2*-orfA) had taken place. This was confirmed by determining the sequences at the boundaries of the integron in R388::Tn*1404*, which were found to correspond exactly to those seen between In3 and the plasmid backbone in R388 (4, 34), indicating that the new cassettes lie within the boundaries of In3. We suggest that R388::Tn*1404* should in future be described as an R388–R151 hybrid.

Cassette array in R388–R151. The 5.3-kb *Bam*HI fragment of the R388–R151 hybrid plasmid containing most of the integron (Fig. 2) was cloned in pACYC184 (8) to give

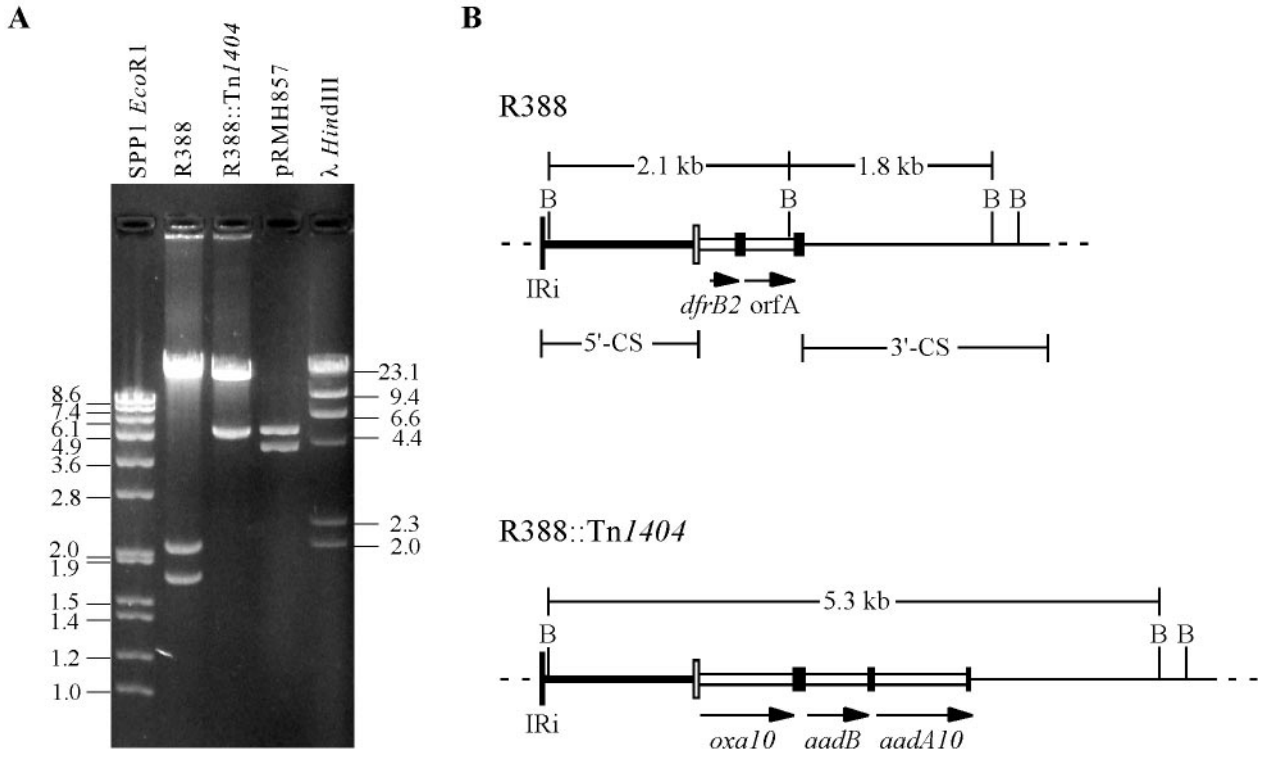


FIG. 2. Structures of R388 and R388::Tn*1404*. (A) *Bam*HI digests of R388, R388::Tn*1404*, and pRM857. Size markers are SPP1 digested with *Eco*RI and bacteriophage λ digested with *Hin*dIII, and the sizes of the fragments (in kilobases) are indicated. (B) Maps of integron In3 in R388 and the cassette array in R388::Tn*1404*. The 5′-CS (thick line) is bounded at one end by IRi and ends with the *attI1* site (narrow open box). Gene cassettes are shown as open boxes and an adjacent filled box representing the 59-be or simple site. The 3′-CS region present is represented by a thin line, and flanking regions are shown as dashed lines. *Bam*HI sites (labeled B) and the sizes of the fragments expected on *Bam*HI digestion are shown.

pRMH857, and the insert was sequenced (GenBank accession no. U37105.2). The 5′-CS has the strong version of the P_c promoter (TTGACA-17 bp-TAAACT), which is also found in In3 (34, 45), and neither the three G residues in In2 that create the P2 promoter nor the 19-bp duplication of *attI1* sequence in In4 (35) was present. As expected, the first cassette was *oxa10* (carbenicillin resistance), and the sequence was identical to that of the sequence with GenBank accession no. U37105.1. The adjacent *aadB* gene cassette (gentamicin, kanamycin, and tobramycin resistance) was found to be identical to the prototype version of this cassette (GenBank accession no. L06418) (6). The third cassette was a novel *aadA* cassette, designated *aadA10*, which confers resistance to streptomycin and spectinomycin.

***aadA10* gene cassette.** The *aadA10* cassette of R388–R151 is 822 bp in length, and in place of the typical 59-be configuration of two simple sites separated by a central region (46), a single simple site is present. The simple site that would be found in the circularized version of this *aadA10* cassette appears to consist of the first 6 bp of the 1L core site separated from the last 6 bp of 1R (found at the beginning of the linear cassette) by a 7-bp region that corresponds to the spacer of the *attI1* simple site (Fig. 1B). Other gene cassettes with similar 1L-*attI1* spacer-1R simple sites are known, and for all of them complete cassettes with a 59-be have been identified (Fig. 1B) (15, 36). It is therefore possible that a longer version of the *aadA10* cassette with a complete 59-be will be discovered in the future.

The sequence of the *aadA10* cassette is most closely related to those of the *aadA6* (88% identical) and *aadA7* (83% identical) cassettes (Fig. 3). There are two possible initiation codons for the *aadA10* gene: an ATG at positions 10 to 12 that is also found in all of the other *aadA* cassettes and a GTG at positions 22 to 24 that is also found in the *aadA7* cassette (Fig. 3). A GTG is also present in this position in the *aadA1* and the *aadA2* cassettes, and a potential ribosome binding site (GAGG) is present upstream of this GTG, which appears to be the major initiation codon for at least *aadA2* (3). In the *aadA7* and *aadA10* cassettes a potential ribosome binding site (GAG) is present in the equivalent position, and it is possible that the GTG codon is also the main initiation codon for these genes. The *aadA10* reading frame extends through the part of the simple site sequence (GTCTAAAACAAAG) found at the end of the cassette and into the adjacent 3′-CS (the sequence with lowercase letters in Fig. 3). An in-frame TAA codon in the 3′-CS would terminate translation in this particular configuration, yielding a 6-amino-acid terminal extension (the sequence with lowercase letters in Fig. 4). The terminal extension would differ if the cassette was in another position. In the *aadA6* cassette, which has a complete 59-be, the reading frame ends within the 59-be, whereas in the *aadA7* cassette, deletion of a

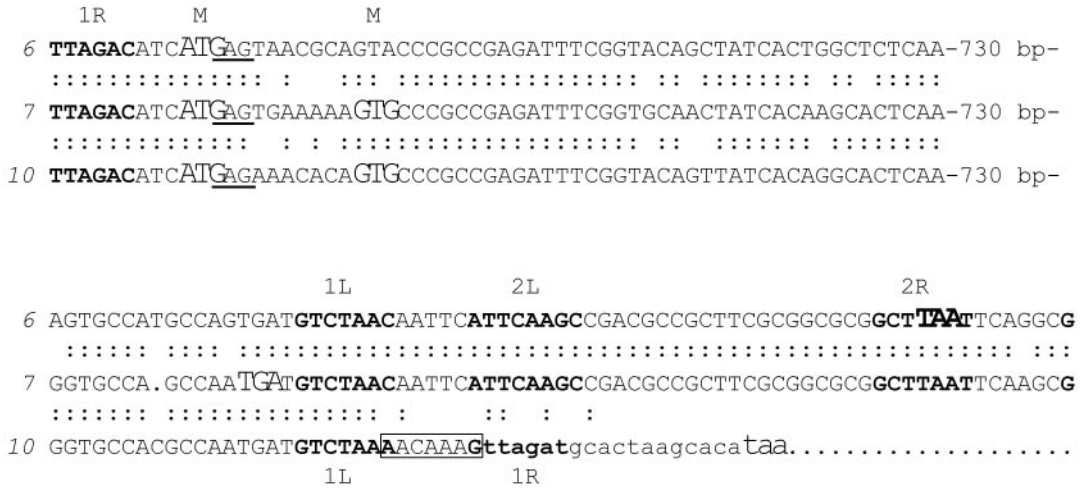


FIG. 3. Alignment of sequences at the ends of the *aadA6*, *aadA7*, and *aadA10* cassettes. Colons indicate identity between aligned sequences. Possible initiation codons (marked by the letter M) and stop codons are indicated by letters in larger type. Potential ribosome binding sites are underlined. Core sites in the 59-be are in boldface type and are labeled as described in the legend to Fig. 1. The *aadA6* and *aadA7* sequences shown end at the cassette boundary, and lowercase letters in the *aadA10* sequence lie outside the cassette boundary and represent the 3′-CS sequence. Bases corresponding to the *attI1* spacer are boxed. The sources of the sequences are as follows: *aadA6*, GenBank accession no. AF140629; *aadA7*, GenBank accession no. AF224733.

single base has brought a TGA codon preceding the start of the 59-be into frame (Fig. 3).

The *aadA10* product, AadA10, is an aminoglycoside (3″)(9)-adenylytransferase [AAD(3″)(9)], as expected from adenylation studies (26). It is most closely related to AadA6 (85% identical; GenBank accession no. AF140629) (32) and AadA7 (80% identical; GenBank accession no. AF224733) (29). AadA10 is also related to all of the other cassette-encoded AadA proteins (59 to 75% identical), and Fig. 4 shows the alignment of the amino acid sequences of these proteins, which fall into groups of more closely related sequences. AadA6, AadA7, and AadA10 are 80 to 85% identical to each other, AadA1 and AadA2 are 86% identical, and AadA4 and AadA5 are 95% identical, while AadA9 is less than 62% identical to any of the other proteins. One hundred twelve amino acids are completely conserved in all of the cassette-encoded AadA proteins (sequences in boldface type in Fig. 4), and at least some of these conserved residues are likely to be important in enzyme function.

Database searches with the sequence of the AadA10 protein also identified several related proteins that are not encoded by a cassette. One of these, from a streptomycin-resistant mutant of *Corynebacterium acetoacidophilum* (GenBank accession no. AJ278607) (J. Deb and G. Karan, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., 2000), is 74% identical to that of AadA10 and differs by only 1 amino acid (V31M in Fig. 4) from AadA2 if the GTG codon is used as the start codon for the *aadA2* gene (position 5 in Fig. 4). The nucleotide sequences of the *aadA2* gene and the *C. acetoacidophilum aadA* gene are also 95% identical over the length of the reading frame, with the last 437 of the total 779 nucleotides being 100% identical. However, this similarity drops away completely on either side of the reading frame, and no vestige of a 59-be is detectable. This high degree of identity suggests that the *C. acetoacidophilum aadA* gene could represent the direct precursor of the one in the *aadA2* cassette.

Other Aad proteins that are not cassette associated are less closely related to AadA10 (29 to 45% identical; 51 to 62% similar). These include the *Salmonella enterica* serovar *choleraesuis* AAD(3″)(9) protein (GenBank accession no. X68089), which confers resistance to both streptomycin and spectinomycin (25); the AAD(9) spectinomycin adenylyltransferases from Tn*554* of *Staphylococcus aureus* (GenBank accession no. X02588) (30), *Enterococcus faecalis* (GenBank accession no. M69221) (24), and *Legionella longbeachae* (GenBank accession no. AF288536); and a number of other proteins (GenBank accession nos. AF408195, AL392149, and AE007309) that are predicted to be aminoglycoside-modifying enzymes on the basis of the similarities of their sequences to that of the *S. aureus* AAD(9) protein. The amino acids that are conserved among all of these proteins are boxed in Fig. 4.

Activity of the *aadA10* simple site. A typical active 59-be consists of two simple sites separated by a central region (46), and deletion of either the LH or the RH simple site of the *aadA1* 59-be has been shown to reduce the activity of this recombination site to undetectable levels in conduction assays (27). Removal of 1L from the *aadB* 59-be was also found to dramatically decrease the activity of this 59-be in conduction assays (19). On the basis of these findings, it seemed unlikely that the single simple site configuration found in the *aadA10* cassette of R388–R151 would function efficiently in recombination. To determine whether this simple site is functional, IntI1-mediated excision of cassettes from pRMH857 was followed by assaying for loss of the associated antibiotic resistance determinants (Table 2). Deletion of the *aadA10* cassette was not detected in over 600 colonies screened, while *aadB*, the central cassette in the array, was frequently excised and the first cassette, *oxa10*, was occasionally lost. Loss of the *aadA10*

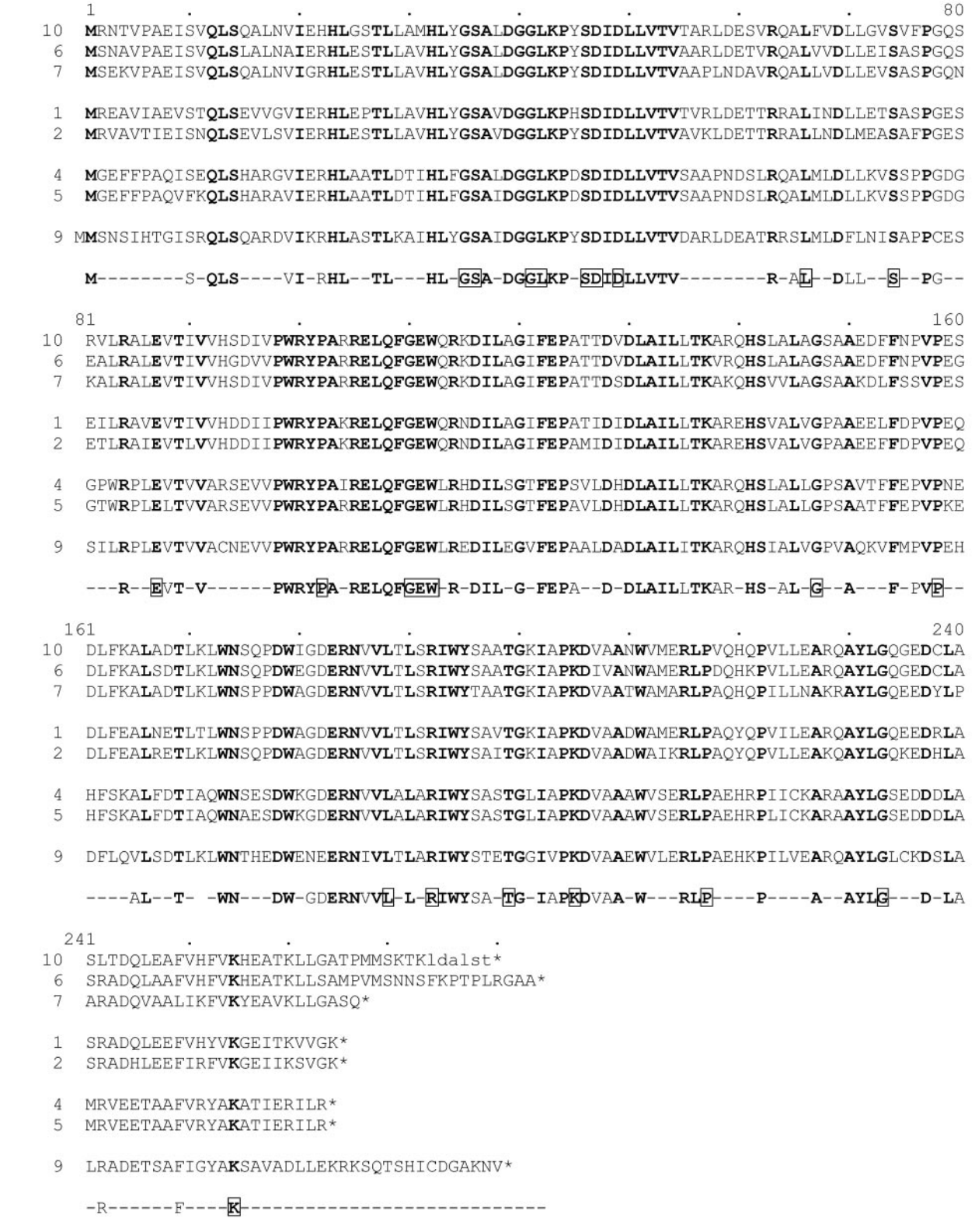


FIG. 4. Alignment of cassette-encoded AadA proteins. The sequences are identified on the left by the numeral in the protein name, and more closely related sequences are grouped. The first ATG after the beginning of the cassette has been used as the start codon, although AadA1, AadA2, AadA7, and AadA9 also have a second potential start codon (GTG) which corresponds to the valine at position 5. Residues identical in seven or all eight (boldface type) sequences are indicated in the consensus sequence shown below. Residues that are also conserved in the Aad proteins from the sequences with GenBank accession nos. X68089, X02588, M69221, AF288536, AF408195, AL392149, and AE007309 are boxed. The protein sequences are translations of DNA sequences in AadA1 (GenBank accession no. X12870), AadA2 (GenBank accession no. X68227), AadA4 (GenBank accession no. Z50802.3), AadA5 (GenBank accession no. AF137361), AadA6 (GenBank accession no. AF140629), AadA7 (GenBank accession no. AF224733), and AadA9 (GenBank accession no. AX135967 or AJ420072). The AadA3 (GenBank accession no. AF047479) and AadA8 (GenBank accession no. AF326210) proteins have not been included in the analysis, as they can be considered hybrids of AadA1 and AadA2.

TABLE 2. IntI1-mediated excision of gene cassettes

Plasmid	Cassette array	% Colonies with cassettes deleted				No. of colonies screened
		1	2	1 and 2	3	
pRMH549	*oxa10-aadB-aadA10*	1.1	25.5	1.1	<0.2	663
pRMH549Δ*oxa10*	*aadB-aadA10*	0.2	<0.1	<0.1	NA[a]	1,069
pRMH549Δ*aadB*	*oxa10-aadA10*	<0.1	<0.1	<0.1	NA	1,034
pRMH328	*aacA4-aadA2*	0.3	30.4	0.5	NA	382

[a] NA, not applicable.

cassette from derivatives of pRMH857 containing only two cassettes (*aadB-aadA10* or *oxa10-aadA10*) was not detected in over 1,000 colonies screened, and the first cassette was lost infrequently. When another array containing two complete gene cassettes, *aacA4-aadA2*, was examined, the first cassette, *aacA4*, was again lost at very low levels, but the *aadA2* cassette in the second position was frequently excised, which is in contrast to the lack of excision of the incomplete *aadA10* cassette from the equivalent position. Together, these results indicate that the simple site present in the *aadA10* cassette is not functional at a significant level, at least for IntI1-mediated excision reactions.

DISCUSSION

We have shown that the resistance genes present on R151-pUZ8 were translocated to R388 by exchange of the gene cassette region between two integrons rather than by transposition. A similar exchange of cassette arrays was recently found to account for the movement of resistance markers from the chromosome of the *P. aeruginosa* Dalgleish strain to R388 via a pUZ8 intermediate, forming the plasmid known as R388::Tn*1405* (34). Although movement of resistance markers from R151-pUZ8 to R388 was *recA* independent in the original study (38), the investigators suggested that the gene exchange may have occurred by homologous recombination between identical regions present in both plasmids, i.e., the 5′ CS and the 3′ CS. An alternate explanation is that IntI1-mediated site-specific recombination may have been involved. If this was the route, two site-specific recombination events must have occurred, one at each extremity of the two cassette arrays.

However, the findings reported here do not preclude the existence of Tn*1404* as a transposon. Philippon et al. (38) also used pUB5573, a sulfonamide-sensitive *Pst*I deletion derivative of R388 that has lost part of the orfA cassette and part of the 3′ CS region, instead of R388 as the recipient for R151-pUZ8 resistance markers. The putative pUB5573::Tn*1404* transconjugants arose in a *rec*$^+$ strain but were about 9.6 kb larger than pUB5573, retained trimethoprim resistance, and had acquired sulfonamide resistance together with the other resistance markers (26, 38). This suggests that a transposable unit carrying all of the resistance determinants of R151 may have translocated to pUB5573 in this case. The size of this unit is similar to those of class 1 integrons such as In5 (9 kb), In2 (11 kb), and In4 (8 kb), which all carry the *sul1* gene (4, 35), and it is possible that Tn*1404* corresponds to a class 1 integron. Although most class 1 integrons lack a complete set of transposition genes and are unable to transpose themselves (4, 35, 37), if they have intact terminal inverted repeats they may be moved in *trans* by appropriate transposition proteins that happen to be present in the same cell. Indeed, integron In33, which corresponds to both Tn*2521* and Tn*1405*, can move by transposition catalyzed by transposition proteins supplied in *trans* (44). Thus, it is possible that movement of an integron from R151-pUZ8 to pUB5573 created pUB5573::Tn*1404*. However, this event could have occurred only if the R151-pUZ8 recombinant carries the appropriate genes for transposition functions. In this regard, it would be interesting to look at the structures of pUB5573::Tn*1404* and of the integron in the original plasmid R151 to determine if transposition genes are present.

The *aadA10* cassette found here may not represent the complete cassette, as a single simple site was found in place of a 59-be. IntI1-mediated excision of this incomplete *aadA10* cassette was not detected, and this is consistent with previous observations that deletion of all or part of either the LH or the RH simple site from a 59-be reduces the activity of the 59-be to undetectable levels (19, 27). Similar observations have been made in the case of the *attI1* recombination site, which consists of one simple site plus two additional, but directly oriented, integrase binding domains (13). In cointegration assays, the *attI1* simple site alone is active at low levels in reactions with a complete *attI1* site but is not an efficient recombination site in reactions with the 59-be (21, 36). Further experiments are needed to determine if single simple sites derived from the 59-be, such as the one found in the *aadA10* cassette, are able to participate in integration reactions.

The loss of the central region of the 59-be appears to have effectively fused the *aadA10* cassette to the 3′-CS, with the consequence of fixing it as part of the class 1 integron backbone. There are four further examples in which the final gene cassette in an array is likely to be fused to the 3′-CS as a result of the replacement of a complete 59-be by a simple site (33, 39, 41). However, the structures of these simple sites differ from that of the one found in the R388–R151 *aadA10* cassette (Fig. 5). The 1L and 1R sites of the original 59-be are present, but they are separated either by the RH spacer found in the complete 59-be or by the LH spacer. Thus, a 59-be can be converted to a single simple site in more than one way. We have previously suggested how simple sites with the *attI1* spacer could have been created (36), and the role of the integron-encoded integrase in these processes deserves investigation.

The other cassette derivatives in which the central part of the 59-be is replaced by the *attI1* spacer sequence have all been fused to a second cassette (Fig. 1B). Although these partial 59-be are presumably also nonfunctional, in these cases the first cassette is likely to retain mobility by moving together with the downstream cassette as a single two-gene unit. Conversion

FIG. 5. Simple sites containing spacers derived from 59-be. The sequences at the ends of the *aadA1* and *aadA2* cassettes with the complete 59-be and derivatives with simple sites are aligned. Colons indicate identity. Core sites are in boldface type and are labeled as described in the legend to Fig. 1, and spacers are boxed. The sequence from the adjacent 3′-CS or gene cassette is indicated with lowercase letters. The stop codons of the *aadA1* and *aadA2* genes are marked by asterisks. The sources of the sequences are as follows: *aadA1*, GenBank accession no. X12870; *aadA2*, GenBank accession no. X68227; *aadA1* and 3′ CS, GenBank accession no. AJ278514; *aadA2* and 3′ CS with RH spacer, GenBank accession no. AF156486; *aadA2* and 3′ CS with LH spacer, GenBank accession no. AF227505; bla_{VIM-2} and 3′ CS, GenBank accession no. AF302086; and bla_{VIM-2}, GenBank accession no. AF191564.

of the remaining 59-be in a fused cassette to a simple site could potentially extend such cassette fusions to include three or more genes. The genes situated in a cassette array usually constitute an operon, as they are transcribed together from the P_c promoter within the integron (10). Furthermore, as the numbers, orders, and identities of the cassettes in the array can be varied by adding or removing cassettes, a vast number of different operons can potentially be created. Conversion of the 59-be within a cassette array to simple sites would reduce the flexibility of an array of functional gene cassettes by fixing the cassette genes in a certain order. This cassette fusion process could therefore have consequences in terms of creating stable operons from the more flexible operons that the cassette arrays within integrons represent. It may also have played a role in the creation of the 3′-CS of class 1 integrons.

It should be noted that the putative Tn*1404* transposon of R151 is not the same as the transposon recently designated Tn*1404* (GenBank accession nos. AF157797 to AF1577801) by Schnabel and Jones (43).

ACKNOWLEDGMENT

S.R.P. was supported by a grant from the Australian National Health and Medical Research Council.

REFERENCES

1. **Avila, P., and F. de la Cruz.** 1988. Physical and genetic map of the IncW plasmid R388. Plasmid **20:**155–157.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Bito, A., and M. Susani.** 1994. Revised analysis of *aadA2* gene of plasmid pSa. Antimicrob. Agents Chemother. **38:**1172–1175.
4. **Brown, H. J., H. W. Stokes, and R. M. Hall.** 1996. The integrons In0, In2, and In5 are defective transposon derivatives. J. Bacteriol. **178:**4429–4437.
5. **Bryan, L. E., M. S. Shahrabadi, and H. M. van den Elzen.** 1974. Gentamicin resistance in *Pseudomonas aeruginosa*: R-factor-mediated resistance. Antimicrob. Agents Chemother. **6:**191–199.
6. **Cameron, F. H., D. J. Groot Obbink, V. A. Ackerman, and R. M. Hall.** 1986. Nucleotide sequence of the AAD(2″) aminoglycoside adenyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538–1 and *dhrfII* in R388. Nucleic Acids Res. **14:**8625–8635.
7. **Centron, D., and P. H. Roy.** 2002. Presence of a group II intron in a multiresistant *Serratia marcescens* strain that harbors three integrons and a novel gene fusion. Antimicrob. Agents Chemother. **46:**1402–1409.
8. **Chang, A. Y. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
9. **Collis, C. M., G. Grammaticopoulos, J. Briton, H. W. Stokes, and R. M. Hall.** 1993. Site-specific insertion of gene cassettes into integrons. Mol. Microbiol. **9:**41–52.
10. **Collis, C. M., and R. M. Hall.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob. Agents Chemother. **39:**155–162.
11. **Collis, C. M., and R. M. Hall.** 1992. Gene cassettes from the insert region of integrons are excised as covalently closed circles. Mol. Microbiol. **6:**2875–2885.
12. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by integron DNA integrase. J. Bacteriol. **174:**1574–1585.
13. **Collis, C. M., M.-J. Kim, H. W. Stokes, and R. M. Hall.** 1998. Binding of the purified integron DNA integrase IntI1 to integron- and cassette-associated recombination sites. Mol. Microbiol. **29:**477–490.
14. **Datta, N., and R. W. Hedges.** 1972. Trimethoprim resistance conferred by W plasmids in *Enterobacteriaceae*. J. Gen. Microbiol. **72:**349–355.
15. **Giakkoupi, P., L. S. Tzouvelekis, A. Tsakris, V. Loukova, D. Sofianou, and E. Tzelepi.** 2000. IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. Antimicrob. Agents Chemother. **44:**2247–2253.
16. **Gravel, A., B. Fournier, and P. H. Roy.** 1998. DNA complexes obtained with integron integrase IntI at the *attI1* site. Nucleic Acids Res. **26:**4347–4355.
17. **Hall, R. M., D. E. Brookes, and H. W. Stokes.** 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. Mol. Microbiol. **5:**1941–1959.
18. **Hall, R. M., H. J. Brown, D. E. Brookes, and H. W. Stokes.** 1994. Integrons found in different locations have identical 5′ ends but variable 3′ ends. J. Bacteriol. **176:**6286–6294.
19. **Hall, R. M., C. M. Collis, M.-J. Kim, S. R. Partridge, G. D. Recchia, and H. W. Stokes.** 1999. Mobile gene cassettes and integrons in evolution. Ann. N. Y. Acad. Sci. **870:**68–80.
20. **Hall, R. M., and C. Vockler.** 1987. The region of the IncN plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found

in IncW plasmids and in Tn*21*-like transposons. Nucleic Acids Res. **15:**7491–7501.
21. **Hansson, K., O. Sköld, and L. Sundström.** 1997. Non-palindromic *attI* sites of integrons are capable of site-specific recombination with one another and with secondary sites. Mol. Microbiol. **26:**441–453.
22. **Hedges, R. W., and M. Matthew.** 1979. Acquisition by *Escherichia coli* of plasmid-borne β-lactamases normally confined to *Pseudomonas* spp. Plasmid **2:**269–278.
23. **Huovinen, P., S. Huovinen, and G. A. Jacoby.** 1988. The sequence of PSE-2 β-lactamase. Antimicrob. Agents Chemother. **32:**134–136.
24. **LeBlanc, D. J., L. N. Lee, and J. M. Inamine.** 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:**1804–1810.
25. **Leung, K. Y., S. R. Ruschkowski, and B. B. Finlay.** 1992. Isolation and characterization of the *aadA* aminoglycoside-resistance gene from *Salmonella choleraesuis.* Mol. Microbiol. **6:**2453–2460.
26. **Lévesque, R. C., and G. A. Jacoby.** 1988. Molecular structure and interrelationships of multiresistance β-lactamase transposons. Plasmid **19:**21–29.
27. **Martinez, E., and F. de la Cruz.** 1990. Genetic elements involved in Tn*21* site-specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. EMBO J. **9:**1275–1281.
28. **Mazel, D., and J. Davies.** 1999. Antibiotic resistance in microbes. Cell. Mol. Life Sci. **56:**742–754.
29. **Mazel, D., B. Dychinco, V. A. Webb, and J. Davies.** 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. Antimicrob. Agents Chemother. **44:**1568–1574.
30. **Murphy, E.** 1985. Nucleotide sequence of a spectinomycin adenyltransferase AAD(9) determinant from *Staphylococcus aureus* and its relationship to AAD(3″)(9). Mol. Gen. Genet. **200:**33–39.
31. **Naas, T., Y. Mikami, T. Imai, L. Poirel, and P. Nordmann.** 2001. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. J. Bacteriol. **183:**235–249.
32. **Naas, T., L. Poirel, and P. Nordmann.** 1999. Molecular characterisation of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, *aadA6*, in *Pseudomonas aeruginosa.* Biochim. Biophys. Acta **1489:**445–451.
33. **Pallecchi, L., M. L. Riccio, J. Docquier, R. Fontana, and G. M. Rossolini.** 2001. Molecular heterogeneity of bla_{VIM-2}-containing integrons from *Pseudomonas aeruginosa* plasmids encoding the VIM-2 metallo-β-lactamase. FEMS Microbiol. Lett. **195:**145–150.
34. **Partridge, S. R., H. J. Brown, and R. M. Hall.** 2002. Characterization and movement of the class 1 integron known as Tn*2521* and Tn*1405*. Antimicrob. Agents Chemother. **46:**1288–1294.
35. **Partridge, S. R., H. J. Brown, H. W. Stokes, and R. M. Hall.** 2001. Transposons Tn*1696* and Tn*21* and their integrons In4 and In2 have independent origins. Antimicrob. Agents Chemother. **45:**1263–1270.
36. **Partridge, S. R., G. D. Recchia, C. Scaramuzzi, C. M. Collis, H. W. Stokes, and R. M. Hall.** 2000. Definition of the *attI1* site of class 1 integrons. Microbiology **146:**2855–2864.
37. **Partridge, S. R., G. D. Recchia, H. W. Stokes, and R. M. Hall.** 2001. Family of class 1 integrons related to In4 from Tn*1696*. Antimicrob. Agents Chemother. **45:**3014–3020.
38. **Philippon, A. M., G. C. Paul, and G. A. Jacoby.** 1983. Properties of PSE-2 β-lactamase and genetic basis for its production in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **24:**362–369.
39. **Poirel, L., I. le Thomas, T. Nass, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44:**622–632.
40. **Recchia, G. D., and R. M. Hall.** 1995. Gene cassettes: a new class of mobile element. Microbiology **141:**3015–3027.
41. **Riccio, M. L., L. Pallecchi, R. Fontana, and G. M. Rossolini.** 2001. In70 of plasmid pAX22, a bla_{VIM-1}-containing integron carrying a new aminoglycoside phosphotransferase gene cassette. Antimicrob. Agents Chemother. **45:**1249–1253.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Schnabel, E. L., and A. L. Jones.** 1999. Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Appl. Environ. Microbiol. **65:**4898–4907.
44. **Sinclair, M. I., and B. W. Holloway.** 1982. A chromosomally located transposon in *Pseudomonas aeruginosa*. J. Bacteriol. **151:**569–579.
45. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669–1683.
46. **Stokes, H. W., D. B. O'Gorman, G. D. Recchia, M. Parsekhian, and R. M. Hall.** 1997. Structure and function of 59-base element recombination sites associated with mobile genetic elements. Mol. Microbiol. **26:**731–745.